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(54) 发明名称
用于生产3-羟基丙酸的组合物和方法

(57) 摘要
本发明涉及用于生产3-羟基丙酸的组合物和方法,具体公开了包含活性3-HP发酵途径的经遗传修饰的酵母细胞以及这些细胞生产3-HP的用途。

1. 一种经遗传修饰的酵母细胞,其选自东方伊萨酵母(*Issatchenkia orientalis*)、郎比可假丝酵母(*Candida lambica*)和博伊丁酵母(*Saccharomyces bulderi*),其包含活性3-HP发酵途径,其中所述细胞包含外源的 β -丙氨酸氨基转移酶(BAAT基因)或4-氨基丁酸氨基转移酶(gabT基因),外源意指所述基因不存在于天然形式的所述酵母细胞的基因组中所述基因的位置,其中所述BAAT基因或gabT基因编码多肽,所述多肽与SEQ ID NO: 20或SEQ ID NO: 21的氨基酸序列有至少99%序列同一性,其中所述多肽源自东方伊萨酵母(*Issatchenkia orientalis*)、克鲁维酵母(*Saccharomyces kluyveri*)、阿维链霉菌(*Streptomyces avermitilis*)或酿酒酵母(*Saccharomyces cerevisiae*)。

2. 根据权利要求1所述的经遗传修饰的酵母细胞,其中所述细胞包含一种或多种选自如下的外源3-HP途径基因:

外源磷酸烯醇丙酮酸羧化酶(PPC)基因;

外源丙酮酸羧化酶(PYC)基因;

外源天冬氨酸氨基转移酶(AAT)基因;

外源天冬氨酸1-脱羧酶(ADC)基因;和

外源3-羟基丙酸脱氢酶(3-HPDH)基因;

外源意指所述基因不存在于天然形式的所述酵母细胞的基因组中所述基因的位置。

3. 根据权利要求2所述的经遗传修饰的酵母细胞,其中所述PYC基因编码多肽,所述多肽与选自SEQ ID NO: 2, 3, 4, 5, 6, 7和8的氨基酸序列具有至少60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%或100%序列同一性。

4. 根据权利要求2或3所述的经遗传修饰的酵母细胞,其中所述AAT基因编码多肽,所述多肽与选自SEQ ID NO: 14, 15和16的氨基酸序列具有至少60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%或100%序列同一性。

5. 根据权利要求2所述的经遗传修饰的酵母细胞,其中所述ADC基因编码多肽,所述多肽与选自SEQ ID NO: 17, 18, 133, 135, 137和139的氨基酸序列具有至少60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%或100%序列同一性。

6. 根据权利要求1所述的经遗传修饰的酵母细胞,其中所述BAAT基因或gabT基因编码多肽,所述多肽与SEQ ID NO: 20或SEQ ID NO: 21的氨基酸序列具有至少95%, 97%, 98%, 99%或100%序列同一性。

7. 根据权利要求2所述的经遗传修饰的酵母细胞,其中所述3-HPDH基因编码多肽,所述多肽与选自SEQ ID NO: 26, 27, 28, 29, 30, 31, 32, 33, 34和129的氨基酸序列具有至少60%, 例如至少65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%或100%序列同一性。

8. 根据权利要求2所述的经遗传修饰的酵母细胞,其中所述PPC基因编码多肽,所述多肽与选自SEQ ID NO: 10, 11和12的氨基酸序列具有至少60%, 例如至少65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%或100%序列同一性。

9. 根据权利要求1所述的经遗传修饰的酵母细胞,其中所述细胞进一步包含选自PDC, ADH, GAL6, CYB2A, CYB2B, GPD, GPP, ALD和PCK基因的天然基因的一个或多个缺失或破坏。

10. 根据权利要求9所述的经遗传修饰的酵母细胞,其中一个或多个缺失或破坏得自一

个或多个外源3-HP途径基因的插入。

11. 根据权利要求1所述的经遗传修饰的酵母细胞, 其中一个或多个外源3-HP途径基因可操作连接到一个或多个外源调节元件上。

12. 根据权利要求1所述的经遗传修饰的酵母细胞, 其中所述细胞能够在含75g/L或更多3-HP的培养基中在小于4的pH生长。

13. 一种产生3-HP的方法, 包括:

(i) 在含至少一种碳源的培养基的存在下培养权利要求1-12中任一项所述的经遗传修饰的酵母细胞; 和

(ii) 从培养物中分离3-HP。

用于生产3-羟基丙酸的组合物和方法

[0001] 本发明申请是基于申请日为2011年11月21日、申请号为201180065738.0 (国际申请号为PCT/US2011/061717)、名称为“用于生产3-羟基丙酸的组合物和方法”的发明专利申请的分案申请。

[0002] 序列表的引用

[0003] 本申请含有计算机可读取形式的序列表,其通过提述并入本文。

背景技术

[0004] 3-羟基丙酸(3-HP)是一种3碳羧酸,其由U.S.Department of Energy鉴定为12种高潜力的建造模块(building block)化学品之一,可以通过发酵制备。3-HP,一种乳酸(2-羟基丙酸)的异构体,的其它名称是包括亚甲基乳酸(ethylene lactic acid)和3-羟基丙酸根(3-hydroxypropionate)。3-HP是有吸引力的可再生的平台化学品,具有由葡萄糖生产的100%理论产率、使其参与多种化学反应的多个功能基团以及低毒性。3-HP可以用作底物,以形成几种商品化学品,例如1,3-丙二醇、丙二酸、丙烯酰胺和丙烯酸。丙烯酸是大体量的化学品(>70亿lbs/年),其用于制备丙烯酸酯和具有超强吸收能力的聚合物,且目前来源于丙烯的催化性氧化。3-HP的发酵生产将提供可持续的石油化学品替代物,作为这些商业上重要的化学品的原料,因而减少了能量消耗、美国对外来原油的依赖性和温室效应气体的产生。

[0005] 细菌可以用于将糖发酵成有机酸。然而,对于大规模的有机酸生产,细菌显示出某些缺点。随着有机酸产生,发酵培养基变得酸性越来越强。事实上,较低的pH条件是优选的,因为所得产物部分或完全是酸性形式的。然而,产生有机酸的大部分细菌在强酸环境中表现不佳,因此或者死亡或者开始产生缓慢以致其随着培养基变得酸性更强而变得经济上不可行。为了防止这种情况,需要对培养基进行缓冲以维持较高的pH。然而,这使得有机酸产物的回收更加困难和昂贵。

[0006] 近些年来,围绕利用酵母将糖发酵成有机酸的兴趣日益增加。酵母在一些工业发酵中用作生物催化剂,并显示出超越细菌的几项优势。尽管很多细菌不能合成其生长和有效代谢糖类所需要的某些氨基酸或蛋白,但大多数酵母菌种能够由无机氮化合物合成其必需的氨基酸或蛋白。酵母还不易受到在细菌中导致生产力或整体发酵运行过程受损的细菌噬菌体感染。

[0007] 虽然酵母是有机酸生产的引人注目的候选者,但其显现出几项困难。首先,酵母中的途径工厂改造通常比细菌中更加困难。酵母中的酶被分隔在胞质、线粒体或过氧化物酶体中,而在细菌中其汇集于胞质中。这意味着可能需要去除靶向信号,以确保生物合成途径的所有酶共存于单个细胞内的相同区室中。还需要对途径中间物在区室之间的转运进行调控,以使到达目标产物的碳流最大化。其次,并非所有的酵母菌种符合大规模经济发酵的必要标准。事实上,仅小部分酵母具有足够高的体积和特定的糖利用与在低pH条件下快速生长的能力的结合。U.S.Department of Energy已经估测,大约2.5g/L/小时的生长速率对于几种有机酸的经济发酵来说是必需的,包括3-HP (<http://www1.eere.energy.gov/>

biomass/pdfs/35523.pdf)。

[0008] 虽然很多酵母菌种天然地将己糖发酵为乙醇,几乎没有任何酵母天然地产生显著产量的有机酸。这导致尝试对多种酵母菌种进行遗传修饰以生产有机酸。先前已经通过破坏或缺失天然的丙酮酸脱羧酶(PDC)基因并插入乳酸脱氢酶(LDH)基因以消除乙醇的产生,从而开发了产生乳酸的经遗传修饰的酵母株(参见例如W099/14335、W000/71738、W002/42471、W003/049525、W003/102152和W003/102201)。这种改变使糖代谢由产生乙醇转为产生乳酸。酵母的发酵产物和途径不同于细菌,因而需要不同的工程改造方法以使产率最大化。增加有机酸产物的产率或纯度可能需要消除或减少的其他天然产物是甘油、醋酸盐和二醇。经遗传改变的酵母株中甘油的减少描述于例如W007/106524中。

[0009] 与乳酸不同,3-HP不是自然界任何已知途径的主要终产物,其在一些细菌和真菌中仅以痕量发现。因此,需要进行更多的遗传工程改造,以生成产生3-HP的酵母。先前对酿酒酵母(*Saccharomyces cerevisiae*)菌株进行了工程改造,以经由乳酸盐中间物产生非常低水平的3-HP(参见W002/042418)。然而,野生型酿酒酵母的耐受水平不足以使其成为生产3-HP的最佳宿主。因此,需要在工业化规模上以更具成本效率的方式生产3-HP的改进的酵母菌株。

发明内容

[0010] 在某些实施方案中,本文提供了经遗传修饰的酵母细胞,其包含由PEP、丙酮酸和/或甘油产生3-HP的活性3-HP发酵途径。在某些实施方案中,本文中提供的细胞含有一个或多个3-HP途径基因,所述基因编码具有PPC、PYC、AAT、ADC、BAAT、gabT、3-HPDH、HIBADH、4-羟基丁酸脱氢酶、ACC、AAM、丙氨酸脱氢酶、醛脱氢酶、BCKA、KGD、4-氨基丁酸转氨酶、 β -丙氨酸-CoA氨解酶、Co-A酰化丙二酸半醛脱氢酶、CoA合成酶、CoA转移酶、甘油脱水酶、IPDA、LDH、乳酰-CoA脱水酶、苹果酸脱羧酶、苹果酸脱氢酶、丙二酰-CoA还原酶、OAA甲酸裂合酶(formatelyase)、OAA脱氢酶、丙酮酸/丙氨酸氨基转移酶、PDH、2-酮酸脱羧酶、3-HP-CoA脱水酶、3-HP-CoA水解酶或3-羟基异丁酰-CoA水解酶活性的酶。

[0011] 在某些实施方案中,本文提供了经遗传修饰的酵母细胞,其包含由PEP或丙酮酸产生3-HP的活性3-HP发酵途径,其中所述细胞含有编码具有PPC、PYC、AAT、ADC、BAAT、gabT、3-HPDH、HIBADH和4-羟基丁酸脱氢酶活性的酶的一个或多个基因。在某些实施方案中,一个或多个3-HP途径基因是外源的,且在這些实施方案中,所述基因可来源于酵母、真菌、细菌、植物、昆虫或哺乳动物来源。例如,细胞可以含有来源于东方伊萨酵母(*I.orientalis*)的酵母PYC基因或来源于类球红细菌(*R.sphaeroides*)、菜豆根瘤菌(*R.etli*)、荧光假单胞菌(*P.fluorescens*)、谷氨酸棒杆菌(*C.glutamicum*)或苜蓿中华根瘤菌(*S.meliloti*)的细菌PYC基因;来源于大肠杆菌(*E.coli*)、嗜热自养甲烷杆菌(*M.thermoautotrophicum*)或产气荚膜梭菌(*C.perfringens*)的细菌PPC基因;来源于东方伊萨酵母或酿酒酵母的酵母AAT基因或来源于大肠杆菌的细菌AAT基因;来源于阿维链霉菌(*S.avermitilis*)、丙酮丁醇梭菌(*C.acetobutylicum*)、幽门螺旋杆菌(*H.pylori*)、地衣芽孢杆菌(*B.licheniformis*)或谷氨酸棒杆菌的细菌ADC基因;来源于东方伊萨酵母或克鲁维酵母(*S.kluyveri*)的酵母BAAT基因或来源于阿维链霉菌的细菌BAAT基因;来源于酿酒酵母的酵母gabT基因或来源于阿维链霉菌的细菌gabT基因;来源于东方伊萨酵母或酿酒酵母的酵母3-HPDH基因或来源于大肠杆

菌或*M.sedula*的细菌3-HPDH基因;来源于粪产碱菌(*A.faecalis*)、恶臭假单胞菌(*P.putida*)或铜绿假单胞菌(*P.aeruginosa*)的细菌HIBADH基因;和/或来源于克鲁维酵母的酵母4-羟基丁酸脱氢酶基因或来源于*R.eutropha*的细菌4-羟基丁酸脱氢酶基因。

[0012] 在某些实施方案中,本文提供了经遗传修饰的酵母细胞,其包含由PEP或丙酮酸产生3-HP的活性3-HP发酵途径,其中所述细胞含有编码具有PPC、苹果酸脱氢酶和苹果酸脱羧酶活性的酶的一个或多个基因。在某些实施方案中,一个或多个3-HP途径基因是外源的,在这些实施方案中,基因可来源于酵母、真菌、细菌、植物、昆虫或哺乳动物来源。

[0013] 在某些实施方案中,本文提供了经遗传修饰的酵母细胞,其包含由PEP或丙酮酸产生3-HP的活性3-HP发酵途径,其中所述细胞含有编码具有PPC、2-酮酸脱羧酶、KGD、BCKA、吡哆丙酮酸脱羧酶、3-HPDH、HIBADH或4-羟基丁酸脱氢酶活性的酶的一个或多个基因。在某些实施方案中,一个或多个3-HP途径基因是外源的,在这些实施方案中,基因可来源于酵母、真菌、细菌、植物、昆虫或哺乳动物来源。

[0014] 在某些实施方案中,本文提供了经遗传修饰的酵母细胞,其包含由PEP或丙酮酸产生3-HP的活性3-HP发酵途径,其中所述细胞含有编码具有PPC、OAA甲酸裂合酶、丙二酰-CoA还原酶、Co-A酰化丙二酸半醛脱氢酶、3-HPDH、HIBADH和4-羟基丁酸脱氢酶活性的酶的一个或多个基因。在某些实施方案中,一个或多个3-HP途径基因是外源的,在这些实施方案中,基因可来源于酵母、真菌、细菌、植物、昆虫或哺乳动物来源。

[0015] 在某些实施方案中,本文提供了经遗传修饰的酵母细胞,其包含由丙酮酸产生3-HP的活性3-HP发酵途径,其中所述细胞含有编码具有PDH、酰基-CoA羧化酶、丙二酰-CoA还原酶、CoA酰化丙二酸半醛脱氢酶、3-HPDH、HIBADH和4-羟基丁酸脱氢酶活性的酶的一个或多个基因。在某些实施方案中,一个或多个3-HP途径基因是外源的,在这些实施方案中,基因可来源于酵母、真菌、细菌、植物、昆虫或哺乳动物来源。

[0016] 在某些实施方案中,本文提供了经遗传修饰的酵母细胞,其包含由丙酮酸产生3-HP的活性3-HP发酵途径,其中所述细胞含有编码具有丙氨酸脱氢酶、丙酮酸/丙氨酸氨基转移酶、丙氨酸2,3氨基变位酶、CoA转移酶、CoA合成酶、 β -丙氨酰-CoA氨解酶、3-HP-CoA脱水酶、3-HP-CoA水解酶、3-羟基异丁酰-CoA水解酶、BAAT、3-HPDH、HIBADH和4-羟基丁酸脱氢酶活性的酶的一个或多个基因。在某些实施方案中,一个或多个3-HP途径基因是外源的,在这些实施方案中,基因可来源于酵母、真菌、细菌、植物、昆虫或哺乳动物来源。

[0017] 在某些实施方案中,本文提供了经遗传修饰的酵母细胞,其包含由丙酮酸产生3-HP的活性3-HP发酵途径,其中所述细胞含有编码具有LDH、CoA转移酶、乳酰-CoA脱水酶、3-HP-CoA脱水酶、3-HP-CoA水解酶和3-羟基异丁酰-CoA水解酶活性的酶的一个或多个基因。在某些实施方案中,一个或多个3-HP途径基因是外源的,在这些实施方案中,基因可来源于酵母、真菌、细菌、植物、昆虫或哺乳动物来源。

[0018] 在某些实施方案中,本文提供了经遗传修饰的酵母细胞,其包含由PEP或丙酮酸产生3-HP的活性3-HP发酵途径,其中所述细胞含有编码具有甘油脱水酶和醛脱氢酶活性的酶的一个或多个基因。在某些实施方案中,一个或多个3-HP途径基因是外源的,在这些实施方案中,基因可来源于酵母、真菌、细菌、植物、昆虫或哺乳动物来源。

[0019] 本文提供的经遗传修饰的酵母细胞可以是任何酵母菌种。在某些实施方案中,细胞是Crabtree阴性的,在这些实施方案的某些中,其属于伊萨酵母属(*Issatchenkia*)、假丝

酵母属 (*Candida*)、克鲁维酵母属 (*Kluyveromyces*)、毕赤酵母属 (*Pichia*)、裂殖酵母属 (*Schizosaccharomyces*)、有孢圆酵母属 (*Torulaspora*)、接合酵母属 (*Zygosaccharomyces*) 或酵母属 (*Saccharomyces*)。在这些实施方案的某些中,细胞可以属于东方伊萨酵母 *P. fermentans* 进化枝或酵母属进化枝,在这些实施方案中,其可以是东方伊萨酵母、郎比可假丝酵母 (*C. lambica*) 或博伊丁酵母 (*S. bulderi*)。在某些实施方案中,酵母细胞可以是3-HP抗性酵母细胞。3-HP抗性可以是细胞的天然特性或是细胞在引入与活性3-HP发酵途径相关的遗传修饰之前、过程中或之后或其组合经过了突变和/或选择所致。在某些实施方案中,酵母细胞可以表现出对除了3-HP、其他发酵产物或副产物和/或多种培养基组分以外的有机酸具有高于由相同菌种的野生型酵母细胞所表现出的耐受性程度。在某些实施方案中,酵母细胞已经经过了突变和/或选择,以致经突变和/或选择的细胞具有高于相同菌种野生型细胞的3-HP抗性程度。在这些实施方案的一些中,细胞在用一个或多个外源3-HP途径基因进行遗传修饰之前已经经过了突变和/或选择。在一些实施方案中,细胞经过了乳酸或3-HP存在下的选择。在一些实施方案中,选择是恒化选择。

[0020] 除了与活性3-HP发酵途径相关的修饰之外,本文提供的细胞可能含有一个或多个天然基因的缺失或破坏。例如,细胞可以含有一个或多个PDC、ADH、GAL6、CYB2A、CYB2B、GPD、GPP、ALD或PCK基因的缺失或破坏。在某些实施方案中,这些缺失或破坏可以与引入与活性3-HP发酵途径相关的一个或多个基因偶联。

[0021] 在某些实施方案中,本文提供了利用本文提供的经遗传修饰的酵母细胞,通过在至少一种碳源存在下培养细胞并从培养基中分离3-HP从而产生3-HP的方法。在这些实施方案的某些中,碳源可以选自葡萄糖、木糖、阿拉伯糖、蔗糖、果糖、纤维素、葡萄糖寡聚物和甘油的一种或多种。

[0022] 具体地,本发明涉及如下各项:

[0023] 1. 包含活性3-HP发酵途径的经遗传修饰的酵母细胞,其中细胞包含选自:

[0024] 外源PPC基因;

[0025] 外源PYC基因;

[0026] 外源AAT基因;

[0027] 外源ADC基因;

[0028] 外源BAAT或gabT基因;和

[0029] 外源3-HPDH基因

[0030] 的一种或多种外源3-HP途径基因。

[0031] 2. 项1的经遗传修饰的酵母细胞,其包含外源PYC基因。

[0032] 3. 项2的经遗传修饰的酵母细胞,其中PYC基因编码与选自SEQ ID NO:2、3、4、5、6、7和8的氨基酸序列具有至少60%,如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。

[0033] 4. 项2或3的经遗传修饰的酵母细胞,其中PYC基因包含与SEQ ID NO:1的核苷酸序列具有至少60%,如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的核苷酸序列。

[0034] 5. 项1-4任一项的经遗传修饰的酵母细胞,其包含外源AAT基因。

[0035] 6. 项5任一项的经遗传修饰的酵母细胞,其中AAT基因编码与选自SEQ ID NO:14、

15和16的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。

[0036] 7. 项5或6的经遗传修饰的酵母细胞，其中AAT基因包含与SEQ ID NO:13的核苷酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的核苷酸序列。

[0037] 8. 项1-7任一项的经遗传修饰的酵母细胞，其包含外源ADC基因。

[0038] 9. 项9的经遗传修饰的酵母细胞，其中ADC基因编码与选自SEQ ID NO:17、18、133、135、137和139的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。

[0039] 10. 项8或9的经遗传修饰的酵母细胞，其中ADC基因包含与选自SEQ ID NO:130、131、132、134、136和138的核苷酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的核苷酸序列。

[0040] 11. 项1-10任一项的经遗传修饰的酵母细胞，其包含外源BAAT基因或外源gabT基因。

[0041] 12. 项11的经遗传修饰的酵母细胞，其中外源BAAT基因或gabT基因编码与选自SEQ ID NO:20、21、22、23、和24的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。

[0042] 13. 项11或12的经遗传修饰的酵母细胞，其中BAAT基因或gabT基因包含与选自SEQ ID NO:19、140、141、和142的核苷酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的核苷酸序列。

[0043] 14. 项11-13任一项的经遗传修饰的酵母细胞，其中所述BAAT基因或gabT为也是gabT基因的BAAT基因。

[0044] 15. 项1-14任一项的经遗传修饰的酵母细胞，其包含外源3-HPDH基因。

[0045] 16. 项15的经遗传修饰的酵母细胞，其中3-HPDH基因编码与选自SEQ ID NO:26、27、28、29、30、31、32、33、34和129的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。

[0046] 17. 项15或16的经遗传修饰的酵母细胞，其中3-HPDH基因包含与选自SEQ ID NO:25、143、144和343的核苷酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的核苷酸序列。

[0047] 18. 项15-17任一项的经遗传修饰的酵母细胞，其中3-HPDH基因也是HIBADH基因。

[0048] 19. 项15-18任一项的经遗传修饰的酵母细胞，其中3-HPDH基因也是4-羟基丁酸脱氢酶基因。

[0049] 20. 项1-19任一项的经遗传修饰的酵母细胞，其包含外源PPC基因。

[0050] 21. 项20的经遗传修饰的酵母细胞，其中PPC基因编码与选自SEQ ID NO:10、11和12的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。

[0051] 22. 项20或21的经遗传修饰的酵母细胞，其中PPC基因包含与SEQ ID NO:9的核苷酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的核苷酸序列。

- [0052] 23. 项1-22任一项的经遗传修饰的酵母细胞,其中所述酵母细胞是Crabtree阴性的。
- [0053] 24. 项1-22任一项的经遗传修饰的酵母细胞,其中酵母细胞属于选自伊萨酵母属(*Issatchenkia*)、假丝酵母属(*Candida*)、克鲁维酵母属(*Kluyveromyces*)、毕赤酵母属(*Pichia*)、裂殖酵母属(*Schizosaccharomyces*)、有孢圆酵母属(*Torulaspora*)、接合酵母属(*Zygosaccharomyces*)和酵母属(*Saccharomyces*)的属。
- [0054] 25. 项24的经遗传修饰的酵母细胞,其中酵母细胞属于选自东方伊萨酵母/发酵毕赤酵母(*I.orientalis*/*P.fermentans*)进化枝和酵母属进化枝的进化枝。
- [0055] 26. 项24的经遗传修饰的酵母细胞,其中酵母细胞选自东方伊萨酵母(*I.orientalis*)、郎比可假丝酵母(*C.lambica*)和博伊丁酵母(*S.bulderi*)。
- [0056] 27. 项1-26任一项的经遗传修饰的酵母细胞,其中所述细胞进一步包含选自PDC、ADH、GAL6、CYB2A、CYB2B、GPD、GPP、ALD和PCK基因的天然基因的一个或多个缺失或破坏。
- [0057] 28. 项27的经遗传修饰的酵母细胞,其中一个或多个缺失或破坏得自一个或多个外源3-HP途径基因的插入。
- [0058] 29. 项1-28任一项的经遗传修饰的酵母细胞,其中一个或多个外源3-HP途径基因可操作连接到一个或多个外源调节元件上。
- [0059] 30. 项1-29任一项的经遗传修饰的酵母细胞,其中细胞能够在含75g/L或更多3-HP的培养基中在小于4的pH下生长。
- [0060] 31. 项1-30任一项的经遗传修饰的酵母细胞,其中细胞是3-HP抗性酵母细胞。
- [0061] 32. 项1-31任一项的经遗传修饰的酵母细胞,其中细胞已经经过了突变和/或选择,以致经突变的和/或选择的细胞相比同种的野生型细胞具有更高级别的对3-HP的抗性。
- [0062] 33. 项32的经遗传修饰的酵母细胞,其中细胞在用了一个或多个外源3-HP途径基因进行遗传修饰之前已经经过了突变和/或选择。
- [0063] 34. 项32或33的经遗传修饰的酵母细胞,其中细胞已经经过了乳酸或3-HP存在下的选择。
- [0064] 35. 项34的经遗传修饰的酵母细胞,其中选择是恒化选择。
- [0065] 36. 生产3-HP的方法,包括:
- [0066] (i) 在含至少一种碳源的培养基的存在下培养项1-35任一项的经遗传修饰的酵母细胞;和
- [0067] (ii) 从培养物中分离3-HP。
- [0068] 37. 项36的方法,其中所述碳源选自葡萄糖、木糖、阿拉伯糖、蔗糖、果糖、纤维素、葡萄糖寡聚物和甘油。
- [0069] 38. 项36或37的方法,其中培养基的pH小于5,如在约1.5至约4.5、约2.0至约4.0或约2.0至约3.5的范围中。
- [0070] 附图简述
- [0071] 图1:所选3-HP发酵途径的概要
- [0072] 图2:质粒pMIBa107
- [0073] 图3:靶向整合技术的示意性展示。
- [0074] 图4:质粒pGMEr125 (a)

- [0075] 图5:质粒pGMRr125 (b)
- [0076] 图6:质粒pGMEr121
- [0077] 图7:质粒pMhCt074
- [0078] 图8:质粒pMhCt083
- [0079] 图9:质粒pMhCt087
- [0080] 图10:质粒pMhCt075
- [0081] 图11:质粒pMhCt077
- [0082] 图12:质粒pMhCt095
- [0083] 图13:质粒pMhCt096
- [0084] 图14:质粒pMeJi310-2
- [0085] 图15:质粒pMeJi312-2
- [0086] 图16:质粒pGMEr126
- [0087] 图17:质粒pGMEr130
- [0088] 图18:质粒pGMEr137
- [0089] 图19:质粒pACN5
- [0090] 图20:质粒pACN23
- [0091] 图21:质粒pHJJ27
- [0092] 图22:质粒pACN43
- [0093] 图23:质粒pHJJ75
- [0094] 图24:质粒pHJJ76
- [0095] 图25:质粒pJLJ49
- [0096] 图26:质粒pJLJ62
- [0097] 图27:质粒pMI458
- [0098] 图28:质粒pCM208
- [0099] 图29:质粒pJY39
- [0100] 图30:质粒pMcTs64
- [0101] 图31:质粒pMcTs65
- [0102] 图32:质粒pJLJ8。

[0103] 发明详述

[0104] 下面的本发明的描述仅仅意在阐述本发明的多个实施方案。因此,所讨论的特定修饰不能视为对本发明范围的限制。对于本领域技术人员来说显而易见的是,可以在不偏离本发明范围的情况下进行多种等价替代、变化和修饰,并且应当理解这种等同的实施方案包含于本文中。

[0105] 本文中引用的所有参考文献作为整体通过提述并入。

[0106] 缩写

[0107] 3-HP,3-羟基丙酸;3-HPA,3-羟基丙醛;3-HPDH,3-羟基丙酸脱氢酶;AAM,丙氨酸2,3-氨基变位酶;AAT,天冬氨酸氨基转移酶;ACC,乙酰-CoA羧化酶;ADC,天冬氨酸1-脱羧酶;AKG, α -酮戊二酸;ALD,醛脱氢酶;BAAT, β -丙氨酸氨基转移酶;BCKA,支链 α -酮酸脱羧酶;bp,碱基对;CYB2,L-(+)-乳酸-细胞色素c氧化还原酶;CYC,异-2-细胞色素c;EMS,甲基磺酸乙

酯(ethane methyl sulfonase);ENO,烯醇酶;gabT,4-氨基丁酸氨基转移酶;GAPDH,甘油醛-3-磷酸脱氢酶;GPD,甘油3-磷酸脱氢酶;GPP,甘油-3-磷酸磷酸酶;HIBADH,3-羟基异丁酸脱氢酶;IPDA,吡啶丙酮酸脱羧酶;KGD, α -酮戊二酸脱羧酶;LDH,乳酸脱氢酶;MAE,苹果酸酶;OAA,草酰乙酸;PCK,磷酸烯醇丙酮酸羧激酶;PDC,丙酮酸脱羧酶;PDH,丙酮酸脱氢酶;PEP,磷酸烯醇丙酮酸;PGK,磷酸甘油酸激酶;PPC,磷酸烯醇丙酮酸羧化酶;PYC,丙酮酸羧化酶;RKI,核糖5-磷酸酮醇异构酶;TAL,转醛醇酶;TEF1,翻译延长因子-1;TEF2,翻译延长因子-2;TKL,转酮醇酶;XDH,木糖醇脱氢酶;XR,木糖还原酶;YP,酵母提取物/蛋白胨。

[0108] 描述

[0109] 本文中提供了用于生产3-HP的经遗传修饰的酵母细胞、制备这些酵母细胞的方法以及利用这些细胞生产3-HP的方法。本文中使用的“3-HP”包括3-羟基丙酸的盐和酸形式。

[0110] 一些3-HP发酵途径是本领域已知的(参见例如US专利号6,852,517;US专利号7,309,597;US公开号2001/0021978;US公开号2008/0199926;W002/42418和W010/031083,所有通过提述并入本文)。3-HP发酵途径经由一系列中间物运行,所述中间物可以包括磷酸烯醇丙酮酸(PEP)、丙酮酸、草酰乙酸(OAA)、天冬氨酸、 β -丙氨酸、丙二酸半醛、苹果酸、丙二酰-CoA、乙酰-CoA、丙氨酸、乳酸、乳酰-CoA、丙烯酰-CoA、甘油、3-羟基丙醛(3-HPA)、 β -丙氨酰-CoA、3-HP-CoA和甘油酸。几条已知的3-HP发酵途径的概述示于图1中。

[0111] 如本文中所公开,对一组来自多个菌种的酵母细胞进行3-HP抗性的测试。基于在含有不同浓度3-HP的培养基中的生长速率和葡萄糖消耗速率,对呈现3-HP抗性的细胞进行进一步评估。基于这些实验,鉴定出了一组用于3-HP生产的理想的宿主细胞。接着对这些宿主细胞进行遗传修饰,以含有活性3-HP发酵途径,获得在低pH条件下产生3-HP的经遗传修饰的酵母细胞。

[0112] 本文在某些实施方案中提供了经遗传修饰的酵母细胞,其具有至少一条由PEP、丙酮酸和/或甘油产生3-HP的活性3-HP发酵途径。本文中使用的具有“活性3-HP发酵途径”的酵母细胞产生催化3-HP发酵途径中的每个反应所必需的活性酶,因此当在至少一种可发酵糖存在时在发酵条件下进行培养时,能够产生可测量的产量的3-HP。具有活性3-HP发酵途径的酵母细胞包含一个或多个3-HP途径基因。本文中使用的“3-HP途径基因”是指编码参与3-HP发酵途径的酶的核苷酸序列编码区。

[0113] 在某些实施方案中,本文中提供的酵母细胞具有经由PEP或丙酮酸、OAA、天冬氨酸、 β -丙氨酸和丙二酸半醛中间物进行的活性3-HP发酵途径(参见例如US公开号2010/0021978,图1)。在这些实施方案中,酵母细胞包含一组3-HP发酵途径基因,其包括丙酮酸羧化酶(PYC)、PEP羧化酶(PPC)、天冬氨酸氨基转移酶(AAT)、天冬氨酸1-脱羧酶(ADC)、 β -丙氨酸氨基转移酶(BAAT)、氨基丁酸氨基转移酶(gabT)、3-HP脱氢酶(3-HPDH)、3-羟基异丁酸脱氢酶(HIBADH)和4-羟基异丁酸脱氢酶基因中的一个或多个。3-HP发酵途径基因还可以包括PEP羧激酶(PCK)基因,其已经修饰以产生优选催化PEP转化为OAA的多肽(天然PCK基因一般产生优选催化OAA至PEP的逆反应的多肽)。

[0114] 在某些实施方案中,本文中提供的酵母细胞具有经由PEP或丙酮酸、OAA和苹果酸中间物进行的活性3-HP发酵途径(参见例如US公开号2010/0021978,图4)。在这些实施方案中,酵母细胞包含一组3-HP发酵途径基因,其包括PPC、PYC、苹果酸脱氢酶和苹果酸脱羧酶基因中的一个或多个。3-HP发酵途径基因还可以包括PCK基因,其已经修饰以产生优选催化

PEP转化为OAA的多肽。

[0115] 在某些实施方案中,本文中提供的酵母细胞具有经由PEP或丙酮酸、OAA和丙二酸半醛中间物进行的活性3-HP发酵途径(参见例如US公开号2010/0021978,图1)。在这些实施方案中,酵母细胞包含一组3-HP发酵途径基因,其包括PPC、PYC、2-酮酸脱羧酶、 α -酮戊二酸(AKG)脱羧酶(KGD)、支链 α -酮酸脱羧酶(BCKA)、吡哆丙酮酸脱羧酶(IPDA)、3-HPDH、HIBADH和4-羟基丁酸脱氢酶基因中的一个或多个。3-HP发酵途径基因还可以包括PCK基因,其已经修饰以产生优选催化PEP转化为OAA的多肽。3-HP发酵途径基因还可以包括PDC基因和/或甲酸苄酯脱羧酶基因,其已经被修饰以编码能够催化OAA转化为丙二酸半醛的多肽。

[0116] 在某些实施方案中,本文中提供的酵母细胞具有经由PEP或丙酮酸、OAA、丙二酰-CoA和丙二酸半醛中间物进行的活性3-HP发酵途径(参见例如US公开号2010/0021978,图2)。在这些实施方案中,酵母细胞包含一组3-HP发酵途径基因,其包括PPC、PYC、OAA甲酸裂合酶、丙二酰-CoA还原酶、CoA酰化丙二酸半醛脱氢酶、3-HPDH、HIBADH和4-羟基丁酸脱氢酶基因中的一个或多个。3-HP发酵途径基因还可以包括PCK基因,其已经修饰以产生优选催化PEP转化为OAA的多肽。此外,3-HP发酵途径基因还可以包括通过修饰2-酮酸脱氢酶基因而获得的OAA脱氢酶基因,以产生催化OAA转化为丙二酰-CoA的多肽。

[0117] 在某些实施方案中,本文中提供的酵母细胞具有经由丙酮酸、乙酰-CoA、丙二酰-CoA和丙二酸半醛中间物进行的活性3-HP发酵途径,其中丙二酸半醛中间物是任选的(参见例如W002/042418,图44)。在这些实施方案中,酵母细胞包含一组3-HP发酵途径基因,其包括丙酮酸脱氢酶(PDH)、乙酰-CoA羧化酶(ACC)、丙二酰-CoA还原酶、CoA酰化丙二酸半醛脱氢酶、3-HPDH、HIBADH和4-羟基丁酸脱氢酶基因中的一个或多个。

[0118] 在某些实施方案中,本文中提供的酵母细胞具有经由丙酮酸、丙氨酸、 β -丙氨酸、 β -丙氨酰-CoA、丙烯酰-CoA、3-HP-CoA和丙二酸半醛中间物进行的活性3-HP发酵途径,其中 β -丙氨酰-CoA、丙烯酰-CoA、3-HP-CoA和丙二酸半醛中间物是任选的(β -丙氨酸能够经由丙二酸半醛中间物或经由 β -丙氨酰-CoA、丙烯酰-CoA和3-HP-CoA中间物转化为3-HP)(参见例如US专利7,309,597,图1)。在这些实施方案中,酵母细胞包含一组3-HP发酵途径基因,其包括丙氨酸脱氢酶、丙酮酸/丙氨酸氨基转移酶、丙氨酸2,3氨基变位酶、CoA转移酶、CoA合成酶、 β -丙氨酰-CoA氨解酶、3-HP-CoA脱水酶、3-HP-CoA水解酶、3-羟基异丁酰-CoA水解酶、BAAT、3-HPDH、HIBADH和4-羟基丁酸脱氢酶基因中的一个或多个。

[0119] 在某些实施方案中,本文中提供的酵母细胞具有经由丙酮酸、乳酸、乳酰-CoA、丙烯酰-CoA和3-HP-CoA中间物进行的活性3-HP发酵途径(参见例如W002/042418,图1)。在这些实施方案中,酵母细胞包含一组3-HP发酵途径基因,其包括LDH、CoA转移酶、CoA合成酶、乳酰-CoA脱水酶、3-HP-CoA脱水酶、3-HP-CoA水解酶和3-羟基异丁酰-CoA水解酶基因中的一个或多个。

[0120] 在某些实施方案中,本文中提供的酵母细胞具有经由甘油和3-HPA中间物进行的活性3-HP发酵途径(参见例如US专利6,852,517)。在这些实施方案中,酵母细胞包含一组3-HP发酵途径基因,其包括甘油脱水酶和醛脱氢酶基因中的一个或多个。

[0121] 在某些实施方案中,本文中提供的酵母细胞具有经由PEP或丙酮酸、OAA、天冬氨酸、 β -丙氨酸、 β -丙氨酰-CoA、丙烯酰-CoA、3-HP-CoA和丙氨酸中间物进行的活性3-HP发酵途径,其中OAA、天冬氨酸和丙氨酸中间物是任选的(PEP或丙酮酸可以经由OAA和天冬氨酸

或经由丙氨酸转化为 β -丙氨酸) (参见W002/042418,图54;US专利7,309,597,图1)。在这些实施方案中,酵母细胞包含一组3-HP发酵途径基因,其包括PPC、PYC、AAT、ADC、CoA转移酶、CoA合成酶、 β -丙氨酰-CoA氨解酶、3-HP-CoA脱水酶、3-HP-CoA水解酶、3-羟基异丁酰-CoA水解酶、丙氨酸脱氢酶、丙酮酸/丙氨酸氨基转移酶和AAM基因中的一个或多个。3-HP发酵途径基因还可以包括PCK基因,其已经修饰以产生优选催化PEP转化为OAA的多肽。

[0122] 本文中提供的酵母细胞中的3-HP发酵途径基因可以是内源的或外源的。本文中关于基因元件例如基因、启动子和终止子序列所使用的“内源的”是指基因元件存在于天然形式的特定酵母细胞的基因组中的特定位置。本文中关于基因元件使用的“外源的”是指基因元件不存在于天然形式的特定酵母细胞的基因组中的特定位置。本文中关于酵母细胞使用的“天然的”是指特定酵母菌种的野生型酵母细胞。本文中关于代谢途径使用的“天然的”是指天然酵母细胞中存在且具有活性的代谢途径。

[0123] 外源基因元件可以具有天然的或非天然的序列。具有天然序列的外源基因元件包含与天然细胞基因组中存在的基因元件相同(除了不影响功能的个体-个体突变以外)的序列(即外源基因元件与内源基因元件相同)。然而,外源元件在宿主细胞基因组中存在的位置与内源元件不同。例如,与内源PYC基因相同的外源PYC基因可以插入到酵母细胞中,产生具有非天然(增加的)数量的PYC基因拷贝。具有非天然序列的外源基因元件包含天然细胞基因组中未发现的序列。例如,来自特定菌种的外源PYC基因可以插入到另一菌种的酵母细胞中。外源基因优选以功能性方式整合到宿主细胞基因组中,这意味着其能够在宿主细胞中产生活性蛋白。然而,在某些实施方案中,外源基因可以作为载体的一部分引入细胞中,所述载体稳定保持于宿主胞质中。

[0124] 在某些实施方案中,本文中提供的酵母细胞包含一个或多个外源3-HP发酵途径基因。在某些实施方案中,本文中公开的经遗传修饰的酵母细胞包含单个外源基因。在其他实施方案中,酵母细胞包含多个外源基因。在这些实施方案中,酵母细胞可包含单个外源基因的多个拷贝和/或两个或多个不同外源基因的拷贝。包含多个外源基因的酵母细胞可包含任意数量的外源基因。例如,这些酵母细胞可包含1至20个外源基因,在某些优选的实施方案中,其可包含1至7个外源基因。外源基因的多个拷贝可整合到单个基因座中,以致其彼此相邻。或者,其可整合到宿主细胞基因组内分离的基因座中。

[0125] 在某些实施方案中,本文中提供的酵母细胞包含一个或多个内源3-HP发酵途径基因。在这些实施方案的某些中,可以对细胞进行工程改造,以过量表达这些内源基因中的一个或多个,这意味着在至少相同的条件下,经修饰的细胞使内源基因以高于天然细胞的水平表达。在这些实施方案的某些中,过量表达的内源基因可以与一个或多个外源调节元件可操作连接。例如,可以将一个或多个天然或非天然外源强效启动子引入细胞中,以致其与一个或多个内源3-HP途径基因可操作连接。

[0126] 本文中提供的酵母细胞中的3-HP发酵途径基因可以与一个或多个调节元件例如启动子或终止子可操作连接。如本文中所使用,术语“启动子”是指位于基因翻译起始密码子上游(即5')的非翻译序列(一般约1至1000碱基对(bp)内,优选约1至500bp内),其调控基因转录的起始。本文中使用的术语“终止子”是指位于基因翻译终止密码子下游(即3')的非翻译序列(一般约1至1000bp内,优选约1至500bp内,尤其是约1至100bp内),其调控基因转录的结束。如果启动子或终止子在基因组中相对于基因的位置使得启动子或终止子(视情

况而定)行使其转录调控功能,则其与基因“可操作连接”。适宜的启动子和终止子描述于例如W099/14335、W000/71738、W002/42471、W003/102201、W003/102152和W003/049525中(所有作为整体通过提述并入)。

[0127] 本文中提供的细胞中与3-HP发酵途径基因连接的调节元件可以是内源的或外源的。例如,外源3-HP发酵途径基因可以插入到酵母细胞中,使其处于内源启动子和/或终止子的转录调控下。或者,外源3-HP发酵途径基因可以与一个或多个外源调节元件连接。例如,可以将外源基因作为基因表达构建体的一部分引入细胞中,所述构建体包含一个或多个外源调节元件。在某些实施方案中,外源调节元件或至少外源调节元件的功能性部分,可包含天然序列。在其他实施方案中,外源调节元件可包含非天然序列。在这些实施方案中,外源调节元件可包含与天然调节元件具有相对较高程度的序列同一性的序列。例如,外源基因可以与天然启动子或终止子具有至少50%、至少60%、至少70%、至少80%或至少90%序列同一性的外源启动子或终止子连接。核苷酸或氨基酸序列的序列同一性百分比可以通过本领域已知的方法计算,例如利用具有缺省参数的BLAST(National Center for Biological Information(NCBI)Basic Local Alignment Search Tool)第2.2.1版软件。例如,利用具有缺省参数的BLAST第2.2.1版运算方法得到至少90%相同性分值的序列被认为具有至少90%的序列同一性。BLAST软件可以从NCBI,Bethesda,Maryland获得。

[0128] 在某些方面中,本文中提供的细胞中与3-HP发酵途径基因连接的调节元件(例如启动子)对于途径基因来说可以是外来的。对于途径基因来说是外来的调节元件是不以天然形式与基因连接的调节元件。本领域技术人员可以领会的是,对于途径基因来说是外来的调节元件可以是内源的或外源的,这取决于途径基因及其与酵母细胞的关系。在一些情况下,内源3-HP发酵途径基因与对于途径基因来说是外来的调节元件(例如启动子)可操作连接。在其他情况下,外源3-HP发酵途径基因与对于途径基因来说是外来的外源调节元件(例如启动子)可操作连接。

[0129] 在其中多个外源基因插入宿主细胞的那些实施方案中,每个外源基因可以处于不同调节元件的控制下,或者两个或多个外源基因可以处于相同调节元件的控制下。例如,当第一外源基因与第一调节元件连接时,第二外源基因也可以与第一调节元件连接,或者其可以与第二调节元件连接。第一和第二调节元件可以是相同的或享有高度的序列同一性,或者其完全无关。

[0130] 本文中提供的酵母细胞中可以与一个或多个3-HP发酵途径基因连接的启动子的示例包括但不限于PDC1、磷酸甘油酸激酶(PGK)、木糖还原酶(XR)、木糖醇脱氢酶(XDH)、L-(+)-乳酸-细胞色素c氧化还原酶(CYB2)、翻译延长因子-1(TEF1)、翻译延长因子-2(TEF2)、烯醇酶(EN01)、甘油醛-3-磷酸脱氢酶(GAPDH)和乳清苷5'-磷酸脱羧酶(URA3)基因的启动子。在这些示例中,3-HP发酵途径基因可以与PDC1、PGK、XR、XDH、CYB2、TEF1、TEF2、EN01、GAPDH或URA3基因的内源或外源启动子连接。当启动子是外源的时,其与PDC1、PGK、XR、XDH、CYB2、TEF1、TEF2、EN01、GAPDH或URA3基因的天然启动子可以是相同的或享有高度的序列同一性(即至少约80%、至少约85%、至少约90%、至少约95%或至少约99%)。

[0131] 本文中提供的酵母细胞中可以与一个或多个3-HP发酵途径基因连接的终止子的示例包括但不限于PDC1、XR、XDH、转醛醇酶(TAL)、转酮醇酶(TKL)、核糖5-磷酸酮醇异构酶(RKI)、CYB2或异-2-细胞色素c(CYC)基因或者半乳糖家族基因(特别是GAL 10终止子)的终

止子。在这些示例中,3-HP发酵途径基因可以与PDC1、XR、XDH、TAL、TKL、RKI、CYB2或CYC基因或者半乳糖家族基因的内源或外源终止子连接。当终止子是外源的时,其与PDC1、XR、XDH、TAL、TKL、RKI、CYB2或CYC基因或者半乳糖家族基因的天然终止子可以是相同的或享有高度的序列同一性(即至少约80%、至少约85%、至少约90%、至少约95%或至少约99%)。在某些实施方案中,3-HP发酵途径基因与包含宿主细胞天然的天然GAL 10基因的功能性部分或者与天然GAL 10终止子享有至少80%、至少85%、至少90%或至少95%序列同一性的序列的终止子连接。

[0132] 外源基因可以通过本领域任何已知的方法插入到酵母宿主细胞中。在优选的实施方案中,基因整合到宿主细胞基因组中。外源基因可以以靶向或随机方式整合到基因组中。在其中基因以靶向方式整合的那些实施方案中,其可以整合到特定基因的基因座,以致外源基因的整合与天然基因的缺失或破坏偶联。例如,外源3-HP途径基因的引入可以与一个或多个编码参与其他发酵产物途径的酶的基因的缺失或破坏偶联。或者,外源基因可以整合到不对应于基因的基因组部分中。

[0133] 靶向整合和/或缺失可以利用整合构建体。本文中使用的术语“构建体”是指用于转化宿主细胞的DNA序列。构建体可以是例如环状质粒或载体、环状质粒或载体的一部分(例如限制性酶消化产物)、线性质粒或载体或者利用质粒或基因组DNA作为模板制备的PCR产物。用外源构建体转化酵母细胞的方法描述于例如W099/14335、W000/71738、W002/42471、W003/102201、W003/102152和W003/049525中。整合构建体可以利用来自插入位点靶标的两条克隆的靶DNA序列进行组装。两条靶DNA序列在天然宿主基因组中可以是相邻的或非相邻的。在本文中,“非相邻的”是指DNA序列在天然基因组中不是互相紧邻的,而是由将要被缺失的区域分隔开。本文中使用的“相邻的”序列在天然基因组中直接互相紧邻。当靶向整合将与靶基因的缺失或破坏偶联时,整合构建体还可以被称为缺失构建体。在缺失构建体中,一条靶序列可包含位于靶基因启动子5'端的区域、所有或部分启动子区域、所有或部分靶基因编码序列或者其一些组合。另一条靶序列可包含位于靶基因终止子3'端的区域、所有或部分终止子区域和/或所有或部分靶基因编码序列。当靶向整合不与天然基因的缺失或破坏偶联时,靶序列的选择使得干扰序列的插入将不会破坏天然基因的表达。制备整合或缺失构建体,以致两条靶序列彼此之间定向于相同方向,如其天然存在于宿主细胞基因组中时。当利用整合或缺失构建体将外源基因引入到宿主细胞中时,将基因表达盒克隆到构建体的两条靶基因序列之间,以使外源基因能够表达。基因表达盒含有外源基因,并且可以进一步包含与外源基因可操作连接的一个或多个调节序列,例如启动子或终止子。缺失构建体还可以构建为不含基因表达盒。设计这种构建体,以在不插入外源基因的情况下使基因序列缺失或破坏。

[0134] 整合或缺失构建体可包含克隆到质粒的两条靶基因序列之间的一个或多个选择标志物盒。选择标志物盒含有使得能够进行转化子选择的至少一个选择标志物基因。“选择标志物基因”是编码经转化的细胞在选择培养基中存活和/或生长所需蛋白的基因,因此能够用于对细胞施加选择压力。成功的转化子将含有选择标志物基因,其给予成功转化的细胞提供选择基础的至少一种特性。通常的选择标志物基因编码下列蛋白:(a)赋予对抗生素或其他毒素的抗性(例如对博来霉素或zeomycin(例如印度斯坦链异壁菌(*Streptoalloteichus hindustanus*) ble基因)、氨基糖苷类例如G418或卡那霉素(例如来

自转座子Tn903的卡那霉素抗性基因)或潮霉素(例如来自大肠杆菌的氨基糖苷类抗生素抗性基因)的抗性), (b) 补充细胞的营养缺陷型缺陷(例如亮氨酸(例如马克斯克鲁维酵母(*K.marxianus*) LEU2基因)、尿嘧啶(例如马克斯克鲁维酵母、酿酒酵母或东方伊萨酵母URA3基因)或色氨酸(例如马克斯克鲁维酵母、酿酒酵母或东方伊萨酵母TRP基因)的缺陷), (c) 能够使细胞合成从简易培养基中不可获得的关键营养成分, 或(d) 赋予细胞在特定碳源上生长的能力(例如来自酿酒酵母的MEL5基因, 其编码 α -半乳糖苷酶(蜜二糖酶)并赋予在蜜二糖作为唯一碳源上生长的能力)。优选的选择标志物包括URA3基因、zeocin抗性基因、G418抗性基因、MEL5基因和潮霉素抗性基因。另一种优选的选择标志物是L-乳酸:亚铁细胞色素c氧化还原酶(CYB2)基因盒, 前提是宿主细胞天然地缺少这种基因或其天然的CYB2基因首先被缺失或破坏。选择标志物与在宿主细胞中可操作的一个或多个启动子和/或终止子序列可操作连接。在某些实施方案中, 这些启动子和/或终止子序列是包含于选择标志物盒中的外源启动子和/或终止子序列。适宜的启动子和终止子如本文中所描述。

[0135] 整合或缺失构建体用于转化宿主细胞。可以利用例如电穿孔和/或化学转化(例如基于氯化钙、醋酸锂等)方法完成转化。可以基于选择标志物的存在或缺失进行选择或筛选, 以鉴定成功的转化子。在成功的转化子中, 在靶位点的基因座的同源重组事件导致靶位点序列的破坏或缺失。当构建体靶向天然基因以供缺失或破坏时, 可以在该重组事件过程中缺失所有或部分天然靶基因、其启动子和/或其终止子。将整合构建体中的表达盒、选择标志物盒和靶序列之间的任何其他基因物质插入到宿主基因组靶序列的对应基因座中。可以进行PCR分析或Southern分析, 以确认发生了期望的插入/缺失。

[0136] 在一些实施方案中, 可以利用来自两个或多个构建体、PCR产物或其组合的DNA进行细胞转化, 而非利用单个构建体或PCR产物。在这些实施方案中, 一个整合片段的3'末端与另一个整合片段的5'末端重叠。在一个实例中, 一个构建体将含有来自靶序列基因座的第一序列和标志物基因盒的非功能性部分, 而另一个将含有来自靶序列基因座的第二序列和标志物基因盒的第二非功能性部分。选择标志物基因盒的部分, 以致其能够组合, 以形成完整的盒。同时用这些碎片转化细胞, 使得形成完整的功能性标志物或结构性基因盒。可以基于由选择标志物给予的特性选择成功的转化子。在另一个实施例中, 选择标志物存在于一个片段上, 而靶序列在另外的片段上, 以使整合片段非常有可能在目标位点整合。在其他实施方案中, 可以利用来自三条线性DNAs的转化整合外源基因物质。在这些实施方案中, 一个片段在5'末端与第二片段并且在3'末端与第三片段重叠。

[0137] 可以设计整合或缺失构建体, 以致选择标志物基因和其调节元件的一些或所有能够由于随后的同源重组事件而自发缺失。完成此的便利方法是设计构建体使得选择标志物基因和/或调节元件的侧翼为重复序列。重复序列是相同的DNA序列, 其对于宿主细胞是天然或非天然的, 并且在构建体上以对于彼此相同或相反的方向取向。重复序列的长度有利地为50至1500bp, 并且无需编码任何物质。包含重复序列允许发生同源重组, 这导致选择标志物基因和一条重复序列的缺失。由于同源重组发生的频率相对较低, 使转化子在非选择培养基上生长数轮, 以使一些细胞中发生自发性同源重组是必需的。已经自发缺失选择标志物基因的细胞可以基于其由选择标志物基因所给予的选择特性的丧失而进行选择或筛选。在某些情况下, 重组酶的表达可以增强重复位点之间的重组。

[0138] 本文中提供的经修饰的酵母细胞中的外源3-HP发酵途径基因可来源于来自任何

适宜来源的来源基因。例如,外源基因可来源于酵母、真菌、细菌、植物、昆虫或哺乳动物来源。如本文中所使用,“来源于”天然来源基因的外源基因编码以下多肽:1)其与由天然基因所编码的多肽相同,2)其与由天然基因所编码的多肽享有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%的序列同一性和/或3)在3-HP发酵途径中与由天然基因所编码的多肽具有相同的功能。例如,来源于东方伊萨酵母PYC基因的PYC基因可以编码包含SEQ ID NO:2的氨基酸序列的多肽,与SEQ ID NO:2的氨基酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的多肽和/或能够催化丙酮酸转化OAA的多肽。来源于天然基因的基因可包含与天然基因的编码区具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的核苷酸序列。在某些实施方案中,来源于天然基因的基因可包含与来源基因的编码区相同的核苷酸序列。例如,来源于东方伊萨酵母PYC基因的PYC基因可包含SEQ ID NO:1的核苷酸序列或与SEQ ID NO:1的核苷酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的核苷酸序列。

[0139] 在本文中提供的经修饰的酵母细胞的某些实施方案中,外源3-HP发酵途径基因源自的天然来源基因产生参与3-HP发酵途径的多肽。然而在其他实施方案中,天然来源基因可以编码不参与3-HP发酵途径或催化3-HP发酵途径中的逆反应的多肽。在这些实施方案中,外源3-HP途径基因相对于天然来源基因将具有已经经过了一个或多个靶向或随机的突变,这引起修饰的活性和/或底物偏好。例如,可以使天然来源基因突变,以产生编码在3-HP发酵途径中期望的反应方向中活性增加和/或非期望的方向中活性降低的多肽的基因。例如,当天然来源基因编码能够同时催化3-HP发酵途径中的正向和逆向反应的多肽时,可以对基因进行修饰,以致所得外源基因在正向反应中活性增加并且在逆向反应中活性降低。类似地,可以使天然来源基因突变,以产生编码与天然多肽具有不同底物偏好的多肽的基因。例如,可以使3-HP途径基因突变,以产生能够作用于天然多肽不偏好或根本不起作用的底物的多肽。在这些实施方案中,由外源3-HP途径基因所编码的多肽可以催化由天然来源基因所编码的多肽完全不能催化的反应。还可以使天然来源基因突变,以致所得3-HP途径基因在一种或多种下游3-HP途径中间物或副产物的存在下在DNA、RNA或蛋白水平上呈现为反馈抑制减少。

[0140] 在本文中提供的经修饰的酵母细胞的某些实施方案中,外源3-HP途径基因可来源于宿主酵母菌种。例如,当宿主细胞是东方伊萨酵母时,外源基因可来源于东方伊萨酵母基因。在这些实施方案中,外源基因可包含与天然基因的编码区相同的核苷酸序列,以致如果处于与野生型细胞中驱动基因表达的启动子不同的启动子的控制下,外源基因掺入到宿主细胞中使得天然基因序列的拷贝数增加和/或基因的调节或表达水平发生改变。在其他实施方案中,外源3-HP途径基因可包含与天然3-HP途径基因的编码区不同的核苷酸序列,然而即使如此仍然编码与由天然3-HP途径基因所编码的多肽相同的多肽。仍在其他实施方案中,外源3-HP途径基因可包含编码与由一个或多个天然3-HP途径基因所编码的多肽具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的多肽的核苷酸序列。在这些实施方案的某些中,外源基因包含与一个或多个天然基因具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少

95%、至少97%或至少99%序列同一性的核苷酸序列。仍在其他实施方案中,外源3-HP基因可以编码与由天然3-HP途径基因所编码的多肽具有少于50%的序列同一性然而即使如此仍然与3-HP发酵途径中的天然多肽具有相同功能(即能够催化相同的反应)的多肽。如果需要,天然来源基因可以经过突变,以提供以通用真核细胞起始密码子(ATG)起始的编码序列,或出于其他目的。

[0141] 在其他实施方案中,外源3-HP途径基因可来源于与宿主酵母细胞菌种不同的菌种。在这些实施方案的某些中,外源3-HP途径基因可来源于与宿主细胞不同的酵母菌种。例如,当宿主细胞是东方伊萨酵母时,外源基因可来源于酿酒酵母。在其他实施方案中,外源3-HP途径基因可来源于真菌、细菌、植物、昆虫或哺乳动物来源。例如,当宿主细胞是东方伊萨酵母时,外源基因可来源于细菌来源,例如大肠杆菌。在外源3-HP途径基因来源于非酵母来源的那些实施方案中,外源基因序列可以经密码子优化,以在酵母宿主细胞中表达。

[0142] 在外源3-HP途径基因来源于宿主细胞菌种以外的其他菌种的那些实施方案中,外源基因可以编码与由来源生物的天然3-HP途径基因所编码的多肽相同的多肽。在这些实施方案的某些中,外源3-HP途径基因可以与来自来源生物的天然3-HP途径基因是相同的。在其他实施方案中,外源基因可以与来自来源生物的天然3-HP途径基因享有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%的序列同一性。在其他实施方案中,外源3-HP途径基因可以编码与由来自来源生物的天然3-HP途径基因所编码的多肽享有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的多肽。在这些实施方案的某些中,外源基因可包含与来自来源生物的一个或多个天然3-HP途径基因具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的核苷酸序列。仍在其他实施方案中,外源3-HP基因可以编码与由来自来源生物的天然3-HP途径基因所编码的多肽具有少于50%的序列同一性然而即使如此仍然与3-HP发酵途径中来自来源生物的天然多肽具有相同功能的多肽。

[0143] 在某些实施方案中,本文中提供的酵母细胞表达一个或多个3-HP途径基因,其编码选自下组的酶:ACC(催化乙酰-CoA转化为丙二酰-CoA),丙氨酸2,3氨基变位酶(AAM,催化丙氨酸转化为 β -丙氨酸),丙氨酸脱氢酶(催化丙酮酸转化为丙氨酸),醛脱氢酶(催化3-HPA转化为3-HP),KGD(催化OAA转化为丙二酸半醛),ATT(催化OAA转化为天冬氨酸),ADC(催化天冬氨酸转化为 β -丙氨酸),BCKA(催化OAA转化为丙二酸半醛),BAAT(催化 β -丙氨酸转化为丙二酸半醛),4-氨基丁酸氨基转移酶(gabT,催化 β -丙氨酸转化为丙二酸半醛), β -丙氨酰-CoA氨解酶(催化 β -丙氨酰-CoA转化为丙烯酰-CoA),Co-A酰化丙二酸半醛脱氢酶(催化丙二酰-CoA转化为丙二酸半醛),CoA合成酶(催化 β -丙氨酸转化为 β -丙氨酰-CoA或乳酸转化为乳酰-CoA),CoA转移酶(催化 β -丙氨酸转化为 β -丙氨酰-CoA和/或乳酸转化为乳酰-CoA),甘油脱水酶(催化甘油转化为3-HPA),IPDA(催化OAA转化为丙二酸半醛),LDH(催化丙酮酸转化为乳酸),乳酰-CoA脱水酶(催化乳酰-CoA转化为丙烯酰-CoA),苹果酸脱羧酶(催化苹果酸转化为3-HP),苹果酸脱氢酶(催化OAA转化为苹果酸),丙二酰-CoA还原酶(催化丙二酰-CoA转化为丙二酸半醛或3-HP),OAA甲酸裂合酶(还被称为丙酮酸-甲酸裂合酶和酮酸甲酸裂合酶,催化OAA转化为丙二酰-CoA),OAA脱氢酶(催化OAA转化为丙二酰-CoA),PPC(催化PEP转化为OAA),丙酮酸/甘氨酸氨基转移酶(催化丙酮酸转化为甘氨酸),PYC(催化丙酮酸

转化为OAA), PDH(催化丙酮酸转化为乙酰-CoA), 2-酮酸脱羧酶(催化OAA转化为丙二酸半醛), 3-HP-CoA脱水酶(也被称为丙烯酰-CoA水合酶, 催化丙烯酰-CoA转化为3-HP-CoA), 3-HPDH(催化丙二酸半醛转化为3-HP), 3-HP-CoA水解酶(催化3-HP-CoA转化为3-HP), HIBADH(催化丙二酸半醛转化为3-HP), 3-羟基异丁酰-CoA水解酶(催化3-HP-CoA转化为3-HP)和4-羟基丁酸脱氢酶(催化丙二酸半醛转化为3-HP)。对于这些酶活性的每个, 圆括号中的目标反应可以是天然或非天然活性的结果。

[0144] 本文中使用的“丙酮酸羧化酶基因”或“PYC基因”是指编码具有丙酮酸羧化酶活性的多肽的任何基因, 意味着能够催化丙酮酸、CO₂、ATP转化为OAA、ADP和磷酸。在某些实施方案中, PYC基因可来源于酵母来源。例如, PYC基因可来源于编码SEQ ID NO:2所示氨基酸序列的东方伊萨酵母PYC基因。在其他实施方案中, 基因可以编码与SEQ ID NO:2的氨基酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的氨基酸序列。在某些实施方案中, 来源于东方伊萨酵母的PYC基因可包含SEQ ID NO:1所示的核苷酸序列或者与SEQ ID NO:1所示的核苷酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的核苷酸序列。在其他实施方案中, PYC基因可来源于细菌来源。例如, PYC基因可来源于仅利用PYC而非PPC(参见下面)进行糖回补(anaplerosis)的少数几个细菌菌种之一, 例如类球红细菌, 或者来源于同时具有PYC和PPC的细菌菌种, 例如菜豆根瘤菌。由类球红细菌和菜豆根瘤菌的PYC基因所编码的氨基酸序列分别示于SEQ ID NO:3和4。PYC基因可来源于编码SEQ ID NO:3或4的氨基酸序列的基因, 或来源于编码与SEQ ID NO:3或4的氨基酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的氨基酸序列的基因。或者, PYC基因可来源于编码不依赖于乙酰-CoA活化的酶的PYC基因, 例如编码SEQ ID NO:5(羧基转移酶亚基)或SEQ ID NO:6(生物素羧化酶亚基)所示氨基酸序列的荧光假单胞菌PYC基因、编码SEQ ID NO:7所示氨基酸序列的谷氨酸棒杆菌PYC基因, 或编码与SEQ ID NO:5、6或7的氨基酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的氨基酸序列的基因。PYC基因还可来源于编码不被天冬氨酸抑制的酶的PYC基因, 例如编码SEQ ID NO:8所示氨基酸序列的苜蓿中华根瘤菌PYC基因(Sauer FEMS Microbiol Rev 29:765 (2005)), 或者来源于编码与SEQ ID NO:8的氨基酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的氨基酸序列的基因。

[0145] 本文中使用的“PEP羧化酶基因”或“PPC基因”是指编码具有PEP羧化酶活性的多肽的任何基因, 意味着能够催化PEP和CO₂转化为OAA和磷酸。在某些实施方案中, PPC基因可来源于细菌PPC基因。例如, PPC基因可来源于大肠杆菌PPC基因, 其编码SEQ ID NO:10所示的氨基酸序列或者与SEQ ID NO:10的氨基酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的氨基酸序列。在某些实施方案中, 来源于大肠杆菌的PPC基因可包含SEQ ID NO:9所示的核苷酸序列或者与SEQ ID NO:9所示的核苷酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的核苷酸序列。在其他实施方案中, PPC基因可来源于在很多古菌(archaea)和有限数量的细菌中发现的“A”型PPC, 其不被乙酰

CoA活化并且较少被天冬氨酸抑制。例如,PPC基因可来源于编码SEQ ID NO:11所示氨基酸序列的嗜热自养甲烷杆菌PPC A基因、编码SEQ ID NO:12所示氨基酸序列的产气荚膜梭菌PPC A基因或编码与SEQ ID NO:11或12的氨基酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的氨基酸序列的基因。在这些实施方案的某些中,基因相对于天然基因已经经过了一个或多个突变,以产生具有改进的特性的酶。例如,基因可以已经进行突变,以编码对天冬氨酸反馈的抗性相对于天然多肽增加的PPC多肽。在其他实施方案中,PPC基因可来源于植物来源。

[0146] 本文中使用的“天冬氨酸氨基转移酶基因”或“AAT基因”是指编码具有天冬氨酸氨基转移酶活性的多肽的任何基因,意味着能够催化OAA转化为天冬氨酸。具有天冬氨酸氨基转移酶活性的酶被分类为EC 2.6.1.1。在某些实施方案中,AAT基因可来源于酵母来源,例如东方伊萨酵母或酿酒酵母。例如,AAT基因可来源于编码SEQ ID NO:14所示氨基酸序列的东方伊萨酵母AAT基因或编码SEQ ID NO:15所示氨基酸序列的酿酒酵母AAT2基因。在其他实施方案中,基因可以编码与SEQ ID NO:14或15的氨基酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的氨基酸序列。在某些实施方案中,来源于东方伊萨酵母的AAT基因可包含SEQ ID NO:13所示的核苷酸序列或者与SEQ ID NO:13所示的核苷酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的核苷酸序列。在其他实施方案中,AAT基因可来源于细菌来源。例如,AAT基因可来源于大肠杆菌aspC基因,其编码包含SEQ ID NO:16所示氨基酸序列的多肽。在其他实施方案中,基因可以编码与SEQ ID NO:16的氨基酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的氨基酸序列。

[0147] 本文中使用的“天冬氨酸脱羧酶”或“ADC基因”是指编码具有天冬氨酸脱羧酶活性的多肽的任何基因,意味着能够催化天冬氨酸转化为 β -丙氨酸。具有天冬氨酸脱羧酶活性的酶被分类为EC 4.1.1.11。在某些实施方案中,ADC基因可来源于细菌来源。由于活性天冬氨酸脱羧酶需要对非活性酶原进行蛋白水解加工,在这些实施方案中,应当选择能够支持由细菌ADC基因所编码的活性酶形成的酵母宿主细胞。

[0148] 在一些实施方案中,ADC基因可来源于编码SEQ ID NO:17所示氨基酸序列的阿维链霉菌panD基因。在一些实施方案中,ADC基因可以编码与SEQ ID NO:17的氨基酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的氨基酸序列。在某些实施方案中,来源于阿维链霉菌的ADC基因可包含SEQ ID NO:130、145、146或147任一项所示的核苷酸序列或者与SEQ ID NO:130、145、146或147任一项所示的核苷酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的核苷酸序列。

[0149] 在其他实施方案中,ADC基因可来源于编码SEQ ID NO:18所示氨基酸序列的丙酮丁醇梭菌panD基因。在一些实施方案中,ADC基因可以编码与SEQ ID NO:18的氨基酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的氨基酸序列。在某些实施方案中,来源于丙酮丁醇梭菌的ADC基因可包含SEQ ID NO:131所示的核苷酸序列或者与SEQ ID NO:131所示的核苷酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%

序列同一性的核苷酸序列。

[0150] 在其他实施方案中,ADC基因可来源于编码SEQ ID NO:133所示氨基酸序列的幽门螺旋杆菌ADC基因。在一些实施方案中,ADC基因可以编码与SEQ ID NO:133的氨基酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的氨基酸序列。在某些实施方案中,来源于幽门螺旋杆菌的ADC基因可包含SEQ ID NO:133所示的核苷酸序列或者与SEQ ID NO:133所示的核苷酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的核苷酸序列。

[0151] 在其他实施方案中,ADC基因可来源于编码SEQ ID NO:135所示氨基酸序列的芽孢杆菌种TS25 (*Bacillus* sp.TS25) ADC基因。在一些实施方案中,ADC基因可以编码与SEQ ID NO:135的氨基酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的氨基酸序列。在某些实施方案中,来源于芽孢杆菌种TS25的ADC基因可包含SEQ ID NO:134所示的核苷酸序列或者与SEQ ID NO:134所示的核苷酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的核苷酸序列。

[0152] 在其他实施方案中,ADC基因可来源于编码SEQ ID NO:137所示氨基酸序列的谷氨酸棒杆菌ADC基因。在一些实施方案中,ADC基因可以编码与SEQ ID NO:137的氨基酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的氨基酸序列。在某些实施方案中,来源于谷氨酸棒杆菌的ADC基因可包含SEQ ID NO:136所示的核苷酸序列或者与SEQ ID NO:136所示的核苷酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的核苷酸序列。

[0153] 在其他实施方案中,ADC基因可来源于编码SEQ ID NO:139所示氨基酸序列的地衣芽孢杆菌ADC基因。在一些实施方案中,ADC基因可以编码与SEQ ID NO:139的氨基酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的氨基酸序列。在某些实施方案中,来源于地衣芽孢杆菌的ADC基因可包含SEQ ID NO:138、148、149、150或151任一项所示的核苷酸序列或者与SEQ ID NO:138、148、149、150或151任一项所示的核苷酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的核苷酸序列。

[0154] 本文中使用的“ β -丙氨酸氨基转移酶基因”或“BAAT基因”是指编码具有 β -丙氨酸氨基转移酶活性的多肽的任何基因,意味着能够催化 β -丙氨酸转化为丙二酸半醛。具有 β -丙氨酸氨基转移酶活性的酶被分类为EC 2.6.1.19。在某些实施方案中,BAAT基因可来源于酵母来源,例如,BAAT基因可来源于pyd4基因的东方伊萨酵母同源物,其编码SEQ ID NO:20所示的氨基酸序列。在一些实施方案中,BAAT基因可以编码与SEQ ID NO:20的氨基酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的氨基酸序列。在某些实施方案中,来源于东方伊萨酵母的BAAT基因可包含SEQ ID NO:19所示的核苷酸序列或者与SEQ ID NO:19所示的核苷酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的核苷酸序列。在其他实施方案中,BAAT基因可来源于编码SEQ ID NO:21所示

氨基酸序列的克鲁维酵母pyd4基因。在一些实施方案中,BAAT基因可以编码与SEQ ID NO: 21的氨基酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的氨基酸序列。在某些实施方案中,来源于克鲁维酵母的BAAT基因可包含SEQ ID NO:142所示的核苷酸序列或者与SEQ ID NO:142所示的核苷酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的核苷酸序列。在其他实施方案中,BAAT基因可来源于细菌来源。例如,BAAT基因可来源于编码SEQ ID NO:22所示氨基酸序列的阿维链霉菌BAAT基因。在一些实施方案中,BAAT基因可以编码与SEQ ID NO:22的氨基酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的氨基酸序列。在某些实施方案中,来源于阿维链霉菌的BAAT基因可包含SEQ ID NO:140所示的核苷酸序列或者与SEQ ID NO:140所示的核苷酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的核苷酸序列。

[0155] BAAT基因还可以是“4-氨基丁酸氨基转移酶”或“gabT基因”,意味着其对4-氨基丁酸以及 β -丙氨酸具有天然活性。或者,BAAT基因可来源于对来自细菌或酵母来源的天然gabT基因的随机或定向工程改造,以编码具有BAAT活性的多肽。例如,BAAT基因可来源于编码SEQ ID NO:23所示氨基酸序列的阿维链霉菌gabT。在一些实施方案中,来源于阿维链霉菌的BAAT基因可以编码与SEQ ID NO:23的氨基酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的氨基酸序列。在其他实施方案中,BAAT基因可来源于编码SEQ ID NO:24所示氨基酸序列的酿酒酵母gabT基因UGA1。在一些实施方案中,来源于酿酒酵母的BAAT基因可以编码与SEQ ID NO:24的氨基酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的氨基酸序列。在某些实施方案中,来源于酿酒酵母的BAAT基因可包含SEQ ID NO:141所示的核苷酸序列或者与SEQ ID NO:141所示的核苷酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的核苷酸序列。

[0156] 本文中使用的“3-HP脱氢酶基因”或“3-HPDH基因”是指编码具有3-HP脱氢酶活性的多肽的任何基因,意味着能够催化丙二酸半醛转化为3-HP。具有3-HP脱氢酶活性的酶如果利用NAD(H)转助因子则被分类为EC 1.1.1.59,如果其利用NADP(H)转助因子则被分类为EC 1.1.1.298。被分类为EC 1.1.1.298的酶也可被称为丙二酸半醛还原酶。

[0157] 在某些实施方案中,3-HPDH基因可来源于酵母来源。例如,3-HPDH基因可来源于YMR226C基因的东方伊萨酵母同源物,其编码SEQ ID NO:26所示的氨基酸序列。在一些实施方案中,3-HPDH基因可以编码与SEQ ID NO:26的氨基酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的氨基酸序列。在某些实施方案中,来源于东方伊萨酵母的3-HPDH基因可包含SEQ ID NO:25所示的核苷酸序列或者与SEQ ID NO:25所示的核苷酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的核苷酸序列。在其他实施方案中,3-HPDH基因可来源于编码SEQ ID NO:129所示氨基酸序列的酿酒酵母YMR226C基因。在一些实施方案中,3-HPDH基因可以编码与SEQ ID NO:129的氨基酸序列具

有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的氨基酸序列。在某些实施方案中,来源于酿酒酵母的3-HPDH基因可包含SEQ ID NO:144所示的核苷酸序列或者与SEQ ID NO:144所示的核苷酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的核苷酸序列。

[0158] 在其他实施方案中,3-HPDH基因可来源于细菌来源。例如,3-HPDH基因可来源于编码SEQ ID NO:27所示氨基酸序列的大肠杆菌ydfG基因。在一些实施方案中,基因可以编码与SEQ ID NO:27的氨基酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的氨基酸序列。在某些实施方案中,来源于大肠杆菌的3-HPDH基因可包含SEQ ID NO:143所示的核苷酸序列或者与SEQ ID NO:143所示的核苷酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的核苷酸序列。在其他实施方案中,3-HPDH基因可来源于编码SEQ ID NO:29所示氨基酸序列的*M.sedula*丙二酸半醛还原酶基因。在一些实施方案中,3-HPDH基因可以编码与SEQ ID NO:29所示的氨基酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的氨基酸序列。在某些实施方案中,来源于*M.sedula*的3-HPDH基因可包含SEQ ID NO:343所示的核苷酸序列或者与SEQ ID NO:343所示的核苷酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的核苷酸序列。

[0159] 本文中使用的“3-羟基异丁酸脱氢酶基因”或“HIBADH基因”是指编码具有3-羟基异丁酸脱氢酶活性的多肽的任何基因,意味着能够催化3-羟基异丁酸转化为丙二酸半醛。具有3-羟基异丁酸脱氢酶活性的酶被分类为EC 1.1.1.31。一些3-羟基异丁酸脱氢酶还具有3-HPDH活性。在某些实施方案中,HIBADH基因可来源于细菌来源。例如,HIBADH基因可来源于编码SEQ ID NO:28所示氨基酸序列的粪产碱菌M3A基因,分别编码SEQ ID NO:30或SEQ ID NO:31所示氨基酸序列的恶臭假单胞菌KT2440或E23440mmsB基因,或者编码SEQ ID NO:32所示氨基酸序列的铜绿假单胞菌PA01mmsB基因。在某些实施方案中,HIBADH基因可以编码与SEQ ID NO:28、30、31或32所示的氨基酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的氨基酸序列。

[0160] 本文中使用的“4-羟基丁酸脱氢酶基因”是指编码具有4-羟基丁酸脱氢酶活性的多肽的任何基因,意味着能够催化4-羟基丁酸转化为琥珀酸半醛。具有4-羟基丁酸脱氢酶活性的酶被分类为EC 1.1.1.61。一些4-羟基丁酸脱氢酶还具有3-HPDH活性。在某些实施方案中,4-羟基丁酸脱氢酶基因可来源于细菌来源。例如,4-羟基丁酸脱氢酶基因可来源于编码SEQ ID NO:33所示氨基酸序列的*R.eutropha* H16 4hbd基因或者编码SEQ ID NO:34所示氨基酸序列的克鲁维梭菌(*C.kluyveri*) DSM 555hbd基因。在其他实施方案中,基因可以编码与SEQ ID NO:33或34所示的氨基酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的氨基酸序列。

[0161] 本文中使用的“PEP羧激酶基因”或“PCK基因”是指编码具有PEP羧激酶活性的多肽的任何基因,意味着能够催化PEP、CO₂和ADP或GDP转化为OAA和ATP或GTP,或者反之亦然。具有PEP羧激酶活性的酶被分类为EC 4.1.1.32 (利用GTP/GDP) 和EC 4.1.1.49 (利用ATP/

ADP)。在某些实施方案中,PCK基因可来源于酵母来源。在其他实施方案中,PCK基因可来源于细菌来源,并且在这些实施方案的某些中,基因可来源于其中PCK反应有利于OAA的产生而非脱羧化占主导的更常见反应形式的细菌中。例如,PCK基因可来源于编码SEQ ID NO:35所示氨基酸序列的产琥珀酸曼氏杆菌(*M.succiniciproducens*)PCK基因、编码SEQ ID NO:36所示氨基酸序列的产琥珀酸厌氧螺菌(*A.succiniciproducens*)PCK基因、编码SEQ ID NO:37所示氨基酸序列的产琥珀酸放线杆菌(*A.succinogenes*)PCK基因或者编码SEQ ID NO:38所示氨基酸序列的*R.eutropha* PCK基因。在其他实施方案中,PCK基因相对于其所来源的天然基因已经经过了一个或多个突变,以致所得基因编码优选催化PEP转化为OAA的多肽。例如,PCK基因可来源于编码SEQ ID NO:39所示氨基酸序列的大肠杆菌K12菌株PCK基因,其中已经使基因突变,以优选催化PEP转化为OAA。在其他实施方案中,PEP向OAA的转化由例如在丙酸菌(例如谢曼氏丙酸杆菌(*P.shermanii*)、伍氏醋酸杆菌(*A.woodii*))中发现的PEP羧基转磷酸酶催化,所述丙酸菌利用无机磷酸盐和二磷酸盐而非ATP/ADP或GTP/GDP。

[0162] 本文中使用的“苹果酸脱氢酶基因”是指编码具有苹果酸脱氢酶活性的多肽的任何基因,意味着能够催化OAA转化为苹果酸。在某些实施方案中,苹果酸脱氢酶基因可来源于细菌或酵母来源。

[0163] 本文中使用的“苹果酸脱羧酶基因”是指编码具有苹果酸脱羧酶活性的多肽的任何基因,意味着能够催化苹果酸转化为3-HP。已知苹果酸脱羧酶活性不能天然发生。因此,苹果酸脱羧酶基因可以通过在编码具有乙酰乳酸脱羧酶活性的多肽的天然来源基因中掺入一个或多个突变而得到。具有乙酰乳酸脱羧酶活性的多肽催化2-羟基-2-甲基-3-氧代丁酸转化为2-乙酰丙酮(2-acetoin),其被分类为EC 4.1.1.5。在某些实施方案中,苹果酸脱羧酶基因可来源于细菌来源。例如,苹果酸脱羧酶基因可来源于编码SEQ ID NO:40所示氨基酸序列的乳酸乳球菌(*L.lactis*)aldB基因、编码SEQ ID NO:41所示氨基酸序列的嗜热链球菌(*S.thermophilus*)aldB基因、编码SEQ ID NO:42所示氨基酸序列的短芽孢杆菌(*B.brevis*)aldB基因或者编码SEQ ID NO:43所示氨基酸序列的产气肠杆菌(*E.aerogenes*)budA基因。

[0164] 本文中使用的“ α -酮戊二酸(AKG)脱羧酶基因”或“KGD基因”是指编码具有 α -酮戊二酸脱羧酶活性的多肽的任何基因,意味着能够催化 α -酮戊二酸(2-氧化戊二酸)转化为琥珀酸半醛。具有 α -酮戊二酸脱羧酶活性的酶被分类为EC 4.1.1.71。KGD基因可以用于得到编码能够催化OAA转化为丙二酸半醛的多肽的基因。这种活性可以在天然KGD基因中找到,或者其可以通过在天然KGD基因中掺入一个或多个突变而得到。在某些实施方案中,KGD基因可来源于细菌来源。例如,KGD基因可来源于编码SEQ ID NO:44所示氨基酸序列的结核分枝杆菌(*M.tuberculosis*)KGD基因、编码SEQ ID NO:45所示氨基酸序列的*B.japonicum* KGD基因或者编码SEQ ID NO:46所示氨基酸序列的*M.loti*(即*Rhizobium loti*)KGD基因。

[0165] 本文中使用的“支链 α -酮酸脱羧酶基因”或“BCKA基因”是指编码具有支链 α -酮酸脱羧酶活性的多肽的任何基因,其能够用于使长度为3至6个碳原子的一类 α -酮酸脱羧。具有BCKA活性的酶被分类为EC 4.1.1.72。BCKA基因可以用于得到编码能够催化OAA转化为丙二酸半醛的多肽的基因。这种活性可以在天然BCKA基因中找到,或者其可以通过在天然BCKA基因中掺入一个或多个突变而得到。在某些实施方案中,BCKA基因可来源于细菌来源。例如,BCKA基因可来源于编码SEQ ID NO:47所示氨基酸序列的乳酸乳球菌kdcA基因。

[0166] 本文中使用的“吡哆丙酮酸脱羧酶基因”或“IPDA基因”是指编码具有吡哆丙酮酸脱羧酶活性的多肽的任何基因,意味着能够催化吡哆丙酮酸转化为吡哆乙醛。具有IPDA活性的酶被分类为EC 4.1.1.74。IPDA基因可以用于得到编码能够催化OAA转化为丙二酸半醛的多肽的基因。这种活性可以在天然IPDA基因中找到,或者其可以通过在天然IPDA基因中掺入一个或多个突变而得到。在某些实施方案中,吡哆丙酮酸脱羧酶基因可来源于酵母、细菌或植物来源。

[0167] 本文中使用的“丙酮酸脱羧酶基因”或“PDC基因”是指编码具有丙酮酸脱羧酶活性的多肽的任何基因,意味着能够催化丙酮酸转化为乙醛。具有PDC活性的酶被分类为EC 4.1.1.1。在优选的实施方案中,掺入到如本文中所提供的经修饰的酵母细胞中的PDC基因相对于其所来源的天然基因已经经过了一个或多个突变,以致所得基因编码能够催化OAA转化为丙二酸半醛的多肽。在某些实施方案中,PDC基因可来源于酵母来源。例如,PDC基因可来源于编码SEQ ID NO:49所示氨基酸序列的东方伊萨酵母PDC基因、编码SEQ ID NO:50所示氨基酸序列的酿酒酵母PDC1基因或者编码SEQ ID NO:51所示氨基酸序列的乳酸克鲁维酵母(*K.lactis*)PDC。在某些实施方案中,来源于东方伊萨酵母PDC基因的PDC基因可包含SEQ ID NO:48所示的核苷酸序列或者与SEQ ID NO:48所示的核苷酸序列具有至少50%、至少60%、至少70%、至少80%、至少85%、至少90%、至少95%、至少97%或至少99%序列同一性的核苷酸序列。在其他实施方案中,PDC基因可来源于细菌来源。例如,PDC基因可来源于编码SEQ ID NO:52所示氨基酸序列的运动发酵单胞菌(*Z.mobilis*)PDC基因或者编码SEQ ID NO:53所示氨基酸序列的巴斯德醋酸杆菌(*A.Pasteurianus*)PDC基因。

[0168] 本文中使用的“甲酸苄酯脱羧酶”基因是指编码具有甲酸苄酯脱羧酶活性的多肽的任何基因,意味着能够催化甲酸苄酯转化为苯甲醛。具有甲酸苄酯脱羧酶活性的酶被分类为EC 4.1.1.7。在优选的实施方案中,掺入到如本文中所提供的经修饰的酵母细胞中的甲酸苄酯脱羧酶基因相对于其所来源的天然基因已经经过了一个或多个突变,以致所得基因编码能够催化OAA转化为丙二酸半醛的多肽。在某些实施方案中,甲酸苄酯脱羧酶基因可来源于细菌来源。例如,甲酸苄酯脱羧酶基因可来源于编码SEQ ID NO:54所示氨基酸序列的恶臭假单胞菌md1C基因、编码SEQ ID NO:55所示氨基酸序列的铜绿假单胞菌md1C基因、编码SEQ ID NO:56所示氨基酸序列的施氏假单胞菌(*P.stutzeri*)dpgB基因或者编码SEQ ID NO:57所示氨基酸序列的荧光假单胞菌ilvB-1基因。

[0169] 本文中使用的“OAA甲酸裂合酶”是指编码具有OAA甲酸裂合酶活性的多肽的任何基因,意味着能够催化酰化酮酸转化为其相应的CoA衍生物。由OAA甲酸裂合酶基因所编码的多肽可以对丙酮酸或对另一种酮酸具有活性。在某些实施方案中,OAA甲酸裂合酶基因编码将OAA转化为丙二酰-CoA的多肽。

[0170] 本文中使用的“丙二酰-CoA还原酶基因”是指编码具有丙二酰-CoA还原酶活性的多肽的任何基因,意味着能够催化丙二酰-CoA转化为丙二酸半醛(也被称为Co-A酰化丙二酸半醛脱氢酶活性)。在某些实施方案中,丙二酰-CoA还原酶基因可来源于双功能性丙二酰-CoA还原酶基因,其还能够催化丙二酸半醛转化为3-HP。在这些实施方案的某些中,丙二酰-CoA还原酶基因可来源于细菌来源。例如,丙二酰-CoA还原酶基因可来源于编码SEQ ID NO:58所示氨基酸序列的橙色绿屈挠菌(*C.aurantiacus*)丙二酰-CoA还原酶基因、编码SEQ ID NO:59所示氨基酸序列的R.castenholzii丙二酰-CoA还原酶基因或者编码SEQ ID NO:

60所示氨基酸序列的赤杆菌属NAP1 (*Erythrobacter* sp.NAP1) 丙二酰-CoA还原酶基因。在其他实施方案中,丙二酰-CoA还原酶基因可来源于编码仅催化丙二酰-CoA转化为丙二酸半醛的多肽的丙二酰-CoA还原酶基因。例如,丙二酰-CoA还原酶基因可来源于编码SEQ ID NO:61所示氨基酸序列的*M.sedula* Msed_0709基因或者编码SEQ ID NO:62所示氨基酸序列的硫化叶菌(*S.tokodaii*) 丙二酰-CoA还原酶。

[0171] 本文中使用的“丙酮酸脱氢酶基因”或“PDH基因”是指编码具有丙酮酸脱氢酶活性的多肽的任何基因,意味着能够催化丙酮酸转化为乙酰-CoA。在某些实施方案中,PDH基因可来源于酵母来源。例如,PDH基因可来源于分别编码SEQ ID NO:63-66所示氨基酸序列的酿酒酵母LAT1、PDA1、PDB1或LPD基因。在其他实施方案中,PDH基因可来源于细菌来源。例如,PDH基因可来源于分别编码SEQ ID NO:67-69所示氨基酸序列的大肠杆菌K12亚株MG1655aceE、aceF或Ipd基因或者分别编码SEQ ID NO:70-73所示氨基酸序列的枯草芽孢杆菌(*B.subtilis*) pdhA、pdhB、pdhC或pdhD基因。

[0172] 本文中使用的“乙酰-CoA羧化酶基因”或“ACC基因”是指编码具有乙酰-CoA羧化酶活性的多肽的任何基因,意味着能够催化乙酰-CoA转化为丙二酰-CoA。具有乙酰-CoA羧化酶活性的酶被分类为EC 6.4.1.2。在某些实施方案中,乙酰-CoA羧化酶基因可来源于酵母来源。例如,乙酰-CoA羧化酶基因可来源于编码SEQ ID NO:74所示氨基酸序列的酿酒酵母ACC1基因。在其他实施方案中,乙酰-CoA羧化酶基因可来源于细菌来源。例如,乙酰-CoA羧化酶基因可来源于分别编码SEQ ID NO:75-78所示氨基酸序列的大肠杆菌accA、accB、accC或accD基因或者分别编码SEQ ID NO:79-82所示氨基酸序列的橙色绿屈挠菌accA、accB、accC或accD基因。

[0173] 本文中使用的“丙氨酸脱氢酶基因”是指编码具有丙氨酸脱氢酶活性的多肽的任何基因,意味着能够催化丙酮酸NAD依赖性还原胺化为丙氨酸。具有丙氨酸脱氢酶活性的酶被分类为EC 1.4.1.1。在某些实施方案中,丙氨酸脱氢酶基因可来源于细菌来源。例如,丙氨酸脱氢酶基因可来源于编码SEQ ID NO:83所示氨基酸序列的枯草芽孢杆菌丙氨酸脱氢酶基因。

[0174] 本文中使用的“丙酮酸/丙氨酸氨基转移酶基因”是指编码具有丙酮酸/丙氨酸氨基转移酶活性的多肽的任何基因,意味着能够催化丙酮酸和L-谷氨酸转化为丙氨酸和2-氧代戊二酸。在某些实施方案中,丙酮酸/丙氨酸氨基转移酶基因来源于酵母来源。例如,丙酮酸/丙氨酸氨基转移酶基因可来源于编码SEQ ID NO:84所示氨基酸序列的粟酒裂殖酵母(*S.pombe*) 丙酮酸/丙氨酸氨基转移酶基因或者编码SEQ ID NO:85所示氨基酸序列的酿酒酵母ALT2基因。

[0175] 本文中使用的“丙氨酸2,3氨基变位酶基因”或“AAM基因”是指编码具有丙氨酸2,3氨基变位酶活性的多肽的任何基因,意味着能够催化丙氨酸转化为β-丙氨酸。已知丙氨酸2,3氨基变位酶活性不能天然发生。因此,丙氨酸2,3氨基变位酶基因可以通过在编码具有类似活性例如赖氨酸2,3氨基变位酶活性(参见例如US专利7,309,597)的多肽的天然来源基因中掺入一个或多个突变而得到。在某些实施方案中,天然来源基因可以是编码SEQ ID NO:86所示氨基酸序列的枯草芽孢杆菌赖氨酸2,3氨基变位酶基因、编码SEQ ID NO:87所示氨基酸序列的牙龈卟啉单胞菌(*P.gingivalis*) 赖氨酸2,3氨基变位酶基因或者编码SEQ ID NO:88所示氨基酸序列的具核梭杆菌(*F.nucleatum*) (ATCC-10953) 赖氨酸2,3氨基变位酶基

因。

[0176] 本文中使用的“CoA转移酶基因”是指编码具有CoA转移酶活性的多肽的任何基因，所述CoA转移酶活性在一个实施例中包括催化 β -丙氨酸转化为 β -丙氨酰-CoA和/或乳酸转化为乳酰-CoA的能力。在某些实施方案中，CoA转移酶基因可来源于酵母来源。在其他实施方案中，CoA转移酶基因可来源于细菌来源。例如，CoA转移酶基因可来源于编码SEQ ID NO: 89所示氨基酸序列的埃氏巨球形菌 (*M. elsdenii*) CoA转移酶基因。

[0177] 本文中使用的“CoA合成酶基因”是指编码具有CoA合成酶活性的多肽的任何基因。在一个实例中，这包括催化 β -丙氨酸转化为 β -丙氨酰-CoA的能力。在另一个实例中，这包括催化乳酸转化为乳酰-CoA的能力。在某些实施方案中，CoA合成酶基因可来源于酵母来源。例如，CoA合成酶基因可来源于酿酒酵母CoA合成酶基因。在其他实施方案中，CoA合成酶基因可来源于细菌来源。例如，CoA合成酶基因可来源于大肠杆菌CoA合成酶、类球红细菌或肠道沙门氏菌 (*S. enterica*) CoA合成酶基因。

[0178] 本文中使用的“ β -丙氨酰-CoA氨解酶基因”是指编码具有 β -丙氨酰-CoA氨解酶活性的多肽的任何基因，意味着能够催化 β -丙氨酰-CoA转化为丙烯酰-CoA。在某些实施方案中， β -丙氨酰-CoA氨解酶基因可来源于细菌来源，例如编码SEQ ID NO: 90所示氨基酸序列的丙酸梭菌 (*C. propionicum*) β -丙氨酰-CoA氨解酶基因。

[0179] 本文中使用的“3-HP-CoA脱水酶基因”或“丙烯酰-CoA水合酶基因”是指编码具有3-HP-CoA脱水酶活性的多肽的任何基因，意味着能够催化丙烯酰-CoA转化为3-HP-CoA。具有3-HP-CoA脱水酶活性的酶被分类为EC4.2.1.116。在某些实施方案中，3-HP-CoA脱水酶基因可来源于酵母或真菌来源，例如编码SEQ ID NO: 91所示氨基酸序列的大豆疫霉根腐菌 (*P. sojae*) 3-HP-CoA脱水酶基因。在其他实施方案中，3-HP-CoA脱水酶基因可来源于细菌来源。例如，3-HP-CoA脱水酶基因可来源于编码SEQ ID NO: 92所示氨基酸序列的橙色绿屈挠菌3-HP-CoA脱水酶基因、编码SEQ ID NO: 93所示氨基酸序列的深红红螺菌 (*R. rubrum*) 3-HP-CoA脱水酶基因或者编码SEQ ID NO: 94所示氨基酸序列的*R. capsulatus* 3-HP-CoA脱水酶基因。仍在其他实施方案中，3-HP-CoA脱水酶基因可来源于哺乳动物来源。例如，3-HP-CoA脱水酶基因可来源于编码SEQ ID NO: 95所示氨基酸序列的智人 (*H. sapiens*) 3-HP-CoA脱水酶基因。

[0180] 本文中使用的“3-HP-CoA水解酶基因”是指编码具有3-HP-CoA水解酶活性的多肽的任何基因，意味着能够催化3-HP-CoA转化为3-HP。在某些实施方案中，3-HP-CoA基因可来源于酵母或真菌来源。在其他实施方案中，3-HP-CoA基因可来源于细菌或哺乳动物来源。

[0181] 本文中使用的“3-羟基异丁酰-CoA水解酶基因”是指编码具有3-羟基异丁酰-CoA水解酶活性的多肽的任何基因，所述3-羟基异丁酰-CoA水解酶活性在一个实例中包括催化3-HP-CoA转化为3-HP的能力。在某些实施方案中，3-羟基异丁酰-CoA水解酶基因可来源于细菌来源，例如编码SEQ ID NO: 96所示氨基酸序列的荧光假单胞菌3-羟基异丁酰-CoA水解酶基因或者编码SEQ ID NO: 97所示氨基酸序列的蜡状芽孢杆菌 (*B. cereus*) 3-羟基异丁酰-CoA水解酶基因。在其他实施方案中，3-羟基异丁酰-CoA水解酶基因可来源于哺乳动物来源，例如编码SEQ ID NO: 98所示氨基酸序列的智人3-羟基异丁酰-CoA水解酶基因。

[0182] 本文中使用的“乳酸脱氢酶基因”或“LDH基因”是指编码具有乳酸脱氢酶活性的多肽的任何基因，意味着能够催化丙酮酸转化为乳酸。在某些实施方案中，LDH基因可来源于

真菌、细菌或哺乳动物来源。

[0183] 本文中使用的“乳酰-CoA脱水酶基因”是指编码具有乳酰-CoA脱水酶活性的多肽的任何基因,意味着能够催化乳酰-CoA转化为丙烯酰-CoA。在某些实施方案中,乳酰-CoA脱水酶基因可来源于细菌来源。例如,乳酰-CoA脱水酶基因可来源于编码SEQ ID NO:99-101所示氨基酸序列的埃氏巨球形菌乳酰-CoA脱水酶E1、E11a或E11b亚基基因。

[0184] 本文中使用的“醛脱氢酶基因”是指编码具有醛脱氢酶活性的多肽的任何基因,所述醛脱氢酶活性在一个实例中包括催化3-HPA转化为3-HP及其相反转化的能力。在某些实施方案中,醛脱氢酶基因可来源于酵母来源,例如编码SEQ ID NO:102所示氨基酸序列的酿酒酵母醛脱氢酶基因或者编码SEQ ID NO:122、124或126所示氨基酸序列的东方伊萨酵母醛脱氢酶基因。在其他实施方案中,醛脱氢酶可来源于细菌来源,例如编码SEQ ID NO:103所示氨基酸序列的大肠杆菌aldH基因或者编码SEQ ID NO:104所示氨基酸序列的肺炎克雷伯氏菌(*K.pneumoniae*)醛脱氢酶基因。

[0185] 本文中使用的“甘油脱水酶基因”是指编码具有甘油脱水酶活性的多肽的任何基因,意味着能够催化甘油转化为3-HPA。在某些实施方案中,甘油脱水酶基因可来源于细菌来源,例如肺炎克雷伯氏菌或弗氏柠檬酸杆菌(*C.freundii*)甘油脱水酶基因。

[0186] 在某些实施方案中,本文中提供的经遗传修饰的酵母细胞进一步包含一个或多个天然基因的缺失或破坏。关于天然基因的“缺失或破坏”是指或者基因的整个编码区被去除(缺失),或者基因的编码区、启动子和/或其终止子区域被修饰(例如通过缺失、插入或突变),以致基因不再产生活性酶,产生显著减少的量(至少减少75%,优选至少减少90%)的活性酶,或产生活性显著降低(至少降低75%,优选至少降低90%)的酶。

[0187] 在某些实施方案中,一个或多个天然基因的缺失或破坏导致一个或多个天然代谢途径的缺失或破坏。关于代谢途径的“缺失或破坏”是指该途径或者无法运行,或者表现出相对于天然途径降低至少75%、至少85%或至少95%的活性。在某些实施方案中,天然代谢途径的缺失或破坏通过掺入导致一个或多个天然基因的表达减少的一个或多个遗传修饰而达到,所述表达减少使得3-HP的产生减少。

[0188] 在某些实施方案中,天然基因的缺失或破坏可以通过强制进化(forced evolution)、诱变或基因工程改造方法,随后进行适宜的选择或筛选以鉴定期望突变体而达到。在某些实施方案中,天然宿主细胞基因的缺失或破坏可以与在宿主细胞中掺入一个或多个外源基因偶联,即可以利用也是缺失构建体的基因表达整合构建体来掺入外源基因。在其他实施方案中,缺失或破坏可以利用不含外源基因的缺失构建体或者通过本领域已知的其他方法而达到。

[0189] 在某些实施方案中,本文中提供的经遗传修饰的酵母细胞包含一个或多个天然基因的缺失或破坏,所述天然基因编码参与乙醇发酵的酶,包括例如丙酮酸脱羧酶(PDC,将丙酮酸转化为乙醛)和/或乙醇脱氢酶(ADH,将乙醛转化为乙醇)基因。这些修饰降低了酵母细胞生产乙醇的能力,从而使3-HP的生产最大化。然而,在某些实施方案中,可以对本文中提供的经遗传修饰的酵母细胞进行工程改造,以同时产生3-HP和乙醇。在那些实施方案中,编码参与乙醇发酵的酶的天然基因优选不被缺失或破坏,并且在某些实施方案中,酵母细胞可包含使乙醇生产增加的一个或多个外源基因。

[0190] 在某些实施方案中,本文中提供的经遗传修饰的酵母细胞包含一个或多个天然基

因的缺失或破坏,所述天然基因编码参与产生另外的发酵产物例如甘油或其他副产物例如乙酸盐或二醇的酶。例如,本文中提供的细胞可包含一个或多个以下基因的缺失或破坏:甘油3-磷酸脱氢酶(GPD,催化二羟基丙酮至甘油3-磷酸的反应)、甘油3-磷酸酶(GPP,催化甘油3-磷酸转化为甘油)、甘油激酶(催化甘油3-磷酸酶转化为甘油)、二羟基丙酮激酶(催化二羟基丙酮磷酸转化为二羟基丙酮)、甘油脱氢酶(催化二羟基丙酮转化为甘油)、醛脱氢酶(ALD,例如,将乙醛转化为乙酸或将3-HP转化为3-HPA)或丁二醇脱氢酶(催化丁二醇转化为乙偶姻和相反转化)基因。

[0191] 在某些实施方案中,本文中提供的经遗传修饰的酵母细胞包含一个或多个天然基因的缺失或破坏,所述天然基因编码催化3-HP发酵途径中的逆反应的酶,包括例如PEP羧激酶(PCK)、具有OAA脱羧酶活性的酶或CYB2A或CYB2B(催化乳酸转化为丙酮酸)。PCK催化PEP转化为OAA和相反转化,但是表现为优选OAA至OPP的反应。为了减少OAA至PEP的转化,一个或多个拷贝的天然PCK基因可以被缺失或破坏。在某些实施方案中,其中一个或多个天然PCK基因已被缺失或破坏的酵母细胞可以表达一个或多个外源PCK基因,所述外源PCK基因已经进行突变,以编码有利于PEP转化为OAA的多肽。OAA脱羧酶催化OAA转化为丙酮酸。已经鉴定出了具有OAA脱羧酶活性的酶,例如大肠杆菌中由eda基因所编码的酶以及酵母和真菌中的苹果酸酶(MAE)。为了降低OAA脱羧酶活性,编码具有OAA脱羧酶活性的酶的一个或多个拷贝的天然基因可以被缺失或破坏。在某些实施方案中,其中一个或多个天然OAA脱羧化基因已被缺失或破坏的酵母细胞可以表达一个或多个外源OAA脱羧化基因,所述外源OAA脱羧化基因已经进行突变,以编码催化丙酮酸转化为OAA的多肽。

[0192] 在某些实施方案中,本文中提供的经遗传修饰的酵母细胞包含一个或多个天然基因的缺失或破坏,所述天然基因编码参与利用3-HP发酵途径产物或中间物的不期望反应的酶。这些基因的示例包括编码将3HP转化为3HP醛的酶的那些,所述3HP醛已知对细菌细胞是有毒性的。

[0193] 在某些实施方案中,本文中提供的经遗传修饰的酵母细胞包含一个或多个天然基因的缺失或破坏,所述天然基因编码对3-HP发酵途径具有中性作用的酶,包括例如GAL6(将半乳糖转化为葡萄糖的GAL系统的负性调节物)。中性基因的缺失或破坏使得能够在不影响天然发酵途径的情况下插入一个或多个外源基因。

[0194] 在某些实施方案中,本文中提供的酵母细胞是3-HP抗性酵母细胞。本文中使用的“3-HP抗性酵母细胞”是指在含有75g/L或更多3-HP的培养基中在小于4.0的pH下呈现至少2.5g/L/hr的平均糖酵解速率的酵母细胞。这些速率和条件代表生产3-HP的经济的过程。在这些实施方案的某些中,酵母细胞可以以其天然形式呈现3-HP抗性。在其他实施方案中,细胞可以在引入与活性3-HP发酵途径相关的遗传修饰之前、过程中或之后已经经过了突变和/或选择(例如恒化选择或重复的系列亚培养),以致经突变和/或选择的细胞具有高于相同菌种野生型细胞的3-HP抗性程度。例如,在一些实施方案中,细胞在用一个或多个外源3-HP途径基因进行遗传修饰之前已经经过了3-HP或乳酸存在下的突变和/或选择。在某些实施方案中,可以对以其天然形式表现出3-HP抗性的细胞进行突变和/或选择。可以在不同水平的3-HP存在下对已经经过了突变和/或选择的细胞进行糖消耗和其他特性的测试,以确定其作为3-HP生产的工业宿主的潜力。除了3-HP抗性,本文中提供的酵母细胞可以已经经过了对一种或多种额外的有机酸(例如乳酸)或者对其他发酵产物、副产物或培养基组分的

抗性的突变和/或选择。

[0195] 可以利用本领域公知的方法进行选择,例如对3-HP或其他化合物的抗性的选择。例如,如上面所提及,选择可以是恒化选择。恒化选择利用使得能够持续培养微生物(例如酵母)的恒化器,其中特定的生长速率和细胞数量可以被独立地控制。持续培养是基本上恒定容积的流动系统,其中持续加入培养基,任何溢出可以持续移除。一旦这种系统处于平衡,细胞数量和营养成分状态保持恒定,并且系统处于稳定状态。恒化器使得能够通过稀释速率和限制性营养成分例如碳源或氮源的浓度变化来调控培养物的群体密度和特定生长速率。通过随着培养物生长而改变条件(例如,降低接种菌株生长所必需的第二碳源的浓度,以及其它),群体中能够在改变的条件下生长较快的微生物将被选择,并且其生长将超过在新条件下表现不佳的微生物。通常这种选择需要在恒化培养物的生长过程中不断增加或减少至少一种培养物组分。恒化器的操作及其在微生物定向进化中的用途是本领域公知的(参见例如Novick Proc Natl Acad Sci USA 36:708-719(1950),Harder J Appl Bacteriol 43:1-24(1977))。其他选择方法包括但不限于例如U.S. 专利号7,629,162中所描述的在选择性条件下重复的系列亚培养。这种方法可以用于替代或附加于上面所描述的利用葡萄糖限制性恒化方法。

[0196] 在如实施例中所公开的3-HP培养基中呈现生长和葡萄糖消耗的最佳组合的酵母菌株是用于与3-HP发酵途径相关的多种遗传修饰的优选宿主细胞。如通过在75g/L或更高3-HP的存在下在pH小于4下的生长所显示,具有相对较高程度的3-HP抗性的有潜力的酵母属包括例如假丝酵母属(*Candida*)、克鲁维酵母属(*Kluyveromyces*)、伊萨酵母属(*Issatchenkia*)、酵母属(*Saccharomyces*)、毕赤酵母属(*Pichia*)、裂殖酵母属(*Schizosaccharomyces*)、有孢圆酵母属(*Torulaspora*)和接合酵母属(*Zygosaccharomyces*)。呈现3-HP抗性的菌种包括东方伊萨酵母(也被称为克鲁斯假丝酵母(*C.krusei*))、郎比可假丝酵母(*C.lambica*) (也被称为发酵毕赤酵母(*Pichia fermentans*))和博伊丁酵母(*S.bulderi*) (也被称为Kazachstania bulderi)。东方伊萨酵母和郎比可假丝酵母来自东方伊萨酵母/发酵毕赤酵母进化枝,而博伊丁酵母来自酵母属进化枝。呈现3-HP抗性的特定菌株包括东方伊萨酵母株24210、PTA-6658、60585和CD1822,博伊丁酵母株MYA-402和MYA-404,以及郎比可假丝酵母株ATCC 38617。

[0197] 其他野生型酵母或真菌可以相同方式测试并进行鉴定,以在如本文中所描述的高水平3-HP存在下具有可接受的生长和葡萄糖利用水平。例如,Gross和Robbins (Hydrobiologia 433(103):91-109)已经汇编了在低pH(<4)的环境中鉴定的81种真菌菌种的一份名单,其能够作为潜力生产宿主进行相关测试。

[0198] 在某些实施方案中,本文中提供的经修饰的酵母细胞通过将一个或多个遗传修饰掺入Crabtree阴性宿主酵母细胞中而制备。在这些实施方案的某些中,宿主酵母细胞属于伊萨酵母属、假丝酵母属或酵母属,在这些实施方案的某些中,宿主酵母细胞属于东方伊萨酵母/发酵毕赤酵母或酵母进化枝。在某些实施方案中,宿主细胞是东方伊萨酵母或郎比可假丝酵母或博伊丁酵母。

[0199] 东方伊萨酵母/发酵毕赤酵母进化枝是含有至少东方伊萨酵母、盔形毕赤酵母(*P.galeiformis*)、毕赤酵母菌种YB-4149(NRRL命名)、嗜酒假丝酵母(*C.ethanolica*)、(*P.deserticola*)、膜璞毕赤酵母(*P.membranifaciens*)和发酵毕赤酵母的最末端的进化

枝。通过对酵母菌种26S核糖体DNA可变D1/D2结构域的分析,鉴定东方伊萨酵母/发酵毕赤酵母进化枝的成员,其中利用由Kurtzman和Robnett在通过提述并入通过提述并入本文的“Identification and Phylogeny of Ascomycetous Yeasts from Analysis of Nuclear Large Subunit (26S) Ribosomal DNA Partial Sequences,”Antonie van Leeuwenhoek 73:331-371,1998中所描述的方法(具体参见第349页)。对成百上千子囊菌26S核糖体DNA可变D1/D2结构域的分析已经揭示,东方伊萨酵母/发酵毕赤酵母进化枝含有非常密切相关的菌种。东方伊萨酵母/发酵毕赤酵母进化枝的成员表现为与进化枝其他成员在26S核糖体DNA可变D1/D2结构域中的相似性高于进化枝外酵母菌种。因此,东方伊萨酵母/发酵毕赤酵母进化枝的其他成员可以通过利用Kurtzman和Robnett的方法比较其相应核糖体DNA的D1/D2结构域并与进化枝其他成员和进化枝外密切相关的菌种作对比而进行鉴定。

[0200] 在某些实施方案中,本文中提供的经修饰的酵母细胞属于伊萨酵母属,在这些实施方案的某些中,酵母细胞是东方伊萨酵母。当最初鉴定时,东方伊萨酵母菌种被命名为库德毕赤酵母(*Pichia kudriavzevii*)。无性型(无性生殖形式)的东方伊萨酵母被称为克鲁斯假丝酵母。东方伊萨酵母菌种的大量其它同义词已经列于别处(Kurtzman and Fell, *The Yeasts, a Taxonomic Study*. Section 35. *Issatchenkia kudryavtsev*, pp 222-223 (1998))。

[0201] 生产3-HP的理想的酵母细胞理想地能够在低pH水平下生长。在低pH下进行发酵的能力降低了下游回收的成本,使得生产更经济。因此,在某些实施方案中,酵母宿主细胞能够在低pH水平下生长(例如在小于7、6、5、4或3的水平下)。

[0202] 除了3-HP抗性和/或低pH生长能力之外,适宜的宿主细胞可以具有一个或多个有利的特性。例如,可以进一步基于糖酵解速率、特定生长速率、热耐受性、对生物质水解产物抑制物的耐受性、过程的总体鲁棒性(robustness)等等对呈现3-HP抗性的潜在宿主细胞进行选择。可以在与3-HP发酵途径相关的任何遗传修饰前对这些标准进行评估,或者在一个或多个这种修饰已经发生后对其进行评估。

[0203] 由于大多数酵母是乙醇的天然生产者,因此将催化由丙酮酸产生乙醇中第一个步骤的酶(PDC)去除或者显著减少,对于产生足够的替代性产物来说是必需的。在Crabtree阳性酵母例如酵母属中,PDC基因的缺失或破坏使得宿主获得对二碳化合物例如乙醇或乙酸盐的营养缺陷型,并且不能在含有葡萄糖的培养基中生长。可以利用乙酸盐非依赖性和葡萄糖耐受性的进行性选择获得能够克服这些局限的突变体(参见例如van Maris *Appl Environ Microbiol* 70:159 (2004))。因此,在某些实施方案中,优选的酵母宿主细胞是Crabtree阴性的酵母细胞,其中PDC缺失菌株能够在葡萄糖上生长,并且保持C2原养型。

[0204] 基因表达水平和/或给定细胞中将要利用的外源基因数目将根据所选择的酵母菌种而变化。对于经全基因组测序的酵母,可以利用全基因组化学计量模型来确定应当表达哪种酶以开发期望的3-HP发酵途径。全基因组化学计量模型量描述于例如Hjersted等,“Genome-scale analysis of *Saccharomyces cerevisiae* metabolism and ethanol production in fed-batch culture,”*Biotechnol. Bioeng.* 2007;和Famili等,“*Saccharomyces cerevisiae* phenotypes can be predicted by using constraint-based analysis of a genome-scale reconstructed metabolic network,”*Proc. Natl. Acad. Sci.* 2003, 100 (23):13134-9。

[0205] 对于不具有已知的基因组序列酵母,通常可以利用例如下面实施例2A中描述的技术获得目标基因的序列(作为过量表达的候选物或者作为插入位点)。可以进行常规的实验设计,以测试多种基因的表达和多种酶的活性,包括在3-HP途径中发挥功能的基因和酶。可进行实验,其中每种酶在酵母中独立表达,和形成酶区块,所述酶区块直至和优选包括所有途径酶,以确立何者为改进的3-HP产生所需(或所期望)。一种说明性实验设计测试每种单独的酶以及每个独特酶对的表达,并且能够进一步测试所有所需酶或者每个独特酶组合的表达。将要领会的是,可以采取多种方法。

[0206] 在某些实施方案中,提供了由如本文中所提供的经遗传修饰的酵母细胞生产3-HP的方法。在某些实施方案中,这些方法包括在至少一种碳源存在下培养如本文中所提供的经遗传修饰的酵母细胞,使细胞能够生产3-HP一段时间,接着从培养物中分离由细胞生产的3-HP。碳源可以是能够被所提供的酵母发酵的任何碳源。碳源可以是十二碳糖例如蔗糖,己糖例如葡萄糖或果糖,葡聚糖(glycan)或其他葡萄糖多聚物,葡萄糖寡聚物例如麦芽糖、麦芽三糖和异麦芽三糖、潘糖,以及果糖寡聚物。如果细胞经修饰以赋予发酵戊糖的能力,则发酵培养基可包含戊糖,例如木糖、木聚糖或木糖的其他寡聚物和/或阿拉伯糖。这些戊糖是含半纤维素的生物质的适宜水解产物。在寡聚糖的情况下,为了将这些消化为相应的单糖以由细胞发酵,向发酵液中加入酶可能是必需的。在某些实施方案中,培养物中可以存在多于一种类型的经遗传修饰的酵母细胞。同样地,在某些实施方案中,培养物中可以存在与经遗传修饰的酵母细胞相同或不同菌种的一种或多种天然酵母细胞。

[0207] 在某些实施方案中,对本文中提供的细胞进行培养以生产3-HP可以分为多个阶段。例如,细胞培养过程可以分为培养期、生产期和回收期。本领域普通技术人员将认识到,这些时期中使用的条件可以基于例如所使用的酵母菌种、酵母所利用的特定3-HP发酵途径、期望产率或其他因素的因素而变化。

[0208] 培养基将通常含有特定细胞所需的营养成分,包括氮源(例如氨基酸、蛋白、无机氮源例如氨或铵盐,等)以及多种维生素、矿物质等。在一些实施方案中,本发明的细胞可以在化学限定性培养基中进行培养。在一个实施例中,培养基含有大约5g/L硫酸铵、大约3g/L磷酸二氢钾、大约0.5g/L硫酸镁、微量元素、维生素以及大约150g/L葡萄糖。可以使pH在培养过程中自由变化,或者如果需要,进行缓冲以防止pH降至预设水平以下或升至预设水平以上。在某些实施方案中,发酵培养基接种足够的作为评估对象的酵母细胞,以产生大约1.0的OD600。除非另有详细说明,本文中使用的OD600是指利用UD600型分光光度计(Beckman Coulter)以1cm路径长度在600nm波长下所测得的光密度值。培养温度可以在大约30-40℃范围内,培养时间可以长达大约120小时。

[0209] 在一个实施例中,生产期过程中,发酵培养基中的细胞浓度通常为大约0.1至20,优选0.1至5,甚至更优选1至3g干燥细胞/升发酵培养基的范围内。根据途径所需,发酵可以在需氧、微氧或厌氧条件下进行。如果需要,氧摄取速率(OUR)可以作为过程控制环节在发酵期间变化(参见例如W003/102200)。在一些实施方案中,本文中提供的经修饰的酵母细胞在微氧条件下进行培养,其特征在于氧摄取速率为2至45mmol/L/hr,例如2至25、2至20、2至15、2至10、10至45、15至40、20至35或25至35mmol/L/hr。在某些实施方案中,当在微氧条件下培养时,本文中提供的经修饰的酵母细胞可以表现尤其出色,所述微氧条件的特征在于氧摄取速率为2至25mmol/L/hr。在生产期过程中,可以对培养基进行缓冲,以致pH保持在大

约3.0至大约7.0或大约4.0至大约6.0的范围内。适宜的缓冲剂是随着酸的形成能够将其中和的碱性物质,包括例如氢氧化钙、碳酸钙、氢氧化钠、氢氧化钾、碳酸钾、碳酸钠、碳酸铵、氨水、氢氧化铵等等。总的来讲,已经用于常规发酵过程中的那些缓冲剂也适用于此处。

[0210] 在利用经缓冲的发酵的那些实施方案中,可以随着酸性发酵产物的形成将其中和为相应的盐。在这些实施方案中,酸的回收包括游离酸的再生。这可以通过去除细胞并用强酸例如硫酸使发酵液酸化来进行。这导致形成盐类副产物。例如,当钙盐用作中和剂并且硫酸用作酸化剂时,产生盐类副产物石膏。将该副产物从肉汤中分离,并利用例如液-液萃取、蒸馏、吸收以及其他技术回收酸(参见例如T.B.Vickroy, Vol. 3, Chapter 38 of *Comprehensive Biotechnology*, (M.Moo-Young编), Pergamon, Oxford, 1985; R.Datta等, *FEMS Microbiol Rev*, 1995, 16:221-231; U.S. 专利Nos. 4,275,234、4,771,001、5,132,456、5,420,304、5,510,526、5,641,406和5,831,122以及W093/00440)。

[0211] 在其他实施方案中,可以允许发酵培养基的pH由3-HP pKa处或以上,通常为4.5或更高的起始pH,降至酸性发酵产物的pKa处或以下,例如低于4.5或4.0,例如在大约1.5至大约4.5的范围内、大约2.0至大约4.0的范围内或大约2.0至大约3.5的范围内。

[0212] 在其他实施方案中,可以进行发酵,以通过在发酵过程之前或初始将发酵液的pH调节至产物酸的pKa处或以下,从而产生产物酸。其后在培养期间,pH可以保持在产物酸的pKa处或以下。在某些实施方案中,pH可以保持在低于4.5或4.0,例如在大约1.5至大约4.5的范围内、大约2.0至大约4.0的范围内或大约2.0至大约3.5的范围内。

[0213] 在本文中提供的方法的某些实施方案中,经遗传修饰的酵母细胞产生相对低水平的乙醇。在某些实施方案中,可以产生率为10%或更低的乙醇,优选产率为2%或更低。在这些实施方案的某些中,产生的乙醇不可检测出。然而在其他实施方案中,3-HP和乙醇可以同时产生。在这些实施方案中,可以产生率为高于10%、高于25%或高于50%的乙醇。

[0214] 在本文中提供的方法的某些实施方案中,3-HP在碳源上的终产率是至少10%、至少20%、至少30%、至少40%、至少50%或高于50%的理论产率。3-HP的浓度或效价将是产率以及碳源起始浓度的函数。在某些实施方案中,在发酵过程中的某些时间点,优选发酵结束时,效价可以达到至少1-3、至少5、至少10、至少20、至少30、至少40、至少50或高于50g/L。在某些实施方案中,可以通过尤其是在生产期过程中改变发酵培养基的温度,从而使3-HP的终产率增加。

[0215] 一旦产生,可以使用本领域已知的任何方法从发酵培养基中分离3-HP。例如,可以利用普通的分离技术从培养液中去除生物质,并且可以利用普通的离析步骤(例如萃取、蒸馏和离子交换步骤)从不含微生物的培养液中获得3-HP。此外,3-HP可以在其产生时进行分离,或者其可以在产物生产期已经结束后从培养液中分离。

[0216] 可以将利用本文中公开的方法产生的3-HP化学性地转化为其他有机化合物。例如,可以使3-HP氢化,以形成1,3丙二醇,这其是一种有价值的聚酯单体。丙二醇还可以利用具有氧化还原酶活性的多肽在体外或体内由3-HP产生。可以利用任何方法使有机酸例如3-HP氢化,例如用于使琥珀酸和/或乳酸氢化的那些。例如,可以利用金属催化剂使3-HP氢化。在另一个实例中,可以利用任何已知进行脱氢反应的方法使3-HP脱氢,从而形成丙烯酸。例如,可以在催化剂(例如金属或无机酸催化剂)存在下对3-HP进行加热,以形成丙烯酸。

[0217] 提供下面的示例来更好地说明所请求保护的本发明,且不应被理解为对本发明范

围的限制。特定材料所被提及的程度,仅仅用于说明的目的,而并不是打算对本发明进行限制。在不行使创造能力和不超出本发明范围的情况下,本领域技术人员可以开发出等同的方法和反应物。应当理解,可以保持在本发明的范围之内,对本文所描述的步骤作出许多变化。发明人的意为这些变化都包括在本发明的范围之内。

实施例

[0218] 培养基和溶液

[0219] TE由10mM Tris碱和1mM EDTA,pH8.0构成。

[0220] 2X YT+amp平板由16g/L胰蛋白胨,10g/L的酵母提取物,5g/L的NaCl,100mg/L的氨苄青霉素和15g/L的Bacto琼脂构成。

[0221] ura选择平板由6.7g含硫酸铵酵母基础氮源,5g酪蛋白氨基酸,100ml 0.5M,pH5的琥珀酸,20g Noble琼脂以及855mL去离子水构成。高压蒸汽灭菌后,加入40mL的无菌50%葡萄糖和2mL 10mg/mL的氯霉素(chloraphenicol),然后倾倒平板。

[0222] ura选择培养基由6.7g含硫酸铵酵母基础氮源,5g酪蛋白氨基酸,100ml 0.5M,pH5的琥珀酸以及855mL去离子水构成。高压蒸汽灭菌后,加入40mL的无菌50%葡萄糖和2mL 10mg/mL的氯霉素。

[0223] YP+10%葡萄糖培养基由500mL YP培养液和100mL无菌50%葡萄糖构成。

[0224] YP培养液由10g/L的酵母提取物,20g/L的蛋白胨构成。

[0225] YPD平板由10g酵母提取物,20g蛋白胨,20g bacto琼脂,以及去离子水加至960ml构成。高压灭菌后,加入40mL的无菌50%葡萄糖,并倾倒平板。

[0226] TAE由4.84g/L的Tris碱,1.14mL/L冰醋酸和2mL/L的0.5M EDTA,pH8.0构成。

[0227] TBE由10.8g/L的Tris碱,5.5g/L的硼酸,和4mL/L的0.5M EDTA,pH8.0构成。

[0228] LiOAc/TE溶液由为8份的无菌水,1份的1M LiOAc以及1份的10×TE构成。

[0229] 10×TE (200mL) 由2.42g Tris碱,4mL 0.5M的EDTA,pH8.0构成,用5M的HCl调整pH值至7.5,用高压蒸汽灭菌器对溶液进行灭菌。

[0230] PEG/LiOAc/TE溶液由8份50%的PEG3350,1份1M的LiOAc,以及1份10×TE构成。

[0231] 50%PEG3350的制备方法为:将100g PEG3350加入到150mL水中,并加热和搅拌直至溶解。然后用水将体积补至200mL,高压蒸汽灭菌。

[0232] ScD F0A平板由275mL的2X-ScD 2X F0A液体培养基和275mL的2XScD 2X F0A平板培养基构成,融化并冷却至65℃。

[0233] 2X-ScD 2X F0A液体培养基由6.66g不含氨基酸的酵母基础氮源,1.54g ura-D0补充物(Clontech,Moutain View,CA,USA),20g葡萄糖,50mg尿嘧啶,以及2g 5-F0A(5-氟乳酸,一水合物;Toronto Research Chemicals,North York,ON,Canada)和水加至1L构成。将得到的溶液过滤除菌。

[0234] 2XScD 2X F0A平板培养基由11g bacto琼脂和275mL水构成。将得到的溶液高压蒸汽灭菌。

[0235] DM2培养基由硫酸铵(5.0g/L),七水硫酸镁(4.50.5g/L),磷酸二氢钾(3.0g/L),微量元素溶液(1mL/L)和维生素溶液(1mL/L)构成。在所有培养基组分溶解之后,用合适的碱(例如,KOH)将培养基的pH调节到所需的初始pH值。

[0236] 微量元素溶液由EDTA (15.0g/L), 七水硫酸锌 (4.5g/L), 脱水氯化镁 (1.0g/L), 六水氯化钴 (II) (0.3g/L), 五水硫酸铜 (II) (0.3g/L), 脱水二钠钼 (disodium molybdenum dehydrate) (0.4g/L), 脱水氯化钙 (4.5g/L), 七水硫酸亚铁 (3g/L), 硼酸 (1.0g/L), 以及碘化钾 (0.1g/L) 构成。

[0237] 维生素溶液由生物素 (D-, 0.05g/L), 泛酸钙 (D+, 1g/L), 烟酸 (5g/L), myo-肌醇 (25g/L), 盐酸吡哆醇 (1g/L), 对氨基苯甲酸 (0.2g/L), 以及盐酸硫胺 (1g/L) 构成。

[0238] DM1X- α -gal 平板由DM1盐, 20g/L的葡萄糖, 微量元素溶液, 维生素溶液, 2ml/L的X- α -gal (16mg/ml), 20g/L的琼脂构成。

[0239] DM1盐溶液由2.28g/L的尿素, 3g/L的磷酸二氢钾, 以及0.5g/L的七水硫酸镁构成。

[0240] Butterfield磷酸缓冲液由1.25ml/L的储备溶液 (26.22g/L磷酸二氢钾和7.78g/L的碳酸钠), 以及5ml/L的氯化镁溶液 (81.1g/L $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$) 构成。将得到的溶液高压蒸汽灭菌, 调节pH至7.2。

[0241] CNB1摇瓶培养基由尿素 (2.3g/L), 七水硫酸镁 (0.5g/L), 磷酸二氢钾 (3.0g/L), 微量元素溶液 (1mL/L) 和维生素溶液 (1mL/L), 葡萄糖 (120.0g/L), 2- (N-吗啉代) 乙磺酸 (MES) (97.6g/L) 构成。在所有培养基组分溶解后, 用合适的碱 (例如, KOH) 将培养基的pH调节为初始pH5.8。

[0242] 表0: 引物序列

[0243]

识别号	SEQ ID NO:	序列 (5'-3')
0611166	152	TAAACGACGGCCAGTGAATTCGCGGGCGGCCGCGAGTCCATCGGTTCTGTCA
0611167	153	CATAAGAAAATCAAAGACAGAAGGCGCGCCTTTGCTAGCATTTTTGTGTTTGCT GTGT
0611168	154	ACACAGCAAAACACAAAAATGCTAGCAAAGGCGCGCCTTCTGTCTTTGATTTCTTATG
0611169	155	GGGGGAAAGAACTACCAATAGGCCTCCTTTAATTCGGAGAAAAATC
0611170	156	GATTTTCTCCGAATTAAGGAGGCCTATTGGTAGTTCTTTCCCCC
0611171	157	AAAATAAACTAGTAAATAAATTAATTATCTAGAGAGGGGGTTATAT
0611172	158	ATATAACCCCTCTCTAGATAATTAATTAATTTATTTACTAGTTATTTT
0611173	159	GACCATGATTACGCCAAGCTCCGCGGGCGGCCGCCAGTCAAAACCTTCTCTC
0611174	160	TAAACGACGGCCAGTGAATTCGCGGGCGGCCGCTTTGAAGGAGCTTGCCA
0611175	161	CTATTCCTTCTCAAATTGCTGTTTAAACGCGTTGAAGATCTATTCTCC
0611176	162	GGAGAATAGATCTTCAACGCGTTTAAACAGCAATTTGAGGAAGGAATAG
0611177	163	AATGTTCAATTTACATTAGATGTTAATTAAGGTCTAGATGTGTTGTTGTGTG
0611178	164	CACACAAACAAACACATCTAGACCTTAATTAACATCTGAATGTAAATGAACATT
0611179	165	GACCATGATTACGCCAAGCTCCGCGGGCGGCCGAATGCCAAGAGTTATGGGGC
0611184	166	GGCTACCCTATATATGGTGAGCG
0611185	167	GGGTCCAAGTTATCCAAGCAG
0611186	168	CCTTAATTAACCGTAAAGTTGTCTCAATG
0611189	169	CAGCAAAACACAAAAATTCTAGAAAATTTAATTAACATCTGAATGTAAATGAAC
0611191	346	CCTTAATTAATTATCCACGGAAGATATGATG
0611195	170	GTTCAATTTACATTAGATGTTAATTAATTTTCTAGAATTTTGTGTTTTGCTG
0611196	171	GCTCTAGATAAAATGCCCTCTTATTCTGTGCGC
0611199	347	GCTCTAGATAAAATGTCCCAAGGTAGAAAAGC
0611225	172	GACTGGATCATTATGACTCC
0611245	272	CGAACCAATTCAAGAAAACCAAC
0611250	173	ACGCCTTGCCAAATGCGGCCGCGAGTCCATCGGTTCTGTGTCAGA
0611251	174	TCAAAGACAGAATTAATTAAGCTCTAGAATTTTGTGTTTTGCTGTGTT
0611252	175	AACACAAAAATTCTAGAGCTTAATTAATTCTGTCTTTGATTTTCTTATG
0611253	176	GTTTAAACCTTTAATTCGGAGAAAATCTGATC
0611254	177	GTTAACGGTACCGAGCTCTAAGTAGTGGTG
0611255	178	AACCGATGGACTCGCGCCGCATTTGGCAAGGCGTATCTAT
0611256	179	GGCGCCAGCAATTTGAGGAAGGAATAGGAG
0611257	180	AACTATTAGATTAATTAAGCTCGCGATGTGTTGTTTGTGTGTTTTGTGTG
0611258	181	CAAACAAACACATCGCGAGCTTAATTAATCTAATAGTTTAATCACAGCTTAT
0611259	182	TACATTATGGTAGCGGCCGCGTGTGACATTTTGATCCACTCG
0611260	183	TGGATCAAAATGTCACACGCGGCCGCTACCATAATGTATGCGTTGAG
0611261	184	GGGCCCTAAAAGTGTGGTGTATTAGA
0611263	185	GCTAGCTCAACAACTCTTTATCAGATTTAGCA

[0244]

0611264	186	GCTAGCGAGGAAAAGAAGTCTAACCTTTGT
0611266	187	TCGCGATAAAATGTCAACTGTGGAAGATCACTCCT
0611267	188	TTAATTAAGCTGCTGGCGCTTCATCTT
0611268	189	TCGCGATAAAATGTCCAGAGGCTTCTTTACTG
0611269	190	TTAATTAAGCTAAAGGTCTCTCACGACAGAG
0611283	191	GTTAACCCTGGTTTAAACATAGCCTCATGAAATCAGCCATT
0611284	192	GGGCCCATATGGCGCCCGGGCGTTGAAGATCTATTCTCCAGCA
0611295	193	GTTTAAACGATTGGTAGTCTTTCCCCCTC
0611296	194	AATAAATTAAGGGCCCTTTATCGCGAGAGGGGGTTATATGTGTAAA
0611297	195	TATAACCCCTCTCGCGATAAAGGGCCCTTAATTTATTTTACTAGTTTAT
0611298	196	GTTTAACTTTTGTAGCACCTCCTGGT
0611376	197	CAGCAAAACACAAAAGCTAGCTAAAATGTTACGTACCATGTTCAAAA
0611377	198	GAAAATCAAAGACAGAAGGCGCGCCTTATGCTGTAACAGCCTGCGG
0611378	199	CAGCAAAACACAAAAGCTAGC TAAA ATGTTAAGAACCATGTTCAAATC
0611379	200	GAAAATCAAAGACAGAAGGCGCGCC TTATGCAGTAACAGCTTGTGGG
0611552	273	TCTGTCCCTTGGCGACGC
0611553	274	CTTTTCAAACAGATAAGTACC
0611554	201	GCATGGTGGTGCAAGCGACG
0611555	202	GGTGTGCTGATTGCTGCTG
0611622	275	ATGGGCTGACCTGAAAATTC
0611631	203	TGTATACAGGATCGAAGAATAGAAG
0611632	204	GAACGTCTACAACGAGGTGAAC
0611661	205	GGCGCGCCTCGCGATAAAATG
0611662	206	AGGGCCCTTAATTAATTATGCAGTAACAGCTT
0611717	207	CGCTACGATACGCTACGATA
0611718	208	CTCCCTTCCCTGATAGAAGG
0611814	209	GGGAGCAATTTGCCACCAGG
0611815	210	CTCCTTCATTTAACTATACCAGACG
0611816	211	GACAGATGTAAGGACCAATAGTGTCC
0611817	212	CCATATCGAAATCTAGCCCGTCC
0611828	213	CATAAGAAAATCAAAGACAGAAGGCGCGCCTTTGCTAGCTTTTTGTGTTT TGCTGTGT
0611954	348	GCTAGCTAAAATGTTTGGTAATATTTCCCA
0611957	349	TTAATTAAGCTATTTATCTAATGATCCTC
0611997	350	TCTAGATAAAATGTCTATTAGTGAAAAATATTTTCTCAAG
0611998	351	TTAATTAAGCTTTTAAATTTTGAAAAAGCTTGATCAATAATGG
0612055	214	ATTGGACACAACATTATAT
0612150	238	ACGCGTCGACTCGACATTTGCTGCAACGGC
0612151	239	CTAGTCTAGATGTTGTTGTTGTTGTCGTT
0612271	215	GTAAACGACGGCCAGTGAATTGTTAACATAGGCTCCAACATCTCG
0612272	216	GGAACCGATGGACTCGCGGCCGC GTGGGATATTGGAAG
0612273	217	GGAGGTGCTACAAAAGGAATTC
0612274	218	GACCATGATTACGCCAAGCTCCGCGGAGTCAAAACCTTCTTCTTACC
0612275	219	GTAAACGACGGCCAGTGAATTC TTTGAAGGAGCTTGCCAAGAAAC
0612276	220	CCTATTCCTTCTCAAAATTGCTG
0612277	221	ATAACTCTTGGCATTGCGGCCGCCAAGTTAGTTAGAGC
0612278	222	ATGACCATGATTACGCCAAGCTCCGCGGCAAGACGGTGTATTAGTGCT TG
0612356	223	CACAAAACACACAAACAAACACAGCTAGCAAAGGCGCGCCATCTAATAG TTAATCACAGCTTA
0612357	224	GCCGTTGCAGCAAATGTCGAGGCCTGTGTGACATTTTGATCCACTCG
0612358	225	CGAGTGGATCAAAATGTCACACAGGCCTCGACATTTGCTGCAACGGC
0612359	226	CATTTTACATTCAGATGTTAATTAATTATCTAGATGTTGTTGTTGTTGTCG
0612360	227	CGACAACAACAACAACATCTAGATAATTAATTAACATCTGAATGTAAATG
0612361	228	GCTCTAACTAACTTGGCGGCCGCTTTTATTATAAAATTATATATTATTCTT
0612366	276	GCTGAAAATATCATTCAGAGCAT
0612367	277	ACTGTTGATGTCGATGCC
0612378	229	AACACACAAACAAACACAGCTAGCTAAAATGTTAAGAACTATGTTTA

[0245]

0612379	230	GATTAACTATTAGATGGCGCGCCTTATGCAGTAACTGCTTGTGGA
0612470	231	CGACGGCCAGTGAATTTCGTTAACCCTGTTTCGATGGGATTCCC
0612471	232	CAGGAACCGATGGACTCGCGGCCGCTCCCTTCTCTAAATGGACTGC
0612472	233	TATATAATTTTATAATAAAAGCGGCCGCACACAGGGGTTTAGTGAAGTC
0612473	234	CATGATTACGCCAAGCTCCGCGGCCATAACTGACATTTATGGTAAGG
0612579	278	TCTGAATGCAGTACGAGTTG
0612695	240	CAAAACACAGCAAAACACAAAAGCTAGCATGTATAGAACCTTGATGAG
0612698	242	CAAAACACACAAACAAACACAGCTAGCATGTACAGAACGTTAATGTCTGC
0612724	241	CAAAGACAGAAAGCGCGCCTTATAAGATGGTTCTCGCTGG
0612725	243	GTGATTAACTATTAGATGGCGCGCCTTACAGGATGGTTCTGGCAGG
0612794	279	GAAGGGGGTCCAAGTTATCC
0612795	235	CCAACAATCTTAATTGGTGAC
0612891	236	GGCTGTTACCGCCTAATTAA
0612893	237	GTTCTTAACATTTTAGCTAGCTG
0612908	280	GATATGGGCGGTAGAGAAGA
0612909	281	GCTCCTTCAAAGGCAACACA
0612910	282	TGAACATCACATGAACGTA
0612911	283	TCAAGGTAGGGTCACTTAAC
0613034	284	TGATTCCTTCAATCACAGGT
0613035	285	AACCGACCTATCGAATGCCT
0613178	286	ACCATGATTACGCCAAGCTTGGTACCTTGGGGTTTACGCTTACAGCGTA CT
0613179	287	TCAACGCCCGGGGGATCTGGATCCGCGGCCGCAAGAAATTCCTTTCTT TTCCCTTTTATA
0613180	288	AACCGATGGACTCCTCGAGGGATCCGCGGCCGCGCATAACAAAATTGTG CCTAACCCA
0613181	289	ACGACTCACTATAGGGCGAATTGGGCCCCGGGAAAAGGAGAGAGAAAA GGAGA
0613183	290	TATGACCATGATTACGCCAAGCTTGGTACCTCCTACAGAAGTAGCACCA GCACCA
0613184	291	GGAACCGATGGACTCCTCGAGGGATCCGCGGCCGCGAGACTACCGTGTA AATAAGAGTACC
0613185	292	TCTTCAACGCCCGGGGGATCTGGATCCGCGGCCGCATTTGATATAAAC GCTTCTATAATA
0613186	293	GTAATACGACTCACTATAGGGCGAATTGGGCCCCAACATCTGCTGCTGTA ATATATTCA
0613241	294	GCATGTCTGTTAACTCTCAAACCAT
0613242	295	TCCATAATCCAATAGATCATCCC
0613243	296	AACACAATGGAACCAACCTAGT
0613416	297	ATTTACACGGTAGTCTGCGGCCGCCATAGCCTCATGAAATCAGCC
0613417	298	CTCTAGGTTCACTGGTTGTTTCTTGGGCTGCCTCCTTCAA
0613418	299	TTGAAGGAGGCAGCCCAAGAAACAACCAAGTGAACCTAGAG
0613419	300	TATTATAGAAGCGTTTATATCAAATGCGGCCGCGGATCCAGATCCCCCG GGGCGTT
0613550	301	AATGATCAACTTGAGAGGTA
0613551	302	CAGGTCTGTTACATAAAGCA
0613688	303	GTTTACGCTCAAATCTCCTCC
0613689	304	GGTACATGAAGCAGGCTTTGAAGG
0613695	305	GATTGTGTCCGTCAGCCTTTGCTC
0613746	306	TACCATATTTTACAGAGGATCA
0613747	307	AGGATGTTCTTGCCTGCAAGT
0614233	308	GATGATATAGTTGATGCTTTCCAAAG
0614626	344	AGGGTACCTTAGTACGAAGG
0614627	345	CTATTCTTACGATGAAGCGG
CM647	262	AATGATCCATGGTCCGAGAG
oACN48	309	GGGCCCCCTTCAATTACGAAATAAAGTGCCGCGG
oACN49	310	GCGGCCGCAATAAATTTAAATAAACGATATCAAAATTC
oACN50	311	CGACGCCAAAGAAGGGCTCAGCCGAAAAAG

[0246]

oACN51	312	CCATTTCTTTTCGGCTGAGCCCTTCTTGCC
oACN52	313	GCGGCCGCAATAACCTCAGGGAGAAGCTTTGGC
oACN53	314	GAGCTCCCAACAAGGTCTCAGTAGGATCG
oACN54	315	CGCCATAAGGAGAGGCTGTAGATTTGTC
oACN55	316	CCAGGACATCTTGCTTGAATGTCC
oANN1	317	GTTCCATCGGGCCCTAAAGGTCTCTCAGACAG
oANN2	318	CAAACACA TCGCGA TAAATGTCCAGAGGCTTC
oANN5	319	GCAAGACCTTGGATCTGAAGGG
oANN6	320	CGAACCAATTCAAGAAAACCAACAG
oANN7	321	GGGCCC GTCCCTTGGCGACGCCCTGATC
oANN8	322	GCGGCCGCTATTTTGTGTTTGTCTGTGTTTTG
oANN9	323	GCGGCCGCC ATCTGAATGTAAATGAACATTAAATG
oANN10	324	GAGCTCCCCAGTTGTTGTTGCAATTAAC
oANN11	325	GAAGAGACGTACAAGATCCGCC
oANN12	326	TAGGAATGGTGCATCATCCAAC
oANN13	327	TTCTTATCTGAAAACCTCGAGTTCGAAAGAAGGTTGAAG
oANN14	328	TTATTGAAATTAATCCAAGGATTCAAGTTGAACATACAATTACTG
oANN15	329	TTTCCAAAAAGTTTCGTGAGTTCGATGGTTGTATGATTATGG
oANN16	330	CCTCTACCACCACCACCAAATG
oANN20	331	GGGAAGAACTAAGAAGAAGTATG
oCA405	260	GCAACTGATGTTACGAATGCG
oCA406	261	TTGCCGTTGCAGCAAATCTC
oHJ1	332	CAATCCTTCTAGAGAGGGGGTTATATGTGTAATATAGAGTTTG
oHJ2	333	TTCTACCCTCGAGATTGGTTCTTCCCCCTCTCAAG
oHJJ116	334	GTGGTCAATCTAGAAAATATGACTGACAAAATCTCCCTAGGTAC
oHJJ117	335	CAATTTTGAGCTGATTCCAATCGTAAAC
oLJ28	264	GCACCAAGAGCAGTTTTCCCATCTATTG
oLJ29	265	CCATATAGTTCTTTTCTAACATCAACATCACACTTC
oLJ30	266	GGAGAATAGATCTTCAACGCTTTAATAAAGTAGTTTG
oLJ31	267	CGTGTTCGCAATAAAACCAATGAC
oLJ43	336	CAAGAGTATCCCCTCTGACAGGAACCGATGG
oLJ44	337	GCTGGAGAATAGATCTTCAACGCCCGG
oLJ45	338	CGGAGAAGGCGTATAAAAGGACACGGAG
oLJ46	339	GGATAAAGTATTACATACGTACAGGATTGTATTAGTGATTTCG
oLJ57	340	CCTCCAGTGTTTTCTCTCTCTCTTTGTTTTTTTTTC
oJY11	268	CCTCGAAGAGCTTGAATTTG
oJY12	269	GTAGTGAATGTCCGGATAAG
oJY13	270	GCAAGGTCATGAGGTTAAAG
oJY14	271	AACACTTATGGCGTCTCCTC
oJY44	341	GGTTAATTAATTTATTTGTACATAAAACACATAAATGTAAAGC
oJY45	342	GAATTCCTTTAATTCGGAGAAAATCTGATCAAGAG
WG26	263	ACGGCAGTATACCCTATCAGG

[0247] 各种序列

[0248] 启动子:在实施例中描述的PDC,TDH3,EN01和PGK1启动子分别来源于东方伊萨酵母的SEQ ID NO:244,245,246和247序列。终止子:实施例中描述的TAL,TKL,RKI和PDC终止子分别来源于东方伊萨酵母的SEQ ID NO:248,249,250和251序列。实施例中描述的URA3启动子,ORF和终止子来源于东方伊萨酵母SEQ ID NO:252序列。

[0249] 实施例1A:基于3-HP抗性选择酵母宿主细胞

[0250] 检测一组野生型酵母株在3-HP存在的情况下的生长能力。

[0251] 通过评估7个野生酵母株(表1,组A)在含有不同水平3-HP的培养基上的生长能力来确定在初筛过程中所使用的3-HP浓度范围。这些实验中使用的3-HP是由丙烯酸水溶液(30%)和CO₂催化剂在200psi和175℃下通过化学合成得到的,如W004/076398中所描述的。用异丙醚在室温下进行逆向萃取以除去残留的丙烯酸(见W005/021470)。

[0252] 细胞在YPD平板上划线,培养过夜。在pH3.0的YPD培养基中制备OD600约为4的细胞浆。该浆状物用于接种到含有不同浓度3-HP(pH3.9)的微滴定孔中,至OD600为0.05。用透气膜覆盖平板,在30℃/300RPM的摇床中孵育过夜。在GENios模型平板读数器(Tecan)中对每个孔测量600nm波长处的光密度,并肉眼观察平板的生长情况。一个或多个菌株所生长的最高3-HP浓度(125g/L)被选为初筛步骤的上限值。

[0253] 初筛

[0254] 对于初筛步骤,利用与范围确定相同的步骤,对生长在含有0g/L,75g/L,100g/L或125g/L 3-HP (pH3.9) 的微滴定板上的89个野生型酵母菌株进行筛选。对于每个菌株都使用新鲜的YPD平板,并且在pH3.0的YPD培养基中制备OD600约为4的细胞浆。该浆状物用于接种到含有不同浓度3-HP (pH3.9) 的微滴定孔中至OD600为0.05。用透气膜覆盖平板,在30℃/300RPM的摇床中孵育过夜。在GENios模型平板读数器中对每个孔测量600nm波长处的光密度,并肉眼观察平板的生长情况。实施类似的步骤,以评估在乳酸浓度为0g/L,30g/L,45g/L和60g/L时的生长情况。表1总结了观察到生长的最高3-HP或乳酸浓度。

[0255] 鉴定出15个能在100g/L的3-HP上生长或者在75g/L的3-HP上生长良好的菌株(表1,B组)。为了进一步缩小菌株的范围,实行了第二微滴定板检测。该检测与第一次的检测类似,但是所使用的3-HP浓度为100g/L,112.5g/L,和125g/L (pH3.9)。从这个检测中鉴定出了11个在75g/L的3-HP上生长良好或在75和112.5g/L的3-HP上都展现出一些生长的菌株(表1,C组)。将这11个菌株进行二次筛选。四株在75g/L的3-HP上生长情况不好以及在112.5g/L的3-HP上没有生长的菌株并没有进行二次筛选。据预测,没有进行二次筛选的菌株在商业发酵中也展示出较差的经济表现。不过可能一个或多个这样的菌株均能满足商业上可行的发酵过程的最低要求。

[0256] 表1:在3-HP或乳酸上的生长

[0257]

酵母菌株	培养物保藏#	组	初始筛选: 3-HP (g/L)	初始筛选 乳酸 (g/L)
白布勃担胞酵母	ATCC 96272		在0g/L 无生长 (NG)	NG
布朗克假丝酵母	ATCC 18735		0	30
博伊丁假丝酵母	PYCC 70-104		0	30
链形假丝酵母	ATCC 20117		0	NG
埃切假丝酵母	PYCC 60-8		0	30
无名假丝酵母	ATCC 20284		0	0
<i>candida fluvistilis</i>	ATCC 38623		0	0
<i>candida geochares</i>	Cargill 1978		75	60
季也蒙假丝酵母	ATCC 20118		0	0
中间假丝酵母	ATCC 20178		0	0
克非假丝酵母	ATCC 44691		75	30
炼乳假丝酵母	ATCC 96927		0	NG
郎比可假丝酵母	ATCC 38617	B, C	75	45
<i>candida methanosorbosa</i>	ATCC 20361		0	NG
<i>candida milleri</i>	ATCC 60591		0	30
<i>candida milleri</i>	ATCC 60592		0	45
<i>candida naeodendra</i>	ATCC 56465		0	NG
近平滑假丝酵母	ATCC 20179		0	0
<i>candida pignliae</i>	ATCC 36592		0	NG
<i>candida pseudolambica</i>	ATCC 96309		0	0
皱褶假丝酵母	ATCC 20306		0	45
休哈塔假丝酵母	NCYC 2389		0	0
<i>candida sonorensis</i>	ATCC 32109	A	0	30
<i>candida sorbophila</i>	Cargill 1973		75	45
<i>candida sorboxylosa</i>	ATCC 24120		0	30
<i>candida sorosivorans</i>	ATCC 38619	B	100	60
纤维假丝酵母	MUCL 47216 or MUCL 31253B		0	NG
粗壮假丝酵母	ATCC 28525	B	75	45
<i>candida vanderwaltii</i>	MUCL 300000		0	45
<i>candida zemplinina</i>	PYCC 04-501	A,B,C	100	60
凝沫假丝酵母	ATCC 20347		75	45
<i>candida matritensis</i>	ATCC 34087		0	NG

[0258]

卡斯特利德巴利酵母	PYCC 70-1022		0	30
汉氏德巴利酵母	ATCC 90624		0	NG
多形德巴利酵母	ATCC 20280		0	0
异常德克拉酵母	ATCC 20277		0	0
郎比可德克拉酵母	ATCC 10563		0	0
<i>Hyphopichia burtonii</i>	ATCC 20030		0	0
东方伊萨酵母	ATCC 24210	B, C	100	45
东方伊萨酵母	ATCC 60585	B, C	100	45
东方伊萨酵母	ATCC PTA-6658	A,B,C	100	60
东方伊萨酵母	CD1822 (Cargill collection – see description below)	A,B,C	100	60
乳酸克鲁维酵母	ATCC 8585	A	0	0
马克斯克鲁维酵母	ATCC 52486	A	75	45
耐热克鲁维酵母	ATCC 52709		0	30
<i>Kodamaea ohmeri</i>	ATCC 20282		0	45
亚罗克鲁维酵母	ATCC 36591		NG	NG
斯达氏油脂酵母	ATCC 12659		0	0
子囊菌油脂酵母	ATCC 56306		NG	NG
美极梅奇酵母菌	ATCC 9889		0	0
<i>Myxozyma kluyveri</i>	ATCC 76214		NG	NG
<i>Hematospora coryli</i>	ATCC 20292		0	NG
嗜霖管囊酵母	NCYC 614		0	30
异常毕赤酵母	ATCC 2102		0	0
发酵毕赤酵母	ATCC 28526		0	30
泌液毕赤酵母	ATCC 28778		NG	NG
杰丁毕赤酵母	ATCC 9950		0	0
膜醭毕赤酵母	NCYC 2696	B,C	125	60
<i>Pichia nakasei</i>	ATCC 24116		0	0
<i>Pichia silvicola</i>	ATCC 16768		0	0
树干毕赤酵母	CBS 6054		0	0
斯地毕赤酵母	ATCC 34024		0	0
<i>Pichia tannicola</i>	ATCC 2261		0	0
帽孢毕赤酵母	ATCC 58362		NG	NG
酿酒酵母	ATCC 96784		75	30
贝酵母	ATCC 90739		0	45
博伊丁酵母	MYA-402	B, C	100	60
博伊丁酵母	MYA-404	B,C	100	45
<i>Saccharomyces capsularis</i>	ATCC 20033		0	0
酿酒酵母	CEN-PK 113-7D	A,B	100	45

[0259]

路氏酵母	NCYC 734		75	45
<i>Saccharomyces crataegeni</i>	MUCL 44417		0	0
<i>Saccharomyces javensis</i>	MUCL 31237		0	45
酿酒酵母	NRRL Y-7290		0	0
葡萄汁酵母	ATCC 76514		0	0
<i>Saccharomyces japonicus</i>	ATCC 10660		0	0
栗酒酵母	NCYC 535	B,C	100	60
戴尔有孢圆酵母	ATCC 52714		75	0
球有孢圆酵母	ATCC 42479	B	75	0
<i>Tickerhamiella occidentalis</i>	CBS 8452		0	30
<i>Yamadazyma guilliermondii</i>	ATCC 90197		0	0
<i>Yamadazyma haplophila</i>	ATCC 20321		0	0
<i>Yamadazyma stipitis</i>	ATCC 201225		0	0
<i>Yarrowia lipolytica</i>	ATCC 46330		0	45
拜耳接合酵母	ATCC 36946		75	45
<i>Zygosaccharomyces bisporus</i>	NCYC 3134		0	45
<i>Zygosaccharomyces kombuchaensis</i>	NCYC2897		0	60
<i>Zygosaccharomyces lentus</i>	NCYC 2928	B,C	100	45
鲁氏接合酵母	ATCC 34890		75	30

[0260] 上面所测试的东方伊萨酵母菌种CD1822是通过在葡萄糖限制恒化器中使东方伊萨酵母ATCC PTA-6658进化91天产生的。用含有15g/L右旋葡萄糖的DM培养基供给该系统，通过将乳酸加入到供给培养基中，在pH=3，稀释率为 0.06h^{-1} 下进行操作。用大概 $2\text{mmol L}^{-1}\text{h}^{-1}$ 的低氧传输速率来维持该条件，而溶解氧的浓度保持恒定在0%的空气饱和度。初始时间点的单菌落分离物在两个摇瓶分析中被表征出。在第一个分析中，菌种的特征在于它们在25g/L的总乳酸存在下，在无需对pH校正的DM1确定培养基(DM1 defined medium)中，能够将葡萄糖发酵成乙醇。在第二分析中，在存在25,32和45g/L的总乳酸的情况下，在无需对pH校正的DM1确定培养基中测定分离物的生长速率。CD1822菌株是基于所测量的发酵速率和生长速率所选择出来的单一分离物。其他进化东方伊萨酵母的方法包括，但不限于在选择条件下重复系列传代培养，如US专利号7629162中所描述。此类方法可以用来代替，或补充上述的葡萄糖限制恒化器法。本领域技术人员可以理解的是，可以使用类似的进化步骤，添加3-HP而不是乳酸来开发改进的3-HP抗性，来产生菌株。此外，在选择前也可以对菌株进行诱变，如本文所述(例如，见实施例1B)。

[0261] 二次筛选

[0262] 对于二次筛选的第一部分，在pH 3.9，含有0g/L,35g/L,或75g/L 3-HP的YPD培养基中测量生长速率。用收获自种子瓶的生物物质接种摇瓶，所述种子瓶过夜生长至OD600为6至10。将250ml生长速率受困的瓶子(50mL工作体积)接种至OD600 0.1，在250rpm和30℃下生长。在分析的整个时间进程中获取样品，并通过OD600对生物物质生长进行分析。绘制所得到的OD600数据，确立生长速率。

[0263] 表2:3-HP中的生长速率(μ)

[0264]	菌株	0g/L 3-HP	35g/L 3-HP	75g/L 3-HP
	东方伊萨酵母ATCC60585	0.56	0.51	0.29
	东方伊萨酵母CD1822	0.62	0.52	0.28
	郎比可假丝酵母ATCC38617	0.65	0.53	0.30
	粗壮假丝酵母	0.51	0.32	0.14
	东方伊萨酵母PTA-6658	0.61	0.53	0.32
	东方伊萨酵母24210	0.58	0.51	0.26
	博伊丁酵母MYA402	0.53	0.45	0.28
	膜酞毕赤酵母	0.41	0.39	0.32
	博伊丁酵母MYA404	0.55	0.44	0.27
	栗酒裂殖酵母	0.41	0.35	0.21
	<i>Zygosaccharomyces lentus</i>	0.61	0.41	0.20

[0265] 对于二次筛选的第二部分,测量pH3.9,含有100g/L葡萄糖和0g/L,35g/L或75g/L 3-HP的YPD培养基中相同10个菌种的葡萄糖消耗量。用收获自种子瓶的生物质接种摇瓶,所述种子瓶过夜生长至OD600为6至10。将250ml生长速率受困的瓶子(50mL工作体积)接种至OD600 0.1,在250rpm和30℃下生长。在分析的整个时间进程中获取样品,并利用Yellow Springs Instruments (YSI)的2700Biochemistry Analyzer分析葡萄糖消耗量。绘制所得到的OD600数据,确立葡萄糖消耗速率。

[0266] 表3:3-HP中的葡萄糖消耗速率(g/L/hr)

	菌株	0 g/L 3-HP	35 g/L 3-HP	75 g/L 3-HP
	东方伊萨酵母ATCC60585	5.5	4.2	3.3
	东方伊萨酵母CD1822	5.5	4.2	4.2
[0267]	郎比可假丝酵母ATCC38617	4.2	4.2	3.5
	粗壮假丝酵母	5.5	2.2	2.1
	东方伊萨酵母PTA-6658	5.5	4.2	4.1
	东方伊萨酵母24210	4.2	4.2	3.8
	博伊丁酵母MYA402	4.2	4.2	4.0
	膜酞毕赤酵母	0.4	2.1	1.2
[0268]	博伊丁酵母MYA404	4.2	4.2	3.8
	栗酒裂殖酵母	2.5	3.1	2.0
	<i>Zygosaccharomyces lentus</i>	3.4	0.8	0.3

[0269] 在75g/L的3-HP (pH3.9) 条件下,有四个菌种(膜酞毕赤酵母,栗酒裂殖酵母,粗壮假丝酵母和*Z.lentus*)并未达到2.5g/L/hr的葡萄糖利用率,而该条件则是经济的发酵过程所必需的。

[0270] 为了鉴定3-HP的主要菌株,将菌株的表现分为三类等级。这些类别中的两个是基于生长速率的不同方面:1)在最高酸浓度上的生长速率和2)所绘制的生长速率相对于酸浓度的斜率。第三个类别是在最高酸浓度时的糖酵解速率。该分级是通过将每个评级的最高和最低值作为标准化的边界,在标准化的范围内完成的。因此,对于每个类别,每个菌株都

获得一个0和1之间的评级,其中1是可能的最高得分。一个菌株的总评级值是三个类别标准化值的总和。所制定权重值中生长速率和糖酵解速率权重相同。在这种情况下,在最高酸浓度下的糖酵解速率权重为50%,而两个生长速率评级每个的权重为25%。每个类别的标准化数值和总和以及权重值都归纳在表4中。

[0271] 表4:3-HP中的菌株级别

菌株	在75g/L 3-HP中的生 长速度	生长速度 斜率	糖酵解速 率	总和值	权重值
东方伊萨酵母ATCC PTA-6658	1.00	0.37	0.97	2.34	0.83
博伊丁酵母MYA402	0.78	0.49	0.94	2.21	0.79
东方伊萨酵母CD1822	0.78	0.21	1.00	1.99	0.75
博伊丁酵母MYA404	0.72	0.42	0.90	2.04	0.74
东方伊萨酵母ATCC60585	0.83	0.44	0.77	2.04	0.70
东方伊萨酵母24210	0.67	0.28	0.90	1.85	0.69
郎比可假丝酵母ATCC38617	0.89	0.19	0.82	1.90	0.68
膜酞毕赤酵母	1.00	1.00	0.23	2.23	0.62
栗酒裂殖酵母	0.39	0.65	0.47	1.51	0.50
粗壮假丝酵母	0	0.14	0.40	0.54	0.24
<i>Zygosaccharomyces lentus</i>	0.33	0	0	0.33	0.08

[0273] 在所检测的菌株中,来自于东方伊萨酵母,郎比可假丝酵母,和博伊丁酵母种的菌株呈现在低pH下作为3-HP生产宿主的最大潜力。

[0274] 利用相同的方法,用pH值为2.85,含有0,25,和50g/L乳酸(~80%游离酸)的培养基,来对初筛中的原始的91个野生型酵母菌株进行筛选,评级和评分。对来自于13个菌株的标准化值和权重和总得分值进行二次筛选。

[0275] 表5:乳酸中的菌株级别

[0276]

菌株	在75g/L 3-HP 中的生长速度	生长速度 斜率	糖酵解速 率	总和值	权重值
郎比可假丝酵母ATCC38617	0.92	1.00	1.00	2.92	0.98
东方伊萨酵母ATCC PTA-6658	0.94	0.95	1.00	2.89	0.97
东方伊萨酵母CD1822	1.00	0.86	1.00	2.86	0.97
东方伊萨酵母24210	0.89	0.73	1.00	2.62	0.91
<i>Candida zemplinina</i>	0.22	0.95	1.00	2.17	0.79
博伊丁酵母MYA404	0.47	0.45	1.00	1.92	0.73
贝酵母	0.08	0.91	0.96	1.95	0.73
博伊丁酵母MYA402	0.50	0.23	1.00	1.73	0.68
<i>Candida milleri</i>	0	0.64	0.92	1.56	0.62
<i>Candida sorosivorans</i>	0.28	0.95	0.59	1.82	0.60
多形德巴利酵母	0.42	0	0.76	1.18	0.49
<i>Candida geochares</i>	0.17	0.27	0.69	1.13	0.46
<i>Saccharomyces javensis</i>	0.11	0.68	0	0.79	0.20

[0277] 对于乳酸,只有*S. javensis*在pH2.85,含有50g/L乳酸的培养基中没能达到2.5g/L/hr的葡萄糖利用率。而东方伊萨酵母,郎比可假丝酵母和博伊丁酵母对3-HP和乳酸都展示出酸耐受,很多菌株只是对一种酸产生了耐受。这些也可以在初筛的结果中看到(表1)。例如,*C. milleri*,皱褶假丝酵母,*C. vanderwaltii*,*K. ohmeri*,贝酵母,*S. javensis*,*Y. lipolytica*,*Z. bisporus*和*Z. kombuchaensis*都表现在45-60g/L乳酸上生长,而不在甚至是所检测的最低浓度的3-HP上生长(35g/L)。因此,菌株对于一种有机酸的耐受性并不能明确地用于预测其对于其他酸类的耐受性。这得到了进一步的凸显,即通过将上述呈现3-HP抗性的菌株与W003/049525中所鉴定的8个优选能作为有机酸生产宿主的菌株列表进行对比。其中那些菌株中的两株(*C. diddensiae*和*C. entomophila*)无法获得来进行测试,其他6株都包括在上述的初筛之中。在这6株中,只有*C. krusei*(作为东方伊萨酵母进行测试)能够在35g/L的3-HP存在的情况下生长。

[0278] 实施例1B: 诱变和选择具有3-HP抗性的突变菌株

[0279] 对在实施例1A中选择出来的酵母细胞进行诱变,并将其暴露于选择压力下,以便鉴定具有高3-HP抗性的突变株。

[0280] 例如,新鲜YP(酵母提取物/蛋白胨)+20g/L的葡萄糖平板或液体培养(OD600为1-4)中的酵母细胞被悬浮于无菌水中,至OD600为10左右。200 μ l该细胞悬液的等分试样被滴加入各试管中,暴露于3 μ l的甲磺酸乙酯(EMS)中,约1小时,这杀灭约65%的细胞。可以使用更高的EMS浓度来提高杀灭率。在暴露之后,用5%的硫代硫酸钠中和细胞,在PBS缓冲液中洗涤,在营养充足的培养基中恢复约4小时,并在选择培养基中培养。同样进行模拟样本(无EMS),以确保条件是选择性的。另外,也可以使用紫外线照射来诱变细胞。

[0281] 0为了选择3-HP抗性的突变菌株,将EMS-处理过的细胞悬液(约 2×10^8 个诱变细胞)的等分试样接种到马铃薯葡萄糖琼脂(PDA)或另外的培养基上,该培养基所含有的3-HP的水平使亲本菌株所不能生长的或生长得很慢。将这些平板孵育几天,直至菌落出现。纯化

单个菌落,在非选择培养基上划线,以消除选择的任何适应性影响,然后在选择培养基上重新测试,以确认抗性的提高。然后通过对产物和底物定期取样进行HPLC分析,在摇瓶中测试抗性菌株。另外,对3-HP抗性的选择可以通过恒化器或系列摇瓶进化来完成。可以再进行几轮诱变和选择。诱变可用于提高天然并不能满足3-HP的生产需求的宿主的抗性,以便它具有用于商业生产3-HP所需的属性。

[0282] 实施例2A:将DNA转化进宿主基因组的步骤

[0283] 将DNA转化进酵母宿主的基因组中,以产生在下面实施例中所述的经修饰的酵母菌株,是基于下述的特定步骤来实施的。

[0284] 将4mL YP+10%葡萄糖的培养基加入到14mL Falcon管中,使用无菌环将所需的菌株接种到该培养基中。培养物在37℃下以250rpm振荡培养过夜(~16hr)。1mL的过夜培养物被加入到250mL含有50mL液体YP+10%葡萄糖培养基的挡板烧瓶中。烧瓶在37℃下以250rpm振荡培养。以约每小时的间隔时间取出少量等量的培养物,并测量OD600。培养物生长至OD600为0.6-1.0。

[0285] 室温下以2279×g离心收集细胞,将沉淀物重悬于25mL无菌水中,然后在室温下以2279×g离心。将沉淀重悬于1mL无菌水中,将重悬细胞转移至1.5mL管中,然后在16100×g下沉淀。将细胞重悬于1mL LiOAc/TE溶液中,然后在16100×g下沉淀。细胞沉淀物重悬于500μl LiOAc/TE溶液中。

[0286] 将下列组分加入到1.5mL管中:100μl上述细胞,10μl新鲜煮沸再冷冻的鲑鱼精子DNA(Agilent Technologies,Santa Clara,CA,USA),以及10μl所需的线性转化DNA。制备用水代替了DNA的对照反应。对于每个转化反应,加入600μl PEG/LiOAc/TE溶液,在30℃下,250rpm的摇床中每个反应在其一侧孵育30分钟。将40μl DMSO加入到每个反应中,然后在42℃的水浴中孵育5分钟。在5400×g下沉淀细胞1分钟。细胞重悬于水中,分为两份,将转化反应的每一半都涂布到ura选择培养平板上。平板被放置在37℃下。经过18至24hr的生长后,一般都可见菌落,这取决于菌株背景。

[0287] 用无菌环将小量的酵母从培养皿转移到含有300μl酵母裂解溶液(EPICENTRE®Biotechnologies,Madison,WI,USA)的1.5mL管中,并使用MasterPure™ Yeast DNA Purification KiT(EPICENTRE®Biotechnologies)根据制造商说明提取基因组DNA。

[0288] 用MasterPure™ Yeast DNA Purification KiT(EPICENTRE®Biotechnologies)制备的基因组DNA被用于PCR反应,以确定在分离的转化体中是否发生了正确的整合事件。PCR反应体系(25μl)包含0.5μl待筛选菌株的基因组DNA,1×Crimson Taq™反应缓冲液(New England Biolabs,Ipswich,MA,USA),有义和反义引物各25pmol,dATP,dCTP,dGTP和dTTP各200μM,以及0.625单位的Crimson Taq™ DNA聚合酶(New England Biolabs)。在EPPENDORF® MASTERCYCLER®(Eppendorf Scientific,Westbury,New York,USA)中进行PCR,其程序为一个循环的95℃ 30秒,接着是30个循环,每个循环95℃ 30秒,50℃ 30秒,68℃最大预期产物的每kbp 1分钟,最后68℃延伸10分钟。热循环之后,在TAE或TBE中用1%的琼脂糖凝胶电泳分离PCR反应产物,观察和解释如之前描述的特异性引物对所产生的条带的大小。

[0289] 实施例2B:插入位点的选择

[0290] 适合于将外源基因掺入到宿主酵母细胞中的插入位点可以是基因的基因座,当从酵母宿主细胞中缺失所述基因的基因座时,将会对3-HP的生产带来有益的或中性的影响。对于所选择的酵母菌株的合适插入位点的非限制性示例描述在本文所进行的实施例中。本领域技术人员可以很容易地应用本发明的教导来使用这些和其他的插入位点,例如,一个或多个PDC的基因座(例如,编码如SEQ ID NO:49所示的氨基酸序列和/或包括如SEQ ID NO:48所示的核苷酸序列的编码区域的东方伊萨酵母PDC基因),ADH基因座(例如,编码如SEQ ID NO:106,108或110所示的氨基酸序列和/或包含如SEQ ID NO:105,107或109所示的核苷酸序列的编码区域的东方伊萨酵母ADH基因),GAL6基因座(例如,编码如SEQ ID NO:112所示的氨基酸序列和/或包含如SEQ ID NO:111所示的核苷酸序列的编码区域的东方伊萨酵母GAL6基因),CYB2A基因座(例如,编码如SEQ ID NO:114所示的氨基酸序列和/或包含如SEQ ID NO:113所示的核苷酸序列的编码区域的东方伊萨酵母CYB2A基因),CYB2B基因座(例如,编码如SEQ ID NO:116所示的氨基酸序列和/或包含如SEQ ID NO:115所示的核苷酸序列的编码区域的东方伊萨酵母CYB2B基因),GPD基因座(例如,编码如SEQ ID NO:118所示的氨基酸序列和/或包含如SEQ ID NO:117所示的核苷酸序列的编码区域的东方伊萨酵母GPD基因),ALD基因座(例如,编码如SEQ ID NO:120所示的氨基酸序列和/或包含如SEQ ID NO:119所示的核苷酸序列的编码区域的东方伊萨酵母ALD同源基因5680,编码如SEQ ID NO:122所示的氨基酸序列和/或包含如SEQ ID NO:121所示的核苷酸序列的编码区域的东方伊萨酵母ALD同源基因42026,编码如SEQ ID NO:124所示的氨基酸序列和/或包含如SEQ ID NO:123所示的核苷酸序列的编码区域的东方伊萨酵母ALD同源基因42426,编码如SEQ ID NO:126所示的氨基酸序列和/或包含如SEQ ID NO:125所示的核苷酸序列的编码区域的东方伊萨酵母ALD同源基因42727),或PCK基因座(编码如SEQ ID NO:128所示的氨基酸序列和/或包含如SEQ ID NO:127所示的核苷酸序列的编码区域的东方伊萨酵母PCK基因)或其同源物。如果这些基因的序列并未公开,则可以利用标准步骤获得其序列,例如基因组测序,基因组或cDNA文库的探针杂交,或者基于已知同源序列设计的简并引物扩增基因片段,然后再进行基因组步行以获得全序列。插入位点的其他合适位置包括不含有开放阅读框的基因间隔区。

[0291] 实施例2C:插入载体,选择标志物盒,基因表达盒和整合构建体的技术

[0292] 产生插入位点载体以将一个或多个外源性基因整合进宿主酵母细胞中。宿主酵母细胞可以是经历过如实施例1中所描述的选择过程的细胞,或其可为未经诱变和/或选择的细胞。

[0293] 为了产生插入位点载体,用宿主基因组DNA作为模板扩增所需插入位点的上游区域(5')和下游区域(3')。上游区域优选大于70bp并小于1.5kbp。将所得的靶序列同时或顺序连接到克隆载体中,以获得含有每条片段的一个拷贝的载体,使得这些片段是连续的或者几乎是连续的。可在片段之间掺入一个独特地限制性位点,使得能够插入基因表达盒和/或选择标志物盒。独特地限制性酶切位点可以掺入至或者靠近上游片段的5'端,以及下游片段的3'端,以允许将来可以将这些位点间的DNA从克隆载体中移除。

[0294] 用于掺入至插入位点载体的选择标志物盒由常规克隆技术所产生。这些选择标志物盒包含可选择性标记的基因,并且也可含有上游启动子和/或下游终止子序列。合适的选

择标志物基因的示例包括URA3, TRP1, HIS, MEL5, CYB2A, LEU2, 和G418基因。侧翼序列可以在启动子/标记基因/终止子序列的任一侧被掺入表达盒, 以允许将来通过重组敲除该标记。这些侧翼序列可以包括直接的或倒置的重复序列(可以是功能性的或非功能性的序列) 或一个或多个loxP位点。

[0295] 基因表达盒利用常规克隆技术所产生。这些基因表达盒含有待过表达的基因, 也可含有上游启动子和/或下游终止子序列。在某些实施方式中, 可以将这些启动子/基因/终止子组合的两个或多个拷贝掺入到单个基因表达盒中。为了促进其在宿主酵母菌株中的表达, 异源基因可以经过密码子优化。本文中所述的选择标志物盒可以克隆进基因表达盒中, 使得它与待过表达的基因以及任何相关的启动子和/或终止子是连续的或几乎连续的。

[0296] 或者, 为了将天然启动子替换为外源启动子, 表达盒可以将待整合的启动子上游的选择盒整合进靶向序列之间。

[0297] 可以利用常规克隆技术将基因表达盒插入到本文所述插入位点载体的两个靶位点序列之间, 以产生基因表达整合构建体。一个或多个选择标志物盒也可以插入到靶序列之间, 无论是作为基因表达盒的一个部分还是独立插入。在某些变化方式中, 基因表达盒的片段可以被克隆到不同的插入位点载体中, 以使得这些整合片段之间有共同的重叠片段。例如, 一个载体可能含有上游插入片段, 启动子, 基因和终止子, 第二个载体可能含有终止子, 选择标志物盒和下游插入片段。在另一个实例中, 为了允许同时插入两个基因, 一个载体可能含有上游插入片段, 启动子, 基因, 终止子以及所有或部分的选择标志物盒, 第二个载体可能含有所有或部分的选择标志物盒, 第二启动子, 基因, 终止子, 以及下游插入片段。

[0298] 为了产生基因敲除构建体, 使用来源于待缺失或破坏的基因上游和下游侧翼区域的靶DNA序列来制作插入位点载体。所选择的靶序列可以包括靶基因的上游和下游侧翼区域和/或所有或部分靶基因编码序列或其调节元件(例如, 启动子或终止子)。一个或多个选择标志物盒可以被掺入到插入位点载体中的两个靶序列之间。当敲除与外源基因的表达偶联时, 还将一个或多个基因表达盒被掺入插入位点载体中。

[0299] 可以通过限制性酶消化来自克隆载体的片段或来自多个载体的重叠片段来使待整合到宿主酵母基因组中的DNA片段线性化。或者, 可以使用PCR, 或者PCR与限制性酶切的组合来产生线性的整合片段。插入位点侧翼区域可以是由于其本身存在于载体模板中或掺入到扩增引物中而掺入到整合片段中。在后一种情况中, 优选侧翼区域最少是70个核苷酸掺入到引物中。

[0300] 合适的插入载体, 选择标志物盒, 基因表达盒, 以及所选酵母菌株的整合构建体的非限制性示例描述于下面的具体实施例中。本领域技术人员能够很容易地应用从这些实施例中得到的教导以及前面的描述, 从而产生其他修饰的能够生产3-HP的酵母菌株。

[0301] 实施例2D: 在adh1202基因座表达外源基因的插入载体的构建

[0302] 构造质粒pMIBa107以允许在东方伊萨酵母adh1202基因座掺入单个基因, 该基因位于PDC启动子和终止子的控制之下, 使用URA3作为选择性标记。PCR扩增有ura选择性标记PDC启动子和终止子, 并克隆进如下所述的pCR4TM4BLUNT **TOPO**® (Invitrogen, La Jolla, CA, USA) 中。含有PDC启动子, 终止子和URA3选择性标记的PCR片段由SOE PCR构建。PDC启动子的扩增引物在PCR产物的3' 端含有PDC终止子的同源物, PDC终止子和URA3选择标志物的扩增引物在产物的5' 端与PDC启动子同源。然后将这两个片段通过SOE PCR置于一起。

[0303] 用引物0611184和0611195从pACN5 (图19) 中扩增PDC启动子。引物0611184将NotI限制性位点引入到PCR产物的5' 端。引物0611195则将XbaI限制性位点引入至PDC启动子后, 并在PCR产物的3' 端引入PDC终止子的同源物。

[0304] 根据制造商说明使用**Platinum®Pfx** DNA聚合酶 (Invitrogen) 来实施扩增反应。每个PCR反应含有0.5 μ l稀释的pACN5 (图19), 引物0611184和0611195各25pM, 1 $\times$ Pfx扩增缓冲液 (Invitrogen), 2mm的MgSO₄, 0.2mM dNTP混合物, 1.25单位的**Platinum®Pfx** DNA聚合酶 (Invitrogen), 终体积为50 μ l。在**EPENDORF® MASTERCYCLER®** (Eppendorf Scientific) 中孵育扩增反应物, 程序为95℃ 2分钟的1个循环, 随后是每个为95℃ 30秒, 55℃ 30秒和72℃ 2分钟的30个循环, 及72℃ 3分钟的最后延伸。

[0305] 以89mM Tris碱-89mM硼酸-2mM EDTA二钠缓冲液 (TBE) 中1%琼脂糖凝胶电泳来纯化PCR产物。一条约700bp的片段被从胶上切下, 并根据制造商的说明使用**QIAQUICK®** Gel Extracttion试剂盒 (Qiagen, Valencia, CA, USA) 从琼脂糖中提取。

[0306] 用引物0611189和0611185从pHJJ76 (图24) 中扩增PDC终止子和URA3选择标志物。引物0611189在PCR产物的5' 端引入了PDC启动子的同源物以及直接在PDC终止子前面引入PacI位点。引物0611185将一个NotI限制性位点引入到PCR产物的3' 端。根据制造商说明使用**Platinum®Pfx** DNA聚合酶 (Invitrogen) 来实施扩增反应。每个PCR反应含有0.5 μ l稀释的pHJJ76, 引物0611189和0611185各25pM, 1 $\times$ Pfx扩增缓冲液 (Invitrogen), 2mm的MgSO₄, 0.2mM dNTP混合物, 1.25单位的**Platinum®Pfx** DNA聚合酶 (Invitrogen), 终体积为50 μ l。在**EPENDORF® MASTERCYCLER®** (Eppendorf Scientific) 中孵育扩增反应物, 程序为95℃ 2分钟的1个循环, 随后是每个为95℃ 30秒, 55℃ 30秒和72℃ 2分钟的30个循环, 及72℃ 3分钟的最后延伸。

[0307] 以使用89mM Tris碱-89mM硼酸-2mM EDTA二钠缓冲液 (TBE) 的1%琼脂糖凝胶电泳来纯化PCR产物。一条约2000bp的片段被从胶上切下, 并根据制造商的说明使用**QIAQUICK®** Gel Extracttion试剂盒 (Qiagen, Valencia, CA, USA) 从琼脂糖中提取。

[0308] 使用SOE-PCR将2000bp的PDC终止子和URA3选择标志物PCR产物以及700bp的PDC启动子PCR产物融合。根据制造商说明使用**Platinum®Pfx** DNA聚合酶 (Invitrogen) 来实施扩增反应。每个PCR反应含有8ng 2000bp的PDC终止子和URA3选择标志物PCR产物, 24ng 700bp的PDC启动子PCR产物, 引物0611184和0611185各50pM, 1 $\times$ Pfx扩增缓冲液 (Invitrogen), 2mm的MgSO₄, 0.2mM dNTP混合物, 2.5单位的**Platinum®Pfx** DNA聚合酶 (Invitrogen), 终体积为100 μ l。在**EPENDORF® MASTERCYCLER®** (Eppendorf Scientific) 中孵育扩增反应反应物, 程序为95℃ 2分钟的1个循环, 随后是每个为95℃ 30秒, 55℃ 30秒和68℃ 3分钟的30个循环, 和68℃ 3分钟的1个循环。

[0309] 以使用89mM Tris碱-89mM硼酸-2mM EDTA二钠缓冲液 (TBE) 的1%琼脂糖凝胶电泳来纯化2700bpPCR产物。约2700bp的片段被从胶上切下, 并根据制造商的说明使用**QIAQUICK®** Gel Extracttion试剂盒 (Qiagen, Valencia, CA, USA) 从琼脂糖中提取。

[0310] 根据使用制造商说明, 用测序用Zero **Blunt®** **TOPO®** PCR克隆试剂盒

(Invitrogen) 将2700bp的PCR产物克隆进pCRTM4BLUNT **TOPO**® (Invitrogen) 载体中。在6μl的反应总体积中,将1μl或4μl 2700bp的PCR产物,1μl盐溶液 (Invitrogen) 和1μl pCRTM4BLUNT **TOPO**® (Invitrogen) 在室温下一起孵育15分钟。根据实验制造商说明,将每个克隆反应中取出2μl转化至One **Shot**® TOP10 Chemically Competent大肠杆菌细胞 (Invitrogen) 中。将转化体涂布在2X YT+amp平板上,37℃孵育过夜。通过NotI消化来筛选所得到的一些转化体,以筛选出所需PCR产物的合适插入。用DNA测序来证实产生所需大小条带的克隆的正确性并将其命名为pMIBa100。

[0311] 质粒pHJJ76 (图24) 含有同源性,以允许将基因整合到adh1202基因座上。用NotI消化质粒pHJJ76,以移除存在于adh1202同源序列内部的URA3选择标志物。用89mM Tris碱-89mM硼酸-2mM EDTA二钠缓冲液 (TBE) 中1%琼脂糖凝胶电泳来纯化消化后的pHJJ76。使用 **QIAQUICK**® Gel Extracttton试剂盒 (Qiagen,Valencia,CA,USA) 从琼脂糖中提取出一条5.2kbp长的片段,然后用T4DNA连接酶连接到一起。根据制造商说明,将连接产物转化至One **Shot**® TOP10 Chemically Competent大肠杆菌细胞 (Invitrogen) 中。将转化体涂布在2X YT+amp平板上,37℃孵育过夜。通过ApaI和SacI消化来筛选所得到的一些转化体。产生所需消化产物的克隆被命名为pHJJ76-no ura。

[0312] 将来自于pMIBa100 (见上) 的PDC启动子和终止子和URA3选择标志物克隆进pHJJ76-no ura中,以产生质粒,在该质粒中,为了在adh1202基因座进行整合,基因可以被置于PDC启动子和终止子的调控之下。用NotI消化pHJJ76-no ura,接着用CIP处理。用 **QIAQUICK**® PCR Purification试剂盒 (Qiagen) 纯化该线性的5.2kbp片段。用NotI消化pMIBa100,并用89mM Tris碱-89mM硼酸-2mM EDTA二钠缓冲液 (TBE) 来实施1%琼脂糖凝胶电泳。约2742bp的片段被从胶上切下,使用 **QIAQUICK**® Gel Extracttton试剂盒 (Qiagen,Valencia,CA,USA) 提取,然后用T4DNA连接酶将其连接至pHJJ76-no ura的5.2kbp片段上。根据制造商说明,将连接产物转化至One **Shot**® TOP10 Chemically Competent大肠杆菌细胞 (Invitrogen) 中。将转化体涂布在2X YT+amp平板上,37℃孵育过夜。通过KpnI和XbaI消化来筛选所得到的一些转化体。产生所需消化产物的克隆命名为pMIBa107 (图2)。

[0313] 实施例2E:在PDC基因座表达多个外源基因的插入载体片段的构造

[0314] 可以使用下列的插入载体片段来产生预定的DNA构建体,所述构建体是将内源性的东方伊萨酵母PDC基因替换为表达多个基因的表达盒,例如,本文所描述的从PDC,EN01和TDH3启动子表达的三个基因。左端构建体 (pMhCt068及其衍生体) 和右端构建体 (pMhCt069及其衍生体) 发生同源重组使URA3蛋白表达,导致该菌株由尿嘧啶营养缺陷型转化为尿嘧啶原养型,而允许对所需要的整合体进行选择。每个左端构建体的5'末端与PDC基因座的上游DNA同源,而每个右端构建体的3'末端则与PDC基因座的下游DNA同源。这些同源区域用于将表达盒靶向PDC基因座。在图3中示意性地示出了该靶向方法,并且可以利用本文所述的多个基因的任意组合来进行改进来靶向任意合适的位点,例如,上述的任意位点,例如ADH基因座 (见下述示例) 或ALD基因座。

[0315] 左端片段的构造

[0316] 对空的左端构建体载体,pMhCt068,进行如下所述多个步骤的克隆。

[0317] 用引物0611166和0611167从东方伊萨酵母的基因组DNA中扩增含有PDC启动子区域和所需的其它限制性位点以及侧翼DNA的PCR片段。

[0318] PCR反应体系 (50 μ L) 含有100ng东方伊萨酵母的基因组DNA (例如, 可使用来自于 **EPICENTRE®** Biotechnologies的MasterPure™ YeastDNA Purification Kit制备), 1X ThermoPol Reaction缓冲液 (New England Biolabs), 引物0611166和0611167每种100pmol, dATP、dCTP、dGTP和dTTP每种200 μ M, 2 μ L 100mM MgSO₄, 以及2个单位的 **Vent_R®** (exo-) DNA聚合酶 (New England Biolabs)。在**EPPENDORF® MASTERCYCLER®** (Eppendorf Scientific) 中进行PCR, 程序为94℃ 2分钟的1个循环, 随后是每个为94℃ 30秒, 54℃ 30秒和72℃ 1分钟的34个循环, 及72℃ 10分钟的最后延伸。热循环后, 以TAE缓冲液中0.9%琼脂糖凝胶电泳来分离PCR反应产物, 其中约780碱基对的PCR产物被从胶上切下并根据制造商的说明使用**NUCLEOSPIN®** Extract II试剂盒 (Macherey-Nagel, Bethlehem, PA, USA) 进行纯化。

[0319] 用引物0611168和0611169从pACN5 (图19) 中扩增含有TAL终止子区域和所需的其它限制性位点以及侧翼DNA的PCR片段。PCR反应体系 (50 μ L) 含有1 μ L pACN5小量制备质粒DNA, 1X iproof™ HF缓冲液 (Bio-Rad Laboratories, Hercules, CA, USA), 引物0611168和0611169每种100pmol, dATP、dCTP、dGTP和dTTP每种200 μ M, 1.5 μ L DMSO, 以及1个单位的 iProof™ High Fidelity DNA聚合酶 (Bio-Rad Laboratories)。在**EPPENDORF® MASTERCYCLER®** (Eppendorf Scientific) 中进行PCR, 程序为98℃ 30秒的1个循环, 随后是每个为98℃ 10秒, 59℃ 20秒和72℃ 45秒的324个循环, 及72℃ 10分钟的最后延伸。热循环后, 以TAE缓冲液中0.9%琼脂糖凝胶电泳来分离PCR反应产物, 其中约460碱基对的PCR产物被从胶上切下并根据制造商的说明使用**NUCLEOSPIN®** Extract II试剂盒 (Macherey-Nagel) 纯化。

[0320] 然后用PCR来构建融合了上述产物的单一扩增产物。PCR反应体系 (50 μ L) 含有125ng包含PCR产物的PDC启动子, 76ng包含PCR产物的TAL终止子, 1X ThermoPol Reaction缓冲液 (New England Biolabs), 引物0611166和0611169每种100pmol, dATP、dCTP、dGTP和dTTP每种200 μ M, 2 μ L 100mM MgSO₄, 以及2个单位的 **Vent_R®** (exo-) DNA聚合酶 (New England Biolabs)。在**EPPENDORF® MASTERCYCLER®** (Eppendorf Scientific) 中进行PCR, 程序为94℃ 2分钟的1个循环, 随后是每个为94℃ 30秒, 54℃ 30秒和72℃ 1分30秒的34个循环, 及72℃ 10分钟的最后延伸。热循环后, 以TAE缓冲液中0.9%琼脂糖凝胶电泳来分离PCR反应产物, 其中约1250碱基对的PCR产物被从胶上切下并根据制造商的说明使用**NUCLEOSPIN®** Extract II试剂盒 (Macherey-Nagel, Bethlehem, PA, USA) 进行纯化。

[0321] 用引物0611170和0611171从pACN43 (图22) 中扩增含有EN01启动子区域和所需的其它限制性位点以及侧翼DNA的PCR片段。PCR反应体系 (50 μ L) 含有1 μ L pACN43小量制备质粒DNA, 1X Phusion HF缓冲液 (New England Biolabs), 引物0611170和0611171每种100pmol, dATP、dCTP、dGTP和dTTP每种200 μ M, 以及1个单位的Phusion™ High Fidelity DNA聚合酶 (New England Biolabs)。在**EPPENDORF® MASTERCYCLER®** (Eppendorf Scientific) 中进行PCR, 程序为98℃ 30秒的1个循环, 随后是每个为98℃ 10秒, 59℃ 20秒

和72℃ 45秒的34个循环,及72℃ 10分钟的最后延伸。热循环后,以TAE缓冲液中0.9%琼脂糖凝胶电泳来分离PCR反应产物,其中约1050碱基对的PCR产物被从胶上切下并根据制造商的说明使用**NUCLEOSPIN®**Extract II试剂盒(Macherey-Nagel)纯化。

[0322] 用引物0611172和0611173从pACN43(图22)中扩增PCR片段以及其它限制性位点和侧翼DNA,该片段含有RKI终止子区域,其后是URA3启动子区域以及URA3ORF的5'末端。PCR反应体系(50μL)含有1μL pACN43小量制备质粒DNA,1X iproof™HF缓冲液(Bio-Rad Laboratories,Hercules,CA,USA),引物0611172和0611173每种100pmol,dATP、dCTP、dGTP和dTTP每种200μM,1.5μL DMSO,以及1个单位的iProof™High Fidelity DNA聚合酶(Bio-Rad Laboratories)。在**EPENDORF® MASTERCYCLER®**(Eppendorf Scientific)中进行PCR,程序为98℃ 30秒的1个循环,随后是每个为98℃ 10秒,59℃ 20秒和72℃ 45秒的34个循环,及72℃ 10分钟的最后延伸。热循环后,以TAE缓冲液中0.9%琼脂糖凝胶电泳来分离PCR反应产物,其中约1400碱基对的PCR产物被从胶上切下并根据制造商的说明使用**NUCLEOSPIN®**Extract II试剂盒(Macherey-Nagel)纯化。

[0323] 用PCR来构建融合了两个上述产物的单一扩增产物。PCR反应体系(50μL)含有93ng 包含PCR产物的EN01启动子(见上),125ng RKI终止子,URA3启动子和包含PCR产物的部分ORF(见上),1X Phusion HF缓冲液(New England Biolabs),引物0611170和0611173每种100pmol,dATP、dCTP、dGTP和dTTP每种200μM,1.5μL DMSO,以及1个单位的Phusion™High Fidelity DNA聚合酶(New England Biolabs)。在**EPENDORF® MASTERCYCLER®**(Eppendorf Scientific)中进行PCR,程序为98℃ 30秒的1个循环,随后是每个为98℃ 10秒,56℃ 20秒和72℃ 2分45秒的34个循环,及72℃ 10分钟的最后延伸。热循环后,以TAE缓冲液中0.9%琼脂糖凝胶电泳来分离PCR反应产物,其中约2460碱基对的PCR产物被从胶上切下并根据制造商的说明使用**NUCLEOSPIN®**Extract II试剂盒(Macherey-Nagel)进行纯化。

[0324] 为了为PCR产物创建接收载体,使用Hind III和EcoRI消化质粒pMhCt017(具有一个无关插入的标准克隆载体pUC19),用10个单位的小牛肠磷酸酶(New England Biolabs)在37℃下处理30分钟,和在TAE缓冲液中通过0.9%的琼脂糖凝胶电泳纯化,约2.6kbp的条带被从胶上切下并根据制造商的说明使用**NUCLEOSPIN®**Extract II试剂盒(Macherey-Nagel)纯化。所得到的Hind III至EcoRI纯化片段与pUC18中发现的相同(Yanisch-Perron,C.,Vieira,J.and Messing,J.(1985)Gene,33,103-119)。

[0325] 用IN-FUSION™ Advantage PCR Cloning KiT(Clontech)将上述纯化的1250bp和2460bp PCR产物插入到消化后的pMhCt017片段中,总反应体积为10μL,由125ng pMhCt017Hind III至EcoRI载体片段,92ng PDC启动子和TAL终止子PCR产物,165ng EN01启动子和URA3启动子以及含有PCR产物的部分ORF,1X In-Fusion反应缓冲液(Clontech)和1μL IN-FUSION™酶(Clontech)构成。将该反应孵育在37℃下15分钟,50℃下15分钟,然后置于冰上。将该反应体系用40μL TE缓冲液稀释,取出2.5μL根据制造商说明用于转化SoloPack Glod SuperCompetent Cells(Agilent Technologies)。将转化体涂布于2X YT+amp平板上,37℃孵育过夜。通过Apa LI消化来筛选所得到的一些转化体,其中有所需PCR产物的正确插入。用DNA测序命名来证实产生所需大小条带的克隆的正确性,并命名为pMhCt068。

[0326] 质粒pMhCt068含有PDC启动子区域,之后是NheI和AscI限制性位点,所述限制性位点可用于本文所述的感兴趣的异位基因的加入,TAL终止子,EN01启动子区域,在其后面的XbaI和PacI限制性位点(该限制性位点用于克隆本文所述的感兴趣的第二异位基因),RKI终止子,东方伊萨酵母URA3启动子和东方伊萨酵母URA3ORF的5'端。发现质粒pMhCt068在进入PDC启动子中大约200bp处有一个A至T的核苷酸改变,在进入PDC启动子的约2/3处有G至T的改变,以及位于NheI限制性位点的5'侧翼有一个提前起始密码子(ATG)。因此,pMhCt068的正确版本如下构建。

[0327] 用引物0611166和0611828从pACN5(图19)中PCR扩增PDC启动子区域,该扩增并没有引入上述不期望的起始密码子。PCR反应体系(50 μ L)含有1 μ L 1至50稀释度的pACN5小量制备质粒DNA,1X ThermoPol Reaction缓冲液(New England Biolabs),引物0611166和0611828每种100pmol,dATP、dCTP、dGTP和dTTP每种200 μ M,2 μ L 100mM MgSO₄,以及2个单位的Vent_R[®](exo-) DNA聚合酶(New England Biolabs)。在EPPENDORF[®] MASTERCYCLER[®](Eppendorf Scientific)中进行PCR,程序为94 $^{\circ}$ C 2分钟的1个循环,随后是每个为94 $^{\circ}$ C 30秒,54 $^{\circ}$ C 30秒和72 $^{\circ}$ C 1分钟的34个循环,及72 $^{\circ}$ C 10分钟的最后延伸。热循环后,以TAE缓冲液中0.9%琼脂糖凝胶电泳来分离PCR反应产物,其中约780碱基对的PCR产物被从胶上切下并根据制造商的说明使用NUCLEOSPIN[®]Extract II试剂盒(Macherey-Nagel)纯化。

[0328] 如上所述的方法将含有PCR产物的PDC启动子融合到含有PCR产物的TAL终止子上。由于TAL终止子PCR片段是由0611168引物所得到的,因此所得到的PCR融合产物应当是混合物,即缺失提前起始密码子的产物和含有不期望的起始密码子的产物。纯化所得到的~1250bp的PCR产物,并如前所述,通过IN-FUSION[™] Advantage PCR Cloning Kit(Clontech)将其与RKI终止子,URA3启动子和含有融合PCR产物和pUC18的部分ORF组合。DNA测序显示产生预期ApaLI消化图谱的克隆的正确性,包括PDC启动子中不存在突变和NheI限制性位点5'端的提前ATG的缺失(这些是期望的),并命名为pMhCt082。

[0329] 右端片段的构造

[0330] 对空的右端构建载体,pMhCt069,进行如下所述多个步骤的克隆。

[0331] 用引物0611174和0611175从pACN43(图22)中扩增含有东方伊萨酵母URA3ORF 3'末端,URA3终止子(URA3终止密码子下游275bp),URA3启动子(当整合进酵母宿主后,用作重复区域以环出标志物)以及期望的其它限制性位点和侧翼DNA的PCR片段。PCR反应体系(50 μ L)含有1 μ L pACN43小量制备质粒DNA,1X Phusion HF缓冲液(New England Biolabs),引物0611174和0611175每种100pmol,dATP、dCTP、dGTP和dTTP每种200 μ M,以及1个单位的Phusion[™] High Fidelity DNA聚合酶(New England Biolabs)。在EPPENDORF[®] MASTERCYCLER[®](Eppendorf Scientific)中进行PCR,程序为98 $^{\circ}$ C 30秒的1个循环,随后是每个为98 $^{\circ}$ C 10秒,59 $^{\circ}$ C 20秒和72 $^{\circ}$ C 45秒的34个循环,及72 $^{\circ}$ C 10分钟的最后延伸。热循环后,以TAE缓冲液中0.9%琼脂糖凝胶电泳来分离PCR反应产物,其中约1210bp的PCR产物被从胶上切下并根据制造商的说明使用NUCLEOSPIN[®] Extract II试剂盒(Macherey-Nagel)纯化。

[0332] 使用引物0611176和0611177从pACN23(图20)中扩增含有TDH3启动子区域和期望

的其它限制性位点和侧翼DNA的PCR片段。PCR反应体系(50 μ L)含有1 μ L pACN23小量制备质粒DNA,1X Phusion HF缓冲液(New England Biolabs),引物0611176和0611177每种100pmol,dATP、dCTP、dGTP和dTTP每种200 μ M,以及1个单位的PhusionTMHigh Fidelity DNA聚合酶(New England Biolabs)。在EPPENDORF® MASTERCYCLER®(Eppendorf Scientific)中进行PCR,程序为98℃ 30秒的1个循环,随后是每个为98℃ 10秒,59℃ 20秒和72℃ 45秒的34个循环,及72℃ 10分钟的最后延伸。热循环后,以TAE缓冲液中0.9%琼脂糖凝胶电泳来分离PCR反应产物,其中约1028碱基对的PCR产物被从胶上切下并根据制造商的说明使用NUCLEOSPIN®Extract II试剂盒(Macherey-Nagel)纯化。

[0333] 用引物0611178和0611179从东方伊萨酵母基因组DNA中扩增PCR片段,所述片段含有东方伊萨酵母PDC基因区域终止密码子的3'端区域(PDC终止子区域)和期望的另外限制性位点以及侧翼DNA。PCR反应体系(50 μ L)含有100ng东方伊萨酵母的基因组DNA,1X iproofTMHF缓冲液(Bio-Rad Laboratories),引物0611178和0611179每种100pmol,dATP、dCTP、dGTP和dTTP每种200 μ M,1.5 μ L DMSO,以及1个单位的iProofTMHigh Fidelity DNA聚合酶(Bio-Rad Laboratories)。在EPPENDORF® MASTERCYCLER®(Eppendorf Scientific)中进行PCR,程序为98℃ 30秒的1个循环,随后是每个为98℃ 10秒,59℃ 20秒和72℃ 45秒的34个循环,及72℃ 10分钟的最后延伸。热循环后,以TAE缓冲液中0.9%琼脂糖凝胶电泳来分离PCR反应产物,其中约938碱基对的PCR产物被从胶上切下并根据制造商的说明使用NUCLEOSPIN®Extract II试剂盒(Macherey-Nagel)纯化。

[0334] 用PCR来构建融合了两个上述最后产物的单一扩增产物。PCR反应体系(50 μ L)含有125ng包含PCR产物的TDH3启动子,114ng含有PCR产物的PDC终止子,1X Phusion HF缓冲液(New England Biolabs),引物0611176和0611179每种100pmol,dATP、dCTP、dGTP和dTTP每种200 μ M,1.5 μ L DMSO,以及1个单位的PhusionTMHigh Fidelity DNA聚合酶(New England Biolabs)。在EPPENDORF® MASTERCYCLER®(Eppendorf Scientific)中进行PCR,程序为98℃ 30秒的1个循环,随后是每个为98℃ 10秒,56℃ 20秒和72℃ 2分30秒的34个循环,及72℃ 10分钟的最后延伸。热循环后,以TAE缓冲液中0.9%琼脂糖凝胶电泳来分离PCR反应产物,其中约1966碱基对的PCR产物被从胶上切下并根据制造商的说明使用NUCLEOSPIN®Extract II试剂盒(Macherey-Nagel)进行纯化。

[0335] 随后用IN-FUSIONTMAdvantage PCR Cloning Kit(Clontech)将上述纯化的1210bp和1966bp PCR融合产物插入到见上所述的Hind III和EcoRI消化后的pMhCt017片段中,总反应体积为10 μ L,由125ng pMhCt017Hind III至EcoRI载体片段,54ng含有URA3ORF 3'末端及其后URA3终止子的PCR产物,200ng TDH3启动子和PDC终止子融合PCR产物,1X In-Fusion反应缓冲液(Clontech)和1 μ L IN-FUSIONTM酶(Clontech)构成。将该反应孵育在37℃下15分钟,50℃下15分钟,然后置于冰上。将该反应体系用40 μ L TE缓冲液稀释,取出2.5 μ L根据制造商说明用于转化SoloPack Glod SuperCompetentCells(Agilent Technologies)。将转化体涂布于2X YT+amp平板上,37℃孵育过夜。通过Apa LI消化来筛选所到一些转化体有所期望的PCR产物合适地插入。用DNA测序来证实产生所需大小条带的克隆的正确性,并命名为pMhCt069。

[0336] 质粒pMhCt069含有东方伊萨酵母URA3标记的3'末端,相应的URA3终止子,URA3启

动子(用于将来将URA3标记环出),TDH3启动子,用于亚克隆所需基因使其异位表达的XbaI和PacI限制性位点,以及PDC位点的3'侧翼区域。

[0337] 实施例2F:在adh9091基因座表达多个外源基因的插入载体片段的构造

[0338] 设计下面的插入载体片段的方法与实施例2E中所描述的类似,是为了将内源性的东方伊萨酵母adh9091基因替换为表达多个本文所述的感兴趣的基因的表达式。

[0339] 左端片段的构造

[0340] 对空的左端构建载体,pGREr125,进行如下所述多个步骤的克隆。

[0341] 含有在东方伊萨酵母adh9091基因座发生同源重组所需的5'端侧翼的构建体,以及PDC启动子/TAL终止子空表达盒被PCR克隆进载体质粒pCR2.1-TOP0(Invitrogen)。用引物0611250和0611251从质粒pACN5(图19)中PCR扩增PDC启动子片段。PCR反应体系(50 μ L)含有15ng pACN5质粒DNA,1X Phusion HF缓冲液(New England Biolabs),引物0611250和0611251每种50pmol,dATP、dCTP、dGTP和dTTP每种200 μ M,1.5 μ L 100%DMSO,以及1个单位的PhusionTMHigh Fidelity DNA聚合酶(New England Biolabs)。在EPPENDORF® MASTERCYCLER®(Eppendorf Scientific)中进行PCR,程序为98℃ 2分钟的1个循环,随后是每个为98℃ 30秒,55℃ 30秒和72℃ 1分30秒的35个循环,及72℃ 7分钟的最后延伸。热循环后,以TBE缓冲液中0.8%琼脂糖凝胶电泳来分离PCR反应产物,其中约900bp的PCR产物被从胶上切下并根据制造商的说明使用QIAQUICK® Gel Extracttton试剂盒(Qiagen)从琼脂糖中纯化。所得到的PCR片段的总长度为约753bp,在该片段的5'末端有NotI限制性位点,其3'末端有PacI和XbaI限制性位点。

[0342] 产生第二PCR片段以使用引物0611252和0611253从质粒pACN5(图19)中扩增TAL终止子区域,所述第二片段含有上述PCR产物的5'同源物,包括XbaI和PacI限制性位点。PCR反应体系(50 μ L)含有15ng质粒pACN5DNA(见上),1X Phusion HF缓冲液(New England Biolabs),引物0611252和0611253每种50pmol,dATP、dCTP、dGTP和dTTP每种200 μ M,1.5 μ L 100%DMSO,以及1个单位的PhusionTMHigh Fidelity DNA聚合酶(New England Biolabs)。在EPPENDORF® MASTERCYCLER®(Eppendorf Scientific)中进行PCR,程序为98℃ 2分钟的1个循环,随后是每个为98℃ 30秒,55℃ 30秒和72℃ 1分30秒的35个循环,有72℃ 7分钟的最后延伸。热循环后,以TBE缓冲液中0.8%琼脂糖凝胶电泳来分离PCR反应产物,其中约900bp的PCR产物被从胶上切下并根据制造商的说明使用QIAQUICK® Gel Extracttton试剂盒(Qiagen)从琼脂糖中纯化。所得到的PCR片段的总长度为约435bp,在该片段的5'末端有PacI和XbaI限制性位点,3'末端有PmeI限制性位点。

[0343] 用引物0611250和0611253通过PCR将753bp的片段和435bp的片段融合,结果得到一条1149bp的片段,其中,PDC启动子在TAL终止子的上游。PCR反应体系(50 μ L)含有125ng 753bp片段,75ng 435bp片段,1X Phusion HF缓冲液(New England Biolabs),引物0611250和0611253每种50pmol,dATP、dCTP、dGTP和dTTP每种200 μ M,1.5 μ L 100%DMSO,以及1个单位的PhusionTMHigh Fidelity DNA聚合酶(New England Biolabs)。在EPPENDORF® MASTERCYCLER®(Eppendorf Scientific)中进行PCR,程序为98℃ 2分钟的1个循环,随后是每个为98℃ 30秒,55℃ 30秒和72℃ 1分钟的35个循环,及72℃ 7分钟的最后延伸。热循环后,以TBE缓冲液中0.8%琼脂糖凝胶电泳来分离PCR反应产物,其中约1149bp对

的PCR产物被从胶上切下并根据制造商的说明使用QIAQUICK®Gel Extracttion试剂盒(Qiagen)从琼脂糖中纯化。

[0344] 产生PCR片段以使用引物0611254和0611255扩增东方伊萨酵母adh9091基因座的5'侧翼,所述第二片段含有上述1149bp PCR产物的3'同源物,包括NotI限制性位点。PCR反应体系(50μL)含有15ng质粒pHJJ27(图21)作为模板DNA,1X Phusion HF缓冲液(New England Biolabs),引物0611254和0611255每种50pmol,dATP、dCTP、dGTP和dTTP每种200μM,1.5μL 100%DMSO,以及1个单位的Phusion™High Fidelity DNA聚合酶(New England Biolabs)。在EPPENDORF® MASTERCYCLER®(Eppendorf Scientific)中进行PCR,程序为98℃ 2分钟的1个循环,随后是每个为98℃ 30秒,55℃ 30秒和72℃ 1分30秒的35个循环,有72℃ 7分钟的最后延伸。热循环后,以TBE缓冲液中0.8%琼脂糖凝胶电泳来分离PCR反应产物,其中约900bp的PCR产物被从胶上切下并根据制造商的说明使用QIAQUICK®Gel Extracttion试剂盒(Qiagen)从琼脂糖中纯化。所得到的PCR片段的总长度为约891bp,在该片段的5'末端有HpaI限制性位点,3'末端有NotI限制性位点。

[0345] 用引物0611254和0611253,利用PCR将891bp片段融合到1149bp的PDC启动子/TAL终止子片段上,产生约2005bp的片段。PCR反应体系(50μL)含有125ng的1149bp片段,95ng的891bp片段,1X Phusion HF缓冲液(New England Biolabs),引物0611254和0611253每种50pmol,dATP、dCTP、dGTP和dTTP每种200μM,1.5μL 100%DMSO,以及1个单位的Phusion™High Fidelity DNA聚合酶(New England Biolabs)。在EPPENDORF® MASTERCYCLER®(Eppendorf Scientific)中进行PCR,程序为98℃ 2分钟的1个循环,随后是每个为98℃ 30秒,55℃ 30秒和72℃ 2分钟的35个循环,及72℃ 7分钟的最后延伸。热循环后,以TBE缓冲液中0.8%琼脂糖凝胶电泳来分离PCR反应产物,其中约2005bp的PCR产物被从胶上切下并根据制造商的说明使用QIAQUICK®Gel Extracttion试剂盒(Qiagen)从琼脂糖中纯化。

[0346] 所得到的2005bp片段,包含用于在adh9091基因座整合的5'侧翼,PDC启动子和TAL终止子,被克隆进pCR2.1-TOPO载体中,使用TOPO TA Cloning Kit(Invitrogen)根据操作说明转化进One-Shot Top10大肠杆菌细胞中。将转化体涂布于2X YT+amp平板上,37℃孵育过夜。通过AvaI消化来筛选到一些转化体有所期望的PCR产物的正确插入。产生所需大小条带的克隆被确认并命名为pGMEr112。质粒pGMEr112包含用于在adh9091基因座进行同源重组的5'侧翼,其后是空的表达盒PDC启动子/TAL终止子。

[0347] 用引物0611283和0611263通过PCR从质粒pHJJ27(图21)中扩增由URA3启动子片段驱动的5'截短的URA3标记基因。PCR反应体系(50μL)含有15ng质粒pHJJ27,1X Phusion HF缓冲液(New England Biolabs),引物0611263和0611283每种50pmol,dATP、dCTP、dGTP和dTTP每种200μM,1.5μL 100%DMSO,以及1个单位的Phusion™High Fidelity DNA聚合酶(New England Biolabs)。在EPPENDORF® MASTERCYCLER®(Eppendorf Scientific)中进行PCR,程序为98℃ 2分钟的1个循环,随后是每个为98℃ 30秒,55℃ 30秒和72℃ 1分30秒的35个循环,及72℃ 7分钟的最后延伸。热循环后,以TBE缓冲液中0.8%琼脂糖凝胶电泳来分离PCR反应产物,其中约900bp的PCR产物被从胶上切下并根据制造商

的说明使用 **QIAQUICK®** Gel Extracttion 试剂盒 (Qiagen) 从琼脂糖中纯化。所得到的 PCR 片段的总长度为约 885bp, 在该片段的 5' 末端有 HpaI 和 PmeI 限制性位点, 3' 末端有 NheI 限制性位点。

[0348] 将所得到的 885bp 片段克隆进 pCR2.1-TOPO 载体中, 使用 TOPO TA Cloning Kit (Invitrogen) 根据制造商说明转化进 One-Shot Top10 大肠杆菌细胞中。将转化体涂布于 2X YT+amp 平板上, 37℃ 孵育过夜。通过 BglI 消化来筛选到一些有所期望的 PCR 产物的正确插入的转化体。产生所需大小条带的克隆被确认并命名为 pGMEr108。质粒 pGMEr108 包括 URA3 启动子, 其后是 URA3 基因的 5' 截短片段, 该片段的侧翼 5' 末端是 HpaI 和 PmeI 限制性位点, 3' 末端则是 NotI 限制性位点。

[0349] 将来自于质粒 pGMEr112 (见上) 的 1998bp 的 HpaI 和 PmeI 限制性片段连接至由 HpaI 和 PmeI 线性化的来自于 pGMEr108 (见上) 的 4806bp 载体上, 所述限制性片段包含 5' adh9091 侧翼, 其后是构建体 PDC 启动子/TAL 终止子。以 TBE 缓冲液中 0.8% 琼脂糖凝胶电泳来分离双限制性酶切反应产物, 其中约 1998bp 的插入片段和 4806bp 的载体片段被从胶上切下并根据制造商的说明使用 **QIAQUICK®** Gel Extracttion 试剂盒 (Qiagen) 从琼脂糖中纯化。使用 1:3 的载体: 插入片段比例来进行连接反应, 特别的, 建立的反应体系有 2μL 4806bp 线性化载体, 6μL 1998bp 插入片段, 9μL 2X Quick Ligation 反应缓冲液以及 1μL Quick T4DNA 连接酶 (New England Biolabs), 根据制造商说明来实施。

[0350] 根据制造商说明, 将 5 μL 连接产物转化到大肠杆菌 **XL10-Gold®** Ultracompetent Cells (Agilent Technologies) 中。将转化体涂布于 2X YT+amp 平板上, 37℃ 孵育过夜。通过 HindIII 消化来筛选到一些有所期望的 PCR 产物的正确插入的转化体。产生所需大小条带的克隆被确认并命名为 pGMEr117。

[0351] 质粒 pGMEr117 包括 5' adh9091 侧翼, 其后是空的表达盒 PDC 启动子/TAL 终止子以及由 URA3 驱动的截短的 5' URA3 基因。此外, 质粒 pGMEr117 有两个不同的 XbaI 限制性位点: 第一个限制性位点位于 PDC 启动子和 TAL 终止子之间 (靠近限制性位点 PacI), 该位点可用于插入感兴趣的基因, 第二个 XbaI 限制性位点则来自于原始 pCR2.1-TOPO 的骨架。为了消除这第二个 XbaI 限制性位点, 用 ApaI 消化质粒 pGMEr117, 然后根据制造商说明用酶 DNA 聚合酶 I, 大 (Klenow) 片段 (New England Biolabs) 处理线性化的质粒。将得到的线性化质粒 (含有平末端) 用限制性酶 EcoRV 消化, 其从含有 XbaI 限制性位点载体中将一条 43bp 的片段切割出来。以 TBE 缓冲液中 0.8% 琼脂糖凝胶电泳来分离限制性酶切反应产物, 并将约 6761bp 的载体片段被从胶上切下并根据制造商的说明使用 **QIAQUICK®** Gel Extracttion 试剂盒 (Qiagen) 从琼脂糖中纯化。建立自连接反应体系, 其具有 3μL 线性化载体, 6μL 灭菌双蒸水, 10μL 2X Quick Ligation 反应缓冲液以及 1μL Quick T4DNA 连接酶 (New England Biolabs), 根据制造商说明来实施。

[0352] 根据制造商说明, 将 5 μL 连接产物转化到大肠杆菌 **XL10-Gold®** Ultracompetent Cells (Agilent Technologies) 中。将转化体涂布于 2X YT+amp 平板上, 37℃ 孵育过夜。通过 XbaI 消化来筛选到一些有所期望的 PCR 产物的正确插入的转化体。产生所需大小条带的克隆被确认并命名为 pGMEr122。

[0353] 用引物 0611295 和 0611296 从质粒 pACN43 (图 22) 中 PCR 扩增 EN01 启动子片段。PCR 反

应体系 (50 μ L) 含有15ng pACN43质粒,1X Phusion HF缓冲液(New England Biolabs),引物0611295和0611296每种50pmol,dATP、dCTP、dGTP和dTTP每种200 μ M,1.5 μ L 100%DMSO(New England Biolabs)以及1个单位的PhusionTMHigh Fidelity DNA聚合酶(New England Biolabs)。在EPPENDORF® MASTERCYCLER®(Eppendorf Scientific)中进行PCR,程序为98℃ 2分钟的1个循环,随后是每个为98℃ 30秒,55℃ 30秒和72℃ 1分钟的35个循环,及72℃ 7分钟的最后延伸。热循环后,以TBE缓冲液中0.8%琼脂糖凝胶电泳来分离PCR反应产物,其中约1009bp的PCR产物被从胶上切下并根据制造商的说明使用QIAQUICK®Gel Extracttion试剂盒(Qiagen)纯化。所得到的PCR片段的总长度约为1009bp,在片段的5'末端具有PmeI限制性位点,在3'末端有ApaI和NruI限制性位点。

[0354] 产生第二PCR片段以使用引物0611297和0611298从质粒pACN43(图22)中扩增RKI终止子,所述第二片段含有上述PCR产物的5'同源物,包括NruI和ApaI限制性位点。PCR反应体系(50 μ L)含有15ng质粒pACN43DNA,1X Phusion HF缓冲液(New England Biolabs),引物0611297和0611298每种50pmol,dATP、dCTP、dGTP和dTTP每种200 μ M,1.5 μ L 100%DMSO(New England Biolabs),以及1个单位的PhusionTMHigh Fidelity DNA聚合酶(New England Biolabs)。在EPPENDORF® MASTERCYCLER®(Eppendorf Scientific)中进行PCR,程序为98℃ 2分钟的1个循环,随后是每个为98℃ 30秒,55℃ 30秒和72℃ 1分钟的35个循环,及72℃ 7分钟的最后延伸。热循环后,以TBE缓冲液中0.8%琼脂糖凝胶电泳来分离PCR反应产物,其中约438bp的PCR产物被从胶上切下并根据制造商的说明使用QIAQUICK®Gel Extracttion试剂盒(Qiagen)从琼脂糖中纯化。所得到的PCR片段的总长度为约438bp,在该片段的5'末端有NruI和ApaI限制性位点,3'末端有PmeI限制性位点。

[0355] 用引物0611295和0611298通过PCR将1009bp的启动子片段和438bp的终止子片段融合在一起,产生一条约1447bp的片段,该片段中EN01启动子位于RKI终止子上游。PCR反应体系(50 μ L)含有125ng 1009bp片段,65ng 438bp片段,1X Phusion HF缓冲液(New England Biolabs),引物0611295和0611298每种50pmol,dATP、dCTP、dGTP和dTTP每种200 μ M,1.5 μ L DMSO(New England Biolabs),以及1个单位的PhusionTMHigh Fidelity DNA聚合酶(New England Biolabs)。在EPPENDORF® MASTERCYCLER®(Eppendorf Scientific)中进行PCR,程序为98℃ 2分钟的1个循环,随后是每个为98℃ 30秒,55℃ 30秒和72℃ 2分钟的35个循环,及72℃ 7分钟的最后延伸。热循环后,以TBE缓冲液中0.8%琼脂糖凝胶电泳来分离PCR反应产物,其中约1447bp对的PCR产物被从胶上切下并根据制造商的说明使用QIAQUICK®Gel Extracttion试剂盒(Qiagen)从琼脂糖中纯化。

[0356] 所得到的1447bp的片段,包含EN01启动子/RKI终止子构建体,克隆进pCR2.1-TOPO载体中,使用TOPO TA Cloning KiT(Invitrogen)根据制造商说明转化进One-Shot Top10大肠杆菌细胞中。将转化体涂布于2X YT+amp平板上,37℃孵育过夜。通过BamHI消化来筛选到一些有所期望的PCR产物的正确插入的转化体。产生所需大小条带的克隆被确认并命名为pGMEr114,其包含空的表达盒EN01启动子/RKI终止子。

[0357] 用限制性酶PmeI在37℃下对质粒pGMEr122和pGMEr114消化3小时。在终止各个消化反应前1小时,向每个消化管中加入1 μ L牛肠碱性磷酸酯酶(New England Biolabs),以使末端去磷酸化防止自连接。所得到的来自于质粒pGMEr122的6761bp载体片段,以及包含

来自于质粒pGMEr144的构建体EN01启动子/TAL终止子(1439bp)的插入片段,在1X TBE缓冲液中通过0.8%琼脂糖凝胶电泳来分离,并从凝胶中切割下来,根据制造商的说明使用QIAQUICK®Gel Extracttion试剂盒(Qiagen)从琼脂糖中纯化。

[0358] 随后制备后续的连接反应,其包括3μL来自于质粒pGMEr122的载体片段,4μL来自于质粒pGMEr114的插入片段,2μL无菌dd水,10μL 2X Quick Ligation反应缓冲液以及1μL Quick T4DNA连接酶(New England Biolabs),根据制造商说明来实施。根据制造商说明,将5μL上述连接反应的等分试样转化到大肠杆菌 XL10-Gold®Ultracompetent Cells (Agilent Technologies)中。将转化体涂布于2X YT+amp平板上,37℃孵育过夜。通过XbaI和BstBI消化来筛选到一些有所期望的PCR产物的正确插入的转化体。产生所需大小条带的克隆通过测序被确认并命名为pGMEr125。EN01启动子/RKI终止子构建体以相反的取向被插入,导致了两个版本的命名为(a)和(b)的质粒pGMEr125(图4和5)。

[0359] 质粒pGMEr125a和pGMEr125b含有PDC启动子区域,TAL终止子,EN01启动子区域,RKI终止子,东方伊萨酵母URA3启动子,相应的URA3标记的5'末端以及东方伊萨酵母adh9091基因座的5'侧翼区域。

[0360] 右端片段的构建

[0361] 对空的右端构建体载体,pGREr121,进行如下所述多个步骤的克隆。

[0362] 用引物0611256和0611257从质粒pACN23(图20)中PCR扩增TDH3启动子片段。PCR反应体系(50μL)含有15ng pACN23质粒DNA,1X Phusion HF缓冲液(New England Biolabs),引物0611256和0611257每种50pmol,dATP、dCTP、dGTP和dTTP每种200μM,1.5μL 100%DMSO(New England Biolabs),以及1个单位的Phusion™High Fidelity DNA聚合酶(New England Biolabs)。在EPPENDORF® MASTERCYCLER®(Eppendorf Scientific)中进行PCR,程序为98℃ 2分钟的1个循环,随后是每个为98℃ 30秒,55℃ 30秒和72℃ 1分30秒的35个循环,及72℃ 7分钟的最后延伸。热循环后,以TBE缓冲液中0.8%琼脂糖凝胶电泳来分离PCR反应产物,其中约994bp的PCR产物被从胶上切下并根据制造商的说明使用QIAQUICK®Gel Extracttion试剂盒(Qiagen)从琼脂糖中纯化。所得到的PCR片段的总长度为约994bp,在该片段的5'末端有SfoI限制性位点,其3'末端有PacI和NruI限制性位点。

[0363] 产生第二PCR片段以使用引物0611258和0611259从质粒pACN23(图20)中扩增TKL终止子,所述第二片段含有上述PCR产物的5'同源物,包括NruI和PacI限制性位点。PCR反应体系(50μL)含有15ng质粒pACN23DNA,1X Phusion HF缓冲液(New England Biolabs),引物0611258和0611259每种50pmol,dATP、dCTP、dGTP和dTTP每种200μM,1.5μL 100%DMSO(New England Biolabs),以及1个单位的Phusion™High Fidelity DNA聚合酶(New England Biolabs)。在EPPENDORF® MASTERCYCLER®(Eppendorf Scientific)中进行PCR,程序为98℃ 2分钟的1个循环,随后是每个为98℃ 30秒,55℃ 30秒和72℃ 1分30秒的35个循环,及72℃ 7分钟的最后延伸。热循环后,以TBE缓冲液中0.8%琼脂糖凝胶电泳来分离PCR反应产物,其中约469bp的PCR产物被从胶上切下并根据制造商的说明使用QIAQUICK®Gel Extracttion试剂盒(Qiagen)从琼脂糖中纯化。所得到的PCR片段的总

长度约为469bp,在该片段的5'末端有NruI和PacI限制性位点,3'末端有NotI限制性位点。

[0364] 用引物0611256和0611259通过PCR将上述994bp和469bp的片段融合在一起,产生一条约1433bp的片段,该片段中TDH3启动子位于TKL终止子上游。PCR反应体系(50 μ L)含有125ng 994bp片段,60ng 469bp片段,1X Phusion HF缓冲液(New England Biolabs),引物0611256和0611259每种50pmol,dATP、dCTP、dGTP和dTTP每种200 μ M,1.5 μ L 100%DMSO(New England Biolabs),以及1个单位的PhusionTMHigh Fidelity DNA聚合酶(New England Biolabs)。在EPPENDORF® MASTERCYCLER® (Eppendorf Scientific)中进行PCR,程序为98℃ 2分钟的1个循环,随后是每个为98℃ 30秒,55℃ 30秒和72℃ 1分钟的35个循环,及72℃ 7分钟的最后延伸。热循环后,以TBE缓冲液中0.8%琼脂糖凝胶电泳来分离PCR反应产物,其中约1433bp的PCR产物被从胶上切下并根据制造商的说明使用QIAQUICK® Gel Extracttion试剂盒(Qiagen)从琼脂糖中纯化。

[0365] 产生第二PCR片段以使用引物0611260和0611261扩增adh9091基因座的3'侧翼,所述第二片段含有上述1433bp的PCR产物3'末端的5'同源物,包括NotI限制性位点。PCR反应体系(50 μ L)含有15ng质粒pHJJ27DNA作为模板DNA,1X Phusion HF缓冲液(New England Biolabs),引物0611260和0611261每种50pmol,dATP、dCTP、dGTP和dTTP每种200 μ M,1.5 μ L 100%DMSO(New England Biolabs),以及1个单位的PhusionTMHigh Fidelity DNA聚合酶(New England Biolabs)。在EPPENDORF® MASTERCYCLER® (Eppendorf Scientific)中进行PCR,程序为98℃ 2分钟的1个循环,随后是每个为98℃ 30秒,55℃ 30秒和72℃ 1分30秒的35个循环,及72℃ 7分钟的最后延伸。热循环后,以TBE缓冲液中0.8%琼脂糖凝胶电泳来分离PCR反应产物,其中约1019bp的PCR产物被从胶上切下并根据制造商的说明使用QIAQUICK® Gel Extracttion试剂盒(Qiagen)从琼脂糖中纯化。所得到的PCR片段的总长度为约1019bp,在该片段的5'末端有NotI限制性位点,3'末端有ApaI限制性位点。

[0366] 用引物0611256和0611261通过PCR将1019bp的片段融合至1433bp的TDH3启动子/TKL终止子片段下游,产生约2405bp的片段。PCR反应体系(50 μ L)含有125ng 1433bp片段,90ng 1019bp片段,1X Phusion HF缓冲液(New England Biolabs),引物0611256和0611261每种50pmol,dATP、dCTP、dGTP和dTTP每种200 μ M,1.5 μ L 100%DMSO(New England Biolabs),以及1个单位的PhusionTMHigh Fidelity DNA聚合酶(New England Biolabs)。在EPPENDORF® MASTERCYCLER® (Eppendorf Scientific)中进行PCR,程序为98℃ 2分钟的1个循环,随后是每个为98℃ 30秒,55℃ 30秒和72℃ 2分钟的35个循环,及72℃ 7分钟的最后延伸。热循环后,以TBE缓冲液中0.8%琼脂糖凝胶电泳来分离PCR反应产物,其中约2405bp的PCR产物被从胶上切下并根据制造商的说明使用QIAQUICK® Gel Extracttion试剂盒(Qiagen)从琼脂糖中纯化。

[0367] 将所得到的2405bp的片段,其包含用于在位于TDH3启动子/TAL终止子构建体下游adh9091基因座发生整合的3'侧翼,克隆进pCR2.1-TOPO载体中,并使用TOPO TA Cloning KiT(Invitrogen)根据制造商说明转化进One-Shot Top10大肠杆菌细胞中。将转化体涂布于2X YT+amp平板上,37℃孵育过夜。通过AvaI消化来筛选到一些有所期望的PCR产物的正确插入的所得的转化体。产生所需大小条带的克隆被确认并命名为pGMEr113。质粒

pGMEr113包含用于在东方伊萨酵母adh9091位点发生同源重组的3'侧翼,及其前面的空的表达盒TDH3启动子/TKL终止子。

[0368] 用引物0611264和0611284从质粒pHJJ27(图21)中扩增URA3ORF的截短的3'片段,URA3终止子和URA3启动子(如上所述,当整合进酵母宿主后,用作重复区域以环出标记)。PCR反应体系(50 μ L)含有15ng pHJJ27质粒DNA,1X Phusion HF缓冲液(New England Biolabs),引物0611264和0611284每种50pmol,dATP、dCTP、dGTP和dTTP每种200 μ M,1.5 μ L 100%DMSO(New England Biolabs),以及1个单位的PhusionTMHigh Fidelity DNA聚合酶(New England Biolabs)。在EPPENDORF® MASTERCYCLER®(Eppendorf Scientific)中进行PCR,程序为98℃ 2分钟的1个循环,随后是每个为98℃ 30秒,55℃ 30秒和72℃ 1分30秒的35个循环,及72℃ 7分钟的最后延伸。热循环后,以TBE缓冲液中0.8%琼脂糖凝胶电泳来分离PCR反应产物,其中约1324bp的PCR产物被从胶上切下并根据制造商的说明使用QIAQUICK®Gel Extracttion试剂盒(Qiagen)纯化。所得到的PCR片段的总长度为约1324bp,在其5'端有NheI限制性位点,3'端则有ApaI和SfoI限制性位点。

[0369] 将上述凝胶纯化的1324bp片段克隆进pCR2.1-TOPO载体中,使用TOPO TA Cloning KiT(Invitrogen)根据制造商说明转化进One-Shot Top10大肠杆菌细胞中。将转化体涂布于2X YT+amp平板上,37℃孵育过夜。通过HindIII消化来筛选到一些有所期望的PCR产物的正确插入的所得的转化体。产生所需大小条带的克隆被确认并命名为pGMEr109。质粒pGMEr109包含URA3ORF的3'片段和URA3终止子,其后是URA3启动子。质粒pGMEr109中URA3基因3'片段的上游部分与克隆进质粒pGMEr108的截短的5' URA3片段的末端具有460bp的同源性。该同源区域允许该基因的这两个部分发生重组,以在用含有这两个片段的构建体对宿主进行共转化时,能产生功能性的选择标志物。

[0370] 用KpnI消化质粒pGMEr109,然后根据制造商说明用酶DNA聚合酶I,大(Klenow)片段(New England Biolabs)进行处理。将得到的线性化质粒(含有平末端)用限制性酶BamHI消化。以TBE缓冲液中0.8%琼脂糖凝胶电泳来分离产物,其中5247bp的载体片段被从胶上切下并根据制造商的说明使用QIAQUICK®Gel Extracttion试剂盒(Qiagen)从琼脂糖中纯化。

[0371] 用BamHI和EcoRV来消化质粒pGMEr113,得到2466bp的片段,该片段具有构建体TDH3启动子/TKL终止子,其后为URA3ORF的截短3'片段和URA3终止子,其后为URA3启动子。以TBE缓冲液中0.8%琼脂糖凝胶电泳来分离双限制性酶切反应产物,其中约2466bp的载体片段被从胶上切下并根据制造商的说明使用QIAQUICK®Gel Extracttion试剂盒(Qiagen)从琼脂糖中纯化。2644bpBamHI和EcoRV消化片段随后被连接进来自质粒pGMEr109的5247bp载体片段。连接反应设置为包含3 μ L 5247bp的线性载体,3 μ L2466bp的插入片段,3 μ L无菌dd水,10 μ L 2X Quick Ligation反应缓冲液以及1 μ L Quick T4DNA连接酶(New England Biolabs),根据制造商说明来实施。

[0372] 根据制造商说明,将5 μ L连接反应产物转化到大肠杆菌 XL10-Gold® Ultracompetent Cells(Agilent Technologies)中。将转化体涂布于2X YT+amp平板上,37℃孵育过夜。通过XbaI和PacI消化来筛选到一些有所期望的PCR产物的正确插入的所得的转化体。产生所需大小条带的克隆通过测序被确认并命名为pGMEr121(图6)。

[0373] 质粒pGMEr121含有东方伊萨酵母URA3标记的3'末端,其后为相应的URA3启动子,TDH3启动子,TKL终止子和adh9091位点的3'侧翼区域。

[0374] 实施例2G:东方伊萨酵母CNB1的构造

[0375] 如下所述,从东方伊萨酵母CD1822中构建东方伊萨酵母CNB1(对于从东方伊萨酵母ATCC PTA-6658中生产东方伊萨酵母CD1822,见实施例1A)。菌株CD1822中含有的URA3基因的两个拷贝都被缺失以允许该基因能够成为遗传工程的选择标志物。URA3是酵母遗传学中的一种多用途标志物,这是由于该基因无论是存在(在尿嘧啶缺失的培养基上生长)还是缺失(在5-氟乳清酸存在的情况下生长)都可以用于选择。通过利用选择盒进行替换来破坏其中一个URA3基因,所述选择盒含有MEL5选择标志物,其侧翼为重复DNA序列。对检测为MEL5选择盒阳性的菌株就MEL5标记基因的缺失进行筛选。然后通过5-氟乳清酸存在的情况下生长来选择第二个URA3基因缺失。

[0376] 用2.8μg经SacI/PspOMI消化的载体pMI458(图27)的DNA转化CD1822。质粒pMI458含有酿酒酵母(*S.cerevisiae*)MEL5基因(SEQ ID NO:255),该基因位于东方伊萨酵母PGK启动子的调控之下(P-loPGK,SEQ ID NO:247),其侧翼分别是与东方伊萨酵母URA3基因上游序列(P-loURA3,SEQ ID NO:253)和下游序列(T-loURA3,SEQ ID NO:254)同源的DNA片段。P-loURA3和T-loURA3的相对取向与在东方伊萨酵母基因组中相同的。在30℃下,5天后获得了大约500个Mel+菌落。通过接种至10mL BFP(Butterfield磷酸缓冲液)管中,取出25μL涂布在DM1-X-α-gal平板上而单菌落分离了10个菌落。然后挑取从各个初步分离物中得到的单个蓝色菌落,置于YPD上以进行进一步分析。

[0377] 使用PCR来从转化体中筛选所期望的基因事件。为了获得在PCR筛选中作为模板使用的基因组DNA,从1.5mL过夜培养物中将细胞旋转沉降于带螺旋帽的微量离心管中,重悬于0.2mL 2%的Triton X-100,1%SDS,100mM NaCl,10mM Tris-HCl,pH为8.0,1mM的乙二胺四乙酸二钠pH 8.0的溶液中。添加用10mM Tris pH8.0/1mM EDTA(Sigma)平衡0.2mL苯酚:氯仿:异戊醇(25:24:1)混合物和0.3g玻璃珠。用Mini-BeadBeater-8(BioSpec)将管子全速振荡2分钟。加入0.2mL TE,简短地旋涡振荡管子。通过在16100×g下离心5分钟来分离水相。将上清液移到新的管中,加入1mL 100%的乙醇。将该管在20℃下放置30分钟,在16100×g下离心5分钟,将液体倒出。风干DNA,重悬于500μL TE中。

[0378] 利用引物oCA405和oCA406来对所期望的5'交叉进行PCR筛选,这将产生1.5kbp的产物。对于所期望的3'交叉的筛选则使用引物WG26和CM647,这产生1.6kbp的产物。用来构建pMI458的URA3区域外的引物(上游或下游远端)是oCA405和CM647,其在野生型中产生3.2kbp的产物,而在pMI458被破坏的等位基因中产生5.0kbp的产物,当选择标志物被环出时产生2.2kbp的产物。这些PCR反应都使用55℃的退火温度。同样可以使用四引物方法通过PCR来筛选URA3开放阅读框的缺失。引物pJLJ28和pJLJ29扩增肌动蛋白基因的800bp片段,引物pJLJ30和pJLJ31扩增URA3基因的600bp片段。在一个反应中使用所有这些引物提供阳性内部对照(肌动蛋白片段)。按照生产商的操作步骤使用来自于Roche的Taq DNA聚合酶,退火温度为61℃。确认MEL5选择盒整合到URA3位点上的是菌株1822ura het MEL-1和1822ura het MEL-2。

[0379] 然后通过允许在KtSEQ1a(SEQ ID NO:256)和KtSEQ1b(SEQ ID NO:257)之间发生重组,将MEL-5标志物从1822ura het MEL-1和1822ura het MEL-2的基因组中移除。MEL+菌

株在YPD培养基中生长过夜,直至OD600约为0.5至2.0。然后在YPD培养基中稀释培养物,使其OD600值回到约0.00001。将200μL培养物稀释液转移到96孔微量滴定板的每个孔中。用具有黏性的覆盖物覆盖平板,在30℃培养箱中孵育,以最大搅拌6-7小时(约6次细胞分裂,取决于菌株的生长速率)。从每个孔中取出100μL(约为~1000cfu每平板)涂布在DM1+X-α-gal培养基上。将平板孵育在30℃下过夜,或者在室温下2天,观察不同的颜色。将白色菌落(推定为mel-)在类似的培养基上划线,如上所述通过PCR进行筛选。发现了两个独立的环出,一个来自于1822ura het MEL-1,保藏为1822ura het MEL-1,另一个来自于1822ura het MEL-2,保藏为1822ura het MEL-2。奇怪的是,所获得的大部分白色菌落均没有给出预期的2.2kbp条带。

[0380] 为了得到ura-衍生体,在YP5D培养基上(YP+100g/L右旋糖)培养1822ura het mel-1和1822ura het mel-1过夜,过夜培养物的等分试样(0.5,5和50μL)涂布于ScD-2X FOA平板上,对FOA-抗性的菌落划线以得到单菌落,通过在ScD-ura平板上涂布来证实ura-表型。从1822ura het mel-1中挑取2个菌落,从1822ura het mel-2中挑取6个菌落以进行进一步分析。这些菌落在YPD中生长过夜,使用上述的酚/氯仿法提取基因组DNA。

[0381] 通过PCR筛选URA3开放阅读框的存在,8个菌株中均不含有URA3基因。1822ura het mel-1的两个ura后代命名为yJLJ3(CNB1)和yJLJ4。基于基因组测序,确定yJLJ3(CNB1)和yJLJ4含有URA3基因及附近透酶基因的缺失;优选只有URA3基因被缺失。为了构建CD1822的ura3营养缺陷型而不破坏该透酶基因,用1μg SacI/ApaI消化的pCM208(图28)转化CD1822。质粒pCM208含有与东方伊萨酵母URA3基因的上游(5' URA侧翼(附近),SEQ ID NO:258)和下游(3' URA侧翼(附近),SEQ ID NO:259)侧翼区域同源的DNA序列。在30℃下培养5天后,获得大约200个Mel+菌落。通过在ScD X-α-gal平板上划线分离出8个蓝色菌落。使用PCR来就期望的基因事件筛选转化体。使用引物oJY11和oJY12来进行5'交叉筛选,这将在所期望的转化体中产生0.9kbp的产物。使用引物oJY13和oJY14来进行3'交叉筛选,这将在所期望的转化体中产生1.0kbp的产物。8个克隆中有3个显示了所期望的PCR产物。这些菌落的MEL标记可被环出,并如上文所述,缺失第二URA3基因。或者,衍生自yJLJ3或yJLJ4的菌株中的URA3和透酶基因的缺失能够在一步转化中被修复,如实施例2H中所述。

[0382] 实施例2H:含有URA3选择标志物的MBin500对照菌株的构建

[0383] 如前所述,由于其URA3基因的纯合缺失,本文所使用的命名为CNB1的东方伊萨酵母菌株,是尿苷营养缺陷型。使用ura修复载体,pJLJ62(图26),能够对URA3基因座进行杂合修复,该载体含有ura修复盒,其由URA3基因及691bp的5'侧翼DNA,1500bp的3'侧翼DNA构成。ura修复盒的侧翼是5'的NotI限制性位点,3'的ApaI限制性位点。使用NotI和ApaI进行限制性消化,以从载体骨架中移除2796bp的ura修复盒。使用QIAquick PCR Purification Kit(Qiagen),根据制造商的说明对消化物进行纯化。将DNA洗脱至玻璃蒸馏水中,用1μg来转化东方伊萨酵母CNB1。在ura选择平板上选择转化体,不需要补充尿苷的单菌落被命名为MBin500。

[0384] 实施例2I:移除URA3选择标志物

[0385] 为了分离其中URA3选择标志物通过存在于整合盒中的两个URA3启动子区域的重组而被移除的菌株,将目的ura+菌株接种至3mL YP+10%葡萄糖的培养基中,在37℃下,以250rpm振荡培养至少4小时至过夜。取出50-100μL培养物涂布在ScD FOA平板上,在37℃下

生长48-60h直到菌落出现。由于FOA可被URA3蛋白转化成毒性化合物,导致ura⁺细胞的死亡,因此可在FOA上生长来选择URA3标记的移除。通过在YPD平板上,37℃下生长来对几个FOA-抗性菌落纯化两次。如本文所述,然后通过PCR对这些纯化的分离物进行URA3适当环出的筛选。

[0386] 实施例2J:对摇瓶中生长的修饰的酵母菌株进行十二烷基磺酸钠聚丙烯酰胺凝胶电泳(SDS-PAGE)分析和酶分析的步骤

[0387] 将4mL ura选择培养基加入到14mL Falcon管中,用无菌环将所期望的菌株接种到该培养基中。在37℃下以250rpm振荡培养该培养物过夜(~16hrs)。对于含有至少一个东方伊萨酵母基因座野生型拷贝的菌株,将500μL过夜培养物加入到125mL含有25mL YP+10%葡萄糖培养基的挡板瓶中。对于pdc Δ/pdc Δ菌株,将1mL过夜培养物加入到125mL含有25mL液体YP+100g/L葡萄糖培养基的挡板瓶中。在37℃下以250rpm振荡培养该瓶培养物。大约每隔一小时从培养物中取出小量等分试液,测量OD₆₀₀。使培养物生长,直至OD₆₀₀值为4至6之间。

[0388] 为了制备用于SDS-PAGE分析的细胞的小样本,从培养物中取出对应2.50D单位的体积的培养物,并置于1.5mL管中。在16100×g下沉淀细胞,移除上清液,细胞沉淀储藏于-20℃直至使用。

[0389] 在室温下以2279×g离心来收获培养瓶中剩余的细胞,将沉淀重悬于12.5mL 0.85M NaCl中,然后在室温下以2279×g离心。将沉淀重悬于1mL 0.85M NaCl中,将重悬细胞转移到2.0mL管中,然后在16100×g下沉淀。然后移除上清液,如果能在一周之内就可以使用以进行酶测定,则将沉淀储藏于-20℃下,或者在-80℃下进行更长期储藏。

[0390] 对于对应2.50D单位的细胞沉淀的SDS-PAGE分析,将细胞重悬于100dH₂O中,然后加入100μL 0.2M NaOH。将样品在室温下孵育5分钟,然后16100×g离心沉淀细胞,重悬于100μL SDS样品缓冲液中(Bio-Rad Laboratories)。将样品在95℃下加热5分钟,简单地离心以沉淀细胞。根据制造商的说明,在Criterion 8-16%Pre-Cast凝胶(Bio-Rad Laboratories)上对1至5μL的上清液进行分析。用InstantBlue™ Coomassie-Based Staining Solution(Expedeon Protein Solutions, San Diego, CA, USA)显色条带。

[0391] 实施例2K:用于产品分析的修饰酵母菌株的摇瓶培养步骤

[0392] 在Ura选择平板上对菌株进行划线以得到单菌落,在30℃下培养1-2天。将来自于Ura选择平板上的1-2个菌落接种到含有50mL CNB1摇瓶培养基的250mL挡板瓶中,以制备种子培养物。种子培养物在30℃下,以200rpm振荡培养约18小时。然后取出培养物的小量等分试液来测定OD₆₀₀值,直至OD₆₀₀达到4至6。使用Uristix® Reagent Strip (Bayer, Elkhart, IN, USA)来测量残余存在的葡萄糖。使用种子瓶培养来接种到含有50mL CNB1摇瓶培养基的125mL挡板瓶中,至OD₆₀₀=0.2。培养物在30℃下,140rpm下振荡孵育20小时。移除培养液的样品以进行如下所述的分析。使用样品的等分试样来测量培养物的光密度(OD),并使用Uristix® Reagent Strip来测量残余的葡萄糖。然后将剩余的样品进行离心,使用上清液进行产品分析。

[0393] 实施例2L:用于产品分析的修饰酵母菌株发酵的步骤

[0394] 使用种子繁殖阶段来培养本文所描述的菌株,然后在2L的生物反应器(Applikon, Foster City, CA, USA)中再进行单阶段发酵。

[0395] 对于种子阶段的制备,将25mL 1X DM2培养基(用KOH调整至所需的pH值)加入到125mL挡板瓶中,然后用无菌环接种目的菌株。在所需的温度下,250rpm振荡培养培养物过夜约16hr。然后每隔约一小时取出培养物的小量等分试液,测定OD600值直至OD600达到4-6。

[0396] 使用Uristix® Reagent StriP (Bayer, Elkhart, IN, USA) 来测量存在的残余的葡萄糖。然后将12mL培养物加入到4mL无菌冷冻的75%甘油中,彻底混匀,在冰上孵育10分钟。随后将培养物和甘油的混合物混匀,等分为10份,每份1.0mL放置于1.8mL冷冻管中 (Thermo Scientific, Rochester, NY, USA), 储藏在-80℃下。

[0397] 使用25mL种子瓶培养接种到含有1.5L DM2培养基的2L生物反应器中。在约30℃-40℃的温度下,控制pH值在2.0-7.0的范围内,在搅拌条件下以及导致氧气吸收速率 (OUR) 在2-45mmol/L/hr范围内的通气条件下,在该生物反应器中进行发酵。在本文所示的实施例中,生物反应器中培养物的温度,pH和OUR分别是30℃,4.0和25-30。

[0398] 定期移除发酵液样品以进行分析。简单地说,使用样品的等分试液测量培养物的光密度 (OD), 葡萄糖浓度和pH。然后将剩下的样品离心。沉淀物储藏于-80℃用于酶分析,上清液用于进行3-HP和其他胞外化合物的分析。除另有指明之外,本文中所报告的所有3-HP生产值均是发酵的48-小时时间点的生产值。在发酵过程中的二氧化碳生成量和氧气消耗是通过确定从生物反应器中排出的气体中的二氧化碳含量和氧气含量来进行测定的。

[0399] 实施例2M:对修饰酵母菌株所产生的3-HP和β-丙氨酸的分析步骤

[0400] 通过在1%甲酸中稀释10倍来酸化培养物样品,并且通过0.46μm的96孔滤板来进行过滤。根据样品中的分析物浓度在水中进行进一步稀释。在样品缓冲液中进一步稀释10倍,该缓冲液为1mM NH₄Ac,0.1%NH₃和5mg/L的一律用¹³C标记的3-HP(作为3-HP的内标),或者1%甲酸和3mg/L一律用¹³C标记的β-丙氨酸(作为β-丙氨酸的内标)。总稀释倍数为约100至1000,取决于β-丙氨酸或3-HP的浓度。

[0401] 将2μL样品注入Agilent 1200HPLC (Agilent) 中,由MassHunter程序与Agilent6410Triple Quad MS/MS检测器控制,使用的仪器设置和栏目在表6中列出。定量离子碎片峰面积与其稳定同位素对应物(来自于内标)之间的比值可用于定量,以消除离子的抑制效应和仪器漂移。在日常检测中,标准偏差为低于5%。

[0402] 表6:用于β-丙氨酸和3-HP分析的LC/MS/MS设置

[0403]

	3-HP (¹³ C 3-HP)	β-丙氨酸(¹³ C β-丙氨酸)
柱	Xbridge HILIC SILICA 3.5μm , 2.1×150mm	Atlantis HILIC SILICA 3μm , 2.1×150mm
洗脱缓冲液	62%乙腈, 0.35m MNH ₄ Ac	38%乙腈, 0.6%甲酸
流速(mL/分钟)	0.30	0.30
柱温	50°C	45°C
保持时间(分钟)	1.07	1.64
运行时间(分钟)	3	3
前驱离子	89(92)	90(93)
用于定量的产物离子	59(61)	72(75)
用于定性的产物离子	41(43)	30(31)
解离电压	50	70
碰撞能量	用于定量时为5; 用于定性时为21	用于定量时为3; 用于定性时为7
极性	阴性	阳性
喷雾器N ₂ 压力(psi)	10	11
N ₂ 流速(L/分钟)	32	35
N ₂ 温度	300°C	340°C
毛细管(V)	4000	4000
Delta EMV	450	400

[0404] 实施例3:表达3-HP发酵途径基因的修饰酵母菌株

[0405] 在酵母宿主细胞中表达编码涉及3-HP不同发酵途径中的酶的一个或多个基因,其为单独表达或组合表达。3-HP途径酶可以从外源基因,内源基因或其一些组合来进行表达。利用基因表达构建体将要表达的外源基因引入酵母细胞中,例如,如实施例2中所描述的表达构建体。外源基因可在一个单一的位点或多个位点整合进宿主酵母的基因组中,并且外源基因的整合伴随着插入位点的目的基因的缺失或破坏,如下所述。

[0406] 实施例3A:表达天冬氨酸/丙二酸半醛途径基因的修饰酵母菌株

[0407] 通过利用PEP和/或丙酮酸,0AA,天冬氨酸,β-丙氨酸,丙二酸半醛中间体的途径来产生3-HP的酵母细胞可以通过表达一个或多个参与该途径的酶来进行工程改造。表达的基因包括一个或多个PPC, PYC, AAT, ADC, BAAT, gabT, 3-HPDH (包括丙二酸半醛还原酶), HIBADH, 或4-羟基丁酸酯脱氢酶基因。

[0408] 所表达的基因可以衍生自宿主细胞的天然基因。例如,当宿主酵母细胞是东方伊萨酵母时,所表达的基因可以衍生自东方伊萨酵母PYC (例如,编码如SEQ ID NO:2所示的氨基酸序列的和/或包括如SEQ ID NO:1所示核苷酸序列编码区的东方伊萨酵母PYC基因), AAT (例如,编码如SEQ ID NO:14所示的氨基酸序列的和/或包括如SEQ ID NO:13所示核苷酸序列编码区的东方伊萨酵母AAT基因), BAAT (例如,编码如SEQ ID NO:20所示的氨基酸序列的和/或包括如SEQ ID NO:19所示核苷酸序列编码区的东方伊萨酵母pyd4同源基因), 或3-HPDH (例如,编码如SEQ ID NO:26所示的氨基酸序列的和/或包括如SEQ ID NO:25所示核苷酸序列编码区的与东方伊萨酵母YMR226C同源的基因)。当酵母宿主细胞是另外的3-HP耐受酵母菌株时,可以使用本领域已知技术来获得基因序列,途径基因的天然同源物能够被外源表达或者与外源调节元件连接来进行表达。天然途径基因可以包括一个或多个PPC,

PYC, AAT, BAAT和/或3-HPDH基因。

[0409] 或者,一个或多个表达的3-HP基因可来自于源基因,该源基因对于宿主细胞而言是非天然的。例如,当酵母宿主细胞是东方伊萨酵母时,可以对该细胞进行工程改造以表达一个或多个非天然的PYC基因,如编码如SEQ ID NO:3所示氨基酸序列的类球红细菌(*R.sphaeroides*) PYC基因,编码如SEQ ID NO:4所示氨基酸序列的菜豆根瘤菌(*R.etli*) PYC基因,编码如SEQ ID NO:5或6所示氨基酸序列的荧光假单胞菌(*P.fluorescens*) PYC基因,编码如SEQ ID NO:7所示氨基酸序列的谷氨酸棒杆菌(*C.glutamicum*) PYC基因,或编码如SEQ ID NO:8所示氨基酸序列的苜蓿中华根瘤菌(*S.meliloti*) PYC基因;一个或多个非天然PPC基因,例如编码如SEQ ID NO:10所示氨基酸序列的大肠杆菌(*E.coli*) PPC基因,编码如SEQ ID NO:11所示氨基酸序列的嗜热碱甲烷杆菌(*M.thermoautotrophicum*) PPC基因,编码如SEQ ID NO:12所示氨基酸序列的产气荚膜梭菌(*C.perfringens*) PPC基因;一个或多个非天然AAT基因,如编码如SEQ ID NO:16所示氨基酸序列的大肠杆菌aspC基因,或编码如SEQ ID NO:15所示氨基酸序列的酿酒酵母(*S.cerevisiae*) AAT2基因;一个或多个非天然ADC基因,如编码如SEQ ID NO:17所示氨基酸序列的(和/或包含如SEQ ID NO:130,145,146或147所示核苷酸序列的编码区的)阿维链霉菌(*S.avermitilis*) PanD基因,编码如SEQ ID NO:18所示氨基酸序列的(和/或包含如SEQ ID NO:131所示核苷酸序列的编码区的)丙酮丁醇梭菌(*C.acetobutylicum*) PanD基因,编码如SEQ ID NO:133所示氨基酸序列的(和/或包含如SEQ ID NO:132所示核苷酸序列的编码区的)幽门螺旋杆菌(*H.pylori*) ADC基因,编码如SEQ ID NO:135所示氨基酸序列的(和/或包含如SEQ ID NO:134所示核苷酸序列的编码区的)芽孢杆菌属(*Bacillus* sp) TS25ADC基因,编码如SEQ ID NO:137所示氨基酸序列的(和/或包含如SEQ ID NO:136所示核苷酸序列的编码区的)谷氨酸棒杆菌(*C.glutamicum*) ADC基因,编码如SEQ ID NO:139所示氨基酸序列的(和/或包含如SEQ ID NO:138,148,149,150或151所示核苷酸序列的编码区的)地衣芽孢杆菌(*B.licheniformis*) ADC基因;一个或多个非天然BAAT或gabT基因,如编码如SEQ ID NO:21所示氨基酸序列的(和/或包含如SEQ ID NO:142所示核苷酸序列的编码区的)克鲁维酵母pyd4基因,编码如SEQ ID NO:22所示氨基酸序列的(和/或包含如SEQ ID NO:140所示核苷酸序列的编码区的)阿维链霉菌BAAT基因,编码如SEQ ID NO:23所示氨基酸序列的阿维链霉菌gabT基因,或编码如SEQ ID NO:24所示氨基酸序列的(和/或包含如SEQ ID NO:141所示核苷酸序列的编码区的)酿酒酵母UGA1基因;一个或多个非天然3-HPDH基因,如编码如SEQ ID NO:27所示氨基酸序列的(和/或包含如SEQ ID NO:143所示核苷酸序列的编码区的)大肠杆菌ydfG基因,或编码如SEQ ID NO:129所示氨基酸序列的(和/或包含如SEQ ID NO:144所示核苷酸序列的编码区的)酿酒酵母YMR226C基因;一个或多个非天然的丙二酸半醛还原酶基因,如编码如SEQ ID NO:29所示氨基酸序列的(和/或包含如SEQ ID NO:343所示核苷酸序列的编码区的)嗜热金属球菌丙二酸半醛还原酶基因;一个或多个非天然HIBADH基因,如编码如SEQ ID NO:28所示氨基酸序列的粪产碱菌(*A.faecalis*) M3A基因,编码分别如SEQ ID NO:30或SEQ ID NO:31所示氨基酸序列的恶臭假单胞菌(*P.putida*) KT2440或E23440mmsB基因,或者编码如SEQ ID NO:32所示氨基酸序列的铜绿假单胞菌(*P.aeruginosa*) PA01 mmsB基因;和/或一个或多个非天然4-羟基丁酸脱氢酶基因,如编码如SEQ ID NO:33所示氨基酸序列的*R.eutropha* (*R.eutropha*) H164hbd基因,或编码如SEQ ID NO:34所示氨基酸序列*C.kluyver* DSM 555hbd基因。

[0410] 实施例3A-0:表达天冬氨酸/丙二酸半醛途径基因的修饰酵母菌株的酶活测定

[0411] 制备用于酶测定的粗制无细胞提取物 (CFE)

[0412] 通过离心来收集本发明所示的来自于摇瓶或生物反应器培养物中的细胞,弃去上清液,如上所述,将细胞沉淀储藏于-80℃下。为了制备CFE,将细胞沉淀物解冻,用磷酸盐缓冲液 (PBS) 洗涤,再一次离心收集。弃去上清液,在2.0mL微量离心管中,将细胞沉淀重悬于大约等体积的裂解缓冲液中,该裂解缓冲液含有1%的蛋白酶抑制剂Cocktail,来自于Sigma的P8215。加入约300μL 0.5mm的氧化锆珠 (BioSpec),在**FastPrep®-24**裂解器 (MP Biomedicals) 中以6/20秒的设定进行3轮细胞裂解。在每轮之间,将样品管置于冰上冷却5分钟。裂解之后,在微量离心管中,4℃下,以最大速度对样品离心15分钟。将上清液转移到新的1.5mL管中,并保持在冰上。根据制造商提供的说明,使用Bio-Rad蛋白质测定试剂 (Bradford测定) 对裂解液中的总蛋白浓度进行确定,用牛血清蛋白作为标准品。

[0413] 丙酮酸羧化酶活性 (PYC) 活性:

[0414] 本文所示细胞的CFE中的丙酮酸羧化酶的活性测定如下。制备储备反应混合溶液,当在测定反应混合物中与CFE组合时,该混合溶液提供的组分的最终浓度如下:Tris (pH8.0),100mM;NaHCO₃,10mM;MgCl₂,5mM;NADH,0.2mM;ATP,1mM;乙酰CoA,1mM;丙酮酸,1mM;生物素(如果测定PYC酶时需要),5μM;牛心苹果酸脱氢酶,0.02单位/mL。将270μL该混合物加入到96孔微量滴定板的孔中,加入30μL经过适当稀释的CFE以开始反应。使用SpectraMax 340PC酶标仪监测340nm处的NADH消耗量。丙酮酸羧化酶的活性表示为nmol NADH消耗/秒/mg蛋白质。

[0415] 磷酸烯醇丙酮酸羧化酶 (PPC) 活性:

[0416] CFE中的磷酸烯醇丙酮酸羧化酶 (PPC) 的活性测定如下。制备储备反应混合溶液,当在测定反应混合物中与CFE组合时,该混合溶液提供的组分的最终浓度如下:Tris (pH8.0),100mM;NaHCO₃,10mM;MgCl₂,5mM;NADH,0.1mM;乙酰CoA,0.5mM;磷酸烯醇丙酮酸,3.3mM;牛心(或猪心)苹果酸脱氢酶,0.02单位/mL。将270μL该混合物加入到96孔微量滴定板的孔中,加入30μL经过适当稀释的CFE以开始反应。使用SpectraMax 340PC酶标仪监测340nm处的NADH消耗量。

[0417] 天冬氨酸转氨酶 (AAT) 活性:

[0418] 本文所示细胞的CFE中的天冬氨酸转氨酶的活性测定如下。制备储备反应混合溶液,当在测定反应混合物中与CFE组合时,该混合溶液提供的组分的最终浓度如下:100mM Tris (pH8.0),100mM;NaHCO₃,10mM;MgCl₂,5mM;NADH,0.1mM;天冬氨酸,1mM;α-酮戊二酸,1mM;苹果酸脱氢酶,0.02单位/mL。在一些测定中,储备反应混合物还含有吡哆醛5'磷酸 (0.1mM)。将270μL该混合物加入到96孔微量滴定板的孔中,加入30μL经过适当稀释的CFE以开始反应。使用SpectraMax 340PC酶标仪监测340nm处的NADH消耗量。天冬氨酸转氨酶的活性表示为nmoles NADH消耗/秒/mg蛋白质。

[0419] 天冬氨酸脱羧酶 (ADC) 活性:

[0420] 本文所示细胞的CFE中的天冬氨酸脱羧酶的活性测定如下。在恒温37℃下,将165μL 100mM NH₄Ac缓冲液 (pH6.8) 和25μL 80mM的天冬氨酸加入到96孔微量滴定板的每个孔中。加入10μL CFE以起始反应。在不同的时间间隔 (5,10,15,20,25,30,40,60分钟),从反应混合物中取出20μL样品,加入到180μL淬灭缓冲液中 (2%的甲酸以及2mg/L的¹³C标记β-丙氨

酸作为内标)。过滤之后,用LC/MS/MS分析样品中的 β -丙氨酸。从 β -丙氨酸相对于时间的曲线图中获取斜率。通过斜率除以反应中总细胞蛋白浓度来计算活性。ADC的活性表示为 μmol β -丙氨酸形成/秒/g蛋白质。

[0421] 在一些具体实验中则使用修改的ADC测定法。在这些情况下,本文所示细胞的CFE中的天冬氨酸转氨酶的活性测定如下。将110 μL 100mM NH_4Ac 缓冲液(pH7.6)和80 μL 25mM的天冬氨酸(用NaOH中和之后)加入到恒温40 $^{\circ}\text{C}$ 的96孔微量滴定板的每个孔中。加入10 μL CFE以起始反应。在不同的时间间隔(2,4,6,8,10分钟),从反应混合物中取出20 μL 样品,加入到180 μL 淬灭缓冲液中(2%的甲酸以及2mg/L的 ^{13}C 标记 β -丙氨酸作为内标或者在2%的甲酸中淬灭,然后按比例1:10转移至含有2mg/L的 ^{13}C 标记 β -丙氨酸作为内标的20%甲醇/80%水中)。过滤之后,用LC/MS/MS分析样品中的 β -丙氨酸。从 β -丙氨酸相对于时间的曲线图中获取斜率。通过斜率除以反应中总细胞蛋白浓度来计算活性。ADC的活性表示为 μmol β -丙氨酸形成/秒/g蛋白质。

[0422] β -丙氨酸转氨酶(BAAT)活性

[0423] CFE中的 β -丙氨酸转氨酶(BAAT)的活性测定如下。在室温下,将190 μL 含有100mM NH_4HCO_3 (pH7.6), 8mM α -酮戊二酸, 0.5mM乙酰CoA, 0.1mM吡哆醛-5'-磷酸和200mM β -丙氨酸的反应混合物加入到96孔微量滴定板中。加入10 μL CFE以起始反应。在2,4,6,8,10,12,15和20分钟时,从反应混合物中取出20 μL 样品,并加入到75 μL 淬灭缓冲液中(2.5%的甲酸)。中和样品,通过加入5 μL 10M的NaOH和50 μL 100mM的 NaCO_3 (pH10)来控制pH值。在注射时将过滤后的样品与OPA(临苯二甲醛)试剂,10mg/mL(Agilent Technologies 5061-3335)混合,以衍生化样品。在HPLC分离之后,通过荧光检测(激发波长340nm;发射波长460nm)对用OPA衍生化的谷氨酸进行定量。将15 μL 样品注射到5 μm 包装的反相Gemini C18分析柱中(Phenomonex150 $\times$ 4.6mm)。在62.5%20mM的磷酸缓冲液(pH7.8)(A)和37.5%甲醇(B)中平衡该柱。线性梯度如下所示:坡增(ramp)至40%B,0-0.3分钟;40%B,0.3-1分钟;坡增至85%B,1-1.75分钟;85%B,1.75-2.25分钟;坡增至37.5%,2.25-3分钟;37.5%B,3-4分钟。流速为2mL/分钟。反应缓冲液中谷氨酸的标准曲线用于确定样品中的浓度。从[谷氨酸]相对于时间的曲线图中获取斜率。通过斜率除以反应中总细胞蛋白浓度来计算活性。

[0424] 3-HP脱氢酶(3-HPDH)活性

[0425] 本文所示细胞的CFE中的3-HP脱氢酶的活性测定如下。将190 μL 稀释于(通常是100倍稀释)100mM NH_4HCO_3 (pH7.6)中的CFE和NADPH加入到恒温37 $^{\circ}\text{C}$ 的96孔微量滴定板的每个孔中。通过加入10 μL 60mM的丙二酸半醛(从在10mM H_2SO_4 中的200mM MSA储备溶液在10mM H_2SO_4 中制备)来起始反应。在1,2,4,6,8,10和12分钟时,从反应混合物中取出每份20 μL 样品,在80 μL 沸水中淬火。冷却之后,用75 μL 含有2mM NH_4Ac (pH6.8)和3mg/L ^{13}C 标记的3-HP的缓冲液与75 μL 淬火混合物进行混合。过滤之后,用LC/MS/MS定量样品中的3-HP。从3-HP相对于时间的曲线图中获取斜率。通过斜率除以反应中总细胞蛋白浓度来计算活性。3-HP脱氢酶的活性表示为nmol NADPH形成/秒/mg蛋白质。

[0426] 在一些具体实施方式中使用修改的3-HPDH测定法。在这些情况下,本文所示细胞的CFE中的3-HPDH的活性测定如下。通过测量在340nm处,NADPH随着时间推移而消失来测量丙二酸半醛的还原。根据Yamada和Jzcoby所发现的方法自己合成丙二酸半醛(Yamada, E.W., Jacoby, W.B., 1960, Direct conversion of malonic semialdehyde to acetyl-

coenzyme A, Journal of Biological Chemistry, Volume 235, Number 3, pp.589-594)。该测定在96孔板中实施,最终体积为200 μ L。将30 μ L CCE加入到170 μ L的测定缓冲液(2mM的丙二酸半醛,100nM Tris pH8.0和0.5mM的NADPH)中来起始反应。在室温下($\sim 25^{\circ}\text{C}$),在微板读数仪(Spectra Max 340PC, Molecular Devices LLC, Sunnyvale, CA)上追踪340nm处的吸光度10分钟。1个单位的3-HPDH活性被定义为在丙二酸半醛存在的情况下,1分钟内氧化1 μ mol的NADPH所需的酶量。

[0427] 实施例3A-1:在adh1202基因座表达天冬氨酸脱羧酶(ADC)的插入载体

[0428] 为了在东方伊萨酵母中表达,对一些天冬氨酸脱羧酶基因进行了密码子优化,并通过GeneArt® (Burlingame, CA, USA)合成,得到了表7中所列出的质粒。通过XbaI和PacI限制性消化,可使合成基因到达载体pMA-T中,也能使其从载体中引出(elicit)。然后将限制性片段克隆进pMIBa107的相同位置中,将基因置于PDC启动子和终止子的控制之下,并允许其在东方伊萨酵母的adh1202基因座发生整合。

[0429] 表7:转化体构建体

[0430]	构建体质粒	基因来源	基因号码	基因SEQ ID NO	整合构建体	转化体
	1051387	幽门螺旋杆菌	P56065	132	pWTY10-0033-1	yWTY1-1 yWTY1-2
	1051391	芽孢杆菌属 TS25	ZY44006.基因3	134	pWTY10-0033-2	yWTY1-5 yWTY1-6
	1051389	谷氨酸棒状杆菌	Q9X4N0	136	pWTY10-0033-3	yWTY1-9 yWTY1-10
	1051388	丙酮丁醇梭菌	P58285	131	pWTY10-0033-4	yWTY1-13 yWTY1-14
	1051390	地衣芽孢杆菌	Q65158	138	pWTY10-0033-5	yWTY1-17 yWTY1-18
	1045172	阿维链霉菌		130	pWTY10-0033-7	yWTY1-25 yWTY1-26

[0431] 质粒1045172,105387,105388,105389,105390,105391用XbaI和PacI进行消化,使用TBE缓冲液进行1.3%琼脂糖凝胶电泳。每个消化反应中,对应于ADC (panD) 基因的400-500bp片段被从凝胶中切割下来,并使用QIAquick® Gel Extract Kit(Qiagen)从琼脂糖中提取。

[0432] 用XbaI和PacI消化质粒pMIBa107,用小牛肠磷酸酶(New England Biolabs)处理,在TBE缓冲液中进行琼脂糖凝胶电泳后,纯化载体条带。将XbaI和PacI消化后的panD片段用T4DNA连接酶和Quick Ligation Kit(New England Biolabs)根据制造商的说明,连接到这个纯化的pMIBa107载体上。根据制造商的说明,将连接产物转化进XL10-GOLD ULTRA细胞中(Agilent Technologies)。将转化体涂布在2X YT+amp平板上,37 $^{\circ}\text{C}$ 孵育过夜。每个反应挑取24个转化体至2X YT+amp平板上。通过ApaI, NcoI和SacI消化筛选出所得到的四个转化体每个的小量制备DNA。通过DNA测序来验证产生所需条带大小的克隆的正确性,并如表7所示被命名。所得到的质粒允许以表达盒正向取向的方式使每个ADC基因整合到adh1202基因座

上。

[0433] 大约10μg的每种整合构建体用ApaI和KpnI消化,使用89mM Tris碱-89mM硼酸-2mM 乙二胺四乙酸钠 (TBE) 缓冲液,在1%琼脂糖凝胶上进行电泳。每个质粒大约4450bp的片段从凝胶中切割下来,根据制造商的说明使用QIAquick® Gel Extract Kit (Qiagen) 提取。发现纯化产物的浓度在39-138ng/ul之间。用0.39-1.4μg来自于整合构建体(用ApaI和KpnI消化)的片段转化进东方伊萨酵母CNB1中,如上所述。将转化体涂布在ura选择培养基上,37℃下孵育,在ura选择培养基上重新划线,37℃下孵育。从URA3+菌落中制备基因组DNA,通过PCR检测以验证整合。使用引物0611718和0611632扩增2.5kbp片段来验证整合。每个PCR反应含有2.5μL基因组DNA,引物0611718和0611632各12.5pm,1X Crimson Taq™ 反应缓冲液 (New England Biolabs),0.2mM dNTP混合物,0.625单位Crimson Taq™ DNA聚合酶 (New England Biolabs),最终体积为25μL。扩增反应孵育在Eppendorf® Mastercycler® (Eppendorf Scientific) 中,程序为1个循环95℃ 30秒;30个循环的95℃ 30秒,50℃ 30秒和68℃ 3分钟;以及1个循环的68℃ 10分钟。

[0434] 对于每个构建体,两个证实为URA3的转化体被命名如表7所示。这些菌株在adh1202对于所示的ADC基因是杂合的,所述基因由来自于东方伊萨酵母的PDC启动子和终止子驱动表达。

[0435] 检测表7中所列出的菌株中(以及MBin500菌株,如上,作为阴性对照)PanD的表达和酶活性。每个菌株的过夜培养物在YPD ON中,37℃下过夜培养,在37℃下,在125mL的挡板瓶中,以1:50稀释到25mL新鲜YPD中,生长至OD600~2-8。然后使用细胞沉淀物来制备CFE,然后如上所述使用该CFE进行ADC活性测定。代表性的结果示于表8中。

[0436] 表8:转化体酶活性数据

菌株	ADC基因的来源	基因SEQ ID NO	ADC活性
MBin500 (对照)	N/A	N/A	0
yWTY1-1 yWTY1-2	幽门螺旋杆菌	132	0
yWTY2-5 yWTY2-6	芽孢杆菌属 TS25	134	0.31 0.15
yWTY3-9 yWTY3-10	谷氨酸棒状杆菌	136	0.31 0.19
yWTY4-13 yWTY4-14	丙酮丁醇梭菌	131	0.37 0.23
yWTY5-17 yWTY5-18	地衣芽孢杆菌	138	0.56 0.61
yWTY7-25 yWTY7-26	阿维链霉菌	130	0.23 0.30

[0438] 接下来,创建yWTY5-17和yWTY7-25的纯合子版本。首先,如上所述分离ura-衍生的yWTY5-17和yWTY7-25。从FOA-抗性菌落中制备基因组DNA,如上所述用PCR进行检查以验证

URA3选择标志物的缺失。使用引物0611718和0611632,对于ura标志存在的整合,扩增出2.4kbp的片段,对于ura标志的缺失,则扩增出1100bp的片段。使用引物0611718和0611632,产生1100bp PCR片段的ura-菌株yWTY5-17和yWTY7-25被分别命名为MIBa331和MIBa332。

[0439] pWTY10-0033-5和pWTY10-0033-7各10μg用ApaI, KpnI和NcoI进行消化,使用89mM Tris碱-89mM硼酸-2mM乙二胺四乙酸钠(TBE)缓冲液,在1%琼脂糖凝胶上进行电泳。每个质粒大约4450bp的片段从凝胶中切割下来,使用QIAquick® Gel Extract Kit (Qiagen)提取。将来自pWTY10-0033-5和pWTY10-0033-7纯化片段分别转化进MIBa331和MIBa332中,如上所述。将转化体涂布在ura选择培养基上,37℃下孵育过夜,然后在ura选择培养基上重新划线,37℃下孵育过夜。制备基因组DNA,实施Crimson Taq™ PCR来验证整合,如上所述。使用引物0611718和0611632,对于ura标志存在的整合,扩增出2.4kbp的片段,对于ura标志的缺失,则扩增出1100bp的片段。使用引物0611718和0611632,产生1100bp和2.4kbp PCR片段的MIBa331和MIBa332转化体被保存并分别命名为MIBa338和MIBa337。MIBa337在adh1202基因座是来自于阿维链霉菌的ADC基因纯合子,MIBa338则在adh1202基因座是来自于地衣芽孢杆菌的ADC基因纯合子。两个菌株都具有各自的ADC位于来自于东方伊萨酵母的PDC启动子和终止子的调控之下。

[0440] 将来自于panD纯合菌株MIBa337和MIBa338与来自于panD杂合菌株yWTY5-17和yWTY7-25的ADC表达和酶活进行比较。培养物在YPD中以37℃下培养过夜,然后在37℃下,在125mL的挡板瓶中,以1:50稀释到25mL新鲜YPD中,生长至OD600~2-8。使用细胞沉淀物来制备CFE,然后如上所述使用该CFE进行ADC活性测定。两个独立实验的代表性的结果示于表9A中。

[0441] 表9A:转化体酶活数据

菌株	panD基因来源	等位基因类型	基因SEQ ID NO	ADC活性(实验1, 实验2)
MBin500 (对照)	N/A	N/A	N/A	0, 0
yWTY5-17	地衣芽孢杆菌	杂合	138	0.6, 0.9
MIBa338	地衣芽孢杆菌	纯合	138	0, 0.22
yWTY7-25	阿维链霉菌	杂合	130	0.19, 0.13
MIBa337	阿维链霉菌	纯合	130	0.28, 0.19

[0443] 第三个独立实验,即从菌株MIBa337和MIBa338制备的CFE中的ADC活性比较的结果示于表9B中。

[0444] 表9B:转化体酶活数据

菌株	panD基因来源	等位基因类型	基因SEQ ID NO	ADC活性
MBin500 (对照)	N/A	N/A	N/A	0
yWTY5-17	地衣芽孢杆菌	杂合	138	0.218
MIBa338	地衣芽孢杆菌	纯合	138	0.453
yWTY7-25	阿维链霉菌	杂合	130	0.087
MIBa337	阿维链霉菌	纯合	130	0.188

[0446] 上述样品的SDS-PAGE分析表明MIBa338中的panD表达量在这些菌株中是最高的。

[0447] 使用本发明所描述的方法,在生物反应器中评估菌株MIBa337和MIBa338的3-HP产生。对照菌株MIBa500没有产生可检测的3-HP(两个独立发酵的平均值)。菌株MIBa337产生了1.33g/L 3-HP(实施了一个发酵),菌株MIBa338产生了3.15g/L 3-HP(三个独立发酵的平均值)。在菌株MIBa337和MIBa338的各个独立发酵中,进一步比较它们的3-HP生产性能和ADC活性(表10)。考虑到这些发酵中细胞量的不同,3-HP生产性能在表10中被记录为3-HP浓度/每单位的细胞量(表示为[g/L 3-HP]/[g/L细胞干重])。该结果显示,使用地衣芽孢杆菌panD基因(菌株MIBa338)要比使用阿维链霉菌的panD基因(菌株MIBa337)具有改进的ADC活性和3-HP生产性能。

[0448] 表10: 菌株MIBa337和MIBa338的3-HP生产性能和ADC活性

发酵时间 (小时)	MIBa337		MIBa338	
	ADC活性(mmlol/min/g 蛋白质)	3HP/DCW	ADC活性(mmlol/min/g蛋 白质)	3HP/DCW
11	0.005	0.00	0.021	0.024
22	0.011	0.05	0.055	0.131
31	0.003	0.04	0.029	0.159
48	0.001	0.05	0.018	0.142

[0450] 实施例3A-2: 在adh1202基因座表达β-丙氨酸转氨酶(BAAT)或3-羟基丙酸脱氢酶(3-HPDH)的插入载体

[0451] 来自于阿维链霉菌的BAAT,来自于酿酒酵母的UGA1,来自于克鲁维酵母的PYD4,来自于酿酒酵母的YMR226c以及来自于大肠杆菌的ydfG,这些基因的东方伊萨酵母密码子优化型式由GENEART®合成,所得到的质粒在下面列出。这些合成基因通过使用XbaI和PacI消化能够进入到载体pMA-T中,也可以从载体中引出。然后将经消化的片段克隆进pMIBa107的相同位置中,将基因置于PDC启动子和终止子的调控之下,并允许其在adh1202基因座发生整合。

[0452] 表11: 转化体构建体

构建体质粒	基因	基因来源	SEQ ID NO	整合构建体	转化体
1045169	gabT (UGA1)	酿酒酵母	141	pMIBa122	MIBa310
1045170	BAAT	阿维链霉菌	140	pMIBa121	MIBa309
1045171	BAAT (PYD4)	克鲁维酵母	142	pMIBa124	MIBa312
1045173	3-HPDH (YMR226c)	酿酒酵母	144	pMIBa123	MIBa311
1045168	3-HPDH (ydfG)	大肠杆菌	143	pMIBa120	MIBa308

[0455] 质粒1054168,1054169,1054170,1054171,1054172和1054173用XbaI和PacI进行

消化,使用89mM Tris碱-89mM硼酸-2mM乙二胺四乙酸钠(TBE)缓冲液,在1%琼脂糖凝胶上进行电泳。将来自于1045168的761bp片段(ydfG),来自于1045169的1430bp片段(UGA1),来自于1045170的1370bp片段(BAAT),来自于1045171的1442bp片段(PYD4)或来自于1045173的814bp片段(YMR226c)从凝胶中切割下来,使用QIAquick®Gel Extract Kit(Qiagen)从琼脂糖中提取。质粒pMIBa107用XbaI和PacI消化,接着用CIP进行处理,产生7.9kbp的线性片段。消化物用QIAQUICK®PCR Purification Kit(Qiagen)进行纯化。如本文所述,然后利用T4DNA连接酶将消化后的ydfG,UGA1,BAAT,PYD4或YMR226c片段连接到pMIBa107上(用XbaI和PacI消化,并用CIP处理)。根据制造商的说明将连接产物转化至One Shot®TOP10Chemically Competent大肠杆菌细胞(Invitrogen)中。将转化体涂布在2X YT+amp平板上,37℃孵育过夜。通过XbaI和PacI消化筛选到所得转化体中。产生所需片段大小的克隆通过DNA测序来确认正确,并分别对于ydfG,BAAT,UGA1,YMR226c或PYD4命名为pMIBa120,pMIBa121,pMIBa122,pMIBa123和pMIBa124。所得到的质粒允许以表达盒正向取向的方式使所需基因整合到adh1202基因组上。

[0456] 表11中的整合构建体用于将为在东方伊萨酵母中表达而密码子优化的目的基因整合到东方伊萨酵母的adh1202基因座上,在PDC启动子和终止子的调控之下。如本文所述,表达盒还包括URA3选择标志物以允许对ura-宿主中的转化体进行选择。表达盒和adh1202同源区域的侧翼是ApaI和KpnI限制性位点,以允许片段从质粒中释放出来。

[0457] 表11中的各个整合构建体各15μg用ApaI,KpnI和NcoI消化,使用89mM Tris碱-89mM硼酸-2mM乙二胺四乙酸钠(TBE)缓冲液,在1%琼脂糖凝胶上进行电泳。用NcoI消化破坏了载体的骨架,使得更容易从琼脂糖凝胶中提取目的片段。将分别来自于pMIBa120,pMIBa121,pMIBa122,pMIBa123和pMIBa124的4884bp,5493bp,5553bp,4937bp和5565bp的片段,从凝胶中切割下来,使用QIAquick®Gel Extract Kit(Qiagen)从琼脂糖中提取。发现纯化后产物的浓度在80-120ng/μL之间。如本文所述,将0.8-1.2μg来自于pMIBa120-4的限制性片段转化进东方伊萨酵母CNB1(ura-)中。然后将转化体涂布在ura选择培养基上,室温下生长60小时。将转化体在ura选择培养基上重新划线,在37℃下孵育过夜。

[0458] 通过菌落PCR对几个转化体各进行检查以验证整合。使用引物对来验证正确的整合,所述引物对检查整合的5'和3'端,并列在下面。引物0611717以反向退火于PDC启动子中,而引物0611225则以正向退火于URA3选择标志物上。引物0611631和0611632分别以正向和反向在整合位点的外面进行退火;引物0611717和0611631在正确的整合体中扩增出976bp的片段;引物0611225和0611632在正确的整合体中扩增出1.4kbp的片段;引物0611631和0611632扩增出2.7kbp的片段则意味着野生型染色体,而如果整合,则扩增出~5kbp的片段。为了构建基因组DNA,将每个转化体的一个菌落在50μL的含0.05U/μL溶细胞酶(Sigma,St.Louis,MO,USA)的TE中,在37℃下孵育30分钟,接着在95℃下孵育10分钟。如本文所述实施PCR以验证整合。使用0611717和0611631产生976bp PCR片段,使用0611225和0611632产生1.4kbp片段,和使用0611631和0611632产生2.7kbp片段的每个杂合整合体的一个转化体被保存,并命名为MIBa308,MIBa309,MIBa310,MIBa311和MIBa312,如表11中所示。

[0459] 转化体MIBa308,MIBa309,MIBa310,MIBa311和MIBa312的培养物在YPD中,37℃下

生长过夜。在125mL挡板瓶中,在37℃以1:50倍将培养物稀释到25mL新鲜YPD中,生长至OD₆₀₀为~4-10。使用本文所述的方法,通过SDS-PAGE来分析细胞样品的蛋白表达。从培养物的细胞沉淀中制备CFE,使用本文所述的方法测量CFE中3HP脱氢酶的活性。通过SDS-PAGE是否存在~53KDa的条带来检测MIBa310和MIBa312中是否分别有UGA1和PYD4的表达,所述条带在没有整合任何基因的菌株中是不存在的。而在这些条件下,SDS-PAGE并不能检测到MIBa309中BAAT的表达。表12A显示了菌株的CFE中3-HP脱氢酶(3-HPDH)的活性。

[0460] 表12A:转化体酶活数据

	菌株	过表达基因	基因来源	基因SEQ ID NO	3-HPDH活性
[0461]	MBin500	N/A	N/A	N/A	0.28,0.24
	MIBa310	gabT (UGA1)	酿酒酵母	141	0.39
	MIBa309	BAAT	阿维链霉菌	140	0.39
	MIBa312	BAAT (PYD4)	克鲁维酵母	142	0.45
[0462]	MIBa311	3-HPDH (YMR226c)	酿酒酵母	144	1.1
	MIBa308	3-HPDH (ydfG)	大肠杆菌	143	0.67

[0463] 在采用改进的测定条件的独立实验中,比较从菌株MBin500(对照),MIBa310,MIBa309和MIBa312中制备的CFE中BAAT的活性。该实验的结果示于表12B中。

[0464] 表12B:转化体酶活数据

	菌株	过表达基因	基因来源	基因SEQ ID NO	BAAT活性
[0465]	MBin500	N/A	N/A	N/A	0.67
	MIBa310	gabT (UGA1)	酿酒酵母	141	9.05
	MIBa309	BAAT	阿维链霉菌	140	0.42
	MIBa312	BAAT (PYD4)	克鲁维酵母	142	105.85

[0466] 质粒pMIBa120-4(如上)含有NotI限制性位点,位于表达盒的侧翼,如下所示:PDC启动子,目的基因(BAAT或3-HPDH),PDC终止子,以及URA3选择标志物。用于在adh1202基因座整合的同源区在NotI限制性位点之外。这些质粒全都具有正向取向的表达盒。

[0467] 构建具有反向取向表达盒的新质粒,以允许方便地筛选纯合整合菌株。用NotI消化质粒pMIBa120-4,使用89mM Tris碱-89mM硼酸-2mM乙二胺四乙酸钠(TBE)缓冲液,在1%琼脂糖凝胶上进行电泳。3.5kbp片段(pMIBa120),4.2kbp片段(pMIBa121),4.2kbp片段(pMIBa122),3.5kbp片段(pMIBa123)和4.2kbp片段(pMIBa124)从凝胶中切割下来,使用QIAquick®Gel Extract Kit(Qiagen)从琼脂糖中提取。使用T4DNA连接酶如上所述,将这些片段分别连接到5.2kbp线性化的用NotI/CIP处理过的pHJJ76-无ura上。根据制造商的说明将连接产物转化至One Shot® TOP10 Chemically Competent大肠杆菌细胞(Invitrogen)中。将转化体涂布在2X YT+amp平板上,37℃孵育过夜。通过XbaI和KpnI消化来筛选数个所得转化体。产生所需片段大小的克隆分别对于UGA1,YMR226C,PYD4,ydfG和BAAT命名为pMIBa131,pMIBa132,pMIBa133,pMIBa134和pMIBa135。所得到的质粒允许以表达盒反向取向的方式使所需基因整合到adh1202基因座上。

[0468] 如本文所述,分离MIBa308-12的ura-衍生体。数个MIBa308-12的FOA抗性菌落是通

过37℃下生长在YPD平板上以纯化两次的菌落。从FOA-抗性菌落中制备基因组DNA,通过PCR进行检查以验证URA3选择标志物的缺失,如本文所述。引物0611631和0611632在整合位点的外部,分别以正向和反向进行退火。引物0611718退火于ura选择标志物上游的PDC终止子上;引物0611632和0611631在野生型染色体上扩增出2.7kbp的片段;引物0611718和0611632对于ura标记存在的整合则扩增出2.4kbp的片段,如果不存在ura标记则扩增出1100bp片段。使用引物0611718和0611632产生1100bp PCR片段,使用0611631和0611632产生2.7kbp片段的MIBa308-12的其中一个ura-菌株被保存,命名为MIBa314 (MIBa310的ura-菌株),MIBa315 (MIBa312的ura-菌株),MIBa316 (MIBa311的ura-菌株),MIBa326 (MIBa308的ura-菌株)和MIBa328 (MIBa309的ura-菌株)。

[0469] pMIBa131,pMIBa132,pMIBa133和pMIBa135各10-15μg,用ApaI,KpnI和NcoI消化,使用89mM Tris碱-89mM硼酸-2mM乙二胺四乙酸钠(TBE)缓冲液,在1%琼脂糖凝胶上进行电泳。用NcoI消化后,将目的片段从琼脂糖凝胶中提取变得更方便。将分别来自于pMIBa131-3,pMIBa135的5553bp,4937bp,5565bp,5493bp片段从凝胶中切割下来,使用QIAQUICKquick®Gel Extract Kit(Qiagen)从琼脂糖中提取。发现纯化后产物的浓度在67-80ng/μL之间。将0.67-0.8μg来自于pMIBa131-3和pMIBa135的限制性片段转化进MIBa314,MIBa316,MIBa315或MIBa328中,如本文所述。将转化体涂布在ura选择培养基上,在37℃下孵育过夜。然后将转化体在ura选择培养基上重新划线,在37℃下孵育过夜。从URA⁺菌落中制备基因组DNA,如上所述使用PCR进行检查以验证第二表达盒的整合,使得菌株对于目的基因是纯合的。如上所述,引物0611718和0611632对于第一个整合扩增出1100bp片段,而引物0611632和0611717则对于反向取向的第二整合扩增出814bp的片段。使用引物0611718和0611632扩增出1100bp,以及使用引物0611717和0611632扩增出814bp的每个品系的URA⁺转化体被命名为MIBa317,MIBa318,MIBa319和MIBa329(见表13)。

[0470] 表13:转化体基因型

菌株	基因型
MIBa317	adh1202Δ::(<i>PDC_{promo}</i> -Opt.ScYMR226c,URA3-Scar)/adh1202Δ::(<i>PDC_{promo}</i> -Opt.ScYMR226c,URA3)ura3-/ura3-
MIBa318	adh1202Δ::(<i>PDC_{promo}</i> -Opt.ScUGA1,URA3-Scar)/adh1202Δ::(<i>PDC_{promo}</i> -Opt.ScUGA1,URA3)ura3-/ura3-
MIBa319	adh1202Δ::(<i>PDC_{promo}</i> -Opt.SkPYD4,URA3-Scar)/adh1202Δ::(<i>PDC_{promo}</i> -Opt.SkPYD4,URA3)ura3-/ura3-
MIBa329	adh1202Δ::(<i>PDC_{promo}</i> -Opt.SaBAAT,URA3-Scar)/adh1202Δ::(<i>PDC_{promo}</i> -Opt.SaBAAT,URA3)ura3-/ura3-

[0472] 确定YMR226c,UGA1,PYD4和BAAT纯合或杂合菌株中的表达和酶活。MIBa309-12,MIBa317-9和MIBa329的过夜培养物在YPD ON中,37℃下生长,然后在37℃在125mL挡板瓶中,以1:50倍将培养物稀释到25mL新鲜YPD中,生长至OD₆₀₀为~4-10。使用本文所述的方法,通过SDS-PAGE来分析细胞样品的蛋白表达。也从培养物的细胞沉淀中制备CFE,使用本文所述的方法测量CFE中3HP脱氢酶的活性。基于SDS-PAGE的结果,MIBa310,MIBa318,MIBa312和MIBa319含有分子量为~53KDa的蛋白(由UGA1或PYD4基因编码的蛋白质的期望大小)。在对菌株MIBa310进行SDS-PAGE分析时并没有观察到对应于该蛋白质的条带。另外,纯合菌株MIBa318和MIBa319中UGA1和PYD4的表达分别比相应的杂合菌株MIBa310或MIBa312中的表达要高(通过SDS-PAGE分析来判断)。在这些条件下在菌株MIBa309或

MIBa329中没有检测(通过SDS-PAGE)到BAAT的表达。表14A显示了菌株MBin500,MIBa311和MIBa317的CFE中,3-HP脱氢酶(3-HPDH)的活性。

[0473] 表14A:转化体酶活性数据

菌株	过表达基因	基因SEQ ID NO	来源	等位类型	3-HPDH活性
MBin500(对照)	N/A	N/A	N/A	N/A	0.13
MIBa311	3-HPDH (YMR226c)	144	酿酒酵母	杂合	1.49
MIBa317	3-HPDH (YMR226c)	144	酿酒酵母	纯合	2.85

[0475] 在使用改进的测定条件的独立实验中,比较从菌株MBin500(对照),MIBa319和MIBa329中制备的CFE中BAAT的活性。该实验的结果示于表14B中。

[0476] 表14B:转化体酶活数据

菌株	过表达基因	基因SEQ ID NO	来源	BAAT活性
MBin500(对照)	N/A	N/A	N/A	0.67
MIBa319	BAAT (PYD4)	142	克鲁维酵母	228.01
MIBa329	BAAT	140	阿维链霉菌	0.38

[0478] 如本文所述,分离菌株MIBa317,MIBa318和MIBa319的ura⁻衍生体。数个MIBa317,MIBa318和MIBa319的FOA抗性菌落是通过37℃下生长在YPD平板上以纯化菌落。从FOA⁻抗性菌落中制备基因组DNA,通过PCR进行检查以验证URA3选择标志物的缺失,如本文中所述。引物0611718和0611632扩增出1100bp片段表明如上述的第一整合,引物0611632和0611717扩增出814bp片段表明在反向取向存在第二整合。引物0611718和0611631扩增出2.6kbp片段表明第二整合具有ura标志,而1200bp片段表明没有ura标志。使用0611718和0611632产生1100bp PCR片段,使用0611632和0611717产生814bp片段,或者使用0611718和0611631产生1200bp MIBa307和MIBa308ura⁻菌株被保存,分别命名为MIBa320和MIBa321。当ura标志从MIBa319中移除时,可能发生基因转换事件,从而产生MIBa322,如PCR所示(用引物0611632和0611631不产生2.7kbp片段,或者用引物0611632和0611717不产生814bp片段,但是用0611718和0611632则扩增出1100bp片段),以至于两个表达盒都是正向取向的。

[0479] 实施例3A-3:在pdc基因座表达天冬氨酸1-脱羧酶(ADC), β -丙氨酸氨基转移酶(BAAT)和3-羟基丙酸脱氢酶(3-HPDH)的插入载体的左端片段的构建

[0480] 含有阿维链霉菌ADC(SEQ ID NO:130)和阿维链霉菌BAAT(SEQ ID NO:140)的左端片段

[0481] 为了允许在EN01启动子和PDC终止子区域之间插入供表达的基因,用XbaI和PacI消化pMhCt068载体,用10个单位的小牛肠磷酸酶(New England Biolabs)在37℃下处理30分钟,在TAE缓冲液中通过0.9%的琼脂糖凝胶电泳纯化,约6.1kbp的条带从凝胶上切下,根据制造商的说明使用NUCLEOSPIN®Extract II Kit(Macherey-Nagel)纯化。

[0482] 然后,用引物0611196和0611186扩增克鲁维酵母BAAT(pyd4)基因(SEQ ID NO:142),所述引物包括后续亚克隆步骤所需的限制性位点。PCR反应体系(50 μ L)包含1 μ L 1比50稀释的小量制备含有克鲁维酵母pyd4基因的质粒,1X Pfx扩增缓冲液(Invitrogen),引

物0611196和0611186每种100pmol, dATP、dCTP、dGTP和dTTP每种200μM, 1.5μL DMSO和2.5个单位的Platinum® Pfx DNA聚合酶(Invitrogen)。在EPPENDORF® MASTERCYCLER® (Eppendorf Scientific)中进行PCR, 程序为95℃ 2分钟的1个循环, 随后是每个为95℃ 30秒, 40.8℃ 30秒和72℃ 1分30秒的34个循环, 及72℃ 5分钟的最后延伸。热循环后, 以TAE缓冲液中0.9%琼脂糖凝胶电泳来分离PCR反应产物, 其中约1428bp的PCR产物被从凝胶上切下并根据制造商的说明使用NUCLEOSPIN® Extract II KiT (Macherey-Nagel) 纯化。

[0483] 上面所产生的pyd4 PCR产物用XbaI和PacI消化, 在TAE缓冲液中通过0.9%琼脂糖凝胶电泳纯化, 约1.4kbp的条带从凝胶上切下, 根据制造商的说明使用NUCLEOSPIN® Extract II KiT (Macherey-Nagel) 纯化。在连接反应中, 将该纯化的DNA克隆进上述被XbaI和PacI限制性消化过的pMhCt068载体中, 所述连接反应(20μL) 含有1X Quick连接缓冲液(New England Biolabs), 100ng XbaI/PacI pMhCt068载体, 70.5ng XbaI/PacI Pyd4插入物, 以及1μL Quick T4DNA连接酶(New England Biolabs)。连接反应在室温下孵育5分钟, 然后在冰上冷却。5μL反应物根据制造商的说明被用于转化Solo Pack Gold SuperCompetent Cells (Agilent Technologies)。转化体被涂布于2X YT+amp平板上并在37℃下孵育过夜。通过XbaI和PacI消化筛选到数个有所需PCR产物的合适插入的所得转化体, 其中确认的一个分离物被命名为“左+pyd4#1”。

[0484] 用引物0611376和0611377 (注意当通过In-Fusion插入到pMhCt068时, 引物0611376会导致在NheI限制性位点的5'末端的“T”碱基被移除, 这将除去存在于初始pMhCt068克隆中的不期望的ATG起始密码子) 扩增编码SEQ ID NO:17的阿维链霉菌ADC, 并且为了在大肠杆菌中表达而经过密码子优化的多聚核苷酸。PCR反应体系(50μL) 包含1μL 1比50稀释的小量制备的含有为大肠杆菌表达经密码子优化的阿维链霉菌ADC基因的质粒, 1X ThermoPol反应缓冲液(New England Biolabs), 引物0611376和0611377每种100pmol, dATP、dCTP、dGTP和dTTP每种200μM, 2μL 100mM MgSO₄, 和2个单位的Vent_R® (exo-) DNA聚合酶(New England Biolabs)。在EPPENDORF® MASTERCYCLER® (Eppendorf Scientific)中进行PCR, 程序为94℃ 2分钟的1个循环, 随后是每个为94℃ 30秒, 54℃ 30秒和72℃ 1分钟的34个循环, 及72℃ 10分钟的最后延伸。热循环后, 以TAE缓冲液中0.9%琼脂糖凝胶电泳来分离PCR反应产物, 其中约420bp的PCR产物被从凝胶上切下并根据制造商的说明使用NUCLEOSPIN® Extract II KiT (Macherey-Nagel) 纯化。

[0485] 然后将“左+pyd4#1”质粒用NheI和AscI消化, 再用10单位的小牛肠磷酸酶(New England Biolabs) 在37℃下处理30分钟, 在TAE缓冲液中通过0.9%的琼脂糖凝胶电泳纯化, 约7.5kbp的条带从凝胶上切下, 根据制造商的说明使用NUCLEOSPIN® Extract II KiT (Macherey-Nagel) 纯化。上述纯化的含有对于大肠杆菌经密码子优化的阿维链霉菌ADC基因的PCR片段, 用NheI和AscI消化, 在TAE缓冲液中通过0.9%的琼脂糖凝胶电泳纯化, 约420bp的条带从凝胶上切下, 根据制造商的说明使用NUCLEOSPIN® Extract II KiT (Macherey-Nagel) 纯化。随后在连接反应中, 将得到的片段连接到线性的“左+pyd4#1”载体中, 所述连接反应(20μL) 含有1X Quick连接缓冲液(New England Biolabs), 100ng NheI/AscI “左+pyd4#1”载体, 31ng NheI和AscI消化过的panD插入片段, 以及1μL Quick T4DNA

连接酶(New England Biolabs)。连接反应在室温下孵育5分钟,然后置于冰上。5μL反应物根据制造商的说明被用于转化Solo Pack Gold SuperCompetent Cells(Agilent Technologies)。转化体被涂布于2X YT+amp平板上并在37℃下孵育过夜。如本文所述,通过AscI和PvuII消化筛选到数个有对于大肠杆菌经密码子优化的阿维链霉菌ADC基因片段的合适插入的所得的转化体。产生所需片段大小的克隆通过DNA测序来确认正确并命名为pMhCt070。

[0486] 质粒pMhCt070用作基础载体,用于添加为在酵母宿主中表达而经过密码子优化的ADC和BAAT同源物。pMhCt070用XbaI和PacI消化,用10个单位的小牛肠磷酸酶(New England Biolabs)在37℃下处理30分钟,在TAE缓冲液中通过0.9%的琼脂糖凝胶电泳纯化,约6.5kbp的条带从凝胶上切下,根据制造商的说明使用**NUCLEOSPIN®**Extract II KiT(Macherey-Nagel)纯化。将如上所述的经XbaI和PacI消化的片段,所述片段含有编码阿维链霉菌BAAT(SEQ ID NO:140),并为了在东方伊萨酵母中表达而经过密码子优化的多核苷酸,连接到pMhCt070切割载体上,如下所示:连接反应(20μL)含有1X Quick连接缓冲液(New England Biolabs),42ng经XbaI和PacI消化的pMhCt070载体,4μL密码子优化的阿维链霉菌BAAT XbaI和PacI消化的插入片段,以及1μL Quick T4 DNA连接酶(New England Biolabs)。连接反应在室温下孵育5分钟,然后置于冰上。5μL反应物根据制造商的说明被用于转化Solo Pack Gold SuperCompetent Cells(Agilent Technologies)。转化体被涂布于2X YT+amp平板上并在37℃下孵育过夜。如本文所述,通过XbaI,PacI和EcoRV消化筛选到数个有所需BAAT ORF的正确插入的所得的转化体。产生所需片段大小的克隆被通过DNA测序确认正确并命名为pMhCt072。

[0487] 将在pMhCt072中的为在大肠杆菌中表达而进行密码子优化的阿维链霉菌ADC基因,用为在东方伊萨酵母中表达而进行的密码子优化的型式(SEQ ID NO:130)进行替换。东方伊萨酵母密码子优化ADC基因(SEQ ID NO:130),连同所需另外的限制性位点和侧翼DNA用引物0611378和0611379来扩增。PCR反应体系(50μL)包含1μL 1比50稀释的小量制备的含有密码子优化的阿维链霉菌panD基因的质粒(**GeneArt®**),1XThermoPol反应缓冲液(New England Biolabs),引物0611378和0611379每种100pmol,dATP、dCTP、dGTP和dTTP每种200μM,2μL 100mM MgSO₄,和2个单位的**Vent_R®**(exo-)DNA聚合酶(New England Biolabs)。在**EPENDORF® MASTERCYCLER®**(Eppendorf Scientific)中进行PCR,程序为94℃ 2分钟的1个循环,随后是每个为94℃ 30秒,54℃ 30秒和72℃ 45秒的34个循环,及72℃ 10分钟的最后延伸。热循环后,以TAE缓冲液中1%琼脂糖凝胶电泳来分离PCR反应产物,其中约420bp的PCR产物被从胶上切下并根据制造商的说明使用**NUCLEOSPIN®**Extract II KiT(Macherey-Nagel)纯化。

[0488] 5μL小量制备的pMhCt072用XbaI和PacI消化,再用10单位的小牛肠磷酸酶(New England Biolabs)在37℃下处理30分钟,在TAE缓冲液中通过1%的琼脂糖凝胶电泳纯化,约7.5kbp的条带从凝胶上切下,根据制造商的说明使用**NUCLEOSPIN®**Extract II KiT(Macherey-Nagel)纯化。在随后的IN-FUSIONTM Advantage PCR Cloning KiT(Clontech)反应中,将含有来自于上面的密码子优化的阿维链霉菌ADC基因的分离PCR产物加入到载体中:10μL反应体积由6μL pMhCt072消化和纯化载体,1μL纯化的密码子优化的

panD PCR产物,1X In-Fusion反应缓冲液(Clontech)和1 μ L IN-FUSIONTM酶(Clontech)构成。反应物在37℃孵育15分钟,在50℃ 15分钟,随后被置于冰上。以40 μ L TE缓冲液稀释反应物并根据制造商的说明使用2.5 μ L来转化Solo Pack Gold Super Competent Cells (Agilent Technologies)。转化体被涂布于2X YT+amp平板上并在37℃下孵育过夜。通过NheI,AscI和ClaI消化筛选到数个合适地插入了所需PCR产物的所得转化体。产生所需片段大小的克隆通过DNA测序来确认正确并命名为pMhCt074(图7)。

[0489] pMhCt074是左端PDC靶向构建体,含有驱动密码子优化的阿维链霉菌ADC (panD, SEQ ID NO:130)表达的PDC启动子,TAL终止子,驱动密码子优化的阿维链霉菌BAAT (SEQ ID NO:140)表达的EN01启动子,RKI终止子,东方伊萨酵母URA3启动子和东方伊萨酵母URA3ORF的5'片段。

[0490] 含有阿维链霉菌ADC (SEQ ID NO:130)和克鲁维酵母BAAT (SEQ ID NO:142)的左端片段

[0491] 为了构建表达克鲁维酵母BAAT (pyd4)的左端DNA构建体,将含有为在东方伊萨酵母中表达而经过密码子优化的克鲁维酵母BAAT序列 (SEQ ID NO:142,如上)的XbaI和PacI消化的片段连接到上面的pMhCt070消化载体上,如下所示:连接反应(20 μ L)含有1X Quick连接缓冲液(New England Biolabs),42ng经XbaI和PacI消化的pMhCt070载体,4 μ L密码子优化的克鲁维酵母BAAT XbaI和PacI消化的插入物,以及1 μ L Quick T4DNA连接酶(New England Biolabs)。连接反应在室温下孵育5分钟,然后置于冰上。5 μ L反应物根据制造商的说明被用于转化Solo Pack Gold Super Competent Cells (Agilent Technologies)。转化体被置于2X YT+amp平板上并在37℃下孵育过夜。如本文所述,通过XbaI,PacI和EcoRV消化筛选到数个有所需pyd4ORF的合适插入所得的转化体。产生所需片段大小的克隆通过DNA测序来确认正确并命名为pMhCt073。

[0492] 质粒pMhCt073含有所需的,为在东方伊萨酵母中表达而经过密码子优化的克鲁维酵母BAAT (pyd4)序列,但是并不含有所需的为在东方伊萨酵母中表达而经过密码子优化的阿维链霉菌ADC (panD)序列。为了移入ORF,5 μ L小量制备的pMhCt073用XbaI和PacI消化,再用10单位的小牛肠磷酸酶(New England Biolabs)在37℃下处理30分钟,在TAE缓冲液中通过1%的琼脂糖凝胶电泳纯化,约7.5kbp的条带从凝胶上切下,根据制造商的说明使用NUCLEOSPIN[®] Extract II KiT (Macherey-Nagel)纯化。在随后的IN-FUSIONTM Advantage PCR Cloning KiT (Clontech)反应中,将含有来自于上面的密码子优化的阿维链霉菌panD基因(如上)的分离PCR产物加入到载体中:10 μ L反应体积由6 μ L pMhCt073消化和纯化载体,1 μ L纯化的密码子优化的panD PCR产物,1X In-Fusion反应缓冲液(Clontech)和1 μ L IN-FUSIONTM酶(Clontech)构成。反应物在37℃孵育15分钟,在50℃ 15分钟,随后被置于冰上。以40 μ L TE缓冲液稀释反应物并根据制造商的说明使用2.5 μ L来转化Solo Pack Gold Super Competent Cells (Agilent Technologies)。转化体被涂布于2X YT+amp平板上并在37℃下孵育过夜。通过NheI,AscI和ClaI消化筛选到数个合适地插入了所需PCR产物所得转化体。产生所需片段大小的克隆通过DNA测序来确认正确并命名为pMhCt076。

[0493] 质粒pMhCt076是左端PDC靶向构建体,含有驱动为在东方伊萨酵母中表达而经密码子优化的阿维链霉菌ADC (panD, SEQ ID NO:130)表达的PDC启动子,TAL终止子,驱动为在东方伊萨酵母中表达而经密码子优化的克鲁维酵母BAAT (SEQ ID NO:142)表达的EN01启动

子, RKI终止子, 东方伊萨酵母URA3启动子和东方伊萨酵母URA3ORF的5' 片段。

[0494] 测序确定, 质粒pMhCt076在到PDC启动子中约200bp处有A至T的核苷酸改变, 以及在PDC启动子的~2/3处有G至T的核苷酸改变, 而这些都是存在于pMhCt068亲本载体中的(如上)。为了解决潜在基因表达改变的担忧, 如下所述克隆一个类似pMhCt076, 但是含有正确PDC启动子的构建体。

[0495] 5μL小量制备的pMhCt082用NheI和PacI消化, 再用10单位的小牛肠磷酸酶(New England Biolabs)在37℃下处理30分钟, 在TAE缓冲液中通过0.9%的琼脂糖凝胶电泳纯化, 约4.7kbp的条带从凝胶上切下, 根据制造商的说明使用**NUCLEOSPIN®Extract II KiT** (Macherey-Nagel) 纯化。4μL小量制备的pMhCt076用NheI和PacI消化, 在TAE缓冲液中通过0.9%的琼脂糖凝胶电泳纯化, 约3.3kbp的条带从凝胶上切下, 根据制造商的说明使用**NUCLEOSPIN®Extract II KiT** (Macherey-Nagel) 纯化。在连接反应中将纯化的4.7kbp载体和3.3kbp插入片段连接起来, 所述反应(20μL) 含有1X Quick连接缓冲液(New England Biolabs), 3μL pMhCt082载体, 6μL pMhCt076插入物, 1μLX Quick T4DNA连接酶(New England Biolabs)。反应物在室温下孵育5分钟, 随后被置于冰上。根据制造商的说明使用5μL该反应来转化**ONE SHOT®TOP10**化学感受态大肠杆菌细胞(Invitrogen)。转化体被涂布于2X YT+amp平板上并在室温下孵育3天。通过StuI和NotI消化来筛选到数个合适地插入了所需PCR产物的所得转化体。产生所需片段大小的克隆通过DNA测序来确认正确并命名为pMhCt083(图8)。

[0496] 质粒pMhCt083与pMhCt076相同, 除了前者含有正确的PDC启动子序列, 而后者如上所述具有一个A至T的核苷酸改变和一个G至T的核苷酸改变。测试表明, 与pMhCt083和pMhCt077相比, 来自于pMhCt076和pMhCt077整合的表达阿维链霉菌panD的菌株中, panD酶活性并没有差异。

[0497] 含有阿维链霉菌ADC (SEQ ID NO:130) 和酿酒酵母gabT (SEQ ID NO:141) 的左端片段

[0498] 4μL小量制备的pMhCt083用XbaI和PacI消化, 用10单位的小牛肠磷酸酶(New England Biolabs)在37℃下处理30分钟, 在TAE缓冲液中通过0.9%的琼脂糖凝胶电泳纯化, 约6.5kbp的条带从凝胶上切下, 根据制造商的说明使用**NUCLEOSPIN®Extract II KiT** (Macherey-Nagel) 纯化。将经XbaI和PacI消化的片段, 所述片段含有编码酿酒酵母gabT并为了在东方伊萨酵母中表达而经过密码子优化 (UGA1, SEQ ID NO:141), 连接到pMhCt083切割载体上, 如下所示: 连接反应(20μL) 含有1X Quick连接缓冲液(New England Biolabs), 1μL经XbaI和PacI消化的纯化pMhCt083载体, 3μL密码子优化的酿酒酵母UGA1XbaI和PacI消化的插入物, 以及1μL Quick T4DNA连接酶(New England Biolabs)。连接反应在室温下孵育5分钟, 然后在冰上冷却。5μL反应物根据制造商的说明被用于转化Solo Pack Gold SuperCompetent Cells (Agilent Technologies)。转化体被涂布于2X YT+amp平板上并在37℃下孵育过夜。如本文所述, 通过XbaI和BglII消化筛选到数个有所需BAAT ORF的合适插入的所得的转化体。产生所需片段大小的克隆通过DNA测序来确认正确并命名为pMhCt087(图9)。

[0499] pMhCt087是左端PDC靶向构建体, 含有驱动为在东方伊萨酵母中表达而经密码子

优化的阿维链霉菌ADC (panD, SEQ ID NO:130) 表达的PDC启动子, TAL终止子, 驱动为在东方伊萨酵母中表达而经密码子优化的酿酒酵母gabT (UGA1, SEQ ID NO:141) 表达的EN01启动子, RKI终止子, 东方伊萨酵母URA3启动子和东方伊萨酵母URA3ORF的5' 片段。

[0500] 实施例3-4: 在pdc基因座表达天冬氨酸1-脱羧酶 (ADC), β -丙氨酸氨基转移酶 (BAAT) 和3-羟基丙酸脱氢酶 (3-HPDH) 的插入载体的右端片段的构建

[0501] 含有大肠杆菌3-HPDH (SEQ ID NO:143) 的右端片段

[0502] 2 μ L小量制备的pMhCt069 (如上) 用XbaI和PacI消化, 再用10单位的小牛肠磷酸酶 (New England Biolabs) 在37°C下处理30分钟, 在TAE缓冲液中通过0.9%的琼脂糖凝胶电泳纯化, 约2.2kbp的条带从凝胶上切下, 根据制造商的说明使用 NUCLEOSPIN® Extract II KiT (Macherey-Nagel) 纯化。将经XbaI和PacI消化的片段, 所述片段含有为了在东方伊萨酵母中表达而经过密码子优化 (ydfG, SEQ ID NO:143) 的大肠杆菌3-HPDH基因, 连接到pMhCt069切割载体上, 如下所示: 连接反应 (20 μ L) 含有1X Quick连接缓冲液 (New England Biolabs), 2 μ L经XbaI和PacI消化的纯化pMhCt069载体, 4 μ L XbaI和PacI消化的密码子优化的大肠杆菌ydfG插入物, 以及1 μ L Quick T4DNA连接酶 (New England Biolabs)。连接反应在室温下孵育5分钟, 然后在冰上冷却。5 μ L反应物根据制造商的说明被用于转化 Solo Pack Gold SuperCompetent Cells (Agilent Technologies)。转化体被涂布于2X YT+amp平板上并在室温下孵育3天。如本文所述, 通过XbaI, PacI和EcoRV消化筛选到数个有所需ydfG ORF的合适插入的所得的转化体。如本发明中所述产生所需片段大小的克隆通过DNA测序来确认正确并命名为pMhCt075 (图10)。

[0503] 质粒pMhCt075含有东方伊萨酵母URA3ORF的3' 片段, 来自于东方伊萨酵母的URA3终止子, 其后是URA3启动子 (用于将来将URA3标志环出), 为了在东方伊萨酵母中表达而经密码子优化的大肠杆菌3-HPDH基因 (ydfG, SEQ ID NO:143), 其由东方伊萨酵母TDH3启动子所驱动, 以及东方伊萨酵母PDC终止子区域。

[0504] 含有酿酒酵母3-HPDH (SEQ ID NO:144) 的右端片段

[0505] 将用XbaI和PacI消化的, 含有为了在东方伊萨酵母中表达而经过密码子优化的酿酒酵母YMR226C基因 (如上) 的片段连接到pMhCt069切割载体中, 如下所示: 连接反应 (20 μ L) 含有1X Quick连接缓冲液 (New England Biolabs), 2 μ L经XbaI和PacI消化的纯化pMhCt069载体, 4 μ L XbaI和PacI消化的密码子优化的酿酒酵母3-HPDH (YMR226C, SEQ ID NO:144) 插入物, 以及1 μ L Quick T4DNA连接酶 (New England Biolabs)。连接反应在室温下孵育5分钟, 然后置于冰上。5 μ L反应物根据制造商的说明被用于转化 Solo Pack Gold SuperCompetent Cells (Agilent Technologies)。转化体被涂布于2X YT+amp平板上并在室温下孵育3天。如本文所述, 通过XbaI, PacI和EcoRV消化筛选到数个有所需YMR226C ORF的合适插入的所得的转化体。产生所需片段大小的克隆通过DNA测序来确认正确并命名为pMhCt077 (图11)。

[0506] 质粒pMhCt077含有东方伊萨酵母URA3ORF的3' 片段, 来自于东方伊萨酵母的URA3终止子, 其后是URA3启动子 (用于将来将URA3标志环出), 为了在东方伊萨酵母中表达而经密码子优化的酿酒酵母3-HPDH基因 (YMR226C, SEQ ID NO:144), 其由东方伊萨酵母TDH3启动子所驱动, 以及东方伊萨酵母PDC终止子区域。

[0507] 实施例3A-5: 在pdc基因座表达天冬氨酸1-脱羧酶 (ADC), β -丙氨酸氨基转移酶

(BAAT) 和3-羟基丙酸脱氢酶 (3-HPDH) 的杂合和纯合酵母菌株

[0508] 上面的实施例3A-3和3A-4描述了为使三种异位基因同时在东方伊萨酵母PDC基因座中靶向表达的各种左端或右端构建体的构建。在转化前,每种构建体(一个所需的左端构建体和一个所需的右端构建体)各约10 μ g用NotI消化,以将所需的转化DNA从pUC18骨架载体中释放出来;对于大多数消化,在NotI消化中也包括限制性酶PvuI。限制性酶PvuI将pUC18载体片段消化为两半,使得用凝胶电泳能够更容易地分离较大的所需DNA片段。含有较大的表达盒的条带可通过凝胶电泳从pUC18骨架DNA中分离出来,并从凝胶中切割,根据制造商的说明使用**NUCLEOSPIN®**Extract II KiT (Macherey-Nagel) 纯化。30 μ L洗脱缓冲液被用于洗脱步骤。等摩尔比的左端和右端构建体,共10 μ L用于转化东方伊萨酵母菌株CNB1或合适的衍生体。在ura选择平板上选择转化体,置于37 $^{\circ}$ C下生长。第二天,挑取出约12个转化体,并在ura选择平板上重新划线以得到单个菌落,在37 $^{\circ}$ C下生长。翌日,从每个起始转化体生成的每条线中挑取单独的菌落,在ura选择平板上重新划线以得到单个菌落。37 $^{\circ}$ C下再生长一个晚上后,从每条线中挑取最终的单菌落,在ura选择平板上重新划线,在37 $^{\circ}$ C下生长过夜。经过这种两轮单菌落纯化和生长之后,制备用于PCR的基因组DNA,所述PCR是为了验证如上所述的所需的靶向整合的发生。为了使用左-和右端构建体靶向到PDC,使用引物0611814,0611554和0611555来进行所需整合事件的验证。引物0611554结合到东方伊萨酵母基因组DNA中正好是PDC终止子区域的3'端,该区域存在于右端PDC靶向构建体中;引物0611555结合到PDC ORF中,进行扩增直至停止;引物0611814结合到存在于右端构建体中的TDH3启动子区域的3'末端附近,并朝3'方向进行扩增。从含有引物0611814和0611554的PCR中产生约1.9kbp的条带表明在PDC基因座发生了所需整合事件。从含引物0611555和0611554的PCR中产生约1.4kbp的条带表明存在野生型PDC基因座。由于该整合事件是二倍体东方伊萨酵母CNB1中的第一个靶向事件,所需整合体对引物0611814和0611554将显示出1.9kbp的条带,对引物0611555和0611554则显示出1.4kbp的条带。对每个质粒具有所需条带谱型的两个独立转化体被命名,如表15中所示。

[0509] 表15:转化体基因型

菌株	具有左端片段的质粒	具有右端片段的质粒	基因型
yMhCt002	pMhCt074	pMhCt075	pdc Δ ::(PDC _{promo} -Opt.SaPanD,ENO1 _{promo} -Opt.SaBAAT,URA3,TDH3 _{promo} -Opt.EcYdfG)/PDC ura3-/ura3-
yMhCt004	pMhCt083	pMhCt077	pdc Δ ::(PDC _{promo} -Opt.SaPanD,ENO1 _{promo} -Opt.SkPyd4,URA3,TDH3 _{promo} -Opt.ScYMR226C)/PDC ura3-/ura3-
yMhCt005	pMhCt087	pMhCt077	pdc Δ ::(PDC _{promo} -Opt.SaPanD,ENO1 _{promo} -Opt.ScUGA1,URA3,TDH3 _{promo} -Opt.ScYMR226c/PDC ura3-/ura3-

[0511] 接下来,如上所述分离yMhCt004或yMhCt005的ura-衍生体。使用引物0611815和0611817,用PCR对每个亲本菌株的几个FOA抗性菌落的基因组DNA进行所需环出事件的筛选。引物0611815在左端构建体的RKI终止子上进行退火,朝着URA3启动子扩增。引物0611817在TDH3启动子中退火,反方向朝着URA3盒扩增。存在828bp的条带意味着只存在所期望的ura3残留位点(在亲本菌株的两个URA3启动子之间发生同源重组后,所留下的单个URA3启动子),而约2.2kbp的条带则意味着存在完整的URA3启动子-URA3ORF-URA3终止子-

URA3启动子盒,这表明所期望的重组事件并没有发生。如上所述,实施Crimson Taq™ DNA聚合酶(New England Biolabs)的PCR反应。来自于亲本菌株yMhCt004的一个FOA抗性菌落,被命名为yMhCt012,来自于亲本菌株yMhCt005的一个FOA抗性菌落,命名为yMhCt007,都具有所期望的828bp条带。

[0512] 接下来转化菌株yMhCt012和yMhCt007,以构建PDC基因被缺失并且分别被panD, pyd4,和YMR226C或者panD,UGA1和YMR226C的表达盒替换的纯合菌株。用来自于pMhCt083和pMhCt077的线性DNA转化菌株yMhCt012,用来自于pMhCt087和pMhCt077的线性DNA转化菌株yMhCt007。经过两轮的单菌落纯化后,用引物0611815和0611817,用PCR筛选每个亲本菌株的几个转化体的基因组DNA,如上所述。来自于各个亲本菌株的两个独立分离的整合体都具有828bp条带(扩增来自于原始靶向PDC基因座的ura3残留区域得到的)和2.2kbp条带(在另一条染色体上发生第二次整合的URA3启动子-URA3ORF-URA3终止子-URA3启动子盒整合所得到的),命名所述整合体,如表16所示。

[0513] 表16:转化体基因型

[0514]	菌株命名	亲本菌株	基因型
	yMhCt013	yMhCt012	pdcΔ::($\text{PDC}_{\text{promo}}$ -Opt.SaPanD,ENO1 promo -Opt.SkPyd4,URA3-Scar,TDH3 promo -Opt.ScYMR226C/ $\text{pdc}\Delta$::($\text{PDC}_{\text{promo}}$ -Opt.SaPanD,ENO1 promo -Opt.SkPyd4,URA3,TDH3 promo -Opt.ScYMR226C)
	yMhCt014		ura3-/ura3-
	yMhCt008	yMhCt007	pdcΔ::($\text{PDC}_{\text{promo}}$ -Opt.SaPanD,ENO1 promo -Opt.ScUGA1,URA3-Scar,TDH3 promo -Opt.ScYMR226C/ $\text{pdc}\Delta$::($\text{PDC}_{\text{promo}}$ -Opt.SaPanD,ENO1 promo -Opt.ScUGA1,URA3,TDH3 promo -Opt.ScYMR226C)
	yMhCt009		ura3-/ura3-

[0515] 如上所述分离yMhCt008的ura-衍生体。使用引物0611815和0611817,用PCR对yMhCt008亲本菌株的几个FOA抗性菌落的基因组DNA进行所需环出事件的筛选。存在828bp的条带意味着只存在所期望的ura3残留位点(在亲本菌株的两个URA3启动子之间发生同源重组后,所留下的单个URA3启动子)。只显示828bp条带的分离物用引物0611555和0611554进行进一步的筛选,如本文所述。从含有引物0611555和0611554的PCR中产生约1.4kbp的条带意味着存在野生型PDC基因座。缺少该条带的分离物,这表明两条染色体上的PDC基因座都丢失,被命名为yMhCt010。

[0516] 在摇瓶中培养菌株,制备CFE,测定天冬氨酸脱羧酶(ADC)活性和3-HP脱氢酶(3-HPDH)活性,如本文所述。这几个测定系列的实验结果(记为试验1-4)如表17中所示。还对来自于表17中试验1的菌株进行SDS-PAGE分析,如本文所述。在SDS-PAGE分析中,试验1中的菌株74/75#1和菌株yMhCt002在27kD处产生了条带,而该条带在试验1的对照菌株中(Mbin500)并不存在。该蛋白质条带的大小与其作为ydfG基因编码的蛋白质的身份是吻合的。

[0517] 表17:转化体酶活数据

[0518]

试验	菌株	过表达基因	等位类型	ADC 活性	3-HPDH 活性
1	MBin500(对照)	N/A	N/A	未检测	0.25
2				0.00	0.12
3				0.00	0.23
4				0.00	0.45
4				0.00	0.54
1	74/75#1	ADC(SEQ ID NO: 130) 3-HPDH (SEQ ID NO: 143) BAAT (SEQ ID NO: 140)	杂合	未检测	0.68
1	yMhCt002	ADC(SEQ ID NO: 130) 3-HPDH (SEQ ID NO: 143) BAAT (SEQ ID NO: 140)	杂合	未检测	0.93
2	yMhCt004	ADC(SEQ ID NO: 130) 3-HPDH (SEQ ID NO: 143) BAAT (SEQ ID NO: 142)	杂合	0.26	1.81
4				0.30	2.20
4				0.24	1.81
2	83/77#2	ADC(SEQ ID NO: 130) 3-HPDH (SEQ ID NO: 143) BAAT (SEQ ID NO: 142)	杂合	0.25	1.80
2	yMhCt005	ADC(SEQ ID NO: 130) 3-HPDH (SEQ ID NO: 144) gabT (SEQ ID NO: 141)	杂合	0.17	1.51
4				0.17	1.26
4				0.14	1.24
2	87/77#2	ADC(SEQ ID NO: 130) 3-HPDH (SEQ ID NO: 144) gabT (SEQ ID NO: 141)	杂合	0.20	1.68
3	yMhCt005	ADC(SEQ ID NO: 130) 3-HPDH (SEQ ID NO: 144) gabT (SEQ ID NO: 141)	杂合	0.16	1.27
3	yMhCt008	ADC(SEQ ID NO: 130)	纯合	0.80	2.29

[0519]	4		3-HPDH (SEQ ID NO: 144) gabT (SEQ ID NO: 141)		0.25	0.85
	4				0.42	1.37
	3	yMhCt009	ADC(SEQ ID NO: 130)	纯合	.045	1.33
	4		3-HPDH (SEQ ID NO: 144)		0.22	0.55
	4		gabT (SEQ ID NO: 141)		0.43	1.15
	4	yMhCt013	ADC(SEQ ID NO: 130)	纯合	0.47	0.65
	4		3-HPDH (SEQ ID NO: 143) BAAT (SEQ ID NO: 142)		0.50	0.82
	4	yMhCt014	ADC(SEQ ID NO: 130)	纯合	0.34	0.51
	4		3-HPDH (SEQ ID NO: 143) BAAT (SEQ ID NO: 142)		0.54	1.06

[0520] 另一个测定系列的实验结果示于表17中(试验2)。表17中试验2的菌株也用SDS-PAGE进行分析,如本文所述。在SDS-PAGE分析中,除Mbin500之外,试验2中的所有菌株在29kD处都具有条带。该蛋白质条带的大小与其作为YMR226c基因编码的蛋白质的身份是吻合的。试验2中的yMhCt005和87/77#2在53kD处具有条带,而该试验中的其他三个样品中却不存在该条带。该蛋白质条带的大小与其作为UGA1基因编码的蛋白质的身份是吻合的。

[0521] 另一个测定系列的实验结果示于表17中(试验3)。表17中试验3的菌株也用SDS-PAGE进行分析,如本文所述。在SDS-PAGE分析中,除Mbin500之外,试验3中的所有菌株在53kD和29kD处都具有条带。该蛋白质条带的大小分别与其作为UGA1和YMR226c基因编码的蛋白质的身份是吻合的。在SDS-PAGE分析中,试验3中的Mbin500和yMhCt005在64kD处具有条带,而该试验中的yMhCt008和yMhCt009中却不存在该条带。该蛋白质条带的大小与其作为东方伊萨酵母酵母CNB1的天然丙酮酸脱羧酶(PDC)基因编码的蛋白质的身份是吻合的。

[0522] 另一个测定系列的实验结果示于表17中(试验4)。表17中试验4的菌株也用SDS-PAGE进行分析,如本文所述。在SDS-PAGE分析中,除Mbin500之外,试验4中的所有菌株在29kD处都具有条带。该蛋白质条带的大小与其作为YMR226c基因编码的蛋白质的身份是吻合的。试验4中的yMhCt005,yMhCt008和yMhCt009在53kD处具有条带,该蛋白质条带的大小与UGA1基因编码的蛋白质是吻合的。试验4中的yMhCt013和yMhCt014菌株在53kD处也有一条微弱的条带,该条带的大小与PYD4基因编码的蛋白质是吻合的。试验4中的Mbin500,yMhCt004和yMhCt005在64kD处具有条带,而该条带在菌株yMhCt008,yMhCt009,yMhCt013和yMhCt014中并不存在。该蛋白质条带的大小与其作为东方伊萨酵母酵母CNB1的天然丙酮酸脱羧酶(PDC)基因编码的蛋白质的身份是吻合的。

[0523] 使用本文所述的方法,在生物反应器中测试菌株Mbin500和yMhCt008,评估3-HP生产。对照菌株Mbin500并没有产生可检测的3-HP(两个独立发酵的平均值)。菌株yMhCt008产生2.45g/L的3-HP(12个独立发酵的平均值)。

[0524] 实施例3A-6:在adh1202基因座表达 β -丙氨酸氨基转移酶(BAAT)或3-羟基丙酸脱氢酶(3-HPDH),在pdc基因座表达天冬氨酸1-脱羧酶(ADC), β -丙氨酸氨基转移酶(BAAT)和3-羟基丙酸脱氢酶(3-HPDH)的酵母菌株

[0525] 用NotI和PvuI消化20 μ g pMhCt077,pMhCt083和pMhCt087(如上),然后以89mM

Tris碱-89mM硼酸-2mM EDTA二钠缓冲液(TBE)中进行1%琼脂糖凝胶电泳。NotI消化片段3815bp,5332bp或5320bp分别来自于pMhCt077,pMhCt083和pMhCt087,从凝胶中切割下来,并使用QIAQUICK®Gel Extract KiT(Qiagen)从琼脂糖中提取。将560ng NotI消化后的pMhCt077和560ng NotI消化后的pMhCt083或pMhCt087转化进菌株MIBa320,MIBa321和MIBa322。MIBa320用pMhCt077/83以及pMhCt077/87组合转化。MIBa321用pMhCt077/87转化,MIBa322用pMhCt077/83转化,如本文所述。将转化体涂布于ura选择培养基上,室温孵育约60小时。转化体在ura选择培养基上重新划线,在37℃下孵育过夜。从URA3+菌落中制备基因组DNA,如本文所述使用PCR检查以验证表达盒的整合。引物对611814和611554扩增出1.9kbp片段表明整合。引物对611555和611554扩增出1.4kbp片段表明是野生型基因座。用611554和611814扩增出1.9kbp PCR片段,用611555和611554扩增出1.4kbp片段的每个品系中的一个URA3+转化体被保存,它们被命名为MIBa323,MIBa324,MIBa325和MIBa327(见表18的基因型)。启动子和终止子来源于东方伊萨酵母基因。

[0526] 表18:转化体基因型

[0527]	菌株名称	基因型
	MIBa323	adh1202Δ::(<i>PDC_{promo}</i> -Opt.ScYMR226c, URA3-Scar)/ adh1202Δ::(<i>PDC_{promo}</i> -Opt.ScYMR226c, URA3-Scar)
	MIBa324	pdcΔ::(<i>PDC_{promo}</i> -Opt.SaPanD, ENO1 _{promo} -Opt.ScUGA1, URA3, TDH3 _{promo} -Opt.ScYMR226c)/ <i>PDC ura3-ura3-</i> adh1202Δ::(<i>PDC_{promo}</i> -Opt.ScYMR226c, URA3-Scar)/ adh1202Δ::(<i>PDC_{promo}</i> -Opt.ScYMR226c, URA3-Scar) pdcΔ::(<i>PDC_{promo}</i> -Opt.SaPanD, ENO1 _{promo} -Opt.SkPyd4, URA3, TDH3 _{promo} -Opt.ScYMR226c)/ <i>PDC ura3-ura3-</i>
[0528]	MIBa325	adh1202Δ::(<i>PDC_{promo}</i> -Opt.ScUGA1, URA3- Scar)/adh1202Δ::(<i>PDC_{promo}</i> -Opt.ScUGA1, URA3-Scar) pdcΔ::(<i>PDC_{promo}</i> -Opt.SaPanD, ENO1 _{promo} -Opt.ScUGA1, URA3, TDH3 _{promo} -Opt.ScYMR226c)/ <i>PDC ura3-ura3-</i>
	MIBa327	adh1202Δ::(<i>PDC_{promo}</i> -Opt.SkPyd4, URA3-Scar)/ adh1202Δ::(<i>PDC_{promo}</i> -Opt.SkPyd4, URA3-Scar) pdcΔ::(<i>PDC_{promo}</i> -Opt.SaPanD, ENO1 _{promo} -Opt.SkPyd4, URA3, TDH3 _{promo} -Opt.ScYMR226c)/ <i>PDC ura3-ura3-</i>

[0529] 菌株在摇瓶培养,如本发明中所述制备CFE并测试3-HP脱氢酶(3-HPDH)活性。结果示于表19中。

[0530] 表19:转化体酶活性数据

[0531]

菌株	过表达的基因	3-HPDH 活性
MBin500 (control)	N/A	0.14
MIBa314	gabT (SEQ ID NO: 141)	0.09
MIBa318	gabT (SEQ ID NO: 141)	0.41
MIBa321	gabT (SEQ ID NO: 141)	0.08
MIBa325	gabT (SEQ ID NO: 141) ADC (SEQ ID NO: 130) 3-HPDH (SEQ ID NO: 144)	1.15
MIBa315	BAAT (SEQ ID NO: 142)	0.12
MIBa319	BAAT (SEQ ID NO: 142)	0.17
MIBa322	BAAT (SEQ ID NO: 142)	0.09
MIBa327	BAAT (SEQ ID NO: 142) ADC (SEQ ID NO: 130) 3-HPDH (SEQ ID NO: 144)	0.98
MIBa316	3-HPDH (SEQ ID NO: 144)	0.48
MIBa317	3-HPDH (SEQ ID NO: 144)	2.15
MIBa320	3-HPDH (SEQ ID NO: 144)	1.07
MIBa323	3-HPDH (SEQ ID NO: 144) ADC (SEQ ID NO: 130) gabT (SEQ ID NO: 141)	2.66
MIBa324	3-HPDH (SEQ ID NO: 144) ADC (SEQ ID NO: 130) BAAT (SEQ ID NO: 142)	2.12

[0532] 如本发明所描述分离MIBa323、MIBa324、MIBa325和MIBa327的Ura⁻衍生体。如本发明所描述的,从FOA抗性菌落制备基因组DNA并以PCR检查来确定失去了URA3选择标志物。引物0611815在左端构建体RKI终止子退火并向URA3启动子扩增,而引物0611817在TDH3启动子退火并反向朝URA3盒扩增。828bp条带的存在表示仅URA3残留位点的存在(在亲本菌株中两个URA3启动子间同源重组后留下的单个URA3启动子),而约2.2kbp条带表示完整URA3启动子-URA3ORF-URA3终止子-URA3启动子盒的存在,表明所需的重组事件没有发生。以引物0611815和0611817获得了828bp PCR片段的MIBa323、MIBa324、MIBa325和MIBa327的Ura⁻菌株被保存并被分别命名为MIBa335、MIBa333、MIBa334和MIBa336。

[0533] 按上面MIBa320-2转化部分中所描述,以来自pMhCt077和pMhCt087的片段转化菌株MIBa333和MIBa334,并以来自pMhCt077和pMhCt083的片段转化菌株MIBa335和MIBa336。如本发明中所描述通过在ura选择培养基上生长来选择转化体。如本发明所描述的,从URA3⁺菌落制备基因组DNA并以PCR检查来确定表达盒的整合。引物0611815在左端构建体RKI终止子退火并向URA3启动子扩增。引物0611817在TDH3启动子退火并反向朝URA3盒扩增。对第一整合所期望的828bp条带的存在表示仅URA3残留位点的存在(在亲本菌株中两个URA3启动子间同源重组后留下的单个URA3启动子),而对第二整合约2.2kbp条带表示完整URA3启动子-URA3ORF-URA3终止子-URA3启动子盒的存在。当ura标志存在时,引物对0611815和0611816扩增625bp片段。当PDC基因座存在时,引物0611555和0611554扩增1.4kbp片段。纯合的整合株不应该能以这些引物扩增片段。保存以引物0611815和0611817扩增出828bp和2.2kbp PCR片段的,以引物0611815和0611816扩增出625bp的,以引物0611555和0611554未扩增出片段的每个品系的一个URA3⁺转化体;这些被命名为MIBa340、MIBa341、MIBa345和

MIBa348 (见表20A)。启动子和终止子来自东方伊萨酵母基因。

[0534] 表20A:转化体基因型

菌株名称	基因型
MIBa345	adh1202Δ::((PDC _{promo} -Opt.ScYMR226c URA3-Scar)/adh1202Δ::((PDC _{promo} -Opt.ScYMR226c URA3-Scar) pdcΔ::((PDC _{promo} -Opt.SaPanD ENO1 _{promo} -Opt.ScUGA1, URA3-Scar, TDH3 _{promo} -Opt.ScYMR226c)/ pdcΔ::((PDC _{promo} -Opt.SaPanD, ENO1 _{promo} -Opt.ScUGA1, URA3, TDH3 _{promo} -Opt.ScYMR226c) ura3-/ura3-
MIBa348	adh1202Δ::((PDC _{promo} -Opt.ScYMR226c URA3-Scar)/adh1202Δ::((PDC _{promo} -Opt.ScYMR226c URA3-Scar) pdcΔ::((PDC _{promo} -Opt.SaPanD, ENO1 _{promo} -Opt.SkPyd4, URA3-Scar, TDH3 _{promo} -Opt.ScYMR226c) / pdcΔ::((PDC _{promo} -Opt.SaPanD, ENO1 _{promo} -
	Opt.SkPyd4, URA3, TDH3 _{promo} -Opt.ScYMR226c) ura3-/ura3-
MIBa340	adh1202Δ::((PDC _{promo} -Opt.ScUGA1 URA3-Scar)/ adh1202Δ::((PDC _{promo} -Opt.ScUGA1 URA3-Scar) pdcΔ::((PDC _{promo} -Opt.SaPanD, ENO1 _{promo} -Opt.ScUGA1, URA3-Scar, TDH3 _{promo} -Opt.ScYMR226c)/ pdcΔ::((PDC _{promo} -Opt.SaPanD, ENO1 _{promo} -Opt.ScUGA1, URA3, TDH3 _{promo} -Opt.ScYMR226c) ura3-/ura3-
MIBa341	adh1202Δ::((PDC _{promo} -Opt.SkPyd4 URA3-Scar)/ adh1202Δ::((PDC _{promo} -Opt.SkPyd4 URA3-Scar) pdcΔ::((PDC _{promo} -Opt.SaPanD, ENO1 _{promo} -Opt.SkPyd4, URA3-Scar, TDH3 _{promo} -Opt.ScYMR226c) / pdcΔ::((PDC _{promo} -Opt.SaPanD, ENO1 _{promo} -Opt.SkPyd4, URA3, TDH3 _{promo} -Opt.ScYMR226c) ura3-/ura3-

[0537] 比较从菌株MBin500 (对照)、MIBa345、MIBa348、MIBa340和MIBa341制备的CFE中的天冬氨酸1-脱羧酶 (ADC), β-丙氨酸氨基转移酶 (BAAT) 和3-HP脱氢酶 (3-HPDH) 活性。该实验的结果显示于表20B中。

[0538] 表20B:转化体酶活性数据

菌株	过表达的基因	基因来源	ADC 活性	BAAT 活性	3-HPDH 活性
MBin500 (对照)	N/A	N/A	0.002	0.61	0.4
MIBa345	YMR226c (SEQ ID NO: 144)	酿酒酵母	0.194	14.37	79.5
	ADC (SEQ ID NO: 130)	阿维链霉菌			
	UGA1 (SEQ ID NO: 141)	酿酒酵母			
MIBa348	YMR226c (SEQ ID NO: 144)	酿酒酵母	0.169	173.67	76.7
	ADC (SEQ ID NO: 130)	阿维链霉菌			
	PYD4 (SEQ ID NO: 142)	克鲁维酵母			
MIBa340	YMR226c (SEQ ID NO: 144)	酿酒酵母	0.239	19.51	64.8
	ADC (SEQ ID NO: 130)	阿维链霉菌			
	UGA1 (SEQ ID NO: 141)	酿酒酵母			
MIBa341	YMR226c (SEQ ID NO: 144)	酿酒酵母	0.22	386.92	65.5
	ADC (SEQ ID NO: 130)	阿维链霉菌			
	PYD4 (SEQ ID NO: 142)	克鲁维酵母			

[0540] 实施例3A-7:具有用于在adh1202基因座表达天冬氨酸1-脱羧酶 (ADC) 的多个核苷酸序列的插入载体左端片段

[0541] 设计构建体以在东方伊萨酵母adh1202基因座掺入编码ADC (SEQ ID NO:17) 的四个核苷酸拷贝。使用与本发明所描述的对PDC基因座所用方法类似的方法,设计左端和右端构建体以允许同源重组。整合载体的一般设计和所需的重组事件在图3中显示。该方法也被用于表达如下面实施例中所描述的可选的ADC (SEQ ID NO:139)。

[0542] 为了防止重组在编码相同ADC序列的多个核苷酸拷贝之间发生,设计四种不同的为在东方伊萨酵母中表达而密码子优化的核苷酸序列 (SEQ ID NO:130、145、146和147) 以编码相同的SEQ ID NO:17的ADC序列。此外,由于构建体的初始设置被设计为靶向东方伊萨

酵母的ald5680基因座, adh1202靶向序列在克隆的后面步骤中被掺入这些载体。ald5680构建体可以被用于靶向东方伊萨酵母CNB1菌株中的第二基因座, 该菌株中在adh1202异位的四个panD拷贝与在ald5680的四个另外的panD拷贝已纯合。

[0543] 如下构建左端ald5680靶向载体。以引物0612271和0612272扩增含紧接着ald5680ORF的5'端的序列, 连同有所需的另外限制性位点和克隆用的侧翼DNA的PCR产物。PCR反应体系(50 μ L)包含1 μ L 1比50稀释的pHJJ75小量制备质粒DNA(图23), 1X iproofTMHF缓冲液(Bio-Rad Laboratories), 引物0612271和0612272每种100pmol, dATP、dCTP、dGTP和dTTP每种200 μ M, 和1个单位的iProofTMHigh Fidelity DNA聚合酶(Bio-Rad Laboratories)。在EPPENDORF® MASTERCYCLER®(Eppendorf Scientific)中进行PCR, 程序为98℃ 30秒的1个循环, 随后是每个为98℃ 10秒, 59℃ 20秒和72℃ 45秒的32个循环, 及72℃ 10分钟的最后延伸。热循环后, 以TAE缓冲液中1%琼脂糖凝胶电泳来分离PCR反应产物, 其中约930碱基对的PCR产物被从凝胶上切下并根据制造商的说明使用NUCLEOSPIN®Extract II KiT(Macherey-Nagel)纯化。

[0544] 为允许纯化更大量的DNA, 使用纯化的930bp PCR产物作为模板DNA来重复上述PCR反应。设置5个50 μ L的反应并以上述条件扩增, 除了以1 μ L纯化的930bp PCR产物替代pHJJ75质粒(如上)作为模板DNA。热循环后, 如上纯化扩增的930bp产物。

[0545] 含PDC promo-optPanD-EN01-UGA1的片段(其含有SEQ ID NO:130的为东方伊萨酵母密码子优化的阿维链霉菌ADC编码序列)通过NotI和EcoRI消化, 从pMhCt087(如上)上切下。10 μ g pMhCt087的中量制备品被以NotI和EcoRI消化并随后通过TAE缓冲液中0.9%琼脂糖凝胶电泳纯化。约4.4kbp的条带被从凝胶上切下并根据制造商的说明使用NUCLEOSPIN®Extract II KiT(Macherey-Nagel)纯化。

[0546] 使用引物0612273和0612274扩增含URA3分裂标志的5'半边, 并有所需的另外限制性位点和克隆用的侧翼DNA的PCR产物。PCR反应体系(50 μ L)包含1 μ L 1比50稀释的pMhCt082小量制备质粒DNA(如上), 1X iproofTMHF缓冲液(Bio-Rad Laboratories), 引物0612273和0612274每种100pmol, dATP、dCTP、dGTP和dTTP每种200 μ M, 和1个单位的iProofTMHigh Fidelity DNA聚合酶(Bio-Rad Laboratories)。在EPPENDORF® MASTERCYCLER®(Eppendorf Scientific)中进行PCR, 程序为98℃ 30秒的1个循环, 随后是每个为98℃ 10秒, 59℃ 20秒和72℃ 45秒的32个循环, 及72℃ 10分钟的最后延伸。热循环后, 以TAE缓冲液中1%琼脂糖凝胶电泳来分离PCR反应产物, 其中约960碱基对的PCR产物被从凝胶上切下并根据制造商的说明使用NUCLEOSPIN®Extract II KiT(Macherey-Nagel)纯化。

[0547] 为构建上述DNA片段的接收载体, 质粒pUC19(Yanisch-Perron, C, Vieira, J. 和Messing, J. (1985) Gene, 33, 103-119)被以Hind III和EcoRI消化, 以10个单位的小牛肠磷酸酶(New England Biolabs)在37℃下处理30分钟, 并以TAE缓冲液中0.9%琼脂糖凝胶电泳纯化, 约2.6kbp的条带被从凝胶上切下并根据制造商的说明使用NUCLEOSPIN®Extract II KiT(Macherey-Nagel)纯化。

[0548] 来自上面的纯化的930bp、4.4kbp和960bp的DNA片段随后被使用IN-FUSIONTM Advantage PCR Cloning KiT(Clontech)在10 μ L总反应体积中插入消化的pUC19片段, 10 μ L总反应体积由150ng以Hind III和EcoRI消化的pUC19载体, 56ng含ald5680侧翼DNA的930bp

DNA, 250ng来自以NotI和EcoRI消化的pMhCt087的PDC promo-optPanD-EN01-UGA1片段, 55ng含URA3分裂标志5' 半边的960bpPCR产物, 1X In-Fusion反应缓冲液(Clontech)和1μL IN-FUSIONTM酶(Clontech)构成。反应物在37℃孵育15分钟, 在50℃ 15分钟, 随后被置于冰上。随后以40μL TE缓冲液稀释反应物并根据制造商的说明使用2.5μL来转化Solo Pack Gold Super Competent Cells(Agilent Technologies)。转化体被涂布于2X YT+amp平板上并在室温下孵育3天。通过SalI和HpaI消化筛选到数个合适插入了所需PCR产物的所得转化体。产生所需片段大小的克隆通过DNA测序来确认正确并命名为pMhCt089。

[0549] 接下来, 以编码SEQ ID NO:17ADC的, 为在东方伊萨酵母中表达而密码子优化的阿维链霉菌panD基因来替代pMhCt089中的UGA10RF。通过GeneArt®在pMA-T载体中合成阿维链霉菌panD型式r1(SEQ ID NO:145)。以XbaI和PacI消化质粒pMA-T, 得到的片段被以TAE缓冲液中0.9%琼脂糖凝胶电泳分离, 约434bp的条带被从胶上切下并根据制造商的说明使用NUCLEOSPIN®Extract II KiT(Macherey-Nagel)纯化。质粒pMhCt089被以XbaI和PacI消化, 以10个单位的小牛肠磷酸酶(New England Biolabs)在37℃下处理30分钟, 而得到的片段被以TAE缓冲液中0.9%琼脂糖凝胶电泳分离, 约4.5kbp的条带被从凝胶上切下并根据制造商的说明使用NUCLEOSPIN®Extract II KiT(Macherey-Nagel)纯化。在含1X Quick连接缓冲液(New England Biolabs), 2μL XbaI/PacI pMhCt089载体, 2μL XbaI/PacI阿维链霉菌panD型式r1插入物, 和1μL Quick T4DNA连接酶(New England Biolabs)的连接体系中(20μL)将pMhCt089载体和阿维链霉菌panD型式r1结合。连接反应物被在室温下孵育5分钟, 并随后被置于冰上。5μL该反应物被用于根据制造商的说明转化ONE SHOT® TOP10化学感受态大肠杆菌细胞(Invitrogen)。转化体被涂布于2X YT+amp平板上并在室温下孵育3天。通过XbaI和PacI消化筛选到数个合适插入了所需PCR产物的所得转化体。产生所需片段大小的克隆通过DNA测序来确认正确并命名为pMhCt092。

[0550] 左端构建体的最后克隆步骤是以adh1202 5' 同源区域替代存在于pMhCt092中的ald5680 5' 同源区域。以引物0612470和0612471扩增含ald12020RF 5' 端序列, 连同有所需的另外限制性位点和克隆用的侧翼DNA的PCR产物。PCR反应体系(50μL)包含1μL 1比50稀释的pGMEr140小量制备质粒DNA(本发明所描述pMIBa107的衍生体, 其中PCR扩增的区域相同), 1X iproofTMHF缓冲液(Bio-Rad Laboratories), 引物0612470和0612471每种100pmol, dATP、dCTP、dGTP和dTTP每种200μM, 和1个单位的iProofTMHigh Fidelity DNA聚合酶(Bio-Rad Laboratories)。在EPPENDORF® MASTERCYCLER®(Eppendorf Scientific)中进行PCR, 程序为98℃ 30秒的1个循环, 随后是每个为98℃ 10秒, 59℃ 20秒和72℃ 30秒的34个循环, 及72℃ 10分钟的最后延伸。热循环后, 以TAE缓冲液中1%琼脂糖凝胶电泳来分离PCR反应产物, 其中约790bp的PCR产物被从凝胶上切下并根据制造商的说明使用NUCLEOSPIN®Extract II KiT(Macherey-Nagel)纯化。

[0551] 为构建上述DNA片段的接收载体, 质粒pMhCt092被以HpaI和NotI消化, 以10个单位的小牛肠磷酸酶(New England Biolabs)在37℃下处理30分钟, 并以TAE缓冲液中0.9%琼脂糖凝胶电泳纯化, 约7.0kbp的条带被从凝胶上切下并根据制造商的说明使用NUCLEOSPIN®Extract II KiT(Macherey-Nagel)纯化。使用IN-FUSIONTM Advantage PCR Cloning KiT(Clontech)在10μL总反应体积中将PCR产物和线性载体联结, 10μL总反应

体积由120ng以HpaI和NotI消化的pMhCt092载体,30ng含adh1202 5'同源区的PCR产物,1X In-Fusion反应缓冲液(Clontech)和1μL IN-FUSIONTM酶(Clontech)构成。反应物在37℃孵育15分钟,在50℃ 15分钟,随后被置于冰上。以40μLTE缓冲液稀释反应物并根据制造商的说明使用2.5μL来转化Solo Pack Gold Super Competent Cells(Agilent Technologies)。转化体被涂布于2X YT+amp平板上并在室温下孵育过夜。通过BamHI和PstI消化筛选到数个合适插入了所需PCR产物的所得转化体。产生所需片段大小的克隆通过DNA测序来确认正确并命名为pMhCt095(图12)。

[0552] 质粒pMhCt095是左端东方伊萨酵母adh1202靶向构建体,其含有驱动为在东方伊萨酵母中表达而密码子优化的阿维链霉菌ADC基因(SEQ ID NO:130)表达的PDC启动子,TAL终止子,驱动为在东方伊萨酵母中表达而密码子优化的第二阿维链霉菌ADC基因(SEQ ID NO:145)表达的EN01启动子,东方伊萨酵母RKI终止子,东方伊萨酵母URA3启动子和东方伊萨酵母URA3ORF的5'片段。

[0553] 实施例3A-8:具有用于在adh1202基因座表达天冬氨酸1-脱羧酶(ADC)的多个核苷酸序列的插入载体右端片段

[0554] 以引物0612275和0612276扩增含东方伊萨酵母URA3ORF 3'片段,连同有所需的另外限制性位点和克隆用的侧翼DNA的PCR产物。PCR反应体系(50μL)包含1μL 1比50稀释的pMhCt069小量制备质粒DNA(如上),1X iproofTMHF缓冲液(Bio-Rad Laboratories),引物0612275和0612276每种100pmol,dATP、dCTP、dGTP和dTTP每种200μM,和1个单位的iProofTMHigh Fidelity DNA聚合酶(Bio-Rad Laboratories)。在EPPENDORF® MASTERCYCLER®(Eppendorf Scientific)中进行PCR,程序为98℃ 30秒的1个循环,随后是每个为98℃ 10秒,59℃ 20秒和72℃ 45秒的32个循环,及72℃ 10分钟的最后延伸。热循环后,以TAE缓冲液中1%琼脂糖凝胶电泳来分离PCR反应产物,其中约1155bp的PCR产物被从胶上切下并根据制造商的说明使用NUCLEOSPIN®Extract II KiT(Macherey-Nagel)纯化。

[0555] 含TDH3启动子,用于异位基因插入的XbaI和PacI限制性位点,和PDC终止子的片段通过以NotI和PmeI消化被从pMhCt069上切下。10μg pMhCt069的中量制备品被以NotI和PmeI消化,并随后通过TAE缓冲液中0.9%琼脂糖凝胶电泳纯化。约1.85kbp的条带被从凝胶上切下并根据制造商的说明使用NUCLEOSPIN®Extract II KiT(Macherey-Nagel)纯化。

[0556] 以引物0612277和0612278扩增含ald56800RF 3'序列,连同有所需的另外限制性位点和克隆用的侧翼DNA的PCR产物。PCR反应体系(50μL)包含1μL 1比50稀释的pHJJ75小量制备质粒DNA(图23),1X iproofTMHF缓冲液(Bio-Rad Laboratories),引物0612277和0612278每种100pmol,dATP、dCTP、dGTP和dTTP每种200μM,和1个单位的iProofTMHigh Fidelity DNA聚合酶(Bio-Rad Laboratories)。在EPPENDORF® MASTERCYCLER®(Eppendorf Scientific)中进行PCR,程序为98℃ 30秒的1个循环,随后是每个为98℃ 10秒,59℃ 20秒和72℃ 45秒的32个循环,及72℃ 10分钟的最后延伸。热循环后,以TAE缓冲液中1%琼脂糖凝胶电泳来分离PCR反应产物,其中约844bp的PCR产物被从凝胶上切下并根据制造商的说明使用NUCLEOSPIN®Extract II KiT(Macherey-Nagel)纯化。

[0557] 来自上面的纯化的1155bp、1.85kbp和844bp的DNA片段随后被使用IN-FUSIONTM Advantage PCR Cloning KiT(Clontech)在10μL总反应体积中插入以EcoRI和Hind III消化的pUC19,10μL总反应体积由150ng以Hind III和EcoRI消化的pUC19载体,66ng含URA3分裂标志3'部分的1155bpDNA;106ng来自以PmeI和NotI消化的并含TDH3启动子,用于异位基因插入的XbaI和PacI限制性位点,和PDC终止子的来自pMhCt069的1.85kbp片段;48ng含3'ald5680侧翼DNA的844bp PCR产物;1X In-Fusion反应缓冲液(Clontech)和1μL IN-FUSIONTM酶(Clontech)构成。反应物在37℃孵育15分钟,在50℃ 15分钟,随后被置于冰上。以40μL TE缓冲液稀释反应物并根据制造商的说明使用2.5μL来转化Solo Pack Gold Super Competent Cells(Agilent Technologies)。转化体被涂布于2X YT+amp平板上并在室温下孵育3天。通过SalI和HpaI消化筛选到数个合适插入了所需PCR产物的所得转化体。产生所需片段大小的克隆通过DNA测序来确认正确并命名为ald5680右#20。

[0558] 如下在TDH3启动子和ald5680右#20的3' ald5680侧翼DNA之间加入TKL终止子,PGK1启动子,XbaI和PacI限制性位点以及PDC终止子区域的较短型式。以引物0612356和0612357扩增TKL终止子连同所需的另外限制性位点和克隆用的侧翼DNA。使用退火温度的温度梯度和在某些反应中的DMSO来扩增所需的PCR产物。准备四个相同的PCR反应体系,每个PCR反应体系(50μL)包含1μL 1比50稀释的pACN23小量制备质粒DNA(图20),1X iproofTMHF缓冲液(Bio-Rad Laboratories),引物0612356和0612357每种100pmol,dATP、dCTP、dGTP和dTTP每种200μM,和1个单位的iProofTMHigh Fidelity DNA聚合酶(Bio-Rad Laboratories)。如上设置第二组四个管,除了每个反应包括加入1.5μL的DMSO。在EPPENDORF® MASTERCYCLER®(Eppendorf Scientific)中进行PCR,程序为98℃ 30秒的1个循环,随后是每个为98℃ 10秒,X℃ 20秒,其中X=47.6℃、51.8℃、57.1℃或62.1℃,和72℃ 45秒的32个循环,及72℃ 10分钟的最后延伸。在每个退火温度进行有和没有DMSO的PCR。热循环后,以TAE缓冲液中1%琼脂糖凝胶电泳来分离每种10μL的PCR反应物。对该凝胶的观察显示有DMSO在两个最高的退火温度进行的,以及没有DMSO在两个最低退火温度进行的PCR反应给出了所需844bp产物的最高产量。将这四个PCR反应物结合,以TAE缓冲液中1%琼脂糖凝胶电泳来分离,其中约844碱基对的PCR产物被从凝胶上切下并根据制造商的说明使用NUCLEOSPIN®Extract II KiT(Macherey-Nagel)纯化。

[0559] 以两步骤过程来完成所需PGK1启动子区域的PCR扩增。首先,以下面引物0612150和0612151扩增之后克隆含PGK1启动子的PCR产物。PCR反应体系(50μL)包含3μL pJLJ49小量制备质粒DNA(图25),1X Pfx扩增缓冲液(Invitrogen),2mm MgSO₄,引物0612150和0612151每种100pmol,dATP、dCTP、dGTP和dTTP每种200μM,和1.25个单位的Platinum®Pfx DNA聚合酶(InvitrogenBio-Rad Laboratories)。在EPPENDORF® MASTERCYCLER®(Eppendorf Scientific)中进行PCR,程序为95℃ 2分钟的1个循环,随后是每个为95℃ 1分钟,55℃ 1分钟和72℃ 3分钟的25个循环,及72℃ 10分钟的最后延伸。热循环后,以TAE缓冲液中1%琼脂糖凝胶电泳来分离PCR反应产物,其中约630bp的PCR产物被从凝胶上切下并根据制造商的说明使用NUCLEOSPIN®Extract II KiT(Macherey-Nagel)纯化。

[0560] 根据制造商的说明,使用测序用Zero Blunt® TOPO®PCR Cloning KiT

(Invitrogen) 将约630bp的PCR产物克隆进pCR4TM BLUNT **TOPO®**载体 (Invitrogen)。在6 μ L总反应体积中,0.5或4 μ L 630bp PCR产物,1 μ L盐溶液 (Invitrogen) 和1 μ L pCR4TM BLUNT **TOPO®** (Invitrogen) 一起在室温孵育15分钟。根据制造商的说明每种克隆反应物2 μ L被转化入ONE **SHOT®**TOP10化学感受态大肠杆菌细胞 (Invitrogen)。转化体被置于LB+kan平板上并在37℃孵育过夜。通过EcoRI消化筛选到数个合适插入了所需PCR产物的所得转化体。产生所需片段大小的克隆通过DNA测序来确认正确并命名为PGK1_in_TOP0。

[0561] 用作PCR模板之前,来自PGK1_in_TOP0的PGK1启动子被如下分离并纯化。25 μ L的PGK1_in_TOP0中量制备品以XbaI和PacI消化并通过TAE缓冲液中0.9%琼脂糖凝胶电泳来纯化,而约640bp的PCR产物被从凝胶上切下并根据制造商的说明使用**NUCLEOSPIN®** Extract II KiT (Macherey-Nagel) 纯化。

[0562] 使用温度梯度以引物0612358和0612359扩增PGK1启动子连同所需的另外限制性位点和克隆用的侧翼DNA。设置八个相同的PCR反应,每个PCR反应体系(50 μ L) 包含20ng通过PGK1_in_TOP0的XbaI和PacI消化得到的纯化PGK1启动子DNA,1X Herculase反应缓冲液 (Agilent Technologies),引物0612358和0612359每种100pmol,dATP、dCTP、dGTP和dTTP每种200 μ M,和2.5个单位的Herculase HotStart DNA聚合酶 (Agilent Technologies)。在**EPPENDORF® MASTERCYCLER®** (Eppendorf Scientific) 中进行PCR,程序为98℃ 30秒的1个循环,随后是每个为98℃ 10秒,X℃ 20秒,其中X=53.7℃、55.4℃、57.6℃、60.0℃、62.4℃、64.8℃、66.9℃、68.6℃,和72℃ 45秒的32个循环,及72℃ 10分钟的最后延伸。热循环后,以TAE缓冲液中1%琼脂糖凝胶电泳来分离每种10 μ L的PCR反应物。该胶的观察显示以最高退火温度进行的PCR反应给出了所需约700bp产物的最高产量。将这四个PCR反应物结合,以TAE缓冲液中1%琼脂糖凝胶电泳来分离,其中约700碱基对的PCR产物被从凝胶上切下并根据制造商的说明使用**NUCLEOSPIN®** Extract II KiT (Macherey-Nagel) 纯化。

[0563] 质粒ald5680右#20包含来自东方伊萨酵母PDC ORF的约870bp的下游区域作为PDC终止子区域。然而,该区域可能比作为终止子适合功能所需的大,而如果保持在其目前的大小,该区域可能起对不想要的PDC基因座重组的催化剂的作用。因此,以引物0612360和0612361扩增以较小型式替换ald5680右#20中PDC终止子的PCR产物。使用退火温度的温度梯度和在某些反应中的DMSO来扩增所需的PCR产物。建立四个相同的PCR反应体系,每个PCR反应体系(50 μ L) 包含1 μ L 1比50稀释的pJLJ49小量制备质粒DNA (图25),1X iproofTMHF缓冲液 (Bio-Rad Laboratories),引物0612360和0612361每种100pmol,dATP、dCTP、dGTP和dTTP每种200 μ M,和1个单位的iProofTMHigh Fidelity DNA聚合酶 (Bio-Rad Laboratories)。如上设置第二组四个管,除了每个反应包括加入1.5 μ L的DMSO。在**EPPENDORF® MASTERCYCLER®** (Eppendorf Scientific) 中进行PCR,程序为98℃ 30秒的1个循环,随后是每个为98℃ 10秒,X℃ 20秒,其中X=47.6℃、51.8℃、57.1℃或62.1℃,和72℃ 45秒的32个循环,及72℃ 10分钟的最后延伸。对于所示的每个退火温度,都实施了有和没有DMSO的PCR反应。热循环后,以TAE缓冲液中1%琼脂糖凝胶电泳来分离每种10 μ L的PCR反应物。对该凝胶的观察显示有DMSO进行的四个PCR反应,无论退火温度,给出了所需338bp产物的最高产量。将这四个PCR反应物结合,以TAE缓冲液中1%琼脂糖凝胶电泳来分离,其中

约338碱基对的PCR产物被从凝胶上切下并根据制造商的说明使用**NUCLEOSPIN®** Extract II KiT (Macherey-Nagel) 纯化。

[0564] 使用PCR构建将约700bp含PGK1的PCR产物的融合到338bp的PDC终止子产物上的单独扩增产物。PCR反应体系(50 μ L) 包含107ng含PGK1的PCR产物, 56ng含PDC终止子的PCR产物, 1X Phusion HF缓冲液(New England Biolabs), 引物0612358和0612361每种100pmol, dATP、dCTP、dGTP和dTTP每种200 μ M, 和1个单位的Phusion[™]High Fidelity DNA聚合酶(New England Biolabs)。在**EPPENDORF® MASTERCYCLER®** (Eppendorf Scientific) 中进行PCR, 程序为98 $^{\circ}$ C 30秒的1个循环, 随后是每个为98 $^{\circ}$ C 10秒, 56 $^{\circ}$ C 20秒和72 $^{\circ}$ C 2分钟45秒的34个循环, 及72 $^{\circ}$ C 10分钟的最后延伸。热循环后, 以TAE缓冲液中0.9%琼脂糖凝胶电泳来分离PCR反应产物, 其中约1020碱基对的PCR产物被从凝胶上切下并根据制造商的说明使用**NUCLEOSPIN®** Extract II KiT (Macherey-Nagel) 纯化。

[0565] 质粒ald5680右#20被以XbaI和NotI消化, 以10个单位的小牛肠磷酸酶(New England Biolabs) 在37 $^{\circ}$ C下处理30分钟, 并以TAE缓冲液中0.9%琼脂糖凝胶电泳纯化。约5.6kbp的条带被从凝胶上切下并根据制造商的说明使用**NUCLEOSPIN®** Extract II KiT (Macherey-Nagel) 纯化。

[0566] 来自上面的纯化的487bp和1020bp的PCR产物随后被使用IN-FUSION[™] Advantage PCR Cloning KiT (Clontech) 在10 μ L总反应体积中插入消化的ald5680右#20片段, 10 μ L总反应体积由150ng以XbaI和NotI消化的ald5680右#20载体, 13ngTKL终止子PCR产物, 28ng PGK1启动子-PDC终止子PCR产物, 1X In-Fusion反应缓冲液(Clontech) 和1 μ L IN-FUSION[™] 酶(Clontech) 构成。反应物在37 $^{\circ}$ C孵育15分钟, 在50 $^{\circ}$ C 15分钟, 随后被置于冰上。以40 μ L TE缓冲液稀释反应物并根据制造商的说明使用2.5 μ L来转化Solo Pack Gold Super Competent Cells (Agilent Technologies)。转化体被涂布于2X YT+amp平板上并在37 $^{\circ}$ C下孵育过夜。通过AccI消化筛选到数个合适插入了所需PCR产物的所得转化体。产生所需片段大小的克隆通过DNA测序来确认正确并命名为pMhCt091。

[0567] 质粒pMhCt091是空的右端东方伊萨酵母ald5680靶向构建体, 其包含东方伊萨酵母URA3 ORF的3' 片段, 随后有用于加入感兴趣基因的NheI和AscI限制性位点的东方伊萨酵母TDH3启动子, 东方伊萨酵母TKL终止子, 随后有用于加入第二感兴趣基因的XbaI和PacI限制性位点的东方伊萨酵母PGK1启动子, 东方伊萨酵母PDC终止子, 和将同源重组靶向3' ald5680基因座的侧翼DNA。

[0568] 通过**GeneArt®**在载体1075328_SaPanD_r5中合成阿维链霉菌panD型式r5 (SEQ ID NO:146)。XbaI和PacI限制性位点被改变为通过PCR克隆进pMhCt091 5' 克隆位点所需的NheI和AscI位点。PCR反应体系(50 μ L) 包含1 μ L 1比50稀释的1075328_SaPanD_r5小量制备质粒DNA(**GeneArt®**), 1X iproof[™] HF缓冲液(Bio-Rad Laboratories), 引物0612378和0612379每种100pmol, dATP、dCTP、dGTP和dTTP每种200 μ M, 1.5 μ L DMSO和1个单位的iproof[™]High Fidelity DNA聚合酶(Bio-Rad Laboratories)。在**EPPENDORF® MASTERCYCLER®** (Eppendorf Scientific) 中进行PCR, 程序为98 $^{\circ}$ C 30秒的1个循环, 随后是每个为98 $^{\circ}$ C 10秒, 59 $^{\circ}$ C 20秒和72 $^{\circ}$ C 30秒的34个循环, 及72 $^{\circ}$ C 10分钟的最后延

伸。热循环后,以TAE缓冲液中0.9%琼脂糖凝胶电泳来分离PCR反应产物,其中约471个碱基对的PCR产物被从凝胶上切下并根据制造商的说明使用**NUCLEOSPIN®Extract II** KiT (Macherey-Nagel) 纯化。

[0569] 质粒pMhCt091 (如上) 被以NheI和AseI消化,以10个单位的小牛肠磷酸酶 (New England Biolabs) 在37℃下处理30分钟,并以TAE缓冲液中0.9%琼脂糖凝胶电泳纯化。约6.9kbp的条带被从凝胶上切下并根据制造商的说明使用**NUCLEOSPIN®Extract II** KiT (Macherey-Nagel) 纯化。来自上面的含panD r5的纯化PCR产物随后被使用IN-FUSIONTM Advantage PCR Cloning KiT (Clontech) 在10μL总反应体积中插入消化的pMhCt091片段,10μL总反应体积由150ng以NheI和AseI消化的pMhCt091载体,19ng panD r5PCR产物,1X In-Fusion反应缓冲液 (Clontech) 和1μL IN-FUSIONTM酶 (Clontech) 构成。反应物在37℃孵育15分钟,在50℃ 15分钟,随后被置于冰上。以40μL TE缓冲液稀释反应物并根据制造商的说明使用2.5μL来转化Solo Pack Gold Super Competent Cells (Agilent Technologies)。转化体被涂布于2X YT+amp平板上并在37℃孵育过夜。通过SmaI消化筛选到数个合适插入了所需PCR产物的所得转化体。产生所需片段大小的克隆通过DNA测序来确认正确并命名为pMhCt093。

[0570] 质粒pMhCt093被以XbaI和PacI消化,以10个单位的小牛肠磷酸酶 (New England Biolabs) 在37℃下处理30分钟,并以TAE缓冲液中0.9%琼脂糖凝胶电泳纯化。约7.4kbp的条带被从凝胶上切下并根据制造商的说明使用**NUCLEOSPIN®Extract II** KiT (Macherey-Nagel) 纯化。通过**GeneArt®**在载体pMA-T中合成为在东方伊萨酵母中表达而被密码子优化的阿维链霉菌panD型式r2 (SEQ ID NO:147)。以XbaI和PacI消化质粒并以TAE缓冲液中0.9%琼脂糖凝胶电泳分离得到的片段。约434bp的条带被从凝胶上切下并根据制造商的说明使用**NUCLEOSPIN®Extract II** KiT (Macherey-Nagel) 纯化。

[0571] 上面纯化的~434bp片段在连接反应体系 (20μL) 中被克隆进XbaI和PacI消化的pMhCt093载体,连接反应体系 (20μL) 含1X Quick连接缓冲液 (New England Biolabs), 2μL以XbaI和PacI消化的pMhCt093载体, 2μL上述的~434bp片段,和1μL Quick T4DNA连接酶 (New England Biolabs)。连接反应物在室温下孵育5分钟,随后管被置于冰上。5μL该反应物被用于根据制造商的说明来转化Solo Pack Gold Super Competent Cells (Agilent Technologies)。转化体被涂布于2X YT+amp平板上并在室温下孵育3天。通过XbaI和PacI消化筛选到数个合适插入了所需PCR产物的所得转化体。选择分离的pMhCt094来进行之后的工作。

[0572] 右端构建体的最后克隆步骤是以adh1202 3' 同源区域替代存在于pMhCt094中的ald5680 3' 同源区域。以引物612472和612473扩增含紧接着ald1202ORF的3' 端的序列,连同有所需的另外限制性位点和克隆用的侧翼DNA的PCR产物。PCR反应体系 (50μL) 包含1μL 1比50稀释的pGMEr140小量制备质粒DNA (如上), 1X iproofTMHF缓冲液 (Bio-Rad Laboratories), 引物0612472和0612473每种100pmol, dATP、dCTP、dGTP和dTTP每种200μM, 和1个单位的iProofTMHigh Fidelity DNA聚合酶 (Bio-Rad Laboratories)。在**EPPENDORF® MASTERCYCLER®** (Eppendorf Scientific) 中进行PCR, 程序为98℃ 30秒的1个循环, 随后是每个为98℃ 10秒, 59℃ 20秒和72℃ 30秒的34个循环, 及72℃

10分钟的最后延伸。热循环后,以TAE缓冲液中1%琼脂糖凝胶电泳来分离PCR反应产物,其中约620碱基对的PCR产物被从凝胶上切下并根据制造商的说明使用**NUCLEOSPIN®** Extract II KiT (Macherey-Nagel) 纯化。

[0573] 为构建上述DNA片段的接收载体,质粒pMhCt094被以SacII和NotI消化,以10个单位的小牛肠磷酸酶(New England Biolabs)在37℃下处理30分钟,并以TAE缓冲液中0.9%琼脂糖凝胶电泳纯化,约7.0kbp的条带被从凝胶上切下并根据制造商的说明使用**NUCLEOSPIN®** Extract II KiT (Macherey-Nagel) 纯化。使用IN-FUSION™ Advantage PCR Cloning KiT (Clontech) 在10μL总反应体积中将PCR产物和线性载体联结,10μL总反应体积由191ng以SacII和NotI消化的pMhCt094载体,36ng含adh1202'同源区的PCR产物,1X In-Fusion反应缓冲液(Clontech)和1μL IN-FUSION™酶(Clontech)构成。反应物在37℃孵育15分钟,在50℃ 15分钟,随后被置于冰上。以40μL TE缓冲液稀释反应物并根据制造商的说明使用2.5μL来转化Solo Pack Gold Super Competent Cells (Agilent Technologies)。转化体被涂布于2X YT+amp平板上并在37℃孵育过夜。通过NsiI和PvuI消化筛选到数个合适插入了所需PCR产物的所得转化体。产生所需片段大小的克隆通过DNA测序来确认正确并命名为pMhCt096(图13)。

[0574] 质粒pMhCt096是右端东方伊萨酵母adh1202靶向构建体,其含有东方伊萨酵母URA3ORF的3'片段,驱动为在东方伊萨酵母中表达而密码子优化的第三阿维链霉菌ADC基因(SEQ ID NO:146)表达的东方伊萨酵母TDH3启动子,东方伊萨酵母TKL终止子,驱动为在东方伊萨酵母中表达而密码子优化的第四阿维链霉菌ADC基因(SEQ ID NO:147)表达的东方伊萨酵母RGK1PGK1启动子,东方伊萨酵母PDC终止子和将同源重组靶向到3' adh1202基因座的侧翼DNA。

[0575] 实施例3A-9在PDC基因座表达天冬氨酸1-脱羧酶(ADC),β-丙氨酸氨基转移酶(BAAT)和3-羟基丙酸脱氢酶(3-HPDH);和在adh1202基因座表达来自四种核苷酸序列的天冬氨酸1-脱羧酶(ADC)的酵母菌株

[0576] 上面的实施例3A-7和3A-8描述了左端和右端构建体的构建,左端和右端构建体是用于在adh1202基因座靶向表达为在东方伊萨酵母中表达而密码子优化的阿维链霉菌ADC基因的四核苷酸变体。在转化进东方伊萨酵母CNB1之前,以HpaI和SacII消化10μg pMhCt095以从pUC19骨架载体中释放所需转化DNA。类似的,以EcoRI和SacII消化10μg pMhCt096以从pUC19骨架载体中释放所需转化DNA。含~5kbp表达盒的条带被通过凝胶电泳从pUC19骨架DNA中分离,从凝胶上切下,并根据制造商的说明使用**NUCLEOSPIN®** Extract II KiT (Macherey-Nagel) 纯化。30μL洗脱缓冲液被用于洗脱步骤。总计10μL的等摩尔比的pMhCt095和pMhCt096线性转化DNA被用于转化菌株yMhCt010(如上)。在ura选择平板上选择转化体并将其置于37℃下生长。翌日挑出约12个转化体并将其再划线到ura选择平板上以获取单菌落并在37℃下生长过夜,随后从每个初始转化体生成的每个划线中挑取单菌落并再划线到ura选择平板上。在37℃下生长再一夜之后,从每个划线中挑取最终单菌落并再划线到ura选择平板上,并37℃下生长过夜。在这第二轮单菌落纯化和生长之后,制备基因组DNA来用于如本发明中所述验证所需靶向整合发生的PCR。使用引物0611718和0611632(如上)来验证pMhCt095和pMhCt096片段正确靶向到adh1202基因座。引物0611718结合到存在于pMhCt096中的PDC终止子区域,而引物0611632结合到靶向区域adh1202基因

座DNA 3'端并以反义方向扩增。以这些引物从PCR中生成约727bp条带表明了 $adh1202$ 基因座所需整合事件的发生。

[0577] PCR反应体系(25 μ L)包含0.5 μ L要被筛选的菌株的基因组DNA,1X Crimson TaqTM反应缓冲液(New England Biolabs),25pmol有义引物,25pmol反义引物,dATP、dCTP、dGTP和dTTP每种200 μ M,和0.625个单位的Crimson TaqTMDNA聚合酶(New England Biolabs)。在EPPENDORF®MASTERCYCLER®(Eppendorf Scientific)中进行PCR,程序为95℃ 30秒的1个循环,随后是每个为95℃ 30秒,50℃ 30秒和68℃ 2.5分钟的30个循环,及68℃ 10分钟的最后延伸。热循环后,以TAE缓冲液中1%琼脂糖凝胶电泳来分离PCR反应产物并如上所述观察和解释条带的大小。给出所需727bp条带的两个独立分离的转化体被命名为yMhCt019或95/96 2(见表21中的基因型)。

[0578] 表21:转化体基因型

	菌株	亲本菌株	基因型
[0579]	yMhCt019	yMhCt010	$adh1202\Delta::(\text{PDC}_{\text{promo}}\text{-Opt.SaPanD r10, ENO1}_{\text{promo}}\text{-Opt.SaPanD r1, URA3, TDH3}_{\text{promo}}\text{-Opt.SaPanD r5, PGK1}_{\text{promo}}\text{-Opt.SaPanD r2})/\text{ADH1202 } \text{cdc}\Delta::(\text{PDC}_{\text{promo}}\text{-Opt.SaPanD ENO1}_{\text{promo}}\text{-Opt.ScUGA1, URA3-Scar, TDH3}_{\text{promo}}\text{-Opt.ScYMR226C})/\text{cdc}\Delta::(\text{PDC}_{\text{promo}}\text{-Opt.SaPanD, ENO1}_{\text{promo}}\text{-Opt.ScUGA1, URA3-Scar, TDH3}_{\text{promo}}\text{-Opt.ScYMR226C}) \text{ura3-}/\text{ura3-}$
	95/96 2		

[0580] 随后如本发明所描述分离yMhCt019的ura⁻衍生体。使用引物0611815和0612795通过PCR筛选到来自yMhCt019数个FOA抗性菌落的基因组DNA有所需环出事件。引物0611815在左端构建体RKI终止子处退火并向URA3启动子扩增,而引物0612795在3' $adh1202$ 同源区域中(pMhCt096的或者内源 $adh1202$ 基因座)反向朝着5'区域退火。如上所述对分离的yMhCt019进行PCR反应,除了延伸阶段的长度被变为3.5分钟。以这些引物生成3.7kbp条带表明所需的环出事件已经发生而仅URA3启动子残留剩余在修饰的 $adh1202$ 基因座,而约5.1kbp的条带会表明完整URA3启动子-URA3ORF-URA3终止子-URA3启动子盒的存在,表示所需重组事件没有发生。给出所需3.7kbp条带的菌株被保存并命名为yMhCt021。

[0581] 为了分离多个在 $adh1202$ 的panD表达盒的纯合的yMhCt021衍生体,如上所述以线性化的pMhCt095和pMhCt096转化yMhCt021。在两轮单菌落纯化和生长之后,制备基因组DNA来用于验证所需靶向整合发生的PCR。使用引物0612891和0612893来验证pMhCt095和pMhCt096片段正确靶向到yMhCt021剩余野生型的 $adh1202$ 基因座。引物0612891在SaPanD r1加上pMhCt095r1终止后的Pac1位点半边的3'区域退火。引物0612893在SaPanD r5的最5'区域退火,包括Nhe1位点和pMhCt096的前导序列,并在反向互补的方向扩增。

[0582] 按照从yMhCt021剩余野生型的 $adh1202$ 基因座上经pMhCt095和pMhCt096的第二重组事件所预期,这些引物生成3.2kbp条带表明完整URA3启动子-URA3ORF-URA3终止子-URA3启动子盒的存在,而约1.7kbp条带表明在其他 $adh1202$ 基因座处URA3残留位点的存在(来自初始整合事件和随后的URA3标记环出)。按上面对分离yMhCt019所描述的进行PCR反应,除了延伸阶段的长度被变为3.5分钟。给出两个这些条带的菌株被命名为yMhCt022(见表22中的基因型)。

[0583] 表22:转化体基因型

菌株	亲本菌株	基因型
[0584] yMhCt022	yMhCt021	adh1202Δ::($P_{DC_{promo}}$ -Opt.SaPanD r10, $ENO1_{promo}$ -Opt.SaPanD r1, URA3, $TDH3_{promo}$ -Opt.SaPanD r5, $PGK1_{promo}$ -Opt.SaPanD r2)/adh1202Δ::($P_{DC_{promo}}$ -Opt.SaPanD r10, $ENO1_{promo}$ -Opt.SaPanD r1, URA3-Scar, $TDH3_{promo}$ -Opt.SaPanD r5, $PGK1_{promo}$ -Opt.SaPanD r2) pdcΔ::($P_{DC_{promo}}$ -Opt.SaPanD, $ENO1_{promo}$ -Opt.ScUGA1, URA3-Scar, $TDH3_{promo}$ -Opt.ScYMR226C)/pdcΔ::($P_{DC_{promo}}$ -Opt.SaPanD, $ENO1_{promo}$ -Opt.ScUGA1, URA3-Scar, $TDH3_{promo}$ -Opt.ScYMR226C) ura3-/ura3-

[0585] 菌株在摇瓶中培养,如本发明中所述制备CFE并测试天冬氨酸脱羧酶(ADC)活性。实验结果在表23A中显示。

[0586] 表23A:转化体酶活性数据

菌株	过表达的基因	ADC 活性
[0587] MBin500 (control)	N/A	0.00
yMhCt019	ADC (SEQ ID NOs: 130, 145, 146, and 147), gabT (SEQ ID NO: 141),	2.18
95/96 2	3-HPDH (SEQ ID NO: 144)	2.52

[0588] 比较了从菌株MBin500(对照),yMhCt019、95/96-2、yMhCt008和yMhCt022制备的CFE中的天冬氨酸1-脱羧酶(ADC), β -丙氨酸氨基转移酶(BAAT)和3-HP脱氢酶(3-HPDH)活性。该实验的结果在表23B中显示。

[0589] 表23B:转化体的酶活性数据

菌株	过表达的基因	基因来源	ADC 活性	BAAT 活性	3HP DH 活性
MBin500 (对照)	N/A	N/A	0.002	0.61	0.4
[0590] yMhCt019	YMR226c (SEQ ID NO: 144)	酿酒酵母	0.789	14.53	72.0
	ADC (SEQ ID NOs: 130, 145, 146 and 147)	阿维链霉菌			
	UGA1 (SEQ ID NO: 141)	酿酒酵母			
95/96-2	YMR226c (SEQ ID NO: 144)	酿酒酵母	0.891	20.42	73.2
	ADC (SEQ ID NOs: 130, 145, 146 and 147)	阿维链霉菌			
	UGA1 (SEQ ID NO: 141)	酿酒酵母			
yMhCt008	YMR226c (SEQ ID NO: 144)	酿酒酵母	0.272	13.14	61.1
	ADC (SEQ ID NO: 130)	阿维链霉菌			
	UGA1 (SEQ ID NO: 141)	酿酒酵母			
yMhCt022	YMR226c (SEQ ID NO: 144)	酿酒酵母	1.233	15.44	66.7
	ADC (SEQ ID NOs: 130, 145, 146 and 147)	阿维链霉菌			
	UGA1 (SEQ ID NO: 141)	酿酒酵母			

[0591] 也如本发明所述通过SDS-PAGE分析了菌株yMhCt019和95/96 2。两个菌株都显示在53kD、29kD、~14kD和~3kD的蛋白条带。53kD和29kD蛋白条带的大小分别与UGA1和YMR226c基因所编码蛋白的大小相符。14和3kD蛋白条带组合的大小与panD基因所编码的经翻译后切割的蛋白相符。在对照菌株Mbin500的SDS-PAGE分析中没有观察到53kD、29kD、14kD和3kD蛋白。

[0592] 使用本发明描述的方法,在生物反应器中评价菌株MBin500和yMhCt019的3-HP产生。对照菌株Mbin500没有产生可检测到的3-HP(两个独立的发酵的平均)。菌株yMhCt019产生5.23g/L的3-HP(三个独立的发酵的平均)。

[0593] 实施例3A-10:在adh1202基因座从四种核苷酸序列表达天冬氨酸1-脱羧酶(ADC)的酵母菌株

[0594] 设计另外的构建体以在adh1202基因座掺入编码来自地衣芽孢杆菌的替代ADC (SEQ ID NO:139)的四个核苷酸拷贝。以与上述方式类似的方式,设计左端和右端构建体以允许在东方伊萨酵母adh1202基因座的同源重组。

[0595] 左端片段的构建

[0596] 质粒pMhCt095(如上)被如本发明中所述以XbaI和PacI消化并以TBE缓冲液中1%琼脂糖凝胶电泳来纯化。约7.3kbp的条带被从凝胶上切下并根据制造商的说明使用 **NUCLEOSPIN®** Extract II KiT (Macherey-Nagel) 纯化。

[0597] 地衣芽孢杆菌天冬氨酸脱羧酶(ADC) PanD基因为为在东方伊萨酵母中表达而被密码子优化(型式1,SEQ ID NO:149)并合成构建入质粒1110206(**GeneArt®**)。质粒1110206被如本发明中所述以XbaI和PacI消化并以TBE缓冲液中1%琼脂糖凝胶电泳来纯化。约380bp的条带被从凝胶上切下并根据制造商的说明使用 **NUCLEOSPIN®** Extract II KiT (Macherey-Nagel) 纯化。

[0598] 使用T4连接酶(New England Biolabs)在10μL总反应体积中将~380bp的纯化片段连接进7.3kbp的pMhCt095线性化载体,10μL总反应体积由60.5ng消化的pMhCt095,6.3ng来自1110206的380bp片段,有10mM ATP的1μL 10X连接缓冲液(New England Biolabs),和1μL T4连接酶(New England Biolabs)构成。反应物在室温下孵育1.5小时,而3μL等份的反应物被用于根据制造商的说明转化进ONE **SHOT®**T0P10化学感受态大肠杆菌细胞(Invitrogen)。转化体被涂布于2X YT+amp平板上并在37℃下孵育过夜。通过使用XbaI和PacI限制性消化筛选到数个有合适插入的所得转化体。产生正确消化条带大小的克隆被命名为pMeJi309。

[0599] 质粒pMeJi309被以NheI和AscI消化并以TBE缓冲液中1%琼脂糖凝胶电泳来纯化。约7.3kbp的条带被从凝胶上切下并根据制造商的说明使用 **NUCLEOSPIN®** Extract II KiT (Macherey-Nagel) 纯化。

[0600] 地衣芽孢杆菌天冬氨酸脱羧酶panD基因为为在东方伊萨酵母中表达而被再次密码子优化(型式2,SEQ ID NO:148)并合成构建入质粒1110205(**GeneArt®**)。在含3μL 1110205,引物0612695和0612724每种25pM,1X pfx扩增缓冲液(Invitrogen),2mm MgSO₄,1.25个单位**Platinum®**pfx DNA聚合酶(Invitrogen)的终体积50μL的混合物中进行PCR。扩增反应物在**EPPENDORF®** **MASTERCYCLER®** (Eppendorf Scientific)中孵育,程序为95℃ 2分钟的1个循环;每个为95℃ 1分钟,55℃ 1分钟和72℃ 1分钟的25个循环;以及72℃ 3分钟的1个循环。

[0601] 扩增反应的PCR产物被以TBE缓冲液中1%琼脂糖凝胶电泳来纯化。约400bp的条带被从凝胶上切下并根据制造商的说明使用 **NUCLEOSPIN®** Extract II KiT (Macherey-Nagel) 纯化。纯化的PCR产物被使用IN-FUSIONTM Advantage PCR Cloning KiT(Clontech)在反应中插入上面消化的pMeJi309载体,反应体系包括93ng pMeJi309载体片段,52ng上述PCR产物,2μL 1X IN-FUSIONTMIn-Fusion反应缓冲液(Clontech)和1μL IN-FUSIONTM酶

(Clontech)。反应物在37℃孵育15分钟,在50℃ 15分钟,随后被置于冰上。2.5μL反应物样品根据制造商的说明转化入ONE **SHOT®**TOP10化学感受态大肠杆菌细胞(Invitrogen)。转化体被涂布于2X YT+amp平板上并在37℃孵育过夜。通过使用XbaI和PacI的限制性消化筛选到数个有合适插入的所得转化体。产生正确消化条带大小的克隆通过DNA测序来确认正确并命名为pMeJi310-2(图14)。

[0602] 右端片段的构建

[0603] 质粒pMhCt096(如上)被以XbaI和PacI消化并以TBE缓冲液中1%琼脂糖凝胶电泳来纯化。约4.8kbp的条带被从凝胶上切下并根据制造商的说明使用**NUCLEOSPIN®** Extract II KiT(Macherey-Nagel)纯化。

[0604] 地衣芽孢杆菌天冬氨酸脱羧酶panD基因为为在东方伊萨酵母中表达而被再次密码子优化(型式3,SEQ ID NO:151)并合成构建入质粒1110208(**GeneArt®**)。质粒1110208被以XbaI和PacI消化并以TBE缓冲液中1%琼脂糖凝胶电泳来纯化。约380bp的条带被从凝胶上切下并根据制造商的说明使用**NUCLEOSPIN®** Extract II KiT(Macherey-Nagel)纯化。

[0605] 使用T4连接酶(New England Biolabs)在10μL总反应体积中将上面380bp的片段连接进7.3kbp的pMhCt096线性化载体,10μL总反应体积由72.2ng消化的pMhCt096,6.9ng来自1110208的380bp片段,有10mM ATP的1μL 10X连接缓冲液,和1μL T4连接酶构成。反应物在室温下孵育1个半小时,而3μL等份的反应物被用于根据制造商的说明转化进ONE **SHOT®**TOP10化学感受态大肠杆菌细胞(Invitrogen)。转化体被涂布于2X YT+amp平板上并在37℃下孵育过夜。通过使用NheI和AscI限制性消化筛选到数个有合适插入的所得转化体。产生正确消化条带大小的克隆被命名为pMeJi311。

[0606] 质粒pMeJi311被以NheI和AscI消化并以TBE缓冲液中1%琼脂糖凝胶电泳来纯化。约7.3kbp的条带被从凝胶上切下并根据制造商的说明使用**NUCLEOSPIN®** Extract II KiT(Macherey-Nagel)纯化。

[0607] 地衣芽孢杆菌天冬氨酸脱羧酶panD基因为为在东方伊萨酵母中表达而被再次密码子优化(型式4,SEQ ID NO:150)并合成构建入质粒1110207(**GeneArt®**)。在含3μL1110207,引物0612698和0612725每种25pM,1X pfx扩增缓冲液(Invitrogen),2mM MgSO₄,1.25个单位**Platinum®**pfx DNA聚合酶(Invitrogen)的终体积50μL的混合物中进行PCR。扩增反应物在**EPPENDORF® MASTERCYCLER®**(Eppendorf Scientific)中孵育,程序为95℃ 2分钟的1个循环;每个为95℃ 1分钟,55℃ 1分钟和72℃ 1分钟的25个循环;以及72℃ 3分钟的1个循环。

[0608] 扩增反应的PCR产物被以TBE缓冲液中1%琼脂糖凝胶电泳来纯化。约400bp的条带被从凝胶上切下并根据制造商的说明使用**NUCLEOSPIN®** Extract II KiT(Macherey-Nagel)纯化。纯化的PCR产物被使用IN-FUSIONTM Advantage PCR Cloning KiT(Clontech)在反应中插入上面消化的pMeJi311载体,反应体系包括65.1ng pMeJi311NheI到AscI消化的载体片段,85ng上述PCR产物,2μL 1X In-Fusion反应缓冲液(Clontech)和1μL IN-FUSIONTM酶(Clontech)。反应物在37℃孵育15分钟,在50℃ 15分钟,随后被置于冰上。2.5μL

反应物样品被根据制造商的说明转化入ONE SHOT® TOP10化学感受态大肠杆菌细胞(Invitrogen)。转化体被涂布于2X YT+amp平板上并在室温孵育2天。通过使用NheI和AseI的限制性消化筛选到数个有合适插入的所得转化体。产生正确消化条带大小的克隆通过DNA测序来确认正确并命名为pMeJi312-2(图15)。

[0609] 左端和右端片段的整合

[0610] 如本发明所述质粒pMeJi310-2被以HpaI和SacII消化而质粒pMeJi312-2被以EcoRI和SacII消化。这些以TBE缓冲液中1%琼脂糖凝胶电泳来纯化,而两个约5kbp的条带被从凝胶上切下并根据制造商的说明使用NUCLEOSPIN® Extract II Kit(Macherey-Nagel)纯化。

[0611] 以消化的pMeJi310-2和pMeJi312-2 DNA转化东方伊萨酵母CNB1,并如本发明中所述通过Crimson Taq(New England Biolabs) PCR来验证正确的基因座靶向和转化。引物0612794和0611245产生约3.17kbp的条带;引物612479和0611632产生约1.48kbp的条带;而引物611248和612795产生约2.3kbp的条带。给出表达盒正确整合预期条带的菌株被命名为MeJi409-2。随后如上所述获得MeJi409-2的ura-衍生体。

[0612] 使用本发明描述的方法,在发酵生物反应器中评价菌株Mbin500和MeJi409-2的3-HP产生。对照菌株Mbin500没有产生可检测到的3-HP(两个独立的发酵的平均)。菌株MeJi409-2(1个发酵)产生4.62g/L。为考虑到相比其他(如,未来)发酵,这些发酵中的细胞质量上的差异,MeJi409-2每单位细胞质量的3-HP浓度(表示为[g/L 3-HP]/[g/L干细胞重])被计算为0.20。

[0613] 实施例3A-11:在adh 9091基因座表达天冬氨酸1-脱羧酶(ADC)和天冬氨酸氨基转移酶(AAT)的酵母菌株

[0614] 使用引物0611268和0611269从东方伊萨酵母基因组DNA中PCR扩增编码SEQ ID NO:14的东方伊萨酵母氨基转移酶(AAT)的核苷酸序列(SEQ ID NO:13)。PCR反应体系(50μL)包含50ng东方伊萨酵母菌株的基因组DNA,1X Phusion HF缓冲液(New England Biolabs),引物0611268和0611269每种50pmol,dATP、dCTP、dGTP和dTTP每种200μM,1.5μL 100%DMSO(New England Biolabs)和1个单位的Phusion High Fidelity DNA聚合酶(New England Biolabs)。在EPPENDORF® MASTERCYCLER®(Eppendorf Scientific)中进行PCR,程序为98℃ 2分钟的1个循环,随后是每个为98℃ 30秒,55℃ 30秒和72℃ 1分钟30秒的35个循环,及72℃ 7分钟的最后延伸。热循环后,以TBE缓冲液中0.8%琼脂糖凝胶电泳来分离PCR反应产物,其中约1278bp的PCR产物被从凝胶上切下并根据制造商的说明使用QIAQUICK® Gel Extraction Kit(Qiagen)纯化。所得PCR片段的总长度约1278bp,在片段5'末端有NruI限制性位点而在其3'末端有PacI限制性位点。

[0615] 上面得到的包含AAT基因CDS(SEQ ID NO:13)的1278bp片段随后被克隆进pCR2.1-TOPO载体并根据制造商的说明转化进One-Shot TOP10大肠杆菌细胞(Invitrogen)。转化体被涂布于2X YT+amp平板上并在37℃孵育过夜。通过Bam HI消化筛选到数个有所需片段的合适插入的所得转化体。产生所需条带大小的克隆通过测序来确认并命名为pGMEr111。

[0616] 质粒pGMEr121和pGMEr111被以限制性酶PacI和NruI双消化。所得的来自质粒pGMEr121的7695bp载体片段,和所得的来自质粒pGMEr111的含AAT编码序列的1272bp插入

片段,被以1XTBE缓冲液中0.8%琼脂糖凝胶电泳来分离,从凝胶上切下,并根据制造商的说明使用**QIAQUICK®**Gel Extraction KiT(Qiagen)纯化。

[0617] 随后以3μL载体片段,4μL插入片段,2μL无菌双蒸水,10μL 2X Quick连接酶缓冲液和1μL Quick T4连接酶(Quick Ligation Kit,New England Biolabs)来建立连接反应体系并根据制造商说明进行反应。5μL等份的上述连接反应物被根据制造商说明转化入**XL10-Gold®**Ultracompetent大肠杆菌细胞(Agilent Technologies)。转化体被涂布于2X YT+amp平板上并在37℃孵育过夜。通过SmaI/PpuMI双消化筛选到数个有所需插入物的合适插入的所得转化体。产生所需条带大小的克隆通过测序来确认并命名为pGMEr126(图16)。

[0618] 质粒pGMEr126包括东方伊萨酵母AAT表达盒,其中基因转录由东方伊萨酵母TDH3启动子和TKL终止子调控,其上游侧翼有截短的URA3编码序列3'区域和URA3启动子;下游侧翼有东方伊萨酵母adh9091基因座3'同源区域。

[0619] 使用引物0611661和0611662从获自**GeneArt®**的pMA-T载体PCR扩增为在东方伊萨酵母中表达而密码子优化的阿维链霉菌panD基因(SEQ ID NO:130)。PCR反应体系(50μL)包含50ng菌株质粒DNA,1X Phusion HF缓冲液(New England Biolabs),引物0611661和0611662每种50pmol,dATP、dCTP、dGTP和dTTP每种200μM,1.5μL 100%DMSO(New England Biolabs)和1个单位的Phusion High Fidelity DNA聚合酶(New England Biolabs)。在**EPPENDORF® MASTERCYCLER®**(Eppendorf Scientific)中进行PCR,程序为98℃ 2分钟的1个循环,随后是每个为98℃ 30秒,55℃ 30秒和72℃ 30秒的35个循环,及72℃ 7分钟的最后延伸。热循环后,以TBE缓冲液中0.8%琼脂糖凝胶电泳来分离PCR反应产物,其中约453bp的PCR产物被从凝胶上切下并根据制造商的说明使用**QIAQUICK®**Gel Extraction KiT(Qiagen)纯化。所得PCR片段的总长度约453bp,在片段5'末端有NruI限制性位点而在3'末端有ApaI限制性位点。

[0620] 包含阿维链霉菌panD基因密码子优化型式的453bp片段被克隆进pCR2.1-TOP0载体并根据制造商的说明转化进One-Shot TOP10大肠杆菌细胞(Invitrogen)。转化体被涂布于2X YT+amp平板上并在37℃孵育过夜。通过EcoRI消化筛选到数个有所需片段的合适插入的所得转化体。产生所需条带大小的克隆通过测序来确认并命名为pGMEr127。

[0621] 质粒pGMEr127和pGMEr125(a)被以限制性酶NruI和ApaI消化。在终止消化反应之前将1μL小牛肠碱性磷酸酯酶(New England Biolabs)加入到pGMEr125(a)消化体系中以将末端去磷酸化并防止自连接。所得的来自质粒pGMEr125(a)(如上)的8188bp载体片段,和来自质粒pGMEr127(如上)的含阿维链霉菌panD基因密码子优化型式(SEQ ID NO:130)的440bp插入片段,被以1XTBE缓冲液中0.8%琼脂糖凝胶电泳来分离,从凝胶上切下,并根据制造商的说明使用**QIAQUICK®**Gel Extraction KiT(Qiagen)纯化。

[0622] 随后以4μL载体片段,4μL插入片段,9μL2X Quick连接酶缓冲液和1μL Quick T4连接酶(New England Biolabs)来建立连接反应体系并根据制造商说明进行反应。5μL等份的上述连接反应物被根据制造商说明转化入**XL10-Gold®**Ultracompetent大肠杆菌细胞(Agilent Technologies)。转化体被涂布于2X YT+amp平板上并在37℃孵育过夜。通过

bamHI消化筛选到数个有所需插入物的合适插入的所得转化体。产生所需条带大小的克隆被确认并命名为pGMEr130(图17)。

[0623] 质粒pGMEr130包含由下面片段制成的构建体:东方伊萨酵母adh9091基因座的5'侧翼,有东方伊萨酵母PDC启动子/TAL终止子的空表达盒,东方伊萨酵母ENO1启动子和RKI终止子调控下的panD表达盒(含SEQ ID NO:130),和东方伊萨酵母URA3启动子调控下的截短的URA3标记基因5'片段。

[0624] 为确定酵母菌株东方伊萨酵母CNB1是否能表达阿维链霉菌panD基因的东方伊萨酵母密码子优化型式(SEQ ID NO:130)和东方伊萨酵母AAT基因(SEQ ID NO:13),构建了表达质粒pGMEr130和pGMEr126。质粒pGMEr130包括(从5'到3')在东方伊萨酵母adh9091基因座构建体基因组整合用的5'侧翼区域,ENO1启动子和RKI终止子调控下的panD表达盒,由URA3启动子驱动的截短的URA3选择标志物5'部分。质粒pGMEr126包括(从5'到3')URA3选择标志物3'部分,TDH3启动子和TKL终止子调控下的AAT基因表达盒,和在东方伊萨酵母adh9091基因座构建体基因组整合用的3'侧翼区域。所有启动子和终止子源自东方伊萨酵母。

[0625] pGMEr126被以限制性酶EcoRI消化,其切割出感兴趣的4758bp片段,而pGMEr130被以限制性酶Hind III消化,构建转化所需的5034bp片段。4758bp和5034bp片段被以1XTBE缓冲液中0.8%琼脂糖凝胶电泳来分离,从凝胶上切下,并根据制造商的说明使用QIAQUICK® Gel Extraction Kit(Qiagen)纯化。

[0626] 培养东方伊萨酵母CNB1并如本发明中所述以约500ng的4758bp和5034bp线性片段共转化。获得8个转化菌株并随后在摇瓶中培养。将得到的培养液用于跑SDS-PAGE,Tris-HCl(Bio-Rad Laboratories)凝胶以检测为在东方伊萨酵母中表达而密码子优化的阿维链霉菌panD基因(SEQ ID NO:130)和东方伊萨酵母AAT基因(SEQ ID NO:13)的表达。阳性菌株被命名为yGMEr008,而如上描述其培养液也被用于确定ADC和AAT活性水平。

[0627] 实施例3A-12:在adh9091基因座表达丙酮酸羧化酶(PYC),天冬氨酸1-脱羧酶(ADC)和天冬氨酸氨基转移酶(AAT)的酵母菌株

[0628] 使用引物0611266和0611267从东方伊萨酵母基因组DNA PCR扩增编码SEQ ID NO:2的东方伊萨酵母丙酮酸脱羧酶(PYC)的核苷酸序列。PCR反应体系(50μL)包含50ng东方伊萨酵母菌株的基因组DNA,1X Phusion HF缓冲液(New England Biolabs),引物0611266和0611267每种50pmol,dATP、dCTP、dGTP和dTTP每种200μM,1.5μL 100%DMSO(New England Biolabs)和1个单位的Phusion High Fidelity DNA聚合酶(New England Biolabs)。在EPPENDORF® MASTERCYCLER®(Eppendorf Scientific)中进行PCR,程序为98℃ 2分钟的1个循环,随后是每个为98℃ 30秒,55℃ 30秒和72℃ 1分钟30秒的35个循环,及72℃ 7分钟的最后延伸。热循环后,以TBE缓冲液中0.8%琼脂糖凝胶电泳来分离PCR反应产物,其中约3557bp的PCR产物被从凝胶上切下并根据制造商的说明使用QIAQUICK® Gel Extraction Kit(Qiagen)纯化。所得PCR片段在片段5'末端有XbaI限制性位点而在其3'末端有PacI限制性位点。

[0629] 得到的包含东方伊萨酵母PYC基因CDS(SEQ ID NO:1)的3557bp片段被克隆进pCR2.1-TOPO载体并根据制造商的说明转化进One-Shot TOP10大肠杆菌细胞

(Invitrogen)。转化体被涂布于2X YT+amp平板上并在37℃孵育过夜。通过EcoRI消化筛选到数个有所需片段的合适插入的所得转化体。产生所需条带大小的6个克隆被确认并命名为pGMEr132.7、pGMEr132.14、pGMEr132.16、pGMEr132.25、pGMEr132.27和pGMEr132.30。序列分析显示质粒pGMEr132.14有合适的PYC CDS但丢失了CDS 5'端的XbaI限制性位点。由于需要该限制性位点以将PYC CDS插入表达质粒pGMEr125,质粒pGMEr132.14的315bp Hind III片段(包含PYC CDS上的5'端,有改变的XbaI位点)被来自质粒pGMEr132.7的315bp Hind III片段所替代,其具有包括正确XbaI位点在内的未变化的PYC CDS 5'端。所得来自pGMEr132.14的7173bp Hind III载体片段,和来自pGMEr132.7的315bp bp Hind III插入物被以1XTBE缓冲液中0.8%琼脂糖凝胶电泳来分离,从凝胶上切下,并根据制造商的说明使用**QIAQUICK®**Gel Extraction Kit(Qiagen)纯化。

[0630] 随后以4μL载体片段,5μL插入片段,10μL2X Quick连接酶缓冲液和1μL Quick T4连接酶(New England Biolabs)来建立连接反应体系并根据制造商说明进行反应。5μL等份的上述连接反应物被根据制造商说明转化入One-Shot TOP10大肠杆菌细胞(Invitrogen)。转化体被涂布于2X YT+amp平板上并在37℃孵育过夜。通过BamHI和XbaI双消化筛选到数个有所需插入物的合适插入和取消的所得转化体。产生所需条带大小的克隆被确认并命名为pGMEr133。

[0631] 为了将东方伊萨酵母PYC CDS插入到质粒pGMEr125(b)中PDC启动子下游。质粒pGMEr125(b)和pGMEr133(如上)被以PacI和XbaI消化。所得的来自质粒pGMEr125(b)的8188bp载体片段,和来自质粒pGMEr133的包含东方伊萨酵母PYC CDS(SEQ ID NO:1)的3553bp插入片段被以1XTBE缓冲液中0.8%琼脂糖凝胶电泳来分离,从凝胶上切下,并根据制造商的说明使用**QIAQUICK®**Gel Extraction Kit(Qiagen)纯化。

[0632] 随后以3μL载体片段,6μL插入片段,10μL2X Quick连接酶缓冲液和1μL Quick T4连接酶(New England Biolabs)来建立连接反应体系并根据制造商说明进行反应。5μL等份的上述连接反应物被根据制造商说明转化入One-Shot TOP10大肠杆菌细胞(Invitrogen)大肠杆菌细胞(Agilent Technologies)。转化体被涂布于2X YT+amp平板上并在37℃孵育过夜。通过BamHI消化筛选到数个有所需片段的合适插入的所得转化体。产生所需条带大小的克隆被确认并命名为pGMEr136。

[0633] 质粒pGMEr136包括东方伊萨酵母adh9091基因座的5'侧翼,东方伊萨酵母PDC启动子和TAL终止子调控下的东方伊萨酵母PYC基因表达盒(SEQ ID NO:1),有东方伊萨酵母EN01启动子/RKI终止子的空表达盒,和URA3启动子调控下的截短的东方伊萨酵母URA3标志基因5'片段。

[0634] 约5μg的质粒pGMEr136(如上)和4μg的质粒pGMEr127被以限制性酶ApaI和NruI消化。所得来自质粒pGMEr136的11729bp载体片段,和所得来自质粒pGMEr127的包含为在东方伊萨酵母中表达而密码子优化的阿维链霉菌panD基因(SEQ ID NO:130)的插入片段(436bp)被根据制造商的说明使用**NucleoSpin®**Extract II(Macherey-Nagel)以1XTBE缓冲液中0.8%琼脂糖凝胶电泳来纯化。

[0635] 随后以5μL载体片段,4μL插入物,9μL2X Quick连接酶缓冲液和1μL Quick T4连接酶(New England Biolabs)来建立连接反应体系。反应物被在室温下孵育1小时。5μL等份的

上述连接反应物被根据制造商说明转化入ONE SHOT®TOP10化学感受态大肠杆菌细胞(Invitrogen)。转化体被涂布于2X YT+amp平板上并在37℃孵育过夜。通过BamHI消化筛选到数个有所需插入物的所得转化体。产生所需条带大小的克隆被选出并命名为pGMEr137(图18)。

[0636] 质粒pGMEr137包含在东方伊萨酵母PDC启动子和TAL终止子基因转录调控下的东方伊萨酵母PYC基因(SEQ ID NO:1),在东方伊萨酵母EN01启动子和RKI终止子转录调控下的为在东方伊萨酵母中表达而密码子优化的阿维链霉菌panD基因(SEQ ID NO:130),随后有URA3标志5'端和东方伊萨酵母adh9091基因座5'侧翼区域的URA3启动子。

[0637] 质粒pGMEr126(如上)被以限制性酶EcoRI消化,其切下感兴趣的4758bp片段;而质粒pGMEr137(如上)被以限制性酶HpaI和NheI消化,构建8400bp片段。4758bp和8400bp的片段被以1XTBE缓冲液中0.8%琼脂糖凝胶电泳来分离,条带被从凝胶上切下并根据制造商的说明使用NucleoSpin®Extract II(Macherey-Nagel)来纯化。培养东方伊萨酵母CNB1并如本发明所述以约500ng 4758bp和8400bp线性片段共转化,得到转化体yGMEr009。

[0638] 实施例3A-13:在PDC基因座表达天冬氨酸1-脱羧酶(ADC),β-丙氨酸氨基转移酶(BAAT)和3-羟基丙酸脱氢酶(3-HPDH);并在adh9091基因座表达丙酮酸羧化酶(PYC),天冬氨酸1-脱羧酶(ADC)和天冬氨酸氨基转移酶(AAT)的酵母菌株

[0639] 为了在已经过表达天冬氨酸氨基转移酶(AAT),β-丙氨酸氨基转移酶(BAAT)和3-HP脱氢酶(3-HPDH)的菌株中增加丙酮酸羧化酶(PYC)和天冬氨酸氨基转移酶(AAT)活性,如上所述以pGMEr137(如上)和pGMEr126(如上)线性片段转化yMhCt010(如上)。在两轮单菌落纯化和生长之后,如上所述制备基因组DNA来用于验证所需靶向整合发生的PCR。使用引物0611814和0612055来确认pGMEr137和pGMEr126片段正确靶向到adh9091基因座。引物0611814在pGMEr126TDH3启动子3'端退火并在3'方向扩增,引物0612055退火于存在于pGMEr126中的在adh90913'侧翼同源区的3',所以以该引物对进行的PCR产物扩增仅在整合DNA通过同源重组靶向正确位点时发生。来自含引物0611814和0612055的PCR的约3066bp条带的存在表示pGMEr126和pGMEr137片段在adh9091基因座发生所需整合。

[0640] 在以pGMEr137和pGMEr126线性片段转化的yMhCt010数个独立转化体两轮单菌落纯化和生长之后,如上所述制备基因组DNA来用于验证所需靶向整合发生的PCR。给出包含引物0611814和0612055的约3066bp PCR条带的三个独立分离的菌株被命名为yMhCt020, GMErIn010#2, GMErIn010#3。这些菌株包含在两个pdc基因座和一个adh9091基因座编码对应ADC(SEQ ID NO:17)的多核苷酸(SEQ ID NO:130);在两个pdc基因座编码对应gabT(SEQ ID NO:24)的多核苷酸(SEQ ID NO:141);在两个pdc基因座编码对应3-HPDH(SEQ ID NO:129)的多核苷酸(SEQ ID NO:144);在一个adh9091基因座编码对应PYC(SEQ ID NO:2)的多核苷酸(SEQ ID NO:1);在一个adh9091基因座编码对应AAT(SEQ ID NO:14)的多核苷酸(SEQ ID NO:13)(见表24)。

[0641] 表24:转化体基因型

[0642]	菌株	亲本菌株	基因型
	yMhCt020	yMhCt010	adh9091Δ::($P_{DC_{promo}}$ - $pycCNB1$, $ENO1_{promo}$ - $SaPanD(reverse)$, $URA3$, $TDH3_{promo}$ - aat)/ $ADH9091$
	GMErin010 #2		$pdca$::($P_{DC_{promo}}$ - $Opt.SaPanD$, $ENO1_{promo}$ - $Opt.ScUGA1$, $URA3-Scar$, $TDH3_{promo}$ - $Opt.ScYMR226C$)/ $pdca$::($P_{DC_{promo}}$ - $Opt.SaPanD$,
	GMErin010 #3		$ENO1_{promo}$ - $Opt.ScUGA1$, $URA3-Scar$, $TDH3_{promo}$ - $Opt.ScYMR226C$) $ura3$ -/ $ura3$ -

[0643] 菌株yMhCt020, GMErin010#2, 和GMErin010#3在摇瓶中培养, 如本发明所述制备CFE并测试PYC、AAT和3-HPDH活性。实验结果在表25中显示。

[0644] 表25: 转化体酶活性数据

[0645]	菌株	过表达的基因	PYC 活性	AAT 活性	ADC 活性	3-HPDH 活性
	MBin500 (对照)	N/A	0.14	0.03	0.00	0.14
	yMhCt008	ADC (SEQ ID NO: 130), gabT (SEQ ID NO: 141), 3-HPDH (SEQ ID NO: 144)	0.22	2.05	0.64	2.95
	GMEr009-2	ADC (SEQ ID NO: 130), PYC (SEQ ID NO: 1), AAT (SEQ ID NO: 13)	1.15	24.62	0.05	0.07
	yMhCt020	ADC (SEQ ID NO: 130), gabT (SEQ ID NO: 141), 3-HPDH (SEQ ID NO: 144), PYC (SEQ ID NO: 1), AAT (SEQ ID NO: 13),	2.74	35.02	0.56	1.68
	GMErin010 #2		1.97	40.32	0.59	1.87
	GMErin010 #3		1.50	23.79	0.42	3.30

[0646] 也如本发明中描述的以SDS-PAGE分析了表25中的菌株。MBin500和GMEr009-2显示在四个其他样品中没有的~64kD蛋白条带。这些蛋白的质量与其作为东方伊萨酵母CNB1中天然丙酮酸脱羧酶的身份相符。菌株yMhCt008、yMhCt020、GMErin010#2和GMErin010#3显示53kD和29kD的条带。这些蛋白的质量分别与UGA1和YMR226c基因所编码的蛋白的质量相符。菌株GMEr009-2、yMhCt020、GMErin010#2, 和GMErin010#3都显示46.3kD的条带。这些蛋白的质量与AAT基因所编码蛋白的质量相符。

[0647] 实施例3A-14: 在两个1202基因座从四个核苷酸序列表达天冬氨酸1-脱羧酶(ADC)的酵母菌株。

[0648] 使用上面描述的FOA逆选择环出方案分离包含编码SEQ ID NO:139地衣芽孢杆菌ADC(如上)的四个核苷酸拷贝的MeJi409-2ura3-衍生体。使用引物0611815和0611817通过PCR筛选亲本菌株MeJi409-2数个FOA抗性菌落的基因组DNA有所需环出事件。引物0611815在左端构建体RKI终止子处退火并向ura3启动子扩增。引物0611817在TDH3启动子处退火并反向朝ura3盒扩增。828bp条带的存在表示如所需的仅ura3残留位点的存在(在亲本菌株中两个URA3启动子间同源重组后留下的单个URA3启动子), 而约2.2kbp条带表示完整URA3启动子-URA3ORF-URA3终止子-URA3启动子盒的存在, 表明所需的重组事件没有发生。如上所述进行以Crimson Taq™ DNA聚合酶(New England Biolabs)的PCR反应。来自亲本菌株MeJi409-2的一个FOA抗性菌落, 命名为MeJi411, 给出了所需的828bp条带。

[0649] 左端和右端片段的整合

[0650] 如本发明中所述质粒pMeJi310-2被以HpaI和SacII消化而质粒pMeJi312-2被以EcoRI和SacII消化。这些被以TBE缓冲液中1%琼脂糖凝胶电泳来纯化, 两个约5kbp的条带

被从凝胶上切下并根据制造商的说明使用**NUCLEOSPIN®**Extract II KiT (Macherey-Nagel) 来纯化。

[0651] 以消化的pMeJi310-2和pMeJi312-2DNA来转化MeJi411,并如本发明所述以Crimson Taq (New England Biolabs) PCR来验证正确的位点靶向和转化。引物0611225和0611632产生了约5kbp的条带;引物0611815和0611632产生了约6kbp有ura标志的条带,和4.5kbp没有该标志的条带。当野生型adh1202基因座仍然完整时引物0611631和0612579得到了约936bp的条带(选择不显示该条带的菌株)。给出表达盒正确整合预期条带的菌株被命名为MeJi412。

[0652] 实施例3A-15:在adh1202基因座表达编码天冬氨酸1-脱羧酶(ADC)的四个核苷酸拷贝的酵母菌株,其中有在PDC启动子的调控下编码天冬氨酸1-脱羧酶(ADC)的两个核苷酸拷贝和在TDH3启动子调控下的两个拷贝。

[0653] 本实施例描述了构建体,其设计为要在adh1202基因座掺入编码SEQ ID NO:139地衣芽孢杆菌ADC的四个核苷酸拷贝,所述拷贝有在东方伊萨酵母PDC启动子调控下的两个拷贝和在东方伊萨酵母TDH31启动子调控下的两个拷贝。以与上面描述方式类似的方式,设计左端和右端构建体以允许在东方伊萨酵母CNB1adh1202基因座的同源重组。

[0654] 左端片段的构建

[0655] 质粒pMeJi310-2(如上,见图14)被以Xba1和Stu1消化随后如本发明中所述以CIP处理并以TBE缓冲液中1%琼脂糖凝胶电泳来纯化。约6.7kbp的条带被从凝胶上切下并根据制造商的说明使用**QIAQUICK®**Gel Extraction KiT (Qiagen) 纯化。

[0656] 通过以Not1的消化,随后以Klenow的补平反应和以Nhe1的后续消化来将PDC启动子从pMeJi310-2上切下。约708bp的条带被从凝胶上切下并根据制造商的说明使用**QIAQUICK®**Gel Extraction KiT (Qiagen) 纯化。

[0657] 使用T4连接酶(New England Biolabs)在10μL总反应体积中将708bp的纯化片段连接进6.7kbp的pMeJi310-2线性化载体,10μL总反应体积由1μL来自pMeJi310-2的6.7kbp片段,1或5μL来自pMeJi310-2的708bp片段,1μL有10mM ATP的10X连接缓冲液(New England Biolabs),和1μL T4连接酶(New England Biolabs)构成。反应物在16℃下孵育过夜,而4μL等份的反应物被用于根据制造商的说明转化进ONE **SHOT®**TOP10化学感受态大肠杆菌细胞(Invitrogen)。转化体被涂布于2X YT+amp平板上并在37℃下孵育过夜。通过使用ApaLI的限制性消化筛选到数个有合适插入的所得转化体。产生正确消化条带大小的克隆被命名为pMIBa137。

[0658] 右端片段的构建

[0659] 质粒pMeJi312-2(如上)被以Xba1和Stu1消化随后如本发明中所述以CIP处理并以TBE缓冲液中1%琼脂糖凝胶电泳来纯化。约6.8kbp的条带被从凝胶上切下并根据制造商的说明使用**QIAQUICK®**Gel Extraction KiT (Qiagen) 纯化。

[0660] 以Pme1和Nhe1消化从pMeJi312-2上切下TDH3启动子。约966bp的条带被从凝胶上切下并根据制造商的说明使用**QIAQUICK®**Gel Extraction KiT (Qiagen) 纯化。

[0661] 使用T4连接酶(New England Biolabs)在10μL总反应体积中将966bp的纯化片段连接进6.8kbp的pMeJi312-2线性化载体,10μL总反应体积由1μL来自pMeJi312-2的6.8kbp

片段,1或5 μ L来自pMeJi312-2的966bp片段,1 μ L有10mM ATP的10X连接缓冲液(New England Biolabs),和1 μ L T4连接酶(New England Biolabs)构成。反应物在16 $^{\circ}$ C下孵育约6小时,而4 μ L等份的反应物被用于根据制造商的说明转化进ONE SHOT[®]TOP10化学感受态大肠杆菌细胞(Invitrogen)。转化体被涂布于2X YT+amp平板上并在37 $^{\circ}$ C下孵育过夜。通过使用Sal1的限制性消化筛选到数个有合适插入的所得转化体。产生正确消化条带大小的克隆被命名为pMIBa136。

[0662] 左端和右端片段的整合

[0663] 如本发明中所述质粒pMIBa137被以Hpa1和Sac11消化而质粒pMIBa136被以EcoRI和Sac11消化。这些被以TBE缓冲液中1%琼脂糖凝胶电泳来纯化,两个约5kbp的条带被从凝胶上切下并根据制造商的说明使用QIAQUICK[®]Gel Extraction KiT(Qiagen)来纯化。

[0664] 以消化的pMIBa137和pMIBa136DNA来转化东方伊萨酵母CNB1,并如本发明所述以Crimson Taq(New England Biolabs)PCR来验证正确的基因座靶向和转化。引物0611717和0611631产生约2.5kbp和955bp的条带;引物0611718和0611632产生约733bp的条带;引物0612794和0611245产生约2.7kbp的条带;引物0611225和0612795产生约4.2kbp的条带。给出表达盒正确整合预期条带的菌株被命名为MIBa351。

[0665] 从MIBa351去除ura标志

[0666] 如上所述分离MIBa351的ura-衍生体。使用引物0611815和0611817通过PCR筛选到MIBa351数个FOA抗性菌落的基因组DNA有所需环出事件。引物0611815在左端构建体RKI终止子处退火并向ura3启动子扩增,而引物0611817在TDH3启动子处退火并反向朝ura3盒扩增。828bp条带的存在表示如所需的仅URA3残留位点的存在(在亲本菌株中两个URA3启动子间同源重组后留下的单个URA3启动子),而约2.2kbp条带表示完整URA3启动子-URA3ORF-URA3终止子-URA3启动子盒的存在,表明所需的重组事件没有发生。如上所述进行用Crimson Taq[™]DNA聚合酶(New England Biolabs)的PCR反应。以上述引物得到828bp片段的FOA抗性菌落被以产生2.7kbp产物的引物0612794和0611245以及测试4kbp产物的引物0611815和0612795进一步测试,以确认编码SEQ ID NO:139的地衣芽孢杆菌ADC的核苷酸序列SEQ ID NO:138的四个拷贝保持完整。一个来自亲本菌株MIBa351的FOA抗性菌落,命名为MIBa353,以所有三个引物组给出了所需的PCR产物。

[0667] 反向表达盒右端片段的构建

[0668] 质粒pMIBa136包含两个朝正向取向的表达盒。为简化同源菌株的筛选,构建了新的质粒,其中pMIBa136的panDbl表达盒被置于反向取向。质粒pMIBa136(如上)被以Not1和Pme1消化,如本发明中所述随后碱性用Klenow的补平反应,并以TBE缓冲液中1%琼脂糖凝胶电泳来纯化。约3.4kbp和4.4kbp的条带被从凝胶上切下并根据制造商的说明使用QIAQUICK[®]Gel Extraction KiT(Qiagen)来纯化。来自pMIBa136的的4.4kbp片段被以CIP处理并根据制造商的说明使用QIAQUICK[®]PCR Purification KiT(Qiagen)来纯化。

[0669] 使用T4连接酶(New England Biolabs)在10 μ L总反应体积中将来自pMIBa136的3.4kbp的纯化片段连接进4.4kbp的pMIBa136载体,10 μ L总反应体积由1 μ L来自pMIBa136的4.4kbp片段,1或5 μ L来自pMIBa136的3.4kbp片段,1 μ L有10mM ATP的10X连接缓冲液(New England Biolabs),和1 μ L T4连接酶(New England Biolabs)构成。反应物在16 $^{\circ}$ C下孵育过

夜,而4 μ L等份的反应物被用于根据制造商的说明转化进ONE SHOT®TOP10化学感受态大肠杆菌细胞(Invitrogen)。转化体被涂布于2X YT+amp平板上并在37℃下孵育过夜。通过使用Pac11和EcoRI的限制性消化筛选到数个有合适插入的所得转化体。产生正确消化条带大小的克隆被命名为pMIBa138。

[0670] 左端和右端片段的整合

[0671] 如本发明中所述质粒pMIBa137被以Hpa1和Sac11消化而质粒pMIBa138被以EcoRI和Sac11消化。这些被以TBE缓冲液中1%琼脂糖凝胶电泳来纯化,两个约5kbp的条带被从凝胶上切下并根据制造商的说明使用QIAQUICK®Gel Extraction KiT(Qiagen)来纯化。

[0672] 以消化的pMIBa137和pMIBa138DNA来转化MIBa353,并使用Phire®Plant Direct PCR KiT(Finnzymes)来通过PCR来验证正确的基因座靶向和转化。引物0611718和0611632产生约733bp的条带(以确认第一整合仍然存在);引物0612367和0611632产生约960bp的条带(以确认整合的第二拷贝);当野生型adh1202基因座仍然存在时引物0611631和06112579产生约936bp的条带(缺少该条带确认野生型adh1202基因座的丧失)。给出表达盒正确整合预期条带的两个菌株被保存并被命名为MIBa355和MIBa356。

[0673] MeJi409-2,MeJi412、MIBa351和MIBa355中天冬氨酸1-脱羧酶活性

[0674] 菌株MeJi409-2,MeJi412、MIBa351和MIBa355被在摇瓶中培养,如本发明中所述制备CFE并测试天冬氨酸1-脱羧酶(ADC)活性。结果显示于表26中。每个菌株的活性为两个独立摇瓶培养物的平均值。也使用本发明描述的方法,在生物反应器中测试菌株MeJi409-2、MeJi412、MIBa351和MIBa355的3-HP产生。这些生物反应器实验的结果也显示于表26中。考虑到这些发酵中的细胞质量的差异,表26中显示的3-HP产生性能被表示为每单位细胞质量的3-HP浓度(表示为g/L 3-HP/g/L干细胞重)。结果显示随着细胞中ADC活性水平的增加,3-HP产生性能增加。

[0675] 表26:转化体ADC活性和3-HP产生数据

[0676]

菌株	过表达的基因	ADC 活性	3-HP/DCW
MBin500	N/A	0	0
MeJi409-2	ADC (SED ID NO: 138)	0.629	0.19
MeJi412		1.151	0.43
MIBa351		0.659	0.32
MIBa355		1.173	0.52

[0677] 实施例3A-16:在PDC基因座表达丙酮酸羧化酶(PYC)的质粒构建

[0678] 如下所述构建用于在PDC基因座整合的,含SEQ ID NO:1核苷酸序列(编码SEQ ID NO:2的东方伊萨酵母PYC)的质粒pANN28。

[0679] 根据制造商的说明使用基因组DNA作为模板来PCR扩增东方伊萨酵母PDC的上游和下游侧翼区域(Pfu聚合酶Stratagene)。引物oANN7和oANN8允许掺入上游区域侧翼独特的限制位点而引物oANN9和oANN10允许掺入下游区域侧翼独特的限制位点。如本发明中所述,PCR产物被以TBE缓冲液中1%琼脂糖凝胶电泳纯化。每个PCR产物约800bp的条带被从凝胶上切下并根据制造商的说明使用Gel Extraction KiT(Qiagen)来纯化。纯化的PCR产物被

克隆进TOP0载体 (Invitrogen) 并根据制造商的说明转化进电感受态大肠杆菌DH10B细胞 (Invitrogen)。使用与用于构建PCR产物的引物相同的引物通过菌落PCR筛选到中的数个有合适插入的所得转化体。产生正确PDC下游侧翼的克隆被命名为pANN04。产生正确PDC上游侧翼的克隆被命名为pANN07。

[0680] 质粒pANN04被以Apa1和Sac1消化 (用作载体/骨架); 质粒pANN04被以Not1和Sac1消化; 质粒pANN07被以Not1和Apa1消化。如本发明中所述, 每个片段被以TBE缓冲液中1%琼脂糖凝胶电泳纯化。对载体约3.5kbp的条带, 和对每个插入物约1kbp的条带被从凝胶上切下并根据制造商的说明使用Gel Extraction KiT (Qiagen) 来纯化。使用T4连接酶 (New England Biolabs) 在10 μ L总反应体积中将纯化产物连接, 10 μ L总反应体积由49ng载体, 120ng下游插入物, 41ng上游插入物, 1 μ L有10mM ATP的10X连接缓冲液 (New England Biolabs), 和1 μ L T4连接酶 (New England Biolabs) 构成。反应物在室温下孵育30分钟, 而2 μ L等份的反应物被根据制造商的说明转化进电感受态大肠杆菌One Shot TOP10细胞 (Invitrogen)。转化体被涂布于LB+卡那霉素平板上并在37 $^{\circ}$ C下孵育过夜。使用引物oANN7和oANN8 (产生约1.7kbp的片段) 通过菌落PCR筛选到数个有合适插入的所得转化体。产生正确插入的克隆被命名为pANN12。

[0681] 来自pGMEr137 (如上) 的东方伊萨酵母PYC编码序列 (SEQ ID NO:1) 被以位点定向诱变修饰, 以消除不改变所编码酶氨基酸序列的三个EcoRI限制性位点。质粒pGMEr137被用作引物oANN13、oANN14和oANN15的模板, 根据制造商的说明来使用Multi Change KiT (Stratagene), 使用这些引物消除上述限制性位点。通过使用EcoRI的限制性消化筛选到数个所得转化体。阳性克隆通过测序进一步确认。得到正确pyc编码序列的克隆被命名为pANN14。

[0682] 质粒pJY39 (图29) 被以Xho1和Pac1消化; 质粒pACN5 (如上, 见图19) 被以Xho1和Xba1消化; 质粒pANN14被以Xba1和Pac1消化。如本发明中所述, 每个片段被以TBE缓冲液中1%琼脂糖凝胶电泳纯化。对载体约8kbp的条带, 对第一插入物约700bp的条带和对编码PYC的第二插入物约3.6kbp的条带被从凝胶上切下并根据制造商的说明使用Gel Extraction KiT (Qiagen) 来纯化。使用T4连接酶 (New England Biolabs) 在10 μ L总反应体积中将纯化产物连接, 10 μ L总反应体积由51ng载体, 49ng第一插入物, 210ng第二插入物, 1 μ L有10mM ATP的10X连接缓冲液 (New England Biolabs), 和1 μ L T4连接酶 (New England Biolabs) 构成。反应物在室温下孵育30分钟, 而2 μ L等份的反应物被根据制造商的说明转化进电感受态大肠杆菌One Shot TOP10细胞 (Invitrogen)。转化体被涂布于LB+卡那霉素平板上并在37 $^{\circ}$ C下孵育过夜。使用引物oJLJ57和oJLJ43 (产生约1kbp的片段), 引物oJLJ45和oANN16 (产生约730bp的片段), 和引物oANN20和oJY45 (产生约1.2kbp的片段) 通过菌落PCR筛选到数个有合适插入的所得转化体。得到正确插入的克隆被命名为pANN15。

[0683] 质粒pANN12和pANN15被以Not1消化。质粒pANN15被另外地以Nco1消化以将骨架进一步片段化并改进所需片段的分离。消化的pANN12被根据制造商的说明使用Qiagen试剂盒纯化。如本发明中所述Not1片段以TBE缓冲液中的琼脂糖凝胶电泳纯化。约5kbp的片段 (来自pANN12) 和约6.3kbp的片段 (来自pANN15) 被根据制造商的说明使用Gel Extraction KiT (Qiagen) 来凝胶纯化。

[0684] 使用T4连接酶 (New England Biolabs) 在10 μ L总反应体积中将来自pANN15的纯化

产物连接进pANN12线性化载体,10 μ L总反应体积由50ng载体,115ng插入物,1 μ L有10mM ATP的10X连接缓冲液(New England Biolabs),和1 μ L T4连接酶(New England Biolabs)构成。反应物在室温下孵育1.5小时,而2 μ L等份的反应物被用于根据制造商的说明转化电感受态大肠杆菌One Shot TOP10细胞(Invitrogen)。转化体被涂布于LB+卡那霉素平板上并在37 $^{\circ}$ C下孵育过夜。使用引物oANN20和oJY45(产生约1.2kbp的片段)通过菌落PCR筛选到数个有合适插入的所得转化体。产生正确插入的克隆被通过以SacI/EcoRI和以SacI/EcoRV的限制性酶消化来进一步筛选以区分插入取向。产生上游PDC侧翼附近ura3标志的克隆被命名为pANN27。产生下游PDC侧翼附近ura3标志的克隆被命名为pANN28。

[0685] 实施例3A-17:在adh1202基因座从四个核苷酸序列表达天冬氨酸1-脱羧酶(ADC)和在pdc基因座表达丙酮酸羧化酶(PYC)的酵母菌株

[0686] 本实施例描述了酵母菌株的构建,该酵母菌株在adh1202基因座表达编码SEQ ID NO:139地衣芽孢杆菌ADC的四个核苷酸拷贝并在pdc基因座表达编码SEQ ID NO:2东方伊萨酵母PYC的核苷酸。

[0687] 从MeJi412去除ura标志

[0688] 如上所述分离MeJi412的ura-衍生体。使用引物0611815和0611817通过菌落PCR筛选到MeJi412数个FOA抗性菌落有所需环出事件。引物0611815在左端构建体RKI终止子处退火并向ura3启动子扩增,而引物0611817在TDH3启动子处退火并反向朝ura3盒扩增。869bp条带的存在表示如所需的仅ura3残留位点的存在(在亲本菌株中两个URA3启动子间同源重组后留下的单个URA3启动子),而约2.6kbp条带表示完整URA3启动子-URA3ORF-URA3终止子-URA3启动子盒的存在,表明所需的重组事件没有发生。如上所述进行用Phire[®]Plant Direct PCR KiT(Finnzymes)的PCR反应。以上述引物产生869bp片段的FOA抗性菌落被以产生3.8kbp产物的引物0612794和0611817以及产生3.7kbp产物的引物0611815和0612795进一步测试,以确认编码SEQ ID NO:139地衣芽孢杆菌ADC的核苷酸序列SEQ ID NO:138四个拷贝保持完整。一个来自亲本菌株MeJi412的FOA抗性菌落,命名为MeJi413,以所有三个引物组给出了所需的PCR产物。

[0689] 片段的整合

[0690] 质粒pANN28(如上)被以AscI和SacI消化并通过TBE缓冲液中的琼脂糖凝胶电泳纯化。约7.1kbp的条带被从凝胶上切下并根据制造商的说明使用Gel Extraction KiT(Qiagen)来纯化。

[0691] 以来自菌株pANN28的消化并纯化片段来转化菌株MeJi413,并根据制造商说明通过菌落PCR(Failsafe,mix E,Epicenter)来验证正确的基因座靶向和转化。引物oANN12和oJLJ44产生约1kbp的条带;引物oANN11和oANN16产生约1.3kbp的条带。给出表达盒正确整合预期条带的菌株被命名为yANN35。

[0692] 随后如上所述分离yANN35的ura-衍生体。使用引物oANN12和oJY44通过菌落PCR筛选到数个FOA抗性菌落有所需环出事件。引物oANN12在下游侧翼区域外退火。引物oJY44在TAL终止子处退火。1.5kbp条带的存在表示如所需的仅ura3残留位点的存在(在亲本菌株中两个URA3启动子间同源重组后留下的单个URA3启动子),而约2.8kbp条带表示完整URA3启动子-URA3 ORF-URA3终止子-URA3启动子盒的存在,表明所需的重组事件没有发生。如上所述进行用Failsafe DNA聚合酶(Epicenter)的PCR反应。对该事件阳性的分离株被进一步使

用引物oANN16和oANN11通过菌落PCR来确认。一个FOA抗性菌落被命名为yANN37。

[0693] 以来自菌株pANN28的消化并纯化片段来转化菌株yANN37,并根据制造商说明通过菌落PCR (Failsafe,mix E,Epicenter) 来验证正确的基因座靶向和转化。使用对PDC基因特异的引物oHJJ116和oHJJ117进行初步筛选。约500bp的条带表示基因的存在并因此是所需整合的阴性结果。对PDC缺失阳性的分离株被以另外的PCR反应进一步确认。引物oANN11和oANN16产生约1.3kbp的条带;引物oANN12和oJY44oJLJ44产生约1kbp的条带;引物oANN12和oJY44产生约1.5kbp的条带和约2.9kbp的条带(分别对应第一个第二整合事件)。

[0694] 此外,如上所述通过菌落PCR来确认之前在adh1202基因座处的整合事件。引物0611631和0611245产生约3.8kbp的条带。引物0611245和oNovo3产生约3kbp的条带。引物0611815和0612795产生约3.6kbp的条带。给出表达盒正确整合预期条带的菌株被命名为yANN41。

[0695] 实施例3A-18:在adh1202基因座从四个核苷酸序列表达天冬氨酸1-脱羧酶(ADC)和在pdc基因座表达丙酮酸羧化酶(PYC)的酵母菌株

[0696] 本实施例描述了酵母菌株的构建,该酵母菌株在adh1202基因座表达编码SEQ ID NO:139地衣芽孢杆菌ADC的四个核苷酸拷贝(有两个在PDC启动子调控下的拷贝和两个在TDH3启动子调控下的拷贝)并在pdc基因座表达编码SEQ ID NO:2东方伊萨酵母PYC的核苷酸。

[0697] 从MIBa355去除ura标志

[0698] 如上所述分离MIBa355的ura-衍生体。使用引物0611815和0611718通过PCR筛选到MIBa355数个FOA抗性菌落的基因组DNA有所需环出事件。约500bp条带的存在表示如所需的仅ura3残留位点的存在(在亲本菌株中两个URA3启动子间同源重组后留下的单个URA3启动子),而约1.9kbp条带表示完整URA3启动子-URA3ORF-URA3终止子-URA3启动子盒的存在,表明所需的重组事件没有发生。根据制造商的说明使用**Phire®**Plant Direct PCR KiT (Finnzymes)进行PCR。以上述引物产生约500bp片段的FOA抗性菌落被以产生3.5kbp产物的引物0611631和0611245以及产生4.5kbp产物的引物0611815和0611632进一步测试,以确认编码SEQ ID NO:139地衣芽孢杆菌ADC的核苷酸序列SEQ ID NO:138四个拷贝保持完整。其也被使用引物0611815和0611817以PCR测试,以确认adh1202处第一修饰的存在。这些PCR引物产生828bp的片段。一个来自亲本菌株MIBa355的FOA抗性菌落,命名为MIBa357,以所有四个引物组给出了所需的PCR产物。

[0699] 质粒pANN28(如上)被以AscI和SacI消化并以TBE缓冲液中的1%琼脂糖凝胶电泳纯化。约7.1kbp的条带被从凝胶上切下并根据制造商的说明使用**NUCLEOSPIN®** Extract II KiT(Macherey-Nagel)纯化。

[0700] MIBa357被以消化的pANN28DNA转化,并使用PhirePlant Direct PCR KiT (Finnzymes)通过PCR验证正确的基因座靶向和转化。引物0611622和0611552产生约850bp条带;引物0611245和0612794产生约2.8kbp的条带;引物0611815和0612795产生约3.9kbp的条带。给出表达盒正确整合预期条带的菌株被命名为McTs241。

[0701] 随后如前所述分离McTs241的ura-衍生体。使用引物0614233和0611554通过PCR筛选到McTs241数个FOA抗性菌落有所需的环出事件,并在减除了ura的平板上缺乏生长。约4.6kbp条带的存在表示如所需的仅ura3残留位点的存在(在亲本菌株中两个URA3启动子间

同源重组后留下的单个URA3启动子),而约5.9kbp条带表示完整URA3启动子-URA3ORF-URA3终止子-URA3启动子盒的存在,表明所需的重组事件没有发生。如上所述进行使用Phire Plant Direct PCR KiT(Finnzymes)的PCR反应。一个来自亲本菌株McTs241的,具有所需环出事件的FOA抗性菌落被命名为McTs247。

[0702] 为构建纯合整合体,McTs247被以消化的pANN28DNA转化,并使用PhirePlant Direct PCR KiT(Finnzymes)通过PCR验证正确的基因座靶向和转化。作为第一次筛选,使用引物0611552和0611553通过PCR来筛选转化体,其仅当pdc基因座完整并因此PDC基因座处PYC的纯合重组没有发生时产生约850bp的条带。对该PCR的条带阴性的那些随后被使用引物0611555和0611554通过另外PCR来筛选。使用这些引物,当PDC完整并因此PDC基因座处无PYC的纯合重组时产物应仅扩增1.4kbp条带。使用产生约850bp条带的引物0611622和0611552;产生约2.8kbp条带的引物0611245和0612794;产生约3.9kbp条带的引物0611815和0612795通过PCR进行转化体的进一步筛选。给出表达盒正确整合预期条带的菌株被命名为McTs253。

[0703] 实施例3A-19:菌株MeJi412、yANN35、yANN41、MIBa355、McTs241和McTs253的PYC活性、ADC活性和3-HP产生性能。

[0704] 菌株MeJi412、yANN35、yANN41、MIBa355、McTs241和McTs253被在摇瓶中生长,如本发明中所述制备CFE并测试丙酮酸羧化酶(PYC)活性和天冬氨酸1-脱羧酶(ADC)活性。结果显示于表27中。也使用本发明描述的方法,在生物反应器中测试菌株MeJi412、yANN35、yANN41、MIBa355、McTs241和McTs253的3-HP产生。这些生物反应器实验的结果也显示于表27中。考虑到这些发酵中的细胞质量的差异,显示的3-HP产生性能被表示为每单位细胞质量的3-HP浓度(表示为g/L 3-HP/g/L干细胞重)。结果显示随着细胞中PYC活性水平的增加,3-HP产生性能增加。

[0705] 表27:转化体PYC和ADC活性和3-HP产生性能

菌株	过表达的基因	PYC 活性	ADC 活性	3-HP/DCW
MeJi412	ADC (SEQ ID NO: 138)	6.8	1.151	0.43
yANN35	ADC (SEQ ID NO: 138) PYC (SEQ ID NO: 1)	47.2	1.090	0.64
yANN41	ADC (SEQ ID NO: 138) PYC (SEQ ID NO: 1)	49.0	1.263	1.30
MIBa355	ADC (SEQ ID NO: 138)	6.9	1.173	0.52
McTs241	ADC (SEQ ID NO: 138) PYC (SEQ ID NO: 1)	24.8	1.119	0.76
McTs253	ADC (SEQ ID NO: 138) PYC (SEQ ID NO: 1)	55.5	1.347	1.30

[0707] 实施例3A-20:在adh1202基因座从四个核苷酸序列表达天冬氨酸1-脱羧酶(ADC)并缺失了β-丙氨酸氨基转移酶(BAAT)的酵母菌株

[0708] 本实施例描述了酵母菌株的构建和性能,所述酵母菌株在adh1202基因座表达编码SEQ ID NO:139地衣芽孢杆菌ADC的四个核苷酸拷贝并缺失了编码SEQ ID NO:20的BAAT(PYD4)的天然东方伊萨酵母基因。

[0709] 东方伊萨酵母BAAT(PYD4)缺失质粒的构建

[0710] 质粒pMIBa 123(如上)被以NotI、KpnI、ApaI消化并如本发明中所述通过TBE缓冲液中1%的琼脂糖凝胶电泳来纯化。约3.6kbp和3.8kbp的两个条带被从凝胶上切下并根据制造商的说明使用NUCLEOSPIN®Extract II KiT(Macherey-Nagel)纯化。这两片

段包含质粒骨架和有SEQ ID NO:144的酿酒酵母3-HPDH基因(YMR226c)的ura选择盒

[0711] 以引物0613178和0613180使用东方伊萨酵母MBin500基因组DNA通过PCR扩增生成上游东方伊萨酵母PYD4同源区的PCR产物。使用引物0613179和0613181通过PCR扩增从MBin500基因组DNA制备下游东方伊萨酵母PYD4同源区片段。每种引物50pmol被用于含0.5μL作为模板的MBin500基因组DNA,dATP、dCTP、dGTP和dTTP每种0.2mM,1XExpand High Fidelity Buffer (Roche),3.5U Expand High Fidelity Enzyme Mix (Roche)的终体积50μL的PCR反应中。在EPPENDORF® MASTERCYCLER®5333 (Eppendorf) 中进行扩增反应,程序为95℃ 3分钟的1个循环;和每个为95℃ 30秒,55℃ 30秒和72℃ 1分钟的30个循环。循环后,反应物被在72℃孵育5分钟并随后在10℃冷却直至进一步处理。使用引物0613178和0613180的约800bp的PCR条带,以及使用引物0613179和0613181的约900bp的PCR条带被以TBE缓冲液中1%的琼脂糖凝胶电泳纯化。条带被从凝胶上切下并根据制造商的说明使用NUCLEOSPIN®Extract II KiT (Macherey-Nagel) 纯化。

[0712] PYD4上游PCR产物,PYD4下游PCR产物,和pMIBa123NotI/KpnI/ApaI消化的质粒被根据制造商说明在用IN-FUSION HD™ (Clontech Laboratories, Inc.) 的反应中组装。来自In-FUSION反应的2μL被根据制造商的说明转化进ONE SHOT®TOP10化学感受态大肠杆菌细胞 (Invitrogen)。在恢复期后,来自转化反应的两份100μL等份被涂布于添加有每mL 100μg氨苄青霉素的150mm 2X YT平板上。平板在37℃孵育过夜。从选择平板上选出推定的重组克隆并使用BIOROBOT®9600 (Qiagen) 从每一个制备质粒DNA。克隆通过限制性消化和测序来分析。有正确序列的质粒通过测序验证并命名为pMcTs61。

[0713] 质粒pMcTs61仍然含PDC启动子,来自酿酒酵母的YMR226c基因,和PDC终止子。为去除这些不需要的片段,pMcTs61被以EcoRI和XhoI消化随后加入Klenow片段以构建平末端。7.1kbp的片段被以TBE缓冲液中1%的琼脂糖凝胶电泳纯化。条带被从凝胶上切下并根据制造商的说明使用NUCLEOSPIN®Extract II KiT (Macherey-Nagel) 纯化。使用T4连接酶 (New England Biolabs, Ipswich, MA, USA) 将消化的平末端质粒连接在一起。反应混合物在总体积20μL中含1X T4连接酶缓冲液,1μL T4DNA连接酶,5μL pMcTs61消化和平末端化的纯化DNA。反应物在室温下孵育2小时。10μL连接反应样品被用于根据制造商的说明转化ONE SHOT®TOP10化学感受态大肠杆菌细胞 (Invitrogen)。在恢复期后,来自转化反应的两份100μL等份被涂布于添加有每mL 100μg氨苄青霉素的150mm 2X YT平板上。平板在37℃孵育过夜。从选择平板上选出推定的重组克隆。通过菌落PCR分析克隆。通过将1个菌落溶解于50μL无菌水中,在95℃加热10分钟,随后在冰上冷却直至使用来制备每个菌落的模板DNA。引物0612911和0612909被用于筛选转化体。如果质粒正确,以这些引物的PCR反应会扩增出1kbp的条带。每种引物10pmol被用于含2μL菌落DNA模板,dATP、dCTP、dGTP和dTTP每种0.1mM,1X Crimson Taq反应缓冲液 (New England Biolabs),1U Crimson Taq DNA聚合酶 (New England Biolabs) 的终体积20μL的PCR反应中。在EPPENDORF® MASTERCYCLER®5333 (Eppendorf) 中进行扩增反应,程序为95℃ 3分钟的1个循环;和每个为95℃ 30秒,55℃ 30秒和72℃ 3分钟的30个循环。循环后,反应物被在72℃孵育5分钟并随后在10℃冷却直至进一步处理。以TAE缓冲液中的溴化乙锭来在1%TAE-琼脂糖凝胶中观察来自5μLPCR反应物的1kbp片段。有正确大小PCR产物的一个转化体被选出并命名

为pMcTs64(图30)。使用BIOROBOT®9600(Qiagen)制备pMcTs64的质粒DNA。

[0714] 使用pMcTs64构建体从MeJi413缺失天然东方伊萨酵母BAAT(PYD4)

[0715] 质粒pMcTs64(如上,见图30)被以ApaI、NcoI、KpnI消化并通过TBE缓冲液中1%的琼脂糖凝胶电泳来纯化。约3.3kbp的条带被从凝胶上切下并根据制造商的说明使用NUCLEOSPIN®Extract II KiT(Macherey-Nagel)纯化。

[0716] 菌株MeJi413(如上)被以消化的pMcTs64DNA转化,并使用Phire Plant Direct PCR KiT(Finnzymes)通过PCR验证正确的基因座靶向和转化。引物0612908和0613242产生约1.7kbp条带;引物0613241和0612909产生约1.5kbp的条带以确认缺失盒的整合;引物0611815和0611632产生约4.2kbp的条带;引物0611817和0611631产生约4.8kbp的条带以确认ADH1202基因座的ADC表达盒仍然完整。给出缺失盒和ADC盒正确整合预期条带的菌株被命名为McTs225。

[0717] 随后如前所述分离McTs225的ura⁻衍生体。使用引物0612911和0612910通过PCR筛选到McTs225数个FOA抗性菌落有所需的环出事件。约1.1kbp条带的存在表示如所需的仅ura3残留位点的存在(在亲本菌株中两个URA3启动子间同源重组后留下的单个URA3启动子),而约2.5kbp条带表示完整URA3启动子-URA3ORF-URA3终止子-URA3启动子盒的存在,表明所需的重组事件没有发生。引物0611815和0611632产生约4.2kbp的条带;引物0611817和0611631产生约4.8kbp的条带以确认adh1202基因座的ADC表达盒仍然完整。如上所述进行使用Phire Plant Direct PCR KiT(Finnzymes)的PCR反应。一个来自亲本菌株McTs225的,具有所需环出事件的FOA抗性菌落被命名为McTs228。

[0718] 为构建编码SEQ ID NO:20的东方伊萨酵母BAAT(PYD4)的天然基因的纯合缺失体,McTs228被以消化的pMcTs64转化,并使用Phire Plant Direct PCR KiT(Finnzymes)通过PCR验证正确的基因座靶向和转化。两组引物被用于通过PCR筛选PYD4位点缺失。仅当PYD4位点完整时,引物0613550和0612910产生约700bp的条带,表明PYD4纯合缺失没有发生。此外转化体被以在PYD4没有被缺失时产生约600bp条带的引物0612911和0613551来筛选。对东方伊萨酵母PYD4基因座阴性的转化体被以产生约3.5kbp和2.1kbp条带的引物0613242和0613243,产生约1.7kbp条带的引物0612908和0613243;产生约950bp条带的引物0612909和0612911来进一步筛选。以产生约4.8kbp条带的引物0611817和0611631以及产生约4.2kbp条带的引物0611815和0612712来确认adh1202处ADC盒仍然完整。给出表达盒正确整合预期条带的菌株被命名为McTs236。

[0719] 随后如前所述分离McTs236的ura⁻衍生体。使用产生约2.1kbp条带的引物0613242和0613243通过PCR筛选到McTs236数个FOA抗性菌落有所需的环出事件。使用产生约3kbp条带的引物0611245和0612794以及产生约3.6kbp条带的引物0611815和0612795来确认adh1202基因座的ADC盒仍然完整。给出表达盒正确整合预期条带的菌株被命名为McTs245。

[0720] 使用本发明描述的方法,在生物反应器中测试菌株MIBa372和McTs245的3-HP产生。考虑到这些发酵中的细胞质量的差异,3-HP产生性能被表示为每单位细胞质量的3-HP浓度(表示为g/L 3-HP/g/L干细胞重)。菌株MIBa372和McTs245的g/L 3-HP/g/L干细胞重分别为1.66和0.16。这些结果表明由于缺失该基因导致3-HP产生性能降低至十分之一,因此是东方伊萨酵母中的天然PYD4基因负责将β-丙氨酸转化成丙二酸半醛。

[0721] 实施例3A-21在adh1202基因座从四个核苷酸序列表达天冬氨酸1-脱羧酶(ADC)并

缺失了3-HP脱氢酶(3-HPDH)的酵母菌株

[0722] 本实施例描述了酵母菌株的构建和性能,所述酵母菌株在adh1202基因座表达编码SEQ ID NO:139地衣芽孢杆菌ADC的四个核苷酸拷贝并缺失了编码SEQ ID NO:26的3-HPDH的天然东方伊萨酵母基因。

[0723] 东方伊萨酵母3-HPDH缺失质粒的构建

[0724] 质粒pMIBa 123(如上)被以Not1、Kpn1、Apa1消化并如本发明中所述通过TBE缓冲液中1%的琼脂糖凝胶电泳来纯化。约3.6kbp和3.8kbp的两个条带被从凝胶上切下并根据制造商的说明使用**NUCLEOSPIN®**Extract II KiT(Macherey-Nagel)纯化。这两片段包含质粒骨架和有SEQ ID NO:144酿酒酵母3-HPDH基因的ura选择盒。

[0725] 使用引物0613183和0613184从如前所述制备的东方伊萨酵母MBin500基因组DNA扩增上游东方伊萨酵母3-HPDH同源区的PCR产物。使用引物0613185和0613186从MBin500基因组DNA扩增下游东方伊萨酵母3-HPDH同源区片段。每种引物50pmol被用于含0.5μL作为模板的MBin500基因组DNA,dATP、dGTP、dCTP、和dTTP每种0.2mM,2%DMSO,1X Phusion HF缓冲液(Finnzymes),2U**Phusion®**Hot Start High Fidelity DNA聚合酶(Finnzymes)的终体积50μL的PCR反应中。在**EPPENDORF® MASTERCYCLER®**5333(Eppendorf Scientific Inc.,Westbury,NY,USA)中进行扩增反应,程序为95℃ 3分钟的1个循环;和每个为95℃ 30秒,55℃ 30秒和72℃ 1分钟的30个循环。循环后,反应物被在72℃孵育5分钟并随后在4℃冷却直至进一步处理。引物0613183和0613184的约640bp的PCR条带,以及引物0613185和0613186的约670bp的PCR条带被以TBE缓冲液中1%的琼脂糖凝胶电泳纯化。条带被从凝胶上切下并根据制造商的说明使用**NUCLEOSPIN®**Extract II KiT(Macherey-Nagel)纯化。

[0726] 东方伊萨酵母3-HPDH上游PCR产物,东方伊萨酵母3-HPDH下游PCR产物,和pMIBa123Not1/Kpn1/Apa1消化的质粒被根据制造商说明在用IN-FUSION HD™(Clontech Laboratories,Inc.)的反应中组装。来自In-FUSION反应的2μL被根据制造商的说明转化进**ONE SHOT®**TOP10化学感受态大肠杆菌细胞(Invitrogen)。在恢复期后,来自转化反应的两份100μL等份被涂布于添加有每mL 100μg氨苄青霉素的150mm 2X YT平板上。平板在37℃孵育过夜。从选择平板上选出推定的重组克隆并使用**BIOROBOT®**9600(Qiagen)从每一个制备质粒DNA。克隆通过限制性消化和测序来分析。有正确序列的质粒通过测序验证并命名为pMcTs60。

[0727] 质粒pMcTs60仍然含PDC启动子,来自酿酒酵母的YMR226c基因,和PDC终止子。为去除这些不需要的片段,pMcTs60被以Not1和Xba1消化,含3-HPDH同源区和质粒骨架片段的约5kbp条带被以TBE缓冲液中1%的琼脂糖凝胶电泳纯化。条带被从凝胶上切下并根据制造商的说明使用**NUCLEOSPIN®**Extract II KiT(Macherey-Nagel)纯化。以两个PCR反应,一个用引物0613416和0613417另一个用引物0613418和0613419来扩增ura3选择盒。每种引物50pmol被用于含0.5μL作为模板的pMcTs60质粒DNA,dATP、dGTP、dCTP和dTTP每种0.2mM,1X Expand High Fidelity Buffer(Roche),3.5U Expand High Fidelity Enzyme Mix(Roche)的终体积50μL的PCR反应中。在**EPPENDORF® MASTERCYCLER®**5333(Eppendorf Scientific Inc.)中进行扩增反应,程序为95℃ 3分钟的1个循环;和每个为

95℃ 30秒,55℃ 30秒和72℃ 1分钟的30个循环。循环后,反应物被在72℃孵育5分钟并随后在4℃冷却直至进一步处理。引物0613416和0613417的约700bp的PCR条带,以及引物0613418和0613419的1kbp的PCR条带被以TBE缓冲液中1%的琼脂糖凝胶电泳纯化。条带被从凝胶上切下并根据制造商的说明使用**NUCLEOSPIN®**Extract II KiT (Macherey-Nagel) 纯化。含东方伊萨酵母3-HPDH同源区的质粒骨架和ura3盒PCR产物被根据制造商说明在用IN-FUSION HD™ (Clontech Laboratories, Inc.) 的反应中组装。来自In-FUSION反应的2μL被根据制造商的说明转化进Solo Pack Gold Super Competent Cells (Stratagene)。在恢复期后,来自转化反应的两份100μL等份被涂布于添加有每mL 100μg氨苄青霉素的150mm 2X YT平板上。平板在37℃孵育过夜。从选择平板上选出推定的重组克隆并使用**BIOROBOT®**9600 (Qiagen) 从每一个制备质粒DNA。克隆通过限制性消化来分析,有正确限制性消化方式的质粒被命名为pMcTs65 (图31)。

[0728] 使用pMcTs65构建体从MeJi413缺失天然东方伊萨酵母3-HPDH

[0729] 质粒pMcTs65 (如上,见图31) 被以ApaI、Sph、KpnI消化并通过TBE缓冲液中1%的琼脂糖凝胶电泳来纯化。约2.9kbp的条带被从凝胶上切下并根据制造商的说明使用**NUCLEOSPIN®**Extract II KiT (Macherey-Nagel) 纯化。

[0730] 菌株MeJi413 (如上) 被以消化的pMcTs65DNA转化,并使用Phire Plant Direct PCR KiT (Finnzymes) 通过PCR验证正确的基因座靶向和转化。引物0613034和0613035产生约2.7kbp条带以确认缺失盒的整合;引物0611815和0611632产生约4.2kbp的条带;引物0611817和0611631产生约4.8kbp的条带以确认adh1202基因座的ADC表达盒仍然完整。给出缺失盒和ADC盒正确整合预期条带的菌株被命名为McTs229。

[0731] 随后如前所述分离McTs229的ura-衍生体。使用引物0613034和0613241通过PCR筛选到McTs229数个FOA抗性菌落有所需的环出事件。约1.4kbp条带的存在表示如所需的仅ura3残留位点的存在 (在亲本菌株中两个URA3启动子间同源重组后留下的单个URA3启动子),而约2.8kbp条带表示完整URA3启动子-URA3ORF-URA3终止子-URA3启动子盒的存在,表明所需的重组事件没有发生。约1.9kbp条带的存在表示存在于这些转化体中的野生型基因盒,因为这些是缺失的杂合。引物0611815和0611632产生约4.2kbp的条带;引物0611631和0612366产生约4.5kbp的条带以确认ADH1202基因座的ADC表达盒仍然完整。如上所述进行使用Phire Plant Direct PCR KiT (Finnzymes) 的PCR反应。一个来自亲本菌株McTs225的,具有所需环出事件的FOA抗性菌落被命名为McTs238。

[0732] 为构建编码SEQ ID NO:26的东方伊萨酵母3-HPDH的天然基因的纯合缺失体,McTs238被以消化的pMcTs65转化,并使用Phire Plant Direct PCR KiT (Finnzymes) 通过PCR验证正确的基因座靶向和转化。两组引物被用于通过PCR筛选YMR226c基因座缺失。当3-HPDH位点完整时引物0613034和0613747产生约500bp的条带以表明3-HPDH纯合缺失没有发生。此外转化体被以引物0613746和0613241来筛选,其在3-HPDH没有被缺失时产生约660bp的条带。对东方伊萨酵母3-HPDH基因座野生型阴性的转化体被以产生约2.8kbp和1.4kbp条带的引物0613034和0613241,产生约1.5kbp条带的引物0612908和0613241;产生约1kbp条带的引物0613034和0612909来进一步筛选。以产生约3kbp条带的引物0611245和0612794以及产生约3.6kbp条带的引物0611815和0612795来确认ADH1202基因座处ADC盒仍然完整。有表达盒正确整合预期条带的菌株被命名为McTs244。

[0733] 随后如前所述分离McTs244的ura-衍生体。使用产生约1.4kbp条带的引物0613034和0613241通过PCR筛选到McTs244数个FOA抗性菌落有所需的环出事件。使用产生约3kbp条带的引物0611245和0612794以及产生约3.6kbp条带的引物0611815和0612795来确认ADH1202基因座的ADC表达盒仍然完整。一个来自亲本菌株McTs244的,具有所需环出事件的FOA抗性菌落被命名为McTs259。

[0734] 使用本发明描述的方法,在生物反应器中测试菌株MIBa372和McTs244的3-HP产生。考虑到这些发酵中的细胞质量的差异,3-HP产生性能被表示为每单位细胞质量的3-HP浓度(表示为g/L 3-HP/g/L干细胞重)。菌株MIBa372和McTs259的g/L 3-HP/g/L干细胞重分别为1.66和<0.1。这些结果表明由于缺失该基因消除了3-HP产生,因此是东方伊萨酵母中的天然3-HPDH基因负责将丙二酸半醛转化为3-HP。

[0735] 实施例3A-22:在pdc基因座表达丙酮酸羧化酶(PYC),天冬氨酸氨基转移酶(AAT), β -丙氨酸氨基转移酶(BAAT),和3-羟基丙酸脱氢酶(3-HPDH);并在adh1202基因座从四个核苷酸序列表达天冬氨酸1-脱氢酶(ADC)的酵母菌株

[0736] 设计另外的构建体以在菌株中东方伊萨酵母pdc基因座掺入编码SEQ ID NO:2的东方伊萨酵母PYC的核苷酸序列SEQ ID NO:1,编码SEQ ID NO:14的东方伊萨酵母ATT的核苷酸序列SEQ ID NO:13,编码SEQ ID NO:21的克鲁维酵母BAAT的核苷酸序列SEQ ID NO:142,和编码SEQ ID NO:129的酿酒酵母3-HPDH的核苷酸序列SEQ ID NO:144,菌株也在adh1202基因座包含编码SEQ ID NO:139的地衣芽孢杆菌ADC的四个核苷酸拷贝,以与上面描述的方式类似的方式,设计左端和右端构建体以允许在东方伊萨酵母CNB1pdc基因座的同源重组。如下所述,这些构建体被制备并转化进MIBa357。

[0737] 左端片段的构建

[0738] 使用质粒pGMEr126(图16)作为模板按制造商的说明通过PCR扩增(Pfu聚合酶,Stratagene)编码SEQ ID NO:14的东方伊萨酵母ATT的核苷酸序列SEQ ID NO:13。引物oANN1和oANN2允许掺入基因编码序列侧翼独特的限制性位点。PCR产物被以TBE缓冲液中1%琼脂糖凝胶电泳纯化。约1.3kbp的条带被从凝胶上切下并根据制造商的说明使用Gel Extraction KiT(Qiagen)纯化。纯化的PCR产物被以ApaI和NruI消化并如本发明中所述被凝胶纯化。

[0739] 质粒pGMEr135(与上面pGMEr136相同,除了EN01启动子/RKI终止子插入物是相反取向)被以ApaI和NruI消化并如本发明中所述以TBE缓冲液中琼脂糖凝胶电泳纯化。约11.7kbp的条带被从凝胶上切下并根据制造商的说明使用Gel Extraction KiT(Qiagen)纯化。

[0740] 使用T4连接酶(New England Biolabs)在10 μ L总反应体积中将纯化的1.3kbpPCR产物连接进11.7kbp的pGMEr135线性化载体,10 μ L总反应体积由49.8ng载体,354ng 1.3kbp插入物,1 μ L有10mM ATP的10X连接缓冲液(New England Biolabs),和1 μ L T4连接酶(New England Biolabs)构成。反应物在室温下孵育30分钟,而2 μ L等份的反应物被根据制造商的说明转化进电感受态大肠杆菌DH10B细胞(Invitrogen)。转化体被涂布于LB+卡那霉素平板上并在37 $^{\circ}$ C下孵育过夜。使用引物oHJ2和oANN1(产生约2.3kbp条带)以及引物oANN5和oANN6(产生约877bp条带)通过菌落PCR筛选到数个有合适插入的所得转化体。PCR扩增aat片段的序列也被确认。产生正确插入和序列的克隆以ApaI和NruI消化并如本发明中所述被

凝胶纯化。约1.3kbp的条带被从凝胶上切下并根据制造商的说明使用Gel Extraction KiT (Qiagen) 纯化。

[0741] 包含编码SEQ ID NO:2的东方伊萨酵母PYC的所需核苷酸序列SEQ ID NO:1的质粒pGMEr137(如上,见图18)被以ApaI和NruI消化并如本发明中所述以TBE缓冲液中琼脂糖凝胶电泳纯化。约11.7kbp的条带被从凝胶上切下并根据制造商的说明使用Gel Extraction KiT (Qiagen) 纯化。

[0742] 使用T4连接酶(New England Biolabs)在10 μ L总反应体积中将纯化的1.3kbpPCR产物连接进11.7kbp的pGMEr137线性化载体,10 μ L总反应体积由49.4ng载体,54ng 1.3kbp插入物,1 μ L有10mM ATP的10X连接缓冲液(New England Biolabs),和1 μ L T4连接酶(New England Biolabs)构成。反应物在室温下孵育30分钟,而2 μ L等份的反应物被用于根据制造商的说明转化进电感受态大肠杆菌DH10B细胞(Invitrogen)。转化体被涂布于LB+卡那霉素平板上并在37 $^{\circ}$ C下孵育过夜。使用引物oHJ2和oANN1(产生约2.3kbp条带)以及引物oANN5和oANN6(产生约877bp条带)通过菌落PCR筛选到数个有合适插入所得转化体。产生正确插入和序列的克隆被命名为pANN02。

[0743] 具有相反朝向AAT编码序列的左端片段的构建

[0744] 质粒pANN02被以PmeI消化并如本发明中所述以TBE缓冲液中琼脂糖凝胶电泳纯化。约10.3kbp的条带和约2.7kbp的条带被从凝胶上切下并根据制造商的说明使用Gel Extraction KiT (Qiagen) 纯化。来自pANN2的10.3kbp载体片段被以CIP(New England Biolabs)去磷酸化并根据制造商的说明使用纯化试剂盒(Qiagen)纯化。使用T4连接酶(New England Biolabs)在10 μ L总反应体积中将来自pANN02的2.7kbp片段连接进来自pANN02的去磷酸化10.3kbp线性化载体,10 μ L总反应体积由36ng载体,28ng插入物,1 μ L有10mM ATP的10X连接缓冲液(New England Biolabs),和1 μ L T4连接酶(New England Biolabs)构成。反应物在室温下孵育30分钟,而2 μ L等份的反应物被根据制造商的说明转化进电感受态大肠杆菌TOP10细胞(Invitrogen)。转化体被涂布于LB+卡那霉素平板上并在37 $^{\circ}$ C下孵育过夜。使用引物oJY44和oHJ1(产生约1.3kbp条带)通过菌落PCR筛选到数个有合适插入的所得转化体。产生正确插入的克隆被命名为pANN5。

[0745] 右端片段的构建

[0746] 如下构建含两个地衣芽孢杆菌ADC编码区和东方伊萨酵母PDC基因座3'靶向侧翼DNA的右端构建体。质粒pMhCt071(与上述pMhCt077相同的质粒,除了酿酒酵母3-HPDH ORF没有为东方伊萨酵母密码子优化)被以PmeI和PacI消化,以10个单位的小牛肠磷酸酶(New England Biolabs)处理,并以TAE缓冲液中0.9%琼脂糖凝胶电泳纯化,约4.7kbp的条带被从凝胶上切下并根据制造商的说明使用NUCLEOSPIN®Extract II KiT (Macherey-Nagel) 纯化。

[0747] 质粒pMeJi312-2(如上,见图15)被以PmeI和PacI消化以提取两个地衣芽孢杆菌ADC表达盒,并以TAE缓冲液中0.9%琼脂糖凝胶电泳纯化,约2.8kbp的条带被从凝胶上切下并根据制造商的说明使用NUCLEOSPIN®Extract II KiT (Macherey-Nagel) 纯化。

[0748] 在含1X Quick连接缓冲液(New England Biolabs),1 μ L 4.7kbp pMhCt071载体片段,3 μ L来自pMeJi312-2的2.8kbp插入物,和1 μ L Quick T4DNA连接酶(New England Biolabs)的连接体系中(20 μ L),将来自pMeJi312-2的含两个地衣芽孢杆菌ADC编码区的片

段连接进线性化的pMhCt071载体片段。连接反应物在室温孵育5分钟,随后将管置于冰上。5 μ L反应物根据制造商的说明被用于转化Solo Pack Gold SuperCompetent Cells (Agilent Technologies)。转化体被涂布于2X YT+amp平板上并在37°C下孵育过夜。通过ApaLI消化筛选到数个有所需片段的正确连接的所得转化体。产生所需片段大小的克隆被保存并命名为pMhCt110。

[0749] 质粒pMhCt110被以XbaI和PacI消化,随后如本发明所述以CIP处理并以TBE缓冲液中1%琼脂糖凝胶电泳纯化。约7.1kbp的片段被从凝胶上切下并根据制造商的说明使用QIAQUICK®Gel Extraction Kit(Qiagen)纯化。

[0750] SEQ ID NO:144的密码子优化酿酒酵母3-HPDH编码序列被通过以XbaI和PacI消化从pMIBa123(如上)上切下并如本发明所述以TBE缓冲液中1%琼脂糖凝胶电泳纯化。约814bp的片段被从凝胶上切下并根据制造商的说明使用QIAQUICK®Gel Extraction Kit(Qiagen)纯化。使用T4连接酶(New England Biolabs)在10 μ L总反应体积中将814bp的纯化片段连接进来自pMhCt110的7.1kbp片段,10 μ L总反应体积由1 μ L消化的pMhCt110,1或7 μ L来自pMIBa123的814bp片段,1 μ L有10mM ATP的10X连接缓冲液(New England Biolabs),和1 μ L T4连接酶(New England Biolabs)构成。反应物在室温下孵育30分钟,而4 μ L等份的反应物被用于根据制造商的说明转化进ONE SHOT®TOP10化学感受态大肠杆菌细胞(Invitrogen)。转化体被涂布于2X YT+amp平板上并在室温下孵育过周末。通过使用XbaI和PacI以及AscI和PacI的2个酶组合的限制性消化筛选到数个有合适插入的所得转化体。从每个消化中产生正确消化条带大小的克隆被命名为pMIBa142。

[0751] 质粒pMIBa142被以AscI消化,随后进行用Klenow的补平反应和以NheI的随后消化以及CIP处理。如本发明所述消化物以TBE缓冲液中1%琼脂糖凝胶电泳来纯化。约7.5kbp的条带被从凝胶上切下并根据制造商的说明使用QIAQUICK®Gel Extraction Kit(Qiagen)来纯化。

[0752] 编码SEQ ID NO:21的克鲁维酵母BAAT的核苷酸序列SEQ ID NO:142通过以PacI的消化被从pMIBa124(如上)上切下,随后进行用Klenow的补平反应和以XbaI的随后消化。如本发明所述消化物以TBE缓冲液中1%琼脂糖凝胶电泳来纯化。约1.4kbp的条带被从凝胶上切下并根据制造商的说明使用QIAQUICK®Gel Extraction Kit(Qiagen)来纯化。

[0753] 使用T4连接酶(New England Biolabs)在10 μ L总反应体积中将来自pMIBa124的1.4kbp纯化片段连接进来自pMIBa142的7.5kbp片段,10 μ L总反应体积由1 μ L消化的pMIBa142,7 μ L来自pMIBa124的1.4kbp片段,1 μ L有10mM ATP的10X连接缓冲液(New England Biolabs),和1 μ L T4连接酶(New England Biolabs)构成。反应物在16°C下孵育2.5小时,而整个连接物被根据制造商的说明转化进Sure细胞(Agilent)。转化体被涂布于2X YT+amp平板上并在37°C下孵育过夜。通过使用StuI和PmeI的限制性消化筛选到数个有合适插入的所得转化体。产生正确消化条带大小的克隆被命名为pMIBa144。

[0754] 左端和右端片段整合进MIBa357

[0755] 如本发明中所述质粒pANN5被以NotI和NheI消化而质粒pMIBa144被以NotI消化。这些被以TBE缓冲液中1%琼脂糖凝胶电泳来纯化。来自pANN5约8.2kbp片段和来自pMIBa144的约6kbp片段被从凝胶上切下并根据制造商的说明使用NUCLEOSPIN®

Extract II KiT (Macherey-Nagel) 纯化。

[0756] 以消化的pANN5和pMIBa144DNA来转化MIBa357,并根据制造商说明使用**Phire®** Plant Direct PCR KiT (Finnzymes) 来通过PCR来验证正确的基因座靶向和转化。引物0611552和0613695产生约4.1kbp的条带(以确认pdc基因座处左半边整合);引物0612358和0611554产生约2.5kbp的条带(以确认pdc基因座处右半边整合);引物0611245和0611631产生约3.5kbp的条带(以确认左半边4XADC整合仍然在adh1202基因座);而引物0611815和0611632产生约4.6kbp的条带(以确认右半边4XADC整合仍然在adh1202基因座)。给出pdc基因座表达盒正确整合预期条带并在adh1202基因座保留表达盒的一个分离株被保存并命名为MIBa360。

[0757] 从MIBa360去除ura标志

[0758] 如上所描述分离MIBa360的ura-衍生体。使用引物0611815和0613689通过PCR筛选到来自MIBa360数个FOA抗性菌落的基因组DNA有所需环出事件。约1.9kbp条带的存在表示如所需地去除了ura标志而有ura3残留位点(在亲本菌株中两个URA3启动子间同源重组后留下的单个URA3启动子),而约3.3kbp条带表示完整URA3启动子-URA3ORF-URA3终止子-URA3启动子盒的存在,表明所需重组事件没有发生。按照制造商的说明使用**Phire®** Plant Direct PCR KiT (Finnzymes) 进行PCR。以上述引物产生约1.9kbp片段的两个FOA抗性菌落被保存并命名为MIBa363和MIBa364。

[0759] 反向表达盒右端片段的构建

[0760] 质粒pMIBa144包含所需朝正向取向前进的ADC和3-HPDH表达盒。为简化同源菌株的筛选,构建了新的质粒,其中pMIBa144的ADC表达盒被置于反向取向。质粒pMIBa144(如上)被以StuI和PmeI消化,如本发明中所述以TBE缓冲液中1%琼脂糖凝胶电泳来纯化。约6.1kbp和2.8kbp的条带被从凝胶上切下并根据制造商的说明使用**QIAQUICK®** Gel Extraction KiT (Qiagen) 来纯化。来自pMIBa144的6.1kbp片段被以CIP处理并根据制造商的说明使用**QIAQUICK®** PCR Purification KiT (Qiagen) 来纯化。

[0761] 使用T4连接酶(New England Biolabs)在10μL总反应体积中将来自pMIBa144的2.8kbp的纯化片段连接进6.1kbp的pMIBa144线性化载体,10μL总反应体积由1μL来自pMIBa144的6.1kbp片段,7μL来自pMIBa144的2.8kbp片段,1μL有10mM ATP的10X连接缓冲液(New England Biolabs),和1μL T4连接酶(New England Biolabs)构成。反应物在16℃下孵育4小时,而4μL等份的反应物被根据制造商的说明转化进ONE **SHOT®** TOP10化学感受态大肠杆菌细胞(Invitrogen)。转化体被涂布于2X YT+amp平板上并在37℃下孵育过夜。通过使用SphI和XbaI的限制性消化筛选到数个有合适插入的所得转化体。产生正确消化条带大小的克隆被命名为pMIBa146。

[0762] 将左端和右端片段整合进MIBa363

[0763] 如本发明中所述质粒pANN5被以NotI和NheI消化而质粒pMIBa146被以NotI消化。这些被以TBE缓冲液中1%琼脂糖凝胶电泳来纯化。来自pANN5约8.2kbp片段和来自pMIBa146的约6.2kbp片段被从凝胶上切下并根据制造商的说明使用**QIAQUICK®** PCR Purification KiT (Qiagen) 纯化。

[0764] 以消化的pANN5和pMIBa144DNA来转化MIBa363,并根据制造商说明使用**Phire®**

Plant Direct PCR KiT (Finnzymes) 或 Kapa Robust DNA 聚合酶来通过 PCR 来验证正确的基因座靶向和转化。为确认在 *pdc* 基因座的整合, 使用了下列引物对。引物 0613689 和 0611815 产生约 1.9kbp 的条带; 引物 0612366 和 0611554 产生约 2.5kbp 的条带; 引物 0613688 和 0611815 产生约 3.2kbp 的条带; 引物 0611622 和 0611552 产生约 945bp 的条带。为检查在 *adh1202* 基因座的整合, 使用了下列引物对。引物 0611245 和 0612794 产生约 2.8kbp 的条带; 引物 0611815 和 0612795 产生约 3.9kbp 的条带。给出在 *pdc* 基因座表达盒正确整合预期条带并在 *adh1202* 基因座保留表达盒的 2 个分离株被保存并命名为 MIBa372 和 MIBa373。

[0765] 从 MIBa372 去除 *ura* 标志物

[0766] 如上所述分离 MIBa372 的 *ura*- 衍生体。使用引物 0611815 和 0613688 通过 PCR 筛选来自 MIBa372 数个 FOA 抗性菌落的基因组 DNA 有所需环出事件。约 2.1kbp 条带的存在表示如需要地去除了 *ura* 标志而有 *ura3* 残留位点 (在亲本菌株中两个 URA3 启动子间同源重组后留下的单个 URA3 启动子), 而约 3.3kbp 条带表示完整 URA3 启动子-URA3ORF-URA3 终止子-URA3 启动子盒的存在, 表明所需重组事件没有发生。也使用确认第一染色体修饰的引物 0611815 和 0613689 (扩增 1.9kbp 片段) 和确认 *pdc* 基因座丢失的 0611552 和 0611553 (如果 *pdc* 基因座存在扩增 850bp 片段) 来通过 PCR 筛选 MIBa372 的 FOA 抗性菌落。按照制造商的说明使用 Phire® Plant Direct PCR KiT (Finnzymes) 进行 PCR。以 0611815 和 0613689 产生约 1.9kbp 片段, 但以 0613688 和 0611815 或者 0611552 和 0611553 不扩增片段的 FOA 抗性菌落被保存并命名为 MIBa375。MIBa375 的基因型显示于表 28 中。

[0767] 表 28: 转化体的基因型

菌株	基因型
MIBa375	$adh1202\Delta::(\text{PDC}_{\text{promo}}\text{-Opt.Blpand PDC}_{\text{promo}}\text{-Opt.Blpand Ura3-Scar TDH3}_{\text{promo}}\text{Opt.Blpand TDH3}_{\text{promo}}\text{Opt.Blpand})/adh1202\Delta::(\text{PDC}_{\text{promo}}\text{-Opt.Blpand PDC}_{\text{promo}}\text{-Opt.Blpand Ura3-Scar [TDH3}_{\text{promo}}\text{Opt.Blpand TDH3}_{\text{promo}}\text{Opt.Blpand)]reverse)}$ $pdc\Delta::(\text{PDC}_{\text{promo}}\text{-CNB1pyc ENO1}_{\text{promo}}\text{-CNB1aat Ura3-Scar TDH3}_{\text{promo}}\text{-OptSkPYD4 PGK1}_{\text{promo}}\text{-OptScYMR226c/ pdc}\Delta::(\text{PDC}_{\text{promo}}\text{-CNB1pyc ENO1}_{\text{promo}}\text{-CNB1aat Ura3-Scar TDH3}_{\text{promo}}\text{-OptSkPYD4 PGK1}_{\text{promo}}\text{-OptScYMR226c ura3-/ura3-}$

[0769] 实施例 3A-23: 缺失了甘油 3-磷酸脱氢酶 (GPD) 基因的酵母菌株。

[0770] 菌株 MIBa375 中 GPD 基因的缺失

[0771] 设计另外的构建体以缺失来自宿主东方伊萨酵母基因组的甘油 3-磷酸脱氢酶基因 (SEQ ID NO:117, 其编码 SEQ ID NO:118 的 GPD) 的两个拷贝, 这些构建体包含与 GPD 基因上游序列同源的约 1003bp 核苷酸序列, 和与 GPD 基因下游序列同源的约 852bp 序列, 有克隆在之间的 T_{PDC} -URA3 标志盒 (PDC 终止子-URA3 启动子-URA3 基因-URA3 终止子-URA3 启动子)。

[0772] 根据制造商的说明书使用 Pfu DNA 聚合酶从东方伊萨酵母 CNB1 基因组 DNA 扩增 GPD 的上游和下游区域。上游区域包含 *Apal* 位点; 使用重叠引物通过 PCR 消除该位点, 所述引物设计有对 *Apal* 识别序列中一个核苷酸的错配。引物对 oACN48/oACN51 以及 oACN49/oACN50 被用于扩增对上游区域的这两个重叠片段。这些 PCR 产物被在 1% 琼脂糖凝胶中分离并从中切下, 并根据制造商的说明使用 Qiaquick Gel Extraction KiT (Qiagen) 纯化, 并被用作以引物 oACN48/oACN49 (具有正向 *Apal*/反向 *Not1* 位点) 的第二轮 PCR 的模板。根据制造商的说明书使用 PfuDNA 聚合酶以引物 oACN52/oACN53 (具有正向 *Not1*/反向 *Sac1* 位点) 从东方伊萨酵

母基因组DNA扩增下游区域。两个PCR产物被凝胶纯化并分别克隆进载体pCR®-BluntII-TOPO® (Invitrogen)。分离株通过质粒DNA的限制性消化被确认具有所需插入物,并通过测序验证。载体pACN58包含克隆的上游片段而pACN59包含下游片段。质粒pACN58被以ApaI/NotI消化以释放上游侧翼,质粒pACN59被以SacI/NotI消化以释放下游侧翼,而pACN59被以ApaI/SacI消化以提供载体骨架。这三个所需的片段被在1%琼脂糖凝胶中分离,切下和纯化,并在使用T4连接酶(New England Biolabs)的3-片段连接反应中连接。连接反应物被转化进大肠杆菌TOP10电感受态细胞,并通过质粒DNA的限制性消化来确认转化体。在该过程中,检测到下游区域中另外的SacI位点,其产生853bp的下游区域(相对于1kbp)。有所需插入物的两个分离株被命名为pACN62和pACN63。

[0773] 使用NotI消化从载体pJLJ8(图32)分离 T_{PDC1} -URA3盒并凝胶纯化。该片段随后被连接进已经被以NotI消化并去磷酸化的pACN62(如上),连接物被转化进大肠杆菌DH10B电感受态细胞(Invitrogen)。使用引物oACN48/oJLJ44和oACN48/oJLJ46通过PCR确认有URA3插入物的菌落。引物oJLJ44在URA3启动子下游末端退火并从 T_{PDC1} -URA3盒向外扩增。引物oJLJ46在PDC终止子5'末端退火并从 T_{PDC1} -URA3盒向外扩增。载体pHJJ56包含面向下游GPD区域的URA3而pHJJ57包含面向上游GPD区域的URA3。

[0774] 通过以KpnI和ApaI消化来将pHJJ56和pHJJ57线性化,而含有缺失盒的片段被通过凝胶提取纯化。线性化的pHJJ56被转化进ura-菌株MIBa375。单菌落被再划线以纯化,并使用引物oJLJ44,oJLJ46,oACN54和oACN55通过PCR测试所需的GPD缺失。在40 μ L Y-裂解缓冲液和2 μ L Zymolyase (ZymoResearch)中37 $^{\circ}$ C裂解细胞30分钟,而1 μ L裂解反应物被用于25 μ L PCR反应。根据制造商的说明书PCR反应使用Failsafe DNA聚合酶和Buffer E,有55 $^{\circ}$ C的退火温度和下列循环过程:94 $^{\circ}$ C 2分钟的1个循环;每个94 $^{\circ}$ C 30秒,55 $^{\circ}$ C 30秒,和72 $^{\circ}$ C 1.5分钟的29个循环;以及72 $^{\circ}$ C 3分钟的1个循环。有一个GPD拷贝敲除的菌株产生约0.9和1.2kbp的条带并被命名为yHJN1和yHJN2。

[0775] 菌株yHJN1和yHJN2在YPD培养基中培养过夜并被置于ScD-2X FOA培养基上以选择URA3标志的缺失。单菌落被在YPD上纯化并贴到ScD-ura和YPD培养基上以确认ura-表型。使用与用来确认第一整合的PCR反应相同的反应来确认Ura-菌落已经保留了敲除。yHJN1的ura-衍生体被命名为yHJN3而yHJN2的ura-衍生体被命名为yHJN4。

[0776] 线性化的pHJJ57被转化进yHJN3和yHJN4,而单菌落被在ScD-ura培养基上纯化。在一个反应中使用引物oJLJ44,oACN54和oACN55,在第二反应中使用引物oJLJ46,oACN54和oACN55通过PCR确认两个GPD拷贝敲除的存在。引物oACN54在GPD上游侧翼序列上游约37bp区域上退火,而oACN55退火至下游侧翼下游约24bp区域。如果两个GPD拷贝被缺失则前面的反应产生约900和1050bp的条带,而后面的反应产生约1025和1200bp的条带。在ScD-ura平板上,有两个GPD拷贝敲除的菌落比有单个拷贝缺失的那些生长更慢。有两个GPD基因拷贝缺失的菌株被命名为yHJN7(源自yHJN3)和yHJN8(源自yHJN4)。

[0777] 使用本发明中描述的方法,在生物反应器中测试菌株MIBa372,yHJN7和yHJN8的甘油生产。菌株MIBa372在48小时中产生29.5g/L甘油。发酵期间菌株yHJN7或yHJN8没有产生可检测的甘油。最终发酵液中甘油的缺失可以为从发酵液中回收和纯化3-HP提供优势。

[0778] 实施例3A-24:在adh1202基因座从四个核苷酸序列表达天冬氨酸1-脱羧酶(ADC),在adh9091基因座表达3-HP脱氢酶(3-HPDH)并缺失了天然3-HP脱氢酶(3-HPDH)的酵母菌株

[0779] 在adh9091基因座整合东方伊萨酵母3-HPDH的质粒构建

[0780] 使用引物0611954和0611957 (对3-HPDH) 或0611997和0611998 (PYD4) 从如前所述制备的MBin500东方伊萨酵母基因组DNA扩增编码SEQ ID NO:26的东方伊萨酵母3-HPDH的SEQ ID NO:25以及编码SEQ ID NO:20的东方伊萨酵母BAAT (PYD4) 的SEQ ID NO:19核苷酸序列。扩增期间引物0611954向3-HPDH 5' 末端加入kozak序列 (TAAA) 和NheI位点, 而引物0611957向3-HPDH 3' 端加入PacI位点。扩增期间引物0611997向PYD4 5' 末端加入kozak序列 (TAAA) 和PacI位点, 而引物0611998向PYD4的3' 端加入PacI位点。每种引物50pmol被用于含50ng作为模板的MBin500基因组DNA, dATP、dGTP、dCTP和dTTP每种0.2mM, 1X Expand High Fidelity Buffer (Roche), 2.6单位Expand High Fidelity DNA聚合酶 (Roche) 的终体积50 μ L的PCR反应中。在EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) 中进行扩增反应, 程序为95°C 3分钟的1个循环; 随后每个为95°C 1分钟, 55°C 1分钟和72°C 1分钟 (3-HPDH PCR) 或2分钟 (PYD4PCR) 的30个循环, 及72°C 5分钟的最终延伸。热循环后, PCR反应产物被以TAE缓冲液中1.0%的琼脂糖凝胶电泳分离, 其中约831bp的3-HPDH或1.4kbp的PYD4 PCR产物被从凝胶上切下并根据制造商的说明使用QIAQUICK® Gel Extraction Kit (Qiagen) 纯化。5 μ L纯化的3-HPDH或PYD4被根据制造商的说明使用TOP0-TA Cloning Kit克隆进pCR2.1 (Invitrogen)。转化物被涂布于2X YT+amp平板上并在37°C下孵育过夜。通过EcoRI消化筛选到数个所得转化体。产生所需插入大小的克隆被通过DNA测序来确认正确。一个含3-HPDH的克隆被命名为pMBin190, 而另一个含PYD4的被命名为pMBin193。

[0781] 质粒pMBin193被以XbaI和PacI消化并在TAE缓冲液中1.0%的琼脂糖凝胶上跑电泳, 其中约1.4kbp PYD4条带被从凝胶上切下并根据制造商的说明使用QIAQUICK® Gel Extraction Kit (Qiagen) 纯化。消化的PYD4片段被使用T4DNA连接酶连接进XbaI和PacI限制性消化的线性pMIBa107质粒 (如上)。连接产物被根据制造商的说明转化进OneShot® TOP10化学感受态大肠杆菌细胞 (Invitrogen)。转化体被涂布于2X YT+amp平板上并在37°C下孵育过夜。通过SnaBI或EcoRI消化筛选到数个的所得转化体。产生所需条带大小的一个克隆被命名为pMBin203。质粒pMBin203含在下列表达盒侧翼的NotI位点: PYD4CDS上游或下游的PDC启动子和终止子, URA3启动子, URA3ORF, 和随后有URA3启动子的URA3终止子。

[0782] 质粒pMBin203被以NotI消化并在TAE缓冲液中1.0%的琼脂糖凝胶上分离, 其中约4.1kbp的片段 (含PDC启动子, PYD4CDS, PDC终止子, 和URA3标志选择标志物) 被从凝胶上切下并根据制造商的说明使用QIAQUICK® Gel Extraction Kit (Qiagen) 纯化。质粒pHJJ27 (含与adh9091基因座同源的5' 和3' 区域; 见图21) 被以NotI消化, 如上所述以CIP处理并在TAE缓冲液中1%琼脂糖凝胶上分离, 其中约5.7kbp的线性质粒被纯化。如上所述来自pMBin203的片段随后被使用T4DNA连接酶连接进NotI限制性消化的pHJJ27。连接产物被根据制造商的说明转化进OneShot® TOP10化学感受态大肠杆菌细胞 (Invitrogen)。转化体被涂布于2X YT+amp平板上并在37°C下孵育过夜。通过PstI消化筛选到数个所得转化体。产生所需条带大小的一个克隆被命名为pMBin204。质粒pMBin204允许PYD4靶向到adh9091基因座。

[0783] 如本发明中所述, 通过NheI和PacI消化编码SEQ ID NO:26的东方伊萨酵母3-HPDH

的SEQ ID NO:25核苷酸序列被从质粒pMBin190(如上)去除,并通过TBE缓冲液中琼脂糖凝胶电泳纯化。约827bp的条带被从凝胶上切下并根据制造商的说明使用**NUCLEOSPIN®** Extract II KiT (Macherey-Nagel) 纯化。质粒pMBin204(如上)被以XbaI和PacI消化并如本发明中所述通过TBE缓冲液中琼脂糖凝胶电泳纯化。约8.4kbp的条带被从凝胶上切下并根据制造商的说明使用**NUCLEOSPIN®** Extract II KiT (Macherey-Nagel) 纯化。

[0784] 使用T4连接酶(New England Biolabs)在20μL总反应体积中将上面纯化的约827bp东方伊萨酵母3-HPDH基因产物连接进8.4kbp的pMBin204线性化载体,20μL总反应体积由1μL8.4kbp载体,10μL827bp插入物,有10mM ATP的2μL10X连接缓冲液(New England Biolabs)和1μL T4连接酶(New England Biolabs)构成。反应物在16℃孵育18小时,10μL等份的反应物被根据制造商的说明转化进One Shot TOP10细胞(Invitrogen)。在恢复期后,来自转化反应的两份100μL等份被涂布于添加有每mL 100μg氨苄青霉素的150mm 2X YT平板上。平板在37℃孵育过夜。从选择平板上选出推定的重组克隆并使用**BIOROBOT®** 9600 (Qiagen) 从每一个制备质粒DNA。克隆通过限制性消化来分析,有正确限制性消化方式的质粒被命名为pMcTs90。

[0785] 在adh9091基因座整合酿酒酵母3-HPDH的质粒构建

[0786] 编码SEQ ID NO:129的酿酒酵母3-HPDH的826bp野生型核苷酸序列被从JGI69基因组DNA PCR扩增,并在基因5'端加上XbaI位点和在基因3'端加上PacI位点。扩增反应按照制造商的说明使用**Platinum®**Pfx DNA聚合酶(Invitrogen)进行。Master PCR反应含1.125μL酿酒酵母基因组DNA,引物611191和611199每种112.5pM,1X Pfx扩增缓冲液(Invitrogen),2mmMgSO₄,0.2mM dNTP混合物,5个单位的**Platinum®**Pfx DNA聚合酶(Invitrogen),终体积200μL。混合物被等分到8个管中并进行梯度PCR。扩增反应物在**EPPENDORF® MASTERCYCLER®** (Eppendorf Scientific Inc.)中孵育,程序为95℃ 2分钟的1个循环;每个为95℃ 30秒,40-55℃梯度30秒,和72℃ 2分钟的30个循环;和72℃ 3分钟的1个循环。

[0787] 如本发明所述826bp野生型YMR226c PCR基因产物被通过TBE缓冲液中1%的琼脂糖凝胶电泳纯化。约826bp片段被从凝胶上切下并使用**QIAQUICK®** Gel Extraction KiT (Qiagen) 从凝胶中提取。PCR产物被以XbaI和PacI在37℃消化过夜并随后使用**QIAQUICK®** PCR Purification KiT (Qiagen) 纯化。

[0788] 质粒pMIBa100(如上)被以XbaI和PacI消化随后以CIP处理,得到约6.8kbp的线性片段。消化物按照制造商的说明使用**QIAQUICK®** PCR Purification KiT (Qiagen) 纯化。

[0789] 使用T4连接酶(New England Biolabs)在10μL总反应体积中将826bp YMR226c纯化并消化的PCR片段连接进6.8kbp pMIBa100线性化载体,10μL总反应体积由1μL来自pMIBa100的6.7kbp片段,1或7μL 826bp YMR226c PCR产物,1μL有10mM ATP的10X连接缓冲液(New England Biolabs),和1μL T4连接酶(New England Biolabs)构成。反应物在16℃下孵育约4小时,而整个反应物被根据制造商的说明转化进Sure化学感受态细胞(Agilent)。转化体被涂布于2X YT+amp平板上并在37℃下孵育过夜。通过使用XbaI和PacI的限制性消化筛选到数个有合适插入的所得转化体。产生正确消化条带大小和DNA序列的

克隆被命名为pMIBa101。

[0790] 质粒pHJJ76-no ura (如上) 被以NotI消化随后以CIP处理。线性5.2kbp片段使用**QIAQUICK®**PCR Purification KiT (Qiagen) 纯化。

[0791] YMR226c表达盒通过以NotI消化被从pMIBa101上切下。约3546bp的条带被从凝胶上切下并按照制造商的说明使用**QIAQUICK®**PCR Purification KiT (Qiagen) 纯化。

[0792] 使用T4连接酶 (New England Biolabs) 在10 μ L总反应体积中将来自pMIBa101的3546bp纯化片段连接进5.2kbp pHJJ76-no ura线性化载体, 10 μ L总反应体积由1 μ L来自pHJJ76-no ura的5.2kbp片段, 1或5 μ L来自pMIBa101的3546bp片段, 1 μ L有10mM ATP的10X连接缓冲液 (New England Biolabs), 和1 μ L T4连接酶 (New England Biolabs) 构成。反应物在16 $^{\circ}$ C下孵育过夜, 而约4 μ L等份的反应物被根据制造商的说明转化进**ONE SHOT®**TOP10化学感受态大肠杆菌细胞 (Invitrogen)。转化体被涂布于2X YT+amp平板上并在37 $^{\circ}$ C下孵育过夜。通过使用XbaI和KpnI的限制性消化筛选到数个有合适插入的所得转化体。产生正确消化条带大小的克隆被命名为pMIBa109。

[0793] 编码SEQ ID NO:129的酿酒酵母3-HPDH的野生型核苷酸序列被通过XbaI和PacI消化被从质粒pMIBa109中去除, 并如本发明所述在TBE缓冲液中通过琼脂糖凝胶电泳纯化。约818bp的条带被从凝胶上切下并根据制造商的说明使用**NUCLEOSPIN®**Extract II KiT (Macherey-Nagel) 纯化。

[0794] 使用T4连接酶 (New England Biolabs) 在20 μ L总反应体积中将纯化的约818bp酿酒酵母3-HPDH基因产物连接进上面8.4kbp的pMBin204线性化载体, 20 μ L总反应体积由1 μ L 8.4kbp载体, 10 μ L 818bp插入物, 2 μ L有10mM ATP的10X连接缓冲液 (New England Biolabs) 和1 μ L T4连接酶 (New England Biolabs) 构成。反应物在16 $^{\circ}$ C孵育18小时, 10 μ L等份的反应物被根据制造商的说明转化进One Shot TOP10细胞 (Invitrogen)。在恢复期后, 来自转化反应的两份100 μ L等份被涂布于添加有每mL 100 μ g氨苄青霉素的150mm 2X YT平板上。平板在37 $^{\circ}$ C孵育过夜。从选择平板上选出推定的重组克隆并使用**BIOROBOT®**9600 (Qiagen) 从每一个制备质粒DNA。克隆通过限制性消化来分析, 有正确限制性消化方式的质粒被命名为pMcTs91。

[0795] 在adh9091基因座整合M.sedula 3-HPDH的质粒构建

[0796] 编码SEQ ID NO:29的M.sedula 3-HPDH的SEQ ID NO:343东方伊萨酵母密码子优化核苷酸序列由**GeneArt®**合成, 得到质粒11AAE2AP。合成的基因被以XbaI和PacI从质粒中消化出来并如本发明所述通过TBE缓冲液中的琼脂糖凝胶电泳纯化。约959bp的条带被从凝胶上切下并根据制造商的说明使用**NUCLEOSPIN®**Extract II KiT (Macherey-Nagel) 纯化。

[0797] 使用T4连接酶 (New England Biolabs) 在20 μ L总反应体积中将纯化的约956bp M.sedula 3-HPDH基因产物连接进上面8.4kbp的pMBin204线性化载体, 20 μ L总反应体积由1 μ L 8.4kbp载体, 16 μ L 956bp插入物, 2 μ L有10mM ATP的10X连接缓冲液 (New England Biolabs) 和1 μ L T4连接酶 (New England Biolabs) 构成。反应物在室温下孵育1小时, 10 μ L等份的反应物被根据制造商的说明转化进Solo Pack Gold Super Competent Cells (Invitrogen)。在恢复期后, 来自转化反应的两份100 μ L等份被涂布于添加有每mL 100 μ g氨

苜青霉素的150mm 2X YT平板上。平板在37℃孵育过夜。从选择平板上选出推定的重组克隆并使用**BIOROBOT®**9600 (Qiagen) 从每一个制备质粒DNA。克隆通过限制性消化来分析, 有正确限制性消化方式的质粒被命名为pMcTs76。

[0798] 在adh9091基因座整合大肠杆菌3-HPDH的质粒构建

[0799] 编码SEQ ID NO:27的大肠杆菌3-HPDH的SEQ ID NO:143东方伊萨酵母密码子优化核苷酸序列由**GeneArt®**合成, 得到质粒1045168。合成的基因被以XbaI和PacI从质粒中消化出来并如本发明所述通过TBE中的琼脂糖凝胶电泳纯化。约761bp的条带被从凝胶上切下并根据制造商的说明使用**NUCLEOSPIN®**Extract II KiT (Macherey-Nagel) 纯化。

[0800] 使用T4连接酶 (New England Biolabs) 在20μL总反应体积中将纯化的约761bp大肠杆菌3-HPDH基因产物连接进上面8.4kbp的pMBin204线性化载体, 20μL总反应体积由1μL 8.4kbp载体, 16μL 761bp插入物, 2μL有10mM ATP的10X连接缓冲液 (New England Biolabs) 和1μL T4连接酶 (New England Biolabs) 组成。反应物在室温下孵育1小时, 10μL等份的反应物被根据制造商的说明转化进Solo Pack Gold Super Competent Cells (InvitrogenAgilent)。在恢复期后, 来自转化反应的两份100μL等份被涂布于添加有每mL 100μg氨苄青霉素的150mm 2X YT平板上。平板在37℃孵育过夜。从选择平板上选出推定的重组克隆并使用**BIOROBOT®**9600 (Qiagen) 从每一个制备质粒DNA。克隆通过限制性消化来分析, 有正确限制性消化方式的质粒被命名为pMcTs77。

[0801] 在McTs259中在adh9091整合3-HPDH片段

[0802] 如本发明中所述质粒pMcTs76、pMcTs77、pMcTs91被以KpnI和ApaI消化, 而质粒pMcTs90被以SacI和ApaI消化。得到的消化产物被通过TBE缓冲液中1%琼脂糖凝胶电泳纯化, 而来自pMcTs76的5.5kbp片段, 来自pMcTs77的5.3kbp片段, 来自pMcTs90的5.4kbp片段和来自pMcTs91的5.4kbp片段被从凝胶上切下并根据制造商的说明使用**NUCLEOSPIN®**Extract II KiT (Macherey-Nagel) 纯化。

[0803] 在adh1202基因座表达编码SEQ ID NO:139的地衣芽孢杆菌ADC的四个核苷酸拷贝并缺失了编码SEQ ID NO:26的3-HPDH (如上) 的天然东方伊萨酵母基因的菌株McTs259, 被以消化的pMcTs76、pMcTs77、pMcTs90或pMcTs91DNA转化。按照制造商的说明使用**Phire®** Plant Direct PCR KiT (Finnzymes) 来通过PCR来验证正确的基因座靶向和转化。为确认在adh9091基因座的整合, 使用下列引物对。引物0614627和0612909对来自pMcTs76的整合片段产生约3.47kbp条带, 对来自pMcTs77的整合片段产生约3.27kbp条带, 对来自pMcTs90的整合片段产生约3.34kbp条带, 对来自pMcTs91的整合片段产生约3.33kbp条带; 引物0612908和0614626产生约1.97kbp条带。为检查在adh1202的整合, 使用了下列引物对。引物0611245和0612794产生约3.0kbp条带而引物0611815和0612795产生约3.6kbp的条带。为检查天然东方伊萨酵母3-HPDH基因的缺失, 使用了下列引物。引物0613034和0613241产生约1.4kbp条带。如表29中所示, 给出在adh9091基因座表达盒正确整合预期条带, 在adh1202基因座保留表达盒并保留东方伊萨酵母3-HPDH位点缺失的分离株被保存并命名为McTs261 (pMcTs76片段), McTs263 (pMcTs77片段), McTs267 (pMcTs90片段) 和McTs269 (pMcTs91片段)。

[0804] 表29: 转化体构建

[0805]

构建体质粒	基因	基因来源	基因产物 SEQ ID NO	整合构建体	转化株
pMbin190	3-HPDH (YMR226c)	东方伊萨酵母	26	pMcTs90	McTs267
11AAE2AP	3-HPDH (Msed_1993)	勤奋金属球菌	29	pMcTs76	McTs261
pMiBa109	3-HPDH (YMR226c)	酿酒酵母	129	pMcTs91	McTs269
1045168	3-HPDH (ydfG)	大肠杆菌	27	pMcTs77	McTs263

[0806] 使用上述摇瓶方法测试转化菌株的3-HP产生。杂合转化体McTs267、McTs269和McTs263分别产生0.149 (+/-0.024), 0.168 (+/-0.052), 0.162 (+/-0.018) g/L 3-HP每g/L干细胞重。天然菌株MeJi412产生0.263 (+/-0.026) g/L 3-HP每g/L干细胞重, 而3-HPDH缺失菌株不产生可检测的3-HP。本试验中杂合转化体McTs261不产生可检测的3-HP。这些结果显示使用外源或内源3-HPDH基因序列, 甚至杂合的3-HPDH转化体也可以恢复一些3-HPDH缺失菌株的3-HPDH活性。

[0807] 实施例3B: 表达苹果酸途径基因的修饰的酵母菌株

[0808] 通过利用PEP、OAA和苹果酸中间体的途径来产生3-HP的酵母细胞被工程改造以表达一个或多个涉及该途径中的酶。表达的基因可以包括一个或多个PPC, 苹果酸脱氢酶和苹果酸脱羧酶基因。表达的基因可以源自对宿主细胞是天然的基因, 或者其可以源自对宿主细胞非天然的来源基因。

[0809] 实施例3C: 表达丙二酸半醛途径基因的修饰的酵母菌株

[0810] 通过利用PEP、OAA和丙二酸半醛中间体的途径来产生3-HP的酵母细胞被工程改造以表达一个或多个涉及该途径中的酶。表达的基因可包括一个或多个PPC, 2-酮酸脱羧酶, KGD, BCKA, 吡哆丙酮酸脱羧酶, 3-HPDH (包括丙二酸半醛还原酶), HIBADH和4-羟基丁酸脱氢酶基因。表达的基因可源自对宿主细胞是天然的基因, 或者其可源自对宿主细胞非天然的来源基因。

[0811] 实施例3D: 表达丙二酰CoA途径基因的修饰的酵母菌株

[0812] 通过利用PEP、OAA, 丙二酰CoA, 和可选的, 丙二酸半醛中间体的途径来产生3-HP的酵母细胞被工程改造以表达一个或多个涉及该途径中的酶。表达的基因可以包括一个或多个PPC, OAA甲酸裂解酶, 丙二酰CoA还原酶, CoA酰化丙二酸半醛脱氢酶, 3-HPDH (包括丙二酸半醛还原酶), HIBADH和4-羟基丁酸脱氢酶基因。表达的基因可以源自对宿主细胞是天然的基因, 或者其可以源自对宿主细胞非天然的来源基因。

[0813] 实施例3E: 表达丙二酰CoA途径基因的修饰的酵母菌株

[0814] 通过利用丙酮酸, 乙酰CoA, 丙二酰CoA, 和可选的, 丙二酸半醛中间体的途径来产生3-HP的酵母细胞被工程改造以表达一个或多个涉及该途径中的酶。表达的基因可以包括一个或多个PDH, 乙酰CoA羧化酶, 丙二酰CoA还原酶, CoA酰化丙二酸半醛脱氢酶, 3-HPDH (包括丙二酸半醛还原酶), HIBADH和4-羟基丁酸脱氢酶基因。表达的基因可以源自对宿主细胞是天然的基因, 或者其可以源自对宿主细胞非天然的来源基因。

[0815] 实施例3F: 表达丙氨酸途径基因的修饰的酵母菌株

[0816] 通过利用丙酮酸, 丙氨酸, β -丙氨酸, 和可选的, 丙二酸半醛, β -丙氨酰CoA, 丙烯酰CoA以及3-HP-CoA中间体的途径来产生3-HP的酵母细胞被工程改造以表达一个或多个涉及

该途径中的酶。表达的基因可以包括一个或多个丙氨酸脱氢酶,丙酮酸/丙氨酸氨基转移酶,丙氨酸2,3氨基变位酶,CoA转移酶,CoA合成酶, β -丙氨酰CoA氨解酶,3-HP-CoA脱水酶,3-HP-CoA水解酶,3-羟异丁酰CoA水解酶,BAAT,3-HPDH(包括丙二酸半醛还原酶),HIBADH和4-羟基丁酸脱氢酶基因。表达的基因可以源自对宿主细胞是天然的基因,或者其可以源自对宿主细胞非天然的来源基因。

[0817] 实施例3G:表达乳酸途径基因的修饰的酵母菌株

[0818] 通过利用丙酮酸,乳酸,乳酰CoA,丙烯酰CoA和3-HP-CoA中间体的途径来产生3-HP的酵母细胞被工程改造以表达一个或多个涉及该途径中的酶。表达的基因可以包括一个或多个LDH,CoA转移酶,CoA合成酶,乳酰CoA脱水酶,3-HP-CoA脱水酶,3-HP-CoA水解酶和3-羟异丁酰CoA水解酶基因。表达的基因可以源自对宿主细胞是天然的基因,或者其可以源自对宿主细胞非天然的来源基因。

[0819] 实施例3H:表达甘油途径基因的修饰的酵母菌株

[0820] 通过利用甘油和3-HPA中间体的途径来产生3-HP的酵母细胞被工程改造以表达一个或多个涉及该途径中的酶。表达的基因可以包括一个或多个甘油脱水酶和醛脱氢酶基因。表达的基因可以源自对宿主细胞是天然的基因,或者其可以源自对宿主细胞非天然的来源基因。

[0821] 实施例3I:表达 β -丙氨酰CoA途径基因的修饰的酵母菌株

[0822] 通过利用PEP或丙酮酸, β -丙氨酸, β -丙氨酰CoA,丙烯酰CoA,3-HP-CoA和可选的OAA,天冬氨酸和丙氨酸中间体的途径来产生3-HP的酵母细胞被工程改造以表达一个或多个涉及该途径中的酶。表达的基因可以包括一个或多个PPC,PYC,AAT,ADC,CoA转移酶,CoA合成酶, β -丙氨酰CoA氨解酶,3-HP-CoA脱水酶,3-HP-CoA水解酶,3-羟异丁酰CoA水解酶,丙氨酸脱氢酶,丙酮酸/丙氨酸氨基转移酶和AAM基因。表达的基因可以源自对宿主细胞是天然的基因,或者其可以源自对宿主细胞非天然的来源基因。

[0823] 在某些方面中,本发明使用的酵母细胞或方法可由下面编号的段落描述:

[0824] [B1]包含活性3-HP发酵途径的经遗传修饰的酵母细胞,其中细胞包含选自:

[0825] 外源PPC基因;

[0826] 外源PYC基因;

[0827] 外源AAT基因;

[0828] 外源ADC基因;

[0829] 外源BAAT或gabT基因;和

[0830] 外源3-HPDH基因

[0831] 的一种或多种外源3-HP途径基因。

[0832] [B2]段落B1的经遗传修饰的酵母细胞,其包含外源AAT基因。

[0833] [B3]段落B1或B2的经遗传修饰的酵母细胞,其包含外源PYC基因。

[0834] [B4]段落B1-B3任一项的经遗传修饰的酵母细胞,其包含外源ADC基因。

[0835] [B5]段落B1-B4任一项的经遗传修饰的酵母细胞,其包含外源BAAT或gabT基因。

[0836] [B6]段落B1-B5任一项的经遗传修饰的酵母细胞,其包含外源3-HPDH基因。

[0837] [B7]段落B1的经遗传修饰的酵母细胞,其包含:

[0838] 外源PYC基因;

- [0839] 外源AAT基因；
- [0840] 外源ADC基因；
- [0841] 外源BAAT或gabT基因；和
- [0842] 外源3-HPDH基因。
- [0843] [B8]段落B1-B7任一项的经遗传修饰的酵母细胞，其包含外源PPC基因。
- [0844] [B9]段落B1-B7任一项的经遗传修饰的酵母细胞，其中外源PYC基因编码与选自SEQ ID NO:2、3、4、5、6、7和8的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。
- [0845] [B10]段落B9的经遗传修饰的酵母细胞，其中外源PYC基因编码与选自SEQ ID NO:2的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。
- [0846] [B11]段落B9的经遗传修饰的酵母细胞，其中外源PYC基因编码与SEQ ID NO:3的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。
- [0847] [B12]段落B9的经遗传修饰的酵母细胞，其中外源PYC基因编码与SEQ ID NO:4的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。
- [0848] [B13]段落B9的经遗传修饰的酵母细胞，其中外源PYC基因编码与SEQ ID NO:5的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。
- [0849] [B14]段落B9的经遗传修饰的酵母细胞，其中外源PYC基因编码与SEQ ID NO:6的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。
- [0850] [B15]段落B9的经遗传修饰的酵母细胞，其中外源PYC基因编码与SEQ ID NO:7的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。
- [0851] [B16]段落B9的经遗传修饰的酵母细胞，其中外源PYC基因编码与SEQ ID NO:8的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。
- [0852] [B17]段落B1-B16任一项的经遗传修饰的酵母细胞，其中外源PYC基因包含与SEQ ID NO:1的核苷酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的核苷酸序列。
- [0853] [B18]段落B1-B17任一项的经遗传修饰的酵母细胞，其中AAT基因编码与选自SEQ ID NO:14、15和16的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。
- [0854] [B19]段落B18的经遗传修饰的酵母细胞，其中AAT基因编码与SEQ ID NO:14的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。
- [0855] [B20]段落B18的经遗传修饰的酵母细胞，其中AAT基因编码与SEQ ID NO:15的氨

基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。

[0856] [B21]段落B18的经遗传修饰的酵母细胞，其中AAT基因编码与SEQ ID NO:16的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。

[0857] [B22]段落B1-B21任一项的经遗传修饰的酵母细胞，其中AAT基因包含与SEQ ID NO:13的核苷酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的核苷酸序列。

[0858] [B23]段落B1-B22任一项的经遗传修饰的酵母细胞，其中ADC基因编码与选自SEQ ID NO:17、18、133、135、137和139的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。

[0859] [B24]段落B23的经遗传修饰的酵母细胞，其中ADC基因编码与SEQ ID NO:17的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。

[0860] [B25]段落B23的经遗传修饰的酵母细胞，其中ADC基因编码与SEQ ID NO:18的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。

[0861] [B26]段落B23的经遗传修饰的酵母细胞，其中ADC基因编码与SEQ ID NO:133的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。

[0862] [B27]段落B23的经遗传修饰的酵母细胞，其中ADC基因编码与SEQ ID NO:135的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。

[0863] [B28]段落B23的经遗传修饰的酵母细胞，其中ADC基因编码与SEQ ID NO:137的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。

[0864] [B29]段落B23的经遗传修饰的酵母细胞，其中ADC基因编码与SEQ ID NO:139的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。

[0865] [B30]段落B1-B29任一项的经遗传修饰的酵母细胞，其中ADC基因包含与选自SEQ ID NO:130、131、132、134、136和138的核苷酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的核苷酸序列。

[0866] [B31]段落B30的经遗传修饰的酵母细胞，其中ADC基因包含与SEQ ID NO:130的核苷酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的核苷酸序列。

[0867] [B32]段落B30的经遗传修饰的酵母细胞，其中ADC基因包含与SEQ ID NO:131的核苷酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的核苷酸序列。

[0868] [B33]段落B30的经遗传修饰的酵母细胞，其中ADC基因包含与SEQ ID NO:132的核

苷酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的核苷酸序列。

[0869] [B34]段落B30的经遗传修饰的酵母细胞，其中ADC基因包含与SEQ ID NO:134的核苷酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的核苷酸序列。

[0870] [B35]段落B30的经遗传修饰的酵母细胞，其中ADC基因包含与SEQ ID NO:136的核苷酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的核苷酸序列。

[0871] [B36]段落B30的经遗传修饰的酵母细胞，其中ADC基因包含与SEQ ID NO:138的核苷酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的核苷酸序列。

[0872] [B37]段落B1-B36任一项的经遗传修饰的酵母细胞，其中外源BAAT或gabT基因编码与选自SEQ ID NO:20、21、22、23和24的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。

[0873] [B38]段落B37的经遗传修饰的酵母细胞，其中外源BAAT或gabT基因编码与SEQ ID NO:20的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。

[0874] [B39]段落B37的经遗传修饰的酵母细胞，其中外源BAAT或gabT基因编码与SEQ ID NO:21的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。

[0875] [B40]段落B37的经遗传修饰的酵母细胞，其中外源BAAT或gabT基因编码与SEQ ID NO:22的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。

[0876] [B41]段落B37的经遗传修饰的酵母细胞，其中外源BAAT或gabT基因编码与SEQ ID NO:23的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。

[0877] [B42]段落B37的经遗传修饰的酵母细胞，其中外源BAAT或gabT基因编码与SEQ ID NO:24的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。

[0878] [B43]段落B1-B42任一项的经遗传修饰的酵母细胞，其中BAAT或gabT基因包含与选自SEQ ID NO:19、140、141和142的核苷酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的核苷酸序列。

[0879] [B44]段落B43的经遗传修饰的酵母细胞，其中BAAT基因或gabT基因包含与SEQ ID NO:19的核苷酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的核苷酸序列。

[0880] [B45]段落B43的经遗传修饰的酵母细胞，其中BAAT基因或gabT基因包含与SEQ ID NO:140的核苷酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的核苷酸序列。

[0881] [B46]段落B43的经遗传修饰的酵母细胞，其中BAAT基因或gabT基因包含与SEQ ID

N0:141的核苷酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的核苷酸序列。

[0882] [B47]段落B43的经遗传修饰的酵母细胞，其中BAAT基因或gabT基因包含与SEQ ID N0:142的核苷酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的核苷酸序列。

[0883] [B48]段落B1-B47任一项的经遗传修饰的酵母细胞，其中所述外源BAAT基因或外源gabT为也是gabT基因的BAAT基因。

[0884] [B49]段落B1-B48任一项的经遗传修饰的酵母细胞，其中3-HPDH基因编码与选自由SEQ ID N0:26、27、28、29、30、31、32、33、34和129组成的组的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。

[0885] [B50]段落B49的经遗传修饰的酵母细胞，其中3-HPDH基因编码与SEQ ID N0:26的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。

[0886] [B51]段落B49的经遗传修饰的酵母细胞，其中3-HPDH基因编码与SEQ ID N0:27的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。

[0887] [B52]段落B49的经遗传修饰的酵母细胞，其中3-HPDH基因编码与SEQ ID N0:28的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。

[0888] [B53]段落B49的经遗传修饰的酵母细胞，其中3-HPDH基因编码与SEQ ID N0:29的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。

[0889] [B54]段落B49的经遗传修饰的酵母细胞，其中3-HPDH基因编码与SEQ ID N0:30的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。

[0890] [B55]段落的B49经遗传修饰的酵母细胞，其中3-HPDH基因编码与SEQ ID N0:31的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。

[0891] [B56]段落B49的经遗传修饰的酵母细胞，其中3-HPDH基因编码与SEQ ID N0:32的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。

[0892] [B57]段落B49的经遗传修饰的酵母细胞，其中3-HPDH基因编码与SEQ ID N0:33的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。

[0893] [B58]段落B49的经遗传修饰的酵母细胞，其中3-HPDH基因编码与SEQ ID N0:34的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。

[0894] [B59]段落B49的经遗传修饰的酵母细胞，其中3-HPDH基因编码与SEQ ID N0:129

的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。

[0895] [B60]段落B1-B59任一项的经遗传修饰的酵母细胞，其中3-HPDH基因包含与选自SEQ ID NO:25、143、144和343的核苷酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的核苷酸序列。

[0896] [B61]段落B60的经遗传修饰的酵母细胞，其中3-HPDH基因包含与SEQ ID NO:25的核苷酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的核苷酸序列。

[0897] [B62]段落B60的经遗传修饰的酵母细胞，其中3-HPDH基因包含与SEQ ID NO:143的核苷酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的核苷酸序列。

[0898] [B63]段落B60的经遗传修饰的酵母细胞，其中3-HPDH基因包含与SEQ ID NO:144的核苷酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的核苷酸序列。

[0899] [B64]段落B60的经遗传修饰的酵母细胞，其中3-HPDH基因包含与SEQ ID NO:343的核苷酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的核苷酸序列。

[0900] [B65]段落B1-B64任一项的经遗传修饰的酵母细胞，其中3-HPDH基因也是HIBADH基因。

[0901] [B66]段落B1-B65任一项的经遗传修饰的酵母细胞，其中3-HPDH基因也是4-羟基丁酸脱氢酶基因。

[0902] [B67]段落B1-B66任一项的经遗传修饰的酵母细胞，其中PPC基因编码与选自SEQ ID NO:10、11和12的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。

[0903] [B68]段落B67的经遗传修饰的酵母细胞，其中PPC基因编码与SEQ ID NO:10的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。

[0904] [B69]段落B67的经遗传修饰的酵母细胞，其中PPC基因编码与SEQ ID NO:11的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。

[0905] [B70]段落B67的经遗传修饰的酵母细胞，其中PPC基因编码与SEQ ID NO:12的氨基酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的多肽。

[0906] [B71]段落B1-B70任一项的经遗传修饰的酵母细胞，其中PPC基因包含与SEQ ID NO:9的核苷酸序列具有至少60%，如至少65%、70%、75%、80%、85%、90%、95%、97%、98%、99%或100%序列同一性的核苷酸序列。

[0907] [B72]段落B1-B71任一项的经遗传修饰的酵母细胞，其中所述酵母细胞是Crabtree阴性的。

[0908] [B73]段落B1-B72任一项的经遗传修饰的酵母细胞，其中酵母细胞属于选自伊萨

酵母、假丝酵母、克鲁维酵母属、毕赤酵母、裂殖酵母、有孢圆酵母属、接合酵母属和酵母属的属。

[0909] [B74]段落B73的经遗传修饰的酵母细胞,其中酵母细胞属于选自东方伊萨酵母/发酵毕赤酵母进化枝和酵母属进化枝的进化枝。

[0910] [B75]段落B73的经遗传修饰的酵母细胞,其中酵母细胞选自东方伊萨酵母、郎比可假丝酵母和博伊丁酵母。

[0911] [B76]段落B1-B75任一项的经遗传修饰的酵母细胞,其中所述细胞进一步包含选自PDC、ADH、GAL6、CYB2A、CYB2B、GPD、GPP、ALD和PCK基因的天然基因的一个或多个缺失或破坏。

[0912] [B77]段落B76的经遗传修饰的酵母细胞,其中一个或多个缺失或破坏得自一个或多个外源3-HP途径基因的插入。

[0913] [B78]段落B1-B77任一项的经遗传修饰的酵母细胞,其中一个或多个外源3-HP途径基因可操作连接到一个或多个外源调节元件上。

[0914] [B79]段落B78的经遗传修饰的酵母细胞,其中一个或多个调节元件对于一个或多个3-HP途径基因是外来的。

[0915] [B80]段落B1-B79任一项的经遗传修饰的酵母细胞,其中外源PYC基因可操作连接到对于PYC基因是外来的外源启动子上。

[0916] [B81]段落B1-B80任一项的经遗传修饰的酵母细胞,其中外源ATT基因可操作连接到对于ATT基因是外来的外源启动子上。

[0917] [B82]段落B1-B81任一项的经遗传修饰的酵母细胞,其中外源ADC基因可操作连接到对于ADC基因是外来的外源启动子上。

[0918] [B83]段落B1-B82任一项的经遗传修饰的酵母细胞,其中外源BAAT或gabT基因可操作连接到对于BAAT或gabT基因是外来的外源启动子上。

[0919] [B84]段落B1-B83任一项的经遗传修饰的酵母细胞,其中外源3-HPDH基因可操作连接到对于3-HPDH基因是外来的外源启动子上。

[0920] [B85]段落B1-B84任一项的经遗传修饰的酵母细胞,其中外源PPC基因可操作连接到对于PPC基因是外来的外源启动子上。

[0921] [B86]段落B1-B85任一项的经遗传修饰的酵母细胞,其中细胞能够在含75g/L或更多3-HP的培养基中在小于4的pH下生长。

[0922] [B87]段落B1-B86任一项的经遗传修饰的酵母细胞,其中细胞是3-HP抗性酵母细胞。

[0923] [B88]段落B1-B87任一项的经遗传修饰的酵母细胞,其中细胞已经经过了突变和/或选择,以致经突变和/或选择的细胞相比同种的野生型细胞具有更程度的3-HP抗性。

[0924] [B89]段落B88的经遗传修饰的酵母细胞,其中细胞在用一个或多个外源3-HP途径基因进行遗传修饰之前已经经过了突变和/或选择。

[0925] [B90]段落B88或B89的经遗传修饰的酵母细胞,其中细胞已经经过了乳酸或3-HP存在下的选择。

[0926] [B91]段落B91的经遗传修饰的酵母细胞,其中选择是恒化选择。

[0927] [B92]生产3-HP的方法,包括:

[0928] (i) 在含至少一种碳源的培养基的存在下培养段落B1-B91任一项的经遗传修饰的酵母细胞;和

[0929] (ii) 从培养物中分离3-HP。

[0930] [B93]段落B92的方法,其中所述碳源选自葡萄糖、木糖、阿拉伯糖、蔗糖、果糖、纤维素、葡萄糖寡聚物和甘油。

[0931] [B94]段落B92或B93的方法,其中培养基的pH小于5,如在约1.5至约4.5、约2.0至约4.0或约2.0至约3.5的范围中。

- [0001] 序列表
- [0002] <110> 卡吉尔公司 (CARGILL, INCORPORATED)
- [0003] Jessen, Holly
- [0004] Rush, Brian
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- [0006] Mastel, Beth
- [0007] Berry, Alan
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- [0009] <120> 用于生产3-羟基丙酸的组合物和方法
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[0121]	Val	Glu	Val	Arg	Asn	Ala	Ile	Leu	Lys	Asp	Ala	Val	Thr	Leu	Ala	Lys	
[0122]			275					280					285				
[0123]	Thr	Ala	Asn	Tyr	Arg	Asn	Ala	Gly	Thr	Ala	Glu	Phe	Leu	Val	Asp	Ser	
[0124]		290					295				300						
[0125]	Gln	Asn	Arg	His	Tyr	Phe	Ile	Glu	Ile	Asn	Pro	Arg	Ile	Gln	Val	Glu	

[0126]	305	310	315	320
[0127]	His Thr Ile Thr Glu Glu Ile Thr Gly Val Asp Ile Val Ala Ala Gln			
[0128]		325	330	335
[0129]	Ile Gln Ile Ala Ala Gly Ala Ser Leu Glu Gln Leu Gly Leu Leu Gln			
[0130]		340	345	350
[0131]	Asn Lys Ile Thr Thr Arg Gly Phe Ala Ile Gln Cys Arg Ile Thr Thr			
[0132]		355	360	365
[0133]	Glu Asp Pro Ala Lys Asn Phe Ala Pro Asp Thr Gly Lys Ile Glu Val			
[0134]		370	375	380
[0135]	Tyr Arg Ser Ala Gly Gly Asn Gly Val Arg Leu Asp Gly Gly Asn Gly			
[0136]	385	390	395	400
[0137]	Phe Ala Gly Ala Val Ile Ser Pro His Tyr Asp Ser Met Leu Val Lys			
[0138]		405	410	415
[0139]	Cys Ser Thr Ser Gly Ser Asn Tyr Glu Ile Ala Arg Arg Lys Met Ile			
[0140]		420	425	430
[0141]	Arg Ala Leu Val Glu Phe Arg Ile Arg Gly Val Lys Thr Asn Ile Pro			
[0142]		435	440	445
[0143]	Phe Leu Leu Ala Leu Leu Thr His Pro Val Phe Ile Ser Gly Asp Cys			
[0144]		450	455	460
[0145]	Trp Thr Thr Phe Ile Asp Asp Thr Pro Ser Leu Phe Glu Met Val Ser			
[0146]	465	470	475	480
[0147]	Ser Lys Asn Arg Ala Gln Lys Leu Leu Ala Tyr Ile Gly Asp Leu Cys			
[0148]		485	490	495
[0149]	Val Asn Gly Ser Ser Ile Lys Gly Gln Ile Gly Phe Pro Lys Leu Asn			
[0150]		500	505	510
[0151]	Lys Glu Ala Glu Ile Pro Asp Leu Leu Asp Pro Asn Asp Glu Val Ile			
[0152]		515	520	525
[0153]	Asp Val Ser Lys Pro Ser Thr Asn Gly Leu Arg Pro Tyr Leu Leu Lys			
[0154]		530	535	540
[0155]	Tyr Gly Pro Asp Ala Phe Ser Lys Lys Val Arg Glu Phe Asp Gly Cys			
[0156]	545	550	555	560
[0157]	Met Ile Met Asp Thr Thr Trp Arg Asp Ala His Gln Ser Leu Leu Ala			
[0158]		565	570	575
[0159]	Thr Arg Val Arg Thr Ile Asp Leu Leu Arg Ile Ala Pro Thr Thr Ser			
[0160]		580	585	590
[0161]	His Ala Leu Gln Asn Ala Phe Ala Leu Glu Cys Trp Gly Gly Ala Thr			
[0162]		595	600	605
[0163]	Phe Asp Val Ala Met Arg Phe Leu Tyr Glu Asp Pro Trp Glu Arg Leu			
[0164]		610	615	620
[0165]	Arg Gln Leu Arg Lys Ala Val Pro Asn Ile Pro Phe Gln Met Leu Leu			
[0166]	625	630	635	640
[0167]	Arg Gly Ala Asn Gly Val Ala Tyr Ser Ser Leu Pro Asp Asn Ala Ile			

[0168]		645		650		655
[0169]	Asp His Phe Val Lys Gln Ala Lys Asp Asn Gly Val Asp Ile Phe Arg					
[0170]		660		665		670
[0171]	Val Phe Asp Ala Leu Asn Asp Leu Glu Gln Leu Lys Val Gly Val Asp					
[0172]		675		680		685
[0173]	Ala Val Lys Lys Ala Gly Gly Val Val Glu Ala Thr Val Cys Tyr Ser					
[0174]		690		695		700
[0175]	Gly Asp Met Leu Ile Pro Gly Lys Lys Tyr Asn Leu Asp Tyr Tyr Leu					
[0176]	705		710		715	720
[0177]	Glu Thr Val Gly Lys Ile Val Glu Met Gly Thr His Ile Leu Gly Ile					
[0178]		725		730		735
[0179]	Lys Asp Met Ala Gly Thr Leu Lys Pro Lys Ala Ala Lys Leu Leu Ile					
[0180]		740		745		750
[0181]	Gly Ser Ile Arg Ser Lys Tyr Pro Asp Leu Val Ile His Val His Thr					
[0182]		755		760		765
[0183]	His Asp Ser Ala Gly Thr Gly Ile Ser Thr Tyr Val Ala Cys Ala Leu					
[0184]		770		775		780
[0185]	Ala Gly Ala Asp Ile Val Asp Cys Ala Ile Asn Ser Met Ser Gly Leu					
[0186]	785		790		795	800
[0187]	Thr Ser Gln Pro Ser Met Ser Ala Phe Ile Ala Ala Leu Asp Gly Asp					
[0188]		805		810		815
[0189]	Ile Glu Thr Gly Val Pro Glu His Phe Ala Arg Gln Leu Asp Ala Tyr					
[0190]		820		825		830
[0191]	Trp Ala Glu Met Arg Leu Leu Tyr Ser Cys Phe Glu Ala Asp Leu Lys					
[0192]		835		840		845
[0193]	Gly Pro Asp Pro Glu Val Tyr Lys His Glu Ile Pro Gly Gly Gln Leu					
[0194]		850		855		860
[0195]	Thr Asn Leu Ile Phe Gln Ala Gln Gln Val Gly Leu Gly Glu Gln Trp					
[0196]	865		870		875	880
[0197]	Glu Glu Thr Lys Lys Lys Tyr Glu Asp Ala Asn Met Leu Leu Gly Asp					
[0198]		885		890		895
[0199]	Ile Val Lys Val Thr Pro Thr Ser Lys Val Val Gly Asp Leu Ala Gln					
[0200]		900		905		910
[0201]	Phe Met Val Ser Asn Lys Leu Glu Lys Glu Asp Val Glu Lys Leu Ala					
[0202]		915		920		925
[0203]	Asn Glu Leu Asp Phe Pro Asp Ser Val Leu Asp Phe Phe Glu Gly Leu					
[0204]		930		935		940
[0205]	Met Gly Thr Pro Tyr Gly Gly Phe Pro Glu Pro Leu Arg Thr Asn Val					
[0206]	945		950		955	960
[0207]	Ile Ser Gly Lys Arg Arg Lys Leu Lys Gly Arg Pro Gly Leu Glu Leu					
[0208]		965		970		975
[0209]	Glu Pro Phe Asn Leu Glu Glu Ile Arg Glu Asn Leu Val Ser Arg Phe					

[0210]	980	985	990
[0211]	Gly Pro Gly Ile Thr Glu Cys Asp Val Ala Ser Tyr Asn Met Tyr Pro		
[0212]	995	1000	1005
[0213]	Lys Val Tyr Glu Gln Tyr Arg Lys Val Val Glu Lys Tyr Gly Asp		
[0214]	1010	1015	1020
[0215]	Leu Ser Val Leu Pro Thr Lys Ala Phe Leu Ala Pro Pro Thr Ile		
[0216]	1025	1030	1035
[0217]	Gly Glu Glu Val His Val Glu Ile Glu Gln Gly Lys Thr Leu Ile		
[0218]	1040	1045	1050
[0219]	Ile Lys Leu Leu Ala Ile Ser Asp Leu Ser Lys Ser His Gly Thr		
[0220]	1055	1060	1065
[0221]	Arg Glu Val Tyr Phe Glu Leu Asn Gly Glu Met Arg Lys Val Thr		
[0222]	1070	1075	1080
[0223]	Ile Glu Asp Lys Thr Ala Ala Ile Glu Thr Val Thr Arg Ala Lys		
[0224]	1085	1090	1095
[0225]	Ala Asp Gly His Asn Pro Asn Glu Val Gly Ala Pro Met Ala Gly		
[0226]	1100	1105	1110
[0227]	Val Val Val Glu Val Arg Val Lys His Gly Thr Glu Val Lys Lys		
[0228]	1115	1120	1125
[0229]	Gly Asp Pro Leu Ala Val Leu Ser Ala Met Lys Met Glu Met Val		
[0230]	1130	1135	1140
[0231]	Ile Ser Ala Pro Val Ser Gly Arg Val Gly Glu Val Phe Val Asn		
[0232]	1145	1150	1155
[0233]	Glu Gly Asp Ser Val Asp Met Gly Asp Leu Leu Val Lys Ile Ala		
[0234]	1160	1165	1170
[0235]	Lys Asp Glu Ala Pro Ala Ala		
[0236]	1175	1180	
[0237]	<210> 3		
[0238]	<211> 1154		
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[0241]	<400> 3		
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[0243]	1 5 10 15		
[0244]	Ile Arg Val Met Arg Ala Ala Asn Glu Met Gly Lys Lys Thr Val Ala		
[0245]	20 25 30		
[0246]	Val Tyr Ala Glu Glu Asp Lys Leu Ser Leu His Arg Phe Lys Ala Asp		
[0247]	35 40 45		
[0248]	Glu Ala Tyr Arg Ile Gly Glu Gly Leu Ser Pro Val Gly Ala Tyr Leu		
[0249]	50 55 60		
[0250]	Ser Ile Pro Glu Ile Ile Arg Val Ala Gln Met Ser Gly Ala Asp Ala		
[0251]	65 70 75 80		

[0252]	Ile His Pro Gly Tyr Gly Leu Leu Ser Glu Asn Pro Asp Phe Val Glu		
[0253]		85	90
[0254]	Ala Cys Asp Ala Ala Gly Ile Ala Phe Ile Gly Pro Lys Ala Glu Thr		95
[0255]		100	105
[0256]	Met Arg Ala Leu Gly Asp Lys Ala Ser Ala Arg Arg Val Ala Met Ala		110
[0257]		115	120
[0258]	Ala Gly Val Pro Val Ile Pro Ala Thr Glu Val Leu Gly Asp Asp Met		125
[0259]		130	135
[0260]	Glu Glu Ile Lys Arg Gln Ala Ala Glu Ile Gly Tyr Pro Leu Met Leu		140
[0261]		145	150
[0262]	Lys Ala Ser Trp Gly Gly Gly Gly Arg Gly Met Arg Pro Ile Thr Ser		155
[0263]		165	170
[0264]	Glu Ala Glu Leu Ala Asp Lys Val Arg Glu Gly Arg Arg Glu Ala Glu		175
[0265]		180	185
[0266]	Ala Ala Phe Gly Asn Gly Glu Gly Tyr Leu Glu Lys Met Ile Gln Arg		190
[0267]		195	200
[0268]	Ala Arg His Val Glu Val Gln Ile Leu Gly Asp Lys Tyr Gly Ala Ile		205
[0269]		210	215
[0270]	Tyr His Leu Tyr Glu Arg Asp Cys Thr Val Gln Arg Arg Asn Gln Lys		220
[0271]		225	230
[0272]	Val Val Glu Arg Ala Pro Ala Pro Tyr Leu Thr Glu Glu Gln Arg Thr		235
[0273]		245	250
[0274]	Glu Ile Cys Glu Leu Gly Arg Arg Ile Cys Ala His Val Asn Tyr Glu		255
[0275]		260	265
[0276]	Cys Ala Gly Thr Val Glu Phe Leu Met Asp Met Asp Ser Glu Lys Phe		270
[0277]		275	280
[0278]	Tyr Phe Ile Glu Val Asn Pro Arg Val Gln Val Glu His Thr Val Thr		285
[0279]		290	295
[0280]	Glu Glu Val Thr Gly Ile Asp Ile Val Gln Ser Gln Ile Arg Ile Ala		300
[0281]		305	310
[0282]	Glu Gly Ala Thr Leu Ala Glu Ala Thr Gly Cys Pro Ser Gln Asp Asp		315
[0283]		325	330
[0284]	Ile Lys Leu Ser Gly His Ala Leu Gln Cys Arg Val Thr Thr Glu Asp		335
[0285]		340	345
[0286]	Pro Gln Asn Asn Phe Ile Pro Asp Tyr Gly Arg Leu Thr Ala Tyr Arg		350
[0287]		355	360
[0288]	Ser Ala Thr Gly Met Gly Ile Arg Leu Asp Gly Gly Thr Ala Tyr Ala		365
[0289]		370	375
[0290]	Gly Gly Val Ile Thr Arg Tyr Tyr Asp Ser Leu Leu Val Lys Val Thr		380
[0291]		385	390
[0292]	Ala Trp Ala Pro Thr Pro Glu Lys Ala Ile Ala Arg Met Asp Arg Ala		395
[0293]		405	410
			415

[0294]	Leu Arg Glu Phe Arg Ile Arg Gly Val Ala Thr Asn Ile Ala Phe Val		
[0295]	420	425	430
[0296]	Glu Asn Leu Leu Lys His Pro Ser Phe Leu Asp Tyr Ser Tyr Thr Thr		
[0297]	435	440	445
[0298]	Lys Phe Ile Asp Thr Thr Pro Asp Leu Phe Asn Phe Lys Pro Arg Arg		
[0299]	450	455	460
[0300]	Asp Arg Ala Thr Lys Ile Leu Thr Tyr Ile Ala Asp Ile Thr Val Asn		
[0301]	465	470	475
[0302]	Gly His Pro Glu Thr Ala Gly Arg Val Arg Pro Ser Ala Glu Leu Lys		
[0303]	485	490	495
[0304]	Asp Pro Lys Ala Pro Glu Pro Lys Gly Ala Pro Gln Pro Gly Thr Arg		
[0305]	500	505	510
[0306]	Thr Leu Leu Glu Glu Lys Gly Pro Gln Ala Val Ala Asp Trp Met Ala		
[0307]	515	520	525
[0308]	Ala Gln Thr Arg Val Leu Met Thr Asp Thr Thr Met Arg Asp Gly His		
[0309]	530	535	540
[0310]	Gln Ser Leu Leu Ala Thr Arg Met Arg Ser Ile Asp Met Ile Lys Val		
[0311]	545	550	555
[0312]	Thr Pro Ala Tyr Ala Ala Asn Leu Gly Gly Leu Phe Ser Val Glu Cys		
[0313]	565	570	575
[0314]	Trp Gly Gly Ala Thr Phe Asp Val Ala Tyr Arg Phe Leu Gln Glu Cys		
[0315]	580	585	590
[0316]	Pro Trp Gln Arg Leu Arg Asp Ile Arg Ala Arg Leu Pro Asn Val Met		
[0317]	595	600	605
[0318]	Thr Gln Met Leu Leu Arg Ala Ser Asn Gly Val Gly Tyr Thr Asn Tyr		
[0319]	610	615	620
[0320]	Pro Asp Asn Val Val Gln Glu Phe Val Arg Gln Ala Ala Glu Thr Gly		
[0321]	625	630	635
[0322]	Val Asp Val Phe Arg Val Phe Asp Ser Leu Asn Trp Val Glu Asn Met		
[0323]	645	650	655
[0324]	Arg Val Ala Met Asp Ala Val Ile Glu Ala Asn Lys Val Cys Glu Gly		
[0325]	660	665	670
[0326]	Thr Ile Cys Tyr Thr Gly Asp Leu Leu Asp Pro Asp Arg Ser Lys Tyr		
[0327]	675	680	685
[0328]	Asp Leu Asn Tyr Tyr Val Gly Met Gly Arg Ala Leu Arg Asp Ala Gly		
[0329]	690	695	700
[0330]	Ala His Val Leu Gly Leu Lys Asp Met Ala Gly Leu Leu Lys Pro Ala		
[0331]	705	710	715
[0332]	Ala Ala Arg Val Leu Val Lys Ala Leu Lys Glu Glu Val Gly Leu Pro		
[0333]	725	730	735
[0334]	Ile His Phe His Thr His Asp Thr Ser Gly Ile Ala Gly Ala Thr Val		
[0335]	740	745	750

[0336]	Leu Ala Ala Cys Asp Ala Gly Val Asp Ala Val Asp Ala Ala Met Asp		
[0337]	755	760	765
[0338]	Ala Phe Ser Gly Gly Thr Ser Gln Pro Cys Leu Gly Ser Ile Val Glu		
[0339]	770	775	780
[0340]	Ala Leu Lys His Thr Asp Arg Asp Thr Gly Leu Asp Ile Ala Ala Ile		
[0341]	785	790	795 800
[0342]	Arg Glu Ile Ser Asp Tyr Trp Gly His Val Arg Gln Gln Tyr Ser Ala		
[0343]	805	810	815
[0344]	Phe Glu Ser Gly Leu Pro Ser Pro Ala Ser Glu Val Tyr Leu His Glu		
[0345]	820	825	830
[0346]	Met Pro Gly Gly Gln Phe Thr Asn Leu Lys Ala Gln Ala Arg Ser Met		
[0347]	835	840	845
[0348]	Gly Leu Glu Glu Arg Trp Ser Glu Val Ala Gln Ala Tyr Ala Asp Ala		
[0349]	850	855	860
[0350]	Asn Arg Met Phe Gly Asp Ile Val Lys Val Thr Pro Ser Ser Lys Val		
[0351]	865	870	875 880
[0352]	Val Gly Asp Met Ala Leu Met Met Val Ala Gln Gly Leu Thr Arg Glu		
[0353]	885	890	895
[0354]	Glu Val Glu Asp Pro Glu Val Glu Val Ser Phe Pro Asp Ser Val Val		
[0355]	900	905	910
[0356]	Asp Met Leu Lys Gly Asn Leu Gly Gln Pro His Gly Gly Trp Pro Glu		
[0357]	915	920	925
[0358]	Pro Ile Leu Lys Lys Val Leu Lys Gly Glu Ala Pro Ser Thr Glu Arg		
[0359]	930	935	940
[0360]	Pro Gly Ala His Leu Pro Pro Val Asp Ile Ala Ala Ala Arg Glu Lys		
[0361]	945	950	955 960
[0362]	Leu Leu Ser Glu Ile Lys Gln Gly Asp Asp Asp Pro Leu Asp Thr Ala		
[0363]	965	970	975
[0364]	Val Asp Ala Glu Asp Leu Asn Gly Tyr Leu Met Tyr Pro Lys Val Phe		
[0365]	980	985	990
[0366]	Thr Asp Tyr Arg Ala Arg His Arg Ile Tyr Gly Pro Val Arg Thr Leu		
[0367]	995	1000	1005
[0368]	Pro Thr Arg Thr Phe Phe Tyr Gly Met Glu Pro Gly Glu Glu Ile		
[0369]	1010	1015	1020
[0370]	Ser Ala Glu Ile Asp Pro Gly Lys Thr Leu Glu Ile Arg Leu Ser		
[0371]	1025	1030	1035
[0372]	Ala Val Gly Glu Thr Ser Asp Asp Gly Asp Ala Lys Val Phe Phe		
[0373]	1040	1045	1050
[0374]	Glu Leu Asn Gly Gln Pro Arg Val Ile Arg Val Ala Asn Arg Ala		
[0375]	1055	1060	1065
[0376]	Val Lys Ala Lys Thr Ala Thr Arg Pro Lys Ala Gln Asp Gly Asn		
[0377]	1070	1075	1080

[0378]	Pro Ala His Val Gly Ala Pro Met Pro Gly Ser Val Ala Ser Val
[0379]	1085 1090 1095
[0380]	Ala Val Ser Ala Gly Gln Lys Val Lys Pro Gly Asp Leu Leu Val
[0381]	1100 1105 1110
[0382]	Thr Ile Glu Ala Met Lys Met Glu Thr Gly Leu His Ala Asp Arg
[0383]	1115 1120 1125
[0384]	Ala Ala Thr Val Lys Ala Val His Val Gly Pro Gly Ala Gln Ile
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[0395]	Arg Val Phe Arg Ala Ala Asn Glu Leu Gly Ile Lys Thr Val Ala Ile
[0396]	20 25 30
[0397]	Trp Ala Glu Glu Asp Lys Leu Ala Leu His Arg Phe Lys Ala Asp Glu
[0398]	35 40 45
[0399]	Ser Tyr Gln Val Gly Arg Gly Pro His Leu Ala Arg Asp Leu Gly Pro
[0400]	50 55 60
[0401]	Ile Glu Ser Tyr Leu Ser Ile Asp Glu Val Ile Arg Val Ala Lys Leu
[0402]	65 70 75 80
[0403]	Ser Gly Ala Asp Ala Ile His Pro Gly Tyr Gly Leu Leu Ser Glu Ser
[0404]	85 90 95
[0405]	Pro Glu Phe Val Asp Ala Cys Asn Lys Ala Gly Ile Ile Phe Ile Gly
[0406]	100 105 110
[0407]	Pro Lys Ala Asp Thr Met Arg Gln Leu Gly Asn Lys Val Ala Ala Arg
[0408]	115 120 125
[0409]	Asn Leu Ala Ile Ser Val Gly Val Pro Val Val Pro Ala Thr Glu Pro
[0410]	130 135 140
[0411]	Leu Pro Asp Asp Met Ala Glu Val Ala Lys Met Ala Ala Ala Ile Gly
[0412]	145 150 155 160
[0413]	Tyr Pro Val Met Leu Lys Ala Ser Trp Gly Gly Gly Gly Arg Gly Met
[0414]	165 170 175
[0415]	Arg Val Ile Arg Ser Glu Ala Asp Leu Ala Lys Glu Val Thr Glu Ala
[0416]	180 185 190
[0417]	Lys Arg Glu Ala Met Ala Ala Phe Gly Lys Asp Glu Val Tyr Leu Glu
[0418]	195 200 205
[0419]	Lys Leu Val Glu Arg Ala Arg His Val Glu Ser Gln Ile Leu Gly Asp

[0420]	210	215	220
[0421]	Thr His Gly Asn Val Val His Leu Phe Glu Arg Asp Cys Ser Val Gln		
[0422]	225	230	235
[0423]	Arg Arg Asn Gln Lys Val Val Glu Arg Ala Pro Ala Pro Tyr Leu Ser		
[0424]	245	250	255
[0425]	Glu Ala Gln Arg Gln Glu Leu Ala Ala Tyr Ser Leu Lys Ile Ala Gly		
[0426]	260	265	270
[0427]	Ala Thr Asn Tyr Ile Gly Ala Gly Thr Val Glu Tyr Leu Met Asp Ala		
[0428]	275	280	285
[0429]	Asp Thr Gly Lys Phe Tyr Phe Ile Glu Val Asn Pro Arg Ile Gln Val		
[0430]	290	295	300
[0431]	Glu His Thr Val Thr Glu Val Val Thr Gly Ile Asp Ile Val Lys Ala		
[0432]	305	310	315
[0433]	Gln Ile His Ile Leu Asp Gly Ala Ala Ile Gly Thr Pro Gln Ser Gly		
[0434]	325	330	335
[0435]	Val Pro Asn Gln Glu Asp Ile Arg Leu Asn Gly His Ala Leu Gln Cys		
[0436]	340	345	350
[0437]	Arg Val Thr Thr Glu Asp Pro Glu His Asn Phe Ile Pro Asp Tyr Gly		
[0438]	355	360	365
[0439]	Arg Ile Thr Ala Tyr Arg Ser Ala Ser Gly Phe Gly Ile Arg Leu Asp		
[0440]	370	375	380
[0441]	Gly Gly Thr Ser Tyr Ser Gly Ala Ile Ile Thr Arg Tyr Tyr Asp Pro		
[0442]	385	390	395
[0443]	Leu Leu Val Lys Val Thr Ala Trp Ala Pro Asn Pro Leu Glu Ala Ile		
[0444]	405	410	415
[0445]	Ser Arg Met Asp Arg Ala Leu Arg Glu Phe Arg Ile Arg Gly Val Ala		
[0446]	420	425	430
[0447]	Thr Asn Leu Thr Phe Leu Glu Ala Ile Ile Gly His Pro Lys Phe Arg		
[0448]	435	440	445
[0449]	Asp Asn Ser Tyr Thr Thr Arg Phe Ile Asp Thr Thr Pro Glu Leu Phe		
[0450]	450	455	460
[0451]	Gln Gln Val Lys Arg Gln Asp Arg Ala Thr Lys Leu Leu Thr Tyr Leu		
[0452]	465	470	475
[0453]	Ala Asp Val Thr Val Asn Gly His Pro Glu Ala Lys Asp Arg Pro Lys		
[0454]	485	490	495
[0455]	Pro Leu Glu Asn Ala Ala Arg Pro Val Val Pro Tyr Ala Asn Gly Asn		
[0456]	500	505	510
[0457]	Gly Val Lys Asp Gly Thr Lys Gln Leu Leu Asp Thr Leu Gly Pro Lys		
[0458]	515	520	525
[0459]	Lys Phe Gly Glu Trp Met Arg Asn Glu Lys Arg Val Leu Leu Thr Asp		
[0460]	530	535	540
[0461]	Thr Thr Met Arg Asp Gly His Gln Ser Leu Leu Ala Thr Arg Met Arg		

[0462]	545	550	555	560
[0463]	Thr Tyr Asp Ile Ala Arg Ile Ala Gly Thr Tyr Ser His Ala Leu Pro			
[0464]		565	570	575
[0465]	Asn Leu Leu Ser Leu Glu Cys Trp Gly Gly Ala Thr Phe Asp Val Ser			
[0466]		580	585	590
[0467]	Met Arg Phe Leu Thr Glu Asp Pro Trp Glu Arg Leu Ala Leu Ile Arg			
[0468]		595	600	605
[0469]	Glu Gly Ala Pro Asn Leu Leu Leu Gln Met Leu Leu Arg Gly Ala Asn			
[0470]		610	615	620
[0471]	Gly Val Gly Tyr Thr Asn Tyr Pro Asp Asn Val Val Lys Tyr Phe Val			
[0472]		625	630	635
[0473]	Arg Gln Ala Ala Lys Gly Gly Ile Asp Leu Phe Arg Val Phe Asp Cys			
[0474]		645	650	655
[0475]	Leu Asn Trp Val Glu Asn Met Arg Val Ser Met Asp Ala Ile Ala Glu			
[0476]		660	665	670
[0477]	Glu Asn Lys Leu Cys Glu Ala Ala Ile Cys Tyr Thr Gly Asp Ile Leu			
[0478]		675	680	685
[0479]	Asn Ser Ala Arg Pro Lys Tyr Asp Leu Lys Tyr Tyr Thr Asn Leu Ala			
[0480]		690	695	700
[0481]	Val Glu Leu Glu Lys Ala Gly Ala His Ile Ile Ala Val Lys Asp Met			
[0482]		705	710	715
[0483]	Ala Gly Leu Leu Lys Pro Ala Ala Ala Lys Val Leu Phe Lys Ala Leu			
[0484]		725	730	735
[0485]	Arg Glu Ala Thr Gly Leu Pro Ile His Phe His Thr His Asp Thr Ser			
[0486]		740	745	750
[0487]	Gly Ile Ala Ala Ala Thr Val Leu Ala Ala Val Glu Ala Gly Val Asp			
[0488]		755	760	765
[0489]	Ala Val Asp Ala Ala Met Asp Ala Leu Ser Gly Asn Thr Ser Gln Pro			
[0490]		770	775	780
[0491]	Cys Leu Gly Ser Ile Val Glu Ala Leu Ser Gly Ser Glu Arg Asp Pro			
[0492]		785	790	795
[0493]	Gly Leu Asp Pro Ala Trp Ile Arg Arg Ile Ser Phe Tyr Trp Glu Ala			
[0494]		805	810	815
[0495]	Val Arg Asn Gln Tyr Ala Ala Phe Glu Ser Asp Leu Lys Gly Pro Ala			
[0496]		820	825	830
[0497]	Ser Glu Val Tyr Leu His Glu Met Pro Gly Gly Gln Phe Thr Asn Leu			
[0498]		835	840	845
[0499]	Lys Glu Gln Ala Arg Ser Leu Gly Leu Glu Thr Arg Trp His Gln Val			
[0500]		850	855	860
[0501]	Ala Gln Ala Tyr Ala Asp Ala Asn Gln Met Phe Gly Asp Ile Val Lys			
[0502]		865	870	875
[0503]	Val Thr Pro Ser Ser Lys Val Val Gly Asp Met Ala Leu Met Met Val			

[0504]	885	890	895
[0505]	Ser Gln Asp Leu Thr Val Ala Asp Val Val Ser Pro Asp Arg Glu Val		
[0506]	900	905	910
[0507]	Ser Phe Pro Glu Ser Val Val Ser Met Leu Lys Gly Asp Leu Gly Gln		
[0508]	915	920	925
[0509]	Pro Pro Ser Gly Trp Pro Glu Ala Leu Gln Lys Lys Ala Leu Lys Gly		
[0510]	930	935	940
[0511]	Glu Lys Pro Tyr Thr Val Arg Pro Gly Ser Leu Leu Lys Glu Ala Asp		
[0512]	945	950	955
[0513]	Leu Asp Ala Glu Arg Lys Val Ile Glu Lys Lys Leu Glu Arg Glu Val		
[0514]	965	970	975
[0515]	Ser Asp Phe Glu Phe Ala Ser Tyr Leu Met Tyr Pro Lys Val Phe Thr		
[0516]	980	985	990
[0517]	Asp Phe Ala Leu Ala Ser Asp Thr Tyr Gly Pro Val Ser Val Leu Pro		
[0518]	995	1000	1005
[0519]	Thr Pro Ala Tyr Phe Tyr Gly Leu Ala Asp Gly Glu Glu Leu Phe		
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[0521]	Ala Asp Ile Glu Lys Gly Lys Thr Leu Val Ile Val Asn Gln Ala		
[0522]	1025	1030	1035
[0523]	Val Ser Ala Thr Asp Ser Gln Gly Met Val Thr Val Phe Phe Glu		
[0524]	1040	1045	1050
[0525]	Leu Asn Gly Gln Pro Arg Arg Ile Lys Val Pro Asp Arg Ala His		
[0526]	1055	1060	1065
[0527]	Gly Ala Thr Gly Ala Ala Val Arg Arg Lys Ala Glu Pro Gly Asn		
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[0529]	Ala Ala His Val Gly Ala Pro Met Pro Gly Val Ile Ser Arg Val		
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[0531]	Phe Val Ser Ser Gly Gln Ala Val Asn Ala Gly Asp Val Leu Val		
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[0533]	Ser Ile Glu Ala Met Lys Met Glu Thr Ala Ile His Ala Glu Lys		
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[0535]	Asp Gly Thr Ile Ala Glu Val Leu Val Lys Ala Gly Asp Gln Ile		
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[0537]	Asp Ala Lys Asp Leu Leu Ala Val Tyr Gly Gly		
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			15

[0546]	Gln Ser Leu Leu Ala Thr Arg Met Arg Thr Glu Asp Met Leu Pro Ile
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[0548]	Cys Asp Lys Leu Asp Lys Val Gly Tyr Trp Ser Leu Glu Cys Trp Gly
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[0550]	Gly Ala Thr Phe Asp Ala Cys Val Arg Phe Leu Lys Glu Asp Pro Trp
[0551]	50 55 60
[0552]	Glu Arg Leu Arg Gln Leu Arg Ala Ala Leu Pro Asn Thr Arg Leu Gln
[0553]	65 70 75 80
[0554]	Met Leu Leu Arg Gly Gln Asn Leu Leu Gly Tyr Arg His Tyr Ser Asp
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[0556]	Asp Val Val Lys Ala Phe Val Ala Lys Ala Ala Val Asn Gly Ile Asp
[0557]	100 105 110
[0558]	Val Phe Arg Ile Phe Asp Ala Met Asn Asp Val Arg Asn Leu Arg Val
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[0560]	Ala Ile Glu Ala Val Lys Ala Ala Gly Lys His Ala Gln Gly Thr Ile
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[0562]	Ala Tyr Thr Thr Ser Pro Val His Thr Ile Asp Ala Phe Val Ala Gln
[0563]	145 150 155 160
[0564]	Ala Lys Gln Met Glu Ala Met Gly Cys Asp Ser Val Ala Ile Lys Asp
[0565]	165 170 175
[0566]	Met Ala Gly Leu Leu Thr Pro Tyr Ala Thr Gly Glu Leu Val Arg Ala
[0567]	180 185 190
[0568]	Leu Lys Ala Glu Gln Ser Leu Pro Val Phe Ile His Ser His Asp Thr
[0569]	195 200 205
[0570]	Ala Gly Leu Ala Ala Met Cys Gln Leu Lys Ala Ile Glu Asn Gly Ala
[0571]	210 215 220
[0572]	Asp His Ile Asp Thr Ala Ile Ser Ser Phe Ala Ser Gly Thr Ser His
[0573]	225 230 235 240
[0574]	Pro Gly Thr Glu Ser Met Val Ala Ala Leu Lys Gly Thr Glu Phe Asp
[0575]	245 250 255
[0576]	Thr Gly Leu Asn Leu Glu Leu Leu Gln Glu Ile Gly Leu Tyr Phe Tyr
[0577]	260 265 270
[0578]	Ala Val Arg Lys Lys Tyr His Gln Phe Glu Ser Glu Phe Thr Ala Val
[0579]	275 280 285
[0580]	Asp Thr Arg Val Gln Val Asn Gln Val Pro Gly Gly Met Ile Ser Asn
[0581]	290 295 300
[0582]	Leu Ala Asn Gln Leu Lys Glu Gln Gly Ala Leu Asn Arg Met Gly Glu
[0583]	305 310 315 320
[0584]	Val Leu Ala Glu Ile Pro Arg Val Arg Glu Asp Leu Gly Phe Pro Pro
[0585]	325 330 335
[0586]	Leu Val Thr Pro Thr Ser Gln Ile Val Gly Thr Gln Ala Phe Phe Asn
[0587]	340 345 350

[0588]	Val Leu Ala Gly Glu Arg Tyr Lys Thr Ile Thr Asn Glu Val Lys Leu
[0589]	355 360 365
[0590]	Tyr Leu Gln Gly Gly Tyr Gly Lys Ala Pro Gly Thr Val Asn Glu Lys
[0591]	370 375 380
[0592]	Leu Arg Arg Gln Ala Ile Gly Ser Glu Glu Val Ile Asp Val Arg Pro
[0593]	385 390 395 400
[0594]	Ala Asp Leu Leu Lys Pro Glu Met Thr Lys Leu Arg Ala Asp Ile Gly
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[0596]	Ala Leu Ala Lys Ser Glu Glu Asp Val Leu Thr Phe Ala Met Phe Pro
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[0598]	Asp Ile Gly Arg Lys Phe Leu Glu Glu Arg Ala Ala Gly Thr Leu Thr
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[0600]	Pro Glu Val Leu Leu Pro Ile Pro Glu Ala Gly Lys Val Ala Ser Ala
[0601]	450 455 460
[0602]	Gly Gly Glu Gly Val Pro Thr Glu Phe Val Ile Asp Val His Gly Glu
[0603]	465 470 475 480
[0604]	Thr Tyr Arg Val Asp Ile Thr Gly Val Gly Val Lys Ala Glu Gly Lys
[0605]	485 490 495
[0606]	Arg His Phe Tyr Leu Ser Ile Asp Gly Met Pro Glu Glu Val Val Phe
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[0608]	Glu Pro Leu Asn Glu Phe Val Gly Gly Gly Ser Ser Lys Arg Lys Gln
[0609]	515 520 525
[0610]	Ala Ser Ala Pro Gly His Val Ser Thr Thr Met Pro Gly Asn Ile Val
[0611]	530 535 540
[0612]	Asp Val Leu Val Lys Glu Gly Asp Thr Val Lys Ala Gly Gln Ala Val
[0613]	545 550 555 560
[0614]	Leu Ile Thr Glu Ala Met Lys Met Glu Thr Glu Val Gln Ala Ala Ile
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[0616]	Ala Gly Lys Val Thr Ala Ile His Val Ala Lys Gly Asp Arg Val Asn
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[0627]	Ile Ala Asn Arg Gly Glu Ile Ala Val Arg Ile Val Arg Ala Cys Ala
[0628]	20 25 30
[0629]	Glu Met Gly Ile Arg Ser Val Ala Ile Tyr Ser Asp Ala Asp Arg His

[0630]	35	40	45
[0631]	Ala Leu His Val Lys Arg Ala Asp Glu Ala His Ser Ile Gly Ala Glu		
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[0633]	Pro Leu Ala Gly Tyr Leu Asn Pro Arg Lys Leu Val Asn Leu Ala Val		
[0634]	65	70	75
[0635]	Glu Thr Gly Cys Asp Ala Leu His Pro Gly Tyr Gly Phe Leu Ser Glu		
[0636]	85	90	95
[0637]	Asn Ala Glu Leu Ala Asp Ile Cys Ala Glu Arg Gly Ile Lys Phe Ile		
[0638]	100	105	110
[0639]	Gly Pro Ser Ala Glu Val Ile Arg Arg Met Gly Asp Lys Thr Glu Ala		
[0640]	115	120	125
[0641]	Arg Arg Ser Met Ile Lys Ala Gly Val Pro Val Thr Pro Gly Thr Glu		
[0642]	130	135	140
[0643]	Gly Asn Val Ser Gly Ile Glu Glu Ala Leu Ser Glu Gly Asp Arg Ile		
[0644]	145	150	155
[0645]	Gly Tyr Pro Val Met Leu Lys Ala Thr Ser Gly Gly Gly Gly Arg Gly		
[0646]	165	170	175
[0647]	Ile Arg Arg Cys Asn Ser Arg Glu Glu Leu Glu Gln Asn Phe Pro Arg		
[0648]	180	185	190
[0649]	Val Ile Ser Glu Ala Thr Lys Ala Phe Gly Ser Ala Glu Val Phe Leu		
[0650]	195	200	205
[0651]	Glu Lys Cys Ile Val Asn Pro Lys His Ile Glu Ala Gln Ile Leu Gly		
[0652]	210	215	220
[0653]	Asp Ser Phe Gly Asn Val Val His Leu Phe Glu Arg Asp Cys Ser Ile		
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[0655]	Gln Arg Arg Asn Gln Lys Leu Ile Glu Ile Ala Pro Ser Pro Gln Leu		
[0656]	245	250	255
[0657]	Thr Pro Glu Gln Arg Ala Tyr Ile Gly Asp Leu Ser Val Arg Ala Ala		
[0658]	260	265	270
[0659]	Lys Ala Val Gly Tyr Glu Asn Ala Gly Thr Val Glu Phe Leu Leu Ala		
[0660]	275	280	285
[0661]	Glu Gly Glu Val Tyr Phe Met Glu Met Asn Thr Arg Val Gln Val Glu		
[0662]	290	295	300
[0663]	His Thr Ile Thr Glu Glu Ile Thr Gly Ile Asp Ile Val Arg Glu Gln		
[0664]	305	310	315
[0665]	Ile Arg Ile Ala Ser Gly Leu Pro Leu Ser Val Lys Gln Glu Asp Ile		
[0666]	325	330	335
[0667]	Gln His Arg Gly Phe Ala Leu Gln Phe Arg Ile Asn Ala Glu Asp Pro		
[0668]	340	345	350
[0669]	Lys Asn Asn Phe Leu Pro Ser Phe Gly Lys Ile Thr Arg Tyr Tyr Ala		
[0670]	355	360	365
[0671]	Pro Gly Gly Pro Gly Val Arg Thr Asp Thr Ala Ile Tyr Thr Gly Tyr		

[0672]	370	375	380
[0673]	Thr Ile Pro Pro Phe Tyr Asp Ser Met Cys Leu Lys Leu Val Val Trp		
[0674]	385	390	395 400
[0675]	Ala Leu Thr Trp Glu Glu Ala Met Asp Arg Gly Leu Arg Ala Leu Asp		
[0676]	405	410	415
[0677]	Asp Met Arg Leu Gln Gly Val Lys Thr Thr Ala Ala Tyr Tyr Gln Glu		
[0678]	420	425	430
[0679]	Ile Leu Arg Asn Pro Glu Phe Arg Ser Gly Gln Phe Asn Thr Ser Phe		
[0680]	435	440	445
[0681]	Val Glu Ser His Pro Glu Leu Thr Asn Tyr Ser Ile Lys Arg Lys Pro		
[0682]	450	455	460
[0683]	Glu Glu Leu Ala Leu Ala Ile Ala Ala Ala Ile Ala Ala His Ala Gly		
[0684]	465	470	475 480
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[0693]	Val Ala Asn Arg Gly Glu Ile Ala Val Arg Ala Phe Arg Ala Ala Leu		
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[0695]	Glu Thr Gly Ala Ala Thr Val Ala Ile Tyr Pro Arg Glu Asp Arg Gly		
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[0697]	Ser Phe His Arg Ser Phe Ala Ser Glu Ala Val Arg Ile Gly Thr Glu		
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[0699]	Gly Ser Pro Val Lys Ala Tyr Leu Asp Ile Asp Glu Ile Ile Gly Ala		
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[0701]	Ala Lys Lys Val Lys Ala Asp Ala Ile Tyr Pro Gly Tyr Gly Phe Leu		
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[0703]	Ser Glu Asn Ala Gln Leu Ala Arg Glu Cys Ala Glu Asn Gly Ile Thr		
[0704]	100	105	110
[0705]	Phe Ile Gly Pro Thr Pro Glu Val Leu Asp Leu Thr Gly Asp Lys Ser		
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[0707]	Arg Ala Val Thr Ala Ala Lys Lys Ala Gly Leu Pro Val Leu Ala Glu		
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[0709]	Ser Thr Pro Ser Lys Asn Ile Asp Glu Ile Val Lys Ser Ala Glu Gly		
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[0711]	Gln Thr Tyr Pro Ile Phe Val Lys Ala Val Ala Gly Gly Gly Gly Arg		
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[0713]	Gly Met Arg Phe Val Ala Ser Pro Asp Glu Leu Arg Lys Leu Ala Thr		

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[0715]	Glu Ala Ser Arg Glu Ala Glu Ala Ala Phe Gly Asp Gly Ala Val Tyr		
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[0717]	Val Glu Arg Ala Val Ile Asn Pro Gln His Ile Glu Val Gln Ile Leu		
[0718]	210	215	220
[0719]	Gly Asp His Thr Gly Glu Val Val His Leu Tyr Glu Arg Asp Cys Ser		
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[0721]	Leu Gln Arg Arg His Gln Lys Val Val Glu Ile Ala Pro Ala Gln His		
[0722]	245	250	255
[0723]	Leu Asp Pro Glu Leu Arg Asp Arg Ile Cys Ala Asp Ala Val Lys Phe		
[0724]	260	265	270
[0725]	Cys Arg Ser Ile Gly Tyr Gln Gly Ala Gly Thr Val Glu Phe Leu Val		
[0726]	275	280	285
[0727]	Asp Glu Lys Gly Asn His Val Phe Ile Glu Met Asn Pro Arg Ile Gln		
[0728]	290	295	300
[0729]	Val Glu His Thr Val Thr Glu Glu Val Thr Glu Val Asp Leu Val Lys		
[0730]	305	310	315
[0731]	Ala Gln Met Arg Leu Ala Ala Gly Ala Thr Leu Lys Glu Leu Gly Leu		
[0732]	325	330	335
[0733]	Thr Gln Asp Lys Ile Lys Thr His Gly Ala Ala Leu Gln Cys Arg Ile		
[0734]	340	345	350
[0735]	Thr Thr Glu Asp Pro Asn Asn Gly Phe Arg Pro Asp Thr Gly Thr Ile		
[0736]	355	360	365
[0737]	Thr Ala Tyr Arg Ser Pro Gly Gly Ala Gly Val Arg Leu Asp Gly Ala		
[0738]	370	375	380
[0739]	Ala Gln Leu Gly Gly Glu Ile Thr Ala His Phe Asp Ser Met Leu Val		
[0740]	385	390	395
[0741]	Lys Met Thr Cys Arg Gly Ser Asp Phe Glu Thr Ala Val Ala Arg Ala		
[0742]	405	410	415
[0743]	Gln Arg Ala Leu Ala Glu Phe Thr Val Ser Gly Val Ala Thr Asn Ile		
[0744]	420	425	430
[0745]	Gly Phe Leu Arg Ala Leu Leu Arg Glu Glu Asp Phe Thr Ser Lys Arg		
[0746]	435	440	445
[0747]	Ile Ala Thr Gly Phe Ile Ala Asp His Pro His Leu Leu Gln Ala Pro		
[0748]	450	455	460
[0749]	Pro Ala Asp Asp Glu Gln Gly Arg Ile Leu Asp Tyr Leu Ala Asp Val		
[0750]	465	470	475
[0751]	Thr Val Asn Lys Pro His Gly Val Arg Pro Lys Asp Val Ala Ala Pro		
[0752]	485	490	495
[0753]	Ile Asp Lys Leu Pro Asn Ile Lys Asp Leu Pro Leu Pro Arg Gly Ser		
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[0755]	Arg Asp Arg Leu Lys Gln Leu Gly Pro Ala Ala Phe Ala Arg Asp Leu		

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[0757]	Arg Glu Gln Asp Ala Leu Ala Val Thr Asp Thr Thr Phe Arg Asp Ala		
[0758]	530	535	540
[0759]	His Gln Ser Leu Leu Ala Thr Arg Val Arg Ser Phe Ala Leu Lys Pro		
[0760]	545	550	555
[0761]	Ala Ala Glu Ala Val Ala Lys Leu Thr Pro Glu Leu Leu Ser Val Glu		
[0762]	565	570	575
[0763]	Ala Trp Gly Gly Ala Thr Tyr Asp Val Ala Met Arg Phe Leu Phe Glu		
[0764]	580	585	590
[0765]	Asp Pro Trp Asp Arg Leu Asp Glu Leu Arg Glu Ala Met Pro Asn Val		
[0766]	595	600	605
[0767]	Asn Ile Gln Met Leu Leu Arg Gly Arg Asn Thr Val Gly Tyr Thr Pro		
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[0769]	Tyr Pro Asp Ser Val Cys Arg Ala Phe Val Lys Glu Ala Ala Ser Ser		
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[0771]	Gly Val Asp Ile Phe Arg Ile Phe Asp Ala Leu Asn Asp Val Ser Gln		
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[0773]	Met Arg Pro Ala Ile Asp Ala Val Leu Glu Thr Asn Thr Ala Val Ala		
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[0775]	Glu Val Ala Met Ala Tyr Ser Gly Asp Leu Ser Asp Pro Asn Glu Lys		
[0776]	675	680	685
[0777]	Leu Tyr Thr Leu Asp Tyr Tyr Leu Lys Met Ala Glu Glu Ile Val Lys		
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[0779]	Ser Gly Ala His Ile Leu Ala Ile Lys Asp Met Ala Gly Leu Leu Arg		
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[0781]	Pro Ala Ala Val Thr Lys Leu Val Thr Ala Leu Arg Arg Glu Phe Asp		
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[0783]	Leu Pro Val His Val His Thr His Asp Thr Ala Gly Gly Gln Leu Ala		
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[0785]	Thr Tyr Phe Ala Ala Ala Gln Ala Gly Ala Asp Ala Val Asp Gly Ala		
[0786]	755	760	765
[0787]	Ser Ala Pro Leu Ser Gly Thr Thr Ser Gln Pro Ser Leu Ser Ala Ile		
[0788]	770	775	780
[0789]	Val Ala Ala Phe Ala His Thr Arg Arg Asp Thr Gly Leu Ser Leu Glu		
[0790]	785	790	795
[0791]	Ala Val Ser Asp Leu Glu Pro Tyr Trp Glu Ala Val Arg Gly Leu Tyr		
[0792]	805	810	815
[0793]	Leu Pro Phe Glu Ser Gly Thr Pro Gly Pro Thr Gly Arg Val Tyr Arg		
[0794]	820	825	830
[0795]	His Glu Ile Pro Gly Gly Gln Leu Ser Asn Leu Arg Ala Gln Ala Thr		
[0796]	835	840	845
[0797]	Ala Leu Gly Leu Ala Asp Arg Phe Glu Leu Ile Glu Asp Asn Tyr Ala		

[0798]	850	855	860
[0799]	Ala Val Asn Glu Met Leu Gly Arg Pro Thr Lys Val Thr Pro Ser Ser		
[0800]	865	870	875 880
[0801]	Lys Val Val Gly Asp Leu Ala Leu His Leu Val Gly Ala Gly Val Asp		
[0802]	885	890	895
[0803]	Pro Ala Asp Phe Ala Ala Asp Pro Gln Lys Tyr Asp Ile Pro Asp Ser		
[0804]	900	905	910
[0805]	Val Ile Ala Phe Leu Arg Gly Glu Leu Gly Asn Pro Pro Gly Gly Trp		
[0806]	915	920	925
[0807]	Pro Glu Pro Leu Arg Thr Arg Ala Leu Glu Gly Arg Ser Glu Gly Lys		
[0808]	930	935	940
[0809]	Ala Pro Leu Thr Glu Val Pro Glu Glu Glu Gln Ala His Leu Asp Ala		
[0810]	945	950	955 960
[0811]	Asp Asp Ser Lys Glu Arg Arg Asn Ser Leu Asn Arg Leu Leu Phe Pro		
[0812]	965	970	975
[0813]	Lys Pro Thr Glu Glu Phe Leu Glu His Arg Arg Arg Phe Gly Asn Thr		
[0814]	980	985	990
[0815]	Ser Ala Leu Asp Asp Arg Glu Phe Phe Tyr Gly Leu Val Glu Gly Arg		
[0816]	995	1000	1005
[0817]	Glu Thr Leu Ile Arg Leu Pro Asp Val Arg Thr Pro Leu Leu Val		
[0818]	1010	1015	1020
[0819]	Arg Leu Asp Ala Ile Ser Glu Pro Asp Asp Lys Gly Met Arg Asn		
[0820]	1025	1030	1035
[0821]	Val Val Ala Asn Val Asn Gly Gln Ile Arg Pro Met Arg Val Arg		
[0822]	1040	1045	1050
[0823]	Asp Arg Ser Val Glu Ser Val Thr Ala Thr Ala Glu Lys Ala Asp		
[0824]	1055	1060	1065
[0825]	Ser Ser Asn Lys Gly His Val Ala Ala Pro Phe Ala Gly Val Val		
[0826]	1070	1075	1080
[0827]	Thr Val Thr Val Ala Glu Gly Asp Glu Val Lys Ala Gly Asp Ala		
[0828]	1085	1090	1095
[0829]	Val Ala Ile Ile Glu Ala Met Lys Met Glu Ala Thr Ile Thr Ala		
[0830]	1100	1105	1110
[0831]	Ser Val Asp Gly Lys Ile Asp Arg Val Val Val Pro Ala Ala Thr		
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[0833]	Lys Val Glu Gly Gly Asp Leu Ile Val Val Val Ser		
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[0842]	Arg Val Phe Arg Ala Ala Asn Glu Leu Gly Leu Lys Thr Val Ala Ile
[0843]	20 25 30
[0844]	Trp Ala Glu Glu Asp Lys Leu Ala Leu His Arg Phe Lys Ala Asp Glu
[0845]	35 40 45
[0846]	Ser Tyr Gln Val Gly Arg Gly Pro His Leu Pro Arg Asp Leu Gly Pro
[0847]	50 55 60
[0848]	Ile Met Ser Tyr Leu Ser Ile Asp Glu Val Ile Arg Val Ala Lys Leu
[0849]	65 70 75 80
[0850]	Ser Gly Ala Asp Ala Ile His Pro Gly Tyr Gly Leu Leu Ser Glu Ser
[0851]	85 90 95
[0852]	Pro Glu Phe Ala Glu Ala Cys Ala Ala Asn Gly Ile Thr Phe Ile Gly
[0853]	100 105 110
[0854]	Pro Lys Pro Glu Thr Met Arg Gln Leu Gly Asn Lys Val Ala Ala Arg
[0855]	115 120 125
[0856]	Asn Leu Ala Ile Ser Ile Gly Val Pro Val Val Pro Ala Thr Glu Pro
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[0858]	Leu Pro Asp Asp Pro Glu Glu Ile Lys Arg Leu Ala Glu Glu Ile Gly
[0859]	145 150 155 160
[0860]	Tyr Pro Val Met Leu Lys Ala Ser Trp Gly Gly Gly Gly Arg Gly Met
[0861]	165 170 175
[0862]	Arg Ala Ile Arg Asp Pro Lys Asp Leu Ile Arg Glu Val Thr Glu Ala
[0863]	180 185 190
[0864]	Lys Arg Glu Ala Lys Ala Ala Phe Gly Lys Asp Glu Val Tyr Leu Glu
[0865]	195 200 205
[0866]	Lys Leu Val Glu Arg Ala Arg His Val Glu Ser Gln Ile Leu Gly Asp
[0867]	210 215 220
[0868]	Thr His Gly Asn Val Val His Leu Phe Glu Arg Asp Cys Ser Ile Gln
[0869]	225 230 235 240
[0870]	Arg Arg Asn Gln Lys Val Val Glu Arg Ala Pro Ala Pro Tyr Leu Asn
[0871]	245 250 255
[0872]	Asp Ala Gln Arg Gln Glu Leu Ala Asp Tyr Ser Leu Lys Ile Ala Arg
[0873]	260 265 270
[0874]	Ala Thr Asn Tyr Ile Gly Ala Gly Thr Val Glu Tyr Leu Met Asp Ser
[0875]	275 280 285
[0876]	Asp Thr Gly Lys Phe Tyr Phe Ile Glu Val Asn Pro Arg Ile Gln Val
[0877]	290 295 300
[0878]	Glu His Thr Val Thr Glu Val Val Thr Gly Ile Asp Ile Val Lys Ala
[0879]	305 310 315 320
[0880]	Gln Ile His Ile Leu Asp Gly Phe Ala Ile Gly Ala Pro Glu Ser Gly
[0881]	325 330 335

[0882]	Val Pro Arg Gln Glu Asp Ile Arg Leu Asn Gly His Ala Leu Gln Cys		
[0883]		340	345
[0884]	Arg Ile Thr Thr Glu Asp Pro Glu Gln Asn Phe Ile Pro Asp Tyr Gly		350
[0885]		355	360
[0886]	Arg Ile Thr Ala Tyr Arg Gly Ala Thr Gly Phe Gly Ile Arg Leu Asp		365
[0887]		370	375
[0888]	Gly Gly Thr Ala Tyr Ser Gly Ala Val Ile Thr Arg Tyr Tyr Asp Pro		380
[0889]		385	390
[0890]	Leu Leu Glu Lys Val Thr Ala Trp Ala Pro Asn Pro Gly Glu Ala Ile		395
[0891]		405	410
[0892]	Gln Arg Met Ile Arg Ala Leu Arg Glu Phe Arg Ile Arg Gly Val Ala		415
[0893]		420	425
[0894]	Thr Asn Leu Thr Phe Leu Glu Ala Ile Ile Ser His Pro Lys Phe His		430
[0895]		435	440
[0896]	Asp Asn Ser Tyr Thr Thr Arg Phe Ile Asp Thr Thr Pro Glu Leu Phe		445
[0897]		450	455
[0898]	Gln Gln Val Lys Arg Gln Asp Arg Ala Thr Lys Leu Leu Thr Tyr Leu		460
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[0900]	Ala Asp Val Thr Val Asn Gly His Pro Glu Val Lys Gly Arg Pro Lys		475
[0901]		485	490
[0902]	Pro Ser Asp Asp Ile Ala Ala Pro Val Val Pro Phe Thr Gly Gly Asp		495
[0903]		500	505
[0904]	Val Lys Pro Gly Thr Lys Gln Arg Leu Asp Gln Leu Gly Pro Lys Lys		510
[0905]		515	520
[0906]	Phe Ala Glu Trp Val Lys Ala Gln Pro Glu Val Leu Ile Thr Asp Thr		525
[0907]		530	535
[0908]	Thr Met Arg Asp Gly His Gln Ser Leu Leu Ala Thr Arg Met Arg Thr		540
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[0910]	Tyr Asp Ile Ala Arg Ile Ala Gly Thr Tyr Ala Arg Ala Leu Pro Asn		555
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[0912]	Leu Phe Ser Leu Glu Cys Trp Gly Gly Ala Thr Phe Asp Val Ser Met		575
[0913]		580	585
[0914]	Arg Phe Leu Thr Glu Asp Pro Trp Glu Arg Leu Ala Met Val Arg Glu		590
[0915]		595	600
[0916]	Gly Ala Pro Asn Leu Leu Leu Gln Met Leu Leu Arg Gly Ala Asn Gly		605
[0917]		610	615
[0918]	Val Gly Tyr Lys Asn Tyr Pro Asp Asn Val Val Lys Tyr Phe Val Arg		620
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[0920]	Gln Ala Ala Lys Gly Gly Ile Asp Val Phe Arg Val Phe Asp Cys Leu		635
[0921]		645	650
[0922]	Asn Trp Val Glu Asn Met Arg Val Ala Met Asp Ala Val Ala Glu Glu		655
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			670

[0924]	Asp Arg Ile Cys Glu Ala Ala Ile Cys Tyr Thr Gly Asp Ile Leu Asn
[0925]	675 680 685
[0926]	Ser Ala Arg Pro Lys Tyr Asp Leu Lys Tyr Tyr Thr Ala Leu Ala Ala
[0927]	690 695 700
[0928]	Glu Leu Glu Lys Ala Gly Ala His Met Ile Ala Val Lys Asp Met Ala
[0929]	705 710 715 720
[0930]	Gly Leu Leu Lys Pro Ala Ala Ala Arg Val Leu Phe Lys Ala Leu Lys
[0931]	725 730 735
[0932]	Glu Ala Thr Gly Leu Pro Ile His Phe His Thr His Asp Thr Ser Gly
[0933]	740 745 750
[0934]	Ile Ala Ala Ala Thr Val Leu Ala Ala Val Glu Ser Gly Val Asp Val
[0935]	755 760 765
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[1045]	Glu Arg Val Glu Thr Ile Arg Lys Leu Ser Lys Ser Ser Arg Ala Gly	
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[1047]	Asn Asp Ala Asn Arg Gln Glu Leu Leu Thr Thr Leu Gln Asn Leu Ser	
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[1055]	Asn Gln Pro Glu Leu Ser Glu Asp Thr Ile Lys Lys Ala Val Glu Ser			
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[1057]	Leu Ser Leu Glu Leu Val Leu Thr Ala His Pro Thr Glu Ile Thr Arg			
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[1059]	Arg Thr Leu Ile His Lys Met Val Glu Val Asn Ala Cys Leu Lys Gln			
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[1061]	Leu Asp Asn Lys Asp Ile Ala Asp Tyr Glu His Asn Gln Leu Met Arg			
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[1063]	Arg Leu Arg Gln Leu Ile Ala Gln Ser Trp His Thr Asp Glu Ile Arg			
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[1071]	Val Pro Val Arg Phe Thr Ser Trp Met Gly Gly Asp Arg Asp Gly Asn			
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[1075]	Trp Lys Ala Thr Asp Leu Phe Leu Lys Asp Ile Gln Val Leu Val Ser			
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[1077]	Glu Leu Ser Met Val Glu Ala Thr Pro Glu Leu Leu Ala Leu Val Gly			
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[1079]	Glu Glu Gly Ala Ala Glu Pro Tyr Arg Tyr Leu Met Lys Asn Leu Arg			
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[1081]	Ser Arg Leu Met Ala Thr Gln Ala Trp Leu Glu Ala Arg Leu Lys Gly			
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[1097]	Ala Glu Thr Arg Glu Val Leu Asp Thr Cys Gln Val Ile Ala Glu Ala					
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[1113]	Leu Phe His Gly Arg Gly Gly Ser Ile Gly Arg Gly Gly Ala Pro Ala					
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[1115]	His Ala Ala Leu Leu Ser Gln Pro Pro Gly Ser Leu Lys Gly Gly Leu					
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[1117]	Arg Val Thr Glu Gln Gly Glu Met Ile Arg Phe Lys Tyr Gly Leu Pro					
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[1123]	Met Asp Glu Leu Ser Val Ile Ser Cys Asp Val Tyr Arg Gly Tyr Val					
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[1125]	Arg Glu Asn Lys Asp Phe Val Pro Tyr Phe Arg Ser Ala Thr Pro Glu					
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[1129]	Pro Thr Gly Gly Val Glu Ser Leu Arg Ala Ile Pro Trp Ile Phe Ala					
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[1131]	Trp Thr Gln Asn Arg Leu Met Leu Pro Ala Trp Leu Gly Ala Gly Thr					
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[1133]	Ala Leu Gln Lys Val Val Glu Asp Gly Lys Gln Ser Glu Leu Glu Ala					

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[1135]	Met Cys Arg Asp Trp Pro Phe Phe Ser Thr Arg Leu Gly Met Leu Glu		
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[1137]	Met Val Phe Ala Lys Ala Asp Leu Trp Leu Ala Glu Tyr Tyr Asp Gln		
[1138]	770	775	780
[1139]	Arg Leu Val Asp Lys Ala Leu Trp Pro Leu Gly Lys Glu Leu Arg Asn		
[1140]	785	790	795
[1141]	Leu Gln Glu Glu Asp Ile Lys Val Val Leu Ala Ile Ala Asn Asp Ser		
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[1143]	His Leu Met Ala Asp Leu Pro Trp Ile Ala Glu Ser Ile Gln Leu Arg		
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[1145]	Asn Ile Tyr Thr Asp Pro Leu Asn Val Leu Gln Ala Glu Leu Leu His		
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[1147]	Arg Ser Arg Gln Ala Glu Lys Glu Gly Gln Glu Pro Asp Pro Arg Val		
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[1149]	Glu Gln Ala Leu Met Val Thr Ile Ala Gly Ile Ala Ala Gly Met Arg		
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[1167]	Leu Arg Leu Thr Leu Arg Val Pro Asn Pro Thr Val Glu Arg Ala Glu		
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[1169]	Ala Lys Ile Leu Leu Glu Thr Leu Glu Ser Ile Pro Arg Ser Tyr Asp		
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[1171]	Thr Ala Ser Leu Phe Tyr Gly Met Asp Ala Ala Pro Val Phe Glu Val		
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[1173]	Ile Leu Pro Met Thr Ser Ser Ser Ser Cys Leu Asn Arg Ile His Ser		
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[1175]	Tyr Tyr Leu Asp Phe Val Lys Gly Lys Glu Arg Leu Gln Leu Ala Asp		

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[1179]	Asn Val Ile Pro Leu Phe Glu Asp His Glu Gly Met Leu Asn Ala Ala			
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[1195]	Val Lys Pro Gly Arg Ala Ala Glu Ile Glu Arg Glu Ser Val Leu Glu			
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[1197]	Ile Ile Ser Ala Tyr Cys Arg Glu Tyr Arg Arg Gln Val Met Asp Leu			
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[1203]	Val Ser Leu Pro Arg Ala Ile Thr Phe Thr Ala Ala Leu Tyr Ser Leu			
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[1205]	Gly Val Pro Pro Glu Leu Leu Gly Phe Asn Ala Leu Ser Ser Gly Asp			
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[1207]	Leu Glu Phe Ile Glu Glu Val Tyr Pro Gly Leu Gly Arg Asp Leu His			
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[1209]	Asp Ala Ala Arg Tyr Ala Asn Pro Glu Ser Pro Phe Leu Ser Pro Glu			
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[1211]	Val Lys Ser Ser Phe Glu Glu Tyr Leu Glu Pro Glu Tyr Asp Glu Gly			
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[1213]	His Met Lys Thr Thr Glu Glu Ile Ile Arg Ala Leu Arg Ile Asn Arg			
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[1215]	Thr Ala Asn Leu Gln Glu Leu Ile Leu Glu Ala Ala Ser Gln Arg Lys			
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[1229]	Phe Glu Gly Lys Leu Thr Pro Tyr His Gln Thr Ser Gln Ile Ala Leu	
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[1233]	Pro Arg Ile Pro Asn Ala Asn Lys Glu Ser Val Phe Arg Gln Leu Met	
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[1235]	Ser Ile Met Ser Ile Ile Glu Thr Asn Val Gln Ser Lys Glu Leu Thr	
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[1237]	Gly Thr Pro Ala Ile Ser Glu Val Val Val Pro Met Ile Glu Thr Gly	
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[1241]	Gly Asn Lys Asn Tyr Lys Thr Lys Leu Asp Leu Asn Ser Val Arg Ile	
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[1243]	Ile Pro Leu Val Glu Asp Val Pro Ala Leu Ala Asn Ile Asp Arg Ile	
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[1245]	Leu Asp Glu His Tyr Glu Ile Glu Lys Ser Lys Gly His Ile Leu Lys	
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[1249]	Leu Ile Ser Gly Val Leu Ser Val Leu Met Ala Val Asp Gly Ala Tyr	
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[1251]	Lys Trp Gly Glu Lys His Gly Val Thr Ile Ser Pro Ile Leu Gly Cys	
[1252]	225	230 235 240
[1253]	Gly Ser Leu Pro Phe Arg Gly His Phe Ser Glu Glu Asn Ile Asp Glu	
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[1255]	Ile Leu Ala Thr Tyr Ser Gly Ile Lys Thr Phe Thr Phe Gln Ser Ala	
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[1257]	Leu Arg Tyr Asp His Gly Glu Glu Ala Thr Lys His Ala Val Arg Glu	
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[1267]	Glu Tyr Asn Arg Glu Val Ala Asn Leu Asp Asn Val Ala Asp Leu Val		
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[1271]	Glu Tyr Ala Val Pro Arg Ala Ile Ser Phe Thr Gly Ala Met Tyr Thr		
[1272]	385	390	395 400
[1273]	Leu Gly Met Pro Pro Glu Leu Met Gly Met Gly Arg Ala Leu Asn Glu		
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[1275]	Ile Lys Thr Lys Tyr Gly Gln Glu Gly Ile Asp Lys Leu Leu Glu Ile		
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[1283]	Tyr Asp Phe Leu Asn Glu Asn Glu Phe Tyr His Thr Leu Leu Lys Thr		
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[1287]	Arg Asn Ser Thr Glu Glu Leu Lys Ile Leu Asn Glu Trp Ile Val Arg		
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[1724]	Gly Ile Met Val Pro Pro Pro Gly Tyr Leu Ala Gly Val Arg Glu Leu
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[1726]	Cys Asp Lys Tyr Gly Ile Val Phe Val Leu Asp Glu Val Met Ala Gly
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[1795]	Gly Thr Tyr Gly Ala Asn Ala Val Ala Cys Ala Ala Ala Cys Ala Thr		
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[1797]	Leu Asp Val Val Arg Asp Glu Lys Leu Val Asp Asn Ala Glu Ala Met		
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[2049]	225				230					235						240	
[2050]	Gly	Glu	Gly	Gly	Leu	Glu	Tyr	Phe	Phe	Asn	Arg	Gly	Phe	Gly	Tyr	Gly	
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[2052]	Ala	Asn	Glu	Trp	Met	His	Thr	Leu	Ala	Lys	Tyr	Asp	Lys	Phe	Pro	Tyr	
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[2063]	<213> 恶臭假单胞菌
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[2067]	Arg Asn Leu Ile Lys Ala Gly His Gln Leu Asn Leu Phe Asp Leu Asn
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[2070]	35 40 45
[2071]	Ser Pro Lys Asp Ala Ala Ala Asn Ser Glu Leu Val Ile Thr Met Leu
[2072]	50 55 60
[2073]	Pro Ala Ala Ala His Val Arg Ser Val Tyr Leu Asn Glu Asp Gly Val
[2074]	65 70 75 80
[2075]	Leu Ala Gly Ile Arg Pro Gly Thr Pro Thr Val Asp Cys Ser Thr Ile
[2076]	85 90 95
[2077]	Asp Pro Gln Thr Ala Arg Asp Val Ser Lys Ala Ala Ala Ala Lys Gly
[2078]	100 105 110
[2079]	Val Asp Met Gly Asp Ala Pro Val Ser Gly Gly Thr Gly Gly Ala Ala
[2080]	115 120 125
[2081]	Ala Gly Thr Leu Thr Phe Met Val Gly Ala Ser Thr Glu Leu Phe Ala
[2082]	130 135 140
[2083]	Ser Leu Lys Pro Val Leu Glu Gln Met Gly Arg Asn Ile Val His Cys
[2084]	145 150 155 160
[2085]	Gly Glu Val Gly Thr Gly Gln Ile Ala Lys Ile Cys Asn Asn Leu Leu
[2086]	165 170 175
[2087]	Leu Gly Ile Ser Met Ile Gly Val Ser Glu Ala Met Ala Leu Gly Asn
[2088]	180 185 190
[2089]	Ala Leu Gly Ile Asp Thr Lys Val Leu Ala Gly Ile Ile Asn Ser Ser
[2090]	195 200 205
[2091]	Thr Gly Arg Cys Trp Ser Ser Asp Thr Tyr Asn Pro Trp Pro Gly Ile
[2092]	210 215 220
[2093]	Ile Glu Thr Ala Pro Ala Ser Arg Gly Tyr Thr Gly Gly Phe Gly Ala
[2094]	225 230 235 240
[2095]	Glu Leu Met Leu Lys Asp Leu Gly Leu Ala Thr Glu Ala Ala Arg Gln
[2096]	245 250 255
[2097]	Ala His Gln Pro Val Ile Leu Gly Ala Val Ala Gln Gln Leu Tyr Gln
[2098]	260 265 270
[2099]	Ala Met Ser Leu Arg Gly Glu Gly Gly Lys Asp Phe Ser Ala Ile Val

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[2110]	Arg Asn Leu Ile Lys Ala Gly His Gln Leu Asn Leu Phe Asp Leu Asn		
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[2112]	Lys Thr Val Leu Ala Glu Leu Ala Glu Leu Gly Gly Gln Ile Ser Pro		
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[2114]	Ser Pro Lys Asp Ala Ala Ala Ser Ser Glu Leu Val Ile Thr Met Leu		
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[2116]	Pro Ala Ala Ala His Val Arg Ser Val Tyr Leu Asn Asp Asp Gly Val		
[2117]	65 70 75 80		
[2118]	Leu Ala Gly Ile Arg Pro Gly Thr Pro Thr Val Asp Cys Ser Thr Ile		
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[2120]	Asp Pro Gln Thr Ala Arg Asp Val Ser Lys Ala Ala Ala Lys Gly		
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[2122]	Val Asp Met Gly Asp Ala Pro Val Ser Gly Gly Thr Gly Gly Ala Ala		
[2123]	115 120 125		
[2124]	Ala Gly Thr Leu Thr Phe Met Val Gly Ala Ser Ala Glu Leu Phe Ala		
[2125]	130 135 140		
[2126]	Ser Leu Lys Pro Val Leu Glu Gln Met Gly Arg Asn Ile Val His Cys		
[2127]	145 150 155 160		
[2128]	Gly Glu Val Gly Thr Gly Gln Ile Ala Lys Ile Cys Asn Asn Leu Leu		
[2129]	165 170 175		
[2130]	Leu Gly Ile Ser Met Ile Gly Val Ser Glu Ala Met Ala Leu Gly Asn		
[2131]	180 185 190		
[2132]	Ala Leu Gly Ile Asp Thr Lys Val Leu Ala Gly Ile Ile Asn Ser Ser		
[2133]	195 200 205		
[2134]	Thr Gly Arg Cys Trp Ser Ser Asp Thr Tyr Asn Pro Trp Pro Gly Ile		
[2135]	210 215 220		
[2136]	Ile Glu Thr Ala Pro Ala Ser Arg Gly Tyr Thr Gly Gly Phe Gly Ala		
[2137]	225 230 235 240		
[2138]	Glu Leu Met Leu Lys Asp Leu Gly Leu Ala Thr Glu Ala Ala Arg Gln		
[2139]	245 250 255		
[2140]	Ala His Gln Pro Val Ile Leu Gly Ala Val Ala Gln Gln Leu Tyr Gln		
[2141]	260 265 270		

[2142]	Ala Met Ser Leu Arg Gly Glu Gly Gly Lys Asp Phe Ser Ala Ile Val
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[2144]	Glu Gly Tyr Arg Lys Lys Asp
[2145]	290 295
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[2149]	<213> 铜绿假单胞菌
[2150]	<400> 32
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[2153]	Ala Ala Asn Leu Leu Lys Ala Gly His Arg Val Asn Val Phe Asp Leu
[2154]	20 25 30
[2155]	Gln Pro Lys Ala Val Leu Gly Leu Val Glu Gln Gly Ala Gln Gly Ala
[2156]	35 40 45
[2157]	Asp Ser Ala Leu Gln Cys Cys Glu Gly Ala Glu Val Val Ile Ser Met
[2158]	50 55 60
[2159]	Leu Pro Ala Gly Gln His Val Glu Ser Leu Tyr Leu Gly Asp Asp Gly
[2160]	65 70 75 80
[2161]	Leu Leu Ala Arg Val Ala Gly Lys Pro Leu Leu Ile Asp Cys Ser Thr
[2162]	85 90 95
[2163]	Ile Ala Pro Glu Thr Ala Arg Lys Val Ala Glu Ala Ala Ala Lys
[2164]	100 105 110
[2165]	Gly Leu Thr Leu Leu Asp Ala Pro Val Ser Gly Gly Val Gly Gly Ala
[2166]	115 120 125
[2167]	Arg Ala Gly Thr Leu Ser Phe Ile Val Gly Gly Pro Ala Glu Gly Phe
[2168]	130 135 140
[2169]	Ala Arg Ala Arg Pro Val Leu Glu Asn Met Gly Arg Asn Ile Phe His
[2170]	145 150 155 160
[2171]	Ala Gly Asp His Gly Ala Gly Gln Val Ala Lys Ile Cys Asn Asn Met
[2172]	165 170 175
[2173]	Leu Leu Gly Ile Leu Met Ala Gly Thr Ala Glu Ala Leu Ala Leu Gly
[2174]	180 185 190
[2175]	Val Lys Asn Gly Leu Asp Pro Ala Val Leu Ser Glu Val Met Lys Gln
[2176]	195 200 205
[2177]	Ser Ser Gly Gly Asn Trp Ala Leu Asn Leu Tyr Asn Pro Trp Pro Gly
[2178]	210 215 220
[2179]	Val Met Pro Gln Ala Pro Ala Ser Asn Gly Tyr Ala Gly Gly Phe Gln
[2180]	225 230 235 240
[2181]	Val Arg Leu Met Asn Lys Asp Leu Gly Leu Ala Leu Ala Asn Ala Gln
[2182]	245 250 255
[2183]	Ala Val Gln Ala Ser Thr Pro Leu Gly Ala Leu Ala Arg Asn Leu Phe

[2184]	260	265	270
[2185]	Ser Leu His Ala Gln Ala Asp Ala Glu His Glu Gly Leu Asp Phe Ser		
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[2187]	Ser Ile Gln Lys Leu Tyr Arg Gly Lys Asp		
[2188]	290	295	
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[2193]	<400> 33		
[2194]	Met Ala Phe Ile Tyr Tyr Leu Thr His Ile His Leu Asp Phe Gly Ala		
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[2196]	Val Ser Leu Leu Lys Ser Glu Cys Glu Arg Ile Gly Ile Arg Arg Pro		
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[2198]	Leu Leu Val Thr Asp Lys Gly Val Val Ala Ala Gly Val Ala Gln Arg		
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[2200]	Ala Ile Asp Ala Met Gln Gly Leu Gln Val Ala Val Phe Asp Glu Thr		
[2201]	50	55	60
[2202]	Pro Ser Asn Pro Thr Glu Ala Met Val Arg Lys Ala Ala Ala Gln Tyr		
[2203]	65	70	75
[2204]	Arg Glu Ala Gly Cys Asp Gly Leu Val Ala Val Gly Gly Gly Ser Ser		
[2205]	85	90	95
[2206]	Ile Asp Leu Ala Lys Gly Ile Ala Ile Leu Ala Thr His Glu Gly Glu		
[2207]	100	105	110
[2208]	Leu Thr Thr Tyr Ala Thr Ile Glu Gly Gly Ser Ala Arg Ile Thr Asp		
[2209]	115	120	125
[2210]	Lys Ala Ala Pro Leu Ile Ala Val Pro Thr Thr Ser Gly Thr Gly Ser		
[2211]	130	135	140
[2212]	Glu Val Ala Arg Gly Ala Ile Ile Ile Leu Asp Asp Gly Arg Lys Leu		
[2213]	145	150	155
[2214]	Gly Phe His Ser Trp His Leu Leu Pro Lys Ser Ala Val Cys Asp Pro		
[2215]	165	170	175
[2216]	Glu Leu Thr Leu Gly Leu Pro Ala Gly Leu Thr Ala Ala Thr Gly Met		
[2217]	180	185	190
[2218]	Asp Ala Ile Ala His Cys Ile Glu Thr Phe Leu Ala Pro Ala Phe Asn		
[2219]	195	200	205
[2220]	Pro Pro Ala Asp Gly Ile Ala Leu Asp Gly Leu Glu Arg Gly Trp Gly		
[2221]	210	215	220
[2222]	His Ile Glu Arg Ala Thr Arg Asp Gly Gln Asp Arg Asp Ala Arg Leu		
[2223]	225	230	235
[2224]	Asn Met Met Ser Ala Ser Met Gln Gly Ala Met Ala Phe Gln Lys Gly		
[2225]	245	250	255

[2226]	Leu Gly Cys Val His Ser Leu Ser His Pro Leu Gly Gly Leu Lys Ile
[2227]	260 265 270
[2228]	Asp Gly Arg Thr Gly Leu His His Gly Thr Leu Asn Ala Val Val Met
[2229]	275 280 285
[2230]	Pro Ala Val Leu Arg Phe Asn Ala Asp Ala Pro Thr Val Val Arg Asp
[2231]	290 295 300
[2232]	Asp Arg Tyr Ala Arg Leu Arg Arg Ala Met His Leu Pro Asp Gly Ala
[2233]	305 310 315 320
[2234]	Asp Ile Ala Gln Ala Val His Asp Met Thr Val Arg Leu Gly Leu Pro
[2235]	325 330 335
[2236]	Thr Gly Leu Arg Gln Met Gly Val Thr Glu Asp Met Phe Asp Lys Val
[2237]	340 345 350
[2238]	Ile Ala Gly Ala Leu Val Asp His Cys His Lys Thr Asn Pro Lys Glu
[2239]	355 360 365
[2240]	Ala Ser Ala Ala Asp Tyr Arg Arg Met Leu Glu Gln Ser Met
[2241]	370 375 380
[2242]	<210> 34
[2243]	<211> 371
[2244]	<212> PRT
[2245]	<213> 克鲁维梭菌
[2246]	<400> 34
[2247]	Met Lys Leu Leu Lys Leu Ala Pro Asp Val Tyr Lys Phe Asp Thr Ala
[2248]	1 5 10 15
[2249]	Glu Glu Phe Met Lys Tyr Phe Lys Val Gly Lys Gly Asp Phe Ile Leu
[2250]	20 25 30
[2251]	Thr Asn Glu Phe Leu Tyr Lys Pro Phe Leu Glu Lys Phe Asn Asp Gly
[2252]	35 40 45
[2253]	Ala Asp Ala Val Phe Gln Glu Lys Tyr Gly Leu Gly Glu Pro Ser Asp
[2254]	50 55 60
[2255]	Glu Met Ile Asn Asn Ile Ile Lys Asp Ile Gly Asp Lys Gln Tyr Asn
[2256]	65 70 75 80
[2257]	Arg Ile Ile Ala Val Gly Gly Gly Ser Val Ile Asp Ile Ala Lys Ile
[2258]	85 90 95
[2259]	Leu Ser Leu Lys Tyr Thr Asp Asp Ser Leu Asp Leu Phe Glu Gly Lys
[2260]	100 105 110
[2261]	Val Pro Leu Val Lys Asn Lys Glu Leu Ile Ile Val Pro Thr Thr Cys
[2262]	115 120 125
[2263]	Gly Thr Gly Ser Glu Val Thr Asn Val Ser Val Ala Glu Leu Lys Arg
[2264]	130 135 140
[2265]	Arg His Thr Lys Lys Gly Ile Ala Ser Asp Glu Leu Tyr Ala Thr Tyr
[2266]	145 150 155 160
[2267]	Ala Val Leu Val Pro Glu Phe Ile Lys Gly Leu Pro Tyr Lys Phe Phe

[2268]		165		170		175
[2269]	Val Thr Ser Ser Val Asp Ala Leu Ile His Ala Thr Glu Ala Tyr Val					
[2270]		180		185		190
[2271]	Ser Pro Asn Ala Asn Pro Tyr Thr Asp Met Phe Ser Val Lys Ala Met					
[2272]		195		200		205
[2273]	Glu Leu Ile Leu Asn Gly Tyr Met Gln Met Val Glu Lys Gly Asn Asp					
[2274]		210		215		220
[2275]	Tyr Arg Val Glu Ile Ile Glu Asp Phe Val Ile Gly Ser Asn Tyr Ala					
[2276]	225		230		235	240
[2277]	Gly Ile Ala Phe Gly Asn Ala Gly Val Gly Ala Val His Ala Leu Ser					
[2278]		245		250		255
[2279]	Tyr Pro Ile Gly Gly Asn Tyr His Val Pro His Gly Glu Ala Asn Tyr					
[2280]		260		265		270
[2281]	Leu Phe Phe Thr Glu Ile Phe Lys Thr Tyr Tyr Glu Lys Asn Pro Asn					
[2282]		275		280		285
[2283]	Gly Lys Ile Lys Asp Val Asn Lys Leu Leu Ala Gly Ile Leu Lys Cys					
[2284]		290		295		300
[2285]	Asp Glu Ser Glu Ala Tyr Asp Ser Leu Ser Gln Leu Leu Asp Lys Leu					
[2286]	305		310		315	320
[2287]	Leu Ser Arg Lys Pro Leu Arg Glu Tyr Gly Met Lys Glu Glu Glu Ile					
[2288]		325		330		335
[2289]	Glu Thr Phe Ala Asp Ser Val Ile Glu Gly Gln Gln Arg Leu Leu Val					
[2290]		340		345		350
[2291]	Asn Asn Tyr Glu Pro Phe Ser Arg Glu Asp Ile Val Asn Thr Tyr Lys					
[2292]		355		360		365
[2293]	Lys Leu Tyr					
[2294]		370				
[2295]	<210> 35					
[2296]	<211> 538					
[2297]	<212> PRT					
[2298]	<213> 产琥珀酸曼氏杆菌(Mannheimia succiniciproducens)					
[2299]	<400> 35					
[2300]	Met Thr Asp Leu Asn Gln Leu Thr Gln Glu Leu Gly Ala Leu Gly Ile					
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[2302]	His Asp Val Gln Glu Val Val Tyr Asn Pro Ser Tyr Glu Leu Leu Phe					
[2303]		20		25		30
[2304]	Ala Glu Glu Thr Lys Pro Gly Leu Glu Gly Tyr Glu Lys Gly Thr Val					
[2305]		35		40		45
[2306]	Thr Asn Gln Gly Ala Val Ala Val Asn Thr Gly Ile Phe Thr Gly Arg					
[2307]		50		55		60
[2308]	Ser Pro Lys Asp Lys Tyr Ile Val Leu Asp Asp Lys Thr Lys Asp Thr					
[2309]	65		70		75	80

[2310]	Val Trp Trp Thr Ser Glu Lys Val Lys Asn Asp Asn Lys Pro Met Ser		
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[2312]	Gln Asp Thr Trp Asn Ser Leu Lys Gly Leu Val Ala Asp Gln Leu Ser		
[2313]		100	105 110
[2314]	Gly Lys Arg Leu Phe Val Val Asp Ala Phe Cys Gly Ala Asn Lys Asp		
[2315]		115	120 125
[2316]	Thr Arg Leu Ala Val Arg Val Val Thr Glu Val Ala Trp Gln Ala His		
[2317]		130	135 140
[2318]	Phe Val Thr Asn Met Phe Ile Arg Pro Ser Ala Glu Glu Leu Lys Gly		
[2319]		145	150 155 160
[2320]	Phe Lys Pro Asp Phe Val Val Met Asn Gly Ala Lys Cys Thr Asn Pro		
[2321]		165	170 175
[2322]	Asn Trp Lys Glu Gln Gly Leu Asn Ser Glu Asn Phe Val Ala Phe Asn		
[2323]		180	185 190
[2324]	Ile Thr Glu Gly Val Gln Leu Ile Gly Gly Thr Trp Tyr Gly Gly Glu		
[2325]		195	200 205
[2326]	Met Lys Lys Gly Met Phe Ser Met Met Asn Tyr Phe Leu Pro Leu Arg		
[2327]		210	215 220
[2328]	Gly Ile Ala Ser Met His Cys Ser Ala Asn Val Gly Lys Asp Gly Asp		
[2329]		225	230 235 240
[2330]	Thr Ala Ile Phe Phe Gly Leu Ser Gly Thr Gly Lys Thr Thr Leu Ser		
[2331]		245	250 255
[2332]	Thr Asp Pro Lys Arg Gln Leu Ile Gly Asp Asp Glu His Gly Trp Asp		
[2333]		260	265 270
[2334]	Asp Glu Gly Val Phe Asn Phe Glu Gly Gly Cys Tyr Ala Lys Thr Ile		
[2335]		275	280 285
[2336]	Asn Leu Ser Ala Glu Asn Glu Pro Asp Ile Tyr Gly Ala Ile Lys Arg		
[2337]		290	295 300
[2338]	Asp Ala Leu Leu Glu Asn Val Val Val Leu Asp Asn Gly Asp Val Asp		
[2339]		305	310 315 320
[2340]	Tyr Ala Asp Gly Ser Lys Thr Glu Asn Thr Arg Val Ser Tyr Pro Ile		
[2341]		325	330 335
[2342]	Tyr His Ile Gln Asn Ile Val Lys Pro Val Ser Lys Ala Gly Pro Ala		
[2343]		340	345 350
[2344]	Thr Lys Val Ile Phe Leu Ser Ala Asp Ala Phe Gly Val Leu Pro Pro		
[2345]		355	360 365
[2346]	Val Ser Lys Leu Thr Pro Glu Gln Thr Lys Tyr Tyr Phe Leu Ser Gly		
[2347]		370	375 380
[2348]	Phe Thr Ala Lys Leu Ala Gly Thr Glu Arg Gly Ile Thr Glu Pro Thr		
[2349]		385	390 395 400
[2350]	Pro Thr Phe Ser Ala Cys Phe Gly Ala Ala Phe Leu Ser Leu His Pro		
[2351]		405	410 415

[2352]	Thr	Gln	Tyr	Ala	Glu	Val	Leu	Val	Lys	Arg	Met	Gln	Glu	Ser	Gly	Ala
[2353]					420				425					430		
[2354]	Glu	Ala	Tyr	Leu	Val	Asn	Thr	Gly	Trp	Asn	Gly	Thr	Gly	Lys	Arg	Ile
[2355]					435				440					445		
[2356]	Ser	Ile	Lys	Asp	Thr	Arg	Gly	Ile	Ile	Asp	Ala	Ile	Leu	Asp	Gly	Ser
[2357]					450				455					460		
[2358]	Ile	Asp	Lys	Ala	Glu	Met	Gly	Ser	Leu	Pro	Ile	Phe	Asp	Phe	Ser	Ile
[2359]					465				470					475		480
[2360]	Pro	Lys	Ala	Leu	Pro	Gly	Val	Asn	Pro	Ala	Ile	Leu	Asp	Pro	Arg	Asp
[2361]					485				490					495		
[2362]	Thr	Tyr	Ala	Asp	Lys	Ala	Gln	Trp	Glu	Glu	Lys	Ala	Gln	Asp	Leu	Ala
[2363]					500				505					510		
[2364]	Gly	Arg	Phe	Val	Lys	Asn	Phe	Glu	Lys	Tyr	Thr	Gly	Thr	Ala	Glu	Gly
[2365]					515				520					525		
[2366]	Gln	Ala	Leu	Val	Ala	Ala	Gly	Pro	Lys	Ala						
[2367]					530				535							
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[2369]	<211> 532															
[2370]	<212> PRT															
[2371]	<213> 产琥珀酸厌氧螺菌(<i>Anaerobiospirillum succiniciproducens</i>)															
[2372]	<400> 36															
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[2375]	Asn	Ile	Val	His	Asn	Pro	Ser	His	Glu	Glu	Leu	Phe	Ala	Ala	Glu	Thr
[2376]					20				25					30		
[2377]	Gln	Ala	Ser	Leu	Glu	Gly	Phe	Glu	Lys	Gly	Thr	Val	Thr	Glu	Met	Gly
[2378]					35				40					45		
[2379]	Ala	Val	Asn	Val	Met	Thr	Gly	Val	Tyr	Thr	Gly	Arg	Ser	Pro	Lys	Asp
[2380]					50				55					60		
[2381]	Lys	Phe	Ile	Val	Lys	Asn	Glu	Ala	Ser	Lys	Glu	Ile	Trp	Trp	Thr	Ser
[2382]					65				70					75		80
[2383]	Asp	Glu	Phe	Lys	Asn	Asp	Asn	Lys	Pro	Val	Thr	Glu	Glu	Ala	Trp	Ala
[2384]					85				90					95		
[2385]	Gln	Leu	Lys	Ala	Leu	Ala	Gly	Lys	Glu	Leu	Ser	Asn	Lys	Pro	Leu	Tyr
[2386]					100				105					110		
[2387]	Val	Val	Asp	Leu	Phe	Cys	Gly	Ala	Asn	Glu	Asn	Thr	Arg	Leu	Lys	Ile
[2388]					115				120					125		
[2389]	Arg	Phe	Val	Met	Glu	Val	Ala	Trp	Gln	Ala	His	Phe	Val	Thr	Asn	Met
[2390]					130				135					140		
[2391]	Phe	Ile	Arg	Pro	Thr	Glu	Glu	Glu	Leu	Lys	Gly	Phe	Glu	Pro	Asp	Phe
[2392]					145				150					155		160
[2393]	Val	Val	Leu	Asn	Ala	Ser	Lys	Ala	Lys	Val	Glu	Asn	Phe	Lys	Glu	Leu

[2394]	165								170				175					
[2395]	Gly	Leu	Asn	Ser	Glu	Thr	Ala	Val	Val	Phe	Asn	Leu	Ala	Glu	Lys	Met		
[2396]	180								185				190					
[2397]	Gln	Ile	Ile	Leu	Asn	Thr	Trp	Tyr	Gly	Gly	Glu	Met	Lys	Lys	Gly	Met		
[2398]	195								200				205					
[2399]	Phe	Ser	Met	Met	Asn	Phe	Tyr	Leu	Pro	Leu	Gln	Gly	Ile	Ala	Ala	Met		
[2400]	210								215				220					
[2401]	His	Cys	Ser	Ala	Asn	Thr	Asp	Leu	Glu	Gly	Lys	Asn	Thr	Ala	Ile	Phe		
[2402]	225								230				235				240	
[2403]	Phe	Gly	Leu	Ser	Gly	Thr	Gly	Lys	Thr	Thr	Leu	Ser	Thr	Asp	Pro	Lys		
[2404]	245								250				255					
[2405]	Arg	Leu	Leu	Ile	Gly	Asp	Asp	Glu	His	Gly	Trp	Asp	Asp	Asp	Gly	Val		
[2406]	260								265				270					
[2407]	Phe	Asn	Phe	Glu	Gly	Gly	Cys	Tyr	Ala	Lys	Val	Ile	Asn	Leu	Ser	Lys		
[2408]	275								280				285					
[2409]	Glu	Asn	Glu	Pro	Asp	Ile	Trp	Gly	Ala	Ile	Lys	Arg	Asn	Ala	Leu	Leu		
[2410]	290								295				300					
[2411]	Glu	Asn	Val	Thr	Val	Asp	Ala	Asn	Gly	Lys	Val	Asp	Phe	Ala	Asp	Lys		
[2412]	305								310				315				320	
[2413]	Ser	Val	Thr	Glu	Asn	Thr	Arg	Val	Ser	Tyr	Pro	Ile	Phe	His	Ile	Lys		
[2414]	325								330				335					
[2415]	Asn	Ile	Val	Lys	Pro	Val	Ser	Lys	Ala	Pro	Ala	Ala	Lys	Arg	Val	Ile		
[2416]	340								345				350					
[2417]	Phe	Leu	Ser	Ala	Asp	Ala	Phe	Gly	Val	Leu	Pro	Pro	Val	Ser	Ile	Leu		
[2418]	355								360				365					
[2419]	Ser	Lys	Glu	Gln	Thr	Lys	Tyr	Tyr	Phe	Leu	Ser	Gly	Phe	Thr	Ala	Lys		
[2420]	370								375				380					
[2421]	Leu	Ala	Gly	Thr	Glu	Arg	Gly	Ile	Thr	Glu	Pro	Thr	Pro	Thr	Phe	Ser		
[2422]	385								390				395				400	
[2423]	Ser	Cys	Phe	Gly	Ala	Ala	Phe	Leu	Thr	Leu	Pro	Pro	Thr	Lys	Tyr	Ala		
[2424]	405								410				415					
[2425]	Glu	Val	Leu	Val	Lys	Arg	Met	Glu	Ala	Ser	Gly	Ala	Lys	Ala	Tyr	Leu		
[2426]	420								425				430					
[2427]	Val	Asn	Thr	Gly	Trp	Asn	Gly	Thr	Gly	Lys	Arg	Ile	Ser	Ile	Lys	Asp		
[2428]	435								440				445					
[2429]	Thr	Arg	Gly	Ile	Ile	Asp	Ala	Ile	Leu	Asp	Gly	Ser	Ile	Asp	Thr	Ala		
[2430]	450								455				460					
[2431]	Asn	Thr	Ala	Thr	Ile	Pro	Tyr	Phe	Asn	Phe	Thr	Val	Pro	Thr	Glu	Leu		
[2432]	465								470				475				480	
[2433]	Lys	Gly	Val	Asp	Thr	Lys	Ile	Leu	Asp	Pro	Arg	Asn	Thr	Tyr	Ala	Asp		
[2434]	485								490				495					
[2435]	Ala	Ser	Glu	Trp	Glu	Val	Lys	Ala	Lys	Asp	Leu	Ala	Glu	Arg	Phe	Gln		

[2436]	500	505	510
[2437]	Lys Asn Phe Lys Lys Phe Glu Ser Leu Gly Gly Asp Leu Val Lys Ala		
[2438]	515	520	525
[2439]	Gly Pro Gln Leu		
[2440]	530		
[2441]	<210> 37		
[2442]	<211> 538		
[2443]	<212> PRT		
[2444]	<213> 产琥珀酸放线杆菌(Actinobacillus succinogenes)		
[2445]	<400> 37		
[2446]	Met Thr Asp Leu Asn Lys Leu Val Lys Glu Leu Asn Asp Leu Gly Leu		
[2447]	1 5 10 15		
[2448]	Thr Asp Val Lys Glu Ile Val Tyr Asn Pro Ser Tyr Glu Gln Leu Phe		
[2449]	20 25 30		
[2450]	Glu Glu Glu Thr Lys Pro Gly Leu Glu Gly Phe Asp Lys Gly Thr Leu		
[2451]	35 40 45		
[2452]	Thr Thr Leu Gly Ala Val Ala Val Asp Thr Gly Ile Phe Thr Gly Arg		
[2453]	50 55 60		
[2454]	Ser Pro Lys Asp Lys Tyr Ile Val Cys Asp Glu Thr Thr Lys Asp Thr		
[2455]	65 70 75 80		
[2456]	Val Trp Trp Asn Ser Glu Ala Ala Lys Asn Asp Asn Lys Pro Met Thr		
[2457]	85 90 95		
[2458]	Gln Glu Thr Trp Lys Ser Leu Arg Glu Leu Val Ala Lys Gln Leu Ser		
[2459]	100 105 110		
[2460]	Gly Lys Arg Leu Phe Val Val Glu Gly Tyr Cys Gly Ala Ser Glu Lys		
[2461]	115 120 125		
[2462]	His Arg Ile Gly Val Arg Met Val Thr Glu Val Ala Trp Gln Ala His		
[2463]	130 135 140		
[2464]	Phe Val Lys Asn Met Phe Ile Arg Pro Thr Asp Glu Glu Leu Lys Asn		
[2465]	145 150 155 160		
[2466]	Phe Lys Ala Asp Phe Thr Val Leu Asn Gly Ala Lys Cys Thr Asn Pro		
[2467]	165 170 175		
[2468]	Asn Trp Lys Glu Gln Gly Leu Asn Ser Glu Asn Phe Val Ala Phe Asn		
[2469]	180 185 190		
[2470]	Ile Thr Glu Gly Ile Gln Leu Ile Gly Gly Thr Trp Tyr Gly Gly Glu		
[2471]	195 200 205		
[2472]	Met Lys Lys Gly Met Phe Ser Met Met Asn Tyr Phe Leu Pro Leu Lys		
[2473]	210 215 220		
[2474]	Gly Val Ala Ser Met His Cys Ser Ala Asn Val Gly Lys Asp Gly Asp		
[2475]	225 230 235 240		
[2476]	Val Ala Ile Phe Phe Gly Leu Ser Gly Thr Gly Lys Thr Thr Leu Ser		
[2477]	245 250 255		

[2478]	Thr Asp Pro Lys Arg Gln Leu Ile Gly Asp Asp Glu His Gly Trp Asp		
[2479]	260	265	270
[2480]	Glu Ser Gly Val Phe Asn Phe Glu Gly Gly Cys Tyr Ala Lys Thr Ile		
[2481]	275	280	285
[2482]	Asn Leu Ser Gln Glu Asn Glu Pro Asp Ile Tyr Gly Ala Ile Arg Arg		
[2483]	290	295	300
[2484]	Asp Ala Leu Leu Glu Asn Val Val Val Arg Ala Asp Gly Ser Val Asp		
[2485]	305	310	315
[2486]	Phe Asp Asp Gly Ser Lys Thr Glu Asn Thr Arg Val Ser Tyr Pro Ile		
[2487]	325	330	335
[2488]	Tyr His Ile Asp Asn Ile Val Arg Pro Val Ser Lys Ala Gly His Ala		
[2489]	340	345	350
[2490]	Thr Lys Val Ile Phe Leu Thr Ala Asp Ala Phe Gly Val Leu Pro Pro		
[2491]	355	360	365
[2492]	Val Ser Lys Leu Thr Pro Glu Gln Thr Glu Tyr Tyr Phe Leu Ser Gly		
[2493]	370	375	380
[2494]	Phe Thr Ala Lys Leu Ala Gly Thr Glu Arg Gly Val Thr Glu Pro Thr		
[2495]	385	390	395
[2496]	Pro Thr Phe Ser Ala Cys Phe Gly Ala Ala Phe Leu Ser Leu His Pro		
[2497]	405	410	415
[2498]	Ile Gln Tyr Ala Asp Val Leu Val Glu Arg Met Lys Ala Ser Gly Ala		
[2499]	420	425	430
[2500]	Glu Ala Tyr Leu Val Asn Thr Gly Trp Asn Gly Thr Gly Lys Arg Ile		
[2501]	435	440	445
[2502]	Ser Ile Lys Asp Thr Arg Gly Ile Ile Asp Ala Ile Leu Asp Gly Ser		
[2503]	450	455	460
[2504]	Ile Glu Lys Ala Glu Met Gly Glu Leu Pro Ile Phe Asn Leu Ala Ile		
[2505]	465	470	475
[2506]	Pro Lys Ala Leu Pro Gly Val Asp Pro Ala Ile Leu Asp Pro Arg Asp		
[2507]	485	490	495
[2508]	Thr Tyr Ala Asp Lys Ala Gln Trp Gln Val Lys Ala Glu Asp Leu Ala		
[2509]	500	505	510
[2510]	Asn Arg Phe Val Lys Asn Phe Val Lys Tyr Thr Ala Asn Pro Glu Ala		
[2511]	515	520	525
[2512]	Ala Lys Leu Val Gly Ala Gly Pro Lys Ala		
[2513]	530	535	
[2514]	<210> 38		
[2515]	<211> 618		
[2516]	<212> PRT		
[2517]	<213> Ralstonis eutropha		
[2518]	<400> 38		
[2519]	Met Asn His Pro Ser Met Gln Gly Thr Thr Ala Leu Asn Val Pro Ala		

[2520]	1	5	10	15
[2521]	Trp Val Arg Asn Gln Lys Leu Val Ala Trp Val Ala Glu Ile Ala Ala			
[2522]	20	25	30	
[2523]	Leu Thr Lys Pro Glu Arg Ile His Trp Cys Asp Gly Ser Gln Glu Glu			
[2524]	35	40	45	
[2525]	Tyr Asp Arg Leu Cys Glu Gln Met Val Ala Ala Gly Thr Leu Lys Arg			
[2526]	50	55	60	
[2527]	Leu Asn Pro Ala Lys Arg Lys Asn Ser Tyr Leu Ala Leu Ser Asp Pro			
[2528]	65	70	75	80
[2529]	Ser Asp Val Ala Arg Val Glu Asp Arg Thr Phe Ile Cys Ser Gln Lys			
[2530]	85	90	95	
[2531]	Lys Glu Asp Ala Gly Pro Thr Asn Asn Trp Val Ala Pro Ala Glu Met			
[2532]	100	105	110	
[2533]	Arg Thr Thr Leu Asn Gly Leu Phe Asp Gly Cys Met Arg Gly Arg Thr			
[2534]	115	120	125	
[2535]	Leu Tyr Val Val Pro Phe Ser Met Gly Pro Leu Gly Ser Pro Ile Ala			
[2536]	130	135	140	
[2537]	His Ile Gly Val Glu Leu Ser Asp Ser Pro Tyr Val Ala Val Asn Met			
[2538]	145	150	155	160
[2539]	Arg Ile Met Thr Arg Met Gly Lys Ala Val Tyr Asp Val Leu Gly Thr			
[2540]	165	170	175	
[2541]	Asp Gly Asp Phe Val Pro Cys Val His Thr Val Gly Lys Pro Leu Ala			
[2542]	180	185	190	
[2543]	Ala Gly Glu Lys Asp Val Pro Trp Pro Cys Asn Pro Thr Lys Tyr Ile			
[2544]	195	200	205	
[2545]	Val His Phe Pro Glu Ser Arg Glu Ile Trp Ser Phe Gly Ser Gly Tyr			
[2546]	210	215	220	
[2547]	Gly Gly Asn Ala Leu Leu Gly Lys Lys Cys Phe Ala Leu Arg Ile Ala			
[2548]	225	230	235	240
[2549]	Ser Thr Met Gly Arg Asp Glu Gly Trp Leu Ala Glu His Met Leu Ile			
[2550]	245	250	255	
[2551]	Leu Gly Val Thr Ser Pro Glu Gly Lys Lys Phe His Val Ala Ala Ala			
[2552]	260	265	270	
[2553]	Phe Pro Ser Ala Cys Gly Lys Thr Asn Phe Ala Met Leu Ile Pro Pro			
[2554]	275	280	285	
[2555]	Lys Gly Phe Glu Gly Trp Lys Val Thr Thr Ile Gly Asp Asp Ile Ala			
[2556]	290	295	300	
[2557]	Trp Ile Lys Pro Gly Lys Asp Gly Arg Leu Tyr Ala Ile Asn Pro Glu			
[2558]	305	310	315	320
[2559]	Ala Gly Tyr Phe Gly Val Ala Pro Gly Thr Ser Glu Lys Thr Asn Phe			
[2560]	325	330	335	
[2561]	Asn Ala Met Ala Thr Leu Lys Glu Asn Val Ile Phe Thr Asn Val Ala			

[2562]	340	345	350
[2563]	Leu Thr Asp Asp Gly Asp Val Trp Trp Glu Gly Met Thr Lys Glu Ala		
[2564]	355	360	365
[2565]	Pro Ala His Leu Thr Asp Trp Gln Gly Lys Asp Trp Thr Pro Glu Ile		
[2566]	370	375	380
[2567]	Ala Lys Ala Thr Gly Ala Lys Ala Ala His Pro Asn Ala Arg Phe Thr		
[2568]	385	390	395
[2569]	Ala Pro Ala Ser Gln Cys Pro Ser Ile Asp Glu Asn Trp Asp Asn Pro		
[2570]	405	410	415
[2571]	Ala Gly Val Pro Ile Asp Ala Phe Ile Phe Gly Gly Arg Arg Ser Thr		
[2572]	420	425	430
[2573]	Thr Val Pro Leu Val Thr Glu Ala Arg Asn Trp Thr Glu Gly Val Tyr		
[2574]	435	440	445
[2575]	Met Ala Ala Thr Met Gly Ser Glu Thr Thr Ala Ala Ala Ala Gly Gln		
[2576]	450	455	460
[2577]	Gln Gly Val Val Arg Arg Asp Pro Phe Ala Met Leu Pro Phe Cys Gly		
[2578]	465	470	475
[2579]	Tyr Asn Met Ser Asp Tyr Phe Gly His Trp Leu Ala Leu Gly Gln Lys		
[2580]	485	490	495
[2581]	Leu Glu Ala Ala Gly Ala Lys Leu Pro Lys Ile Tyr Cys Val Asn Trp		
[2582]	500	505	510
[2583]	Phe Arg Lys Asp Ala Asp Gly Asn Phe Val Trp Pro Gly Phe Gly Glu		
[2584]	515	520	525
[2585]	Asn Met Arg Val Leu Ser Trp Met Ile Asp Arg Val Glu Gly Lys Gly		
[2586]	530	535	540
[2587]	Glu Gly Ala Glu His Val Phe Gly Thr Ser Pro Arg Tyr Glu Asp Leu		
[2588]	545	550	555
[2589]	Asn Trp Ser Gly Val Glu Phe Ser Val Ala Gln Phe Thr Gln Val Thr		
[2590]	565	570	575
[2591]	Ser Ile Asp Ala Asp Ala Trp Lys Gln Glu Leu Ala Leu His Asp Glu		
[2592]	580	585	590
[2593]	Leu Phe Thr Gln Leu Lys His Asn Leu Pro Gln Ala Leu Ala Glu Ala		
[2594]	595	600	605
[2595]	Arg Ala Ala Leu Gly Lys Arg Leu Glu Gly		
[2596]	610	615	
[2597]	<210> 39		
[2598]	<211> 540		
[2599]	<212> PRT		
[2600]	<213> 大肠杆菌		
[2601]	<400> 39		
[2602]	Met Arg Val Asn Asn Gly Leu Thr Pro Gln Glu Leu Glu Ala Tyr Gly		
[2603]	1	5	10
			15

[2604]	Ile Ser Asp Val His Asp Ile Val Tyr Asn Pro Ser Tyr Asp Leu Leu
[2605]	20 25 30
[2606]	Tyr Gln Glu Glu Leu Asp Pro Ser Leu Thr Gly Tyr Glu Arg Gly Val
[2607]	35 40 45
[2608]	Leu Thr Asn Leu Gly Ala Val Ala Val Asp Thr Gly Ile Phe Thr Gly
[2609]	50 55 60
[2610]	Arg Ser Pro Lys Asp Lys Tyr Ile Val Arg Asp Asp Thr Thr Arg Asp
[2611]	65 70 75 80
[2612]	Thr Phe Trp Trp Ala Asp Lys Gly Lys Gly Lys Asn Asp Asn Lys Pro
[2613]	85 90 95
[2614]	Leu Ser Pro Glu Thr Trp Gln His Leu Lys Gly Leu Val Thr Arg Gln
[2615]	100 105 110
[2616]	Leu Ser Gly Lys Arg Leu Phe Val Val Asp Ala Phe Cys Gly Ala Asn
[2617]	115 120 125
[2618]	Pro Asp Thr Arg Leu Ser Val Arg Phe Ile Thr Glu Val Ala Trp Gln
[2619]	130 135 140
[2620]	Ala His Phe Val Lys Asn Met Phe Ile Arg Pro Ser Asp Glu Glu Leu
[2621]	145 150 155 160
[2622]	Ala Gly Phe Lys Pro Asp Phe Ile Val Met Asn Gly Ala Lys Cys Thr
[2623]	165 170 175
[2624]	Asn Pro Gln Trp Lys Glu Gln Gly Leu Asn Ser Glu Asn Phe Val Ala
[2625]	180 185 190
[2626]	Phe Asn Leu Thr Glu Arg Met Gln Leu Ile Gly Gly Thr Trp Tyr Gly
[2627]	195 200 205
[2628]	Gly Glu Met Lys Lys Gly Met Phe Ser Met Met Asn Tyr Leu Leu Pro
[2629]	210 215 220
[2630]	Leu Lys Gly Ile Ala Ser Met His Cys Ser Ala Asn Val Gly Glu Lys
[2631]	225 230 235 240
[2632]	Gly Asp Val Ala Val Phe Phe Gly Leu Ser Gly Thr Gly Lys Thr Thr
[2633]	245 250 255
[2634]	Leu Ser Thr Asp Pro Lys Arg Arg Leu Ile Gly Asp Asp Glu His Gly
[2635]	260 265 270
[2636]	Trp Asp Asp Asp Gly Val Phe Asn Phe Glu Gly Gly Cys Tyr Ala Lys
[2637]	275 280 285
[2638]	Thr Ile Lys Leu Ser Lys Glu Ala Glu Pro Glu Ile Tyr Asn Ala Ile
[2639]	290 295 300
[2640]	Arg Arg Asp Ala Leu Leu Glu Asn Val Thr Val Arg Glu Asp Gly Thr
[2641]	305 310 315 320
[2642]	Ile Asp Phe Asp Asp Gly Ser Lys Thr Glu Asn Thr Arg Val Ser Tyr
[2643]	325 330 335
[2644]	Pro Ile Tyr His Ile Asp Asn Ile Val Lys Pro Val Ser Lys Ala Gly
[2645]	340 345 350

[2646]	His Ala Thr Lys Val Ile Phe Leu Thr Ala Asp Ala Phe Gly Val Leu
[2647]	355 360 365
[2648]	Pro Pro Val Ser Arg Leu Thr Ala Asp Gln Thr Gln Tyr His Phe Leu
[2649]	370 375 380
[2650]	Ser Gly Phe Thr Ala Lys Leu Ala Gly Thr Glu Arg Gly Ile Thr Glu
[2651]	385 390 395 400
[2652]	Pro Thr Pro Thr Phe Ser Ala Cys Phe Gly Ala Ala Phe Leu Ser Leu
[2653]	405 410 415
[2654]	His Pro Thr Gln Tyr Ala Glu Val Leu Val Lys Arg Met Gln Ala Ala
[2655]	420 425 430
[2656]	Gly Ala Gln Ala Tyr Leu Val Asn Thr Gly Trp Asn Gly Thr Gly Lys
[2657]	435 440 445
[2658]	Arg Ile Ser Ile Lys Asp Thr Arg Ala Ile Ile Asp Ala Ile Leu Asn
[2659]	450 455 460
[2660]	Gly Ser Leu Asp Asn Ala Glu Thr Phe Thr Leu Pro Met Phe Asn Leu
[2661]	465 470 475 480
[2662]	Ala Ile Pro Thr Glu Leu Pro Gly Val Asp Thr Lys Ile Leu Asp Pro
[2663]	485 490 495
[2664]	Arg Asn Thr Tyr Ala Ser Pro Glu Gln Trp Gln Glu Lys Ala Glu Thr
[2665]	500 505 510
[2666]	Leu Ala Lys Leu Phe Ile Asp Asn Phe Asp Lys Tyr Thr Asp Thr Pro
[2667]	515 520 525
[2668]	Ala Gly Ala Ala Leu Val Ala Ala Gly Pro Lys Leu
[2669]	530 535 540
[2670]	<210> 40
[2671]	<211> 236
[2672]	<212> PRT
[2673]	<213> 乳酸乳球菌
[2674]	<400> 40
[2675]	Met Ser Glu Ile Thr Gln Leu Phe Gln Tyr Asn Thr Leu Gly Ala Leu
[2676]	1 5 10 15
[2677]	Met Ala Gly Leu Tyr Glu Gly Thr Met Thr Ile Gly Glu Leu Leu Lys
[2678]	20 25 30
[2679]	His Gly Asp Leu Gly Ile Gly Thr Leu Asp Ser Ile Asp Gly Glu Leu
[2680]	35 40 45
[2681]	Ile Val Leu Asp Gly Lys Ala Tyr Gln Ala Lys Gly Asp Lys Thr Ile
[2682]	50 55 60
[2683]	Val Glu Leu Thr Asp Asp Ile Lys Val Pro Tyr Ala Ala Val Val Pro
[2684]	65 70 75 80
[2685]	His Gln Ala Glu Val Val Phe Lys Gln Lys Phe Thr Val Ser Asp Lys
[2686]	85 90 95
[2687]	Glu Leu Glu Asp Arg Ile Glu Ser Tyr Phe Asp Gly Gln Asn Leu Phe

[2688]	100	105	110
[2689]	Arg Ser Ile Lys Ile Thr Gly Lys Phe Pro Lys Met His Val Arg Met		
[2690]	115	120	125
[2691]	Ile Pro Arg Ala Lys Ser Gly Thr Lys Phe Val Glu Val Ser Gln Asn		
[2692]	130	135	140
[2693]	Gln Pro Glu Tyr Thr Glu Glu Asn Ile Lys Gly Thr Ile Val Gly Ile		
[2694]	145	150	155
[2695]	Trp Thr Pro Glu Met Phe His Gly Val Ser Val Ala Gly Tyr His Leu		
[2696]	165	170	175
[2697]	His Phe Ile Ser Glu Asp Phe Thr Phe Gly Gly His Val Leu Asp Phe		
[2698]	180	185	190
[2699]	Ile Ile Asp Asn Gly Thr Val Glu Ile Gly Ala Ile Asp Gln Leu Asn		
[2700]	195	200	205
[2701]	Gln Ser Phe Pro Val Gln Asp Arg Lys Phe Leu Phe Ala Asp Leu Asp		
[2702]	210	215	220
[2703]	Ile Glu Ala Leu Lys Lys Asp Ile Asp Val Ala Glu		
[2704]	225	230	235
[2705]	<210> 41		
[2706]	<211> 239		
[2707]	<212> PRT		
[2708]	<213> 嗜热链球菌		
[2709]	<400> 41		
[2710]	Met Ser Glu Ala Ile Lys Leu Phe Gln Tyr Asn Thr Leu Gly Ala Leu		
[2711]	1	5	10
[2712]	Met Ala Gly Leu Tyr Gly Gly Thr Leu Thr Val Gly Glu Leu Leu Glu		
[2713]	20	25	30
[2714]	His Gly Asp Leu Gly Leu Gly Thr Leu Asp Ser Ile Asp Gly Glu Leu		
[2715]	35	40	45
[2716]	Ile Val Leu Asp Gly Lys Ala Tyr Gln Ala Lys Gly Ser Glu Gly Lys		
[2717]	50	55	60
[2718]	Val Glu Val Val Glu Val Ser Pro Asp Glu Lys Val Pro Tyr Ala Ala		
[2719]	65	70	75
[2720]	Val Val Pro His Gln Ala Glu Val Ile Phe Arg Gln Arg Tyr Glu Met		
[2721]	85	90	95
[2722]	Thr Asp Lys Glu Leu Glu Asp Arg Ile Glu Ser Tyr Tyr Asp Gly Val		
[2723]	100	105	110
[2724]	Asn Leu Phe Arg Ser Ile Lys Ile Lys Gly His Phe Lys His Met His		
[2725]	115	120	125
[2726]	Val Arg Met Ile Pro Lys Ser Asn Ala Asp Ile Lys Phe Ala Asp Val		
[2727]	130	135	140
[2728]	Ala Thr Arg Gln Pro Glu Tyr Glu Val Asp Asp Ile Ser Gly Thr Ile		
[2729]	145	150	155

[2730]	Val Gly Ile Trp Thr Pro Glu Met Phe His Gly Val Ser Val Ala Gly
[2731]	165 170 175
[2732]	Tyr His Leu His Phe Ile Ser Asp Asp Leu Thr Phe Gly Gly His Val
[2733]	180 185 190
[2734]	Met Asp Phe Val Ile Glu Asn Gly Ile Ile Glu Val Gly Pro Val Asp
[2735]	195 200 205
[2736]	Gln Leu Asp Gln Arg Phe Pro Val Gln Asp Arg Gln Tyr Leu Phe Ala
[2737]	210 215 220
[2738]	Lys Phe Asn Val Asp Glu Met Arg Lys Asp Ile Thr Lys Ala Glu
[2739]	225 230 235
[2740]	<210> 42
[2741]	<211> 285
[2742]	<212> PRT
[2743]	<213> 短芽孢杆菌
[2744]	<400> 42
[2745]	Met Lys Lys Asn Ile Ile Thr Ser Ile Thr Ser Leu Ala Leu Val Ala
[2746]	1 5 10 15
[2747]	Gly Leu Ser Leu Thr Ala Phe Ala Ala Thr Thr Ala Thr Val Pro Ala
[2748]	20 25 30
[2749]	Pro Pro Ala Lys Gln Glu Ser Lys Pro Ala Val Ala Ala Asn Pro Ala
[2750]	35 40 45
[2751]	Pro Lys Asn Val Leu Phe Gln Tyr Ser Thr Ile Asn Ala Leu Met Leu
[2752]	50 55 60
[2753]	Gly Gln Phe Glu Gly Asp Leu Thr Leu Lys Asp Leu Lys Leu Arg Gly
[2754]	65 70 75 80
[2755]	Asp Met Gly Leu Gly Thr Ile Asn Asp Leu Asp Gly Glu Met Ile Gln
[2756]	85 90 95
[2757]	Met Gly Thr Lys Phe Tyr Gln Ile Asp Ser Thr Gly Lys Leu Ser Glu
[2758]	100 105 110
[2759]	Leu Pro Glu Ser Val Lys Thr Pro Phe Ala Val Thr Thr His Phe Glu
[2760]	115 120 125
[2761]	Pro Lys Glu Lys Thr Thr Leu Thr Asn Val Gln Asp Tyr Asn Gln Leu
[2762]	130 135 140
[2763]	Thr Lys Met Leu Glu Glu Lys Phe Glu Asn Lys Asn Val Phe Tyr Ala
[2764]	145 150 155 160
[2765]	Val Lys Leu Thr Gly Thr Phe Lys Met Val Lys Ala Arg Thr Val Pro
[2766]	165 170 175
[2767]	Lys Gln Thr Arg Pro Tyr Pro Gln Leu Thr Glu Val Thr Lys Lys Gln
[2768]	180 185 190
[2769]	Ser Glu Phe Glu Phe Lys Asn Val Lys Gly Thr Leu Ile Gly Phe Tyr
[2770]	195 200 205
[2771]	Thr Pro Asn Tyr Ala Ala Ala Leu Asn Val Pro Gly Phe His Leu His

[2772]	210	215	220
[2773]	Phe Ile Thr Glu Asp Lys Thr Ser Gly Gly His Val Leu Asn Leu Gln		
[2774]	225	230	235
[2775]	Phe Asp Asn Ala Asn Leu Glu Ile Ser Pro Ile His Glu Phe Asp Val		
[2776]	245	250	255
[2777]	Gln Leu Pro His Thr Asp Asp Phe Ala His Ser Asp Leu Thr Gln Val		
[2778]	260	265	270
[2779]	Thr Thr Ser Gln Val His Gln Ala Glu Ser Glu Arg Lys		
[2780]	275	280	285
[2781]	<210> 43		
[2782]	<211> 259		
[2783]	<212> PRT		
[2784]	<213> 产气肠杆菌		
[2785]	<400> 43		
[2786]	Met Met Met His Ser Ser Ala Cys Asp Cys Glu Ala Ser Leu Cys Glu		
[2787]	1	5	10
[2788]	Thr Leu Arg Gly Phe Ser Ala Gln His Pro Asp Ser Val Ile Tyr Gln		
[2789]	20	25	30
[2790]	Thr Ser Leu Met Ser Ala Leu Leu Ser Gly Val Tyr Val Gly Glu Thr		
[2791]	35	40	45
[2792]	Thr Ile Ala Asp Leu Leu Ala His Gly Asp Phe Gly Leu Gly Thr Phe		
[2793]	50	55	60
[2794]	Asn Glu Leu Asp Gly Glu Met Ile Ala Phe Ser Ser Gln Val Tyr Gln		
[2795]	65	70	75
[2796]	Leu Arg Ala Asp Gly Ser Ala Arg Ala Ala Lys Pro Glu Gln Lys Thr		
[2797]	85	90	95
[2798]	Pro Phe Ala Val Met Thr Trp Phe Gln Pro Gln Tyr Arg Lys Thr Phe		
[2799]	100	105	110
[2800]	Asn Gly Pro Val Ser Arg Gln Gln Ile His Asp Val Ile Asp Gln Gln		
[2801]	115	120	125
[2802]	Ile Pro Ser Asp Asn Leu Phe Cys Val Arg Ile Asp Gly Asn Phe Arg		
[2803]	130	135	140
[2804]	His Ala His Thr Arg Thr Val Pro Arg Gln Thr Pro Pro Tyr Arg Ala		
[2805]	145	150	155
[2806]	Met Thr Asp Val Leu Asp Asp Gln Pro Val Phe Arg Phe Asn Gln Arg		
[2807]	165	170	175
[2808]	Glu Gly Val Leu Val Gly Phe Arg Thr Pro Gln His Met Gln Gly Ile		
[2809]	180	185	190
[2810]	Asn Val Ala Gly Tyr His Glu His Phe Ile Thr Asp Asp Arg Gln Gly		
[2811]	195	200	205
[2812]	Gly Gly His Leu Leu Asp Tyr Gln Leu Glu Ser Gly Val Leu Thr Phe		
[2813]	210	215	220

[2814]	Gly Glu Ile His Lys Leu Met Ile Asp Leu Pro Ala Asp Ser Ala Phe		
[2815]	225	230	235 240
[2816]	Leu Gln Ala Asn Leu His Pro Ser Asn Leu Asp Ala Ala Ile Arg Ala		
[2817]		245	250 255
[2818]	Val Glu Asn		
[2819]	<210> 44		
[2820]	<211> 1231		
[2821]	<212> PRT		
[2822]	<213> 结核分枝杆菌		
[2823]	<400> 44		
[2824]	Met Ala Asn Ile Ser Ser Pro Phe Gly Gln Asn Glu Trp Leu Val Glu		
[2825]	1	5	10 15
[2826]	Glu Met Tyr Arg Lys Phe Arg Asp Asp Pro Ser Ser Val Asp Pro Ser		
[2827]		20	25 30
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[2834]	Ala Gly Asn Gly Val Val Ala Ala Leu Ala Ala Lys Thr Ala Val Pro		
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[2836]	Pro Pro Ala Glu Gly Asp Glu Val Ala Val Leu Arg Gly Ala Ala Ala		
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[2842]	Ile Asn Asn Gln Leu Lys Arg Thr Arg Gly Gly Lys Ile Ser Phe Thr		
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[2844]	His Leu Leu Gly Tyr Ala Leu Val Gln Ala Val Lys Lys Phe Pro Asn		
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[2846]	Met Asn Arg His Tyr Thr Glu Val Asp Gly Lys Pro Thr Ala Val Thr		
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[2848]	Pro Ala His Thr Asn Leu Gly Leu Ala Ile Asp Leu Gln Gly Lys Asp		
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[2850]	Gly Lys Arg Ser Leu Val Val Ala Gly Ile Lys Arg Cys Glu Thr Met		
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[2852]	Arg Phe Ala Gln Phe Val Thr Ala Tyr Glu Asp Ile Val Arg Arg Ala		
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[2858]	Met Pro Gly Gln Gly Ala Ile Ile Gly Val Gly Ala Met Glu Tyr Pro
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[2862]	Gly Lys Leu Ile Thr Leu Thr Ser Thr Tyr Asp His Arg Ile Ile Gln
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[2864]	Gly Ala Glu Ser Gly Asp Phe Leu Arg Thr Ile His Glu Leu Leu Leu
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[2866]	Ser Asp Gly Phe Trp Asp Glu Val Phe Arg Glu Leu Ser Ile Pro Tyr
[2867]	340 345 350
[2868]	Leu Pro Val Arg Trp Ser Thr Asp Asn Pro Asp Ser Ile Val Asp Lys
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[2870]	Asn Ala Arg Val Met Asn Leu Ile Ala Ala Tyr Arg Asn Arg Gly His
[2871]	370 375 380
[2872]	Leu Met Ala Asp Thr Asp Pro Leu Arg Leu Asp Lys Ala Arg Phe Arg
[2873]	385 390 395 400
[2874]	Ser His Pro Asp Leu Glu Val Leu Thr His Gly Leu Thr Leu Trp Asp
[2875]	405 410 415
[2876]	Leu Asp Arg Val Phe Lys Val Asp Gly Phe Ala Gly Ala Gln Tyr Lys
[2877]	420 425 430
[2878]	Lys Leu Arg Asp Val Leu Gly Leu Leu Arg Asp Ala Tyr Cys Arg His
[2879]	435 440 445
[2880]	Ile Gly Val Glu Tyr Ala His Ile Leu Asp Pro Glu Gln Lys Glu Trp
[2881]	450 455 460
[2882]	Leu Glu Gln Arg Val Glu Thr Lys His Val Lys Pro Thr Val Ala Gln
[2883]	465 470 475 480
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[2886]	Phe Leu Gln Thr Lys Tyr Val Gly Gln Lys Arg Phe Ser Leu Glu Gly
[2887]	500 505 510
[2888]	Ala Glu Ser Val Ile Pro Met Met Asp Ala Ala Ile Asp Gln Cys Ala
[2889]	515 520 525
[2890]	Glu His Gly Leu Asp Glu Val Val Ile Gly Met Pro His Arg Gly Arg
[2891]	530 535 540
[2892]	Leu Asn Val Leu Ala Asn Ile Val Gly Lys Pro Tyr Ser Gln Ile Phe
[2893]	545 550 555 560
[2894]	Thr Glu Phe Glu Gly Asn Leu Asn Pro Ser Gln Ala His Gly Ser Gly
[2895]	565 570 575
[2896]	Asp Val Lys Tyr His Leu Gly Ala Thr Gly Leu Tyr Leu Gln Met Phe
[2897]	580 585 590

[2898]	Gly Asp Asn Asp Ile Gln Val Ser Leu Thr Ala Asn Pro Ser His Leu		
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[2900]	Glu Ala Val Asp Pro Val Leu Glu Gly Leu Val Arg Ala Lys Gln Asp		
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[2902]	Leu Leu Asp His Gly Ser Ile Asp Ser Asp Gly Gln Arg Ala Phe Ser		
[2903]	625	630	635 640
[2904]	Val Val Pro Leu Met Leu His Gly Asp Ala Ala Phe Ala Gly Gln Gly		
[2905]	645	650	655
[2906]	Val Val Ala Glu Thr Leu Asn Leu Ala Asn Leu Pro Gly Tyr Arg Val		
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[2908]	Gly Gly Thr Ile His Ile Ile Val Asn Asn Gln Ile Gly Phe Thr Thr		
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[2910]	Ala Pro Glu Tyr Ser Arg Ser Ser Glu Tyr Cys Thr Asp Val Ala Lys		
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[2912]	Met Ile Gly Ala Pro Ile Phe His Val Asn Gly Asp Asp Pro Glu Ala		
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[2914]	Cys Val Trp Val Ala Arg Leu Ala Val Asp Phe Arg Gln Arg Phe Lys		
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[2921]	770	775	780
[2922]	Arg Gly Asp Ile Ser Met Lys Glu Ala Glu Asp Ala Leu Arg Asp Tyr		
[2923]	785	790	795 800
[2924]	Gln Gly Gln Leu Glu Arg Val Phe Asn Glu Val Arg Glu Leu Glu Lys		
[2925]	805	810	815
[2926]	His Gly Val Gln Pro Ser Glu Ser Val Glu Ser Asp Gln Met Ile Pro		
[2927]	820	825	830
[2928]	Ala Gly Leu Ala Thr Ala Val Asp Lys Ser Leu Leu Ala Arg Ile Gly		
[2929]	835	840	845
[2930]	Asp Ala Phe Leu Ala Leu Pro Asn Gly Phe Thr Ala His Pro Arg Val		
[2931]	850	855	860
[2932]	Gln Pro Val Leu Glu Lys Arg Arg Glu Met Ala Tyr Glu Gly Lys Ile		
[2933]	865	870	875 880
[2934]	Asp Trp Ala Phe Gly Glu Leu Leu Ala Leu Gly Ser Leu Val Ala Glu		
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[2936]	Gly Lys Leu Val Arg Leu Ser Gly Gln Asp Ser Arg Arg Gly Thr Phe		
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[2938]	Ser Gln Arg His Ser Val Leu Ile Asp Arg His Thr Gly Glu Glu Phe		
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[2940]	Thr Pro Leu Gln Leu Leu Ala Thr Asn Ser Asp Gly Ser Pro Thr Gly
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[2942]	Gly Lys Phe Leu Val Tyr Asp Ser Pro Leu Ser Glu Tyr Ala Ala Val
[2943]	945 950 955 960
[2944]	Gly Phe Glu Tyr Gly Tyr Thr Val Gly Asn Pro Asp Ala Val Val Leu
[2945]	965 970 975
[2946]	Trp Glu Ala Gln Phe Gly Asp Phe Val Asn Gly Ala Gln Ser Ile Ile
[2947]	980 985 990
[2948]	Asp Glu Phe Ile Ser Ser Gly Glu Ala Lys Trp Gly Gln Leu Ser Asn
[2949]	995 1000 1005
[2950]	Val Val Leu Leu Leu Pro His Gly His Glu Gly Gln Gly Pro Asp
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[2955]	1040 1045 1050
[2956]	His Leu Leu Arg Arg His Ala Leu Asp Gly Ile Gln Arg Pro Leu
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[2958]	Ile Val Phe Thr Pro Lys Ser Met Leu Arg His Lys Ala Ala Val
[2959]	1070 1075 1080
[2960]	Ser Glu Ile Lys Asp Phe Thr Glu Ile Lys Phe Arg Ser Val Leu
[2961]	1085 1090 1095
[2962]	Glu Glu Pro Thr Tyr Glu Asp Gly Ile Gly Asp Arg Asn Lys Val
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[2965]	1115 1120 1125
[2966]	Ala Arg Lys Ala Lys Asp Asn Arg Asn Asp Leu Ala Ile Val Arg
[2967]	1130 1135 1140
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[2969]	1145 1150 1155
[2970]	Leu Asp Arg Tyr Glu Asn Val Lys Glu Phe Phe Trp Val Gln Glu
[2971]	1160 1165 1170
[2972]	Glu Pro Ala Asn Gln Gly Ala Trp Pro Arg Phe Gly Leu Glu Leu
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[2974]	Pro Glu Leu Leu Pro Asp Lys Leu Ala Gly Ile Lys Arg Ile Ser
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[2976]	Arg Arg Ala Met Ser Ala Pro Ser Ser Gly Ser Ser Lys Val His
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[2987]	Gln	Gly	Thr	Asn	Ala	Thr	Tyr	Ile	Asp	Glu	Ile	Tyr	Ala	Arg	Tyr	Glu				
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[2989]	Lys	Asp	Pro	Ser	Ser	Val	Asp	Ala	Glu	Trp	Gln	Glu	Phe	Phe	Lys	Ser				
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[2997]	Ile	Ala	Ala	Lys	Ala	Gln	Ala	Lys	Gly	Ala	Asp	Ile	Ser	Ser	Ala	Asp				
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[3009]	Thr	Tyr	Cys	Gln	Thr	Leu	Gly	Val	Glu	Phe	Met	His	Ile	Ser	Asn	Ala				
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[3016]				245						250				255						
[3017]	Lys	Arg	Phe	Gly	Leu	Asp	Gly	Ala	Glu	Ser	Leu	Ile	Pro	Ala	Leu	Glu				
[3018]			260					265					270							
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[3020]			275					280					285							
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[3025]	Pro Asp Ala Val Glu Gly Ser Gly Asp Val Lys Tyr His Leu Gly Ala			
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[3027]	Ser Ser Asp Arg Glu Phe Asp Gly Asn Arg Ile His Leu Ser Leu Thr			
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[3029]	Ala Asn Pro Ser His Leu Glu Ile Val Asp Pro Val Val Leu Gly Lys			
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[3031]	Val Arg Ala Lys Gln Asp Gln His Gly Asp Pro Pro Asp Met Arg Ile			
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[3033]	Ser Val Met Pro Leu Leu Met His Gly Asp Ala Ala Phe Ala Gly Gln			
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[3035]	Gly Val Val Ala Glu Cys Phe Gly Leu Ser Asp Leu Lys Gly Tyr Arg			
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[3037]	Thr Gly Gly Ser Val His Phe Ile Val Asn Asn Gln Ile Gly Phe Thr			
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[3039]	Thr Tyr Pro Arg Tyr Ser Arg Ser Ser Pro Tyr Pro Ser Asp Val Ala			
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[3041]	Lys Met Ile Asp Ala Pro Ile Phe His Val Asn Gly Asp Asp Pro Glu			
[3042]		450	455	460
[3043]	Ala Val Val Phe Ala Ala Lys Val Ala Thr Glu Phe Arg Gln Lys Phe			
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[3045]	His Lys Pro Val Val Ile Asp Met Phe Cys Tyr Arg Arg His Gly His			
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[3047]	Asn Glu Gly Asp Glu Pro Ala Phe Thr Gln Pro Val Met Tyr Lys Lys			
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[3051]	Ser Glu Gly Val Met Thr Glu Gly Glu Val Asp Lys Ala Lys Ala Asp			
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[3053]	Trp Arg Ala Arg Leu Asp Ala Glu Phe Glu Ala Gly Thr Ser Tyr Lys			
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[3055]	Pro Asn Lys Ala Asp Trp Leu Asp Gly Lys Trp Ala Gly Phe Lys Ile			
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[3057]	Ala Asp Gln Glu Glu Asp Ala Arg Arg Gly Val Thr Gly Val Asp Ile			
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[3059]	Thr Ala Leu Lys Asp Ile Gly Arg Lys Ile Thr Lys Val Pro Asp Gly			
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[3061]	Phe Arg Val His Arg Thr Ile Gln Arg Phe Leu Glu Asn Arg Ser Lys			
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[3063]	Ala Ile Asp Ser Gly Ala Gly Ile Asp Trp Ala Thr Gly Glu Ala Leu			
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[3065]	Ala Phe Cys Ser Leu Leu Asn Glu Asn His His Val Arg Leu Ser Gly			

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[3067]	Gln Asp Ser Glu Arg Gly Thr Phe Ser Gln Arg His Ser Val Leu Ile					
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[3069]	Asp Gln Glu Asp Glu Ser Arg Tyr Thr Pro Phe Asn His Leu Gly His					
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[3071]	Glu Gln Gly His Tyr Glu Val Ile Asn Ser Leu Leu Ser Glu Glu Ala					
[3072]		690		695		700
[3073]	Val Leu Gly Phe Glu Tyr Gly Tyr Ser Leu Ala Glu Pro Asn Thr Leu					
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[3075]	Thr Leu Trp Glu Ala Gln Phe Gly Asp Phe Ala Asn Gly Ala Gln Val					
[3076]		725		730		735
[3077]	Val Phe Asp Gln Phe Ile Ser Ser Gly Glu Arg Lys Trp Leu Arg Met					
[3078]		740		745		750
[3079]	Ser Gly Leu Val Cys Leu Leu Pro His Gly Tyr Glu Gly Gln Gly Pro					
[3080]		755		760		765
[3081]	Glu His Ser Ser Ala Arg Leu Glu Arg Tyr Leu Gln Met Cys Ala Glu					
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[3083]	Asp Asn Met Gln Val Val Tyr Pro Thr Thr Pro Ala Asn Tyr Phe His					
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[3085]	Val Leu Arg Arg Gln Leu His Arg Glu Ile Arg Lys Pro Leu Ile Leu					
[3086]		805		810		815
[3087]	Met Thr Pro Lys Ser Leu Leu Arg His Lys Arg Ala Val Ser Arg Leu					
[3088]		820		825		830
[3089]	Glu Glu Leu Ala Lys Gly Thr Thr Phe His Arg Ile Leu Tyr Asp Asp					
[3090]		835		840		845
[3091]	Ala Gln Met Leu Pro Thr Asp Ala Ile Lys Leu Val Pro Asp Glu Lys					
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[3093]	Ile Arg Arg Ile Val Leu Cys Ser Gly Lys Val Tyr Tyr Asp Leu Tyr					
[3094]	865		870		875	880
[3095]	Glu Glu Arg Glu Lys Arg Gly Ile Asp Asp Ile Tyr Leu Met Arg Val					
[3096]		885		890		895
[3097]	Glu Gln Leu Tyr Pro Val Pro Leu Lys Ala Leu Val Ala Glu Leu Ser					
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[3099]	Arg Phe Lys Lys Ala Glu Val Val Trp Cys Gln Glu Glu Pro Arg Asn					
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[3101]	Met Gly Ala Trp His Phe Ile Glu Pro Tyr Leu Glu Trp Val Leu Asn					
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[3103]	Gln Val Asn Gly Val Ser Arg Arg Pro Arg Tyr Val Gly Arg Ala Ala					
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[3105]	Ser Ala Ala Thr Ala Thr Gly Leu Met Ser Lys His Gln Ala Gln Leu					
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[3130]	Arg Ala Tyr Arg Met Arg Gly His Leu His Ala Asn Leu Asp Pro Leu	
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[3132]	Gly Ile Ala Lys Pro Leu Glu Asp Tyr Asn Glu Leu Ser Pro Glu Asn	
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[3150]	Val Leu Gly Met Ala His Arg Gly Arg Leu Asn Val Leu Ser Gln Val
[3151]	290 295 300
[3152]	Met Ala Lys Pro His Arg Ala Ile Phe His Glu Phe Lys Gly Gly Ser
[3153]	305 310 315 320
[3154]	Ala Ala Pro Asp Glu Val Glu Gly Ser Gly Asp Val Lys Tyr His Leu
[3155]	325 330 335
[3156]	Gly Ala Ser Ser Asp Arg Glu Phe Asp Gly Asn Lys Val His Leu Ser
[3157]	340 345 350
[3158]	Leu Thr Ala Asn Pro Ser His Leu Glu Ile Val Asp Pro Val Val Met
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[3160]	Gly Lys Ala Arg Ala Lys Gln Asp Tyr Leu Phe Gly Arg Gly Arg Glu
[3161]	370 375 380
[3162]	Glu Ile Val Pro Leu Glu Glu Arg Ala Lys Val Leu Pro Leu Leu Leu
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[3166]	Gly Leu Ser Gly Leu Arg Gly His Arg Val Ala Gly Thr Leu His Phe
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[3168]	Ile Ile Asn Asn Gln Ile Gly Phe Thr Thr Asn Pro Arg Phe Ser Arg
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[3174]	Val Ala Ile Glu Phe Arg Met Lys Phe His Lys Pro Val Val Val Asp
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[3186]	Asp Gly Ala Trp Ser Gly Leu Arg Thr Ala Asp Asn Gln Asp Glu Gln
[3187]	580 585 590
[3188]	Arg Arg Gly Lys Thr Ala Val Pro Val Lys Thr Leu Lys Glu Ile Gly
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[3190]	Lys Lys Leu Thr Glu Val Pro Lys Gly Phe Glu Ala His Lys Thr Ile
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[3194]	Ile Asp Trp Ser Thr Ala Glu Ala Leu Ala Phe Gly Ala Ile Leu Leu		
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[3196]	Asp Gly Asn Pro Ile Arg Leu Ser Gly Gln Asp Ser Glu Arg Gly Thr		
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[3198]	Phe Ser Gln Arg His Ser Val Leu Tyr Asp Gln Arg Asp Glu Thr Arg		
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[3200]	Tyr Ile Pro Leu Asn Asn Leu Ser Ala Ala Gln Ala Gly Tyr Glu Val		
[3201]	690	695	700
[3202]	Ile Asn Ser Met Leu Ser Glu Glu Ala Val Leu Gly Phe Glu Tyr Gly		
[3203]	705	710	715 720
[3204]	Tyr Ser Leu Ala Glu Pro Lys Ala Leu Thr Leu Trp Glu Ala Gln Phe		
[3205]		725	730 735
[3206]	Gly Asp Phe Ala Asn Gly Ala Gln Val Val Phe Asp Gln Phe Ile Ser		
[3207]		740	745 750
[3208]	Ser Gly Glu Arg Lys Trp Leu Arg Met Ser Gly Leu Val Cys Leu Leu		
[3209]		755	760 765
[3210]	Pro His Gly Tyr Glu Gly Gln Gly Pro Glu His Ser Ser Ala Arg Leu		
[3211]	770	775	780
[3212]	Glu Arg Phe Leu Gln Leu Cys Ala Glu Asp Asn Met Gln Val Ala Asn		
[3213]	785	790	795 800
[3214]	Cys Thr Thr Pro Ala Asn Tyr Phe His Ile Leu Arg Arg Gln Leu Lys		
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[3216]	Arg Asp Phe Arg Lys Pro Leu Ile Leu Met Thr Pro Lys Ser Leu Leu		
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[3218]	Arg His Lys Arg Ala Val Ser Thr Leu Pro Glu Ile Ser Gly Glu Ser		
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[3220]	Ser Phe His Arg Leu Leu Trp Asp Asp Ala Gln Leu Leu Pro Asn Gln		
[3221]	850	855	860
[3222]	Pro Ile Lys Leu Thr Lys Asp Ser Lys Ile Arg Arg Val Val Leu Cys		
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[3224]	Ser Gly Lys Val Tyr Tyr Asp Leu Tyr Glu Glu Arg Glu Lys Arg Gly		
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[3226]	Ile Asn Asp Ile Tyr Leu Leu Arg Val Glu Gln Leu Tyr Pro Phe Pro		
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[3228]	Ala Lys Ala Leu Ile Thr Glu Leu Ser Arg Phe Arg Asn Ala Glu Met		
[3229]		915	920 925
[3230]	Val Trp Cys Gln Glu Glu Pro Lys Asn Met Gly Ala Trp Ser Phe Ile		
[3231]	930	935	940
[3232]	Asp Pro Tyr Leu Glu Trp Val Leu Ala His Ile Asp Ala Lys His Gln		
[3233]	945	950	955 960

[3234]	Arg Val Arg Tyr Thr Gly Arg Pro Ala Ala Ala Ser Pro Ala Thr Gly
[3235]	965 970 975
[3236]	Leu Met Ser Lys His Leu Ala Gln Leu Ala Ala Leu Leu Glu Asp Ala
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[3238]	Leu Gly Glu
[3239]	995
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[3247]	Ile Glu Glu Ile Phe Gly Val Pro Gly Asp Tyr Asn Leu Gln Phe Leu
[3248]	20 25 30
[3249]	Asp Gln Ile Ile Ser Arg Glu Asp Met Lys Trp Ile Gly Asn Ala Asn
[3250]	35 40 45
[3251]	Glu Leu Asn Ala Ser Tyr Met Ala Asp Gly Tyr Ala Arg Thr Lys Lys
[3252]	50 55 60
[3253]	Ala Ala Ala Phe Leu Thr Thr Phe Gly Val Gly Glu Leu Ser Ala Ile
[3254]	65 70 75 80
[3255]	Asn Gly Leu Ala Gly Ser Tyr Ala Glu Asn Leu Pro Val Val Glu Ile
[3256]	85 90 95
[3257]	Val Gly Ser Pro Thr Ser Lys Val Gln Asn Asp Gly Lys Phe Val His
[3258]	100 105 110
[3259]	His Thr Leu Ala Asp Gly Asp Phe Lys His Phe Met Lys Met His Glu
[3260]	115 120 125
[3261]	Pro Val Thr Ala Ala Arg Thr Leu Leu Thr Ala Glu Asn Ala Thr Tyr
[3262]	130 135 140
[3263]	Glu Ile Asp Arg Val Leu Ser Gln Leu Leu Lys Glu Arg Lys Pro Val
[3264]	145 150 155 160
[3265]	Tyr Ile Asn Leu Pro Val Asp Val Ala Ala Ala Lys Ala Glu Lys Pro
[3266]	165 170 175
[3267]	Ala Leu Ser Leu Glu Lys Glu Ser Ser Thr Thr Asn Thr Thr Glu Gln
[3268]	180 185 190
[3269]	Val Ile Leu Ser Lys Ile Glu Glu Ser Leu Lys Asn Ala Gln Lys Pro
[3270]	195 200 205
[3271]	Val Val Ile Ala Gly His Glu Val Ile Ser Phe Gly Leu Glu Lys Thr
[3272]	210 215 220
[3273]	Val Thr Gln Phe Val Ser Glu Thr Lys Leu Pro Ile Thr Thr Leu Asn
[3274]	225 230 235 240
[3275]	Phe Gly Lys Ser Ala Val Asp Glu Ser Leu Pro Ser Phe Leu Gly Ile

[3276]		245		250		255
[3277]	Tyr Asn Gly Lys Leu Ser Glu Ile Ser Leu Lys Asn Phe Val Glu Ser					
[3278]		260		265		270
[3279]	Ala Asp Phe Ile Leu Met Leu Gly Val Lys Leu Thr Asp Ser Ser Thr					
[3280]		275		280		285
[3281]	Gly Ala Phe Thr His His Leu Asp Glu Asn Lys Met Ile Ser Leu Asn					
[3282]		290		295		300
[3283]	Ile Asp Glu Gly Ile Ile Phe Asn Lys Val Val Glu Asp Phe Asp Phe					
[3284]	305		310		315	320
[3285]	Arg Ala Val Val Ser Ser Leu Ser Glu Leu Lys Gly Ile Glu Tyr Glu					
[3286]		325		330		335
[3287]	Gly Gln Tyr Ile Asp Lys Gln Tyr Glu Glu Phe Ile Pro Ser Ser Ala					
[3288]		340		345		350
[3289]	Pro Leu Ser Gln Asp Arg Leu Trp Gln Ala Val Glu Ser Leu Thr Gln					
[3290]		355		360		365
[3291]	Ser Asn Glu Thr Ile Val Ala Glu Gln Gly Thr Ser Phe Phe Gly Ala					
[3292]		370		375		380
[3293]	Ser Thr Ile Phe Leu Lys Ser Asn Ser Arg Phe Ile Gly Gln Pro Leu					
[3294]	385		390		395	400
[3295]	Trp Gly Ser Ile Gly Tyr Thr Phe Pro Ala Ala Leu Gly Ser Gln Ile					
[3296]		405		410		415
[3297]	Ala Asp Lys Glu Ser Arg His Leu Leu Phe Ile Gly Asp Gly Ser Leu					
[3298]		420		425		430
[3299]	Gln Leu Thr Val Gln Glu Leu Gly Leu Ser Ile Arg Glu Lys Leu Asn					
[3300]		435		440		445
[3301]	Pro Ile Cys Phe Ile Ile Asn Asn Asp Gly Tyr Thr Val Glu Arg Glu					
[3302]		450		455		460
[3303]	Ile His Gly Pro Thr Gln Ser Tyr Asn Asp Ile Pro Met Trp Asn Tyr					
[3304]	465		470		475	480
[3305]	Ser Lys Leu Pro Glu Thr Phe Gly Ala Thr Glu Asp Arg Val Val Ser					
[3306]		485		490		495
[3307]	Lys Ile Val Arg Thr Glu Asn Glu Phe Val Ser Val Met Lys Glu Ala					
[3308]		500		505		510
[3309]	Gln Ala Asp Val Asn Arg Met Tyr Trp Ile Glu Leu Val Leu Glu Lys					
[3310]		515		520		525
[3311]	Glu Asp Ala Pro Lys Leu Leu Lys Lys Met Gly Lys Leu Phe Ala Glu					
[3312]		530		535		540
[3313]	Gln Asn Lys					
[3314]	545					
[3315]	<210> 48					
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[3317]	<212> DNA					

[3318]	<213> 东方伊萨酵母
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[3322]	gttgaaggca ttagatgggt cggtaacgct aacgagttga atgccggcta cgaagctgat 180
[3323]	ggttatgcaa gaatcaatgg atttgcattc ctaatcacca cttttggtgt cggatgaattg 240
[3324]	tctgccgtca atgccattgc aggttcttat gctgaacacg tccattgat ccatattgtt 300
[3325]	ggtatgcctt cttgtctgc tatgaagaac aacttgttgt tacaccatac cttgggtgac 360
[3326]	acaagattcg acaacttcac cgaaatgtca aagaaaatca gtgcaaaggt tgaaattgtt 420
[3327]	tacgatttgg aatcagctcc aaaattaatt aataactga ttgaaaccgc ttatcacaca 480
[3328]	aagagaccag tctacttggg acttcttcc aactttgtg atgaattggt tccagcggca 540
[3329]	ttagttaagg aaaacaagt acatttagaa gaacctctaa acaaccccg tgcgaagaa 600
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[3331]	gacgcttgct ctgcaagaca taacatttct aaggaagtga gagagttggc taaattgact 720
[3332]	aaattccctg tcttcaccac cccaatgggt aaatctactg ttgatgaaga tgatgaagaa 780
[3333]	ttctttggct tatacttggg ttctctatct gctccagatg ttaaggacat tgttgccca 840
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[3347]	gaagataaga ttagagtcac tgaattatc ttggatgaat tcgatgtcc agaaatcttg 1680
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[3349]	<210> 49
[3350]	<211> 575
[3351]	<212> PRT
[3352]	<213> 东方伊萨酵母
[3353]	<400> 49
[3354]	Met Thr Asp Lys Ile Ser Leu Gly Thr Tyr Leu Phe Glu Lys Leu Lys
[3355]	1 5 10 15
[3356]	Glu Ala Gly Ser Tyr Ser Ile Phe Gly Val Pro Gly Asp Phe Asn Leu
[3357]	20 25 30
[3358]	Ala Leu Leu Asp His Val Lys Glu Val Glu Gly Ile Arg Trp Val Gly
[3359]	35 40 45

[3360]	Asn Ala Asn Glu Leu Asn Ala Gly Tyr Glu Ala Asp Gly Tyr Ala Arg
[3361]	50 55 60
[3362]	Ile Asn Gly Phe Ala Ser Leu Ile Thr Thr Phe Gly Val Gly Glu Leu
[3363]	65 70 75 80
[3364]	Ser Ala Val Asn Ala Ile Ala Gly Ser Tyr Ala Glu His Val Pro Leu
[3365]	85 90 95
[3366]	Ile His Ile Val Gly Met Pro Ser Leu Ser Ala Met Lys Asn Asn Leu
[3367]	100 105 110
[3368]	Leu Leu His His Thr Leu Gly Asp Thr Arg Phe Asp Asn Phe Thr Glu
[3369]	115 120 125
[3370]	Met Ser Lys Lys Ile Ser Ala Lys Val Glu Ile Val Tyr Asp Leu Glu
[3371]	130 135 140
[3372]	Ser Ala Pro Lys Leu Ile Asn Asn Leu Ile Glu Thr Ala Tyr His Thr
[3373]	145 150 155 160
[3374]	Lys Arg Pro Val Tyr Leu Gly Leu Pro Ser Asn Phe Ala Asp Glu Leu
[3375]	165 170 175
[3376]	Val Pro Ala Ala Leu Val Lys Glu Asn Lys Leu His Leu Glu Glu Pro
[3377]	180 185 190
[3378]	Leu Asn Asn Pro Val Ala Glu Glu Glu Phe Ile His Asn Val Val Glu
[3379]	195 200 205
[3380]	Met Val Lys Lys Ala Glu Lys Pro Ile Ile Leu Val Asp Ala Cys Ala
[3381]	210 215 220
[3382]	Ala Arg His Asn Ile Ser Lys Glu Val Arg Glu Leu Ala Lys Leu Thr
[3383]	225 230 235 240
[3384]	Lys Phe Pro Val Phe Thr Thr Pro Met Gly Lys Ser Thr Val Asp Glu
[3385]	245 250 255
[3386]	Asp Asp Glu Glu Phe Phe Gly Leu Tyr Leu Gly Ser Leu Ser Ala Pro
[3387]	260 265 270
[3388]	Asp Val Lys Asp Ile Val Gly Pro Thr Asp Cys Ile Leu Ser Leu Gly
[3389]	275 280 285
[3390]	Gly Leu Pro Ser Asp Phe Asn Thr Gly Ser Phe Ser Tyr Gly Tyr Thr
[3391]	290 295 300
[3392]	Thr Lys Asn Val Val Glu Phe His Ser Asn Tyr Cys Lys Phe Lys Ser
[3393]	305 310 315 320
[3394]	Ala Thr Tyr Glu Asn Leu Met Met Lys Gly Ala Val Gln Arg Leu Ile
[3395]	325 330 335
[3396]	Ser Glu Leu Lys Asn Ile Lys Tyr Ser Asn Val Ser Thr Leu Ser Pro
[3397]	340 345 350
[3398]	Pro Lys Ser Lys Phe Ala Tyr Glu Ser Ala Lys Val Ala Pro Glu Gly
[3399]	355 360 365
[3400]	Ile Ile Thr Gln Asp Tyr Leu Trp Lys Arg Leu Ser Tyr Phe Leu Lys
[3401]	370 375 380

[3402]	Pro Arg Asp Ile Ile Val Thr Glu Thr Gly Thr Ser Ser Phe Gly Val		
[3403]	385	390	395 400
[3404]	Leu Ala Thr His Leu Pro Arg Asp Ser Lys Ser Ile Ser Gln Val Leu		
[3405]	405	410	415
[3406]	Trp Gly Ser Ile Gly Phe Ser Leu Pro Ala Ala Val Gly Ala Ala Phe		
[3407]	420	425	430
[3408]	Ala Ala Glu Asp Ala His Lys Gln Thr Gly Glu Gln Glu Arg Arg Thr		
[3409]	435	440	445
[3410]	Val Leu Phe Ile Gly Asp Gly Ser Leu Gln Leu Thr Val Gln Ser Ile		
[3411]	450	455	460
[3412]	Ser Asp Ala Ala Arg Trp Asn Ile Lys Pro Tyr Ile Phe Ile Leu Asn		
[3413]	465	470	475 480
[3414]	Asn Arg Gly Tyr Thr Ile Glu Lys Leu Ile His Gly Arg His Glu Asp		
[3415]	485	490	495
[3416]	Tyr Asn Gln Ile Gln Pro Trp Asp His Gln Leu Leu Leu Lys Leu Phe		
[3417]	500	505	510
[3418]	Ala Asp Lys Thr Gln Tyr Glu Asn His Val Val Lys Ser Ala Lys Asp		
[3419]	515	520	525
[3420]	Leu Asp Ala Leu Met Lys Asp Glu Ala Phe Asn Lys Glu Asp Lys Ile		
[3421]	530	535	540
[3422]	Arg Val Ile Glu Leu Phe Leu Asp Glu Phe Asp Ala Pro Glu Ile Leu		
[3423]	545	550	555 560
[3424]	Val Ala Gln Ala Lys Leu Ser Asp Glu Ile Asn Ser Lys Ala Ala		
[3425]	565	570	575
[3426]	<210> 50		
[3427]	<211> 563		
[3428]	<212> PRT		
[3429]	<213> 酿酒酵母		
[3430]	<400> 50		
[3431]	Met Ser Glu Ile Thr Leu Gly Lys Tyr Leu Phe Glu Arg Leu Lys Gln		
[3432]	1	5	10 15
[3433]	Val Asn Val Asn Thr Val Phe Gly Leu Pro Gly Asp Phe Asn Leu Ser		
[3434]	20	25	30
[3435]	Leu Leu Asp Lys Ile Tyr Glu Val Glu Gly Met Arg Trp Ala Gly Asn		
[3436]	35	40	45
[3437]	Ala Asn Glu Leu Asn Ala Ala Tyr Ala Ala Asp Gly Tyr Ala Arg Ile		
[3438]	50	55	60
[3439]	Lys Gly Met Ser Cys Ile Ile Thr Thr Phe Gly Val Gly Glu Leu Ser		
[3440]	65	70	75 80
[3441]	Ala Leu Asn Gly Ile Ala Gly Ser Tyr Ala Glu His Val Gly Val Leu		
[3442]	85	90	95
[3443]	His Val Val Gly Val Pro Ser Ile Ser Ala Gln Ala Lys Gln Leu Leu		

[3444]	100	105	110
[3445]	Leu His His Thr Leu Gly Asn Gly Asp Phe Thr Val Phe His Arg Met		
[3446]	115	120	125
[3447]	Ser Ala Asn Ile Ser Glu Thr Thr Ala Met Ile Thr Asp Ile Ala Thr		
[3448]	130	135	140
[3449]	Ala Pro Ala Glu Ile Asp Arg Cys Ile Arg Thr Thr Tyr Val Thr Gln		
[3450]	145	150	155
[3451]	Arg Pro Val Tyr Leu Gly Leu Pro Ala Asn Leu Val Asp Leu Asn Val		
[3452]	165	170	175
[3453]	Pro Ala Lys Leu Leu Gln Thr Pro Ile Asp Met Ser Leu Lys Pro Asn		
[3454]	180	185	190
[3455]	Asp Ala Glu Ser Glu Lys Glu Val Ile Asp Thr Ile Leu Ala Leu Val		
[3456]	195	200	205
[3457]	Lys Asp Ala Lys Asn Pro Val Ile Leu Ala Asp Ala Cys Cys Ser Arg		
[3458]	210	215	220
[3459]	His Asp Val Lys Ala Glu Thr Lys Lys Leu Ile Asp Leu Thr Gln Phe		
[3460]	225	230	235
[3461]	Pro Ala Phe Val Thr Pro Met Gly Lys Gly Ser Ile Asp Glu Gln His		
[3462]	245	250	255
[3463]	Pro Arg Tyr Gly Gly Val Tyr Val Gly Thr Leu Ser Lys Pro Glu Val		
[3464]	260	265	270
[3465]	Lys Glu Ala Val Glu Ser Ala Asp Leu Ile Leu Ser Val Gly Ala Leu		
[3466]	275	280	285
[3467]	Leu Ser Asp Phe Asn Thr Gly Ser Phe Ser Tyr Ser Tyr Lys Thr Lys		
[3468]	290	295	300
[3469]	Asn Ile Val Glu Phe His Ser Asp His Met Lys Ile Arg Asn Ala Thr		
[3470]	305	310	315
[3471]	Phe Pro Gly Val Gln Met Lys Phe Val Leu Gln Lys Leu Leu Thr Thr		
[3472]	325	330	335
[3473]	Ile Ala Asp Ala Ala Lys Gly Tyr Lys Pro Val Ala Val Pro Ala Arg		
[3474]	340	345	350
[3475]	Thr Pro Ala Asn Ala Ala Val Pro Ala Ser Thr Pro Leu Lys Gln Glu		
[3476]	355	360	365
[3477]	Trp Met Trp Asn Gln Leu Gly Asn Phe Leu Gln Glu Gly Asp Val Val		
[3478]	370	375	380
[3479]	Ile Ala Glu Thr Gly Thr Ser Ala Phe Gly Ile Asn Gln Thr Thr Phe		
[3480]	385	390	395
[3481]	Pro Asn Asn Thr Tyr Gly Ile Ser Gln Val Leu Trp Gly Ser Ile Gly		
[3482]	405	410	415
[3483]	Phe Thr Thr Gly Ala Thr Leu Gly Ala Ala Phe Ala Ala Glu Glu Ile		
[3484]	420	425	430
[3485]	Asp Pro Lys Lys Arg Val Ile Leu Phe Ile Gly Asp Gly Ser Leu Gln		

[3486]	435	440	445
[3487]	Leu Thr Val Gln Glu Ile Ser Thr Met Ile Arg Trp Gly Leu Lys Pro		
[3488]	450	455	460
[3489]	Tyr Leu Phe Val Leu Asn Asn Asp Gly Tyr Thr Ile Glu Lys Leu Ile		
[3490]	465	470	475
[3491]	His Gly Pro Lys Ala Gln Tyr Asn Glu Ile Gln Gly Trp Asp His Leu		
[3492]	485	490	495
[3493]	Ser Leu Leu Pro Thr Phe Gly Ala Lys Asp Tyr Glu Thr His Arg Val		
[3494]	500	505	510
[3495]	Ala Thr Thr Gly Glu Trp Asp Lys Leu Thr Gln Asp Lys Ser Phe Asn		
[3496]	515	520	525
[3497]	Asp Asn Ser Lys Ile Arg Met Ile Glu Ile Met Leu Pro Val Phe Asp		
[3498]	530	535	540
[3499]	Ala Pro Gln Asn Leu Val Glu Gln Ala Lys Leu Thr Ala Ala Thr Asn		
[3500]	545	550	555
[3501]	Ala Lys Gln		
[3502]	<210> 51		
[3503]	<211> 563		
[3504]	<212> PRT		
[3505]	<213> 乳酸克鲁维酵母(Kluyveromyces lactis)		
[3506]	<400> 51		
[3507]	Met Ser Glu Ile Thr Leu Gly Arg Tyr Leu Phe Glu Arg Leu Lys Gln		
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[3509]	Val Glu Val Gln Thr Ile Phe Gly Leu Pro Gly Asp Phe Asn Leu Ser		
[3510]	20	25	30
[3511]	Leu Leu Asp Asn Ile Tyr Glu Val Pro Gly Met Arg Trp Ala Gly Asn		
[3512]	35	40	45
[3513]	Ala Asn Glu Leu Asn Ala Ala Tyr Ala Ala Asp Gly Tyr Ala Arg Leu		
[3514]	50	55	60
[3515]	Lys Gly Met Ser Cys Ile Ile Thr Thr Phe Gly Val Gly Glu Leu Ser		
[3516]	65	70	75
[3517]	Ala Leu Asn Gly Ile Ala Gly Ser Tyr Ala Glu His Val Gly Val Leu		
[3518]	85	90	95
[3519]	His Val Val Gly Val Pro Ser Val Ser Ser Gln Ala Lys Gln Leu Leu		
[3520]	100	105	110
[3521]	Leu His His Thr Leu Gly Asn Gly Asp Phe Thr Val Phe His Arg Met		
[3522]	115	120	125
[3523]	Ser Ser Asn Ile Ser Glu Thr Thr Ala Met Ile Thr Asp Ile Asn Thr		
[3524]	130	135	140
[3525]	Ala Pro Ala Glu Ile Asp Arg Cys Ile Arg Thr Thr Tyr Val Ser Gln		
[3526]	145	150	155
[3527]	Arg Pro Val Tyr Leu Gly Leu Pro Ala Asn Leu Val Asp Leu Thr Val		

[3528]	165	170	175
[3529]	Pro Ala Ser Leu Leu Asp Thr Pro Ile Asp Leu Ser Leu Lys Pro Asn		
[3530]	180	185	190
[3531]	Asp Pro Glu Ala Glu Glu Glu Val Ile Glu Asn Val Leu Gln Leu Ile		
[3532]	195	200	205
[3533]	Lys Glu Ala Lys Asn Pro Val Ile Leu Ala Asp Ala Cys Cys Ser Arg		
[3534]	210	215	220
[3535]	His Asp Ala Lys Ala Glu Thr Lys Lys Leu Ile Asp Leu Thr Gln Phe		
[3536]	225	230	235
[3537]	Pro Ala Phe Val Thr Pro Met Gly Lys Gly Ser Ile Asp Glu Lys His		
[3538]	245	250	255
[3539]	Pro Arg Phe Gly Gly Val Tyr Val Gly Thr Leu Ser Ser Pro Ala Val		
[3540]	260	265	270
[3541]	Lys Glu Ala Val Glu Ser Ala Asp Leu Val Leu Ser Val Gly Ala Leu		
[3542]	275	280	285
[3543]	Leu Ser Asp Phe Asn Thr Gly Ser Phe Ser Tyr Ser Tyr Lys Thr Lys		
[3544]	290	295	300
[3545]	Asn Ile Val Glu Phe His Ser Asp Tyr Thr Lys Ile Arg Ser Ala Thr		
[3546]	305	310	315
[3547]	Phe Pro Gly Val Gln Met Lys Phe Ala Leu Gln Lys Leu Leu Thr Lys		
[3548]	325	330	335
[3549]	Val Ala Asp Ala Ala Lys Gly Tyr Lys Pro Val Pro Val Pro Ser Glu		
[3550]	340	345	350
[3551]	Pro Glu His Asn Glu Ala Val Ala Asp Ser Thr Pro Leu Lys Gln Glu		
[3552]	355	360	365
[3553]	Trp Val Trp Thr Gln Val Gly Glu Phe Leu Arg Glu Gly Asp Val Val		
[3554]	370	375	380
[3555]	Ile Thr Glu Thr Gly Thr Ser Ala Phe Gly Ile Asn Gln Thr His Phe		
[3556]	385	390	395
[3557]	Pro Asn Asn Thr Tyr Gly Ile Ser Gln Val Leu Trp Gly Ser Ile Gly		
[3558]	405	410	415
[3559]	Phe Thr Thr Gly Ala Thr Leu Gly Ala Ala Phe Ala Ala Glu Glu Ile		
[3560]	420	425	430
[3561]	Asp Pro Lys Lys Arg Val Ile Leu Phe Ile Gly Asp Gly Ser Leu Gln		
[3562]	435	440	445
[3563]	Leu Thr Val Gln Glu Ile Ser Thr Met Ile Arg Trp Gly Leu Lys Pro		
[3564]	450	455	460
[3565]	Tyr Leu Phe Val Leu Asn Asn Asp Gly Tyr Thr Ile Glu Arg Leu Ile		
[3566]	465	470	475
[3567]	His Gly Glu Thr Ala Gln Tyr Asn Cys Ile Gln Asn Trp Gln His Leu		
[3568]	485	490	495
[3569]	Glu Leu Leu Pro Thr Phe Gly Ala Lys Asp Tyr Glu Ala Val Arg Val		

[3570]	500	505	510
[3571]	Ser Thr Thr Gly Glu Trp Asn Lys Leu Thr Thr Asp Glu Lys Phe Gln		
[3572]	515	520	525
[3573]	Asp Asn Thr Arg Ile Arg Leu Ile Glu Val Met Leu Pro Thr Met Asp		
[3574]	530	535	540
[3575]	Ala Pro Ser Asn Leu Val Lys Gln Ala Gln Leu Thr Ala Ala Thr Asn		
[3576]	545	550	555
[3577]	Ala Lys Asn		560
[3578]	<210> 52		
[3579]	<211> 568		
[3580]	<212> PRT		
[3581]	<213> 运动发酵单胞菌 (<i>Zymomonas mobilis</i>)		
[3582]	<400> 52		
[3583]	Met Ser Tyr Thr Val Gly Thr Tyr Leu Ala Glu Arg Leu Val Gln Ile		
[3584]	1	5	10
[3585]	Gly Leu Lys His His Phe Ala Val Ala Gly Asp Tyr Asn Leu Val Leu		15
[3586]	20	25	30
[3587]	Leu Asp Asn Leu Leu Leu Asn Lys Asn Met Glu Gln Val Tyr Cys Cys		
[3588]	35	40	45
[3589]	Asn Glu Leu Asn Cys Gly Phe Ser Ala Glu Gly Tyr Ala Arg Ala Lys		
[3590]	50	55	60
[3591]	Gly Ala Ala Ala Ala Val Val Thr Tyr Ser Val Gly Ala Leu Ser Ala		
[3592]	65	70	75
[3593]	Phe Asp Ala Ile Gly Gly Ala Tyr Ala Glu Asn Leu Pro Val Ile Leu		80
[3594]	85	90	95
[3595]	Ile Ser Gly Ala Pro Asn Asn Asn Asp His Ala Ala Gly His Val Leu		
[3596]	100	105	110
[3597]	His His Ala Leu Gly Lys Thr Asp Tyr His Tyr Gln Leu Glu Met Ala		
[3598]	115	120	125
[3599]	Lys Asn Ile Thr Ala Ala Ala Glu Ala Ile Tyr Thr Pro Glu Glu Ala		
[3600]	130	135	140
[3601]	Pro Ala Lys Ile Asp His Val Ile Lys Thr Ala Leu Arg Glu Lys Lys		
[3602]	145	150	155
[3603]	Pro Val Tyr Leu Glu Ile Ala Cys Asn Ile Ala Ser Met Pro Cys Ala		160
[3604]	165	170	175
[3605]	Ala Pro Gly Pro Ala Ser Ala Leu Phe Asn Asp Glu Ala Ser Asp Glu		
[3606]	180	185	190
[3607]	Ala Ser Leu Asn Ala Ala Val Glu Glu Thr Leu Lys Phe Ile Ala Asn		
[3608]	195	200	205
[3609]	Arg Asp Lys Val Ala Val Leu Val Gly Ser Lys Leu Arg Ala Ala Gly		
[3610]	210	215	220
[3611]	Ala Glu Glu Ala Ala Val Lys Phe Ala Asp Ala Leu Gly Gly Ala Val		

[3612]	225	230	235	240
[3613]	Ala Thr Met Ala Ala Ala Lys Ser Phe Phe Pro Glu Glu Asn Pro His			
[3614]		245	250	255
[3615]	Tyr Ile Gly Thr Ser Trp Gly Glu Val Ser Tyr Pro Gly Val Glu Lys			
[3616]		260	265	270
[3617]	Thr Met Lys Glu Ala Asp Ala Val Ile Ala Leu Ala Pro Val Phe Asn			
[3618]		275	280	285
[3619]	Asp Tyr Ser Thr Thr Gly Trp Thr Asp Ile Pro Asp Pro Lys Lys Leu			
[3620]		290	295	300
[3621]	Val Leu Ala Glu Pro Arg Ser Val Val Val Asn Gly Ile Arg Phe Pro			
[3622]	305	310	315	320
[3623]	Ser Val His Leu Lys Asp Tyr Leu Thr Arg Leu Ala Gln Lys Val Ser			
[3624]		325	330	335
[3625]	Lys Lys Thr Gly Ala Leu Asp Phe Phe Lys Ser Leu Asn Ala Gly Glu			
[3626]		340	345	350
[3627]	Leu Lys Lys Ala Ala Pro Ala Asp Pro Ser Ala Pro Leu Val Asn Ala			
[3628]		355	360	365
[3629]	Glu Ile Ala Arg Gln Val Glu Ala Leu Leu Thr Pro Asn Thr Thr Val			
[3630]		370	375	380
[3631]	Ile Ala Glu Thr Gly Asp Ser Trp Phe Asn Ala Gln Arg Met Lys Leu			
[3632]	385	390	395	400
[3633]	Pro Asn Gly Ala Arg Val Glu Tyr Glu Met Gln Trp Gly His Ile Gly			
[3634]		405	410	415
[3635]	Trp Ser Val Pro Ala Ala Phe Gly Tyr Ala Val Gly Ala Pro Glu Arg			
[3636]		420	425	430
[3637]	Arg Asn Ile Leu Met Val Gly Asp Gly Ser Phe Gln Leu Thr Ala Gln			
[3638]		435	440	445
[3639]	Glu Val Ala Gln Met Val Arg Leu Lys Leu Pro Val Ile Ile Phe Leu			
[3640]		450	455	460
[3641]	Ile Asn Asn Tyr Gly Tyr Thr Ile Glu Val Met Ile His Asp Gly Pro			
[3642]	465	470	475	480
[3643]	Tyr Asn Asn Ile Lys Asn Trp Asp Tyr Ala Gly Leu Met Glu Val Phe			
[3644]		485	490	495
[3645]	Asn Gly Asn Gly Gly Tyr Asp Ser Gly Ala Gly Lys Gly Leu Lys Ala			
[3646]		500	505	510
[3647]	Lys Thr Gly Gly Glu Leu Ala Glu Ala Ile Lys Val Ala Leu Ala Asn			
[3648]		515	520	525
[3649]	Thr Asp Gly Pro Thr Leu Ile Glu Cys Phe Ile Gly Arg Glu Asp Cys			
[3650]		530	535	540
[3651]	Thr Glu Glu Leu Val Lys Trp Gly Lys Arg Val Ala Ala Ala Asn Ser			
[3652]	545	550	555	560
[3653]	Arg Lys Pro Val Asn Lys Leu Leu			

[3654]	565
[3655]	<210> 53
[3656]	<211> 557
[3657]	<212> PRT
[3658]	<213> 巴斯德醋酸杆菌(Acetobacter pasteurianus)
[3659]	<400> 53
[3660]	Met Thr Tyr Thr Val Gly Met Tyr Leu Ala Glu Arg Leu Val Gln Ile
[3661]	1 5 10 15
[3662]	Gly Leu Lys His His Phe Ala Val Gly Gly Asp Tyr Asn Leu Val Leu
[3663]	20 25 30
[3664]	Leu Asp Gln Leu Leu Leu Asn Lys Asp Met Lys Gln Ile Tyr Cys Cys
[3665]	35 40 45
[3666]	Asn Glu Leu Asn Cys Gly Phe Ser Ala Glu Gly Tyr Ala Arg Ser Asn
[3667]	50 55 60
[3668]	Gly Ala Ala Ala Ala Val Val Thr Phe Ser Val Gly Ala Ile Ser Ala
[3669]	65 70 75 80
[3670]	Met Asn Ala Leu Gly Gly Ala Tyr Ala Glu Asn Leu Pro Val Ile Leu
[3671]	85 90 95
[3672]	Ile Ser Gly Ala Pro Asn Ser Asn Asp Gln Gly Thr Gly His Ile Leu
[3673]	100 105 110
[3674]	His His Thr Ile Gly Lys Thr Asp Tyr Ser Tyr Gln Leu Glu Met Ala
[3675]	115 120 125
[3676]	Arg Gln Val Thr Cys Ala Ala Glu Ser Ile Thr Asp Ala His Ser Ala
[3677]	130 135 140
[3678]	Pro Ala Lys Ile Asp His Val Ile Arg Thr Ala Leu Arg Glu Arg Lys
[3679]	145 150 155 160
[3680]	Pro Ala Tyr Leu Asp Ile Ala Cys Asn Ile Ala Ser Glu Pro Cys Val
[3681]	165 170 175
[3682]	Arg Pro Gly Pro Val Ser Ser Leu Leu Ser Glu Pro Glu Ile Asp His
[3683]	180 185 190
[3684]	Thr Ser Leu Lys Ala Ala Val Asp Ala Thr Val Ala Leu Leu Lys Asn
[3685]	195 200 205
[3686]	Arg Pro Ala Pro Val Met Leu Leu Gly Ser Lys Leu Arg Ala Ala Asn
[3687]	210 215 220
[3688]	Ala Leu Ala Ala Thr Glu Thr Leu Ala Asp Lys Leu Gln Cys Ala Val
[3689]	225 230 235 240
[3690]	Thr Ile Met Ala Ala Ala Lys Gly Phe Phe Pro Glu Asp His Ala Gly
[3691]	245 250 255
[3692]	Phe Arg Gly Leu Tyr Trp Gly Glu Val Ser Asn Pro Gly Val Gln Glu
[3693]	260 265 270
[3694]	Leu Val Glu Thr Ser Asp Ala Leu Leu Cys Ile Ala Pro Val Phe Asn
[3695]	275 280 285

[3696]	Asp Tyr Ser Thr Val Gly Trp Ser Gly Met Pro Lys Gly Pro Asn Val
[3697]	290 295 300
[3698]	Ile Leu Ala Glu Pro Asp Arg Val Thr Val Asp Gly Arg Ala Tyr Asp
[3699]	305 310 315 320
[3700]	Gly Phe Thr Leu Arg Ala Phe Leu Gln Ala Leu Ala Glu Lys Ala Pro
[3701]	325 330 335
[3702]	Ala Arg Pro Ala Ser Ala Gln Lys Ser Ser Val Pro Thr Cys Ser Leu
[3703]	340 345 350
[3704]	Thr Ala Thr Ser Asp Glu Ala Gly Leu Thr Asn Asp Glu Ile Val Arg
[3705]	355 360 365
[3706]	His Ile Asn Ala Leu Leu Thr Ser Asn Thr Thr Leu Val Ala Glu Thr
[3707]	370 375 380
[3708]	Gly Asp Ser Trp Phe Asn Ala Met Arg Met Thr Leu Ala Gly Ala Arg
[3709]	385 390 395 400
[3710]	Val Glu Leu Glu Met Gln Trp Gly His Ile Gly Trp Ser Val Pro Ser
[3711]	405 410 415
[3712]	Ala Phe Gly Asn Ala Met Gly Ser Gln Asp Arg Gln His Val Val Met
[3713]	420 425 430
[3714]	Val Gly Asp Gly Ser Phe Gln Leu Thr Ala Gln Glu Val Ala Gln Met
[3715]	435 440 445
[3716]	Val Arg Tyr Glu Leu Pro Val Ile Ile Phe Leu Ile Asn Asn Arg Gly
[3717]	450 455 460
[3718]	Tyr Val Ile Glu Ile Ala Ile His Asp Gly Pro Tyr Asn Tyr Ile Lys
[3719]	465 470 475 480
[3720]	Asn Trp Asp Tyr Ala Gly Leu Met Glu Val Phe Asn Ala Gly Glu Gly
[3721]	485 490 495
[3722]	His Gly Leu Gly Leu Lys Ala Thr Thr Pro Lys Glu Leu Thr Glu Ala
[3723]	500 505 510
[3724]	Ile Ala Arg Ala Lys Ala Asn Thr Arg Gly Pro Thr Leu Ile Glu Cys
[3725]	515 520 525
[3726]	Gln Ile Asp Arg Thr Asp Cys Thr Asp Met Leu Val Gln Trp Gly Arg
[3727]	530 535 540
[3728]	Lys Val Ala Ser Thr Asn Ala Arg Lys Thr Thr Leu Ala
[3729]	545 550 555
[3730]	<210> 54
[3731]	<211> 528
[3732]	<212> PRT
[3733]	<213> 恶臭假单胞菌
[3734]	<400> 54
[3735]	Met Ala Ser Val His Gly Thr Thr Tyr Glu Leu Leu Arg Arg Gln Gly
[3736]	1 5 10 15
[3737]	Ile Asp Thr Val Phe Gly Asn Pro Gly Ser Asn Glu Leu Pro Phe Leu

[3738]	20	25	30
[3739]	Lys Asp Phe Pro Glu Asp Phe Arg Tyr Ile Leu Ala Leu Gln Glu Ala		
[3740]	35	40	45
[3741]	Cys Val Val Gly Ile Ala Asp Gly Tyr Ala Gln Ala Ser Arg Lys Pro		
[3742]	50	55	60
[3743]	Ala Phe Ile Asn Leu His Ser Ala Ala Gly Thr Gly Asn Ala Met Gly		
[3744]	65	70	75
[3745]	Ala Leu Ser Asn Ala Trp Asn Ser His Ser Pro Leu Ile Val Thr Ala		
[3746]	85	90	95
[3747]	Gly Gln Gln Thr Arg Ala Met Ile Gly Val Glu Ala Leu Leu Thr Asn		
[3748]	100	105	110
[3749]	Val Asp Ala Ala Asn Leu Pro Arg Pro Leu Val Lys Trp Ser Tyr Glu		
[3750]	115	120	125
[3751]	Pro Ala Ser Ala Ala Glu Val Pro His Ala Met Ser Arg Ala Ile His		
[3752]	130	135	140
[3753]	Met Ala Ser Met Ala Pro Gln Gly Pro Val Tyr Leu Ser Val Pro Tyr		
[3754]	145	150	155
[3755]	Asp Asp Trp Asp Lys Asp Ala Asp Pro Gln Ser His His Leu Phe Asp		
[3756]	165	170	175
[3757]	Arg His Val Ser Ser Ser Val Arg Leu Asn Asp Gln Asp Leu Asp Ile		
[3758]	180	185	190
[3759]	Leu Val Lys Ala Leu Asn Ser Ala Ser Asn Pro Ala Ile Val Leu Gly		
[3760]	195	200	205
[3761]	Pro Asp Val Asp Ala Ala Asn Ala Asn Ala Asp Cys Val Met Leu Ala		
[3762]	210	215	220
[3763]	Glu Arg Leu Lys Ala Pro Val Trp Val Ala Pro Ser Ala Pro Arg Cys		
[3764]	225	230	235
[3765]	Pro Phe Pro Thr Arg His Pro Cys Phe Arg Gly Leu Met Pro Ala Gly		
[3766]	245	250	255
[3767]	Ile Ala Ala Ile Ser Gln Leu Leu Glu Gly His Asp Val Val Leu Val		
[3768]	260	265	270
[3769]	Ile Gly Ala Pro Val Phe Arg Tyr His Gln Tyr Asp Pro Gly Gln Tyr		
[3770]	275	280	285
[3771]	Leu Lys Pro Gly Thr Arg Leu Ile Ser Val Thr Cys Asp Pro Leu Glu		
[3772]	290	295	300
[3773]	Ala Ala Arg Ala Pro Met Gly Asp Ala Ile Val Ala Asp Ile Gly Ala		
[3774]	305	310	315
[3775]	Met Ala Ser Ala Leu Ala Asn Leu Val Glu Glu Ser Ser Arg Gln Leu		
[3776]	325	330	335
[3777]	Pro Thr Ala Ala Pro Glu Pro Ala Lys Val Asp Gln Asp Ala Gly Arg		
[3778]	340	345	350
[3779]	Leu His Pro Glu Thr Val Phe Asp Thr Leu Asn Asp Met Ala Pro Glu		

[3780]	355	360	365
[3781]	Asn Ala Ile Tyr Leu Asn Glu Ser Thr Ser Thr Thr Ala Gln Met Trp		
[3782]	370	375	380
[3783]	Gln Arg Leu Asn Met Arg Asn Pro Gly Ser Tyr Tyr Phe Cys Ala Ala		
[3784]	385	390	395
[3785]	Gly Gly Leu Gly Phe Ala Leu Pro Ala Ala Ile Gly Val Gln Leu Ala		
[3786]	405	410	415
[3787]	Glu Pro Glu Arg Gln Val Ile Ala Val Ile Gly Asp Gly Ser Ala Asn		
[3788]	420	425	430
[3789]	Tyr Ser Ile Ser Ala Leu Trp Thr Ala Ala Gln Tyr Asn Ile Pro Thr		
[3790]	435	440	445
[3791]	Ile Phe Val Ile Met Asn Asn Gly Thr Tyr Gly Ala Leu Arg Trp Phe		
[3792]	450	455	460
[3793]	Ala Gly Val Leu Glu Ala Glu Asn Val Pro Gly Leu Asp Val Pro Gly		
[3794]	465	470	475
[3795]	Ile Asp Phe Arg Ala Leu Ala Lys Gly Tyr Gly Val Gln Ala Leu Lys		
[3796]	485	490	495
[3797]	Ala Asp Asn Leu Glu Gln Leu Lys Gly Ser Leu Gln Glu Ala Leu Ser		
[3798]	500	505	510
[3799]	Ala Lys Gly Pro Val Leu Ile Glu Val Ser Thr Val Ser Pro Val Lys		
[3800]	515	520	525
[3801]	<210> 55		
[3802]	<211> 528		
[3803]	<212> PRT		
[3804]	<213> 铜绿假单胞菌		
[3805]	<400> 55		
[3806]	Met Lys Thr Val His Ser Ala Ser Tyr Glu Ile Leu Arg Arg His Gly		
[3807]	1	5	10
[3808]	Leu Thr Thr Val Phe Gly Asn Pro Gly Ser Asn Glu Leu Pro Phe Leu		
[3809]	20	25	30
[3810]	Lys Asp Phe Pro Glu Asp Phe Arg Tyr Ile Leu Gly Leu His Glu Gly		
[3811]	35	40	45
[3812]	Ala Val Val Gly Met Ala Asp Gly Phe Ala Leu Ala Ser Gly Arg Pro		
[3813]	50	55	60
[3814]	Ala Phe Val Asn Leu His Ala Ala Ala Gly Thr Gly Asn Gly Met Gly		
[3815]	65	70	75
[3816]	Ala Leu Thr Asn Ala Trp Tyr Ser His Ser Pro Leu Val Ile Thr Ala		
[3817]	85	90	95
[3818]	Gly Gln Gln Val Arg Ser Met Ile Gly Val Glu Ala Met Leu Ala Asn		
[3819]	100	105	110
[3820]	Val Asp Ala Gly Gln Leu Pro Lys Pro Leu Val Lys Trp Ser His Glu		
[3821]	115	120	125

[3822]	Pro	Ala	Cys	Ala	Gln	Asp	Val	Pro	Arg	Ala	Leu	Ser	Gln	Ala	Ile	Gln
[3823]	130							135					140			
[3824]	Thr	Ala	Ser	Leu	Pro	Pro	Arg	Ala	Pro	Val	Tyr	Leu	Ser	Ile	Pro	Tyr
[3825]	145					150						155				160
[3826]	Asp	Asp	Trp	Ala	Gln	Pro	Ala	Pro	Ala	Gly	Val	Glu	His	Leu	Ala	Ala
[3827]					165					170					175	
[3828]	Arg	Gln	Val	Ser	Gly	Ala	Ala	Leu	Pro	Ala	Pro	Ala	Leu	Leu	Ala	Glu
[3829]					180					185					190	
[3830]	Leu	Gly	Glu	Arg	Leu	Ser	Arg	Ser	Arg	Asn	Pro	Val	Leu	Val	Leu	Gly
[3831]					195					200					205	
[3832]	Pro	Asp	Val	Asp	Gly	Ala	Asn	Ala	Asn	Gly	Leu	Ala	Val	Glu	Leu	Ala
[3833]					210					215					220	
[3834]	Glu	Lys	Leu	Arg	Met	Pro	Ala	Trp	Val	Ala	Pro	Ser	Ala	Ser	Arg	Cys
[3835]	225					230						235				240
[3836]	Pro	Phe	Pro	Thr	Arg	His	Ala	Cys	Phe	Arg	Gly	Val	Leu	Pro	Ala	Ala
[3837]					245						250					255
[3838]	Ile	Ala	Gly	Ile	Ser	Arg	Leu	Leu	Asp	Gly	His	Asp	Leu	Ile	Leu	Val
[3839]					260					265					270	
[3840]	Val	Gly	Ala	Pro	Val	Phe	Arg	Tyr	His	Gln	Phe	Ala	Pro	Gly	Asp	Tyr
[3841]					275					280					285	
[3842]	Leu	Pro	Ala	Gly	Ala	Glu	Leu	Val	Gln	Val	Thr	Cys	Asp	Pro	Gly	Glu
[3843]					290					295					300	
[3844]	Ala	Ala	Arg	Ala	Pro	Met	Gly	Asp	Ala	Leu	Val	Gly	Asp	Ile	Ala	Leu
[3845]	305					310						315				320
[3846]	Thr	Leu	Glu	Ala	Leu	Leu	Glu	Gln	Val	Arg	Pro	Ser	Ala	Arg	Pro	Leu
[3847]					325						330				335	
[3848]	Pro	Glu	Ala	Leu	Pro	Arg	Pro	Pro	Ala	Leu	Ala	Glu	Glu	Gly	Gly	Pro
[3849]					340					345					350	
[3850]	Leu	Arg	Pro	Glu	Thr	Val	Phe	Asp	Val	Ile	Asp	Ala	Leu	Ala	Pro	Arg
[3851]					355					360					365	
[3852]	Asp	Ala	Ile	Phe	Val	Lys	Glu	Ser	Thr	Ser	Thr	Val	Thr	Ala	Phe	Trp
[3853]					370					375					380	
[3854]	Gln	Arg	Val	Glu	Met	Arg	Glu	Pro	Gly	Ser	Tyr	Phe	Phe	Pro	Ala	Ala
[3855]	385					390						395				400
[3856]	Gly	Gly	Leu	Gly	Phe	Gly	Leu	Pro	Ala	Ala	Val	Gly	Ala	Gln	Leu	Ala
[3857]					405						410				415	
[3858]	Gln	Pro	Arg	Arg	Gln	Val	Ile	Gly	Ile	Ile	Gly	Asp	Gly	Ser	Ala	Asn
[3859]					420						425				430	
[3860]	Tyr	Gly	Ile	Thr	Ala	Leu	Trp	Ser	Ala	Ala	Gln	Tyr	Arg	Val	Pro	Ala
[3861]					435						440				445	
[3862]	Val	Phe	Ile	Ile	Leu	Lys	Asn	Gly	Thr	Tyr	Gly	Ala	Leu	Arg	Trp	Phe
[3863]					450					455					460	

[3864]	Ala Gly Val Leu Glu Val Pro Asp Ala Pro Gly Leu Asp Val Pro Gly
[3865]	465 470 475 480
[3866]	Leu Asp Phe Cys Ala Ile Ala Arg Gly Tyr Gly Val Glu Ala Leu His
[3867]	485 490 495
[3868]	Ala Ala Thr Arg Glu Glu Leu Glu Gly Ala Leu Lys His Ala Leu Ala
[3869]	500 505 510
[3870]	Ala Asp Arg Pro Val Leu Ile Glu Val Pro Thr Gln Thr Ile Glu Pro
[3871]	515 520 525
[3872]	<210> 56
[3873]	<211> 526
[3874]	<212> PRT
[3875]	<213> 施氏假单胞菌(Pseudomonas stutzeri)
[3876]	<400> 56
[3877]	Met Ala Ser Val His Ser Ile Thr Tyr Glu Leu Leu Arg Arg Gln Gly
[3878]	1 5 10 15
[3879]	Ile Asp Thr Val Phe Gly Asn Pro Gly Ser Asn Glu Leu Pro Phe Leu
[3880]	20 25 30
[3881]	Lys Asp Phe Pro Glu Asp Phe Arg Tyr Ile Leu Ala Leu Gln Glu Ala
[3882]	35 40 45
[3883]	Cys Val Val Gly Ile Ala Asp Gly Tyr Ala Gln Ala Ser Arg Lys Pro
[3884]	50 55 60
[3885]	Ala Phe Ile Asn Leu His Ser Ala Ala Gly Thr Gly Asn Ala Met Gly
[3886]	65 70 75 80
[3887]	Ala Met Ser Asn Ala Trp Asn Cys His Ser Pro Leu Ile Val Thr Ala
[3888]	85 90 95
[3889]	Gly Gln Gln Asn Arg Ala Met Ile Gly Val Glu Ala Leu Leu Thr Asn
[3890]	100 105 110
[3891]	Val Asp Ala Ala Ser Leu Pro Arg Pro Leu Val Lys Trp Ser Tyr Glu
[3892]	115 120 125
[3893]	Pro Ala Ser Ala Ala Glu Val Pro His Ala Met Ser Arg Ala Ile His
[3894]	130 135 140
[3895]	Met Ala Ser Met Ala Pro Arg Gly Pro Val Tyr Leu Ser Val Pro Tyr
[3896]	145 150 155 160
[3897]	Asp Asp Trp Asp Lys Glu Ala Asp Pro Gln Ser His His Leu Tyr Asp
[3898]	165 170 175
[3899]	Arg Ser Val Asn Ser Ala Val Arg Leu Asn Asp Gln Asp Leu Glu Val
[3900]	180 185 190
[3901]	Leu Val Glu Ala Leu Asn Ser Ala Ser Asn Pro Ala Ile Val Leu Gly
[3902]	195 200 205
[3903]	Pro Asp Val Asp Ser Ala Asn Ala Asn Ala Asp Cys Val Thr Leu Ala
[3904]	210 215 220
[3905]	Glu Arg Leu Lys Ala Pro Val Trp Val Ala Pro Ser Ala Pro Arg Cys

[3906]	225	230	235	240
[3907]	Pro Phe Pro Thr Arg His Pro Cys Phe Arg Gly Leu Met Pro Ala Gly			
[3908]		245	250	255
[3909]	Ile Ala Ala Ile Ser Gln Leu Leu Glu Gly His Asp Val Val Leu Val			
[3910]		260	265	270
[3911]	Ile Gly Ala Pro Val Phe Arg Tyr His Gln Tyr Asp Pro Gly Gln Tyr			
[3912]		275	280	285
[3913]	Leu Lys Pro Gly Thr Arg Leu Ile Ser Ile Thr Cys Asp Pro Leu Glu			
[3914]		290	295	300
[3915]	Ala Ala Arg Ala Pro Met Gly Asp Ala Ile Val Ala Asp Ile Gly Thr			
[3916]	305	310	315	320
[3917]	Met Thr Ala Ala Leu Ala Ser Arg Ile Gly Glu Ser Glu Arg Gln Leu			
[3918]		325	330	335
[3919]	Pro Ala Val Leu Pro Ser Pro Glu Arg Val Asn Gln Asp Ala Gly Arg			
[3920]		340	345	350
[3921]	Leu Arg Pro Glu Thr Val Phe Asp Thr Leu Asn Glu Met Ala Pro Glu			
[3922]		355	360	365
[3923]	Asp Ala Ile Tyr Leu Asn Glu Ser Thr Ser Thr Thr Ala Gln Met Trp			
[3924]		370	375	380
[3925]	Gln Arg Leu Asn Met Arg Asn Pro Gly Ser Tyr Tyr Phe Cys Ala Ala			
[3926]	385	390	395	400
[3927]	Gly Gly Leu Gly Phe Ala Leu Pro Ala Ala Ile Gly Val Gln Leu Ala			
[3928]		405	410	415
[3929]	Glu Pro Asp Arg Gln Val Ile Ala Val Ile Gly Asp Gly Ser Ala Asn			
[3930]		420	425	430
[3931]	Tyr Ser Ile Ser Ala Leu Trp Thr Ala Ala His Tyr Asn Ile Pro Ala			
[3932]		435	440	445
[3933]	Ile Phe Leu Ile Met Asn Asn Gly Thr Tyr Gly Ala Leu Arg Trp Phe			
[3934]		450	455	460
[3935]	Ala Gly Val Leu Glu Ala Glu Asn Val Pro Gly Leu Asp Val Pro Gly			
[3936]	465	470	475	480
[3937]	Ile Asp Phe Cys Ala Ile Ala Lys Gly Tyr Gly Ile Pro Ala Leu Lys			
[3938]		485	490	495
[3939]	Ala Asp Asn Leu Glu Gln Leu Lys Gly Ser Ile His Glu Ala Leu Ser			
[3940]		500	505	510
[3941]	Ala Lys Gly Pro Val Leu Ile Glu Val Ser Thr Val Ser Leu			
[3942]		515	520	525
[3943]	<210> 57			
[3944]	<211> 528			
[3945]	<212> PRT			
[3946]	<213> 荧光假单胞菌			
[3947]	<400> 57			

[3948]	Met	Lys	Thr	Val	His	Ser	Ala	Ser	Tyr	Asp	Ile	Leu	Arg	Gln	Gln	Gly
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[3950]	Leu	Thr	Thr	Val	Phe	Gly	Asn	Pro	Gly	Ser	Asn	Glu	Leu	Pro	Phe	Leu
[3951]				20					25					30		
[3952]	Lys	Gly	Phe	Pro	Glu	Asp	Phe	Arg	Tyr	Ile	Leu	Gly	Leu	His	Glu	Gly
[3953]			35					40					45			
[3954]	Ala	Val	Val	Gly	Met	Ala	Asp	Gly	Phe	Ala	Leu	Ala	Ser	Gly	Gln	Pro
[3955]		50					55					60				
[3956]	Ala	Phe	Val	Asn	Leu	His	Ala	Ala	Ala	Gly	Thr	Gly	Asn	Gly	Met	Gly
[3957]	65					70				75					80	
[3958]	Ala	Leu	Thr	Asn	Ala	Trp	Tyr	Ser	His	Ser	Pro	Leu	Val	Ile	Thr	Ala
[3959]				85					90						95	
[3960]	Gly	Gln	Gln	Val	Arg	Ser	Met	Ile	Gly	Val	Glu	Ala	Met	Leu	Ala	Asn
[3961]				100					105						110	
[3962]	Val	Asp	Ala	Pro	Gln	Leu	Pro	Lys	Pro	Leu	Val	Lys	Trp	Ser	Ala	Glu
[3963]			115					120					125			
[3964]	Pro	Ala	Cys	Ala	Glu	Asp	Val	Pro	Arg	Ala	Leu	Ser	Gln	Ala	Ile	His
[3965]		130					135					140				
[3966]	Met	Ala	Asn	Gln	Ala	Pro	Lys	Gly	Pro	Val	Tyr	Leu	Ser	Ile	Pro	Tyr
[3967]	145					150				155					160	
[3968]	Asp	Asp	Trp	Ala	Arg	Pro	Ala	Pro	Ala	Gly	Val	Glu	His	Leu	Ala	Arg
[3969]				165					170						175	
[3970]	Arg	Gln	Val	Ala	Thr	Ala	Gly	Leu	Pro	Ser	Ala	Ala	Gln	Leu	Arg	Ser
[3971]			180						185						190	
[3972]	Leu	Val	Gln	Arg	Leu	Ala	Ala	Ala	Arg	Asn	Pro	Val	Leu	Val	Leu	Gly
[3973]			195					200					205			
[3974]	Pro	Asp	Val	Asp	Gly	Ser	Arg	Ser	Asn	His	Leu	Ala	Val	Gln	Leu	Ala
[3975]		210					215					220				
[3976]	Glu	Lys	Leu	Arg	Met	Pro	Ala	Trp	Val	Ala	Pro	Ser	Ala	Ser	Arg	Cys
[3977]	225					230				235					240	
[3978]	Pro	Phe	Pro	Thr	Arg	His	Pro	Ser	Phe	Arg	Gly	Val	Leu	Pro	Ala	Ala
[3979]				245					250						255	
[3980]	Ile	Ala	Gly	Ile	Ser	Arg	Cys	Leu	Ala	Asp	His	Asp	Leu	Ile	Leu	Val
[3981]				260					265						270	
[3982]	Val	Gly	Ala	Pro	Val	Phe	Arg	Tyr	His	Gln	Phe	Ala	Pro	Gly	Asp	Tyr
[3983]			275					280					285			
[3984]	Leu	Pro	Ala	Gly	Thr	Glu	Leu	Leu	His	Ile	Thr	Cys	Asp	Pro	Gly	Glu
[3985]		290					295					300				
[3986]	Ala	Ala	Arg	Ala	Pro	Met	Gly	Asp	Ala	Leu	Val	Gly	Asp	Ile	Val	Glu
[3987]	305					310				315					320	
[3988]	Thr	Leu	Gln	Ala	Leu	Val	Trp	Ala	Leu	Pro	Asp	Cys	Asp	Arg	Pro	Gln
[3989]				325					330						335	

[3990]	Pro Gln Ala Leu Pro Pro Ala Ala Pro Val Glu Glu Leu Gly Gly Leu
[3991]	340 345 350
[3992]	Leu Arg Pro Glu Thr Val Phe Asp Val Ile Asp Glu Leu Ala Pro Lys
[3993]	355 360 365
[3994]	Asp Ala Ile Tyr Val Lys Glu Ser Thr Ser Thr Val Gly Ala Phe Trp
[3995]	370 375 380
[3996]	Gln Arg Val Glu Met Arg Glu Pro Gly Ser Tyr Tyr Phe Pro Ala Ala
[3997]	385 390 395 400
[3998]	Gly Gly Leu Gly Phe Gly Leu Pro Ala Ala Val Gly Val Gln Leu Ala
[3999]	405 410 415
[4000]	Arg Pro Glu Arg Arg Val Ile Gly Val Ile Gly Asp Gly Ser Ala Asn
[4001]	420 425 430
[4002]	Tyr Gly Ile Thr Ala Leu Trp Thr Ala Ala Gln Tyr Gln Ile Pro Val
[4003]	435 440 445
[4004]	Val Phe Ile Ile Leu Lys Asn Gly Thr Tyr Gly Ala Leu Arg Trp Phe
[4005]	450 455 460
[4006]	Ala Gly Val Leu Gln Val Ser Asp Ala Pro Gly Leu Asp Val Pro Gly
[4007]	465 470 475 480
[4008]	Leu Asp Phe Cys Ala Ile Gly Arg Gly Tyr Gly Val His Ser Val Gln
[4009]	485 490 495
[4010]	Ala Asn Thr Arg Glu Ala Phe Ala Gln Ala Leu Ser Glu Ala Leu Ala
[4011]	500 505 510
[4012]	Gly Asp Arg Pro Val Leu Ile Glu Val Pro Thr Leu Thr Ile Glu Pro
[4013]	515 520 525
[4014]	<210> 58
[4015]	<211> 1220
[4016]	<212> PRT
[4017]	<213> 橙色绿屈挠菌 (Chloroflexus aurantiacus)
[4018]	<400> 58
[4019]	Met Ala Thr Gly Glu Ser Met Ser Gly Thr Gly Arg Leu Ala Gly Lys
[4020]	1 5 10 15
[4021]	Ile Ala Leu Ile Thr Gly Gly Ala Gly Asn Ile Gly Ser Glu Leu Thr
[4022]	20 25 30
[4023]	Arg Arg Phe Leu Ala Glu Gly Ala Thr Val Ile Ile Ser Gly Arg Asn
[4024]	35 40 45
[4025]	Arg Ala Lys Leu Thr Ala Leu Ala Glu Met Gln Ala Glu Ala Gly Val
[4026]	50 55 60
[4027]	Pro Ala Lys Arg Ile Asp Leu Glu Val Met Asp Gly Ser Asp Pro Val
[4028]	65 70 75 80
[4029]	Ala Val Arg Ala Gly Ile Glu Ala Ile Val Ala Arg His Gly Gln Ile
[4030]	85 90 95
[4031]	Asp Ile Leu Val Asn Asn Ala Gly Ser Ala Gly Ala Gln Arg Arg Leu

[4032]	100	105	110
[4033]	Ala Glu Ile Pro Leu Thr Glu	Ala Glu Leu Gly Pro Gly	Ala Glu Glu
[4034]	115	120	125
[4035]	Thr Leu His Ala Ser Ile Ala	Asn Leu Leu Gly Met Gly	Trp His Leu
[4036]	130	135	140
[4037]	Met Arg Ile Ala Ala Pro His	Met Pro Val Gly Ser Ala	Val Ile Asn
[4038]	145	150	155
[4039]	Val Ser Thr Ile Phe Ser Arg	Ala Glu Tyr Tyr Gly Arg	Ile Pro Tyr
[4040]	165	170	175
[4041]	Val Thr Pro Lys Ala Ala Leu	Asn Ala Leu Ser Gln Leu	Ala Ala Arg
[4042]	180	185	190
[4043]	Glu Leu Gly Ala Arg Gly Ile	Arg Val Asn Thr Ile Phe	Pro Gly Pro
[4044]	195	200	205
[4045]	Ile Glu Ser Asp Arg Ile Arg	Thr Val Phe Gln Arg Met	Asp Gln Leu
[4046]	210	215	220
[4047]	Lys Gly Arg Pro Glu Gly Asp	Thr Ala His His Phe Leu	Asn Thr Met
[4048]	225	230	235
[4049]	Arg Leu Cys Arg Ala Asn Asp	Gln Gly Ala Leu Glu Arg	Arg Phe Pro
[4050]	245	250	255
[4051]	Ser Val Gly Asp Val Ala Asp	Ala Ala Val Phe Leu Ala	Ser Ala Glu
[4052]	260	265	270
[4053]	Ser Ala Ala Leu Ser Gly Glu	Thr Ile Glu Val Thr His	Gly Met Glu
[4054]	275	280	285
[4055]	Leu Pro Ala Cys Ser Glu Thr	Ser Leu Leu Ala Arg Thr	Asp Leu Arg
[4056]	290	295	300
[4057]	Thr Ile Asp Ala Ser Gly Arg	Thr Thr Leu Ile Cys Ala	Gly Asp Gln
[4058]	305	310	315
[4059]	Ile Glu Glu Val Met Ala Leu	Thr Gly Met Leu Arg Thr	Cys Gly Ser
[4060]	325	330	335
[4061]	Glu Val Ile Ile Gly Phe Arg	Ser Ala Ala Ala Leu Ala	Gln Phe Glu
[4062]	340	345	350
[4063]	Gln Ala Val Asn Glu Ser Arg	Arg Leu Ala Gly Ala Asp	Phe Thr Pro
[4064]	355	360	365
[4065]	Pro Ile Ala Leu Pro Leu Asp	Pro Arg Asp Pro Ala Thr	Ile Asp Ala
[4066]	370	375	380
[4067]	Val Phe Asp Trp Ala Gly Glu	Asn Thr Gly Gly Ile His	Trp Ile Leu
[4068]	385	390	395
[4069]	Pro Ala Thr Ser His Glu Pro	Ala Pro Cys Val Ile Glu	Val Asp Asp
[4070]	405	410	415
[4071]	Glu Arg Val Leu Asn Phe Leu	Ala Asp Glu Ile Thr Gly	Thr Ile Val
[4072]	420	425	430
[4073]	Ile Ala Ser Arg Leu Ala Arg	Tyr Trp Gln Ser Gln Arg	Leu Thr Pro

[4074]	435	440	445
[4075]	Gly Ala Arg Ala Arg Gly Pro Arg Val Ile Phe Leu Ser Asn Gly Ala		
[4076]	450	455	460
[4077]	Asp Gln Asn Gly Asn Val Tyr Gly Arg Ile Gln Ser Ala Ala Ile Gly		
[4078]	465	470	475 480
[4079]	Gln Leu Ile Arg Val Trp Arg His Glu Ala Glu Leu Asp Tyr Gln Arg		
[4080]	485	490	495
[4081]	Ala Ser Ala Ala Gly Asp His Val Leu Pro Pro Val Trp Ala Asn Gln		
[4082]	500	505	510
[4083]	Ile Val Arg Phe Ala Asn Arg Ser Leu Glu Gly Leu Glu Phe Ala Cys		
[4084]	515	520	525
[4085]	Ala Trp Thr Ala Gln Leu Leu His Ser Gln Arg His Ile Asn Glu Ile		
[4086]	530	535	540
[4087]	Thr Leu Asn Ile Pro Ala Asn Ile Ser Ala Thr Thr Gly Ala Arg Ser		
[4088]	545	550	555 560
[4089]	Ala Ser Val Gly Trp Ala Glu Ser Leu Ile Gly Leu His Leu Gly Lys		
[4090]	565	570	575
[4091]	Val Ala Leu Ile Thr Gly Gly Ser Ala Gly Ile Gly Gly Gln Ile Gly		
[4092]	580	585	590
[4093]	Arg Leu Leu Ala Leu Ser Gly Ala Arg Val Met Leu Ala Ala Arg Asp		
[4094]	595	600	605
[4095]	Arg His Lys Leu Glu Gln Met Gln Ala Met Ile Gln Ser Glu Leu Ala		
[4096]	610	615	620
[4097]	Glu Val Gly Tyr Thr Asp Val Glu Asp Arg Val His Ile Ala Pro Gly		
[4098]	625	630	635 640
[4099]	Cys Asp Val Ser Ser Glu Ala Gln Leu Ala Asp Leu Val Glu Arg Thr		
[4100]	645	650	655
[4101]	Leu Ser Ala Phe Gly Thr Val Asp Tyr Leu Ile Asn Asn Ala Gly Ile		
[4102]	660	665	670
[4103]	Ala Gly Val Glu Glu Met Val Ile Asp Met Pro Val Glu Gly Trp Arg		
[4104]	675	680	685
[4105]	His Thr Leu Phe Ala Asn Leu Ile Ser Asn Tyr Ser Leu Met Arg Lys		
[4106]	690	695	700
[4107]	Leu Ala Pro Leu Met Lys Lys Gln Gly Ser Gly Tyr Ile Leu Asn Val		
[4108]	705	710	715 720
[4109]	Ser Ser Tyr Phe Gly Gly Glu Lys Asp Ala Ala Ile Pro Tyr Pro Asn		
[4110]	725	730	735
[4111]	Arg Ala Asp Tyr Ala Val Ser Lys Ala Gly Gln Arg Ala Met Ala Glu		
[4112]	740	745	750
[4113]	Val Phe Ala Arg Phe Leu Gly Pro Glu Ile Gln Ile Asn Ala Ile Ala		
[4114]	755	760	765
[4115]	Pro Gly Pro Val Glu Gly Asp Arg Leu Arg Gly Thr Gly Glu Arg Pro		

[4116]	770	775	780
[4117]	Gly Leu Phe Ala Arg Arg Ala Arg Leu Ile Leu Glu Asn Lys Arg Leu		
[4118]	785	790	795 800
[4119]	Asn Glu Leu His Ala Ala Leu Ile Ala Ala Arg Thr Asp Glu Arg		
[4120]	805	810	815
[4121]	Ser Met His Glu Leu Val Glu Leu Leu Leu Pro Asn Asp Val Ala Ala		
[4122]	820	825	830
[4123]	Leu Glu Gln Asn Pro Ala Ala Pro Thr Ala Leu Arg Glu Leu Ala Arg		
[4124]	835	840	845
[4125]	Arg Phe Arg Ser Glu Gly Asp Pro Ala Ala Ser Ser Ser Ser Ala Leu		
[4126]	850	855	860
[4127]	Leu Asn Arg Ser Ile Ala Ala Lys Leu Leu Ala Arg Leu His Asn Gly		
[4128]	865	870	875 880
[4129]	Gly Tyr Val Leu Pro Ala Asp Ile Phe Ala Asn Leu Pro Asn Pro Pro		
[4130]	885	890	895
[4131]	Asp Pro Phe Phe Thr Arg Ala Gln Ile Asp Arg Glu Ala Arg Lys Val		
[4132]	900	905	910
[4133]	Arg Asp Gly Ile Met Gly Met Leu Tyr Leu Gln Arg Met Pro Thr Glu		
[4134]	915	920	925
[4135]	Phe Asp Val Ala Met Ala Thr Val Tyr Tyr Leu Ala Asp Arg Asn Val		
[4136]	930	935	940
[4137]	Ser Gly Glu Thr Phe His Pro Ser Gly Gly Leu Arg Tyr Glu Arg Thr		
[4138]	945	950	955 960
[4139]	Pro Thr Gly Gly Glu Leu Phe Gly Leu Pro Ser Pro Glu Arg Leu Ala		
[4140]	965	970	975
[4141]	Glu Leu Val Gly Ser Thr Val Tyr Leu Ile Gly Glu His Leu Thr Glu		
[4142]	980	985	990
[4143]	His Leu Asn Leu Leu Ala Met Tyr Leu Glu Arg Tyr Gly Ala Arg Gln		
[4144]	995	1000	1005
[4145]	Val Trp Ile Val Glu Thr Glu Thr Gly Ala Glu Thr Met Arg Arg		
[4146]	1010	1015	1020
[4147]	Leu Leu His Asp His Val Glu Ala Gly Arg Leu Met Thr Ile Val		
[4148]	1025	1030	1035
[4149]	Ala Gly Asp Gln Ile Glu Ala Ala Ile Asp Gln Ala Ile Thr Arg		
[4150]	1040	1045	1050
[4151]	Tyr Gly Arg Pro Gly Pro Val Val Cys Thr Pro Phe Arg Pro Leu		
[4152]	1055	1060	1065
[4153]	Pro Thr Val Pro Leu Val Gly Arg Lys Asp Ser Asp Trp Ser Thr		
[4154]	1070	1075	1080
[4155]	Val Leu Ser Glu Ala Glu Phe Ala Glu Leu Cys Glu His Gln Leu		
[4156]	1085	1090	1095
[4157]	Thr His His Phe Arg Val Ala Arg Lys Ile Ala Leu Ser Asp Gly		

[4158]	1100	1105	1110
[4159]	Ala Ser Leu Ala Leu Val Thr Pro Glu Thr Thr Ala Thr Ser Thr		
[4160]	1115	1120	1125
[4161]	Thr Glu Gln Phe Ala Leu Ala Asn Phe Ile Lys Thr Thr Leu His		
[4162]	1130	1135	1140
[4163]	Ala Phe Thr Ala Thr Ile Gly Val Glu Ser Glu Arg Thr Ala Gln		
[4164]	1145	1150	1155
[4165]	Arg Ile Leu Ile Asn Gln Val Asp Leu Thr Arg Arg Ala Arg Ala		
[4166]	1160	1165	1170
[4167]	Glu Glu Pro Arg Asp Pro His Glu Arg Gln Gln Glu Leu Glu Arg		
[4168]	1175	1180	1185
[4169]	Phe Ile Glu Ala Val Leu Leu Val Thr Ala Pro Leu Pro Pro Glu		
[4170]	1190	1195	1200
[4171]	Ala Asp Thr Arg Tyr Ala Gly Arg Ile His Arg Gly Arg Ala Ile		
[4172]	1205	1210	1215
[4173]	Thr Val		
[4174]	1220		
[4175]	<210> 59		
[4176]	<211> 1229		
[4177]	<212> PRT		
[4178]	<213> Roseiflexus castenholzii		
[4179]	<400> 59		
[4180]	Met Ser Thr Val Arg Arg Leu Glu Gly Lys Val Ala Leu Ile Thr Gly		
[4181]	1 5 10 15		
[4182]	Gly Ala Gly Asn Ile Gly Glu Val Ile Thr Arg Arg Phe Leu Ala Glu		
[4183]	20 25 30		
[4184]	Gly Ala Thr Val Val Ile Thr Gly Arg Asn Ala Glu Lys Leu Ala Val		
[4185]	35 40 45		
[4186]	Tyr Arg Arg Arg Leu Ile Asp Glu Glu Arg Val Ala Pro Glu Arg Val		
[4187]	50 55 60		
[4188]	Val Ala Leu Arg Met Asp Gly Ser Asp Ile Ala Gln Val Arg Ala Gly		
[4189]	65 70 75 80		
[4190]	Val Ala Gln Ile Val His Gly Gly Thr Asp Val Pro Ile Pro Leu His		
[4191]	85 90 95		
[4192]	Arg Ile Asp Ile Leu Val Asn Asn Ala Gly Ser Ala Gly Pro Arg Arg		
[4193]	100 105 110		
[4194]	Arg Leu Val Asp Ile Pro Leu Glu Pro Ser Glu Val Gln Pro Pro Asp		
[4195]	115 120 125		
[4196]	Ser Glu Thr Leu Ala Gln Ala Val Gly Asn Leu Val Gly Ile Thr Trp		
[4197]	130 135 140		
[4198]	Asn Leu Thr Arg Ala Ala Ala Pro His Met Pro Ser Gly Ser Ser Val		
[4199]	145 150 155 160		

[4200]	Ile Asn Ile Ser Thr Ile Phe Ser Arg Thr Asp Tyr Tyr Gly Arg Ile		
[4201]		165	170
[4202]	Ala Tyr Val Ala Pro Lys Ala Ala Leu Asn Ala Leu Ser Asp Gly Leu		175
[4203]		180	185
[4204]	Ala Arg Glu Leu Gly Val Arg Gly Ile Arg Val Asn Thr Ile Tyr Pro		190
[4205]		195	200
[4206]	Gly Pro Ile Glu Ser Glu Arg Ile Tyr Thr Met Phe Gln Ala Met Asp		205
[4207]		210	215
[4208]	Ala Leu Lys Gly Gln Pro Glu Gly Asp Thr Ala Ser Gly Phe Leu Arg		220
[4209]		225	230
[4210]	Met Met Arg Leu Ser Arg Ile Asp Gln Asn Gly Glu Val Val Lys Arg		235
[4211]		245	250
[4212]	Phe Pro Ser Pro Val Asp Val Ala Asn Thr Ala Val Phe Leu Ala Ser		240
[4213]		260	265
[4214]	Asp Glu Ser Ala Ala Phe Thr Gly His Ala Phe Glu Val Thr His Gly		270
[4215]		275	280
[4216]	Met Glu Val Pro Thr Glu Ser Arg Thr Thr Phe Val Ser Arg Pro Gly		285
[4217]		290	295
[4218]	Leu Arg Ser Val Asp Ala Thr Gly Lys Val Ile Leu Ile Cys Ala Gly		300
[4219]		305	310
[4220]	Asp Gln Val Asp Asp Ala Val Ala Leu Ala Asp Thr Leu Arg Ser Cys		315
[4221]		325	330
[4222]	Arg Ala Thr Val Val Ile Gly Phe Arg Asp Pro Arg Ala Leu Glu Lys		320
[4223]		340	345
[4224]	Ala Ser Val Leu Leu Arg Glu Pro Arg His Ala Leu Ala Ala Asp Met		350
[4225]		355	360
[4226]	Tyr Gly Arg Pro Thr Met Thr Ala Glu Ala Arg Leu Val Arg Leu Asp		365
[4227]		370	375
[4228]	Pro Leu Asp Pro Arg Ala Ala Ala Gln Thr Leu Glu Gln Ile His Ala		380
[4229]		385	390
[4230]	Glu Leu Gly Ala Ile His His Ala Val Val Leu Pro Gly Gln Ser Arg		395
[4231]		405	410
[4232]	His Ala Pro Ser Ala Ser Leu Ile Glu Val Asp Asp Gln Val Val Glu		415
[4233]		420	425
[4234]	Arg Phe Leu His Gln Glu Leu Val Gly Thr Ile Ala Leu Ala Arg Glu		430
[4235]		435	440
[4236]	Leu Ala Arg Phe Trp Glu Glu Tyr Pro Ser Gly Ser Ser Met His Arg		445
[4237]		450	455
[4238]	Val Leu Phe Val Ser Asn Pro Asp Asp Gln Gln Gly Asn Gln Tyr Ser		460
[4239]		465	470
[4240]	His Ile Leu Arg Ala Ala Val Glu Gln Leu Val Arg Val Trp Arg His		475
[4241]		485	490

[4242]	Glu Ser Glu Tyr Asp Ser Val Asn Pro Ala His Gln Gln Glu Gly Gln		
[4243]		500	505
[4244]	Ser Ser Ala Ala Val Trp Ala Asn Gln Leu Ile Arg Tyr Val Asn Asn		510
[4245]		515	520
[4246]	Glu Met Ala Asn Leu Asp Phe Thr Cys Ala Trp Val Ala Lys Leu Leu		525
[4247]		530	535
[4248]	Gly Ser Asp Arg Arg Ile Ala Glu Ile Asn Leu Tyr Leu Pro Glu Glu		540
[4249]		545	550
[4250]	Ile Val Gly Thr Ile Gly Val His Asn Pro Gly Phe Gly Trp Ala Glu		555
[4251]		565	570
[4252]	Ser Leu Phe Gly Leu His Met Gly Lys Val Ala Leu Ile Thr Gly Gly		575
[4253]		580	585
[4254]	Ser Ala Gly Ile Gly Gly Gln Ile Gly Arg Leu Leu Ala Leu Ser Gly		590
[4255]		595	600
[4256]	Ala His Val Met Leu Ala Ala Arg Asn Ala Asp Gln Leu Glu Gln Met		605
[4257]		610	615
[4258]	Arg Ala Ser Ile Val Arg Glu Val Arg Asp Ala Ser Tyr Pro Asp Ala		620
[4259]		625	630
[4260]	Glu Ser Arg Val Ala Ile Phe Pro Gly Ser Asp Val Ser Asp Ile Asp		635
[4261]		645	650
[4262]	Gly Leu Glu Arg Leu Val Asn His Thr Val Arg Val Phe Gly Lys Val		655
[4263]		660	665
[4264]	Asp Tyr Leu Ile Asn Asn Ala Gly Ile Ala Gly Ala Glu Glu Met Val		670
[4265]		675	680
[4266]	Ile Asp Met Pro Val Asp Ala Trp Arg His Thr Leu Arg Ala Asn Leu		685
[4267]		690	695
[4268]	Ile Ser Asn Tyr Ala Leu Leu Arg Arg Leu Ala Pro Gln Met Lys Ala		700
[4269]		705	710
[4270]	Ala Gly Gly Ala Tyr Val Leu Asn Val Ser Ser Tyr Phe Gly Gly Glu		715
[4271]		725	730
[4272]	Lys Tyr Val Ala Ile Pro Tyr Pro Asn Arg Ser Asp Tyr Ala Val Ser		735
[4273]		740	745
[4274]	Lys Ala Gly Gln Arg Ala Met Val Glu Ser Leu Ala Arg Phe Leu Gly		750
[4275]		755	760
[4276]	Pro Glu Ile Gln Ile Asn Ala Ile Ala Pro Gly Pro Val Glu Gly Glu		765
[4277]		770	775
[4278]	Arg Leu Lys Gly Ala Gly Ser Arg Pro Gly Leu Phe Met Arg Arg Ala		780
[4279]		785	790
[4280]	Arg Leu Ile Leu Glu Asn Lys Arg Leu Asn Glu Val Phe Ala Ala Leu		795
[4281]		805	810
[4282]	Leu Ala Ala Arg His Glu Gly Ala Thr Ile Ala Asp Leu Leu Pro Asp		815
[4283]		820	825
			830

[4284]	Leu Phe Ala Asn Asp Ile Gln Ser Ile Ala Asn Ser Ala Ala Met Pro
[4285]	835 840 845
[4286]	Ala Pro Leu Arg Arg Leu Ala Thr Met Leu Arg Glu Thr Ser Asp Ala
[4287]	850 855 860
[4288]	Gly Gly Ser Ala Gln Ser Tyr Leu Met Asn Ala Thr Ile Ala Arg Lys
[4289]	865 870 875 880
[4290]	Leu Leu Asn Arg Leu Glu Asn Gly Gly Tyr Ile Thr Leu His Asp Arg
[4291]	885 890 895
[4292]	Arg Ala Leu Thr Val Glu Pro Pro Glu Pro Phe Phe Thr Glu Ala Gln
[4293]	900 905 910
[4294]	Ile Glu Arg Glu Ala Ile Lys Val Arg Asp Gly Ile Leu Gly Met Leu
[4295]	915 920 925
[4296]	His Leu Gln Arg Met Pro Thr Glu Phe Asp Val Ala Leu Ala Thr Val
[4297]	930 935 940
[4298]	Phe Tyr Leu Ala Asp Arg Asn Val Thr Gly Glu Thr Phe His Pro Ser
[4299]	945 950 955 960
[4300]	Gly Gly Leu Arg Phe Glu Arg Thr Val Thr Glu Gly Glu Leu Phe Gly
[4301]	965 970 975
[4302]	Lys Pro Gly Gln Gln Arg Leu Glu Arg Leu Lys Gly Ser Val Val Tyr
[4303]	980 985 990
[4304]	Leu Ile Gly Glu His Leu Arg Gln His Leu Val Leu Leu Ala Arg Thr
[4305]	995 1000 1005
[4306]	Phe Leu Asp Glu Ile His Val Ala Arg Val Val Leu Leu Thr Glu
[4307]	1010 1015 1020
[4308]	Thr Thr Gln Ala Ala Thr Asp Leu Ala Ala Glu Leu Ser Asp Tyr
[4309]	1025 1030 1035
[4310]	Glu Ala Ala Gly Arg Phe Val Val Ile Pro Thr Cys Gly Asp Ile
[4311]	1040 1045 1050
[4312]	Glu Gly Gly Ile Asp Arg Ala Met Ala Glu Tyr Gly Arg Pro Gly
[4313]	1055 1060 1065
[4314]	Pro Val Ile Ser Thr Pro Phe Arg Pro Leu Pro Asp Arg Ala Leu
[4315]	1070 1075 1080
[4316]	Ser Ala Arg Asn Gly Asp Trp Ser Ser Val Leu Thr Thr Ala Glu
[4317]	1085 1090 1095
[4318]	Phe Glu Glu Leu Val Glu Gln Gln Ile Thr His His Phe Arg Val
[4319]	1100 1105 1110
[4320]	Ala Arg Lys Ala Gly Leu Ile Glu Gly Ala Asn Val Thr Leu Val
[4321]	1115 1120 1125
[4322]	Thr Pro Pro Thr Ser Ala Arg Ser Thr Ser Glu Glu Phe Ala Leu
[4323]	1130 1135 1140
[4324]	Ala Asn Phe Val Lys Thr Thr Leu His Ala Leu Thr Ala Thr Ala
[4325]	1145 1150 1155

[4326]	Gly Ala Glu Ser Glu Arg Thr Val Pro His Val Pro Val Asn Gln		
[4327]	1160	1165	1170
[4328]	Val Asp Leu Thr Arg Arg Ala Arg Ser Glu Glu Pro Arg Thr Pro		
[4329]	1175	1180	1185
[4330]	Ser Glu Glu Glu Glu Glu Leu Gln Arg Phe Val Asn Ala Val Leu		
[4331]	1190	1195	1200
[4332]	Leu Thr Ser Ala Pro Leu Pro Thr Pro Leu Glu Ser Arg Tyr Arg		
[4333]	1205	1210	1215
[4334]	Ala Arg Ile Tyr Arg Gly Asn Ala Ile Thr Val		
[4335]	1220	1225	
[4336]	<210> 60		
[4337]	<211> 1217		
[4338]	<212> PRT		
[4339]	<213> Erythrobacter sp.		
[4340]	<400> 60		
[4341]	Met Ser Lys Glu Gly Asn Ala Ala Lys Gly Arg Leu Glu Gly Lys Val		
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[4343]	Ala Leu Ile Thr Gly Ala Ala Gly Asn Leu Gly Asn Glu Ile Ser Arg		
[4344]	20	25	30
[4345]	Ala Phe Ala Arg Glu Gly Ala Phe Val Val Met Thr Gly Arg Thr Glu		
[4346]	35	40	45
[4347]	Glu Arg Ile Ser Ala Ala Arg Glu Gln Leu Ile Ala Asp Thr Gly Val		
[4348]	50	55	60
[4349]	Ala Pro Glu Arg Ile Asp Thr Ala Val Leu Asp Gly Gly Asn Pro Asp		
[4350]	65	70	75
[4351]	Ser Ile Arg Ala Ala Met Ala Lys Leu Arg Lys Glu Tyr Gly Arg Ile		
[4352]	85	90	95
[4353]	Asp Ile Leu Ile Asn Asn Ala Gly Ser Ala Gly Pro Lys Gln Pro Leu		
[4354]	100	105	110
[4355]	His Asn Val Pro Leu Ser Pro Gln Glu Met Glu Ala Cys Gly Asp Thr		
[4356]	115	120	125
[4357]	Glu Thr Val Arg Asp Ala Met Leu Asn Ile Leu Gly Val Thr Trp Asn		
[4358]	130	135	140
[4359]	Met Ala Arg Ile Val Ala Pro Met Met Pro Val Gly Gly Ala Met Val		
[4360]	145	150	155
[4361]	Asn Ile Ser Thr Ile Phe Ser His Thr Arg Tyr Tyr Gly Arg Thr Ala		
[4362]	165	170	175
[4363]	Tyr Val Val Pro Lys Ala Ala Leu Asn Ala Leu Ser Asn Gln Leu Ala		
[4364]	180	185	190
[4365]	Ser Glu Leu Gly Pro Arg Gly Ile Arg Val Asn Thr Val Phe Pro Gly		
[4366]	195	200	205
[4367]	Pro Ile Glu Ser Asp Arg Ile Arg Thr Val Phe Ala Ala Met Asp Glu		

[4368]	210	215	220
[4369]	Val Gln Ser Gln Pro Lys Asp Thr Thr Ala Asn Tyr Phe Thr Gly Arg		
[4370]	225	230	235
[4371]	Met Ala Leu Thr Arg Ser Val Asn Gly Lys Val Asp Gly Lys Pro Leu		
[4372]		245	250
[4373]	Pro Asn Pro Lys Asp Ile Ala Gly Thr Cys Leu Phe Leu Ala Ser Glu		
[4374]		260	265
[4375]	Glu Ala Ala Gly Ile Ala Gly Glu Glu Val Asp Val Thr His Gly Leu		
[4376]		275	280
[4377]	Ser Ala Asn Arg Thr Ser Ala Ser Thr Tyr Met Thr Arg Pro Ser Met		
[4378]	290	295	300
[4379]	Arg Ser Leu Asp Gly Ala Gly Leu Asn Ile Phe Ile Val Ser Gly Glu		
[4380]	305	310	315
[4381]	Asn Trp Asp Asp Ala Leu Val Ala Ala His Thr Leu Ile Gly Ser Gly		
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[4383]	Ala Lys Val Arg Leu Gly Leu Ala Arg Asn Ala Asp Val Ala Gln Ala		
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[4385]	Asn Ala Arg Leu Lys Ala Gln Gly Ile Gly Glu Glu Leu Thr Val Thr		
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[4387]	Arg Phe Asn Arg Ala Glu Pro Asp Ala Met Glu Asp Ala Leu Ala Ala		
[4388]	370	375	380
[4389]	Phe Ser Gly Asp Val Asp Gly Ala Ile Thr Gly Ala Ile Ile Leu Pro		
[4390]	385	390	395
[4391]	Val Lys Pro Ser Gly His Phe Thr Gly Ser Leu Leu Ala Ala Asp Asp		
[4392]		405	410
[4393]	Asp Thr Val Thr Lys Phe Met Asp Thr Glu Leu Val Gly Ala Ile Ala		
[4394]		420	425
[4395]	Val Ser Arg Ser Leu Ala Arg Tyr Trp His Gly Arg Glu Asp Leu Gln		
[4396]		435	440
[4397]	Ser Pro Pro Arg Cys Val Phe Met Thr Asn Pro Gly Asp Pro Leu Gly		
[4398]	450	455	460
[4399]	Asn Ser Phe Ala Ser Val Leu Ser Ala Gly Ile Thr Gln Leu Ile Arg		
[4400]	465	470	475
[4401]	Ile Trp Arg Asp Glu Glu Arg Val Gln Ala Gly Asn Gly Ser Thr Glu		
[4402]		485	490
[4403]	His Ala Val Trp Ser Asn Gln Ile Val Arg His Thr Asn Thr Glu Asp		
[4404]		500	505
[4405]	Glu Asn Thr Arg Phe Ala Ser Gly His Ala Thr Arg Val Leu Phe Arg		
[4406]		515	520
[4407]	Glu Gln His Ile Ala Glu Ile Asp Leu Lys Leu Pro Ala Asn Ile Ser		
[4408]	530	535	540
[4409]	Glu Glu Thr Gly Ser Arg Lys Ala Met Val Gly Phe Ala Glu Asn Ile		

[4410]	545	550	555	560
[4411]	Thr Gly Leu His	Leu Gly Lys Val	Ala Phe Ile Thr	Gly Gly Ser Ala
[4412]		565	570	575
[4413]	Gly Ile Gly Gly	Gln Val Ala Arg	Leu Leu Ala Leu	Ala Gly Ala Lys
[4414]		580	585	590
[4415]	Val Met Met Val	Ala Arg Arg Glu	Ser Glu Leu Val	Ala Ala Arg Asp
[4416]		595	600	605
[4417]	Arg Ile Val Gly	Glu Leu Gln Asp	Ile Gly Phe Ala	Gly Val Glu Arg
[4418]	610	615	620	
[4419]	Arg Val Lys Tyr	Met Ala Asp Ile	Asp Val Ser Asp	Phe Ala Ser Leu
[4420]	625	630	635	640
[4421]	Asp Lys Ala Val	Asp Ala Thr Leu	Glu Glu Phe Gly	Arg Ile Asp Tyr
[4422]		645	650	655
[4423]	Leu Ile Asn Asn	Ala Gly Val Ala	Gly Ala Glu Asp	Met Val Ile Asp
[4424]		660	665	670
[4425]	Met Glu Pro Glu	Ala Trp Arg Phe	Thr Leu Asp Ala	Asn Leu Ile Ser
[4426]		675	680	685
[4427]	Asn Tyr His Leu	Met Gln Arg Val	Val Pro Leu Met	Lys Glu Gln Gly
[4428]	690	695	700	
[4429]	Ser Gly Tyr Val	Leu Asn Val Ser	Ser Tyr Phe Gly	Gly Glu Lys Phe
[4430]	705	710	715	720
[4431]	Leu Ala Val Ala	Tyr Pro Asn Arg	Ala Asp Tyr Gly	Leu Ser Lys Ala
[4432]		725	730	735
[4433]	Gly Gln Arg Ala	Met Val Glu Ala	Phe Ser Pro Phe	Leu Gly Pro Glu
[4434]		740	745	750
[4435]	Val Gln Cys Asn	Ala Ile Ala Pro	Gly Pro Val Asp	Gly Asp Arg Leu
[4436]		755	760	765
[4437]	Ser Gly Thr Gly	Gly Lys Pro Gly	Leu Phe Gln Arg	Arg Ala Lys Leu
[4438]	770	775	780	
[4439]	Ile Leu Glu Asn	Lys Arg Leu Asn	Ala Val Tyr Ser	Ala Val Ile His
[4440]	785	790	795	800
[4441]	Ala Ile Arg Glu	Gly Gly Asp Ala	Ala Lys Ile Leu	Thr Arg Leu Ser
[4442]		805	810	815
[4443]	Arg Asn Ser Thr	Ser Thr Leu Ser	His Asp Ala Glu	Ala Pro Glu Glu
[4444]		820	825	830
[4445]	Leu Arg Lys Leu	Ala Leu Asp Phe	Ala Ser Gln Gly	Asp Gly Leu Cys
[4446]		835	840	845
[4447]	Thr Trp Asp Gln	Tyr Leu Leu Thr	Asp Ala Met Ala	Gln Arg Leu Leu
[4448]		850	855	860
[4449]	Val Arg Leu Gln	Leu Gly Gly Phe	Leu Leu Gly Ser	Asn Glu Trp Ala
[4450]	865	870	875	880
[4451]	Ser Leu Ser Ser	Ser Glu Gln Thr	Trp Leu Lys Leu	Ser Pro Pro Asp

[4452]		885		890		895
[4453]	Asp Lys Pro Phe Leu Pro Ala Ala Gln Val Asp Lys Val Ala Asn Gly					
[4454]		900		905		910
[4455]	Val Gly Lys Gly Val Ile Ser Gln Leu His Leu Gly Ala Met Pro Thr					
[4456]		915		920		925
[4457]	Glu Ala Glu Val Ala Gln Ala Thr Val Phe Phe Leu Ala Asp Arg Ala					
[4458]		930		935		940
[4459]	Val Ser Gly Glu Thr Phe Met Pro Ser Gly Gly Leu Arg Val Glu Arg					
[4460]	945		950		955	960
[4461]	Ser Asn Thr Glu Arg Glu Met Phe Gly Ser Pro Lys Gln Glu Arg Ile					
[4462]		965		970		975
[4463]	Asp Lys Met Lys Gly Lys Thr Val Trp Ile Ile Gly Glu His Leu Ser					
[4464]		980		985		990
[4465]	Asp Tyr Val Ala Ala Thr Ile Glu Glu Leu Val Ser Gly Cys Gly Val					
[4466]		995		1000		1005
[4467]	Ala Lys Val Val Leu Ile Ala Lys Asp Lys Ser Gly Glu Lys Ala					
[4468]		1010		1015		1020
[4469]	Val Arg Asp Gln Leu Pro Asn Asp Leu Ser Lys Asp Ala Leu Glu					
[4470]		1025		1030		1035
[4471]	Val Leu Ile Ala Gly Asp Gly Leu Glu Glu Ala Met Asp Glu Ala					
[4472]		1040		1045		1050
[4473]	Leu Gly His Trp Gly Lys Pro Thr Thr Val Leu Ser Met Pro Gly					
[4474]		1055		1060		1065
[4475]	Glu Pro Leu Pro Asp His Leu Phe Glu Gly Gly Asn Pro Leu Ser					
[4476]		1070		1075		1080
[4477]	Thr Lys Asp Phe Ala His Met Val Glu Ala Asn Ile Thr Arg His					
[4478]		1085		1090		1095
[4479]	Tyr Arg Val Thr Arg Lys Ala Ser Leu Tyr Asp Gly Cys Gln Val					
[4480]		1100		1105		1110
[4481]	Val Leu Val Ser Pro Asp Val Pro Tyr Gly Ser Asp Gly Pro Gly					
[4482]		1115		1120		1125
[4483]	Val Ala Leu Ala Asn Phe Val Lys Thr Ser Leu His Ala Phe Thr					
[4484]		1130		1135		1140
[4485]	Ala Thr Val Ala Val Glu Asn Glu Arg Leu Val His Asp Val Pro					
[4486]		1145		1150		1155
[4487]	Val Asn Gln Ile Asn Leu Thr Arg Arg Val Ser Ser Glu Glu Pro					
[4488]		1160		1165		1170
[4489]	Arg Asp Ala Asp Glu His Ala Glu Glu Leu Arg Arg Phe Thr Arg					
[4490]		1175		1180		1185
[4491]	Ala Val Leu Leu Val Gly Ala Pro Leu Pro Asp Ala Gln Asp Ser					
[4492]		1190		1195		1200
[4493]	Arg Tyr Arg Ser Lys Ile Tyr Arg Gly Thr Ser Met Thr Val					

[4494]	1205	1210	1215
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[4500]	Met Arg Arg Thr Leu Lys Ala Ala Ile Leu Gly Ala Thr Gly Leu Val		
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[4502]	Gly Ile Glu Tyr Val Arg Met Leu Ala Asp His Pro Tyr Ile Lys Pro		
[4503]	20 25 30		
[4504]	Thr Tyr Leu Ala Gly Lys Gly Ser Val Gly Lys Pro Tyr Gly Glu Ile		
[4505]	35 40 45		
[4506]	Val Arg Trp Gln Thr Val Gly Asn Val Pro Lys Glu Val Ala Asn Gln		
[4507]	50 55 60		
[4508]	Glu Val Lys Pro Thr Asp Pro Lys Leu Met Asp Asp Val Asp Ile Ile		
[4509]	65 70 75 80		
[4510]	Phe Ser Pro Leu Pro Gln Gly Ala Ala Gly Pro Val Glu Glu Gln Phe		
[4511]	85 90 95		
[4512]	Ala Lys Leu Gly Phe Asn Val Ile Ser Asn Ser Pro Asp His Arg Phe		
[4513]	100 105 110		
[4514]	Asp Met Asp Val Pro Met Ile Ile Pro Glu Val Asn Pro His Thr Val		
[4515]	115 120 125		
[4516]	Thr Leu Ile Asp Glu Gln Arg Lys Arg Arg Asp Trp Lys Gly Phe Ile		
[4517]	130 135 140		
[4518]	Val Thr Thr Pro Leu Cys Thr Ala Gln Gly Ala Ala Ile Pro Leu Thr		
[4519]	145 150 155 160		
[4520]	Pro Ile Tyr Gln Asn Phe Lys Met Ser Gly Val Met Ile Thr Thr Met		
[4521]	165 170 175		
[4522]	Gln Ser Leu Ser Gly Ala Gly Tyr Pro Gly Ile Ala Ser Leu Asp Ile		
[4523]	180 185 190		
[4524]	Val Asp Asn Ala Leu Pro Leu Gly Asp Gly Tyr Asp Ala Lys Thr Val		
[4525]	195 200 205		
[4526]	Lys Glu Ile Thr Arg Ile Leu Ser Glu Val Lys Arg Asn Val Gln Glu		
[4527]	210 215 220		
[4528]	Pro Gly Val Asn Glu Ile Thr Leu Asp Ala Thr Thr His Arg Ile Ala		
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[4530]	Thr Ile His Gly His Tyr Glu Val Ala Tyr Val Thr Phe Lys Glu Asp		
[4531]	245 250 255		
[4532]	Thr Asp Val Arg Lys Val Met Glu Ser Met Glu Ser Phe Lys Gly Glu		
[4533]	260 265 270		
[4534]	Pro Gln Asp Leu Lys Leu Pro Thr Ala Pro Glu Lys Pro Ile Ile Val		
[4535]	275 280 285		

[4536]	Thr Thr Gln Asp Ala Arg Pro Gln Val Phe Phe Asp Arg Trp Ala Gly
[4537]	290 295 300
[4538]	Asn Pro Pro Gly Met Ser Val Val Val Gly Arg Leu Lys Gln Val Asn
[4539]	305 310 315 320
[4540]	Pro Arg Thr Ile Arg Phe Val Ser Leu Ile His Asn Thr Val Arg Gly
[4541]	325 330 335
[4542]	Ala Ala Gly Gly Gly Val Leu Thr Ala Glu Leu Leu Val Glu Lys Gly
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[4544]	Tyr Ile Asp Lys Arg
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[4550]	<400> 62
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[4553]	Gly Ile Glu Tyr Val Arg Met Leu Ser Asn His Pro Tyr Ile Lys Pro
[4554]	20 25 30
[4555]	Ala Tyr Leu Ala Gly Lys Gly Ser Val Gly Lys Pro Tyr Gly Glu Val
[4556]	35 40 45
[4557]	Val Arg Trp Gln Thr Val Gly Gln Val Pro Lys Glu Ile Ala Asp Met
[4558]	50 55 60
[4559]	Glu Ile Lys Pro Thr Asp Pro Lys Leu Met Asp Asp Val Asp Ile Ile
[4560]	65 70 75 80
[4561]	Phe Ser Pro Leu Pro Gln Gly Ala Ala Gly Pro Val Glu Glu Gln Phe
[4562]	85 90 95
[4563]	Ala Lys Glu Gly Phe Pro Val Ile Ser Asn Ser Pro Asp His Arg Phe
[4564]	100 105 110
[4565]	Asp Pro Asp Val Pro Leu Leu Val Pro Glu Leu Asn Pro His Thr Ile
[4566]	115 120 125
[4567]	Ser Leu Ile Asp Glu Gln Arg Lys Arg Arg Glu Trp Lys Gly Phe Ile
[4568]	130 135 140
[4569]	Val Thr Thr Pro Leu Cys Thr Ala Gln Gly Ala Ala Ile Pro Leu Gly
[4570]	145 150 155 160
[4571]	Ala Ile Phe Lys Asp Tyr Lys Met Asp Gly Ala Phe Ile Thr Thr Ile
[4572]	165 170 175
[4573]	Gln Ser Leu Ser Gly Ala Gly Tyr Pro Gly Ile Pro Ser Leu Asp Val
[4574]	180 185 190
[4575]	Val Asp Asn Ile Leu Pro Leu Gly Asp Gly Tyr Asp Ala Lys Thr Ile
[4576]	195 200 205
[4577]	Lys Glu Ile Phe Arg Ile Leu Ser Glu Val Lys Arg Asn Val Asp Glu

[4578]	210	215	220
[4579]	Pro Lys Leu Glu Asp Val Ser Leu Ala Ala Thr Thr His Arg Ile Ala		
[4580]	225	230	235
[4581]	Thr Ile His Gly His Tyr Glu Val Leu Tyr Val Ser Phe Lys Glu Glu		
[4582]		245	250
[4583]	Thr Ala Ala Glu Lys Val Lys Glu Thr Leu Glu Asn Phe Arg Gly Glu		
[4584]		260	265
[4585]	Pro Gln Asp Leu Lys Leu Pro Thr Ala Pro Ser Lys Pro Ile Ile Val		
[4586]		275	280
[4587]	Met Asn Glu Asp Thr Arg Pro Gln Val Tyr Phe Asp Arg Trp Ala Gly		
[4588]	290	295	300
[4589]	Asp Ile Pro Gly Met Ser Val Val Val Gly Arg Leu Lys Gln Val Asn		
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[4591]	Lys Arg Met Ile Arg Leu Val Ser Leu Ile His Asn Thr Val Arg Gly		
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[4593]	Ala Ala Gly Gly Gly Ile Leu Ala Ala Glu Leu Leu Val Glu Lys Gly		
[4594]		340	345
[4595]	Tyr Ile Glu Lys		
[4596]	355		
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[4604]	Leu Thr Arg Ser Leu Arg Leu Gln Leu Arg Cys Tyr Ala Ser Tyr Pro		
[4605]		20	25
[4606]	Glu His Thr Ile Ile Gly Met Pro Ala Leu Ser Pro Thr Met Thr Gln		
[4607]		35	40
[4608]	Gly Asn Leu Ala Ala Trp Thr Lys Lys Glu Gly Asp Gln Leu Ser Pro		
[4609]	50	55	60
[4610]	Gly Glu Val Ile Ala Glu Ile Glu Thr Asp Lys Ala Gln Met Asp Phe		
[4611]	65	70	75
[4612]	Glu Phe Gln Glu Asp Gly Tyr Leu Ala Lys Ile Leu Val Pro Glu Gly		
[4613]		85	90
[4614]	Thr Lys Asp Ile Pro Val Asn Lys Pro Ile Ala Val Tyr Val Glu Asp		
[4615]		100	105
[4616]	Lys Ala Asp Val Pro Ala Phe Lys Asp Phe Lys Leu Glu Asp Ser Gly		
[4617]		115	120
[4618]	Ser Asp Ser Lys Thr Ser Thr Lys Ala Gln Pro Ala Glu Pro Gln Ala		
[4619]	130	135	140

[4620]	Glu Lys Lys Gln Glu Ala Pro Ala Glu Glu Thr Lys Thr Ser Ala Pro		
[4621]	145	150	155 160
[4622]	Glu Ala Lys Lys Ser Asp Val Ala Ala Pro Gln Gly Arg Ile Phe Ala		
[4623]		165 170	175
[4624]	Ser Pro Leu Ala Lys Thr Ile Ala Leu Glu Lys Gly Ile Ser Leu Lys		
[4625]		180 185	190
[4626]	Asp Val His Gly Thr Gly Pro Arg Gly Arg Ile Thr Lys Ala Asp Ile		
[4627]		195 200	205
[4628]	Glu Ser Tyr Leu Glu Lys Ser Ser Lys Gln Ser Ser Gln Thr Ser Gly		
[4629]	210	215	220
[4630]	Ala Ala Ala Ala Thr Pro Ala Ala Ala Thr Ser Ser Thr Thr Ala Gly		
[4631]	225	230	235 240
[4632]	Ser Ala Pro Ser Pro Ser Ser Thr Ala Ser Tyr Glu Asp Val Pro Ile		
[4633]		245 250	255
[4634]	Ser Thr Met Arg Ser Ile Ile Gly Glu Arg Leu Leu Gln Ser Thr Gln		
[4635]		260 265	270
[4636]	Gly Ile Pro Ser Tyr Ile Val Ser Ser Lys Ile Ser Ile Ser Lys Leu		
[4637]		275 280	285
[4638]	Leu Lys Leu Arg Gln Ser Leu Asn Ala Thr Ala Asn Asp Lys Tyr Lys		
[4639]	290	295	300
[4640]	Leu Ser Ile Asn Asp Leu Leu Val Lys Ala Ile Thr Val Ala Ala Lys		
[4641]	305	310	315 320
[4642]	Arg Val Pro Asp Ala Asn Ala Tyr Trp Leu Pro Asn Glu Asn Val Ile		
[4643]		325 330	335
[4644]	Arg Lys Phe Lys Asn Val Asp Val Ser Val Ala Val Ala Thr Pro Thr		
[4645]		340 345	350
[4646]	Gly Leu Leu Thr Pro Ile Val Lys Asn Cys Glu Ala Lys Gly Leu Ser		
[4647]		355 360	365
[4648]	Gln Ile Ser Asn Glu Ile Lys Glu Leu Val Lys Arg Ala Arg Ile Asn		
[4649]	370	375	380
[4650]	Lys Leu Ala Pro Glu Glu Phe Gln Gly Gly Thr Ile Cys Ile Ser Asn		
[4651]	385	390	395 400
[4652]	Met Gly Met Asn Asn Ala Val Asn Met Phe Thr Ser Ile Ile Asn Pro		
[4653]		405 410	415
[4654]	Pro Gln Ser Thr Ile Leu Ala Ile Ala Thr Val Glu Arg Val Ala Val		
[4655]		420 425	430
[4656]	Glu Asp Ala Ala Ala Glu Asn Gly Phe Ser Phe Asp Asn Gln Val Thr		
[4657]		435 440	445
[4658]	Ile Thr Gly Thr Phe Asp His Arg Thr Ile Asp Gly Ala Lys Gly Ala		
[4659]		450 455	460
[4660]	Glu Phe Met Lys Glu Leu Lys Thr Val Ile Glu Asn Pro Leu Glu Met		
[4661]	465	470	475 480

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 [4670] Leu Gly Ala Val Leu Arg Thr Pro Thr Arg Ile Gly His Val Arg Thr
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 [4674] Gly Ser Asp Thr Val Gln Ile Glu Leu Pro Glu Ser Ser Phe Glu Ser
 [4675] 50 55 60
 [4676] Tyr Met Leu Glu Pro Pro Asp Leu Ser Tyr Glu Thr Ser Lys Ala Thr
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 [4678] Leu Leu Gln Met Tyr Lys Asp Met Val Ile Ile Arg Arg Met Glu Met
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 [4680] Ala Cys Asp Ala Leu Tyr Lys Ala Lys Lys Ile Arg Gly Phe Cys His
 [4681] 100 105 110
 [4682] Leu Ser Val Gly Gln Glu Ala Ile Ala Val Gly Ile Glu Asn Ala Ile
 [4683] 115 120 125
 [4684] Thr Lys Leu Asp Ser Ile Ile Thr Ser Tyr Arg Cys His Gly Phe Thr
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 [4686] Phe Met Arg Gly Ala Ser Val Lys Ala Val Leu Ala Glu Leu Met Gly
 [4687] 145 150 155 160
 [4688] Arg Arg Ala Gly Val Ser Tyr Gly Lys Gly Gly Ser Met His Leu Tyr
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 [4692] Leu Gly Ala Gly Leu Ala Phe Ala His Gln Tyr Lys Asn Glu Asp Ala
 [4693] 195 200 205
 [4694] Cys Ser Phe Thr Leu Tyr Gly Asp Gly Ala Ser Asn Gln Gly Gln Val
 [4695] 210 215 220
 [4696] Phe Glu Ser Phe Asn Met Ala Lys Leu Trp Asn Leu Pro Val Val Phe
 [4697] 225 230 235 240
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 [4701] 260 265 270
 [4702] Lys Val Asn Gly Met Asp Ile Leu Ala Val Tyr Gln Ala Ser Lys Phe
 [4703] 275 280 285

[4704]	Ala Lys Asp Trp Cys Leu Ser Gly Lys Gly Pro Leu Val Leu Glu Tyr
[4705]	290 295 300
[4706]	Glu Thr Tyr Arg Tyr Gly Gly His Ser Met Ser Asp Pro Gly Thr Thr
[4707]	305 310 315 320
[4708]	Tyr Arg Thr Arg Asp Glu Ile Gln His Met Arg Ser Lys Asn Asp Pro
[4709]	325 330 335
[4710]	Ile Ala Gly Leu Lys Met His Leu Ile Asp Leu Gly Ile Ala Thr Glu
[4711]	340 345 350
[4712]	Ala Glu Val Lys Ala Tyr Asp Lys Ser Ala Arg Lys Tyr Val Asp Glu
[4713]	355 360 365
[4714]	Gln Val Glu Leu Ala Asp Ala Ala Pro Pro Pro Glu Ala Lys Leu Ser
[4715]	370 375 380
[4716]	Ile Leu Phe Glu Asp Val Tyr Val Lys Gly Thr Glu Thr Pro Thr Leu
[4717]	385 390 395 400
[4718]	Arg Gly Arg Ile Pro Glu Asp Thr Trp Asp Phe Lys Lys Gln Gly Phe
[4719]	405 410 415
[4720]	Ala Ser Arg Asp
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[4729]	Ala Pro Thr Ser Phe Val Arg Pro Ser Ala Ala Ala Ala Ala Leu Arg
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[4731]	Phe Ser Ser Thr Lys Thr Met Thr Val Arg Glu Ala Leu Asn Ser Ala
[4732]	35 40 45
[4733]	Met Ala Glu Glu Leu Asp Arg Asp Asp Asp Val Phe Leu Ile Gly Glu
[4734]	50 55 60
[4735]	Glu Val Ala Gln Tyr Asn Gly Ala Tyr Lys Val Ser Lys Gly Leu Leu
[4736]	65 70 75 80
[4737]	Asp Arg Phe Gly Glu Arg Arg Val Val Asp Thr Pro Ile Thr Glu Tyr
[4738]	85 90 95
[4739]	Gly Phe Thr Gly Leu Ala Val Gly Ala Ala Leu Lys Gly Leu Lys Pro
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[4741]	Ile Val Glu Phe Met Ser Phe Asn Phe Ser Met Gln Ala Ile Asp His
[4742]	115 120 125
[4743]	Val Val Asn Ser Ala Ala Lys Thr His Tyr Met Ser Gly Gly Thr Gln
[4744]	130 135 140
[4745]	Lys Cys Gln Met Val Phe Arg Gly Pro Asn Gly Ala Ala Val Gly Val

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[4747]	Gly Ala Gln His Ser Gln Asp Phe Ser Pro Trp Tyr Gly Ser Ile Pro			
[4748]		165	170	175
[4749]	Gly Leu Lys Val Leu Val Pro Tyr Ser Ala Glu Asp Ala Arg Gly Leu			
[4750]		180	185	190
[4751]	Leu Lys Ala Ala Ile Arg Asp Pro Asn Pro Val Val Phe Leu Glu Asn			
[4752]		195	200	205
[4753]	Glu Leu Leu Tyr Gly Glu Ser Phe Glu Ile Ser Glu Glu Ala Leu Ser			
[4754]		210	215	220
[4755]	Pro Glu Phe Thr Leu Pro Tyr Lys Ala Lys Ile Glu Arg Glu Gly Thr			
[4756]	225	230	235	240
[4757]	Asp Ile Ser Ile Val Thr Tyr Thr Arg Asn Val Gln Phe Ser Leu Glu			
[4758]		245	250	255
[4759]	Ala Ala Glu Ile Leu Gln Lys Lys Tyr Gly Val Ser Ala Glu Val Ile			
[4760]		260	265	270
[4761]	Asn Leu Arg Ser Ile Arg Pro Leu Asp Thr Glu Ala Ile Ile Lys Thr			
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[4763]	Val Lys Lys Thr Asn His Leu Ile Thr Val Glu Ser Thr Phe Pro Ser			
[4764]		290	295	300
[4765]	Phe Gly Val Gly Ala Glu Ile Val Ala Gln Val Met Glu Ser Glu Ala			
[4766]	305	310	315	320
[4767]	Phe Asp Tyr Leu Asp Ala Pro Ile Gln Arg Val Thr Gly Ala Asp Val			
[4768]		325	330	335
[4769]	Pro Thr Pro Tyr Ala Lys Glu Leu Glu Asp Phe Ala Phe Pro Asp Thr			
[4770]		340	345	350
[4771]	Pro Thr Ile Val Lys Ala Val Lys Glu Val Leu Ser Ile Glu			
[4772]		355	360	365
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[4780]	Thr Val Arg Thr Leu Thr Ile Asn Lys Ser His Asp Val Val Ile Ile			
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[4782]	Gly Gly Gly Pro Ala Gly Tyr Val Ala Ala Ile Lys Ala Ala Gln Leu			
[4783]		35	40	45
[4784]	Gly Phe Asn Thr Ala Cys Val Glu Lys Arg Gly Lys Leu Gly Gly Thr			
[4785]		50	55	60
[4786]	Cys Leu Asn Val Gly Cys Ile Pro Ser Lys Ala Leu Leu Asn Asn Ser			
[4787]	65	70	75	80

[4788]	His	Leu	Phe	His	Gln	Met	His	Thr	Glu	Ala	Gln	Lys	Arg	Gly	Ile	Asp
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[4790]	Val	Asn	Gly	Asp	Ile	Lys	Ile	Asn	Val	Ala	Asn	Phe	Gln	Lys	Ala	Lys
[4791]					100				105						110	
[4792]	Asp	Asp	Ala	Val	Lys	Gln	Leu	Thr	Gly	Gly	Ile	Glu	Leu	Leu	Phe	Lys
[4793]					115				120						125	
[4794]	Lys	Asn	Lys	Val	Thr	Tyr	Tyr	Lys	Gly	Asn	Gly	Ser	Phe	Glu	Asp	Glu
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[4797]					145				150						155	
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[4800]	Glu	Val	Thr	Pro	Phe	Pro	Gly	Ile	Glu	Ile	Asp	Glu	Glu	Lys	Ile	Val
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[4802]	Ser	Ser	Thr	Gly	Ala	Leu	Ser	Leu	Lys	Glu	Ile	Pro	Lys	Arg	Leu	Thr
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[4808]	Ala	Ser	Met	Asp	Gly	Glu	Val	Ala	Lys	Ala	Thr	Gln	Lys	Phe	Leu	Lys
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[4811]					260				265							270
[4812]	Arg	Asn	Asp	Asp	Lys	Asn	Val	Val	Glu	Ile	Val	Val	Glu	Asp	Thr	Lys
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[4820]	Lys	Phe	Pro	His	Ile	Lys	Val	Val	Gly	Asp	Val	Thr	Phe	Gly	Pro	Met
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[4823]					355				360							365
[4824]	Lys	Thr	Gly	His	Gly	His	Val	Asn	Tyr	Asn	Asn	Ile	Pro	Ser	Val	Met
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[4826]	Tyr	Ser	His	Pro	Glu	Val	Ala	Trp	Val	Gly	Lys	Thr	Glu	Glu	Gln	Leu
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[4833]		435	440
[4834]	Pro Asn Ala Gly Glu Met Ile Ala Glu Ala Gly Leu Ala Leu Glu Tyr		445
[4835]		450	455
[4836]	Gly Ala Ser Ala Glu Asp Val Ala Arg Val Cys His Ala His Pro Thr		460
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[4850]	Ala Gln Tyr Leu Ile Asp Gln Leu Leu Ala Glu Ala Arg Lys Gly Gly		30
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[4852]	Val Asn Val Ala Ala Gly Thr Gly Ile Ser Asn Tyr Ile Asn Thr Ile		45
[4853]		50	55
[4854]	Pro Val Glu Glu Gln Pro Glu Tyr Pro Gly Asn Leu Glu Leu Glu Arg		60
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[4858]	Ala Ser Lys Lys Asp Leu Glu Leu Gly Gly His Met Ala Ser Phe Gln		95
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[4860]	Ser Ser Ala Thr Ile Tyr Asp Val Cys Phe Asn His Phe Phe Arg Ala		110
[4861]		115	120
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[4867]		165	170
[4868]	Ser Tyr Pro His Pro Lys Leu Met Pro Glu Phe Trp Gln Phe Pro Thr		175
[4869]		180	185
[4870]	Val Ser Met Gly Leu Gly Pro Ile Gly Ala Ile Tyr Gln Ala Lys Phe		190
[4871]		195	200
			205

[4872]	Leu Lys Tyr Leu Glu His Arg Gly Leu Lys Asp Thr Ser Lys Gln Thr		
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[4874]	Val Tyr Ala Phe Leu Gly Asp Gly Glu Met Asp Glu Pro Glu Ser Lys		
[4875]	225	230	235 240
[4876]	Gly Ala Ile Thr Ile Ala Thr Arg Glu Lys Leu Asp Asn Leu Val Phe		
[4877]	245	250	255
[4878]	Val Ile Asn Cys Asn Leu Gln Arg Leu Asp Gly Pro Val Thr Gly Asn		
[4879]	260	265	270
[4880]	Gly Lys Ile Ile Asn Glu Leu Glu Gly Ile Phe Glu Gly Ala Gly Trp		
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[4882]	Asn Val Ile Lys Val Met Trp Gly Ser Arg Trp Asp Glu Leu Leu Arg		
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[4884]	Lys Asp Thr Ser Gly Lys Leu Ile Gln Leu Met Asn Glu Thr Val Asp		
[4885]	305	310	315 320
[4886]	Gly Asp Tyr Gln Thr Phe Lys Ser Lys Asp Gly Ala Tyr Val Arg Glu		
[4887]	325	330	335
[4888]	His Phe Phe Gly Lys Tyr Pro Glu Thr Ala Ala Leu Val Ala Asp Trp		
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[4892]	Lys Ile Tyr Ala Ala Phe Lys Lys Ala Gln Glu Thr Lys Gly Lys Ala		
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[4894]	Thr Val Ile Leu Ala His Thr Ile Lys Gly Tyr Gly Met Gly Asp Ala		
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[4896]	Ala Glu Gly Lys Asn Ile Ala His Gln Val Lys Lys Met Asn Met Asp		
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[4902]	His Thr Tyr Leu His Ala Gln Arg Gln Lys Leu His Gly Tyr Leu Pro		
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[4905]	465	470	475 480
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[4910]	Lys Asp Arg Leu Val Pro Ile Ile Ala Asp Glu Ala Arg Thr Phe Gly		
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[4914]	Gln Tyr Thr Pro Gln Asp Arg Glu Gln Val Ala Tyr Tyr Lys Glu Asp		
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[4916]	Glu Lys Gly Gln Ile Leu Gln Glu Gly Ile Asn Glu Leu Gly Ala Gly		
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[4918]	Cys Ser Trp Leu Ala Ala Ala Thr Ser Tyr Ser Thr Asn Asn Leu Pro		
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[4922]	Gly Asp Leu Cys Trp Ala Ala Gly Asp Gln Gln Ala Arg Gly Phe Leu		
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[4924]	Ile Gly Gly Thr Ser Gly Arg Thr Thr Leu Asn Gly Glu Gly Leu Gln		
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[4926]	His Glu Asp Gly His Ser His Ile Gln Ser Leu Thr Ile Pro Asn Cys		
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[4928]	Ile Ser Tyr Asp Pro Ala Tyr Ala Tyr Glu Val Ala Val Ile Met His		
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[4930]	Asp Gly Leu Glu Arg Met Tyr Gly Glu Lys Gln Glu Asn Val Tyr Tyr		
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[4932]	Tyr Ile Thr Thr Leu Asn Glu Asn Tyr His Met Pro Ala Met Pro Glu		
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[4934]	Gly Ala Glu Glu Gly Ile Arg Lys Gly Ile Tyr Lys Leu Glu Thr Ile		
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[4936]	Glu Gly Ser Lys Gly Lys Val Gln Leu Leu Gly Ser Gly Ser Ile Leu		
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[4938]	Arg His Val Arg Glu Ala Ala Glu Ile Leu Ala Lys Asp Tyr Gly Val		
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[4940]	Gly Ser Asp Val Tyr Ser Val Thr Ser Phe Thr Glu Leu Ala Arg Asp		
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[4942]	Gly Gln Asp Cys Glu Arg Trp Asn Met Leu His Pro Leu Glu Thr Pro		
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[4944]	Arg Val Pro Tyr Ile Ala Gln Val Met Asn Asp Ala Pro Ala Val Ala		
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[4946]	Ser Thr Asp Tyr Met Lys Leu Phe Ala Glu Gln Val Arg Thr Tyr Val		
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[4948]	Pro Ala Asp Asp Tyr Arg Val Leu Gly Thr Asp Gly Phe Gly Arg Ser		
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[4952]	Val Val Val Ala Ala Leu Gly Glu Leu Ala Lys Arg Gly Glu Ile Asp		
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[4954]	Lys Lys Val Val Ala Asp Ala Ile Ala Lys Phe Asn Ile Asp Ala Asp		
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[4967]	Ser Leu Ile Thr Val Glu Gly Asp Lys Ala Ser Met Glu Val Pro Ser
[4968]	35 40 45
[4969]	Pro Gln Ala Gly Ile Val Lys Glu Ile Lys Val Ser Val Gly Asp Lys
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[4973]	Ala Asp Ala Ala Pro Ala Gln Ala Glu Glu Lys Lys Glu Ala Ala Pro
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[4975]	Ala Ala Ala Pro Ala Ala Ala Ala Ala Lys Asp Val Asn Val Pro Asp
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[4982]	145 150 155 160
[4983]	Lys Val Asn Val Gly Asp Lys Val Ser Thr Gly Ser Leu Ile Met Val
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[4986]	180 185 190
[4987]	Glu Ala Ala Pro Ala Ala Ala Pro Ala Pro Ala Ala Gly Val Lys Glu
[4988]	195 200 205
[4989]	Val Asn Val Pro Asp Ile Gly Gly Asp Glu Val Glu Val Thr Glu Val
[4990]	210 215 220
[4991]	Met Val Lys Val Gly Asp Lys Val Ala Ala Glu Gln Ser Leu Ile Thr
[4992]	225 230 235 240
[4993]	Val Glu Gly Asp Lys Ala Ser Met Glu Val Pro Ala Pro Phe Ala Gly
[4994]	245 250 255
[4995]	Val Val Lys Glu Leu Lys Val Asn Val Gly Asp Lys Val Lys Thr Gly
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[4997]	Ser Leu Ile Met Ile Phe Glu Val Glu Gly Ala Ala Pro Ala Ala Ala

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[5001]	Ala Pro Ala Ala Ala Pro Ala Ala Lys Ala Glu Gly Lys Ser Glu Phe		
[5002]	305	310	315 320
[5003]	Ala Glu Asn Asp Ala Tyr Val His Ala Thr Pro Leu Ile Arg Arg Leu		
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[5005]	Ala Arg Glu Phe Gly Val Asn Leu Ala Lys Val Lys Gly Thr Gly Arg		
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[5007]	Lys Gly Arg Ile Leu Arg Glu Asp Val Gln Ala Tyr Val Lys Glu Ala		
[5008]	355	360	365
[5009]	Ile Lys Arg Ala Glu Ala Ala Pro Ala Ala Thr Gly Gly Gly Ile Pro		
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[5011]	Gly Met Leu Pro Trp Pro Lys Val Asp Phe Ser Lys Phe Gly Glu Ile		
[5012]	385	390	395 400
[5013]	Glu Glu Val Glu Leu Gly Arg Ile Gln Lys Ile Ser Gly Ala Asn Leu		
[5014]	405	410	415
[5015]	Ser Arg Asn Trp Val Met Ile Pro His Val Thr His Phe Asp Lys Thr		
[5016]	420	425	430
[5017]	Asp Ile Thr Glu Leu Glu Ala Phe Arg Lys Gln Gln Asn Glu Glu Ala		
[5018]	435	440	445
[5019]	Ala Lys Arg Lys Leu Asp Val Lys Ile Thr Pro Val Val Phe Ile Met		
[5020]	450	455	460
[5021]	Lys Ala Val Ala Ala Ala Leu Glu Gln Met Pro Arg Phe Asn Ser Ser		
[5022]	465	470	475 480
[5023]	Leu Ser Glu Asp Gly Gln Arg Leu Thr Leu Lys Lys Tyr Ile Asn Ile		
[5024]	485	490	495
[5025]	Gly Val Ala Val Asp Thr Pro Asn Gly Leu Val Val Pro Val Phe Lys		
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[5027]	Asp Val Asn Lys Lys Gly Ile Ile Glu Leu Ser Arg Glu Leu Met Thr		
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[5029]	Ile Ser Lys Lys Ala Arg Asp Gly Lys Leu Thr Ala Gly Glu Met Gln		
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[5031]	Gly Gly Cys Phe Thr Ile Ser Ser Ile Gly Gly Leu Gly Thr Thr His		
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[5033]	Phe Ala Pro Ile Val Asn Ala Pro Glu Val Ala Ile Leu Gly Val Ser		
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[5037]	Leu Met Leu Pro Ile Ser Leu Ser Phe Asp His Arg Val Ile Asp Gly		
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[5039]	Ala Asp Gly Ala Arg Phe Ile Thr Ile Ile Asn Asn Thr Leu Ser Asp		

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[5052]	Val Ile Val Glu Arg Tyr Asn Thr Leu Gly Gly Val Cys Leu Asn Val		
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[5054]	Gly Cys Ile Pro Ser Lys Ala Leu Leu His Val Ala Lys Val Ile Glu		
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[5056]	Glu Ala Lys Ala Leu Ala Glu His Gly Ile Val Phe Gly Glu Pro Lys		
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[5058]	Thr Asp Ile Asp Lys Ile Arg Thr Trp Lys Glu Lys Val Ile Asn Gln		
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[5060]	Leu Thr Gly Gly Leu Ala Gly Met Ala Lys Gly Arg Lys Val Lys Val		
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[5062]	Val Asn Gly Leu Gly Lys Phe Thr Gly Ala Asn Thr Leu Glu Val Glu		
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[5064]	Gly Glu Asn Gly Lys Thr Val Ile Asn Phe Asp Asn Ala Ile Ile Ala		
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[5066]	Ala Gly Ser Arg Pro Ile Gln Leu Pro Phe Ile Pro His Glu Asp Pro		
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[5068]	Arg Ile Trp Asp Ser Thr Asp Ala Leu Glu Leu Lys Glu Val Pro Glu		
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[5070]	Arg Leu Leu Val Met Gly Gly Gly Ile Ile Gly Leu Glu Met Gly Thr		
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[5072]	Val Tyr His Ala Leu Gly Ser Gln Ile Asp Val Val Glu Met Phe Asp		
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[5074]	Gln Val Ile Pro Ala Ala Asp Lys Asp Ile Val Lys Val Phe Thr Lys		
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[5084]	Val Asp Asp Arg Gly Phe Ile Arg Val Asp Lys Gln Leu Arg Thr Asn		
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[5086]	Val Pro His Ile Phe Ala Ile Gly Asp Ile Val Gly Gln Pro Met Leu		
[5087]	305	310	315
[5088]	Ala His Lys Gly Val His Glu Gly His Val Ala Ala Glu Val Ile Ala		
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[5092]	Thr Glu Pro Glu Val Ala Trp Val Gly Leu Thr Glu Lys Glu Ala Lys		
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[5100]	Asn Gly Gly Glu Leu Leu Gly Glu Ile Gly Leu Ala Ile Glu Met Gly		
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[5102]	Cys Asp Ala Glu Asp Ile Ala Leu Thr Ile His Ala His Pro Thr Leu		
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[5104]	His Glu Ser Val Gly Leu Ala Ala Glu Val Phe Glu Gly Ser Ile Thr		
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[5106]	Asp Leu Pro Asn Pro Lys Ala Lys Lys Lys		
[5107]	465	470	
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[5127]	Trp His Gly Leu Pro Leu Tyr Gln Ala Phe Leu Phe Ser Arg Gly His		
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[5129]	Phe Arg Gly Asn Gln Met Pro Asp Asp Val Asn Ala Leu Ser Pro Gln		
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[5133]	Leu Lys Lys Arg Gly Lys Lys Ala Val Ala Ile Thr Tyr Thr Gly Asp		
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[5139]	Ile Ser Thr Pro Val Glu Lys Gln Ser Ala Ala Glu Thr Ile Ala Gln		
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[5141]	Lys Ala Val Ala Ala Gly Ile Val Gly Val Gln Val Asp Gly Met Asp		
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[5143]	Pro Leu Ala Val Tyr Ala Ala Thr Ala Glu Ala Arg Glu Arg Ala Ile		
[5144]	245	250	255
[5145]	Asn Gly Glu Gly Pro Thr Leu Ile Glu Thr Leu Thr Phe Arg Tyr Gly		
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[5147]	Pro His Thr Met Ala Gly Asp Asp Pro Thr Lys Tyr Arg Thr Lys Glu		
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[5149]	Ile Glu Asn Glu Trp Glu Gln Lys Asp Pro Leu Val Arg Phe Arg Ala		
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[5151]	Phe Leu Glu Asn Lys Gly Leu Trp Ser Glu Glu Glu Glu Ala Lys Val		
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[5185]	145					150					155				160	
[5186]	Ala	Ile	Arg	Asp	Asn	Asp	Pro	Val	Val	Phe	Leu	Glu	His	Met	Lys	Leu
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[5189]			180						185						190	
[5190]	Leu	Gly	Lys	Ala	Asp	Val	Lys	Arg	Glu	Gly	Thr	Asp	Leu	Ser	Ile	Ile
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[5193]		210					215					220				
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[5218]	35	40 45
[5219]	Pro Ser Pro Val Lys Gly Lys Val Leu Glu Leu Lys Val Glu Glu Gly	
[5220]	50	55 60
[5221]	Thr Val Ala Thr Val Gly Gln Thr Ile Ile Thr Phe Asp Ala Pro Gly	
[5222]	65	70 75 80
[5223]	Tyr Glu Asp Leu Gln Phe Lys Gly Ser Asp Glu Ser Asp Asp Ala Lys	
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[5225]	Thr Glu Ala Gln Val Gln Ser Thr Ala Glu Ala Gly Gln Asp Val Ala	
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[5227]	Lys Glu Glu Gln Ala Gln Glu Pro Ala Lys Ala Thr Gly Ala Gly Gln	
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[5229]	Gln Asp Gln Ala Glu Val Asp Pro Asn Lys Arg Val Ile Ala Met Pro	
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[5231]	Ser Val Arg Lys Tyr Ala Arg Glu Lys Gly Val Asp Ile Arg Lys Val	
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[5233]	Thr Gly Ser Gly Asn Asn Gly Arg Val Val Lys Glu Asp Ile Asp Ser	
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[5235]	Phe Val Asn Gly Gly Ala Gln Glu Ala Ala Pro Gln Glu Thr Ala Ala	
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[5237]	Pro Gln Glu Thr Ala Ala Lys Pro Ala Ala Ala Pro Ala Pro Glu Gly	
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[5239]	Glu Phe Pro Glu Thr Arg Glu Lys Met Ser Gly Ile Arg Lys Ala Ile	
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[5241]	Ala Lys Ala Met Val Asn Ser Lys His Thr Ala Pro His Val Thr Leu	
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[5243]	Met Asp Glu Val Asp Val Thr Asn Leu Val Ala His Arg Lys Gln Phe	
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[5245]	Lys Gln Val Ala Ala Asp Gln Gly Ile Lys Leu Thr Tyr Leu Pro Tyr	
[5246]	260	265 270
[5247]	Val Val Lys Ala Leu Thr Ser Ala Leu Lys Lys Phe Pro Val Leu Asn	
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[5249]	Thr Ser Ile Asp Asp Lys Thr Asp Glu Val Ile Gln Lys His Tyr Phe	

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[5253]	Val Lys Asn Ala Asp Arg Lys Ser Val Phe Glu Ile Ser Asp Glu Ile		
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[5255]	Asn Gly Leu Ala Thr Lys Ala Arg Glu Gly Lys Leu Ala Pro Ala Glu		
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[5257]	Met Lys Gly Ala Ser Cys Thr Ile Thr Asn Ile Gly Ser Ala Gly Gly		
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[5259]	Gln Trp Phe Thr Pro Val Ile Asn His Pro Glu Val Ala Ile Leu Gly		
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[5261]	Ile Gly Arg Ile Ala Glu Lys Ala Ile Val Arg Asp Gly Glu Ile Val		
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[5263]	Ala Ala Pro Val Leu Ala Leu Ser Leu Ser Phe Asp His Arg Met Ile		
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[5265]	Asp Gly Ala Thr Ala Gln Asn Ala Leu Asn His Ile Lys Arg Leu Leu		
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[5267]	Asn Asp Pro Gln Leu Ile Leu Met Glu Ala		
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[5276]	Ala Gly Pro Gly Gly Tyr Val Ala Ala Ile Arg Ala Ala Gln Leu Gly		
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[5278]	Gln Lys Val Thr Val Val Glu Lys Ala Thr Leu Gly Gly Val Cys Leu		
[5279]		35	40
[5280]	Asn Val Gly Cys Ile Pro Ser Lys Ala Leu Ile Asn Ala Gly His Arg		
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[5282]	Tyr Glu Asn Ala Lys His Ser Asp Asp Met Gly Ile Thr Ala Glu Asn		
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[5284]	Val Thr Val Asp Phe Thr Lys Val Gln Glu Trp Lys Ala Ser Val Val		
[5285]		85	90
[5286]	Asn Lys Leu Thr Gly Gly Val Ala Gly Leu Leu Lys Gly Asn Lys Val		
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[5288]	Asp Val Val Lys Gly Glu Ala Tyr Phe Val Asp Ser Asn Ser Val Arg		
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[5290]	Val Met Asp Glu Asn Ser Ala Gln Thr Tyr Thr Phe Lys Asn Ala Ile		
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140

[5292]	Ile Ala Thr Gly Ser Arg Pro Ile Glu Leu Pro Asn Phe Lys Tyr Ser		
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[5294]	Glu Arg Val Leu Asn Ser Thr Gly Ala Leu Ala Leu Lys Glu Ile Pro		
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[5296]	Lys Lys Leu Val Val Ile Gly Gly Gly Tyr Ile Gly Thr Glu Leu Gly		
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[5298]	Thr Ala Tyr Ala Asn Phe Gly Thr Glu Leu Val Ile Leu Glu Gly Gly		
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[5300]	Asp Glu Ile Leu Pro Gly Phe Glu Lys Gln Met Ser Ser Leu Val Thr		
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[5302]	Arg Arg Leu Lys Lys Lys Gly Asn Val Glu Ile His Thr Asn Ala Met		
[5303]	225	230	235 240
[5304]	Ala Lys Gly Val Glu Glu Arg Pro Asp Gly Val Thr Val Thr Phe Glu		
[5305]		245	250 255
[5306]	Val Lys Gly Glu Glu Lys Thr Val Asp Ala Asp Tyr Val Leu Ile Thr		
[5307]		260	265 270
[5308]	Val Gly Arg Arg Pro Asn Thr Asp Glu Leu Gly Leu Glu Gln Val Gly		
[5309]		275	280 285
[5310]	Ile Glu Met Thr Asp Arg Gly Ile Val Lys Thr Asp Lys Gln Cys Arg		
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[5312]	Thr Asn Val Pro Asn Ile Tyr Ala Ile Gly Asp Ile Ile Glu Gly Pro		
[5313]	305	310	315 320
[5314]	Pro Leu Ala His Lys Ala Ser Tyr Glu Gly Lys Ile Ala Ala Glu Ala		
[5315]		325	330 335
[5316]	Ile Ala Gly Glu Pro Ala Glu Ile Asp Tyr Leu Gly Ile Pro Ala Val		
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[5318]	Val Phe Ser Glu Pro Glu Leu Ala Ser Val Gly Tyr Thr Glu Ala Gln		
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[5320]	Ala Lys Glu Glu Gly Leu Asp Ile Val Ala Ala Lys Phe Pro Phe Ala		
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[5322]	Ala Asn Gly Arg Ala Leu Ser Leu Asn Glu Thr Asp Gly Phe Met Lys		
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[5324]	Leu Ile Thr Arg Lys Glu Asp Gly Leu Val Ile Gly Ala Gln Ile Ala		
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[5326]	Gly Ala Ser Ala Ser Asp Met Ile Ser Glu Leu Ser Leu Ala Ile Glu		
[5327]		420	425 430
[5328]	Gly Gly Met Thr Ala Glu Asp Ile Ala Met Thr Ile His Ala His Pro		
[5329]		435	440 445
[5330]	Thr Leu Gly Glu Ile Thr Met Glu Ala Ala Glu Val Ala Ile Gly Ser		
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[5332]	Pro Ile His Ile Val Lys		
[5333]	465	470	

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[5349]	Glu	Asn	Pro	Cys	Leu	Pro	Glu	Leu	Leu	Ala	Ser	Ser	Gln	Arg	Lys	Ile
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[5359]	Pro	Glu	Asp	Ala	Leu	Glu	Lys	Ala	Lys	Leu	Ile	Gly	Phe	Pro	Val	Met
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[5369]	Phe	Gly	Arg	Asp	Cys	Ser	Ile	Gln	Arg	Arg	His	Gln	Lys	Ile	Ile	Glu
[5370]				245					250						255	
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[5372]				260					265					270		
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[5374]			275					280					285			
[5375]	Thr	Val	Glu	Tyr	Leu	Tyr	Ser	Pro	Lys	Asp	Asp	Lys	Phe	Tyr	Phe	Leu

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[5379]	Ser Gly Val Asn Leu Pro Ala Thr Gln Leu Gln Ile Ala Met Gly Ile		
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[5381]	Pro Met His Met Ile Ser Asp Ile Arg Lys Leu Tyr Gly Leu Asp Pro		
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[5383]	Thr Gly Thr Ser Tyr Ile Asp Phe Lys Asn Leu Lys Arg Pro Ser Pro		
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[5385]	Lys Gly His Cys Ile Ser Cys Arg Ile Thr Ser Glu Asp Pro Asn Glu		
[5386]	370	375	380
[5387]	Gly Phe Lys Pro Ser Thr Gly Lys Ile His Glu Leu Asn Phe Arg Ser		
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[5389]	Ser Ser Asn Val Trp Gly Tyr Phe Ser Val Gly Asn Asn Gly Ala Ile		
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[5391]	His Ser Phe Ser Asp Ser Gln Phe Gly His Ile Phe Ala Val Gly Asn		
[5392]		420 425	430
[5393]	Asp Arg Gln Asp Ala Lys Gln Asn Met Val Leu Ala Leu Lys Asp Phe		
[5394]		435 440	445
[5395]	Ser Ile Arg Gly Glu Phe Lys Thr Pro Ile Glu Tyr Leu Ile Glu Leu		
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[5397]	Leu Glu Thr Arg Asp Phe Glu Ser Asn Asn Ile Ser Thr Gly Trp Leu		
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[5399]	Asp Asp Leu Ile Leu Lys Asn Leu Ser Ser Asp Ser Lys Leu Asp Pro		
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[5401]	Thr Leu Ala Ile Ile Cys Gly Ala Ala Met Lys Ala Tyr Val Phe Thr		
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[5403]	Glu Lys Val Arg Asn Lys Tyr Leu Glu Leu Leu Arg Arg Gly Gln Val		
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[5409]	Phe Ile Leu Ser Ile Asn Lys Ser Gln Cys Glu Val Asn Val Gln Lys		
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[5411]	Leu Ser Ser Asp Cys Leu Leu Ile Ser Val Asp Gly Lys Cys His Thr		
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[5413]	Val Tyr Trp Lys Asp Asp Ile Arg Gly Thr Arg Leu Ser Ile Asp Ser		
[5414]		595 600	605
[5415]	Asn Thr Ile Phe Leu Glu Ala Glu Leu Asn Pro Thr Gln Val Ile Ser		
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[5417]	Pro Thr Pro Gly Lys Leu Val Lys Tyr Leu Val Arg Ser Gly Asp His		

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[5419]	Val Phe Ala Gly Gln Gln Tyr Ala Glu Ile Glu Ile Met Lys Met Gln			
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[5421]	Met Pro Leu Val Ala Lys Ser Asp Gly Val Ile Glu Leu Leu Arg Gln			
[5422]		660	665	670
[5423]	Pro Gly Ser Ile Ile Glu Ala Gly Asp Val Ile Ala Lys Leu Thr Leu			
[5424]		675	680	685
[5425]	Asp Ser Pro Ser Lys Ala Asn Glu Ser Ser Leu Tyr Arg Gly Glu Leu			
[5426]		690	695	700
[5427]	Pro Val Leu Gly Pro Pro Leu Ile Glu Gly Ser Arg Pro Asn His Lys			
[5428]	705	710	715	720
[5429]	Leu Arg Val Leu Ile Asn Arg Leu Glu Asn Ile Leu Asn Gly Tyr His			
[5430]		725	730	735
[5431]	Glu Asn Ser Gly Ile Glu Thr Thr Leu Lys Glu Leu Ile Lys Ile Leu			
[5432]		740	745	750
[5433]	Arg Asp Gly Arg Leu Pro Tyr Ser Glu Trp Asp Ser Gln Ile Ser Thr			
[5434]		755	760	765
[5435]	Val Arg Asn Arg Leu Pro Arg Gln Leu Asn Glu Gly Leu Gly Asn Leu			
[5436]		770	775	780
[5437]	Val Lys Lys Ser Val Ser Phe Pro Ala Lys Glu Leu His Lys Leu Met			
[5438]	785	790	795	800
[5439]	Lys Arg Tyr Leu Glu Glu Asn Thr Asn Asp His Val Val Tyr Val Ala			
[5440]		805	810	815
[5441]	Leu Gln Pro Leu Leu Lys Ile Ser Glu Arg Tyr Ser Glu Gly Leu Ala			
[5442]		820	825	830
[5443]	Asn His Glu Cys Glu Ile Phe Leu Lys Leu Ile Lys Lys Tyr Tyr Ala			
[5444]		835	840	845
[5445]	Val Glu Lys Ile Phe Glu Asn His Asp Ile His Glu Glu Arg Asn Leu			
[5446]		850	855	860
[5447]	Leu Asn Leu Arg Arg Lys Asp Leu Thr Asn Leu Lys Lys Ile Leu Cys			
[5448]	865	870	875	880
[5449]	Ile Ser Leu Ser His Ala Asn Val Val Ala Lys Asn Lys Leu Val Thr			
[5450]		885	890	895
[5451]	Ala Ile Leu His Glu Tyr Glu Pro Leu Cys Gln Asp Ser Ser Lys Met			
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[5453]	Ser Leu Lys Phe Arg Ala Val Ile His Asp Leu Ala Ser Leu Glu Ser			
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[5455]	Lys Trp Ala Lys Glu Val Ala Val Lys Ala Arg Ser Val Leu Leu Arg			
[5456]		930	935	940
[5457]	Gly Ile Phe Pro Pro Ile Lys Lys Arg Lys Glu His Ile Lys Thr Leu			
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[5459]	Leu Gln Leu His Ile Lys Asp Thr Gly Ala Glu Asn Ile His Ser Arg			

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[5461]	Asn Ile Tyr Ser Cys Met Arg Asp Phe Gly Asn Leu Ile His Ser Asn					
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[5463]	Leu Ile Gln Leu Gln Asp Leu Phe Phe Phe Phe Gly His Gln Asp Thr					
[5464]		995		1000		1005
[5465]	Ala Leu Ser Ser Ile Ala Ser Glu Ile Tyr Ala Arg Tyr Ala Tyr					
[5466]		1010		1015		1020
[5467]	Gly Asn Tyr Gln Leu Lys Ser Ile Lys Ile His Lys Gly Ala Pro					
[5468]		1025		1030		1035
[5469]	Asp Leu Leu Met Ser Trp Gln Phe Ser Ser Leu Arg Asn Tyr Leu					
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[5471]	Val Asn Ser Asp Gly Glu Ser Asp Glu Phe Thr Lys Leu Ser Lys					
[5472]		1055		1060		1065
[5473]	Pro Pro Ser Thr Ser Gly Lys Ser Ser Ala Asn Ser Phe Gly Leu					
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[5475]	Leu Val Asn Met Arg Ala Leu Glu Ser Leu Glu Lys Thr Leu Asp					
[5476]		1085		1090		1095
[5477]	Glu Val Tyr Glu Gln Ile His Ile Pro Glu Glu Arg Leu Ser Ser					
[5478]		1100		1105		1110
[5479]	Gly Glu Asn Ser Leu Ile Val Asn Ile Leu Ser Pro Ile Arg Tyr					
[5480]		1115		1120		1125
[5481]	Arg Ser Glu Asn Asp Leu Ile Lys Thr Leu Lys Ile Lys Leu His					
[5482]		1130		1135		1140
[5483]	Glu Asn Glu Arg Gly Leu Ser Lys Leu Lys Val Asn Arg Ile Thr					
[5484]		1145		1150		1155
[5485]	Phe Ala Phe Ile Ala Ala Asn Ala Pro Ala Val Lys Phe Tyr Ser					
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[5487]	Phe Asp Gly Thr Thr Tyr Asp Glu Ile Ser Gln Ile Arg Asn Met					
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[5489]	Asp Pro Ser Tyr Glu Ala Pro Leu Glu Leu Gly Lys Met Ser Asn					
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[5493]	Phe Glu Gly Ile Ser Lys Phe Thr Pro Leu Asp Lys Arg Phe Phe					
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[5495]	Val Arg Lys Ile Ile Asn Ser Phe Met Tyr Asn Asp Gln Lys Thr					
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[5497]	Thr Glu Glu Asn Leu Lys Ala Glu Ile Asn Ala Gln Val Val Tyr					
[5498]		1250		1255		1260
[5499]	Met Leu Glu His Leu Gly Ala Val Asp Ile Ser Asn Ser Asp Leu					
[5500]		1265		1270		1275
[5501]	Asn His Ile Phe Leu Ser Phe Asn Thr Val Leu Asn Ile Pro Val					

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[5503]	His Arg Leu Glu Glu Ile Val Ser Thr Ile Leu Lys Thr His Glu		
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[5505]	Thr Arg Leu Phe Gln Glu Arg Ile Thr Asp Val Glu Ile Cys Ile		
[5506]	1310	1315	1320
[5507]	Ser Val Glu Cys Leu Glu Thr Lys Lys Pro Ala Pro Leu Arg Leu		
[5508]	1325	1330	1335
[5509]	Leu Ile Ser Asn Lys Ser Gly Tyr Val Val Lys Ile Glu Thr Tyr		
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[5511]	Tyr Glu Lys Ile Gly Lys Asn Gly Asn Leu Ile Leu Glu Pro Cys		
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[5513]	Ser Glu Gln Ser His Tyr Ser Gln Lys Ser Leu Ser Leu Pro Tyr		
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[5515]	Ser Val Lys Asp Trp Leu Gln Pro Lys Arg Tyr Lys Ala Gln Phe		
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[5517]	Met Gly Thr Thr Tyr Val Tyr Asp Phe Pro Gly Leu Phe His Gln		
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[5519]	Ala Ala Ile Gln Gln Trp Lys Arg Tyr Phe Pro Lys His Lys Leu		
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[5521]	Asn Asp Ser Phe Phe Ser Trp Val Glu Leu Ile Glu Gln Asn Gly		
[5522]	1430	1435	1440
[5523]	Asn Leu Ile Lys Val Asn Arg Glu Pro Gly Leu Asn Asn Ile Gly		
[5524]	1445	1450	1455
[5525]	Met Val Ala Phe Glu Ile Met Val Gln Thr Pro Glu Tyr Pro Glu		
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[5527]	Gly Arg Asn Met Ile Val Ile Ser Asn Asp Ile Thr Tyr Asn Ile		
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[5529]	Gly Ser Phe Gly Pro Arg Glu Asp Leu Phe Phe Asp Arg Val Thr		
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[5531]	Asn Tyr Ala Arg Glu Arg Gly Ile Pro Arg Ile Tyr Leu Ala Ala		
[5532]	1505	1510	1515
[5533]	Asn Ser Gly Ala Lys Leu Gly Ile Ala Glu Glu Leu Ile Pro Leu		
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[5535]	Phe Arg Val Ala Trp Asn Asp Pro Ser Asp Pro Thr Lys Gly Phe		
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[5537]	Gln Tyr Leu Tyr Leu Ala Pro Lys Asp Met Gln Leu Leu Lys Asp		
[5538]	1550	1555	1560
[5539]	Ser Gly Lys Gly Asn Ser Val Val Val Glu His Lys Met Val Tyr		
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[5541]	Gly Glu Glu Arg Tyr Ile Ile Lys Ala Ile Val Gly Phe Glu Glu		
[5542]	1580	1585	1590
[5543]	Gly Leu Gly Val Glu Cys Leu Gln Gly Ser Gly Leu Ile Ala Gly		

[5544]	1595	1600	1605
[5545]	Ala Thr Ser Lys Ala Tyr Arg Asp Ile Phe Thr Ile Thr Ala Val		
[5546]	1610	1615	1620
[5547]	Thr Cys Arg Ser Val Gly Ile Gly Ser Tyr Leu Val Arg Leu Gly		
[5548]	1625	1630	1635
[5549]	Gln Arg Thr Ile Gln Val Glu Asp Lys Pro Ile Ile Leu Thr Gly		
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[5553]	Asn Leu Gln Ile Gly Gly Thr Gln Ile Met Tyr Lys Asn Gly Ile		
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[5557]	Met Thr Trp Leu Ser Tyr Val Pro Ala Lys Arg Asp Met Ser Pro		
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[5559]	Pro Leu Leu Glu Thr Met Asp Arg Trp Asp Arg Asp Val Asp Phe		
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[5565]	Lys Asp Ser Phe Phe Glu Thr Leu Ser Gly Trp Ala Lys Gly Val		
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[5567]	Ile Val Gly Arg Ala Arg Leu Gly Gly Ile Pro Val Gly Val Ile		
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[5569]	Ala Val Glu Thr Lys Thr Ile Glu Glu Ile Ile Pro Ala Asp Pro		
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[5571]	Ala Asn Leu Asp Ser Ser Glu Phe Ser Val Lys Glu Ala Gly Gln		
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[5573]	Val Trp Tyr Pro Asn Ser Ala Phe Lys Thr Ala Gln Thr Ile Asn		
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[5579]	Leu Lys Tyr Gly Ser Phe Ile Val Asp Ala Leu Val Asp Tyr Lys		
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[5589]	Thr Met Ile Arg Leu Asp Ser Thr Tyr Gly His Leu Arg Arg Thr		
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[5591]	Leu Thr Glu Lys Lys Leu Ser Leu Glu Lys Gln Asn Asp Leu Thr		
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[5593]	Lys Arg Leu Lys Ile Arg Glu Arg Gln Leu Ile Pro Ile Tyr Asn		
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[5595]	Gln Ile Ser Ile Gln Phe Ala Asp Leu His Asp Arg Ser Thr Arg		
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[5604]	2045	2050	2055
[5605]	Tyr Asn Asp Leu Asp Val Asn Asp Asp Arg Ala Val Val Glu Phe		
[5606]	2060	2065	2070
[5607]	Ile Glu Arg Asn Ser Lys Lys Ile Asp Lys Asn Ile Glu Glu Phe		
[5608]	2075	2080	2085
[5609]	Glu Ile Ser Leu Leu Ile Asp Glu Leu Lys Lys Lys Phe Glu Asp		
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[5611]	Arg Arg Gly Asn Ile Val Leu Glu Glu Leu Thr Arg Leu Val Asp		
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[5624]	Asp Ile Asn Ile Asp Glu Glu Val His Arg Leu Arg Glu Lys Ser Val		
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[5626]	Glu Leu Thr Arg Lys Ile Phe Ala Asp Leu Gly Ala Trp Gln Ile Ala		
[5627]	50 55 60		

[5628]	Gln Leu Ala Arg His Pro Gln Arg Pro Tyr Thr Leu Asp Tyr Val Arg		
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[5630]	Leu Ala Phe Asp Glu Phe Asp Glu Leu Ala Gly Asp Arg Ala Tyr Ala		
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[5632]	Asp Asp Lys Ala Ile Val Gly Gly Ile Ala Arg Leu Asp Gly Arg Pro		
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[5634]	Val Met Ile Ile Gly His Gln Lys Gly Arg Glu Thr Lys Glu Lys Ile		
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[5636]	Arg Arg Asn Phe Gly Met Pro Ala Pro Glu Gly Tyr Arg Lys Ala Leu		
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[5638]	Arg Leu Met Gln Met Ala Glu Arg Phe Lys Met Pro Ile Ile Thr Phe		
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[5640]	Ile Asp Thr Pro Gly Ala Tyr Pro Gly Val Gly Ala Glu Glu Arg Gly		
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[5642]	Gln Ser Glu Ala Ile Ala Arg Asn Leu Arg Glu Met Ser Arg Leu Gly		
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[5644]	Val Pro Val Val Cys Thr Val Ile Gly Glu Gly Gly Ser Gly Gly Ala		
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[5646]	Leu Ala Ile Gly Val Gly Asp Lys Val Asn Met Leu Gln Tyr Ser Thr		
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[5648]	Tyr Ser Val Ile Ser Pro Glu Gly Cys Ala Ser Ile Leu Trp Lys Ser		
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[5650]	Ala Asp Lys Ala Pro Leu Ala Ala Glu Ala Met Gly Ile Ile Ala Pro		
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[5652]	Arg Leu Lys Glu Leu Lys Leu Ile Asp Ser Ile Ile Pro Glu Pro Leu		
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[5654]	Gly Gly Ala His Arg Asn Pro Glu Ala Met Ala Ala Ser Leu Lys Ala		
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[5656]	Gln Leu Leu Ala Asp Leu Ala Asp Leu Asp Val Leu Ser Thr Glu Asp		
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[5658]	Leu Lys Asn Arg Arg Tyr Gln Arg Leu Met Ser Tyr Gly Tyr Ala		
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[5667]	Gly Ile Ser Glu Leu Glu Ile Ser Glu Gly Glu Glu Ser Val Arg Ile		
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[5673]	Ala Thr Val Pro Ser Met Glu Ala Pro Ala Ala Ala Glu Ile Ser Gly		
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[5675]	His Ile Val Arg Ser Pro Met Val Gly Thr Phe Tyr Arg Thr Pro Ser		
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[5692]	Ile Leu Arg Ala Cys Lys Glu Leu Gly Ile Lys Thr Val Ala Val His		
[5693]	20	25	30
[5694]	Ser Ser Ala Asp Arg Asp Leu Lys His Val Leu Leu Ala Asp Glu Thr		
[5695]	35	40	45
[5696]	Val Cys Ile Gly Pro Ala Pro Ser Val Lys Ser Tyr Leu Asn Ile Pro		
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[5698]	Ala Ile Ile Ser Ala Ala Glu Ile Thr Gly Ala Val Ala Ile His Pro		
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[5704]	Met Gly Asp Lys Val Ser Ala Ile Ala Ala Met Lys Lys Ala Gly Val		
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[5706]	Pro Cys Val Pro Gly Ser Asp Gly Pro Leu Gly Asp Asp Met Asp Lys		
[5707]	130	135	140
[5708]	Asn Arg Ala Ile Ala Lys Arg Ile Gly Tyr Pro Val Ile Ile Lys Ala		
[5709]	145	150	155
[5710]	Ser Gly Gly Gly Gly Gly Arg Gly Met Arg Val Val Arg Gly Asp Ala		
[5711]	165	170	175

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[5723]			260						265					270		
[5724]	Gly	Thr	Phe	Glu	Phe	Leu	Phe	Glu	Asn	Gly	Glu	Phe	Tyr	Phe	Ile	Glu
[5725]		275						280					285			
[5726]	Met	Asn	Thr	Arg	Ile	Gln	Val	Glu	His	Pro	Val	Thr	Glu	Met	Ile	Thr
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[5736]	Ser	His	Ile	Tyr	Ala	Gly	Tyr	Thr	Val	Pro	Pro	Tyr	Tyr	Asp	Ser	Met
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[5758]	Cys Asp His His Met Arg Met Thr Ala Arg Asn Arg Leu His Ser Leu
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[5760]	Leu Asp Glu Gly Ser Leu Val Glu Leu Gly Ser Glu Leu Glu Pro Lys
[5761]	65 70 75 80
[5762]	Asp Val Leu Lys Phe Arg Asp Ser Lys Lys Tyr Lys Asp Arg Leu Ala
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[5764]	Ser Ala Gln Lys Glu Thr Gly Glu Lys Asp Ala Leu Val Val Met Lys
[5765]	100 105 110
[5766]	Gly Thr Leu Tyr Gly Met Pro Val Val Ala Ala Ala Phe Glu Phe Ala
[5767]	115 120 125
[5768]	Phe Met Gly Gly Ser Met Gly Ser Val Val Gly Ala Arg Phe Val Arg
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[5770]	Ala Val Glu Gln Ala Leu Glu Asp Asn Cys Pro Leu Ile Cys Phe Ser
[5771]	145 150 155 160
[5772]	Ala Ser Gly Gly Ala Arg Met Gln Glu Ala Leu Met Ser Leu Met Gln
[5773]	165 170 175
[5774]	Met Ala Lys Thr Ser Ala Ala Leu Ala Lys Met Gln Glu Arg Gly Leu
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[5776]	Pro Tyr Ile Ser Val Leu Thr Asp Pro Thr Met Gly Gly Val Ser Ala
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[5778]	Ser Phe Ala Met Leu Gly Asp Leu Asn Ile Ala Glu Pro Lys Ala Leu
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[5780]	Ile Gly Phe Ala Gly Pro Arg Val Ile Glu Gln Thr Val Arg Glu Lys
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[5782]	Leu Pro Pro Gly Phe Gln Arg Ser Glu Phe Leu Ile Glu Lys Gly Ala
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[5784]	Ile Asp Met Ile Val Arg Arg Pro Glu Met Arg Leu Lys Leu Ala Ser
[5785]	260 265 270
[5786]	Ile Leu Ala Lys Leu Met Asn Leu Pro Ala Pro Asn Pro Glu Ala Pro
[5787]	275 280 285
[5788]	Arg Glu Gly Val Val Val Pro Pro Val Pro Asp Gln Glu Pro Glu Ala
[5789]	290 295 300
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[5791]	<211> 282
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[5795]	Met Glu Glu Thr Ala Ile Pro Gln Ser Leu Thr Pro Trp Asp Arg Val

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[5801]	Asp Asp Pro Ala Met Val Gly Gly Met Ala Thr Phe Ala Gly Gln Thr			
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[5803]	Val Met Val Ile Gly His Gln Lys Gly Asn Asp Thr Arg Glu Asn Met			
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[5805]	Arg Arg Asn Phe Gly Met Pro His Pro Glu Gly Tyr Arg Lys Ala Gln			
[5806]	85	90	95	
[5807]	Arg Leu Met Arg His Ala Glu Lys Phe Gly Leu Pro Val Ile Cys Phe			
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[5809]	Val Asp Thr Pro Ala Ala Asp Pro Thr Lys Ser Ser Glu Glu Arg Gly			
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[5811]	Gln Ala Asn Ala Ile Ala Glu Ser Ile Met Leu Met Thr Thr Leu Arg			
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[5813]	Val Pro Ser Ile Ala Val Val Ile Gly Glu Gly Gly Ser Gly Gly Ala			
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[5815]	Leu Ala Ile Ser Val Ala Asp Arg Ile Leu Met Gln Glu Asn Ala Ile			
[5816]	165	170	175	
[5817]	Tyr Ser Val Ala Pro Pro Glu Ala Ala Ala Ser Ile Leu Trp Arg Asp			
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[5819]	Ala Ala Lys Ala Pro Glu Ala Ala Arg Ala Leu Lys Leu Thr Ala Ala			
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[5821]	Asp Leu Tyr Asp Leu Arg Ile Ile Asp Glu Val Ile Pro Glu Pro Pro			
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[5823]	Gly Gly Ala His Ala Asp Arg Leu Thr Ala Ile Thr Thr Val Gly Glu			
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[5827]	Leu Leu Arg Glu Arg Tyr Arg Lys Tyr Arg Ser Met Gly Gln Tyr Gln			
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[5829]	Glu Gln Gln Met Asp Phe Phe Gly Arg Met			
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[5846]	Pro	Val	Thr	Pro	Phe	Val	Gln	Pro	Pro	Pro	Ala	Pro	Glu	Gly	Pro	Pro
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[5848]	Val	Glu	Met	Pro	Ala	Gly	His	Thr	Ile	Thr	Ala	Pro	Met	Val	Gly	Thr
[5849]				100						105					110	
[5850]	Phe	Tyr	Ala	Ala	Pro	Ser	Pro	Arg	Asp	Arg	Pro	Phe	Val	Gln	Glu	Gly
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[5911]	Asp Ser His Leu Tyr Ser Gly Tyr Thr Pro Pro Gly Thr Tyr Asp Ser		
[5912]	370	375	380
[5913]	Leu Leu Ala Lys Ile Ile Thr Phe Gly Asp Thr Arg Asp Glu Ala Leu		
[5914]	385	390	395
[5915]	Asn Arg Met Arg Arg Ala Leu Asn Glu Cys Val Ile Thr Gly Ile Lys		
[5916]	405	410	415
[5917]	Thr Thr Ile Pro Phe Gln Leu Ala Leu Ile Asp Asp Pro Glu Phe Arg		
[5918]	420	425	430
[5919]	Ala Gly Arg Ile His Thr Gly Tyr Val Ala Glu Leu Leu Arg Gln Trp		
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[5922]	450	455
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[5928]	Met Lys Glu Phe Phe Arg Leu Ser Arg Lys Gly Phe Thr Gly Arg Glu	
[5929]	1 5 10 15	
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[5934]	Val Cys Pro Lys Cys Gly His His Met Arg Leu Ser Ala His Glu Trp	
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[5938]	Leu Pro Thr Asp Pro Leu Gly Phe Val Thr Asp Glu Glu Ser Tyr Ala	
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[5944]	Ala Asp Phe Ser Phe Met Gly Ala Ser Met Gly Ser Val Tyr Gly Glu	
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[5946]	Lys Met Ala Arg Ser Ala Glu Arg Ala Ala Glu Leu Gly Val Pro Leu	
[5947]	145 150 155 160	
[5948]	Leu Thr Ile Asn Thr Ser Gly Gly Ala Arg Gln Gln Glu Gly Val Ile	
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[5950]	Gly Leu Met Gln Met Ala Lys Val Thr Met Ala Leu Thr Arg Leu Ala	
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[5952]	Asp Ala Gly Gln Pro His Ile Ala Leu Leu Val Asp Pro Cys Tyr Gly	
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[5954]	Gly Val Thr Ala Ser Tyr Pro Ser Val Ala Asp Ile Ile Ile Ala Glu	
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[5956]	Pro Gly Ala Asn Ile Gly Phe Ala Gly Lys Arg Leu Ile Glu Gln Ile	
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[5958]	Met Arg Gln Lys Leu Pro Ala Gly Phe Gln Thr Ala Glu Phe Met Leu	
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[5960]	Glu His Gly Met Ile Asp Met Val Val Pro Arg Ser Glu Met Arg Asp	
[5961]	260 265 270	
[5962]	Thr Leu Ala Arg Ile Leu Arg Leu Tyr Arg Gln Arg Ser Thr Ser Pro	
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[5975]	Ala Leu Thr Pro Gly Gly Val Ser Gln Leu Ile Ser Asn Gly His Arg
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[5983]	Tyr Val Tyr Phe Arg Lys Gly Leu Val Leu Phe Thr Tyr Leu His Leu
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[5985]	Ala Ala Glu Pro Glu Leu Ala Gln Ala Leu Lys Asp Lys Gly Val Thr
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[5987]	Ala Ile Ala Tyr Glu Thr Val Ser Glu Gly Arg Thr Leu Pro Leu Leu
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[5989]	Thr Pro Met Ser Glu Val Ala Gly Arg Met Ala Ala Gln Ile Gly Ala
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[5991]	Gln Phe Leu Glu Lys Pro Lys Gly Gly Lys Gly Ile Leu Leu Ala Gly
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[5993]	Val Pro Gly Val Ser Arg Gly Lys Val Thr Ile Ile Gly Gly Gly Val
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[5995]	Val Gly Thr Asn Ala Ala Lys Met Ala Val Gly Leu Gly Ala Asp Val
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[5997]	Thr Ile Ile Asp Leu Asn Ala Asp Arg Leu Arg Gln Leu Asp Asp Ile
[5998]	195 200 205
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[6001]	Asp Ala Val Ala Glu Ala Asp Leu Leu Ile Cys Ala Val Leu Ile Pro
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[6003]	Gly Ala Lys Ala Pro Thr Leu Val Thr Glu Glu Met Val Lys Gln Met
[6004]	245 250 255
[6005]	Lys Pro Gly Ser Val Ile Val Asp Val Ala Ile Asp Gln Gly Gly Ile

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[6009]	Lys His Gly Val Val His Tyr Ala Val Ala Asn Met Pro Gly Ala Val		
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[6011]	Pro Arg Thr Ser Thr Ile Ala Leu Thr Asn Val Thr Val Pro Tyr Ala		
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[6013]	Leu Gln Ile Ala Asn Lys Gly Ala Val Lys Ala Leu Ala Asp Asn Thr		
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[6015]	Ala Leu Arg Ala Gly Leu Asn Thr Ala Asn Gly His Val Thr Tyr Glu		
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[6017]	Ala Val Ala Arg Asp Leu Gly Tyr Glu Tyr Val Pro Ala Glu Lys Ala		
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[6019]	Leu Gln Asp Glu Ser Ser Val Ala Gly Ala		
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[6030]	Leu Asn Gln Gln Val Phe Lys Ala Asn Tyr Ala Val Arg Gly Ala Leu		
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[6032]	Ala Ile Leu Ala Asp Glu Ile Gln Asp Asp Leu Leu Glu Asn Pro Ser		
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[6034]	Ser Tyr Pro Phe Ser Glu Ile Val Tyr Ala Asn Ile Gly Asn Pro Gln		
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[6036]	Gln Met Gly Gln Ser Pro Ile Thr Phe Val Arg Gln Val Leu Ser Leu		
[6037]	85	90	95
[6038]	Cys Gln Tyr Pro Thr Leu Leu Asp His Ala Glu Glu Lys Trp Phe Gln		
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[6040]	Asn Leu Phe Pro Thr Asp Val Val Gln Arg Ser Lys Met Leu Leu Lys		
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[6042]	Glu Ser Gly Ser Leu Gly Ala Tyr Ser Ala Ser Gln Gly Ile Pro Leu		
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[6044]	Val Arg Arg His Val Ala Asp Phe Ile Arg Ala Arg Asp Gly Phe Asp		
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[6046]	Cys Glu Pro Ser Asp Ile Tyr Leu Thr Ser Gly Ala Ser His Ala Ala		
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[6050]	Val	Pro	Ala	Pro	Gln	Tyr	Pro	Leu	Tyr	Gly	Ala	Gln	Ile	Asp	Leu	Met
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[6054]	Ile	Asp	Phe	Asp	Gln	Phe	Lys	Lys	Ser	Phe	Asp	Glu	Ala	Ser	Lys	Lys
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[6071]															330	
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[6073]															340	
[6074]	Asp	Leu	Phe	Ile	Lys	Glu	Val	Asp	Glu	Ile	His	Glu	Ala	Leu	Arg	Leu
[6075]															355	
[6076]	Gln	Cys	Arg	Gln	Leu	Tyr	Glu	Gly	Thr	Lys	Arg	Met	Lys	Arg	Val	Ser
[6077]															360	
[6078]	Cys	Leu	Glu	Pro	His	Gly	Ala	Met	Tyr	Leu	His	Pro	Ser	Val	Ser	Leu
[6079]															370	
[6080]	Pro	Glu	Lys	Leu	Ile	Thr	Thr	Ala	Lys	Ala	Gln	Lys	Ile	Gln	Pro	Asp
[6081]															375	
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[6093]	<213> 酿酒酵母															
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[6101]	Arg	Ala	Asp	Glu	Leu	Lys	Glu	Glu	Leu	Lys	Lys	Asn	Pro	Glu	Val	Leu
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[6115]	Pro	Ala	Thr	Pro	Glu	Asp	Ile	Tyr	Leu	Thr	Thr	Gly	Ala	Ser	Ser	Ala
[6116]					165					170					175	
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[6120]			195					200				205				
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[6123]	Ser	Thr	Asn	Ser	Asp	Glu	Ile	Glu	Lys	Val	Val	Gln	Asp	Ala	Leu	Lys
[6124]	225					230					235					240
[6125]	Lys	Gln	Ile	Arg	Pro	Ser	Val	Leu	Ile	Val	Ile	Asn	Pro	Gly	Asn	Pro
[6126]					245					250					255	
[6127]	Thr	Gly	Ala	Val	Leu	Ser	Glu	Glu	Thr	Ile	Ala	Arg	Ile	Cys	Leu	Ile
[6128]				260					265					270		
[6129]	Ala	Ala	Lys	Tyr	Gly	Ile	Thr	Ile	Ile	Ser	Asp	Glu	Val	Tyr	Gln	Glu
[6130]			275					280					285			
[6131]	Asn	Ile	Phe	Asn	Asp	Val	Lys	Phe	His	Ser	Met	Lys	Lys	Val	Leu	Arg

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[6133]	Lys Leu Gln His Leu Tyr Pro Gly Lys Phe Asp Asn Val Gln Leu Ala		
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[6135]	Ser Leu His Ser Ile Ser Lys Gly Phe Met Asp Glu Cys Gly Gln Arg		
[6136]		325	330
[6137]	Gly Gly Tyr Met Glu Ile Ile Gly Phe Ser Gln Glu Ile Arg Asp Ala		
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[6139]	Leu Phe Lys Leu Met Ser Ile Ser Ile Cys Ser Val Val Thr Gly Gln		
[6140]		355	360
[6141]	Ala Val Val Asp Leu Met Val Lys Pro Pro Gln Pro Gly Asp Glu Ser		
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[6143]	Tyr Glu Gln Asp His Asp Glu Arg Leu Lys Ile Phe His Glu Met Arg		
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[6145]	Thr Arg Ala Asn Leu Leu Tyr Glu Thr Phe Lys Glu Leu Glu Gly Ile		
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[6147]	Glu Cys Gln Lys Pro Gln Gly Ala Met Tyr Leu Phe Pro Arg Leu Val		
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[6149]	Leu Pro Lys Lys Ala Leu Cys Glu Ser Glu Arg Leu Gly Ile Glu Pro		
[6150]		435	440
[6151]	Asp Glu Phe Tyr Cys Thr Ser Leu Leu Glu Ser Thr Gly Ile Cys Thr		
[6152]	450	455	460
[6153]	Val Pro Gly Ser Gly Phe Gly Gln Arg Pro Gly Thr Tyr His Val Arg		
[6154]	465	470	475
[6155]	Thr Thr Phe Leu Ala Pro Gly Thr Lys Trp Ile Gln Asp Trp Lys Glu		
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[6157]	Phe His Gln Asp Phe Phe Ser Lys Tyr Arg Asn		
[6158]		500	505
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[6163]	<400> 86		
[6164]	Met Lys Asn Lys Trp Tyr Lys Pro Lys Arg His Trp Lys Glu Ile Glu		
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[6166]	Leu Trp Lys Asp Val Pro Glu Glu Lys Trp Asn Asp Trp Leu Trp Gln		
[6167]		20	25
[6168]	Leu Thr His Thr Val Arg Thr Leu Asp Asp Leu Lys Lys Val Ile Asn		
[6169]		35	40
[6170]	Leu Thr Glu Asp Glu Glu Glu Gly Val Arg Ile Ser Thr Lys Thr Ile		
[6171]		50	55
[6172]	Pro Leu Asn Ile Thr Pro Tyr Tyr Ala Ser Leu Met Asp Pro Asp Asn		
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[6174]	Pro Arg Cys Pro Val Arg Met Gln Ser Val Pro Leu Ser Glu Glu Met		
[6175]		85	90
[6176]	His Lys Thr Lys Tyr Asp Leu Glu Asp Pro Leu His Glu Asp Glu Asp		95
[6177]		100	105
[6178]	Ser Pro Val Pro Gly Leu Thr His Arg Tyr Pro Asp Arg Val Leu Phe		110
[6179]		115	120
[6180]	Leu Val Thr Asn Gln Cys Ser Met Tyr Cys Arg Tyr Cys Thr Arg Arg		125
[6181]		130	135
[6182]	Arg Phe Ser Gly Gln Ile Gly Met Gly Val Pro Lys Lys Gln Leu Asp		140
[6183]		145	150
[6184]	Ala Ala Ile Ala Tyr Ile Arg Glu Thr Pro Glu Ile Arg Asp Cys Leu		155
[6185]		165	170
[6186]	Ile Ser Gly Gly Asp Gly Leu Leu Ile Asn Asp Gln Ile Leu Glu Tyr		175
[6187]		180	185
[6188]	Ile Leu Lys Glu Leu Arg Ser Ile Pro His Leu Glu Val Ile Arg Ile		190
[6189]		195	200
[6190]	Gly Thr Arg Ala Pro Val Val Phe Pro Gln Arg Ile Thr Asp His Leu		205
[6191]		210	215
[6192]	Cys Glu Ile Leu Lys Lys Tyr His Pro Val Trp Leu Asn Thr His Phe		220
[6193]		225	230
[6194]	Asn Thr Ser Ile Glu Met Thr Glu Glu Ser Val Glu Ala Cys Glu Lys		235
[6195]		245	250
[6196]	Leu Val Asn Ala Gly Val Pro Val Gly Asn Gln Ala Val Val Leu Ala		240
[6197]		260	265
[6198]	Gly Ile Asn Asp Ser Val Pro Ile Met Lys Lys Leu Met His Asp Leu		255
[6199]		275	280
[6200]	Val Lys Ile Arg Val Arg Pro Tyr Tyr Ile Tyr Gln Cys Asp Leu Ser		285
[6201]		290	295
[6202]	Glu Gly Ile Gly His Phe Arg Ala Pro Val Ser Lys Gly Leu Glu Ile		300
[6203]		305	310
[6204]	Ile Glu Gly Leu Arg Gly His Thr Ser Gly Tyr Ala Val Pro Thr Phe		315
[6205]		325	330
[6206]	Val Val Asp Ala Pro Gly Gly Gly Gly Lys Ile Ala Leu Gln Pro Asn		320
[6207]		340	345
[6208]	Tyr Val Leu Ser Gln Ser Pro Asp Lys Val Ile Leu Arg Asn Phe Glu		335
[6209]		355	360
[6210]	Gly Val Ile Thr Ser Tyr Pro Glu Pro Glu Asn Tyr Ile Pro Asn Gln		365
[6211]		370	375
[6212]	Ala Asp Ala Tyr Phe Glu Ser Val Phe Pro Glu Thr Ala Asp Lys Lys		380
[6213]		385	390
[6214]	Glu Pro Ile Gly Leu Ser Ala Ile Phe Ala Asp Lys Glu Val Ser Phe		395
[6215]		405	410

[6216]	Thr Pro Glu Asn Val Asp Arg Ile Lys Arg Arg Glu Ala Tyr Ile Ala
[6217]	420 425 430
[6218]	Asn Pro Glu His Glu Thr Leu Lys Asp Arg Arg Glu Lys Arg Asp Gln
[6219]	435 440 445
[6220]	Leu Lys Glu Lys Lys Phe Leu Ala Gln Gln Lys Lys Gln Lys Glu Thr
[6221]	450 455 460
[6222]	Glu Cys Gly Gly Asp Ser Ser
[6223]	465 470
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[6233]	Asp Gln Leu Lys Lys Tyr Val Thr Leu Thr Ala Glu Glu Glu Glu Gly
[6234]	35 40 45
[6235]	Val Lys Glu Ser Leu Lys Val Leu Arg Met Ala Ile Thr Pro Tyr Tyr
[6236]	50 55 60
[6237]	Leu Ser Leu Ile Asp Pro Glu Asn Pro Asn Cys Pro Ile Arg Lys Gln
[6238]	65 70 75 80
[6239]	Ala Ile Pro Thr His Gln Glu Leu Val Arg Ala Pro Glu Asp Gln Val
[6240]	85 90 95
[6241]	Asp Pro Leu Ser Glu Asp Glu Asp Ser Pro Val Pro Gly Leu Thr His
[6242]	100 105 110
[6243]	Arg Tyr Pro Asp Arg Val Leu Phe Leu Ile Thr Asp Lys Cys Ser Met
[6244]	115 120 125
[6245]	Tyr Cys Arg His Cys Thr Arg Arg Arg Phe Ala Gly Gln Lys Asp Ala
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[6247]	Ser Ser Pro Ser Glu Arg Ile Asp Arg Cys Ile Asp Tyr Ile Ala Asn
[6248]	145 150 155 160
[6249]	Thr Pro Thr Val Arg Asp Val Leu Leu Ser Gly Gly Asp Ala Leu Leu
[6250]	165 170 175
[6251]	Val Ser Asp Glu Arg Leu Glu Tyr Ile Leu Lys Arg Leu Arg Glu Ile
[6252]	180 185 190
[6253]	Pro His Val Glu Ile Val Arg Ile Gly Ser Arg Thr Pro Val Val Leu
[6254]	195 200 205
[6255]	Pro Gln Arg Ile Thr Pro Gln Leu Val Asp Met Leu Lys Lys Tyr His
[6256]	210 215 220
[6257]	Pro Val Trp Leu Asn Thr His Phe Asn His Pro Asn Glu Val Thr Glu

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[6259]	Glu Ala Val Glu Ala Cys Glu Arg Met Ala Asn Ala Gly Ile Pro Leu			
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[6261]	Gly Asn Gln Thr Val Leu Leu Arg Gly Ile Asn Asp Cys Thr His Val			
[6262]		260	265	270
[6263]	Met Lys Arg Leu Val His Leu Leu Val Lys Met Arg Val Arg Pro Tyr			
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[6265]	Tyr Ile Tyr Val Cys Asp Leu Ser Leu Gly Ile Gly His Phe Arg Thr			
[6266]		290	295	300
[6267]	Pro Val Ser Lys Gly Ile Glu Ile Ile Glu Asn Leu Arg Gly His Thr			
[6268]	305	310	315	320
[6269]	Ser Gly Tyr Ala Val Pro Thr Phe Val Val Asp Ala Pro Gly Gly Gly			
[6270]		325	330	335
[6271]	Gly Lys Ile Pro Val Met Pro Asn Tyr Val Val Ser Gln Ser Pro Arg			
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[6273]	His Val Val Leu Arg Asn Tyr Glu Gly Val Ile Thr Thr Tyr Thr Glu			
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[6275]	Pro Glu Asn Tyr His Glu Glu Cys Asp Cys Glu Asp Cys Arg Ala Gly			
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[6277]	Lys His Lys Glu Gly Val Ala Ala Leu Ser Gly Gly Gln Gln Leu Ala			
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[6279]	Ile Glu Pro Ser Asp Leu Ala Arg Lys Lys Arg Lys Phe Asp Lys Asn			
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[6292]	Gly Val Val Arg Thr Leu Glu Thr Leu Arg Met Ala Ile Thr Pro Tyr			
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[6294]	Tyr Phe Ser Leu Ile Asp Met Asn Ser Asp Arg Cys Pro Ile Arg Lys			
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[6296]	Gln Ala Ile Pro Thr Ile Gln Glu Ile His Gln Ser Asp Ala Asp Leu			
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[6298]	Leu Asp Pro Leu His Glu Asp Glu Asp Ser Pro Val Pro Gly Leu Thr			
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[6300]	His Arg Tyr Pro Asp Arg Val Leu Leu Leu Ile Thr Asp Met Cys Ser
[6301]	115 120 125
[6302]	Met Tyr Cys Arg His Cys Thr Arg Arg Arg Phe Ala Gly Ser Ser Asp
[6303]	130 135 140
[6304]	Asp Ala Met Pro Met Asp Arg Ile Asp Lys Ala Ile Glu Tyr Ile Ala
[6305]	145 150 155 160
[6306]	Lys Thr Pro Gln Val Arg Asp Val Leu Leu Ser Gly Gly Asp Ala Leu
[6307]	165 170 175
[6308]	Leu Val Ser Asp Lys Lys Leu Glu Ser Ile Ile Gln Lys Leu Arg Ala
[6309]	180 185 190
[6310]	Ile Pro His Val Glu Ile Ile Arg Ile Gly Ser Arg Thr Pro Val Val
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[6312]	Leu Pro Gln Arg Ile Thr Pro Glu Leu Cys Asn Met Leu Lys Lys Tyr
[6313]	210 215 220
[6314]	His Pro Ile Trp Leu Asn Thr His Phe Asn His Pro Gln Glu Val Thr
[6315]	225 230 235 240
[6316]	Pro Glu Ala Lys Lys Ala Cys Glu Met Leu Ala Asp Ala Gly Val Pro
[6317]	245 250 255
[6318]	Leu Gly Asn Gln Thr Val Leu Leu Arg Gly Ile Asn Asp Ser Val Pro
[6319]	260 265 270
[6320]	Val Met Lys Arg Leu Val His Asp Leu Val Met Met Arg Val Arg Pro
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[6322]	Tyr Tyr Ile Tyr Gln Cys Asp Leu Ser Met Gly Leu Glu His Phe Arg
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[6324]	Thr Pro Val Ser Lys Gly Ile Glu Ile Ile Glu Gly Leu Arg Gly His
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[6327]	325 330 335
[6328]	Gly Gly Lys Thr Pro Val Met Pro Gln Tyr Val Ile Ser Gln Ser Pro
[6329]	340 345 350
[6330]	His Arg Val Val Leu Arg Asn Phe Glu Gly Val Ile Thr Thr Tyr Thr
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[6332]	Glu Pro Glu Asn Tyr Thr His Glu Pro Cys Tyr Asp Glu Glu Lys Phe
[6333]	370 375 380
[6334]	Glu Lys Met Tyr Glu Ile Ser Gly Val Tyr Met Leu Asp Glu Gly Leu
[6335]	385 390 395 400
[6336]	Lys Met Ser Leu Glu Pro Ser His Leu Ala Arg His Glu Arg Asn Lys
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[6338]	Lys Arg Ala Glu Ala Glu Gly Lys Lys
[6339]	420 425
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[6341]	<211> 517

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[6349]	Pro	Glu	Ala	Leu	Thr	Lys	Ala	Leu	Glu	Lys	Arg	Phe	Leu	Asp	Thr	Asn
[6350]					35					40					45	
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[6353]	Asp	Gly	Arg	Ala	Ala	Glu	His	Leu	Ala	His	Thr	Gly	Leu	Leu	Lys	Arg
[6354]	65					70					75					80
[6355]	Ala	Ile	Ile	Gly	His	Trp	Gln	Thr	Val	Pro	Ala	Ile	Gly	Lys	Leu	Ala
[6356]					85						90					95
[6357]	Val	Glu	Asn	Lys	Ile	Glu	Ala	Tyr	Asn	Phe	Ser	Gln	Gly	Thr	Leu	Val
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[6359]	His	Trp	Phe	Arg	Ala	Leu	Ala	Gly	His	Lys	Leu	Gly	Val	Phe	Thr	Asp
[6360]					115						120					125
[6361]	Ile	Gly	Leu	Glu	Thr	Phe	Leu	Asp	Pro	Arg	Gln	Leu	Gly	Gly	Lys	Leu
[6362]					130						135					140
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[6364]	145					150					155					160
[6365]	His	Glu	Gln	Leu	Phe	Tyr	Pro	Thr	Phe	Pro	Val	Asn	Val	Ala	Phe	Leu
[6366]					165						170					175
[6367]	Arg	Gly	Thr	Tyr	Ala	Asp	Glu	Ser	Gly	Asn	Ile	Thr	Met	Asp	Glu	Glu
[6368]					180						185					190
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[6370]					195						200					205
[6371]	Gly	Gly	Lys	Val	Val	Val	Gln	Val	Lys	Asp	Val	Val	Ala	His	Gly	Ser
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[6373]	Leu	Asp	Pro	Arg	Met	Val	Lys	Ile	Pro	Gly	Ile	Tyr	Val	Asp	Tyr	Val
[6374]	225					230					235					240
[6375]	Val	Val	Ala	Ala	Pro	Glu	Asp	His	Gln	Gln	Thr	Tyr	Asp	Cys	Glu	Tyr
[6376]					245						250					255
[6377]	Asp	Pro	Ser	Leu	Ser	Gly	Glu	His	Arg	Ala	Pro	Glu	Gly	Ala	Thr	Asp
[6378]					260						265					270
[6379]	Ala	Ala	Leu	Pro	Met	Ser	Ala	Lys	Lys	Ile	Ile	Gly	Arg	Arg	Gly	Ala
[6380]					275						280					285
[6381]	Leu	Glu	Leu	Thr	Glu	Asn	Ala	Val	Val	Asn	Leu	Gly	Val	Gly	Ala	Pro
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[6387]	Ala Arg Phe Gly Ser Ser Arg Asn Ala Asp Ala Ile Ile Asp His Thr			
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[6389]	Tyr Gln Phe Asp Phe Tyr Asp Gly Gly Gly Leu Asp Ile Ala Tyr Leu			
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[6391]	Gly Leu Ala Gln Cys Asp Gly Ser Gly Asn Ile Asn Val Ser Lys Phe			
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[6393]	Gly Thr Asn Val Ala Gly Cys Gly Gly Phe Pro Asn Ile Ser Gln Gln			
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[6395]	Thr Pro Asn Val Tyr Phe Cys Gly Thr Phe Thr Ala Gly Gly Leu Lys			
[6396]		405	410	415
[6397]	Ile Ala Val Glu Asp Gly Lys Val Lys Ile Leu Gln Glu Gly Lys Ala			
[6398]		420	425	430
[6399]	Lys Lys Phe Ile Lys Ala Val Asp Gln Ile Thr Phe Asn Gly Ser Tyr			
[6400]		435	440	445
[6401]	Ala Ala Arg Asn Gly Lys His Val Leu Tyr Ile Thr Glu Arg Cys Val			
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[6403]	Phe Glu Leu Thr Lys Glu Gly Leu Lys Leu Ile Glu Val Ala Pro Gly			
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[6405]	Ile Asp Ile Glu Lys Asp Ile Leu Ala His Met Asp Phe Lys Pro Ile			
[6406]		485	490	495
[6407]	Ile Asp Asn Pro Lys Leu Met Asp Ala Arg Leu Phe Gln Asp Gly Pro			
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[6411]	<210> 90			
[6412]	<211> 145			
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[6418]	Ala His Tyr Thr Gly Asn Leu Val Asn Gly Ala Arg Ile Val Asn Gln			
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[6420]	Trp Gly Asp Val Gly Thr Glu Leu Met Val Tyr Val Asp Gly Asp Ile			
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[6422]	Ser Leu Phe Leu Gly Tyr Lys Asp Ile Glu Phe Thr Ala Pro Val Tyr			
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[6424]	Val Gly Asp Phe Met Glu Tyr His Gly Trp Ile Glu Lys Val Gly Asn			
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[6426]	Gln Ser Tyr Thr Cys Lys Phe Glu Ala Trp Lys Val Ala Thr Met Val
[6427]	85 90 95
[6428]	Asp Ile Thr Asn Pro Gln Asp Thr Arg Ala Thr Ala Cys Glu Pro Pro
[6429]	100 105 110
[6430]	Val Leu Cys Gly Arg Ala Thr Gly Ser Leu Phe Ile Ala Lys Lys Asp
[6431]	115 120 125
[6432]	Gln Arg Gly Pro Gln Glu Ser Ser Phe Lys Glu Arg Lys His Pro Gly
[6433]	130 135 140
[6434]	Glu
[6435]	145
[6436]	<210> 91
[6437]	<211> 260
[6438]	<212> PRT
[6439]	<213> Phytophthora sojae
[6440]	<400> 91
[6441]	Met Ala Ala Glu Tyr Glu Ser Ile Leu Thr Glu Val Arg Gly Lys Val
[6442]	1 5 10 15
[6443]	Ala Ile Ile Thr Leu Asn Arg Pro Lys Ala Leu Asn Ala Leu Cys Ser
[6444]	20 25 30
[6445]	Pro Leu Ile Glu Glu Leu Asn Gly Ala Ala His Ala Phe Asp Ala Asp
[6446]	35 40 45
[6447]	Pro Ser Ile Gly Ala Ile Val Ile Thr Gly Ser Gly Ser Lys Ala Phe
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[6449]	Ala Ala Gly Ala Asp Ile Lys Glu Met Ala Thr Lys Thr Phe Val Asp
[6450]	65 70 75 80
[6451]	Ala Tyr Lys Ser Asn Met Phe Ala Asn Trp Gly Asp Ile Thr Lys Val
[6452]	85 90 95
[6453]	Ser Lys Pro Val Ile Ala Ala Val Asn Gly Tyr Ala Leu Gly Gly Gly
[6454]	100 105 110
[6455]	Cys Glu Leu Ala Met Leu Cys Asp Leu Ile Ile Ala Gly Asp Ser Ala
[6456]	115 120 125
[6457]	Lys Phe Gly Gln Pro Glu Ile Thr Leu Gly Thr Ile Pro Gly Cys Gly
[6458]	130 135 140
[6459]	Gly Thr Gln Arg Leu Ile Arg Ala Val Gly Lys Ser Lys Ala Met Glu
[6460]	145 150 155 160
[6461]	Met Ile Leu Thr Gly Asn Met Ile Asp Ala Gln Gln Ala Glu Arg Asp
[6462]	165 170 175
[6463]	Gly Leu Val Ala Arg Val Val Pro Ala Asp Gln Leu Leu Asp Glu Ala
[6464]	180 185 190
[6465]	Leu Lys Thr Ala Asn Lys Ile Ala Ser Phe Ser Gln Pro Val Val Lys
[6466]	195 200 205
[6467]	Met Ala Lys Glu Ala Val Asn Ala Ala Tyr Glu Gln Ser Leu Gln Glu

[6468]	210	215	220
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[6471]	Asp Gln Lys Glu Gly Met Ala Ala Phe Val Glu Lys Arg Lys Ala Asp		
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[6480]	Met Ser Glu Glu Ser Leu Val Leu Ser Thr Ile Glu Gly Pro Ile Ala		
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[6488]	Gly Ala Asp Ile Lys Ala Met Ala Asn Ala Thr Pro Ile Asp Met Leu		
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[6490]	Thr Ser Gly Met Ile Ala Arg Trp Ala Arg Ile Ala Ala Val Arg Lys		
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[6492]	Pro Val Ile Ala Ala Val Asn Gly Tyr Ala Leu Gly Gly Gly Cys Glu		
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[6494]	Leu Ala Met Met Cys Asp Ile Ile Ile Ala Ser Glu Asn Ala Gln Phe		
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[6496]	Gly Gln Pro Glu Ile Asn Leu Gly Ile Ile Pro Gly Ala Gly Gly Thr		
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[6498]	Gln Arg Leu Thr Arg Ala Leu Gly Pro Tyr Arg Ala Met Glu Leu Ile		
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[6500]	Leu Thr Gly Ala Thr Ile Ser Ala Gln Glu Ala Leu Ala His Gly Leu		
[6501]		165	170 175
[6502]	Val Cys Arg Val Cys Pro Pro Glu Ser Leu Leu Asp Glu Ala Arg Arg		
[6503]		180	185 190
[6504]	Ile Ala Gln Thr Ile Ala Thr Lys Ser Pro Leu Ala Val Gln Leu Ala		
[6505]		195	200 205
[6506]	Lys Glu Ala Val Arg Met Ala Ala Glu Thr Thr Val Arg Glu Gly Leu		
[6507]		210	215 220
[6508]	Ala Ile Glu Leu Arg Asn Phe Tyr Leu Leu Phe Ala Ser Ala Asp Gln		
[6509]	225	230	235 240

[6510]	Lys Glu Gly Met Gln Ala Phe Ile Glu Lys Arg Ala Pro Asn Phe Ser
[6511]	245 250 255
[6512]	Gly Arg
[6513]	<210> 93
[6514]	<211> 288
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[6516]	<213> Rhodospirillum rubrum
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[6518]	Met Ala Ala Ala Pro Gly Pro Pro Ala Ala Arg Pro Arg Ala Ala Arg
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[6520]	Gln Ser Arg Met Ile Leu Pro Pro Leu Arg Glu Gln Ala Gln Met Ala
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[6522]	Tyr Glu Asn Ile Leu Val Glu Thr Asn Gly Lys Val Gly Ile Val Thr
[6523]	35 40 45
[6524]	Leu Asn Arg Pro Lys Ala Leu Asn Ala Leu Ser Ala Gly Leu Val Arg
[6525]	50 55 60
[6526]	Asp Leu Gly Ala Ala Leu Asp Ala Phe Glu Ala Asp Val Asn Val His
[6527]	65 70 75 80
[6528]	Val Ile Val Leu Thr Gly Ser Asp Lys Ala Phe Ala Ala Gly Ala Asp
[6529]	85 90 95
[6530]	Ile Lys Glu Met Ala Glu Lys Ser Tyr Met Asp Ala Tyr Leu Glu Asp
[6531]	100 105 110
[6532]	Phe Ile Thr Lys Gly Trp Glu Arg Val Thr Thr Cys Arg Lys Pro Ile
[6533]	115 120 125
[6534]	Ile Ala Ala Val Ala Gly Phe Ala Leu Gly Gly Gly Cys Glu Met Ala
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[6536]	Met Met Cys Asp Phe Ile Ile Ala Ala Gln Asn Ala Lys Phe Gly Gln
[6537]	145 150 155 160
[6538]	Pro Glu Ile Asn Leu Gly Thr Leu Pro Gly Ala Gly Gly Thr Gln Arg
[6539]	165 170 175
[6540]	Leu Thr Arg Phe Val Gly Lys Ser Lys Ala Met Asp Met Cys Leu Thr
[6541]	180 185 190
[6542]	Gly Arg Met Met Asp Ala Asp Glu Ala Trp Lys Cys Gly Leu Val Ser
[6543]	195 200 205
[6544]	Arg Ile Val Pro Val Asp Asp Leu Lys Asp Glu Val Leu Lys Ile Ala
[6545]	210 215 220
[6546]	Glu Ala Ile Ala Asp Lys Ser Leu Pro Ile Thr Met Met Val Lys Glu
[6547]	225 230 235 240
[6548]	Ala Val Asn Ala Ala Tyr Glu Thr Thr Leu Ala Gln Gly Val Arg Phe
[6549]	245 250 255
[6550]	Glu Arg Arg Leu Phe Gln Ala Ser Phe Ala Thr Asp Asp Gln Lys Glu
[6551]	260 265 270

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[6561]	Ile Arg Leu Asn Arg Pro Glu Ala Leu Asn Ala Leu Asn Ser Gln Leu
[6562]	20 25 30
[6563]	Leu Gly Glu Leu Ala Ala Ala Leu Ser Thr Leu Asp Ala Asp Pro Ala
[6564]	35 40 45
[6565]	Val Arg Cys Phe Val Leu Thr Gly Ser Asp Lys Ala Phe Ala Ala Gly
[6566]	50 55 60
[6567]	Ala Asp Ile Lys Glu Met Ala Asp Lys Ser Phe Val Asp Met Leu Lys
[6568]	65 70 75 80
[6569]	Leu Asp Phe Phe Gly Thr Glu Gly Asp Ala Ile Leu Arg Thr Arg Lys
[6570]	85 90 95
[6571]	Pro Val Ile Ala Ala Val Ala Gly Tyr Ala Leu Gly Gly Gly Cys Glu
[6572]	100 105 110
[6573]	Leu Ala Met Met Cys Asp Phe Ile Leu Cys Ala Glu Asn Ala Lys Phe
[6574]	115 120 125
[6575]	Gly Gln Pro Glu Ile Asn Leu Gly Val Val Ala Gly Ile Gly Gly Thr
[6576]	130 135 140
[6577]	Gln Arg Leu Thr Arg Phe Val Gly Lys Ser Lys Ser Met Glu Met His
[6578]	145 150 155 160
[6579]	Leu Thr Gly Arg Phe Met Asp Ala Ala Glu Ala Glu Arg Ser Gly Leu
[6580]	165 170 175
[6581]	Val Ser Arg Val Leu Pro Leu Ala Asp Leu Leu Pro Glu Ala Leu Ala
[6582]	180 185 190
[6583]	Thr Ala Arg Lys Ile Ala Glu Lys Ser Ala Ile Ala Thr Met Val Ala
[6584]	195 200 205
[6585]	Lys Asp Cys Val Asn Arg Ala Tyr Glu Thr Thr Leu Arg Glu Gly Val
[6586]	210 215 220
[6587]	Leu Tyr Glu Arg Arg Val Phe His Ala Leu Phe Ala Thr Glu Asp Gln
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[6589]	Lys Glu Gly Met Ala Ala Phe Ala Glu Lys Arg Pro Ala Lys Phe Ala
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 [6603] Leu Ile Gln Leu Asn Arg Pro Lys Ala Leu Asn Ala Leu Cys Asp Gly
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 [6609] Gly Ala Asp Ile Lys Glu Met Gln Asn Leu Ser Phe Gln Asp Cys Tyr
 [6610] 100 105 110
 [6611] Ser Ser Lys Phe Leu Lys His Trp Asp His Leu Thr Gln Val Lys Lys
 [6612] 115 120 125
 [6613] Pro Val Ile Ala Ala Val Asn Gly Tyr Ala Phe Gly Gly Gly Cys Glu
 [6614] 130 135 140
 [6615] Leu Ala Met Met Cys Asp Ile Ile Tyr Ala Gly Glu Lys Ala Gln Phe
 [6616] 145 150 155 160
 [6617] Ala Gln Pro Glu Ile Leu Ile Gly Thr Ile Pro Gly Ala Gly Gly Thr
 [6618] 165 170 175
 [6619] Gln Arg Leu Thr Arg Ala Val Gly Lys Ser Leu Ala Met Glu Met Val
 [6620] 180 185 190
 [6621] Leu Thr Gly Asp Arg Ile Ser Ala Gln Asp Ala Lys Gln Ala Gly Leu
 [6622] 195 200 205
 [6623] Val Ser Lys Ile Cys Pro Val Glu Thr Leu Val Glu Glu Ala Ile Gln
 [6624] 210 215 220
 [6625] Cys Ala Glu Lys Ile Ala Ser Asn Ser Lys Ile Val Val Ala Met Ala
 [6626] 225 230 235 240
 [6627] Lys Glu Ser Val Asn Ala Ala Phe Glu Met Thr Leu Thr Glu Gly Ser
 [6628] 245 250 255
 [6629] Lys Leu Glu Lys Lys Leu Phe Tyr Ser Thr Phe Ala Thr Asp Asp Arg
 [6630] 260 265 270
 [6631] Lys Glu Gly Met Thr Ala Phe Val Glu Lys Arg Lys Ala Asn Phe Lys
 [6632] 275 280 285
 [6633] Asp Gln
 [6634] 290
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[6636]	<211>	399
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[6638]	<213>	荧光假单胞菌
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[6640]	Met Arg Val Val Leu Cys Lys Phe Ala Leu Leu Arg Ser His Asp Gly	
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[6642]	Ser Ser Ala Asn Ile Leu Ile Lys Asn Asn Lys Ser Arg Glu Leu Ser	
[6643]	20	25 30
[6644]	Met Thr Ala Gln Val Ser Thr Glu Ala Ser His Ala Ala Ile Leu Gln	
[6645]	35	40 45
[6646]	Asp Glu Val Leu Ala Glu Val Arg Asn His Ile Gly His Leu Thr Leu	
[6647]	50	55 60
[6648]	Asn Arg Pro Ala Gly Leu Asn Ala Leu Thr Leu Gln Met Val Arg Ser	
[6649]	65	70 75 80
[6650]	Leu Thr Ser Gln Leu Gln Ala Trp Ser Asp Asp Pro Gln Val Tyr Ala	
[6651]	85	90 95
[6652]	Val Val Leu Arg Gly Ala Gly Glu Lys Ala Phe Cys Ala Gly Gly Asp	
[6653]	100	105 110
[6654]	Ile Arg Ser Leu Tyr Asp Ser Phe Lys Asn Gly Asp Thr Leu His Gln	
[6655]	115	120 125
[6656]	Asp Phe Phe Val Glu Glu Tyr Ala Leu Asp Leu Ala Ile His His Tyr	
[6657]	130	135 140
[6658]	Arg Lys Pro Val Leu Ala Leu Met Asp Gly Phe Val Leu Gly Gly Gly	
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[6660]	Met Gly Leu Val Gln Gly Ala Asp Leu Arg Val Val Thr Glu Arg Ser	
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[6662]	Arg Leu Ala Met Pro Glu Val Ala Ile Gly Tyr Phe Pro Asp Val Gly	
[6663]	180	185 190
[6664]	Gly Ser Tyr Phe Leu Pro Arg Ile Pro Gly Glu Leu Gly Ile Tyr Leu	
[6665]	195	200 205
[6666]	Gly Val Thr Gly Val Gln Ile Arg Ala Ala Asp Ala Leu Tyr Cys Gly	
[6667]	210	215 220
[6668]	Leu Ala Asp Trp Tyr Leu Asp Ser His Lys Leu Ala Asp Leu Asp Gln	
[6669]	225	230 235 240
[6670]	Lys Leu Asp Asn Leu Arg Trp His Asp Ser Pro Leu Lys Asp Leu Gln	
[6671]	245	250 255
[6672]	Gly Ala Leu Ala Arg Leu Ala Val Gln Gln Leu Pro Asp Ala Pro Leu	
[6673]	260	265 270
[6674]	Ala Ala Leu Arg Pro Ala Ile Asp His Phe Phe Ala Leu Pro Asp Val	
[6675]	275	280 285
[6676]	Pro Ser Ile Val Glu Gln Leu Gln Gln Val Thr Val Ala Asp Ser His	
[6677]	290	295 300

[6678]	Glu Trp Ala Leu Asn Thr Val Ser Leu Met Gln Thr Arg Ser Pro Leu
[6679]	305 310 315 320
[6680]	Ala Met Ala Val Thr Leu Glu Met Leu Arg Arg Gly Arg Arg Leu Ser
[6681]	325 330 335
[6682]	Leu Glu Gln Cys Phe Ala Leu Glu Leu His Leu Asp Arg Gln Trp Phe
[6683]	340 345 350
[6684]	Glu Arg Gly Asp Leu Ile Glu Gly Val Arg Ala Leu Ile Ile Asp Lys
[6685]	355 360 365
[6686]	Asp Lys Ser Pro Arg Trp Asn Pro Pro Thr Leu His Gly Leu Ala Leu
[6687]	370 375 380
[6688]	Asn His Val Glu Ser Phe Phe His His Phe Glu Lys Val Val Lys
[6689]	385 390 395
[6690]	<210> 97
[6691]	<211> 351
[6692]	<212> PRT
[6693]	<213> 蜡状芽孢杆菌
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[6695]	Met Thr Glu Gln Val Leu Phe Ser Val Ser Glu Asn Gly Val Ala Thr
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[6697]	Ile Thr Leu Asn Arg Pro Lys Ala Leu Asn Ser Leu Ser Tyr Asp Met
[6698]	20 25 30
[6699]	Leu Gln Pro Ile Gly Gln Lys Leu Lys Glu Trp Glu His Asp Glu Arg
[6700]	35 40 45
[6701]	Ile Ala Leu Ile Val Leu Lys Gly Ala Gly Thr Lys Gly Phe Cys Ala
[6702]	50 55 60
[6703]	Gly Gly Asp Ile Lys Thr Leu Tyr Glu Ala Arg Ser Asn Glu Val Ala
[6704]	65 70 75 80
[6705]	Leu Gln His Ala Glu Arg Phe Phe Glu Glu Glu Tyr Glu Ile Asp Thr
[6706]	85 90 95
[6707]	Tyr Ile Tyr Gln Tyr Thr Lys Pro Ile Ile Ala Cys Leu Asp Gly Ile
[6708]	100 105 110
[6709]	Val Met Gly Gly Gly Val Gly Leu Thr Asn Gly Ala Lys Tyr Arg Ile
[6710]	115 120 125
[6711]	Val Thr Glu Arg Thr Lys Trp Ala Met Pro Glu Met Asn Ile Gly Phe
[6712]	130 135 140
[6713]	Phe Pro Asp Val Gly Ala Ala Tyr Phe Leu Asn Lys Ala Pro Gly Tyr
[6714]	145 150 155 160
[6715]	Thr Gly Arg Phe Val Ala Leu Thr Ala Ser Ile Leu Lys Ala Ser Asp
[6716]	165 170 175
[6717]	Val Leu Phe Ile Asn Ala Ala Asp Tyr Phe Met Thr Ser Asp Ser Leu
[6718]	180 185 190
[6719]	Pro Glu Phe Leu Thr Glu Leu Glu Ser Val Asn Trp His Lys Glu Asp

[6720]	195	200	205
[6721]	Asp Val His Thr Asn Leu Lys Glu Val Ile Arg Thr Phe Ala Thr Ala		
[6722]	210	215	220
[6723]	Pro Asn Leu Glu Ser Glu Leu Ala Pro Ser Leu Glu Val Ile Asn Ser		
[6724]	225	230	235
[6725]	His Phe Ala Phe Asp Thr Ile Glu Glu Ile Ile His Ser Leu Glu Lys		
[6726]	245	250	255
[6727]	Asp Glu Ser Ser Phe Ala Leu Lys Thr Lys Glu Ile Leu Leu Ser Lys		
[6728]	260	265	270
[6729]	Ser Pro Ile Ser Leu Lys Val Thr Leu Lys Gln Phe Ile Asp Gly Gln		
[6730]	275	280	285
[6731]	Asp Lys Ser Val Glu Glu Cys Phe Ala Thr Asp Leu Ile Leu Ala Lys		
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[6733]	Asn Phe Met Arg His Glu Asp Phe Phe Glu Gly Val Arg Ser Val Val		
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[6735]	Val Asp Lys Asp Gln Asn Pro Asn Tyr Lys Tyr Lys Gln Leu Ser Asp		
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[6746]	Ile Leu His His Leu Arg Met Ser Lys His Thr Asp Ala Ala Glu Glu		
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[6748]	Val Leu Leu Glu Lys Lys Gly Cys Ala Gly Val Ile Thr Leu Asn Arg		
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[6750]	Pro Lys Phe Leu Asn Ala Leu Thr Leu Asn Met Ile Arg Gln Ile Tyr		
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[6752]	Pro Gln Leu Lys Lys Trp Glu Gln Asp Pro Glu Thr Phe Val Ile Ile		
[6753]	65	70	75
[6754]	Ile Lys Gly Ala Gly Gly Lys Ala Phe Cys Ala Gly Gly Asp Ile Arg		
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[6756]	Val Ile Ser Glu Ala Glu Lys Ala Lys Gln Lys Ile Ala Pro Val Phe		
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[6758]	Phe Arg Glu Glu Tyr Met Leu Asn Asn Ala Val Gly Ser Cys Gln Lys		
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[6760]	Pro Tyr Val Ala Leu Ile His Gly Ile Thr Met Gly Gly Gly Val Gly		
[6761]	130	135	140

[6762]	Leu Ser Val His Gly Gln Phe Arg Val Ala Thr Glu Lys Cys Leu Phe			
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[6764]	Ala Met Pro Glu Thr Ala Ile Gly Leu Phe Pro Asp Val Gly Gly Gly			
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[6766]	Tyr Phe Phe Ala Thr Thr Pro Arg Lys Thr Trp Leu Leu Pro Cys Ile			
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[6768]	Asn Gly Phe Arg Leu Lys Gly Arg Asp Val Tyr Arg Ala Gly Ile Ala			
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[6770]	Thr His Phe Val Asp Ser Glu Lys Leu Ala Met Leu Glu Glu Asp Leu			
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[6772]	Leu Ala Leu Lys Ser Pro Ser Lys Glu Asn Ile Ala Ser Val Leu Glu			
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[6774]	Asn Tyr His Thr Glu Ser Lys Ile Asp Arg Asp Lys Ser Phe Ile Leu			
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[6776]	Glu Glu His Met Asp Lys Ile Asn Ser Cys Phe Ser Ala Asn Thr Val			
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[6778]	Glu Glu Ile Ile Glu Asn Leu Gln Gln Asp Gly Ser Ser Phe Ala Leu			
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[6780]	Glu Gln Leu Lys Val Ile Asn Lys Met Ser Pro Thr Ser Leu Lys Ile			
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[6784]	Leu Thr Met Glu Tyr Arg Leu Ser Gln Ala Cys Met Arg Gly His Asp			
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[6805]	Ala Thr Gly Tyr Gly Arg Phe Asn Val Asp Cys Ala Lys Gly Glu Val		
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[6807]	Ser Glu Ile Thr Cys His Ala Lys Gly Ala Leu Phe Glu Cys Pro Gly		
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[6809]	Thr Thr Thr Ile Leu Asp Ile Gly Gly Gln Asp Val Lys Ser Ile Lys		
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[6813]	Ala Ala Gly Thr Gly Arg Phe Leu Asp Val Met Ser Lys Val Leu Glu		
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[6815]	Ile Pro Met Ser Glu Met Gly Asp Trp Tyr Phe Lys Ser Lys His Pro		
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[6817]	Ala Ala Val Ser Ser Thr Cys Thr Val Phe Ala Glu Ser Glu Val Ile		
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[6819]	Ser Leu Leu Ser Lys Asn Val Pro Lys Glu Asp Ile Val Ala Gly Val		
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[6821]	His Gln Ser Ile Ala Ala Lys Ala Cys Ala Leu Val Arg Arg Val Gly		
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[6823]	Val Gly Glu Asp Leu Thr Met Thr Gly Gly Gly Ser Arg Asp Pro Gly		
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[6825]	Val Val Asp Ala Val Ser Lys Glu Leu Gly Ile Pro Val Arg Val Ala		
[6826]	225	230	235
[6827]	Leu His Pro Gln Ala Val Gly Ala Leu Gly Ala Ala Leu Ile Ala Tyr		
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[6840]	Lys Lys Glu Gly Arg Leu Val Cys Trp Ser Ala Ser Val Ala Pro Pro		
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[6842]	Glu Phe Cys Thr Ala Met Asp Ile Ala Ile Val Tyr Pro Glu Thr His		
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[6844]	Ala Ala Gly Ile Gly Ala Arg His Gly Ala Pro Ala Met Leu Glu Val		
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[6853]					130					135					140	
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[6855]	145					150					155				160	
[6856]	Val	Pro	Phe	Asn	His	Glu	Phe	Pro	Val	Thr	Lys	His	Ala	Lys	Gln	Tyr
[6857]					165					170					175	
[6858]	Ile	Val	Gly	Glu	Phe	Lys	His	Ala	Ile	Lys	Gln	Leu	Glu	Asp	Leu	Cys
[6859]					180					185					190	
[6860]	Gly	Arg	Pro	Phe	Asp	Tyr	Asp	Lys	Phe	Phe	Glu	Val	Gln	Lys	Gln	Thr
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[6869]					260					265					270	
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[6877]					325					330					335	
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[6897]	His Pro Gln Lys Thr Val Leu Asn Tyr Lys Lys Gln Gly Lys Gly Leu
[6898]	20 25 30
[6899]	Val Gly Met Met Pro Tyr Tyr Ala Pro Glu Glu Ile Val Tyr Ala Ala
[6900]	35 40 45
[6901]	Gly Tyr Leu Pro Val Gly Met Phe Gly Ser Gln Asn Pro Gln Ile Ser
[6902]	50 55 60
[6903]	Ala Ala Arg Thr Tyr Leu Pro Pro Phe Ala Cys Ser Leu Met Gln Ala
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[6905]	Asp Met Glu Leu Gln Leu Asn Gly Thr Tyr Asp Cys Leu Asp Ala Val
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[6907]	Ile Phe Ser Val Pro Cys Asp Thr Leu Arg Cys Met Ser Gln Lys Trp
[6908]	100 105 110
[6909]	His Gly Lys Ala Pro Val Ile Val Phe Thr Gln Pro Gln Asn Arg Lys
[6910]	115 120 125
[6911]	Ile Arg Pro Ala Val Asp Phe Leu Lys Ala Glu Tyr Glu His Val Arg
[6912]	130 135 140
[6913]	Thr Glu Leu Gly Arg Ile Leu Asn Val Lys Ile Ser Asp Leu Ala Ile
[6914]	145 150 155 160
[6915]	Gln Glu Ala Ile Lys Val Tyr Asn Glu Asn Arg Gln Val Met Arg Glu
[6916]	165 170 175
[6917]	Phe Cys Asp Val Ala Ala Gln Tyr Pro Gln Ile Phe Thr Pro Ile Lys
[6918]	180 185 190
[6919]	Arg His Asp Val Ile Lys Ala Arg Trp Phe Met Asp Lys Ala Glu His
[6920]	195 200 205
[6921]	Thr Ala Leu Val Arg Glu Leu Ile Asp Ala Val Lys Lys Glu Pro Val
[6922]	210 215 220
[6923]	Gln Pro Trp Asn Gly Lys Lys Val Ile Leu Ser Gly Ile Met Ala Glu
[6924]	225 230 235 240
[6925]	Pro Asp Glu Phe Leu Asp Ile Phe Ser Glu Phe Asn Ile Ala Val Val
[6926]	245 250 255
[6927]	Ala Asp Asp Leu Ala Gln Glu Ser Arg Gln Phe Arg Thr Asp Val Pro
[6928]	260 265 270
[6929]	Ser Gly Ile Asp Pro Leu Glu Gln Leu Ala Gln Gln Trp Gln Asp Phe

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[6933]	Leu Ile Asp Met Thr Lys Lys Tyr Asn Ala Asp Ala Val Val Ile Cys		
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[6935]	Met Met Arg Phe Cys Asp Pro Glu Glu Phe Asp Tyr Pro Ile Tyr Lys		
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[6937]	Pro Glu Phe Glu Ala Ala Gly Val Arg Tyr Thr Val Leu Asp Leu Asp		
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[6939]	Ile Glu Ser Pro Ser Leu Glu Gln Leu Arg Thr Arg Ile Gln Ala Phe		
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[6956]	Ile Asn Pro Ser Thr Glu Glu Glu Ile Cys His Ile Tyr Glu Gly Arg		
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[6958]	Glu Asp Asp Val Glu Glu Ala Val Gln Ala Ala Asp Arg Ala Phe Ser		
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[6960]	Asn Gly Ser Trp Asn Gly Ile Asp Pro Ile Asp Arg Gly Lys Ala Leu		
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[6962]	Tyr Arg Leu Ala Glu Leu Ile Glu Gln Asp Lys Asp Val Ile Ala Ser		
[6963]	115 120 125		
[6964]	Ile Glu Thr Leu Asp Asn Gly Lys Ala Ile Ser Ser Ser Arg Gly Asp		
[6965]	130 135 140		
[6966]	Val Asp Leu Val Ile Asn Tyr Leu Lys Ser Ser Ala Gly Phe Ala Asp		
[6967]	145 150 155 160		
[6968]	Lys Ile Asp Gly Arg Met Ile Asp Thr Gly Arg Thr His Phe Ser Tyr		
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[6970]	Thr Lys Arg Gln Pro Leu Gly Val Cys Gly Gln Ile Ile Pro Trp Asn		
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[6975]			210					215					220			
[6976]	Leu	Tyr	Val	Ser	Lys	Tyr	Ile	Pro	Gln	Ala	Gly	Ile	Pro	Pro	Gly	Val
[6977]	225					230					235					240
[6978]	Ile	Asn	Ile	Val	Ser	Gly	Phe	Gly	Lys	Ile	Val	Gly	Glu	Ala	Ile	Thr
[6979]					245					250					255	
[6980]	Asn	His	Pro	Lys	Ile	Lys	Lys	Val	Ala	Phe	Thr	Gly	Ser	Thr	Ala	Thr
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[6982]	Gly	Arg	His	Ile	Tyr	Gln	Ser	Ala	Ala	Ala	Gly	Leu	Lys	Lys	Val	Thr
[6983]			275							280					285	
[6984]	Leu	Glu	Leu	Gly	Gly	Lys	Ser	Pro	Asn	Ile	Val	Phe	Ala	Asp	Ala	Glu
[6985]	290					295							300			
[6986]	Leu	Lys	Lys	Ala	Val	Gln	Asn	Ile	Ile	Leu	Gly	Ile	Tyr	Tyr	Asn	Ser
[6987]	305					310					315					320
[6988]	Gly	Glu	Val	Cys	Cys	Ala	Gly	Ser	Arg	Val	Tyr	Val	Glu	Glu	Ser	Ile
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[6990]	Tyr	Asp	Lys	Phe	Ile	Glu	Glu	Phe	Lys	Ala	Ala	Ser	Glu	Ser	Ile	Lys
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[6993]			355							360					365	
[6994]	Gln	Met	Gln	Leu	Asn	Lys	Ile	Leu	Lys	Tyr	Val	Asp	Ile	Gly	Lys	Asn
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[6997]	385					390					395					400
[6998]	Tyr	Phe	Ile	Lys	Pro	Thr	Val	Phe	Gly	Asp	Val	Lys	Glu	Asp	Met	Arg
[6999]				405						410					415	
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[7002]	Lys	Ser	Ala	Asp	Glu	Val	Ile	Asn	Met	Ala	Asn	Asp	Ser	Glu	Tyr	Gly
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[7008]	Phe	His	His	Ala	Val	Pro	Phe	Gly	Gly	Phe	Asn	Ala	Ser	Gly	Leu	Gly
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[7010]	Arg	Glu	Met	Ser	Val	Asp	Ala	Leu	Gln	Asn	Tyr	Leu	Gln	Val	Lys	Ala
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[7012]	Val	Arg	Ala	Lys	Leu	Asp	Glu									
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[7017]	<213>	大肠杆菌
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[7022]	20	25 30
[7023]	Glu Asn Glu Thr Phe Glu Thr Val Asp Pro Val Thr Gln Ala Pro Leu	
[7024]	35	40 45
[7025]	Ala Lys Ile Ala Arg Gly Lys Ser Val Asp Ile Asp Arg Ala Met Ser	
[7026]	50	55 60
[7027]	Ala Ala Arg Gly Val Phe Glu Arg Gly Asp Trp Ser Leu Ser Ser Pro	
[7028]	65	70 75 80
[7029]	Ala Lys Arg Lys Ala Val Leu Asn Lys Leu Ala Asp Leu Met Glu Ala	
[7030]	85	90 95
[7031]	His Ala Glu Glu Leu Ala Leu Leu Glu Thr Leu Asp Thr Gly Lys Pro	
[7032]	100	105 110
[7033]	Ile Arg His Ser Leu Arg Asp Asp Ile Pro Gly Ala Ala Arg Ala Ile	
[7034]	115	120 125
[7035]	Arg Trp Tyr Ala Glu Ala Ile Asp Lys Val Tyr Gly Glu Val Ala Thr	
[7036]	130	135 140
[7037]	Thr Ser Ser His Glu Leu Ala Met Ile Val Arg Glu Pro Val Gly Val	
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[7039]	Ile Ala Ala Ile Val Pro Trp Asn Phe Pro Leu Leu Leu Thr Cys Trp	
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[7041]	Lys Leu Gly Pro Ala Leu Ala Ala Gly Asn Ser Val Ile Leu Lys Pro	
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[7043]	Ser Glu Lys Ser Pro Leu Ser Ala Ile Arg Leu Ala Gly Leu Ala Lys	
[7044]	195	200 205
[7045]	Glu Ala Gly Leu Pro Asp Gly Val Leu Asn Val Val Thr Gly Phe Gly	
[7046]	210	215 220
[7047]	His Glu Ala Gly Gln Ala Leu Ser Arg His Asn Asp Ile Asp Ala Ile	
[7048]	225	230 235 240
[7049]	Ala Phe Thr Gly Ser Thr Arg Thr Gly Lys Gln Leu Leu Lys Asp Ala	
[7050]	245	250 255
[7051]	Gly Asp Ser Asn Met Lys Arg Val Trp Leu Glu Ala Gly Gly Lys Ser	
[7052]	260	265 270
[7053]	Ala Asn Ile Val Phe Ala Asp Cys Pro Asp Leu Gln Gln Ala Ala Ser	
[7054]	275	280 285
[7055]	Ala Thr Ala Ala Gly Ile Phe Tyr Asn Gln Gly Gln Val Cys Ile Ala	

[7056]	290	295	300
[7057]	Gly Thr Arg Leu Leu Leu Glu Glu Ser Ile Ala Asp Glu Phe Leu Ala		
[7058]	305	310	315 320
[7059]	Leu Leu Lys Gln Gln Ala Gln Asn Trp Gln Pro Gly His Pro Leu Asp		
[7060]	325	330	335
[7061]	Pro Ala Thr Thr Met Gly Thr Leu Ile Asp Cys Ala His Ala Asp Ser		
[7062]	340	345	350
[7063]	Val His Ser Phe Ile Arg Glu Gly Glu Ser Lys Gly Gln Leu Leu Leu		
[7064]	355	360	365
[7065]	Asp Gly Arg Asn Ala Gly Leu Ala Ala Ala Ile Gly Pro Thr Ile Phe		
[7066]	370	375	380
[7067]	Val Asp Val Asp Pro Asn Ala Ser Leu Ser Arg Glu Glu Ile Phe Gly		
[7068]	385	390	395 400
[7069]	Pro Val Leu Val Val Thr Arg Phe Thr Ser Glu Glu Gln Ala Leu Gln		
[7070]	405	410	415
[7071]	Leu Ala Asn Asp Ser Gln Tyr Gly Leu Gly Ala Ala Val Trp Thr Arg		
[7072]	420	425	430
[7073]	Asp Leu Ser Arg Ala His Arg Met Ser Arg Arg Leu Lys Ala Gly Ser		
[7074]	435	440	445
[7075]	Val Phe Val Asn Asn Tyr Asn Asp Gly Asp Met Thr Val Pro Phe Gly		
[7076]	450	455	460
[7077]	Gly Tyr Lys Gln Ser Gly Asn Gly Arg Asp Lys Ser Leu His Ala Leu		
[7078]	465	470	475 480
[7079]	Glu Lys Phe Thr Glu Leu Lys Thr Ile Trp Ile Ser Leu Glu Ala		
[7080]	485	490	495
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[7084]	<213> 肺炎克雷伯氏菌 (<i>Klebsiella pneumoniae</i>)		
[7085]	<400> 104		
[7086]	Met Asn Phe Gln His Leu Ala Tyr Trp Gln Glu Lys Ala Lys Asn Leu		
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[7088]	Ala Ile Glu Thr Arg Leu Phe Ile Asn Gly Glu Tyr Cys Ala Ala Ala		
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[7090]	Asp Asn Thr Thr Phe Glu Thr Ile Asp Pro Ala Ala Gln Gln Thr Leu		
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[7092]	Ala Gln Val Ala Arg Gly Lys Lys Ala Asp Val Glu Arg Ala Val Lys		
[7093]	50	55	60
[7094]	Ala Ala Arg Gln Ala Phe Asp Asn Gly Asp Trp Ser Gln Ala Ser Pro		
[7095]	65	70	75 80
[7096]	Ala Gln Arg Lys Ala Ile Leu Thr Arg Phe Ala Asp Leu Met Glu Ala		
[7097]	85	90	95

[7098]	His Arg Glu Glu Leu Ala Leu Leu Glu Thr Leu Asp Thr Gly Lys Pro		
[7099]	100	105	110
[7100]	Ile Arg His Ser Leu Arg Asp Asp Ile Pro Gly Ala Ala Arg Ala Ile		
[7101]	115	120	125
[7102]	Arg Trp Tyr Ala Glu Ala Leu Asp Lys Val Tyr Gly Glu Val Ala Pro		
[7103]	130	135	140
[7104]	Thr Gly Ser Asn Glu Leu Ala Met Ile Val Arg Glu Pro Ile Gly Val		
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[7128]	Pro Asp Thr Thr Met Gly Met Leu Ile Asp Asn Thr His Ala Asp Asn		
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[7130]	Val His Ser Phe Ile Arg Gly Gly Glu Ser Gln Ser Thr Leu Phe Leu		
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[7166]	tacgttagac ctctaggtaa ggttgttctt gttggtttac cagcaggcgc acaagtcaaa 840
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[7182]	Asp Leu His Ala Trp Lys Gly Asp Trp Pro Leu Ala Thr Lys Leu Pro		
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[7259]	Ala Lys Leu Pro Leu Val Gly Gly His Glu Gly Ala Gly Ile Val Val
[7260]	85 90 95
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[7293]	Thr Leu Pro Glu Ile Tyr Glu Leu Met Glu Gln Gly Lys Ile Leu Gly		
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[7439]	Phe His Ala Pro Glu Tyr Val Ser Leu Leu Asn Asp Pro Arg Asn Lys		
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[7441]	Tyr Asn Lys Met Val Gln Val Glu Arg Leu Gly Asn Val Ala Gly Gly		
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[7443]	Glu Pro Val Ala Tyr Leu Asn Leu Glu Ile Glu Lys Leu Ser Gln Ala		
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[7867]	Ser Leu Lys Leu Leu Thr Gly Gly Lys Arg Ile Gly Asn Lys Gly Tyr		
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[7969]	Phe Ile Phe Ser Glu Cys Thr Asp Asp Met Thr Ile Met Lys Glu Glu		
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[7973]	Ala Ile Glu Arg Ala Asn Ala Thr Thr Tyr Gly Leu Gly Ala Ala Leu		
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[7979]	Pro Phe Gly Gly Ser Lys Met Ser Gly Ile Gly Arg Glu Leu Gly Pro		

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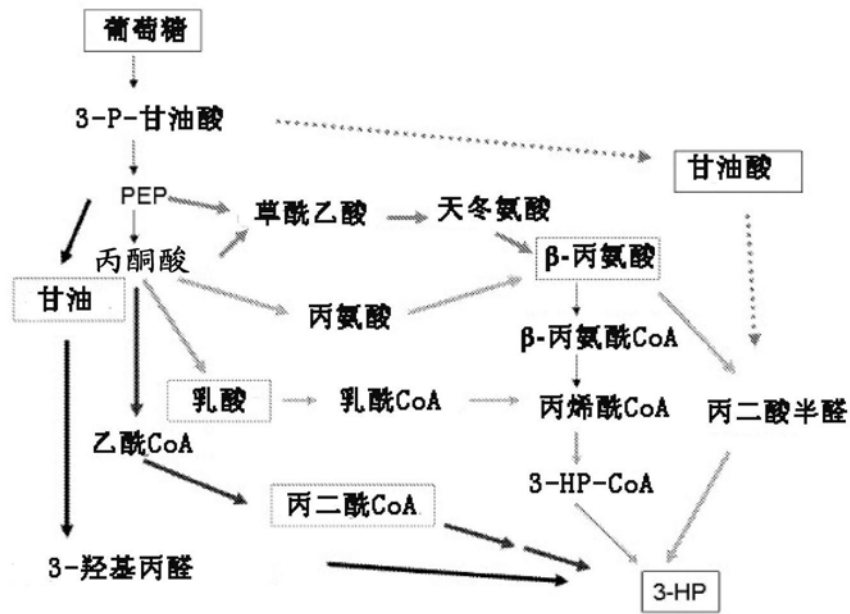


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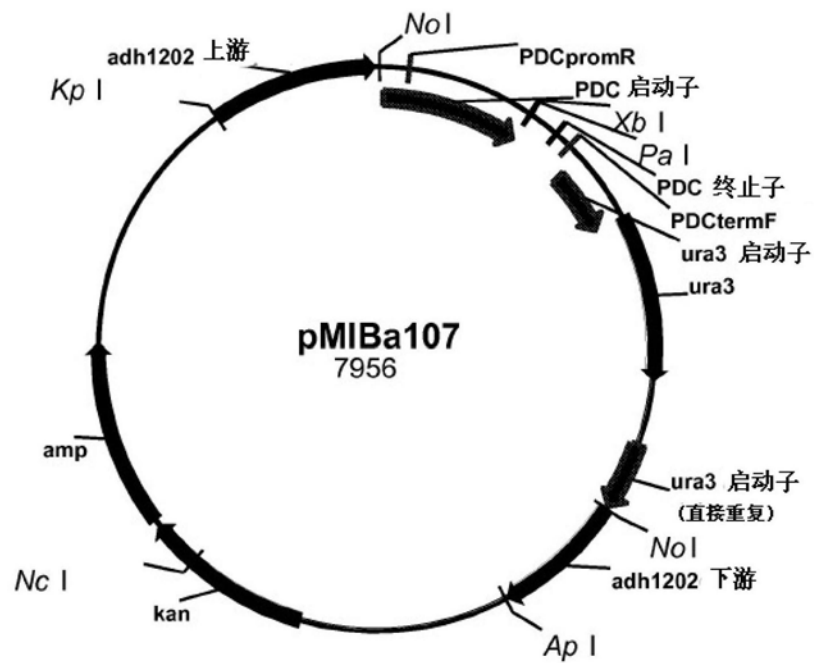


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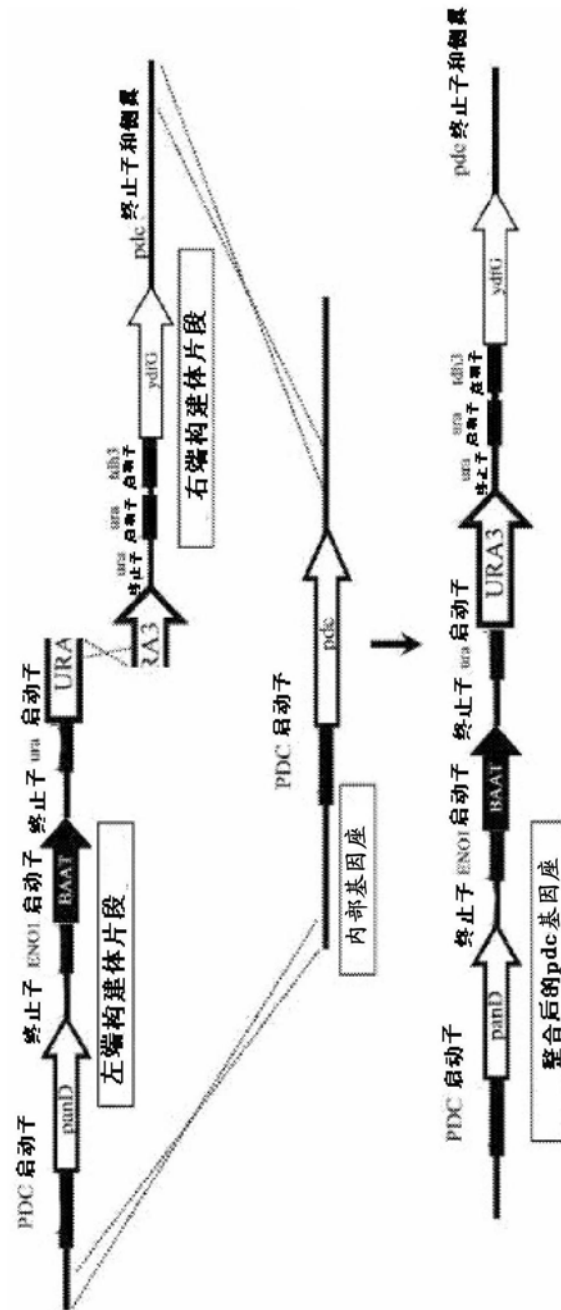


图3

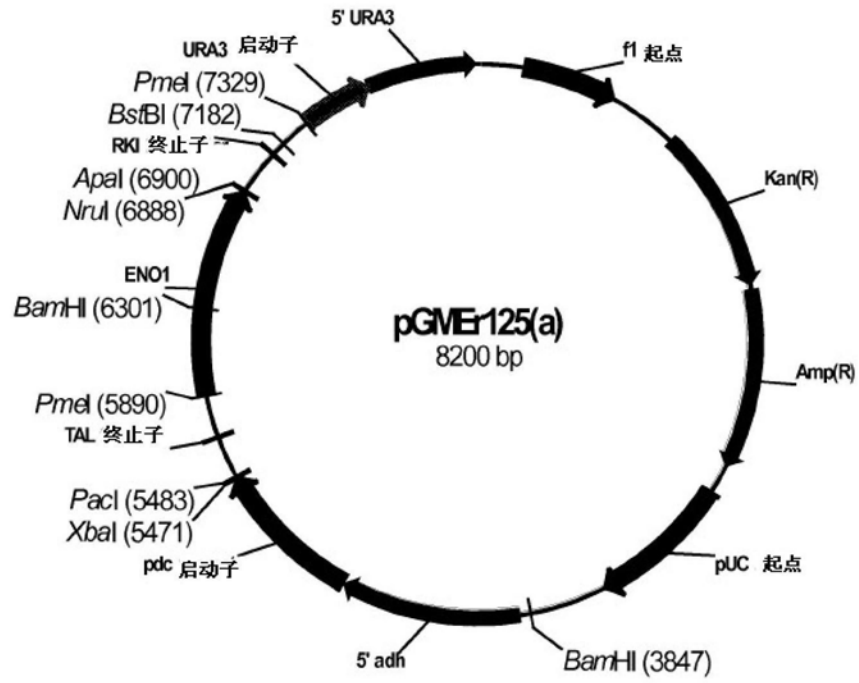


图4

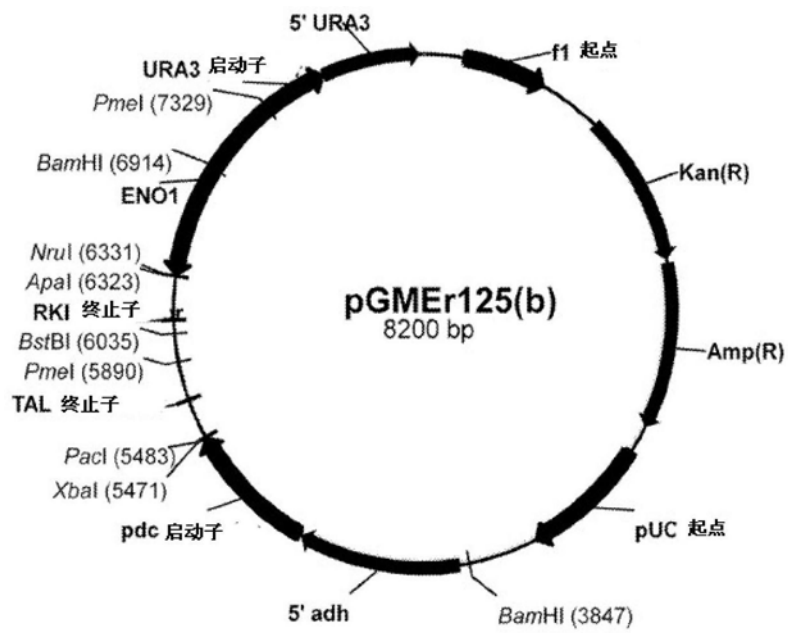


图5

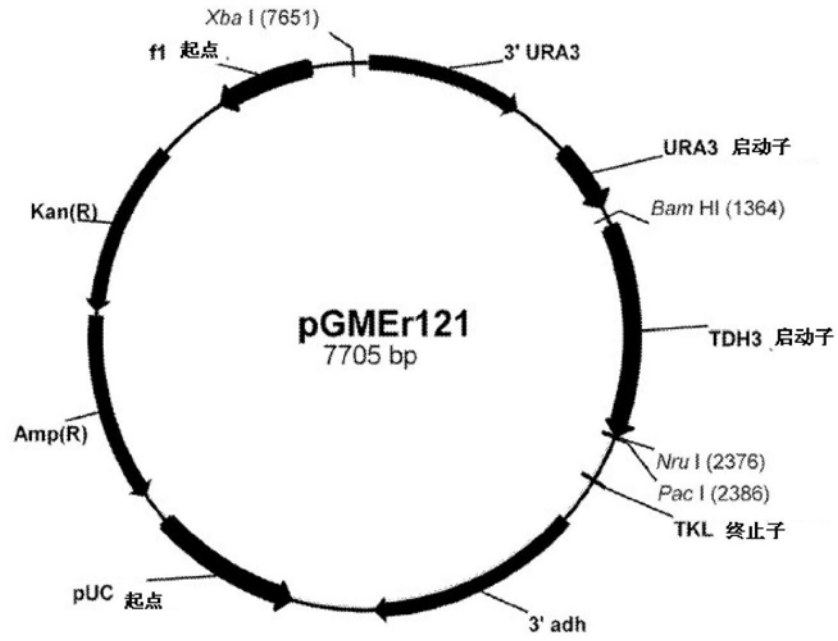


图6

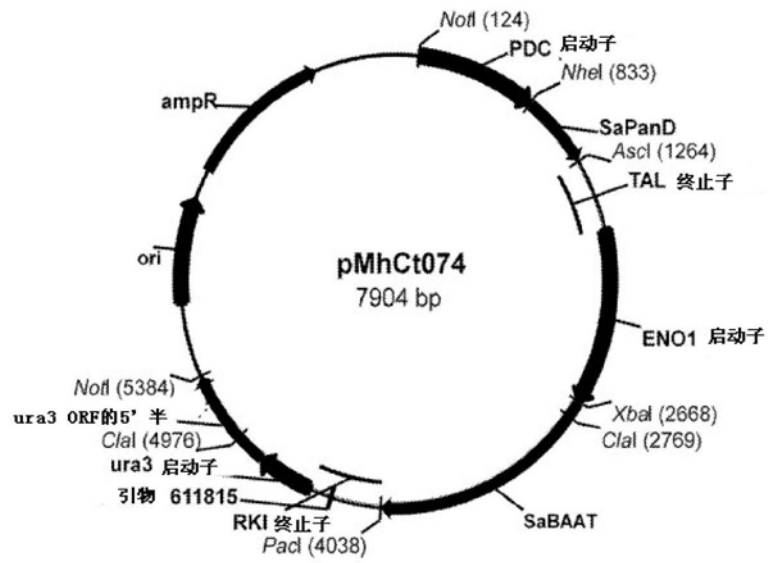


图7

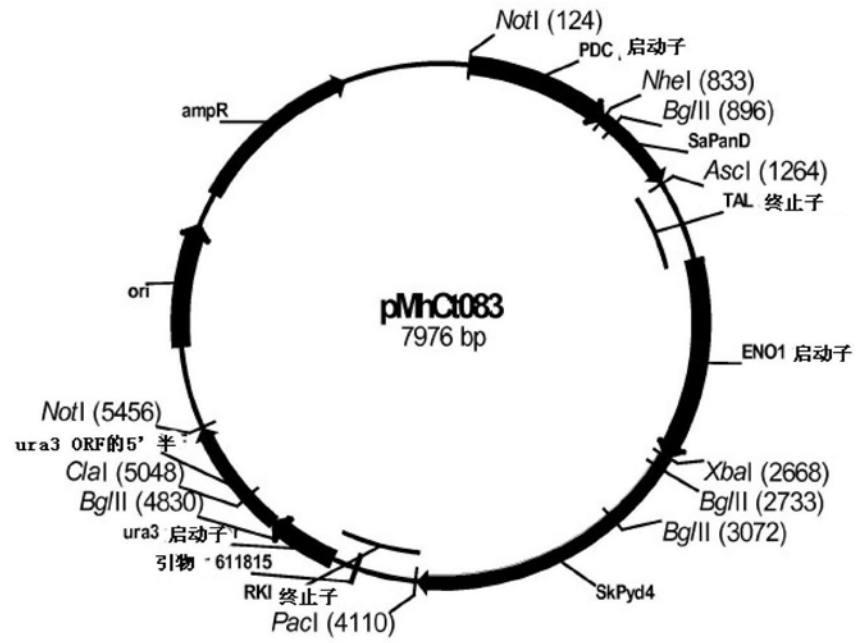


图8

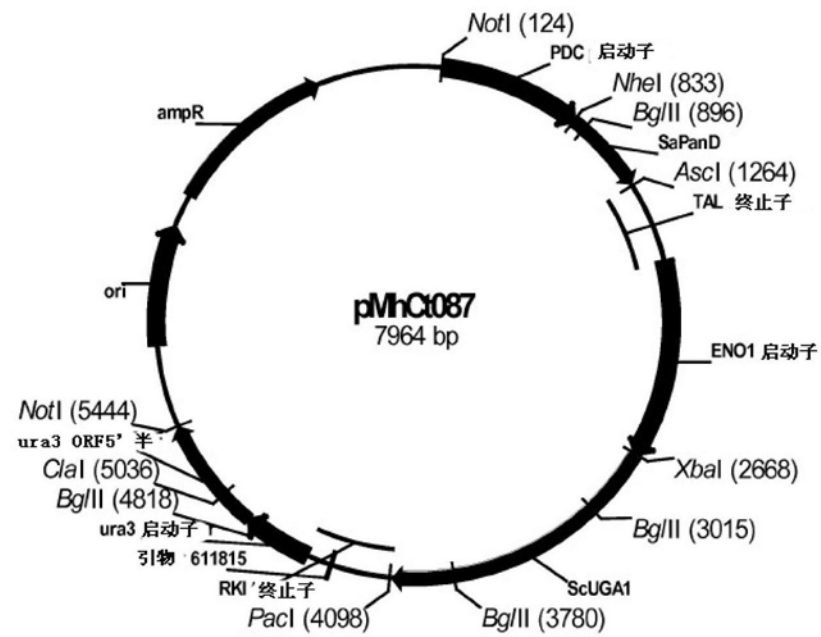


图9

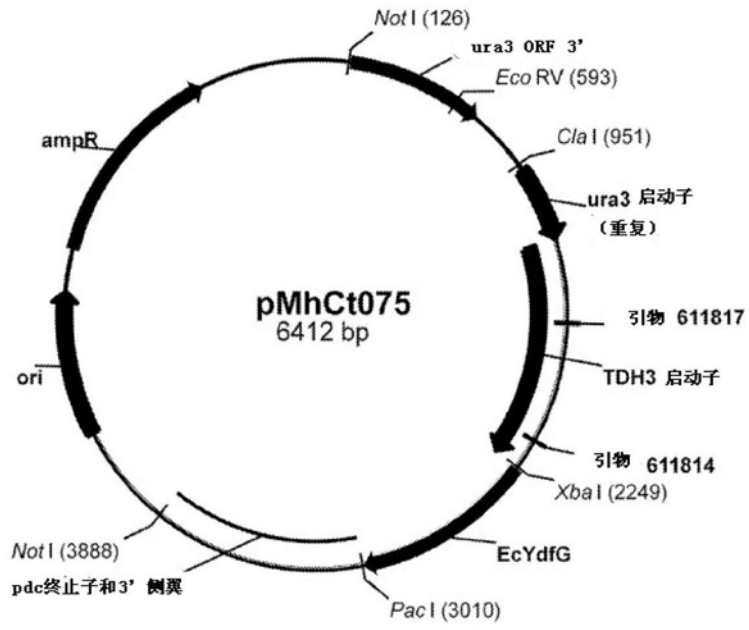


图10

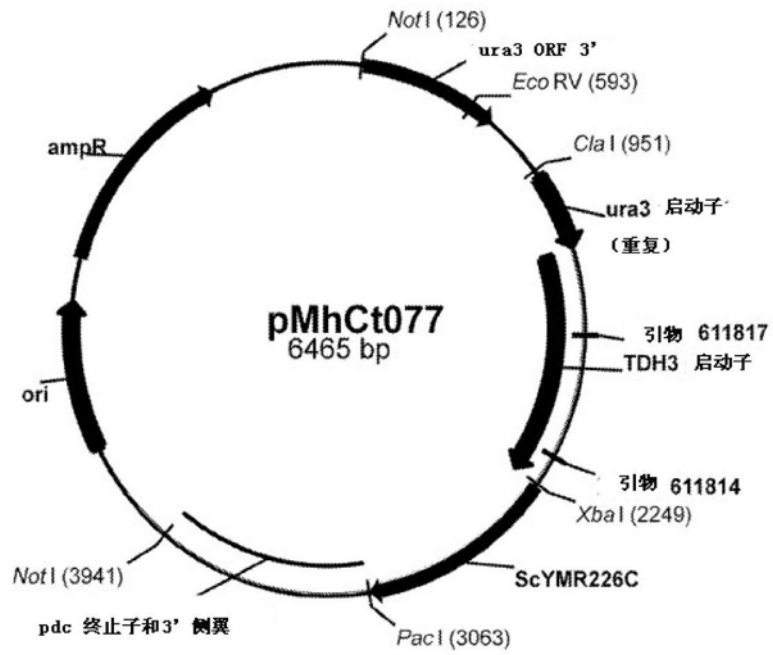


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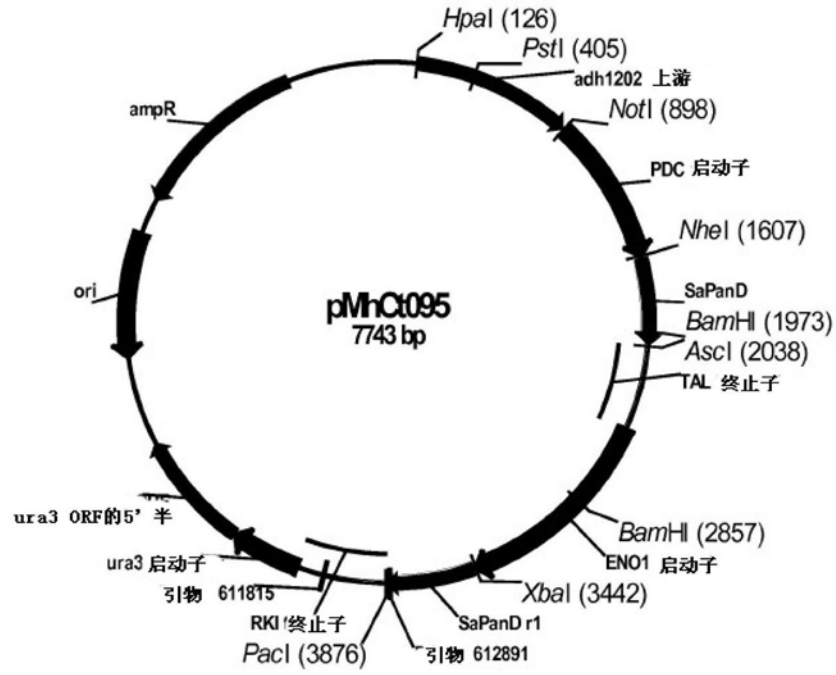


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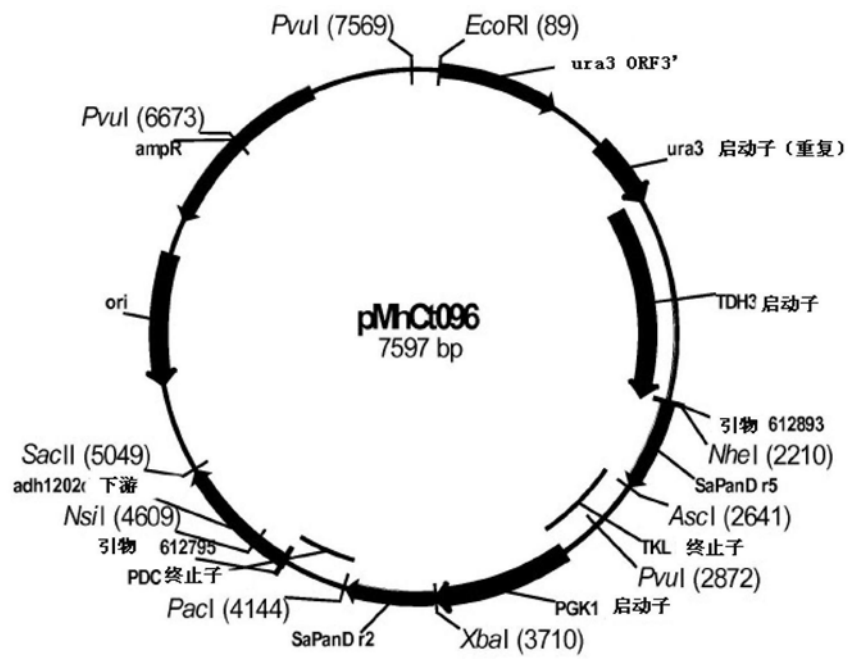


图13

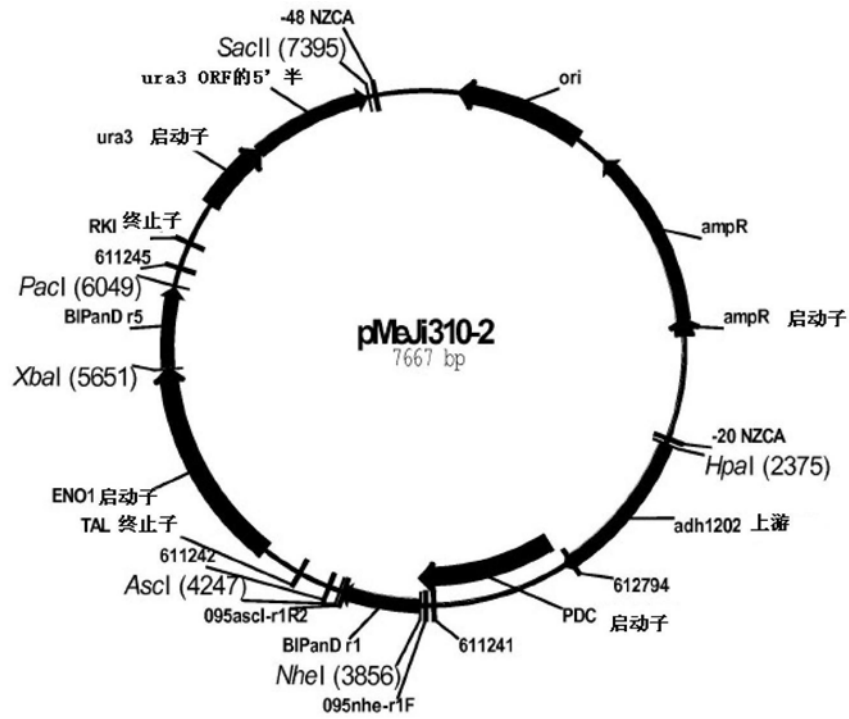


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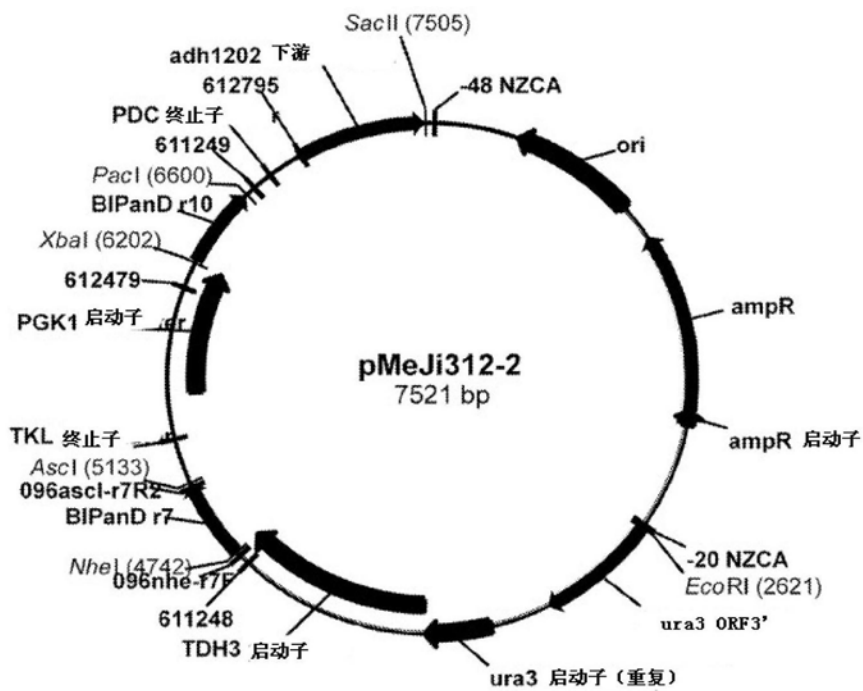


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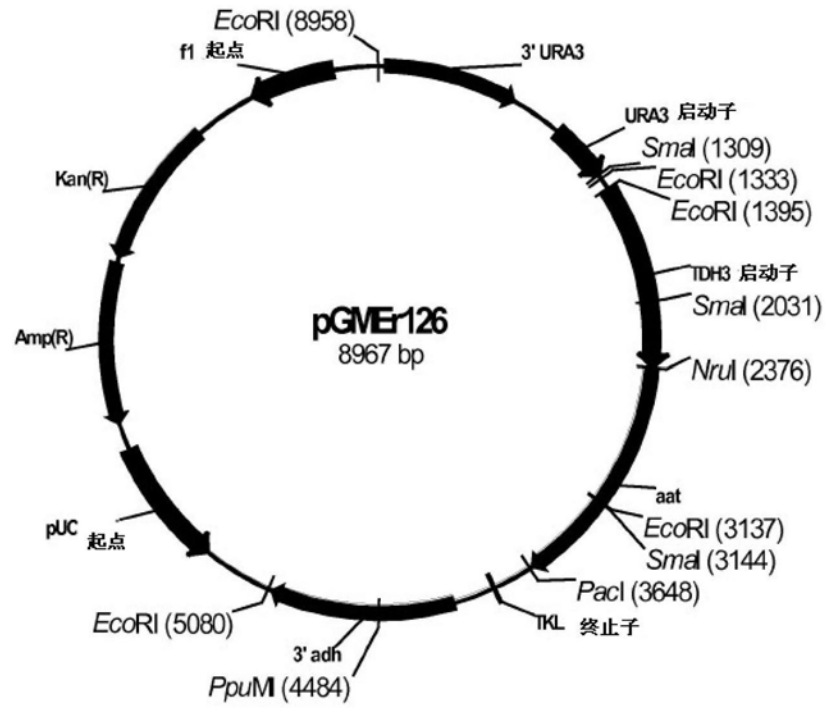


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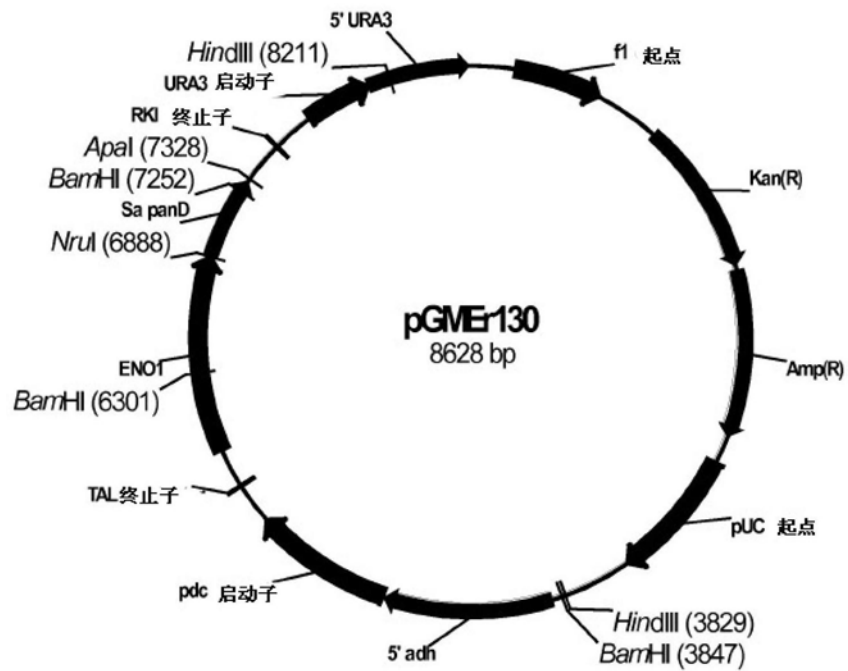


图17

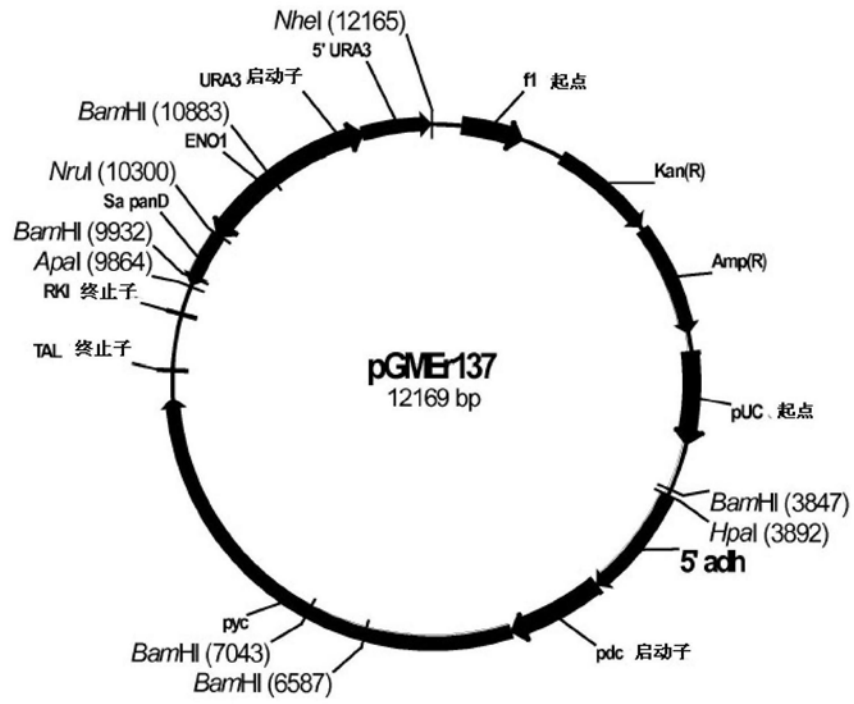


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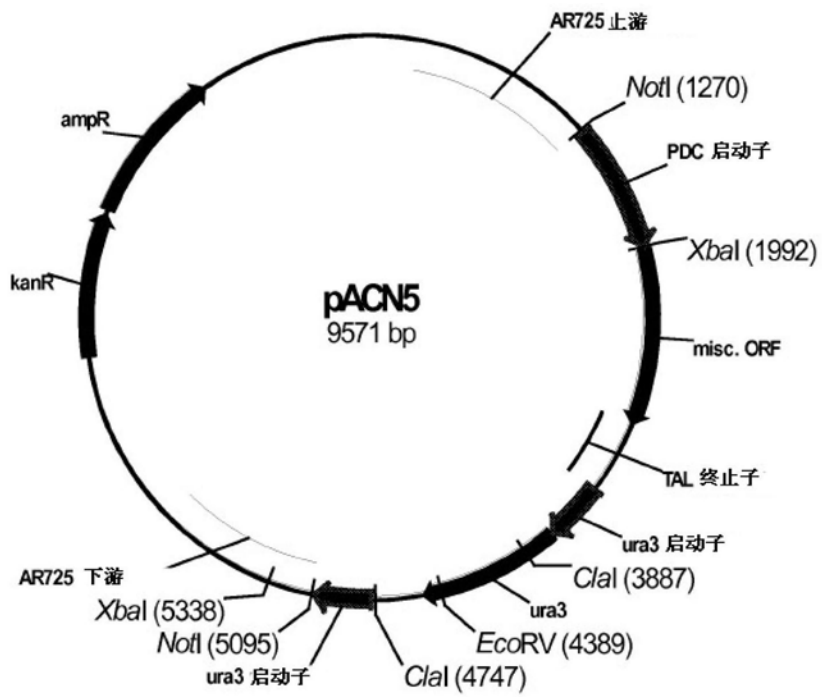


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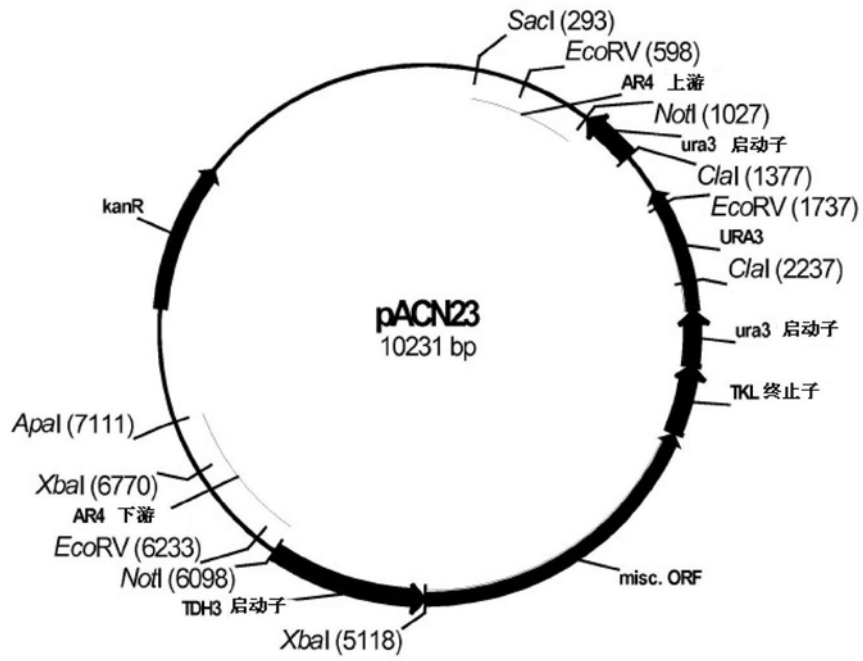


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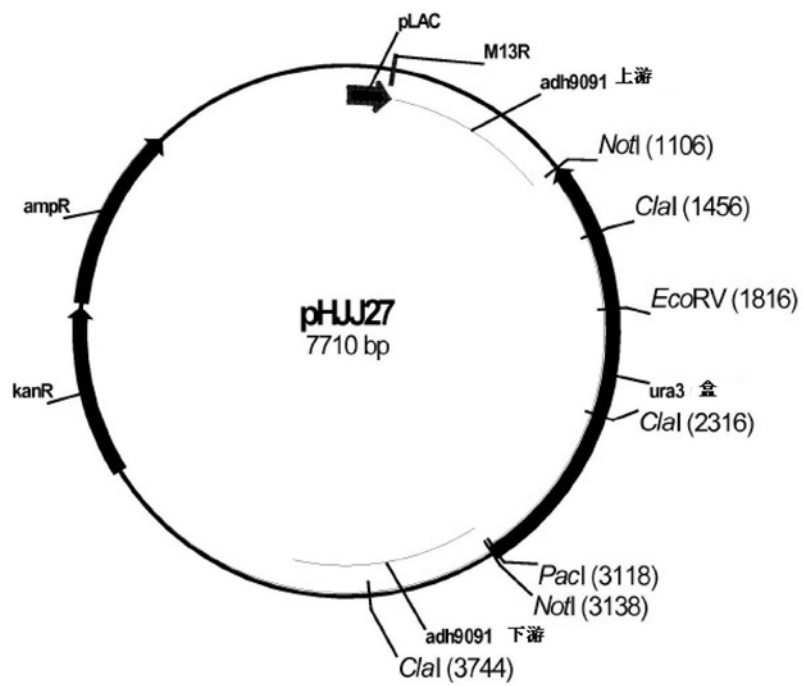


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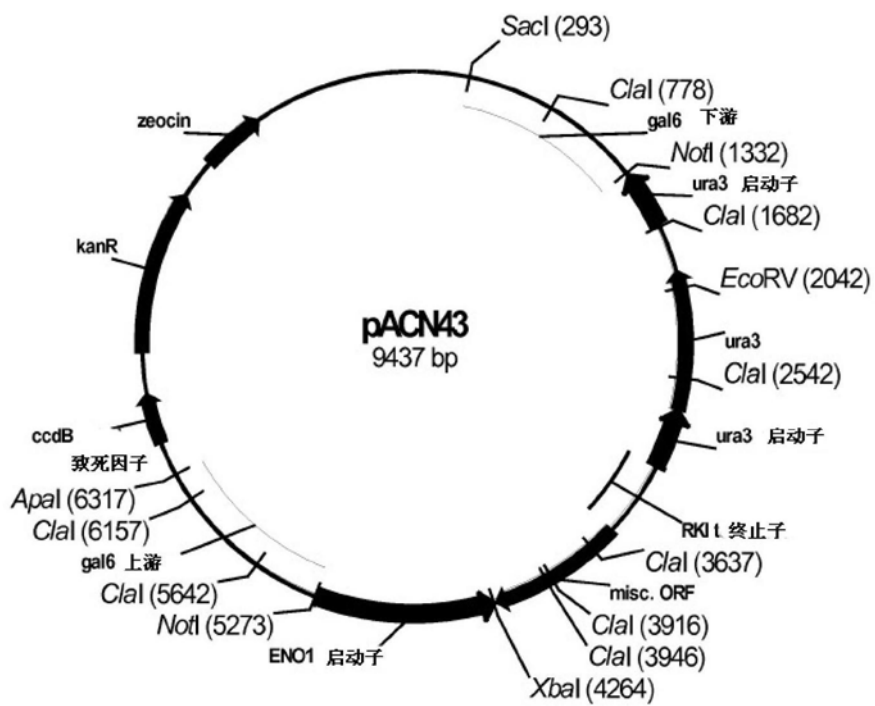


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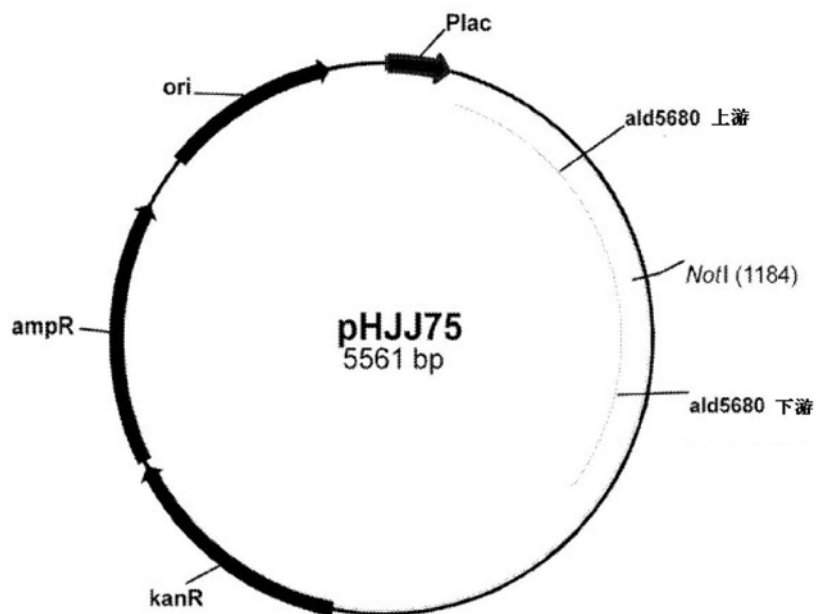


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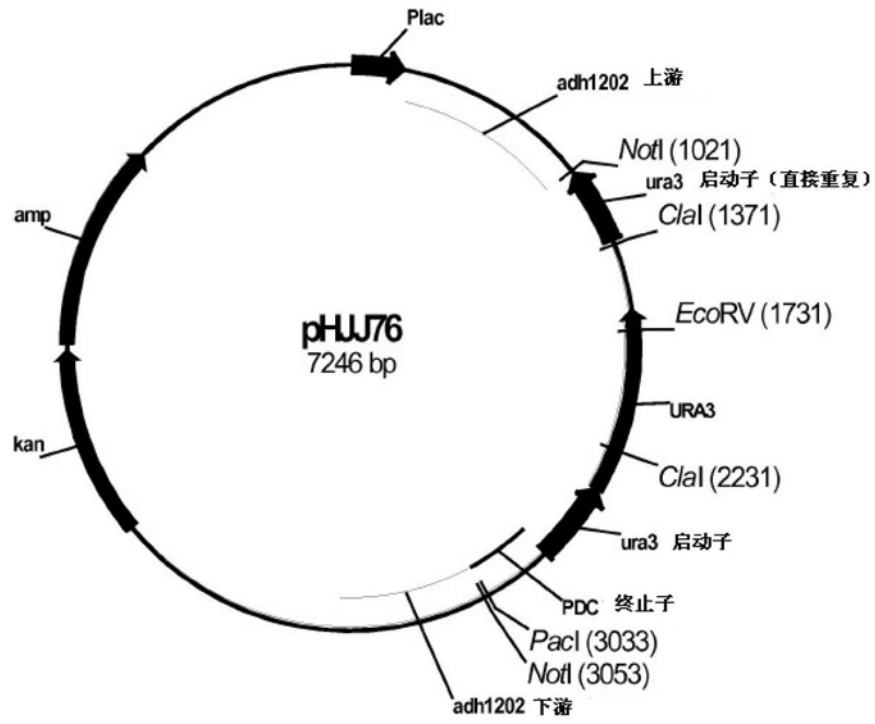


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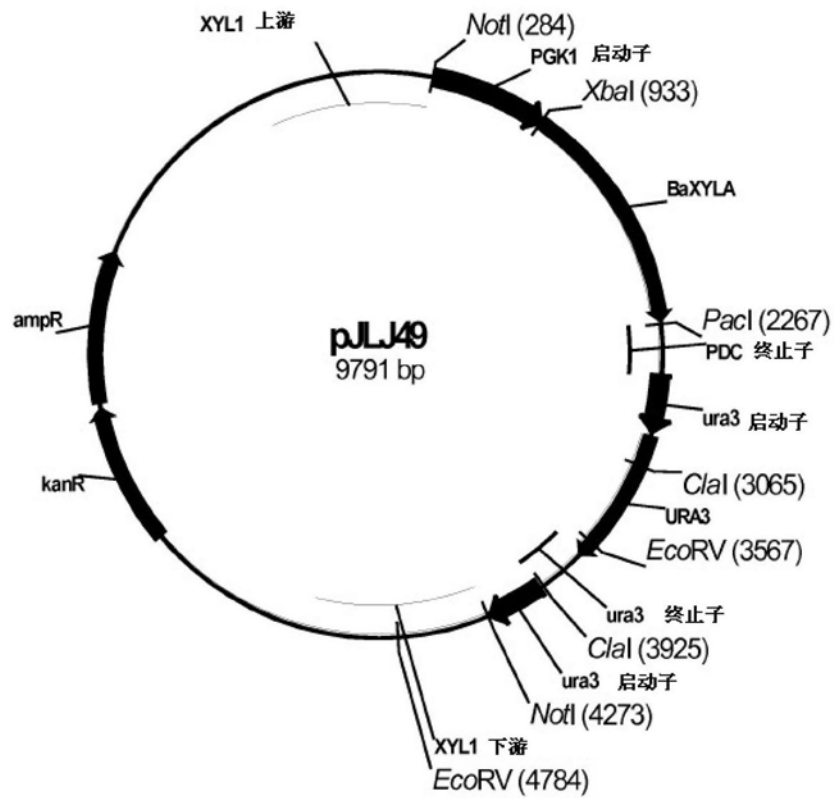


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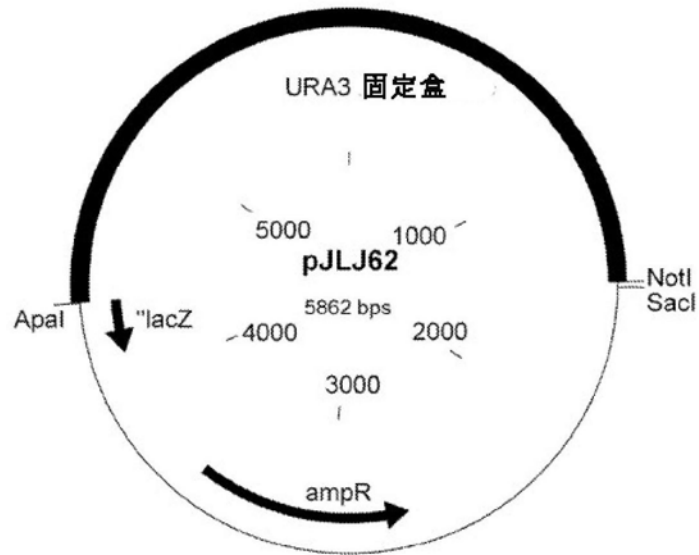


图26

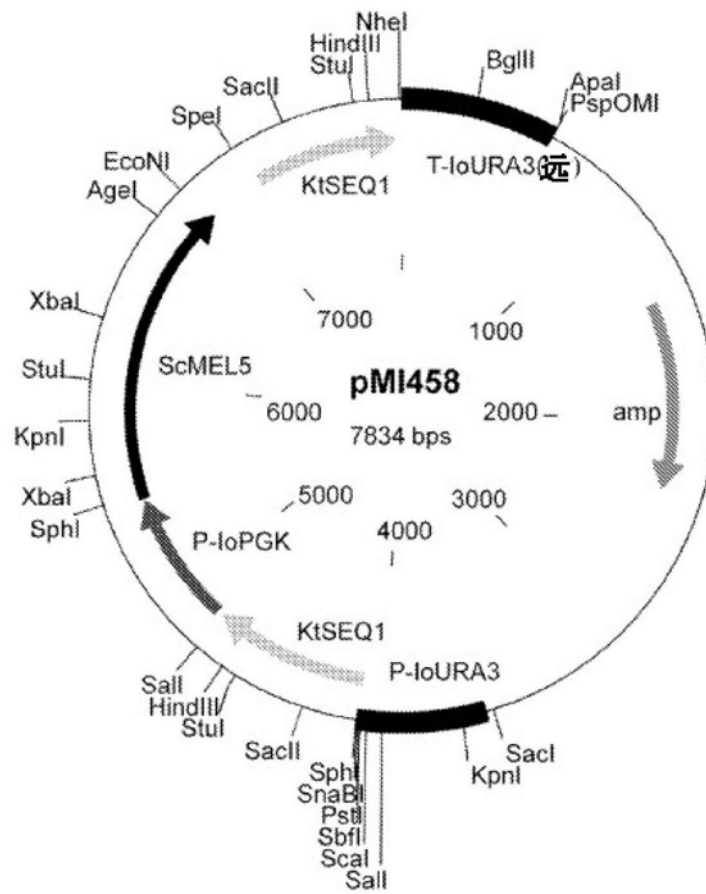


图27

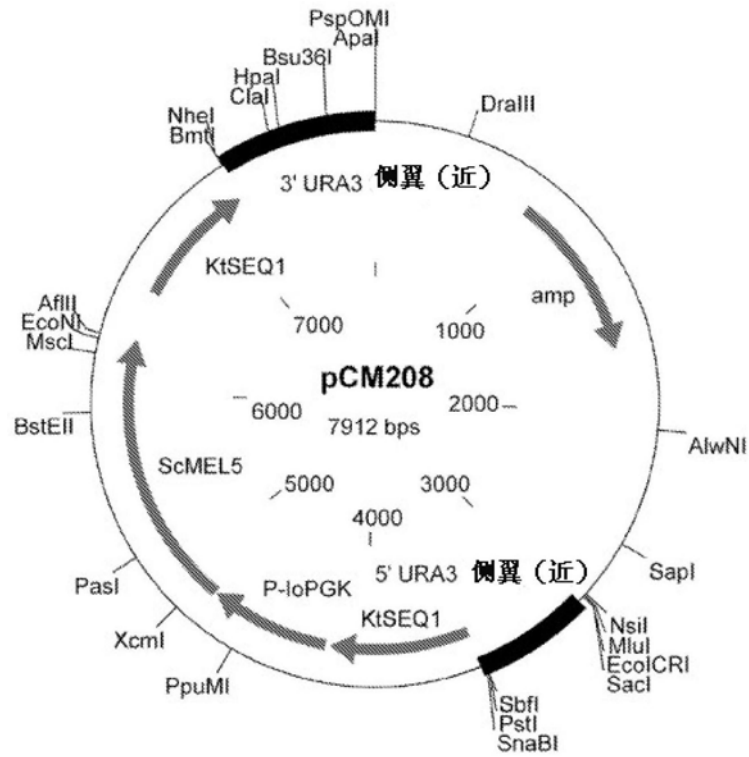


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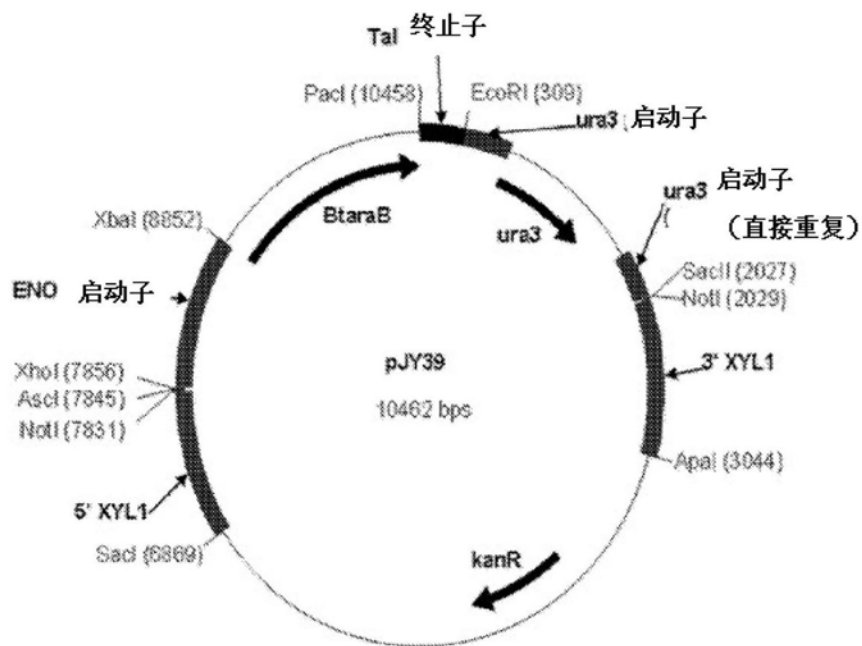


图29

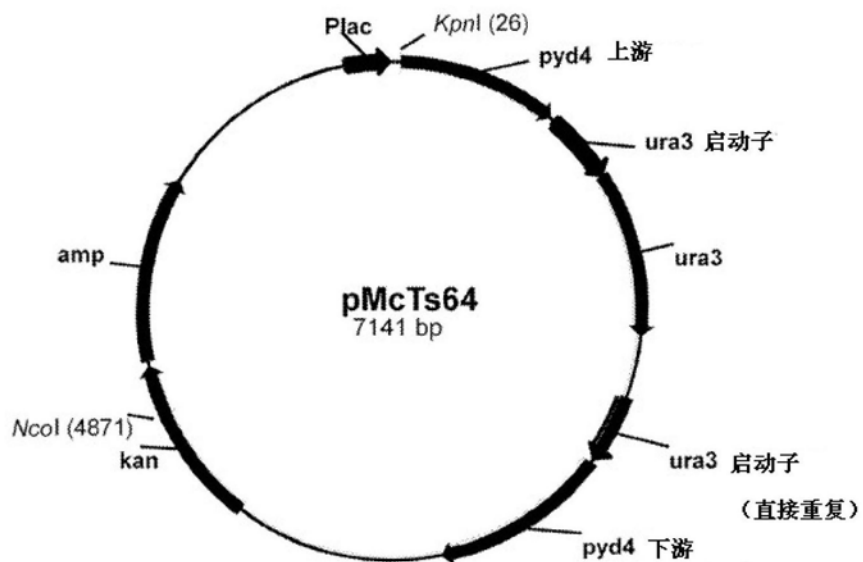


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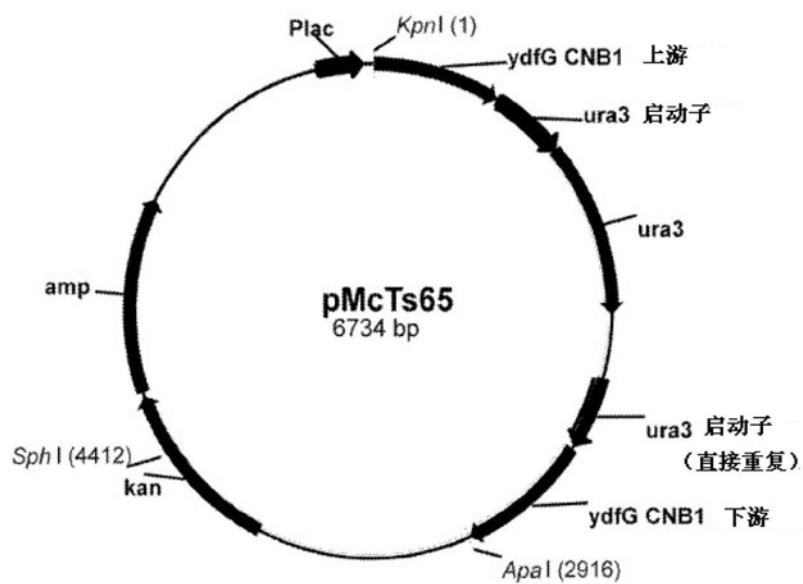


图31

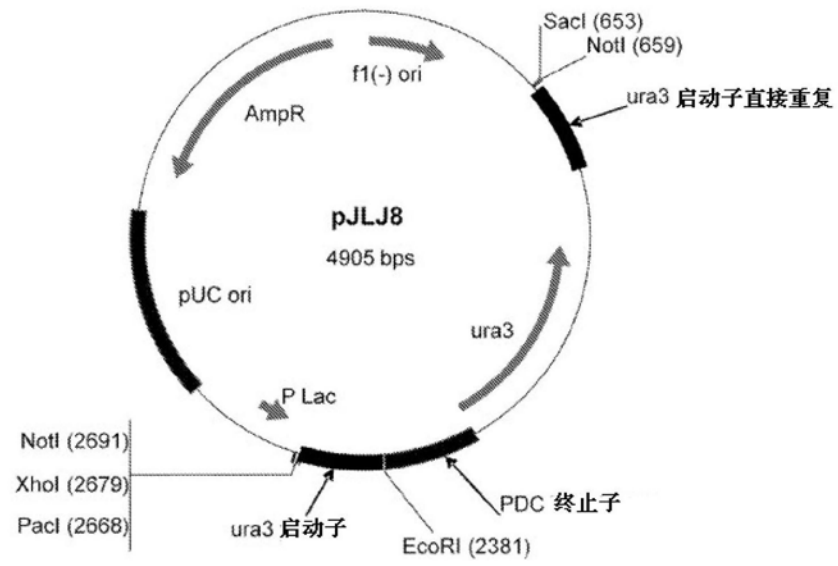


图32