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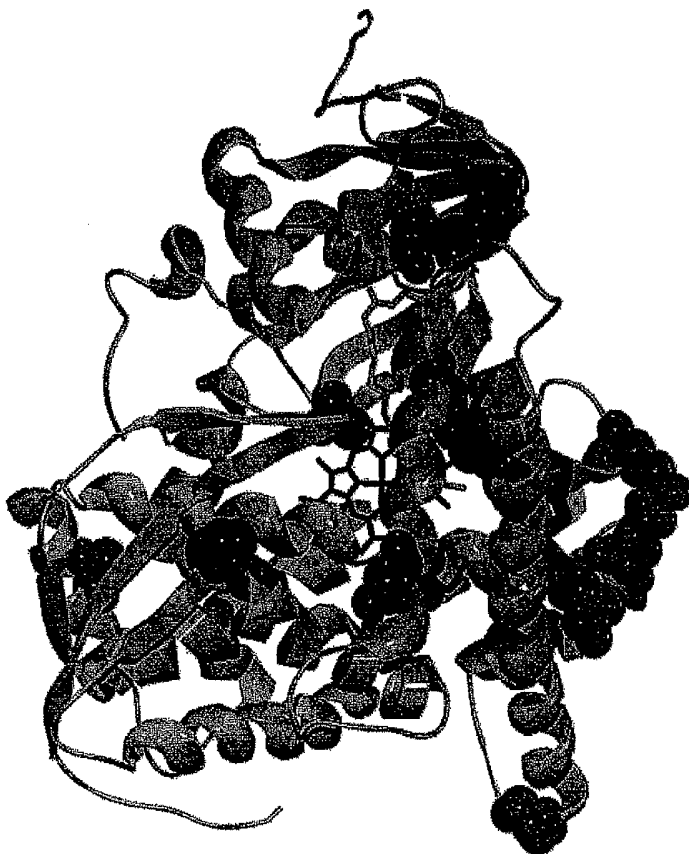
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(54) Title: REGIO- AND ENANTIOSELECTIVE ALKANE HYDROXYLATION WITH MODIFIED CYTOCHROME P450



(57) Abstract: Cytochrome P450 BM-3 from *Bacillus megaterium* was engineered using a combination of directed evolution and site-directed mutagenesis to hydroxylate linear alkanes regio- and enantioselectively using atmospheric dioxygen as an oxidant. Mutant 9-10A-A328V hydroxylates octane primarily at the 2-position to form *S*-2-octanol (40% ee). Another mutant, 1-12G, hydroxylates alkanes larger than hexane primarily at the 2-position, but forms *R*-2-alcohols (40-55% ee). These biocatalysts are highly active for alkane substrates and support thousands of product turnovers. These regio- and enantio-selectivities are retained in whole-cell biotransformations with *E. coli*, where the engineered P450s can be expressed at high levels and the expensive cofactor is supplied endogenously.



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REGIO- AND ENANTIOSELECTIVE ALKANE HYDROXYLATION WITH MODIFIED CYTOCHROME P450

Government Rights

[0001] This invention was made with government support under Grant Number BES 9981770 awarded by National Science Foundation. The government has certain rights in the invention.

Field of the Invention

[0002] The invention relates to variants of cytochrome P450 enzymes that display altered and improved enantio- and regioselectivity in their hydroxylation of alkanes. The invention also relates to novel variants of cytochrome P450 enzymes that are capable of hydroxylating ethanes.

Background of the Invention

[0003] Cytochrome P450s are a large superfamily of enzymes that primarily hydroxylate substrates using dioxygen, although other redox-type reactions, including some reductions, have been reported. One variant, cytochrome P450 BM-3 is found in the bacterium *Bacillus megaterium* (EC 1.14.14.1). This variant, also known as CYP102, is a water-soluble, catalytically self-sufficient P450 containing a monooxygenase domain (64 kD) and a reductase domain (54 kD) in a single polypeptide chain (Narhi and Fulco, Journal of Biological Chemistry, 261 (16): 7160-7169 (1986) and Journal of Biological Chemistry, 262 (14): 6683-6690 (1987); Miura and Fulco, Biochimica et Biophysica ACTA, 388 (3): 305-317 (1975); Ruettinger et al., 1989). The minimum requirements for activity of the BM-3 variant are substrate, dioxygen and the cofactor nicotinamide adenine dinucleotide phosphate (NADPH). Nucleotide and amino acid sequences for P450 BM-3 can be found in, the GenBank database under the accession Nos. J04832 (SEQ ID NO: 1) and P14779 (SEQ ID NO: 2), respectively.

[0004] P450 BM-3 hydroxylates fatty acids of chain lengths between C12 and C18 at subterminal positions, and the regioselectivity of oxygen insertion depends on the chain length (Miura and Fulco, Biochimica et Biophysica ACTA 388 (3): 305-317 (1975); Boddupalli et al., Journal of Biological Chemistry 265 (8): 4233-4239 (1990)). The natural substrates of P450 BM-3 are hydroxylated at their ω -1, ω -2, and ω -3 positions using atmospheric dioxygen and nicotinamide adenine dinucleotide phosphate (NADPH)

as shown in **FIG. 1**. (Ost et al., *Biochemistry*, 40, 13430-13438 (2001)). Substrate is bound and hydroxylated in a hydrophobic binding pocket that is positioned directly above a heme cofactor which is located in its own domain of the protein. A single peptide chain connects this heme domain to the reductase domain of the protein where NADPH is reduced and flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) cofactors are used to transfer electrons to the heme active site for catalysis. The resulting products of the catalysis can be seen in **FIG. 1**. The hydroxylation of myristic acid by cytochrome P450 BM-3 results in 53.6% ω -1 hydroxylation product, 24.5% ω -2 hydroxylation product, and 20.0% ω -3 hydroxylation product. However, none of these substrates of P450 BM-3 are alkanes.

[0005] The optimal chain length of saturated fatty acid substrates for P450 BM-3 is 14-16 carbons, and the enzyme was initially believed to have no activity towards fatty acids smaller than C12 (Miura and Fulco, *Biochimica et Biophysica ACTA*, 388 (3): 305-317 (1975)). The activity of P450 BM-3 on saturated fatty acids follows the order C15 = C16 > C14 > C17 > C13 > C18 > C12 (Oliver et al., *Biochemical Journal*, 327: 537-544 Part 2 (1997)). On the C16 fatty acid, $k_{cat}=81\text{ s}^{-1}$ and $K_m=1.4\times 10^{-6}\text{ M}$ ($k_{cat}/K_m=6.0\times 10^7\text{ M}^{-1}\text{ s}^{-1}$). With the C12 fatty acid, $k_{cat}=26\text{ s}^{-1}$, $K_m=1.36\times 10^{-6}\text{ M}$ and $k_{cat}/K_m=1.9\times 10^5\text{ M}^{-1}\text{ s}^{-1}$ (Oliver et al., *Biochemical Journal*, 327: 537-544 Part 2 (1997)). P450 BM-3 is also known to hydroxylate the corresponding fatty acid amides and alcohols and forms epoxides from unsaturated fatty acids (Miura and Fulco, *Biochimica et Biophysica ACTA*, 388 (3): 305-317 (1975); Capdevila et al., *J. Biol. Chem.* 271:22663-22671 (1996); Graham-Lorence et al., *J. Biol. Chem.*, 272:1127-1135 (1997); Ruettinger and Fulco, *Journal of Biological Chemistry*, 256 (11): 5728-5734 (1981)). The enzyme was reported to be inactive towards alkanes and methyl esters lacking the polar functionality of the natural substrates (Miura and Fulco, *Biochimica et Biophysica ACTA*, 388 (3): 305-317 (1975)). However, there were indications that P450 BM-3 could accept shorter-chain alkanes, although with very low activity (Munro et al., *Biochem Soc Trans*, 21 (4): 411S (1993)). However, wild type BM-3 was ineffective in its ability to hydroxylate alkanes, as the turnover of the enzyme was less than 100 total, and the rate was reported to be at 80 min^{-1} .

[0006] Additionally, relative to other enzymes that hydroxylate linear alkanes, wild type BM-3 was also ineffective. For example, *Pseudomonas oleovorans* is able to oxidize n-alkanes using hydroxylase machinery comprising an integral membrane

oxygenase (omega-hydroxylase), a soluble NADH-dependent reductase and a soluble metalloprotein (rubredoxin) which transfers electrons from the reductase to the hydroxylase (Staijen et al., European Journal of Biochemistry, 267 (7): 1957-1965 (2000)). The omega-hydroxylase has been cloned from *P. oleovorans* into *E. coli*, where it has been expressed and purified (Shanklin et al., Proceedings of the National Academy of Sciences of the United States of America, 94 (7): 2981-2986 (1997)). The specific activity of this omega-hydroxylase for octane (5.2 units/mg hydroxylase (Shanklin et al., Proceedings of the National Academy of Sciences of the United States of America, 94 (7): 2981-2986 (1997)) is about 13 times greater than that of P450 BM-3 (0.4 units/mg enzyme). (The specific activity of the complete *P. oleovorans* system, including the rubredoxin and the reductase, is less than 5.2 units/mg). Thus, wildtype P450 BM-3 was inefficient relative to this (and other) naturally occurring enzymes for alkane hydroxylation.

[0007] While the wild-type P450 was found to be ineffective in alkane hydroxylation, this inefficiency has been overcome in previous work by one of the Inventors. In this work, directed evolution was used to convert wild type BM-3 into a fast, but non-selective, alkane hydroxylase, dubbed "139-3." (Farinas et al., *Adv. Synth. Catal.*, 343, 601-606 (2001); Glieder et al., *Nature Biotech.*, 20, 1135-1139 (2002)). The P450 139-3 was found to have an increased oxidation activity towards alkanes, and was found to be active on alkanes as small as propane. In comparison to the P450 BM-3, the evolved 139-3 protein has 11 amino acid substitutions in its heme domain.

Summary of the Invention

[0008] Cytochrome P450 BM-3 from *Bacillus megaterium* was engineered using a combination of directed evolution and site-directed mutagenesis to hydroxylate linear alkanes regio- and enantioselectively using atmospheric dioxygen as an oxidant. Mutant 9-10A-A328V hydroxylates octane primarily at the 2-position to form *S*-2-octanol (40% ee). Another mutant, 1-12G, hydroxylates alkanes larger than hexane primarily at the 2-position, but forms *R*-2-alcohols (40-55% ee). These biocatalysts are highly active for alkane substrates and support thousands of product turnovers. These regio- and enantio-selectivities are retained in whole-cell biotransformations with *E. coli*, where the engineered P450s can be expressed at high levels and the expensive cofactor is supplied endogenously.

[0009] One embodiment is an isolated mutant P450 enzyme with a first mutation that allows the mutant P450 enzyme to hydroxylate an alkane to produce a first product with a first hydroxylation profile. Without the mutation, the enzyme would hydroxylate an alkane to produce a second product with a different hydroxylation profile.

[0010] In another embodiment a method of making a mutant P450 enzyme having altered selective hydroxylation abilities is provided. The method involves providing a first mutant P450 that is capable of alkane hydroxylation of a substrate to produce a product with a first hydroxylation profile, and modifying at least one amino acid in the first mutant P450 to produce a second mutant P450, wherein said second mutant P450 is capable of alkane hydroxylation of the substrate to produce a product with a second hydroxylation profile.

[0011] In another embodiment, a method of making a P450 enzyme with regioselective alkane hydroxylation activity is provided. The method involves selecting residues to alter to reduce the volume of the active site of a P450 enzyme, replacing small hydrophobic residues in the active site with larger hydrophobic residues to create a mutant P450, testing the resulting mutant P450 for regioselectivity, and repeating the steps if no such P450 mutant is made. These steps result in the creation of a P450 enzyme with regioselective alkane hydroxylation activity.

[0012] In another embodiment, a method of making a P450 enzyme with enantioselective alkane hydroxylation activity is provided. The method involves selecting residues to alter to reduce the volume of the active site of a P450 enzyme, replacing small hydrophobic residues in the active site with larger hydrophobic residues to create a mutant P450, testing the resulting mutant P450 for enantioselectivity, and repeating the steps if no such P450 mutant is made. These steps result in the creation of a P450 enzyme with enantioselective alkane hydroxylation activity.

[0013] In another embodiment, a method for making a P450 enzyme with alkane hydroxylation activity towards ethane is provided. The method involves selecting residues to alter to reduce the volume of the active site of a P450 enzyme, replacing small hydrophobic residues in the active site with larger hydrophobic residues to create a mutant P450, testing the resulting mutant P450 for ethane hydroxylation activity, and repeating the steps if no such P450 mutant is made. These steps result in the creation of a P450 enzyme with ethane hydroxylation activity.

[0014] In another embodiment, an isolated nucleic acid encoding a cytochrome P450 mutant that has a higher capability than the corresponding wild-type cytochrome P450 to oxidize at least one substrate selected from an alkane comprising a carbon-chain of no more than 8 carbons is provided. The wild-type cytochrome P450 comprises an amino acid sequence identical to **SEQ ID NO: 2**, and the cytochrome P450 mutant is at least 80% identical to the sequence in **SEQ ID NO: 2**. The mutant P450 has an amino acid substitution at a residue corresponding to a core residue selected from V78, H236, and E252. Additionally, the mutant has an amino acid substitution at a residue corresponding to a selective hydrolysis residue of **SEQ ID NO: 2** selected from R47C, A82L, K94I, P142S, C205V, S226R, A290V, and A328V.

[0015] In another embodiment, a method of creating a regio- and enantioselective hydroxylation P450 is provided. The method involves performing directed evolution on a P450 to obtain a mutant P450, and modifying an active site of said mutant P450 so as to reduce the size of the active site of the mutant P450. This results in the creation of a regio- and enantioselective hydroxylation P450.

[0016] In another embodiment, an isolated mutant P450 enzyme with regioselective alkane hydroxylation activity that has a higher degree of regiospecificity for the hydroxylation of octane than the wild-type and 139-3 mutant is provided.

[0017] In another embodiment, an isolated mutant P450 enzyme that predominantly hydroxylates a substrate at a first position, having an altered enantiospecificity for alkanes, is provided. The mutant P450 enzyme comprises a selective hydroxylation mutation which allows the mutant P450 enzyme with the selective hydroxylation mutation to predominantly hydroxylate the substrate at a second position.

[0018] In another embodiment, a method of hydroxylating an alkane in a selective manner is provided. The method involves providing an isolated mutant P450 enzyme that has selective hydroxylation activity towards an alkane, and contacting the isolated mutant P450 with said alkane. This allows the isolated mutant P450 hydroxylates said alkane in a selective manner.

Brief Description of the Drawings

[0019] **FIG. 1** is an illustration of a prior art general hydroxylation reaction of myristic acid catalyzed by cytochrome P450s.

[0020] **FIG. 2A** is a bar graph displaying the products of hexane catalysis of various BM-3 mutants.

[0021] **FIG. 2B** is a bar graph displaying the products of heptane catalysis of various BM-3 mutants.

[0022] **FIG. 2C** is a bar graph displaying the products of octane catalysis of various BM-3 mutants.

[0023] **FIG. 2D** is a bar graph displaying the products of nonane catalysis of various BM-3 mutants.

[0024] **FIG. 2E** is a bar graph displaying the products of decane catalysis of various BM-3 mutants.

[0025] **FIG. 3A** is a reaction schematic of the hydroxylation of dimethyl ether to produce formaldehyde.

[0026] **FIG. 3B** is a reaction schematic of Purpald with formaldehyde to form a purple colored adduct upon air oxidation (the first two compounds are colorless).

[0027] **FIG. 4** is an illustration of A328 and A82 in the active site of wild type cytochrome P450 BM-3.

[0028] **FIG. 5A** is a GC/FID analysis of the (-)-menthyl carbonate diastereomers of the 2-octanol produced by 9-10A-A328V BM-3 catalyzed alkane oxidation.

[0029] **FIG. 5B** is a GC/FID analysis of the (-)-menthyl carbonate diastereomers of the 2-octanol produced by 1-12G BM-3 catalyzed alkane oxidation.

[0030] **FIG. 6A** is a GC/FID analysis of the octane hydroxylation product distributions using 9-10A-A328V as a purified protein.

[0031] **FIG. 6B** is a GC/FID analysis of the octane hydroxylation product distributions using 9-10A-A328V from a whole cell.

[0032] **FIG. 7** is a depiction of a P450 molecule with the point mutations for the 1-12G mutant displayed as space filling structures.

Detailed Description of the Preferred Embodiment

[0033] Embodiments of the invention include mutant and altered forms of cytochrome P450 proteins. In one embodiment, mutants of cytochrome P450 BM-3 from *Bacillus megaterium* were engineered using an initial mutant P450 and a combination of directed evolution and site-directed mutagenesis, as discussed more completely below. The starting mutant was a P450 enzyme with 11 mutations that allowed it to hydroxylate alkanes to produce certain amounts of particular enantiomeric and regiospecific alkane products. The starting mutant was then engineered to display altered regio- and

enantioselectivity towards various substrates (*e.g.*, the new mutants have an altered hydroxylation profile). The resulting enzymes were found to be capable of hydroxylating linear alkanes in an altered regio- and enantioselective manners. The turnover number was high. Each of the resulting P450 mutants produced regio- and enantiomeric products in different amounts. Simply put, the products of the initial P450 mutant were hydroxylated at particular positions in particular amounts, and the products of the new P450 mutants were hydroxylated at these particular positions, or in novel positions, in different amounts. Thus, by choosing the proper mutant enzyme, with these described characteristics, one can regio- and enantioselectively hydroxylate substrates in a desired manner. This provides tremendous benefits for specifically hydroxylating target substrates in a predefined manner.

[0034] Embodiments of the invention include mutant P450s with regio- and/or enantioselectivity. For example, one mutant P450, 9-10A-A328V, was found to hydroxylate octane primarily at the 2-position to form *S*-2-octanol (40% ee). Another mutant P450, 1-12G, was found to hydroxylate alkanes larger than hexane primarily at the 2-position, but also formed *R*-2-alcohols (40-55% ee). These two mutants were discovered to have enhanced and altered regio- and enantiospecificity compared to other P450 enzymes, including the original 139-3 mutant from which they were derived.

[0035] Embodiments of the invention further include mutant P450s that are capable of hydroxylating substrates as small as ethane. For example, one of the discovered mutants, termed "1-12G" was advantageously found capable of hydroxylating ethane as a substrate.

[0036] Another embodiment of the invention includes regio- and enantioselective enzymes that are retained in whole-cell biotransformations with *E. coli*, where the engineered P450 enzymes are expressed at high levels, and the required cofactor is supplied endogenously.

[0037] Other embodiments of the invention include methods of using these mutants for the selective hydroxylation of alkanes to product well characterized products in known quantities. As the mutant enzymes produce known products in a known amount, all that is required to create a desired product is to select an appropriate mutant P450 enzyme that catalyzes a reaction to produce a desired enantio- or regiospecific product and then apply the substrate to the enzyme under conditions which allow for

catalysis. Methods of selecting and isolating the desired product from the products created are also known and disclosed herein.

[0038] Embodiments of the invention also include methods of creating mutant P450s that are capable of hydroxylating alkanes in a regio- and enantioselective manner.

Definitions

[0039] Unless defined otherwise herein, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Singleton et al. (2001) Dictionary of Microbiology and Molecular Biology, third edition, John Wiley and Sons (New York), and Hale and Marham (1991) The Harper Collins Dictionary of Biology, Harper Perennial, N.Y. provide one of skill with a general dictionary of many of the terms used in this invention. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described. For purposes of the present invention, the following terms are defined below.

[0040] Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

[0041] The term "identical" in the context of two nucleic acid or polypeptide sequences refers to the residues in the two sequences which are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins or peptides it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g. charge or hydrophobicity) and therefore do not change the functional properties of the molecule. Where sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Means for making this adjustment are well-known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a

score between zero and 1. The scoring of conservative substitutions is calculated, e.g., according to the algorithm of Meyers and Miller, *Computer Applic. Biol. Sci.*, 4: 11-17 (1988) e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, Calif., USA).

[0042] A “mutant” form of a protein or DNA molecule is a form that is altered from its wild-type composition. Mutant proteins typically have amino acid substitutions at one or more positions. Mutant DNA molecules typically have nucleotide substitutions in one or more positions. Mutant forms of a protein or DNA molecule can have the same, or altered, functions in comparison to the wild-type. For ease of discussion, mutants may be referred to by their variation from the single amino acid code from which the mutation arose. For example, in one format the mutant is referred to as XPOSY, where “X” refers to the single letter code of the amino acid in the original sequence, “POS” refers to the position of the mutation in the sequence, and Y refers to the single letter code for the new amino acid appearing at the mutation’s position. For example, V175I would mean that in the original protein, the amino acid at position 175 is a valine (“V”), but in the mutant, the valine is replaced with an isoleucine (“I”).

[0043] As used herein, a “core mutation” is a mutation of a wild-type cytochrome P450 protein that provides the protein with enhanced alkane hydroxylase activity. In one embodiment, the cytochrome P450 protein is a P450 BM-3 protein. It should be realized that any mutation, or set of mutations, that enhance the ability of a cytochrome P450 protein to hydroxylate alkanes are considered core mutations.

[0044] A “core mutant” is a cytochrome P450 protein that has been altered to contain one or more core mutations. In one embodiment, a core mutant is the cytochrome P450 139-3 protein which was derived from mutations of P450 BM-3, and includes V78A, H138Y, T175I, V178I, A184V, H236Q, E252G, R255S, A295T, and L353V core mutations. In one embodiment, those mutations that revert the amino acid sequence back to the wild type sequence for the selective hydroxylation mutations are not considered core mutations. Examples of which are H138, V178, and A295. Thus, in one embodiment, when the core mutations are combined with a selective mutation, the core mutations will consist of V78A, T175I, A184V, H236Q, E252G, R255S, and L353V.

[0045] As used herein, the terms “selective hydroxylation mutations” or “selective mutations” are used interchangeably and refer to mutations that provide a P450 protein with altered regio- or enantio-selectivity towards substrates. A protein having

such mutations is termed a “selective hydroxylation mutant” or a “selective mutant”. In one embodiment, the target substrate of such mutants is an alkane. Examples of types or categories of selective hydroxylation mutants are discussed below, particularly in Tables 1-4. The selective hydroxylation mutations may simply alter the selectivity of the P450 towards a single substrate, or across many substrates. The selective mutation may alter both the selectivity and increase the functional ability of the enzyme, so that more regio or enantioselective end product is produced.

[0046] Non-limiting general examples of selective hydroxylation mutations include cytochrome P450 139-3 proteins having one or more of the following additional mutations: A328V, L75I, F87I and A82L. Non-limiting examples of selective hydroxylation mutants showing altered or enhanced regioselective hydroxylation include cytochrome P450 139-3 proteins having one or more of the following additional mutations: A82I, and T260L. Examples of altered and enhanced regioselective and enantioselective mutants of cytochrome P450 139-3 can be found within Tables 1-4 and **FIGs. 2A-2E**.

[0047] In some embodiments, more than a single mutation may be required in order for the desired result to occur, in such situations, each of the required mutations will be considered as either core, selective, or both, as appropriate. Mutants may also be both enantioselective and regioselective.

[0048] An enzyme is “regioselective” if the product that results from the enzymatic reaction is positioned in an altered position. In one embodiment, the enzyme is an alkane hydroxylase and the hydroxylation reaction results in a hydroxyl group positioned in an altered position. This means that while the original P450 may have created a first amount of product A and a second amount of product B, the regioselective enzyme could produce a third amount of product A and a fourth amount of product B. Thus, while the initial 139-3 mutant could be considered regioselective for particular substrates, the regioselective mutants described herein display different regioselectivity from the 139-3 mutant. In one embodiment, the product of a regioselective hydroxylase contains a hydroxy group at the 2 position predominantly, rather than the 1 or the 3 position. In another embodiment, a distribution of hydroxyl groups in the final product that differs from the product of the wild-type enzyme is sufficient to demonstrate that the enzyme is regioselective. In another embodiment, an increase of 1, 1-2, 2-5, 5-10, 10-20, 20-30, 30-40, 40-50, 50-60, 60-70, 70-80, 80-90, 90-100, 100-200, 200-500 percent or

more in the concentration of one product over another product is sufficient to demonstrate that the enzyme is regioselective. In one embodiment, an enzyme is regioselective when its selectivity is greater than the wild-type or 139-3 mutant P450 regioselectivity as shown in Table 2.

[0049] An enzyme is enantioselective if the hydroxylation reaction of the enzyme results in a high amount of one particular enantiomeric product compared to other possible enantiomeric products. An enzyme that has an altered enantioselectivity means that while the original P450 may have created a first amount of enantiomeric product A and a second amount of enantiomeric product B, the enantioselective enzyme could produce a third amount of enantiomeric product A and a fourth amount of enantiomeric product B. Thus, while the initial 139-3 mutant could be considered enantioselective for particular substrates, the enantioselective mutants described herein display different enantioselectivity from the 139-3 mutant. In one embodiment, the enzyme is a mutant form of the wild-type P450 BM-3 enzyme. In another embodiment, the enzyme is a mutant of cytochrome P450 139-3 enzyme. In one embodiment, the positioning of a hydroxy group in the final product is predominantly at the 2 S position. In another embodiment, the positioning of the hydroxy group in the final product is at the 2 R position predominantly. In yet another embodiment, the distribution of positions in the final product is different from the wild type enzyme in a quantity sufficient to demonstrate that the enzyme is differently enantioselective. In another embodiment, an increase of 1, 1-2, 2-5, 5-10, 10-20, 20-30, 30-40, 40-50, 50-60, 60-70, 70-80, 80-90, 90-100, 100-200, 200-500, or more in the concentration of one product over another product is sufficient to demonstrate that the enzyme is enantioselective.

[0050] In one embodiment, the regioselective and enantioselective mutations are so characterized because of their activity to particular lengths of alkane substrate. In another embodiment, the regio- and enantioselective mutations display improved specificity compared to the 139-3 mutant, either in general or for a particular substrate. For example, while the 139-3 mutation may be regioselective for octanes (61% produced as a 2-alcohol), it is not as regioselective as the 9-10A-A328V mutant (80%) or 1-12G mutant (82%) as described in more detail below. Thus, the terms can be terms of degree, in some embodiments. In one embodiment, any increase in selectivity, either enantio-, regio-, or both, is sufficient as long as it is a measurable increase. For example, it may increase by 1, 1-5, 5-10, 10-20, 20-25, 25-30, 30-32, 32-40, 40-50, 50-100, 100-500

percent, or more, over the wild-type or over the 139-3 BM-3 mutant. Alternatively, the regio- or enantioselectivity may only apply as an absolute; thus, an enzyme will only be regio- or enantioselective if the resulting mutant has a regio- or enantioselectivity where the 139-3 or wild-type BM-3 had none. This could happen in at least two ways. Either the selectivity of the 139-3 mutant is effectively random, or the mutant BM-3 is active on a new substrate, and thus any selectivity would be more than the initial amount of selectivity of zero. The level of activity is also important. Even though the wild type P450 may display a negligible amount of activity on alkanes, such activity, and any resulting regio- or enantioselectivity from the enzyme would not qualify the wild type P450 BM-3 as a regio- or enantioselective P450. This is because some effective, or substantial level of activity of the P450 on alkanes is still required. Such substantial or effective levels are discussed below; however, the wild type rate of catalysis in light of the total turnover is not substantial.

[0051] A “consistent” selective mutant is a selective mutant that displays a consistent bias of selectivity of product produced for more than one starting substrate. Thus, for example, the mutant 9-10A-A328V, discussed below, is a consistent regioselective mutant for hexane, heptane, octane, nonane, and decane, as the products from hexane, heptane, octane, nonane, and decane all result predominantly in the 2-alcohol. In contrast, the 139-3 mutant results in more of the 3-alcohol for heptane substrates, but more of the 2-alcohol for the octane substrate. Thus, in one embodiment, a mutant P450 is a consistent regioselective enzyme if the largest amount of product produced from hexane, heptane and octane is the 2-alcohol. In one embodiment, the majority of each of the products is made at the same position.

[0052] An mutant enzyme or protein is “improved” if its activity is altered or enhanced from its parent composition. For example, an improved P450 139-3 protein is one that contains mutations and also exhibits regioselectivity, across substrates, or for an individual substrate, at a level that is above the regio- or enantioselectivity of the P450 139-3 protein. In one embodiment, the improved activity is for a particular substrate, such as ethane, propane, hexane, heptane, octane, nonane, and/or, decane. In one embodiment, the improved regio- or enantioselectivity provides the mutant with the ability to more effectively produce regio-selective products. For example, an increase of 1, 1-5, 5-10, 10-15, 15-100, 100-300, or more percent more effective than the wild-type or 139-3 mutant in converting the substrate to a single product is an improved regio- or

enantiospecific enzyme. Definitions and distinctions between the 139-3 mutant and the mutants described herein can be found in Tables 2, 3, and 4.

[0053] Another form of an “improved” mutant is one that effectively has a greater ability to efficiently produce a regio- or enantiospecific product. Thus, while the percentage of each product may not be very high, the efficiency of the formation of the products is great enough so that the desired product can be made in substantial amounts. Thus, while a wild type P450 BM-3 may have a product distribution of 17% 2-octanol, 40% 3-octanol, and 43% 4-octanol, it may have a relatively slow catalytic rate of 80 min⁻¹ and less than about 100 total turnovers. Improved mutants include those enzymes that have a higher catalytic rate, and/or higher turnover than the unimproved enzyme.

[0054] “Predominant” denotes the species of product that is the largest percent of the products made. Thus, given 4 products, three of which are equal, the fourth, if greater than the other three would be the predominant product.

[0055] A “hydroxylation profile” of a product is a description of the number and position of hydroxyl groups in the product. Thus, for example, an alkane hydroxylase enzyme typically creates products having a defined hydroxylation profile, such that hydroxyl groups are placed at certain positions on particular percentages of the final reaction products. Altering or modifying the hydroxylation profile of a product means changing the positions, or proportions, of hydroxyl groups in the final reaction products. . In another example, all of the products listed in Table 2 are used for the members of hydroxylation profile. For example, 1-alcohol, 2-alcohol, 3-alcohol, 4-alcohol and ketones may make up the hydroxylation profile. Thus, Table 2 denotes the hydroxylation profiles of each of the mutants for substrates hexane through decane, in this embodiment. A “variant” is distinguished from a mutant. A variant P450 has at least one amino acid or nucleic acid difference from the wild-type P450. A “variant” of a P450 mutant typically contains all of the mutant positions, plus additional changes in the amino or nucleic acid sequence. Thus, while the description “variant” will encompass sequences with changes, a variant of a P450 mutant will still maintain the amino acid or nucleotide changes that define the P450 mutant. For examples, a protein that has one or more core mutations along with additional changes in its DNA or protein sequence is a “variant” core mutant. Similarly, a protein that has one or more selectivity mutations along with additional changes in its DNA or protein sequence is a “variant” selectivity mutant. Variant P450s can vary in the number and the types of residue replacements. In one embodiment, a

variant is any amino acid sequence that is 100-99, 99-98, 98-95, 95-90, 90-80, 80-70, 70-60, 60-50, or 50-30 percent identical to its original amino acid sequence. In another embodiment, a variant is any amino acid sequence that is 100-99, 99-98, 98-95, 95-90, 90-80, 80-70, 70-60, 60-50, or 50-30 percent similar to the amino acid sequence of BM-3 P450. In another embodiment, a variant is any amino acid sequence that is 100-99, 99-98, 98-95, 95-90, 90-80, 80-70, 70-60, 60-50, or 50-30 percent similar to the amino acid sequence of cytochrome P450 139-3. In another embodiment the sequence of comparison is a consensus sequence of known BM-3 proteins. These can apply, as appropriate, to both the amino acid sequence and nucleic acid sequences. In another embodiment, a variant is a nucleic acid sequence that is capable of hybridizing to the disclosed BM-3 sequence under highly stringent or moderately stringent conditions. Highly stringent conditions are those that are at least as stringent as, for example, 4XSSC at 65° C., or 4XSSC and 50% formamide at 42° C.

[0056] The “active site” of the enzyme includes those residues which interact with the substrate in the binding and catalysis of the substrate. As appreciated by one of skill in the art, the precise residues involved in the active site may vary according to the substrate. The active site may be defined through mutagenesis studies or through protein structures which will reveal which part of the enzyme is most closely interacting with the substrate. In one embodiment, the active site is defined as those residues within a certain distance of the bound substrate or where the bound substrate would be positioned. For example, residues within 0-1, 1-2, 2-4, 4-5, 5-6, 6-7, 7-8, or 8-10 angstroms of the bound substrate, or the points at which the substrate will bind, are part of the active site. In another embodiment, the active site includes those residues within a certain distance of the heme group. For example, residues within 0-1, 1-2, 2-4, 4-5, 5-6, 6-7, 7-8, or 8-10 angstroms of the heme group, in the substrate bound or substrate free conformation, are part of the active site. In one embodiment, the active site is defined by the herein discussed crystal structures. In another embodiment, the active site includes the residues or mutants discussed herein which resulted in changes in activity of the P450 enzyme, consistent with a mutation in an active site. In one embodiment, the amino acids of the active site includes the amino acids at positions 75, 78, 82, 87, 88, 260, 328 of CYP102A1, or equivalent positions in variant proteins. In one embodiment, the area of the active site, but not all of the residues, is shown in **FIG. 4**. Amino acid A 328 is

shown on the left, and A82 is displayed in the upper right. Palmitoyl glycine is displayed above the heme and between the two residues.

[0057] An alkane is typically defined as a non-aromatic saturated hydrocarbon with the sequence of $C_nH_{(2n+2)}$. For the purposes of this application and determining whether or not an enzyme is active with a particular substrate, an “alkane” does not encompass fatty acids that are the traditional targets for P450s.

[0058] The proteins of the present invention further include “conservative amino acid substitution variants” (i.e., conservative) of the proteins herein described. As used herein, a conservative variant refers to at least one alteration in the amino acid sequence that does not adversely affect the biological functions of the protein. A substitution, insertion or deletion is said to adversely affect the protein when the altered sequence prevents or disrupts a biological function associated with the protein. For example, the overall charge, structure or hydrophobic-hydrophilic properties of the protein can be altered without adversely affecting a biological activity. Accordingly, the amino acid sequence can often be altered, for example to render the peptide more hydrophobic or hydrophilic, without adversely affecting the biological activities of the protein.

[0059] The proteins of the present invention are preferably in isolated form. As used herein, a protein is said to be isolated when physical, mechanical or chemical methods are employed to remove the protein from cellular constituents that are normally associated with the protein. A skilled artisan can readily employ standard purification methods to obtain an isolated protein.

[0060] Homology or identity at the amino acid or nucleotide level is determined by BLAST (Basic Local Alignment Search Tool) analysis using the algorithm employed by the programs blastp, blastn, blastx, tblastn and tblastx (Karlin et al., (1990) Proc. Natl. Acad. Sci. USA 87, 2264-2268 and Altschul, (1993) J. Mol. Evol. 36, 290-300) which are tailored for sequence similarity searching. The approach used by the BLAST program is to first consider similar segments between a query sequence and a database sequence, then to evaluate the statistical significance of all matches that are identified and finally to summarize only those matches which satisfy a preselected threshold of significance. For a discussion of basic issues in similarity searching of sequence databases (see Altschul et al., (1994) Nature Genetics 6, 119-129). The search parameters for histogram, descriptions, alignments, expect (i.e., the statistical significance

threshold for reporting matches against database sequences), cutoff, matrix and filter are at the default settings. The default scoring matrix used by blastp, blastx, tblastn, and tblastx is the BLOSUM62 matrix (Henikoff et al., (1992) Proc. Natl. Acad. Sci. USA 89, 10915-10919). For blastn, the scoring matrix is set by the ratios of M (i.e., the reward score for a pair of matching residues) to N (i.e., the penalty score for mismatching residues), wherein the default values for M and N are 5 and -4, respectively.

[0061] "Stringent conditions" are those that (1) employ low ionic strength and high temperature for washing, for example, 0.5 M sodium phosphate buffer at pH 7.2, 1 mM EDTA at pH 8.0 in 7% SDS at either 65° C. or 55° C., or (2) employ during hybridization a denaturing agent such as formamide, for example, 50% formamide with 0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.05 M sodium phosphate buffer at pH 6.5 with 0.75 M NaCl, 0.075 M sodium citrate at 42° C. Another example is use of 50% formamide, 5.times.SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate at pH 6.8, 0.1% sodium pyrophosphate, 5X Denhardt's solution, sonicated salmon sperm DNA (50 µg/ml), 0.1% SDS and 10% dextran sulfate at 55° C., with washes at 55° C. in 0.2x.SSC and 0.1% SDS. A skilled artisan can readily determine and vary the stringency conditions appropriately to obtain a clear and detectable hybridization signal.

[0062] As used herein, a nucleic acid molecule is said to be "isolated" when the nucleic acid molecule is substantially separated from contaminant nucleic acid encoding other polypeptides from the source of nucleic acid.

[0063] Embodiments of the present invention further include fragments of any one of the encoding nucleic acids molecules. As used herein, a fragment of an encoding nucleic acid molecule refers to a small portion of the entire protein coding sequence. The size of the fragment will be determined by the intended use. For example, if the fragment is chosen so as to encode an active portion of the protein, the fragment will need to be large enough to encode the functional region(s) of the protein. For instance, fragments of the invention include fragments of DNA encoding mutant P450 BM-3 proteins that maintain altered or enhanced enantioselectivity and regioselectivity.

[0064] The encoding nucleic acid molecules of the present invention may further be modified so as to contain a detectable label for diagnostic and probe purposes. A variety of such labels are known in the art and can readily be employed with the encoding molecules herein described. Suitable labels include, but are not limited to,

fluorescent-labeled, biotin-labeled, radio-labeled nucleotides and the like. A skilled artisan can employ any of the art known labels to obtain a labeled encoding nucleic acid molecule.

A. Directed Evolution in General

[0065] One technique to improve the alkane-oxidation ability of wild-type or parent cytochrome P450 enzymes, including P450 BM-3, is directed evolution. General methods for generating libraries and isolating and identifying improved proteins according to the invention using directed evolution are described briefly below. More extensive descriptions can be found in, for example, Arnold (Accounts of Chemical Research, 31(3): 125-131 (1998)); U.S. Pat. Nos. 5,741,691; 5,811,238; 5,605,793 and 5,830,721; and International Applications WO 98/42832, WO 95/22625, WO 97/20078, WO 95/41653 and WO 98/27230.

[0066] The basic steps in directed evolution are (1) the generation of mutant libraries of polynucleotides from a parent or wild-type sequence; (2) (optionally) expression of the mutant polynucleotides to create a mutant polypeptide library; (3) screening/selecting the polynucleotide or polypeptide library for a desired property of a polynucleotide or polypeptide; and (4) selecting mutants which possess a higher level of the desired property; and (5) repeating steps (1) to (5) using the selected mutant(s) as parent(s) until one or more mutants displaying a sufficient level of the desired activity have been obtained. The property can be, but is not limited to, alkane oxidation capability and enantio- and regiospecificity.

[0067] The parent protein or enzyme to be evolved can be a wild-type protein or enzyme, or a variant or mutant. The parent polynucleotide can be retrieved from any suitable commercial or non-commercial source. The parent polynucleotide can correspond to a full-length gene or a partial gene, and may be of various lengths. Preferably the parent polynucleotide is from 50 to 50,000 base pairs. It is contemplated that entire vectors containing the nucleic acid encoding the parent protein of interest may be used in the methods of this invention.

[0068] Any method can be used for generating mutations in the parent polynucleotide sequence to provide a library of evolved polynucleotides, including error-prone polymerase chain reaction, cassette mutagenesis (in which the specific region optimized is replaced with a synthetically mutagenized oligonucleotide), oligonucleotide-directed mutagenesis, parallel PCR (which uses a large number of different PCR reactions

that occur in parallel in the same vessel, such that the product of one reaction primes the product of another reaction), random mutagenesis (e.g., by random fragmentation and reassembly of the fragments by mutual priming); site-specific mutations (introduced into long sequences by random fragmentation of the template followed by reassembly of the fragments in the presence of mutagenic oligonucleotides); parallel PCR (e.g., recombination on a pool of DNA sequences); sexual PCR; and chemical mutagenesis (e.g., by sodium bisulfite, nitrous acid, hydroxylamine, hydrazine, formic acid, or by adding nitrosoguanidine, 5-bromouracil, 2-aminopurine, and acridine to the PCR reaction in place of the nucleotide precursor; or by adding intercalating agents such as proflavine, acriflavine, quinacrine); irradiation (X-rays or ultraviolet light, and/or subjecting the polynucleotide to propagation in a host cell that is deficient in normal DNA damage repair function); or DNA shuffling (e.g., in vitro or in vivo homologous recombination of pools of nucleic acid fragments or polynucleotides). Any one of these techniques can also be employed under low-fidelity polymerization conditions to introduce a low level of point mutations randomly over a long sequence, or to mutagenize a mixture of fragments of unknown sequence.

[0069] Once the evolved polynucleotide molecules are generated they can be cloned into a suitable vector selected by the skilled artisan according to methods well known in the art. If a mixed population of the specific nucleic acid sequence is cloned into a vector it can be clonally amplified by inserting each vector into a host cell and allowing the host cell to amplify the vector and/or express the mutant or variant protein or enzyme sequence. Any one of the well-known procedures for inserting expression vectors into a cell for expression of a given peptide or protein may be utilized. Suitable vectors include plasmids and viruses, particularly those known to be compatible with host cells that express oxidation enzymes or oxygenases. *E. coli* is one exemplary preferred host cell. Other exemplary cells include other bacterial cells such as *Bacillus* and *Pseudomonas*, archaeobacteria, yeast cells such as *Saccharomyces cerevisiae*, insect cells and filamentous fungi such as any species of *Aspergillus* cells. For some applications, plant, human, mammalian or other animal cells may be preferred. Suitable host cells may be transformed, transfected or infected as appropriate by any suitable method including electroporation, CaCl₂ mediated DNA uptake, fungal infection, microinjection, microprojectile transformation, viral infection, or other established methods.

[0070] The mixed population of polynucleotides or proteins may then be tested or screened to identify the recombinant polynucleotide or protein having a higher level of the desired activity or property. The mutation/screening steps can then be repeated until the selected mutant(s) display a sufficient level of the desired activity or property. Briefly, after the sufficient level has been achieved, each selected protein or enzyme can be readily isolated and purified from the expression system, or media, if secreted. It can then be subjected to assays designed to further test functional activity of the particular protein or enzyme. Such experiments for various proteins are well known in the art, and are described below and in the Examples below.

[0071] The evolved enzymes can be used in biocatalytic processes for, e.g., alkane hydroxylation. The enzyme mutants can be used in biocatalytic processes for production of chemicals from hydrocarbons. Furthermore, the enzyme mutants can be used in live cells or in dead cells, or it can be partially purified from the cells. One preferred process would be to use the enzyme mutants in any of these forms (except live cells) in an organic solvent, in liquid or even gas phase, or for example in a super-critical fluid like CO₂. The organic solvent would dissolve high concentrations of the non-polar substrate, so that the enzyme could work efficiently on that substrate.

[0072] Recycling the cofactor can present difficulties for such a process. However, cofactor recycling methods well known in the art can be applied. For example, an enzyme capable of regenerating the cofactor, using a second substrate can be used. Alternatively, the enzyme can be used in living cells, and the cofactor recycling can be accomplished by feeding the cells the appropriate substrate(s). The NADPH and oxygen can also be replaced by a peroxide, for example hydrogen peroxide, butyl peroxide or cumene peroxide, or by another oxidant. Mutations that enhance the efficiency of peroxide-based oxidation by BM-3 or other cytochrome P450 enzymes can serve to enhance the peroxide shunt activity of the enzyme mutants described here. The mutations described here can be combined with such mutations, for example, and tested for their contributions to peroxide-driven alkane and alkene oxidation.

Screening Assays

[0073] In a broad aspect, a screening method to detect oxidation comprises combining, in any order, substrate, oxygen donor, and test oxidation enzyme. The assay components can be placed in or on any suitable medium, carrier or support, and are combined under predetermined conditions. The conditions are chosen to facilitate, suit,

promote, investigate or test the oxidation of the substrate by the oxygen donor in the presence of the test enzyme, and may be modified during the assay. The amount of oxidation product, i.e., oxidized substrate, is thereafter detected using a suitable method. Further, as described in WO 99/60096, a screening method can comprise a coupling enzyme such as horseradish peroxidase to enable or enhance the detection of successful oxidation. In some embodiments, one or more cofactors, coenzymes and additional or ancillary proteins may be used to promote or enhance activity of the test oxidation enzyme, coupling enzyme, or both.

[0074] In one embodiment, it is not necessary to recover test enzyme from host cells that express them, because the host cells are used in the screening method, in a so-called "whole cell" assay. In this embodiment, substrate, oxygen donor, and other components of the screening assay, are supplied to the transformed host cells or to the growth media or support for the cells. In one form of this approach, the test enzyme is expressed and retained inside the host cell, and the substrate, oxygen donor, and other components are added to the solution or plate containing the cells and cross the cell membrane and enter the cell. Alternatively, the host cells can be lysed so that all intracellular components, including any recombinantly expressed intracellular enzyme mutant, can be in direct contact with any added substrate, oxygen donor, and other components.

[0075] Resulting oxygenated products are detected by suitable means. For example, the oxidation product may be a colored, luminescent, or fluorescent compound, so that transformed host cells that produce more active oxidation enzymes "light up" in the assay and can be readily identified, and can be distinguished or separated from cells which do not "light up" as much and which produce inactive enzymes, less active enzymes, or no enzymes. A fluorescent reaction product can be achieved, for example, by using a coupling enzyme, such as laccase or horseradish peroxidase, which forms fluorescent polymers from the oxidation product. A chemiluminescent agent, such as luminol, can also be used to enhance the detectability of the luminescent reaction product, such as the fluorescent polymers. Detectable reaction products also include color changes, such as colored materials that absorb measurable visible or UV light.

[0076] To improve the activity of P450 BM-3 or other cytochrome P450 enzymes towards alkanes by directed evolution, a rapid, reproducible screen that is sensitive to small changes (<2-fold) in activity is desirable (Arnold, Accounts of

Chemical Research, 31(3): 125-131 March (1998)). Therefore, an alkane analog such as p-nitrophenoxy octane (8-pnpane), can be prepared that generates yellow color upon hydroxylation. This "surrogate" substrate with a C8 backbone and a p-nitrophenyl moiety is an analog of octane, and allows use of a colorimetric assay to conveniently screen large numbers of P450 BM-3 or other cytochrome P450 mutants for increased hydroxylation activity in microtiter plates (Schwaneberg et al., 1999; Schwaneberg et al., 2001). Hydroxylation of 8-pnpane generates an unstable hemiacetal which dissociates to form (yellow) p-nitrophenolate and the corresponding aldehyde. The hydroxylation kinetics of hundreds of mutants can then be monitored simultaneously in the wells of a microtiter plate using a plate reader (Schwaneberg et al., 2001). This method is particularly suitable for detecting P450 mutants with improved alkane-oxidation activity.

[0077] Enzyme mutants displaying improved levels of the desired activity or property in the screening assay(s) can then be expressed in higher amounts, retrieved, optionally purified, and further tested for the activity or property of interest.

Activity Assays

[0078] The cytochrome P450 mutants created by directed evolution and selected for a desired property or activity can be further evaluated by any suitable test or tests known in the art to be useful to assess the property or activity. For example, the enzyme mutants can be evaluated for their alkane-oxidation capability, regio- and enantiomeric specificity.

[0079] An assay for alkane-oxidation capability essentially comprises contacting the cytochrome P450 mutant with a specific amount of alkane substrate, or a substrate which is an alkane analog such as 8-pnpane, in the presence of an oxygen donor, and any other components (e.g., NADPH) that are necessary or desirable to include in the reaction mixture, such as NADPH and buffering agents. After a sufficient incubation time, the amount of oxidation product formed, or, alternatively, the amount of intact non-oxidized substrate remaining, is estimated. For example, the amount of oxidation product and/or substrate could be evaluated chromatographically, e.g., by mass spectroscopy (MS) coupled to high-pressure liquid chromatography (HPLC) or gas chromatography (GC) columns, or spectrophotometrically, by measuring the absorbance of either compound at a suitable wavelength. By varying specific parameters in such assays, the Michaelis-Menten constant (K_m) and/or maximum catalytic rate (V_{max}) can be derived for each substrate as is well known in the art. Preferred substrates include, but are not limited to,

methane, ethane, propane, butane, pentane, hexane, heptane, octane, and cyclohexane. In addition, HPLC and GC techniques, particularly when coupled to MS, can be used to determine not only the amount of oxidized product, but also the identity of the product. For example, octane can be oxidized to octanol where the hydroxyl group is positioned on any of the carbon atoms in the octanol molecule. The substrates may also be used to determine regio- and enantiomeric specificity of the P450 enzymes.

[0080] Alkene-oxidation can be evaluated by methods similar to those described for alkanes, simply by replacing an alkane with the corresponding alkene, and designing an assay which promotes and detects epoxide formation of the alkene. For example, an assay which detects NADPH consumption may be used. Preferred alkene substrates include ethene, propene, butene, pentene, hexene, heptene, and octene.

B. Directed Evolution for the Creation of BM-3 139-3 and the Analysis of the 139-3 Mutant

[0081] Five rounds of directed evolution starting with wild type cytochrome P450 BM-3 as the parent yielded the alkane hydroxylase 139-3. (Farinas et al., *Adv. Synth. Catal.*, 343, 601-606 (2001), see also, U.S. Pat. Publication 20030100744, Filed July 22, 2002 to Farinas, et al). In each round, a library of randomly-mutated BM-3 enzymes was screened for octane hydroxylation activity on the "surrogate" substrate *p*-nitrophenyl octyl ether. Hydroxylation of this substrate at the carbon atom containing the *p*-nitrophenoxy moiety resulted in the formation of *p*-nitrophenolate, which was used for colorimetric identification of active mutants. Active mutants were then tested for octane hydroxylation activity, and the most active ones were used as parents for subsequent rounds of evolution. In some cases, several active mutants were isolated from a single round of screening and recombined using DNA shuffling to obtain the parent for the next round. In these and the evolution experiments described in this work, random mutagenesis and recombination was applied only to the heme domain (residues 1-429) of the P450; the reductase domain was left untouched. As discussed above, the alkane hydroxylase mutant, 139-3, contained 11 amino acid substitutions in its heme domain.

[0082] The fairly even distribution of 139-3's hydroxylated alkane products, however, suggested that its active site was rather large and that its alkane substrates are "loosely" bound. This is consistent with the fact that the surrogate substrate used to select these mutants is quite large relative to octane, the intended substrate. Additionally, the

one active site mutation, V78A, likely results in an increase in the size of the substrate binding site due to the decreased size of the amino acid side chain at that residue.

[0083] The 139-3 P450 BM-3 mutant exhibited significant activity on propane, despite the fact that small alkane substrates were not used to screen the mutant libraries in the directed evolution experiments. Because of this, as well as other factors, it was reasoned that decreasing the volume of the active site of the 139-3 mutant, using a combination of directed evolution and site-directed mutagenesis might further enhance this activity. Additionally, engineering the active site in this way might also confer regioselectivity towards longer alkanes — if the substrates are bound more tightly, fewer hydroxylation products may be possible. Of course this involves a delicate balance of decreasing the size of the active site as any alteration could result in making the active site so small as to prevent binding of the substrate. Additionally, the alterations could also prevent any activity by the enzyme as well.

[0084] The 11 mutations in the 139-3 P450 BM-3 are core mutations. In one embodiment, all eleven of these mutations are preferably in the final P450 mutant in order for it to catalyze the oxidation of an alkane. In another embodiment, only 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 of these mutations must be present. In another embodiment, the mutant P450 protein only includes eight core mutations, as follows: V78A, T175I, A184V, H236Q, E252G, R255S, and L353V. In another embodiment, the aforementioned mutations are not included, however similar amino acid or amino acids are preferably included so that alkane activity is conferred upon the enzyme. For example, each core mutation might be replaced with a conservative variant of the core mutant amino acid. Without intending to be limited by theory, it is possible that the change in these amino acids result in the 139-3 P450 BM-3 being able to catalyze the hydroxylation of alkanes. In one embodiment, only V78A is required as a core mutation. Thus, an enzyme with the V78A mutation will effectively hydroxylate alkanes. As discussed further below, additional mutations give the P450 mutant the ability to hydroxylate the alkane enantio- and regioselectively.

C. Mutation of the 139-3 P450 Enzyme for Regio- and Enantioselective Mutants

[0085] Initially, two rounds of directed evolution was performed using 139-3 as the parent and propane as the screening substrate. A BM-3-catalyzed hydroxylation reaction results in the oxidation of one equivalent of NADPH for each equivalent of hydroxylated substrate. Using a 96-well plate reader, the rate of NADPH oxidation in the

presence of BM-3-containing cell lysate and substrate was monitored spectrophotometrically at 340 nm to quickly identify mutants with high activity towards any given substrate. (Glieder et al., *Nature Biotech.*, 20:1135-1139 (2002)).

1. 1st round

[0086] A library of P450 BM-3 mutants was generated by performing a first round of directed evolution, wherein P450 BM-3 mutant 139-3 was combined with 15 other unsequenced P450 mutants of the same generation that also exhibited increased activity towards *p*-nitrophenyl octyl ether and octane. The library was transformed into *E. coli* (DH5 α) competent cells, over-expressed, and lysed according to standard protocols developed by our laboratory. Aliquots of the cell-free extract of each mutant were transferred to 96-well plates where NADPH consumption was monitored in the presence of propane. Mutants identified from the screening process were then grown up and purified for comparative analysis using gas chromatography.

2. 2nd round

[0087] Mutant J was selected from the first round of directed evolution, based upon its increased rate of propane oxidation. This mutant was then used as the parent for the second round of evolution, the library for which was generated by error-prone PCR under conditions designed to yield 1 to 2 mutations in the heme domain of the P450 on average per gene.

[0088] Mutant 9-10A was selected from this library for its increased propane hydroxylation rate. The properties of these mutants are detailed in Tables 1-3 and **FIG. 2**. Neither mutant J, nor mutant 9-10A acquired active-site mutations and showed no major changes in regioselectivity towards longer alkanes.

Table 1**Amino Acid Mutation in P450 BM-3 Mutants Accumulated Each Generation**

Position (WT)	DNA Mutation	139-3 aa	J aa	9-10A aa	9-10A-A82L Aa	9-10A-A328V aa	1-12G aa
RV47	C142T			C	C	C	C
V78	T236C	A	A	A	A	A	A
A82	247-249 ^a				L		L
K94	A284T			I	I	I	I
F107	C324T	F	F	F	F	F	F
H138		Y					
P142	C427T			S	S	S	S
T175	C527T	I	I	I	I	I	I
V178		I					
A184	C554T	V	V	V	V	V	V
F205	T617G		C	C	C	C	C
S226	C681G		R	R	R	R	R
H236	T711G	Q	Q	Q	Q	Q	Q
E252	A758G	G	G	G	G	G	G
R255	C766A	S	S	S	S	S	S
A290	C872T		V	V	V	V	V
A295		T					
A328	C986T					V	V
L353	C1060G	V	V	V	V	V	V
E372	A1119G		E	E	E	E	E

a A82L DNA mutation was GCA to CTT.

TABLE 2
Product Distributions (% total Alcohols^a) and %ee of Selected Products

Mutant	product	hex- ane	%ee ^c	hep- tane	%ee ^c	Oct ane	%ee ^c	nonane	%ee ^c	decane	%ee ^c
139-3	1- alcohol	0		0		1		0		0	
	2- alcohol	14	14(<i>S</i>)	30	15(<i>S</i>)	61	58(<i>S</i>)	30	83(<i>S</i>)	15	
	3- alcohol	86	39(<i>S</i>)	42	15(<i>S</i>)	20		50		37	
	4- alcohol			29		17		21		49	
	ketones ^b	<1		3		5		5		7	
J	1- alcohol	0		1		1		0		2	
	2- alcohol	23	20(<i>S</i>)	29	12(<i>S</i>)	52	57(<i>S</i>)	29	65(<i>S</i>)	16	
	3- alcohol	77	46(<i>S</i>)	42	11(<i>S</i>)	25		48		35	
	4- alcohol			28		22		23		48	
	ketones ^b	<1		2		5		5		5	
9-10a	1- alcohol	0		1		1		0		1	
	2- alcohol	6	14(<i>S</i>)	26	7(<i>S</i>)	53	50(<i>S</i>)	39	60(<i>S</i>)	16	
	3- alcohol	95	41(<i>S</i>)	41	8(<i>S</i>)	20		59		32	
	4- alcohol			33		26		3		51	

Mutant	product	hex- ane	%ee ^c	hep- tane	%ee ^c	Oct ane	%ee ^c	nonane	%ee ^c	decane	%ee ^c
9-10A-A82L	ketones ^b	<1		3		5		5		6	
	1- alcohol	0		0		0		1		0	
	2- alcohol	35	39(<i>S</i>)	27	4(<i>S</i>)	22	10(<i>S</i>)	16	7(<i>S</i>)	21	
	3- alcohol	65	42(<i>S</i>)	46	30(<i>S</i>)	25	17(<i>R</i>)	16		19	
	4- alcohol			29		53		67		60	
	ketones ^b	<1		2		5		5		5	
9-10A-A328V	1- alcohol	6		14		10		3		1	
	2- alcohol	64	21(<i>R</i>)	62	15(<i>R</i>)	80	40(<i>S</i>)	76	0	79	5(<i>S</i>)
	3- alcohol	30		17		8		19		17	
	4- alcohol			6		2		3		2	
	ketones ^b	<1		<1		<1		<1		<1	
	1- alcohol	9		5		5		3		1	
1-12G	2- alcohol	77	4(<i>R</i>)	76	40(<i>R</i>)	82	39(<i>R</i>)	86	52(<i>R</i>)	86	55(<i>R</i>)
	3- alcohol	14		15		11		7		9	
	4- alcohol			3		3		5		4	

Mutant	product	hex- ane	%ee ^c	hep- tane	%ee ^c	Oct ane	%ee ^c	nonane	%ee ^c	decane	%ee ^c
	ketones ^b	<1		<1		<1		<1		<1	

- a Product distribution for each alcohol determined by ratio of a specific alcohol product to the total amount of all alcohol products. Errors are at most $\pm 1\%$.
- b Product distribution for ketones was similar to alcohol product distribution. The numbers reported here are the total amount of all ketones to total products (alcohols and ketones).
- c Favored enantiomer is listed in parentheses. Errors are at most $\pm 5\%$.

Table 3
Catalytic Properties of Mutants of P450 BM-3

Mutant	Substrate	Max. rate (min ⁻¹) ^a	Total Turnover
139-3	octane	2000	1000
	propane	100	500
J	octane	3000	3000
	propane	600	800
9-10A	octane	3000	3000
	propane	500	1100
9-10A-A82L	octane	1500	6000
	propane	200	2360
9-10A-A328V	octane	1000	2000
	propane	300	100
1-12G	octane	400	7500
	propane	20	6020

- a Rate units are measured by NADPH depletion as nmol NADPH oxidized/min/nmol protein

3. 3rd round

[0089] Mutant 9-10A was used to parent a third random-mutagenesis library. In addition to screening for increased propane oxidation activity using NADPH consumption rates, a second screen was applied to this library to assess the amount of propane hydroxylation products generated by each mutant. This screen depended upon the surrogate substrate dimethyl ether, which is similar in size and C-H bond strength to propane. Upon hydroxylation, dimethyl ether forms formaldehyde, which can be detected with Purpald dye (Hopps, H.B. *Aldrichim. Acta*, 33, 28-30 (2000)) (**FIG. 3A**, showing the hydroxylation of the surrogate substrate dimethyl ether produces formaldehyde and **FIG. 3B**, showing that purpald reacts with formaldehyde to form a purple adduct upon air oxidation).

[0090] The third round of evolution did not produce a mutant with either increased propane hydroxylation activity or more propane hydroxylation products. A possible explanation for this may be that further increases in activity require two or more simultaneous, or coupled, genetic mutations. Such events occur with very low probability and will not be found in screening a few thousand clones. Therefore, two residues were identified in the active site of mutant 9-10A as targets to modify by site-directed mutagenesis. The effect of these changes on alkane hydroxylation activity and product regioselectivity was then examined.

4. 4th round

[0091] Crystal structures of wildtype P450 BM-3 with and without substrate reveal large conformational changes upon substrate binding at the active site (Haines et al., *Biochemistry*, 40 (45):13456-13465 (2001); Li and Poulos, 1997; Paulsen and Ornstein, *Proteins-Structure Function and Genetics*, 21 (3):237-243 (1995); and Chang and Loew, (*Biochemistry*, 39 (10):2484-2498 (2000)). The substrate free structure displays an open access channel with 17 to 21 ordered water molecules. Substrate recognition serves as a conformational trigger to close the channel, which dehydrates the active site, increases the redox potential, and allows dioxygen to bind to the heme.

[0092] A tyrosine (Tyr51) at the entrance to the substrate-binding pocket makes a hydrogen bond to the carboxylate group of the substrate in the crystal structure of the enzyme bound with palmitoleic acid (Li and Poulos, 1997). Arg 47, also at the

entrance to the binding pocket, may form an ionic interaction as well. Nonpolar alkane substrates must rely solely on hydrophobic partitioning into the enzyme's extended substrate channel, and poor substrate recognition may contribute to P450 BM-3's sluggish activity on octane and other alkanes or alkenes.

[0093] Using the crystal structures of heme domain of wild type BM-3 containing a bound substrate (**FIG. 4**), two residues were identified that could influence substrate binding. Alanine 328 sits in the substrate binding pocket of BM-3 directly above the heme cofactor and is the closest residue in the protein to the proximal side of the heme iron. This residue and its mutation to valine in the wild type enzyme had been reported to affect substrate binding and turnover rates on fatty acids. (Peterson et al., In *Sixth International Symposium on Cytochrome P450 Biodiversity*: University of California, Los Angeles, 2002). **FIG. 4** shows the position of A328 and A82 in the active site of wild type cytochrome P450 BM-3. The illustration was made from the coordinates of the crystal structure 1JPZ. The substrate is palmitoyl glycine and the terminal end (ω) of the substrate is indicated.

[0094] Site-directed mutagenesis was used to change alanine 328 in 9-10A into the larger hydrophobic residue valine and determined the activity of this mutant (termed 9-10A-A328V) towards several alkanes. Neither the propane hydroxylation activity nor the total propane turnovers of this mutant improved relative to its parent, but a dramatic shift in its regioselective hydroxylation of longer alkanes was discovered. Wild type and all mutants of BM-3 generated by directed evolution were found to hydroxylate longer alkanes, such as heptane, octane, and nonane and form roughly equivalent distributions of 2-, 3-, and 4- alcohols. Mutant 9-10A-A328V, on the other hand, formed primarily (>80%) 2-alcohols with these substrates. With octane, the resulting 2-alcohol was ~70% *S*-2-octanol (40% ee) (Tables 2, 3, **FIG. 2**, in **FIG. 2**, the first bar represents the 1-alcohol formed, the second bar represents the 2-alcohol formed and the third bar represents the 3-alcohol formed). Other alkanes, however, were not hydroxylated enantioselectively.

[0095] The second side chain in the BM-3 active site that was selected for alteration is located near the active site of the protein formed after the conformational change associated with substrate binding occurs. In the crystal structure of BM-3 with the bound substrate palmitoyl glycine, the residue alanine 92 is located within 3.5 Å of the terminal end of the substrate. (Haines et al. *Biochemistry*, 40, 13456-13465 (2001)).

Given the proximity of this residue to the substrate, it is possible that changing this residue to a larger hydrophobic side chain could result in a decreased active site volume upon substrate binding. Lacking information to choose an appropriate residue, a small library containing the four large hydrophobic amino acids, leucine, isoleucine, valine, and phenylalanine at position 82 was prepared, and the library was screened using dimethyl ether and Purpald. Mutant 9-10A-A82L was identified from this screen, and subsequent gas chromatographic analysis of reaction mixtures using this mutant revealed that it supported more turnovers with propane than the 9-10A parent. Additionally, the hydroxylation of longer alkanes using this mutant revealed a shift in product regioselectivity, but in this case favoring the formation of primarily 3- and 4-alcohols.

5. 5th round

[0096] The heme domain genes of J, 9-10A, 9-10A-A328V, and A82L 9-10A were recombined using DNA shuffling to generate a library and the library was then screened for improved propane activity using the NADPH consumption screen in the presence of propane and the dimethyl ether/Purpald screen. The mutant with the highest activity, 1-12G, was selected from this library and its alkane hydroxylation activity was determined.

[0097] Surprisingly, 1-12G contained all of the mutations introduced into the recombination library. 1-12G is the double mutant A328V/A82L of 9-10A. Additionally, all of the background mutants present in the 139-9 original parent were also present in 1-12 G. Like the 9-10A-A328V, 1-12G hydroxylates alkanes at the 2-position (>80%). However, chiral GC analysis of these products revealed that 1-12G is enantioselective for the *R*-2-alcohols (40-55% ee), of heptane, octane, nonane, and decane (Tables 2, 3, **FIG. 2**). "ee" represents the difference in the two products created divided by the sum of the two products. The addition the A82L mutation to 9-10A-A328V mutant shifted the substrate octane in the active site such that the opposite enantiomer of its 2-alcohol was apparently favored. The direct regio- and enantioselective hydroxylation activity towards linear alkanes exhibited by this mutant at these high rates and total turnover numbers was surprising. The A82L mutation also increased the stability of the enzyme, bringing the total turnovers for propane and octane to well over 5000.

[0098] As the 9-10A-A328V and the 1-12G mutants hydroxylate alkanes at the 2-position, the mutations that these mutants possess compared to the 139-3 mutant represent the regioselective hydroxylation mutations, as shown in Table 1. Additionally, as the 9-10A-A328V mutant is enantioselective over the 139-3 mutant, in creating the S-2 octanol, the mutations that are different between 9-10A-A328V and the 139-3 mutant represent the enantioselective mutants, and in particular the S-enantioselective mutants, as shown in Table 1. Additionally, as the 1-12G mutant is enantioselective over the 139-3 mutant in creating the R-2 octanol, the mutations that are different between 1-12G and the 139-3 mutant represent enantioselective mutants, and in particular the R-enantioselective mutants. These differences, as well as any others that are similarly discovered, represent the regio and enantioselective mutants.

[0099] Accordingly, those amino acid positions (or corresponding positions in a different P450) that should be changed are illustrated in Table 1, so that one of ordinary skill in the art can produce a regioselective and/or enantioselective P450 enzyme. In one embodiment, the amino acids are identical to those described in Table 1. In another embodiment, the residues are conservative variants of those described in Table 1. In one embodiment, all of the residues that are characterized as regio- or enantioselective are required in order to have a P450 protein that is regio- or enantioselective. In another embodiment, only 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 of these residues are required. The most important residues and guidance of which combination of residues or which additional residues can be changed for the same selectivity will be guided by the results presented herein, the tables showing the key residues, the crystal structure of P450, and the knowledge of one of ordinary skill in the art.

[0100] There are some residues that might be classified as core residues, but are not present in either of the mutants 9-10A-A328V or 1-12. For example, residues H138, V178, and A295 are core residues of P450 BM-3 as mutation of them results in a P450 enzyme with enhanced alkane hydroxylation activity. However, these three residues are not mandatory for the altered or enhanced enantio- and regioselectivity of P450s, as these residues were not present on at least the 9-10A-A328V and 1-12G mutants.

D. Characterization of the activity of 139-9 BM-3 Mutants

[0101] To measure the alkane hydroxylation activity of the BM-3 mutants, ethanol solutions of liquid alkanes (hexane to decane) were added to buffer solutions of

the enzyme such that the total amount of ethanol in the reaction mixture was one percent. Several solvents, including ethanol, methanol, acetone, and dimethyl sulfoxide, were tested and ethanol was shown to support the most product turnovers. Reactions with liquid alkanes that contained no co-solvent produced no detectable products. In the absence of substrate, NADPH has been reported to inactivate BM-3 by over-reducing the flavin cofactors in its reductase domain. (Daff et al., *Biochemistry*, 36, 13816-13823 (1997)). To avoid this problem, substrate was added to the enzyme first and incubated for a few seconds before adding NADPH.

[0102] Dioxygen was not added to these reactions, limiting the amount of possible product formed to the 225-250 μM of oxygen present in air-saturated buffer. The addition of excess dioxygen to the reactions by direct bubbling or rapid stirring did not increase and often decreased the total product turn over, possibly by denaturing the protein. Reaction mixtures for all of our BM-3 mutants containing 0.5-1.0 μM enzyme produced 225-250 μM products, indicating that NADPH oxidation is tightly coupled to product formation in all of these systems. At very low concentrations of BM-3, the FMN cofactor has been reported to diffuse out of the protein and inactivate it. (Strobel et al., *Cytochrome P450: Structure, Mechanism, and Biochemistry (Second Edition)*). Given this fact and problems with measuring total turnover numbers using less than ~ 50 nM protein (>5000 turnovers, given the 250 μM limit on product formation in the reactions), in an optimized reaction environment, it is likely that mutant 1-12G is capable greater than the approximately 7000 turnovers reported here.

[0103] Reactions using propane did not contain co-solvent because of potential competition between the solvent and the small substrate. These reactions were performed in propane saturated buffer under an atmosphere of propane and dioxygen (provided by a balloon filled with the two gases). The addition of this atmosphere ensured that both gaseous substrates were saturated in the reaction solution, since a balloon of just propane or oxygen would dilute the concentration of the other gas. Total turnovers determined with this system were not dependent on oxygen concentration in the balloon (data not shown), illustrating that only the original 250 μM of dioxygen in the buffer was available to the reaction. Additionally, we discovered that NADPH could neither be purchased nor easily purified in a form that contains less than 2-3% ethanol, which interferes with our analysis of the reaction products. For this reason, an NADPH-

regeneration system based upon isocitrate dehydrogenase was used for propane reactions. (Schwaneberg et al., *Biomol. Screening*, 6, 111-117 (2001)).

[0104] The alkane hydroxylation product distributions from mutant 9-10A-A328V clearly show its selectivity towards the 2-position. The fact that only 2-octanol is formed enantioselectively but not 2-heptanol or 2-nonanol suggests that a substrate-protein contact specific to the terminal methyl group of octane induces its enantioselectivity towards this single substrate. Since the other non-regioselective mutants 139-3, J, and 9-10A also exhibit this enantioselectivity towards octane, this contact probably functions independently of the 328th residue. If this contact could be mimicked by changing other residues in the binding pocket, then it might be possible to engineer a mutant enantioselective for the *S*-2-position of other alkanes.

[0105] The addition of the A82L mutation to the 9-10A-A328V mutant overcame the enzyme's preference for octane, but in the process shifted the substrate in the binding pocket such that *R*-2-alcohols were the favored products (**FIG. 5**). This residue was selected for its proximity to the terminal end of the substrate in a crystal structure, but it is not clear if the larger leucine side chain "pushes" the substrate further up the active site or blocks the channel such that the substrate is flipped relative to its position in the A328V mutant. In addition to its effect on substrate selectivity, the A82L mutation both with and without the A328V mutation conferred approximately an order of magnitude increase in stability as determined by total turnover number.

E. Whole Cell Biocatalysis.

[0106] Two of the major obstacles to implementing a biocatalytic process are the need for large amounts of purified protein and expensive cofactors. Lysates of *E. Coli* (DH5 α) containing the overexpressed cytochrome P450 BM-3 mutants exhibit the same activity as purified protein, but still require the addition of expensive NADPH.

[0107] An isocitrate dehydrogenase-based NADPH regenerating system can be used to perform reactions using both cell lysate and purified protein with results indistinguishable from using NADPH alone. However, it is possible that the current embodiments could be used in an *E. coli* system as a whole cell catalyst since the alkane substrates and alcohol products should be permeable to the cell membrane. To test this hypothesis, whole cell cultures of DH5 α overexpressing the 9-10A-A328V and the 1-12G mutants were prepared.

[0108] While the system was not optimized, the cells were prepared and fed octane according to a published procedure wherein *E. coli* K27 cells expressing wild-type BM-3 was used with dmyristic acid as a substrate. (Schneider et al., *Asymmetry.*, 9, 2833-2844 (1998); Schneider et al., *Biotech. Bioeng.*, 64, 333-340 (1999)). Isopropyl- β -D-thiogalactopyranoside (IPTG) was used to induce growth of the proteins (as the genes are *tac* promoted genes), in place of the fatty acid sensitive promoter used in the published system. Extraction of these whole cell reactions with methylene chloride and analysis of the products revealed that both the regio- and enantioselectivity of the alkane hydroxylation products were preserved using the whole cell system (**FIG. 6**). These whole cell reaction conditions, similar in principal to the two-phase whole cell systems described by Schneider, et al. for AlkB octane hydroxylation, provide a cost-effective use of these *E. coli* cells for alkane hydroxylation. (Mathys et al., *Biotech. Bioeng.*, 64, 459-477, (1999)).

F. Alternative Substrates

[0109] The alkanes shown above were perhaps the most difficult substrates to hydroxylate selectively as they have very little in the way of features which can be used to direct hydroxylation. As such, the active site mutants of 9-10A will be more selective on substrates with rigid shapes and functional groups that can only be bound in our active site in a single conformation.

[0110] In some embodiments, the regio- and enantioselective enzymes will also allow the regio and enantioselective hydroxylation of nonfatty acids that are not considered alkanes. For example, the enzymes are able to specifically hydroxylate alkanes with other functional groups, alkenes, cyclic carbon groups of various sizes. In one embodiment, the mutant enzymes can regio- and enantioselectively hydroxylate any carbon which can be hydroxylated, and which is large enough to allow a limited number of binding positions in the binding site. For example, cyclized carbon groups, being more rigid than alkanes, will allow regio- and enantioselective hydroxylation.

G. Ethane hydroxylation

[0111] The ability to modify BM-3 such that it can bind small alkane substrates tightly enough to hydroxylate them selectively also suggests that practical ethane and methane hydroxylation chemistry is possible with this system. Variant 1-12G is active (approximately slightly less than 100 total turnovers) on ethane. This ethane

activity appears to be the first reported by a cytochrome P450 enzyme. More broadly, it appears that the 1-12G variant is the first P450 capable of binding ethane. The particulars of how 1-12G interacts with the ethane substrate are discussed in more detail in the examples below.

H. Additional Mutants

[0112] In addition to the mutants described above, additional mutations may be added to any of the above mutants in order to obtain enzymes with greater hydroxylation activity or altered or a higher degree of enantio- or regiospecificity. As there is a crystal structure available for P450 BM-3, and as the above mutants may be encompassed into models of the mutant enzyme, additional mutations may be made in a selective manner to particular areas of the protein. In particular, mutations at the active site that appear to further reduce or constrain a substrate should lead to an enzyme with the desired characteristics. Examples of such mutants and the resulting characteristics can be found in Table 4. The background for the mutants on Table 4 was the 9-10A mutant. The total turnover rates for the mutants are comparable to that of 9-10A (or up to 50% better).

TABLE 4

%1-oct Product	%2-oct Product	%3-oct Product	%4-oct Product	avored	mutation
2.7	42.2	18.4	16.8	2-octanol	L75I
3.3	46.1	18.4	23.7	2-octanol	L75W
3.6	22.2	27.4	44.5	4-octanol	A78T
4.0	36.8	17.1	31.2	mix	A78F
5.4	48.1	18.2	20.1	2-octanol	A78S
5.1	23.1	25.1	43.9	mix	A82T
5.6	39.9	19.2	25.5	2-octanol	A82S
4.3	25.7	19.5	47.6	4-octanol	A82F
3.7	11.2	20.6	58.8	4-octanol	A82I
4.5	25.5	25.8	42.3	4-octanol	A82C
6.6	52.4	16.8	22.7	2-octanol	A82G
8.3	70.0	5.9	2.7	2-octanol	F87I
4.5	52.0	13.0	4.2	2-octanol	F87V
6.8	55.2	21.7	11.9	2-octanol	F87L
3.6	50.8	20.7	22.2	2-octanol	T88C
7.0	66.7	11.2	9.7	2-octanol	T260L
5.7	38.5	25.5	29.0	mix	T260S
5.6	28.8	22.9	41.6	4-octanol	T260N
5.6	87.7	3.5	0.0	2-octanol	A328F
24.4	70.6	0.0	0.0	2-octanol	A328M
13.1	86.9	0.0	0.0	2-octanol	A328L

[0113] As can be seen from the results in Table 4, these mutations, selected to minimize the space of the active site, resulted in varied regioselective enzymes, including enzymes that are capable of hydroxylating alkanes at the fourth position. A favored position is defined as one in which at least 40% of the product exists. These same mutations and similar selective processes are useful for the other mutants described herein and for mutants resulting from the processes described herein. The product percentages may not add up to 100% for each mutant because there are some overoxidation products, e.g. the corresponding ketones, for some of the mutants. One especially surprising result was that the mutant proteins exhibited highly diverse and varying regio- and enantioselective properties. Thus, by using directed evolution as described above, and then selectively altering the active site amino acids so as to minimize the manner in which the substrate may bind to the active site, large numbers of very diverse, but desirable proteins can be achieved. Thus, in one embodiment, methods for creating such libraries,

and the libraries themselves are contemplated. Examples of such mutants are disclosed in **SEQ ID NOS: 3-54.**

I. Variants of 139-3 other Mutants.

[0114] One embodiment provides for a novel variant P450 BM-3 cytochrome P450 oxygenase in which one or more of the amino acid residues listed in Table 1A, which are not core residues, have been conserved. In one embodiment, the conserved residue is one that is different between either 9-10A-A82L and 139-3, 9-10-A328V and 139-3, and 1-12G and 139-3. Conservation of an amino acid residue can show that the residue has an important function for the oxygenase activity and/or stability of the P450 enzyme. Thus, the P450 BM-3 mutations identified herein to improve alkane-oxidation activity can simply be translated onto such non-P450 BM-3 enzymes to yield improved properties according to the invention.

[0115] Any method can be used to "translate" the P450 BM-3 mutation onto another cytochrome P450 enzyme, and such methods are well known in the art. For example, sequence alignment software such as SIM (alignment of two protein sequences), LALIGN (finds multiple matching subsegments in two sequences), Dotlet (a Java applet for sequence comparisons using the dot matrix method); CLUSTALW (available via the World Wide Web as freeware), ALIGN (at Genestream (IGH)), DIALIGN (multiple sequence alignment based on segment-to-segment comparison, at University of Bielefeld, Germany), Match-Box (at University of Namur, Belgium), MSA (at Washington University), Multalin (at INRA or at PBIL), MUSCA (multiple sequence alignment using pattern discovery, at IBM), and AMAS (Analyse Multiply Aligned Sequences).

[0116] A person of skill in the art can choose suitable settings, or simply use standard default settings, in these programs to align P450 BM-3 with another cytochrome P450 enzyme. U.S. Pat. Publication No. 20030100744 has a representative sequence alignment (*e.g.*, in FIG. 20 and Table 2.) The sequence alignments of P450 BM-3 with other cytochrome P450 enzymes can be taken from the literature, and amino acid residues corresponding to the mutated amino acid residues of the invention identified. For example, such information can be derived from *de Montellano Cytochrome P450: Structure, Mechanism, and Biochemistry* (Plenum Press, New York 1995), see, especially, FIG. 1 on page 187).

[0117] While some P450 enzymes may not share significant sequence similarities, particular domains such as the heme-containing domains of P450s do display close structural similarity. Therefore, the positions of the various mutations described here can be translated to similar positions in different P450 enzymes having very low sequence similarity to P450 BM-3 using molecular modeling of those P450s based on sequence homology. Examples of using such techniques to model various P450s based on sequence homology with P450 BM-3 are available (Lewis et al., 1999). The same mutations described here, when placed in their corresponding positions in other P450 structures (as determined by modeling) would confer similar improvements in alkane oxidation activity.

[0118] An example of such a structure is demonstrated in **FIG. 7**. The P450 is displayed in a ribbon format, while the locations and shapes of the point mutations are displayed in space filling structures.

[0119] In one embodiment, the P450 variants will have at least one of the novel mutations described herein. In one embodiment, the P450 variant will have at least one of the mutations that is different between the 139-3 parent and one of the 3rd, 4th, or 5th generation enzymes, as shown in Table 1. In one embodiment, the variant will have all 8 of the primary core mutations from the 139-3 parent, as well as at least one mutation from later generations. In one embodiment, the later generation is selected from the following: J, 9-10A, 9-10A-A82L, 9-10A-A328V, 1-12G. In one embodiment, a variant will have one of the “selective hydroxylation mutations.” In another embodiment, the variant will have at least one of the mutations described in Table 4. In another embodiment, a variant will have at least one of the core mutations and at least one selective hydroxylation mutation. A functional variant is a variant that functions as the mutant functions, but has different mutations that allow it to so function. Such variants are described herein, as the process for identifying such variants has been fully described in the making of the mutant itself.

EXAMPLES

[0120] All liquid alkane substrates, product standards, and solvents were from Sigma-Aldrich, Inc. (St. Louis, MO). The gases propane and dimethyl ether were from Advanced Gas Technologies (Palm, PA). Isocitrate dehydrogenase and NADP⁺ was from Sigma-Aldrich, Inc. (St. Louis, MO). NADPH was from Biocatalytics, Inc. (Pasadena, CA).

EXAMPLE 1

[0121] This example demonstrates one method by which recombination of the P450 BM-3 mutants may occur, as done in the first 1st library recombination. The first generation of mutants was created by StEP recombination of mutant 139-3 (V78A, H138Y, T175I, V178I, A194V, H236Q, E252G, R255S, A290V, A295T) with 15 other mutants from the same generation. (Glieder et al., *Nature Biotech.*, 20, 1135-1139 (2002)), Zhao et al., *Nature Biotechnology*, 16, 258-261 (1998)). A mutant, J, (V78A, T175I, A184V, F205C, S226R, H236Q, E252G, R255S, A290V, L353V) was isolated based on its increased NADPH depletion rate using propane as a substrate. Table 2 displays the enantio- and regiospecific qualities of the J mutant. For example, the J mutant produces less of the 2-alcohol than the 139-3 mutant does. However, the J mutant creates more of the 3-alcohol than the 139-3 mutant does. Table 3 displays the rate of NADPH depletion as 3000 min⁻¹ for octane and 600 min⁻¹ for propane. These values are clearly higher than the 2000 and 100 rates for the 139-3 mutant P450. The distribution of the products can be seen in **FIG. 2** and in Table 2. This distribution of products is also known as the hydroxylation profile. Here, the profile for, for example, octane is 1%, 52%, 25%, 22%, and 5%, for the 1-alcohol, 2-alcohol, 3-alcohol, 4-alcohol, and ketone respectively. The hydroxylation profile is also 57% (S) ee. As can be seen comparing these results to the 139-3 results for octane, the J mutant clearly has an altered hydroxylation profile. For example, for hexane, the J mutant produces 23% of the product in the 2-alcohol form, while the 139-3 mutant only produces 14% of hexanes in the 2-alcohol form.

EXAMPLE 2

[0122] This example demonstrates one method by which random mutagenesis of P450 BM-3 may be achieved, as described in the 2nd and 3rd library steps above. The second and the third generation were created by error-prone PCR using the Genemorph kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol, using approximately

50 ng ($\times$ ng for third) of plasmid DNA as template and primers BamHI-forw (5'-ggaaacaggatccatcgatgc-3'; **SEQ ID NO: 55**) and SacI-rev (5'-gtgaaggaataccgccaagc-3'; **SEQ ID NO: 56**). Mutant 9-10A (R47C, V78A, K94I, P142S, T175I, A184V, F205C, S226R, H236Q, E252G, R255S, AS90V, L353V) was isolated from the 2nd generation based on increased NADPH consumption and NADP⁺ formation using propane as a substrate. No increase in activity was observed in the products of the third library. Table 3 displays the catalytic properties of the 9-10A mutant produced from this step. While the rate of octane synthesis has not changed, relative to the J mutant, the rate of propane synthesis has actually decreased, although both are still substantially above the rates for the 139-3 mutant. Of course, the total turnover for 9-10A, 1100, is greater than the total turnover for J, 800. The distribution of the products can be seen in **FIG. 2** and in Table 2.

EXAMPLE 3

[0123] This example demonstrates one method by which site directed mutagenesis may be performed, as described above in the 3rd and 4th generations. Base substitution mutations were introduced into mutant 9-10A by PCR overlap extension mutagenesis. (Higuchi et al., *Nucleic Acids Res.*, 16, 7351-7367 (1988)). Position A82 was mutated to L, I, V and F using mutagenic primers A82forw (5'-ggagacgggtatttacaagc-3'; **SEQ ID NO: 57**) and A82rev (6'-gcttgtaaataacccgtctccaanaaaatcacg-3'; **SEQ ID NO: 58**). Position A328 was mutated to V using mutagenic primers A328V forw (5'-gcttatggccaactgttctctgc-3'; **SEQ ID NO: 59**) and A328V rev (5'-gcaggaacagttggccataagc-3'; **SEQ ID NO: 60**). For each mutation, two separate PCRs were performed, each using a perfectly complementary primer (BamHI-forw and SacI-rev) at the end of the sequence and a mutagenic primer. The resulting two overlapping fragments that contain the base substitution were then annealed together in a second PCR to amplify the complete mutated gene. Mutant 9-10A-A82L was isolated based on increased turnover of dimethyl ether. The properties of the resulting mutants, 9-10A-A82L and 9-10A-A328V can be observed in Tables 2 and 3. 9-10A-A82L demonstrated a decrease in catalytic rate for octane and propane, while it displayed a significant increase in the total turnover rate for octane and propane. It also showed a regioselectivity that favors the 4-alcohol product, at least for the octane, nonane, and decane products, in contrast to the 139-3 mutant.

[0124] On the other hand, the 9-10A-A328V mutant showed both a decrease in rate and turnover, as compared to the 9-10A mutant. However, this mutant displayed a

high degree of regioselectivity, as shown in Table 2 and **FIG. 2** (identified as A328V), especially for the 2-alcohol. The distribution of the products for the hydroxylation profiles can be seen in **FIG. 2** and in Table 2. For example, as can be observed in Table 2, while the initial 139-3 mutant produced only 30% of its product of nonane in the 2-alcohol form, the 9-10A-A328V mutant produced 76% of its product in that form. Thus, the hydroxylation profile of this mutant is different from the 139-3 mutant.

EXAMPLE 4

[0125] This example demonstrates how recombination of P450 BM-3 mutants can achieve desirable mutants, as shown in the 5th step above. The last generation of mutants was created by recombination of mutants 139-1, J, 9-10A, 9-10A-A82L and 9-10A-A328V by DNA shuffling. (Stemmer, W.P.C. *Nature* 1994, 370, 389-391; Stemmer, W.P.C. *Proc. Natl. Acad. Sci. USA* 1994, 91, 10747-10751). Mutant 1-12G was isolated based on increased turnover of both dimethyl ether and propane. Mutant 1-12G has all of the mutations from the 9-10A-A328V mutant, with an additional mutation at position A82L. Interestingly, the rate of catalysis of this mutant is only 400 min⁻¹ and 20 min⁻¹ for octane and propane. However, the total turnover, as shown in Table 3 (7500 for octane and 6020 for propane), is much higher than for any of the other mutants. Additionally, as can be observed in **FIG. 2**, the bias towards the production of 2-alcohol products is much greater than for any of the other mutants as well, as high as 86% for decane. As discussed below, this mutant is also capable of hydroxylating ethane, something the wild type and 139-3 mutant are effectively unable to do. Again, the data in Table 2 demonstrate the altered hydroxylation profile of the 1-12G mutant. For example, where the hydroxylation profile of the 139-3 mutant for octane was 1, 61, 20, 17, and 5 percent for the 1-, 2-, 3-, 4-alcohols and ketone respectively, the same values for the 1-12G mutant were 5, 82, 11, 3, and 1. Not only has the hydroxylation profile changed, but the enzyme produces a larger percent of the 2-alcohol than it did before. Additionally, the hydroxylation profile of the enantiomeric products for octane has changed significantly between 139-3 and 1-12G, as the 139-3 results in 58(S), while 1-12G results in 39(R).

EXAMPLE 5

[0126] This example demonstrates how one may express and purify the P450 BM-3 protein and relevant mutants. P450 BM-3 was expressed and purified as described previously. (Glieder et al., *Nature Biotech.*, 20, 1135-1139 (2002)). The P450 BM-3 gene or mutants thereof, which include a silent mutation to introduce a SacI site 130 bp

upstream of the end of the heme domain, was cloned behind the double *tac* promoter of the expression vector pCWori (pBM3_WT18-6) (Farinas, et al., Adv. Synth. Catal., 343:601-606 (2001)). *E. coli* DH5 α , transformed with these plasmids, was used for expression of P450 BM-3 on a 500 mL scale as well as for expression in 96-well plates.

[0127] For protein production, supplemented terrific broth (TB) medium (600 mL, 100 μ g/mL ampicillin, 50 μ g/mL thymine) was inoculated with an overnight culture (1 mL) and incubated at 40°C and 350 rpm. After 12 hours of incubation, the rotation speed was lowered to 200 rpm, α -aminolevulonic acid hydrochloride (ALA; 0.5 mM) was added and expression was induced by addition of isopropyl- β -D-thiogalactoside (IPTG; 1 mM). Cells were harvested 20 to 24 hours after induction by centrifugation. The enzymes were purified following published procedures. (See, Farinas et al. Adv. Synth. Catal., 343:601-606 (2001)). Enzyme concentration was measured in triplicated by CO-difference spectra. (Omura et al., *Journal of Biological Chemistry*, 239, 2370-2378 (1964)). The characteristics of the enzymes were then tested, as described in the following examples.

EXAMPLE 6

[0128] This example demonstrates one method by which cell lysates can be prepared for high-throughput screening. Single colonies were picked and inoculated by a Qpix robot (Genetix, Beaverton, OR) into 1-mL deep-well plates containing Luria-Bertani (LB) medium (350 μ L, supplemented with 100 mg/mL ampicillin). The plates were incubated at 30°C, 250 rpm, and 80% relative humidity. After 24 hours, clones from this preculture were inoculated using a 96-pin replicator into 2-mL deep-well plates containing TB medium (400 μ L, supplemented with 100mg/mL ampicillin, 10 μ M IPTG and 0.5 mM ALA). The cultures were grown at 30°C, 250 rpm, and 80% relative humidity for another 24 hours. Cells were then pelleted and stored frozen at -20°C until they were resuspended in 500 μ L 0.1 M phosphate buffer (0.1 M, pH=8, 500 μ L, containing 0.5 mg/mL lysozyme, 2 Units/mL DnaseI, and 10 mM MgCl₂). After 60 min at 37°C, the lysates were centrifuged and the supernatant was diluted for activity measurements in 96 well microtiter plates, thus preparing the cell lysates for screening, as described in the next example.

EXAMPLE 7

[0129] This example demonstrates methods for the high-throughput determination of enzymatic activity. The first mutant library was screened for NADPH depletion using propane as substrate. 170 μL of phosphate buffer (0.1 M, pH 8.0), saturated with propane was added to 30 μL of *E. coli* supernatant. The reaction was initiated by addition of 50 μL NADPH (0.8 mM) and NADPH oxidation was monitored at 340 nm for five min using a Spectramax Plus microtiter plate reader (Molecular Devices, Sunnyvale, CA).

[0130] The second library was additionally screened for NADP^+ formation using propane as substrate as described earlier. (Glieder et al., *Nature Biotech.*, 20:1135-1139 (2002); Tsotsou et al. *Biosens. Bioelectron.*, 17, 119-131 (2002)). In brief, residual NADPH was destroyed with acid after an appropriate amount of time followed by conversion of NADP^+ to a highly fluorescent alkali product at high pH which was then measured fluorometrically. The results of the screens were used to select the J mutant and 9-10A mutant discussed above.

EXAMPLE 8

[0131] This example demonstrates direct methods for the high-throughput determination of enzymatic activity. For direct measurement of product formation a screen based on the demethylation of dimethyl ether was used in the later generations. To 30 μL of *E. coli* supernatant, 120 μL of phosphate buffer (0.1 M, pH-8) saturated with dimethyl ether was added. After 2 min of incubation at room temperature NADPH (50 μL , 1.0 mM) was added and NADPH depletion was monitored as previously described. Purpald (168 mM in 2 M NaOH) was added 15 min after initiating the reaction to form a purple product with formaldehyde, generated after demethylation of the substrate. The purple color was read approximately 15 min later at 500 nm using a Spectramax Plus microtiter plate reader (Molecular Devices, Sunnyvale, CA). The results demonstrated that the third round of mutagenesis did not produce a mutant with improved results.

EXAMPLE 9

[0132] This example demonstrates one method by which one can determine the enzyme kinetics of an enzyme. The enzymes were purified and quantified as described above. Initial rates of NADPH consumption were measured at 25°C in a BioSpec-1601 UV/VIS spectrophotometer (Shimadzu, Columbia, MD). For the liquid

alkanes, substrate stock solutions in ethanol (10 μ L) were added to the protein solution (100 nM, final concentration) and incubated for 2 min before initiating the reaction by addition of 200 μ L NADPH (0.8 mM) and the absorption at 340 nm was monitored. Rates for any given substrate concentration were determined in triplicate. Results are shown in Table 3. The 9-10A mutant has the highest rate, while the 1-12G mutant has the lowest rate.

EXAMPLE 10

[0133] This example demonstrates one method by which one can determine if the enzyme is capable of alkane hydroxylation reactions. Reactions with the liquid alkanes hexane, heptane, octane, nonane, and decane were performed in closed 20 mL scintillation vials and stirred at low speed using magnetic stirring bars. In a typical reaction, purified protein (or cell lyse) was added to 4.45 mL of 0.1 M potassium phosphate buffer pH=8.0 such that the total protein concentration equaled 50 nM. The substrates were added to this solution as 50 μ L of 400mM ethanol solutions to give 4 mM total substrate and 1% ethanol. After a few seconds, 500 μ L of 5mM NADPH in 0.1 M potassium phosphate buffer pH=8.0 was added to the reaction and the vial was capped. After 1-2 hours of stirring at room temperature, a 1.5 mL aliquot of the reaction was removed from the vial and quenched with 300 μ L of chloroform in a 2 mL microcentrifuge tube. An internal standard containing 15 μ L of 100 mM 1-pentanol or 3-octanol was added to the tube. The tube was vortexed and then centrifuged at 14,000 rpm for 2 minutes in a microcentrifuge. The chloroform layer was removed with a pipet and analyzed by gas chromatography for total turnover numbers and product distributions. Control reactions were performed by repeating these steps without the addition of substrate and revealed no background levels of these specific products. The samples were analyzed as described below.

EXAMPLE 11

[0134] This example demonstrates one method by which a chiral analysis of the products produced herein may be performed. Chiral analysis of liquid alkane hydroxylation products was performed with a slight modification of an existing method, starting with extracting 9 mL alkane reactions (using the reaction conditions above) with 2 mL methylene chloride in a 15 mL centrifuge tube (Westley et al., *J. Org. Chem.*, 33, 3978-3980 (1968)). After centrifugation at 4000 rpm for 15 minutes, the organic layer

was removed with a pipet and dried over a small amount of anhydrous magnesium sulfate. The magnesium sulfate was removed by filtration, and 1 μ L of pyridine and 2.5 μ L (-)-menthyl chloroformate was added. After one hour, 1 mL of deionized water was added to the reaction. After vortexing and letting the layers separate, the organic phase was removed with a pipet and dried with anhydrous magnesium sulfate. The drying agent was again removed with a pipet filter and the remaining solution analyzed by gas chromatography. Control reactions were performed by repeating these steps without the addition of substrate and revealed no background levels of these specific products. Results are summarized in Table 2 and in **FIGs. 2A-2E**. As can be observed in Table 2, which demonstrates that while the 9-10A-A328V mutant was biased in its production of S octane and R hexane and R heptane, the 1-12G mutant was biased in its production of the R enantiomer regardless of the initial substrate used. The data comparing the products from 9-10A-A328V and 1-12G can be observed in **FIGs. 5A** and **5B** respectively. **FIG. 5** shows a graph from a GC analysis of the (-)-menthyl carbonate diastereomers of the 2-octanol produced by mutant BM-3 catalyzed alkane oxidation. S-2-octanol elutes at 18.4 (18.393 and 18.410) minutes, R-2-octanol elutes at 18.6 (18.553 and 18.575) minutes.

EXAMPLE 12

[0135] This example demonstrates one method by which propane hydroxylation may be performed and monitored. Propane hydroxylation reactions were performed in 25 mL Schlenk flasks and no co-solvent was used in the reaction. In a typical reaction, enzyme (either purified or in cell lysate) was added to 4.5 mL of propane saturated 0.1M potassium phosphate buffer pH=8.0 to a final concentration of 500-100 nM. To this mixture, 500 μ L of NADPH-regeneration system containing 1 mM NADP⁺, 100 mM sodium isocitrate, and 20 Units/mL isocitrate dehydrogenase was quickly added. The flask was topped with a balloon filled with equal amounts of propane and dioxygen. After stirring for two hours at room temperature, the propane hydroxylation products were derivatized to alkyl nitrites using a published method. (Nguyen et al., *Analytical Sciences*, 17, 639-643 (2001)). To the reaction mixture, 0.3 g of sodium nitrite and 2 mL 10 μ M chloroform in hexane was added and the mixture was cooled on ice. While on ice, 0.2 mL concentrated sulfuric acid was added. The flask was stoppered with a rubber stopper and stirred on ice for 15 minutes. The reaction was rinsed into a separatory funnel with 20 mL of deionized water. The organic phase was washed twice with 20 mL of water and analyzed by gas chromatography. Control reactions were performed by

repeating these steps without the addition of substrate to correct for background levels of propanol. Results are summarized in Table 3.

EXAMPLE 13

[0136] This example demonstrates how the mutant enzymes described herein can be useful in whole cell reactions. The procedure for whole cell reactions of *E. coli* (DH5 α) overexpressing mutants 9-10A-A328V and 1-12G was similar to Witholt's published method. (Schneider et al., *Tetrahedron Asymmetry*, 9, 2833-2844 (1998)). An overnight culture of cells (in 3 mL LB with 100 μ g/mL ampicillin) was used to inoculate 75 mL of M9 minimal medium containing 0.5% w/v glucose, 0.2 mM calcium chloride, 5 mM magnesium sulfate, and 100 μ g/mL ampicillin. The culture was then shaken for 24 hours at 37°C and 250 rpm. The cells were collected by centrifugation at 3500 rpm for 10 minutes and resuspended in 20 mL of 0.2M potassium phosphate buffer pH-7.4 containing 0.5% glucose, 100 μ g/mL ampicillin, 1 mM IPTG, and 0.5 mM α -aminolevulonic acid, 5 mM alkane (from a 500 mM stock of alkane in dimethyl sulfoxide). This mixture was shaken for 8 hours at 37°C and 250 rpm. Product distributions were measured by gas chromatography after extracting this culture with 1 mL of chloroform. Chiral analysis of the reaction products was performed by extracting the culture with 2 mL of methylene chloride and derivatizing the organic layer with (-)-menthyl chloroformate. The results are demonstrated in **FIG. 6A** and **FIG. 6B** for the resulting products from the purified protein and the whole cell respectively. 3-octanol elutes at about 7.7 minutes, 2-octanol elutes at about 7.8 minutes, and 1 octanol elutes at about 9.0 minutes.

EXAMPLE 14

[0137] This example demonstrates how gas chromatography can be used for identification and quantification of analytes. Identification and quantification of analytes were performed using purchased standards and 5 point calibration curves with internal standards. All analyses were injected at a volume of 1.0 μ L and performed at least in triplicate. Analysis of hydroxylation products were performed on a Hewlett Packard 5890 Series II Plus gas chromatograph with both a flame ionization (FID) and electron capture detector (ECD) and fitted with a HP-7673 autosampler system. Direct analysis of hexane, heptane, octane, nonane, and decane hydroxylation products was performed on an HP-5 capillary column (crosslinked 5% phenyl methyl siloxane, 30 m length, 0.32 mm ID, 0.25

µm film thickness) connected to the FID detector. A typical temperature program for separating the alcohol products is 250°C injector, 300°C detector, 50°C oven for 3 minutes, 10°C/minute to 200°C, 25°C/minute to 250°C, 250°C for 3 minutes. The (-)-menthyl chloroformate derivatized chiral products were separated as diastereomers on a CycloSil-B chiral capillary column (Agilent Technologies, 30 m length, 0.32 mm ID, 0.25 µm film thickness) connected to the FID detector. Each pair of diastereomers required a different temperature program to fully resolve the pair, but a typical program is as follows: chiral heptanol analysis — 250°C injector, 300°C detector, 100°C oven for 1 minute, then 10°C/minute gradient to 180°C, hold at 180°C for 10 minutes, 10°C/minute gradient to 250°C, then 250°C for 3 minutes. The propyl nitrite products were analyzed with an HP-1 capillary column (crosslinked 1% phenyl methyl siloxane, 30 m length, 0.32 mm ID, 0.25 µm film thickness) connected to an ECD detector. The temperature program for separating 1- and 2-propyl nitrites was 250°C injector, 300°C detector, 30°C oven for 3 minutes, 20°C/minute gradient to 200°C, 200°C for 5 minutes. Results can be observed in **FIG. 2**, **FIG. 5**, **FIG. 6**, and Tables 2 and 3.

EXAMPLE 15

[0138] This example demonstrates that further rounds of directed evolution of the 9-10A-A328V and 1-12G biocatalysts will allow the enzyme to support alkane hydroxylation that is even more enantioselective. For example, chiral lipases and alcohol dehydrogenases are available that selectively convert one 2-alcohol enantiomer over the other. These enzymes can be coupled to a screening protocol to screen for mutant BM-3 enzymes that form one enantiomeric alcohol product over another. For example, lipases that are capable of enantioselective transesterification reactions in which a single enantiomer of an alcohol, such as *S*-2-octanol, can be used to replace the alcohol component of an ester substrate. The replaced alcohol component in the ester can be a chromophore, such as *p*-nitrophenolate, or can be reacted with a dye to form a chromophore, such as vinyl alcohol. This will then allow the presence of the chiral alcohol product to be detected colorimetrically in the presence of the lipase and the ester substrate, as discussed in greater detail in Konarzycka-Bessier et al., *Angewandte Chemie International English Edition*, 42(12): 1418-1420 (2003). Alternatively, alcohol dehydrogenases that selectively oxidize chiral alcohols can be incorporated into a coupled screening system.

Equivalents

[0139] The foregoing description and Examples detail certain preferred embodiments of the invention and describes the best mode contemplated by the inventors. It will be appreciated, however, that no matter how detailed the foregoing may appear in text, the invention may be practiced in many ways and the invention should be construed in accordance with the appended claims and any equivalents thereof.

WHAT IS CLAIMED IS:

1. An isolated mutant P450 enzyme having a first mutation, wherein said mutant P450 enzyme hydroxylates alkanes to produce a first product with a first hydroxylation profile, and wherein in the absence of said mutation said enzyme hydroxylates alkanes to produce a second product with a different hydroxylation profile.
2. The isolated mutant P450 of Claim 1, wherein said enzyme is a mutant of the cytochrome P450 BM-3 enzyme.
3. The isolated mutant P450 enzyme of Claim 1, wherein the mutant comprises at least one core mutation.
4. The isolated mutant P450 enzyme of Claim 3, wherein the core mutant is a V78A mutation.
5. The isolated mutant P450 enzyme of Claim 3, wherein the mutant further comprises at least 7 core mutations.
6. The isolated mutant P450 enzyme of Claim 3, wherein the core mutation comprises a mutation selected from the group consisting of: V78A, T175I, A184V, H236Q, E252G, R255S, L353V, conservative variants of the aforementioned mutations, and combinations thereof.
7. The isolated mutant P450 enzyme of Claim 6, wherein the mutant is a P450 139-3 mutant.
8. The isolated mutant P450 enzyme of Claim 1, wherein the core mutation comprises a mutation selected from the group consisting of: V78A, T175I, A184V, H236Q, E252G, R255S, L353V, H138Y, V178I, A295T, conservative variants of the aforementioned mutations, and combinations thereof.
9. The isolated mutant P450 enzyme of Claim 1, wherein said enzyme exhibits selective hydroxylation, and comprises amino acid mutations selected from the group consisting of: R47C, K94I, P142S, F205C, S226R, A290V, conservative variants of the aforementioned mutations, and combinations thereof.
10. The isolated mutant P450 enzyme of Claim 1, wherein the selective hydroxylation mutation is selected from the group consisting of: A328V, A82L, R47C, K94I, P142S, F205C, F87I, T88C, S226R, A290V, conservative variants of the aforementioned mutations, and some combination thereof.

11. The isolated mutant P450 enzyme of Claim 10, wherein said enzyme is derived from the cytochrome P450 139-3 enzyme and comprises at least one selective mutation.

12. The mutant BM-3 P450 enzyme of Claim 1, wherein the mutant P450 enzyme has an altered enantiospecificity compared to the enantiospecificity of mutant 139-3.

13. The mutant BM-3 P450 enzyme of Claim 1, wherein the mutant P450 enzyme has an altered regiospecificity compared to the regiospecificity of mutant 139-3.

14. The enantioselective enzyme of Claim 12 or 13, wherein the selective hydroxylation mutation is selected from the group consisting of: R47C, F87I, A82L, A328V, conservative variants of the aforementioned mutations, and combinations thereof.

15. The mutant BM-3 P450 enzyme of Claim 14, wherein the selective hydroxylation mutation is selected from the group consisting of: A328V and A82L.

16. The mutant BM-3 P450 enzyme of Claim 14, wherein the selective hydroxylation mutation is A82L.

17. The isolated mutant P450 enzyme of Claim 1, wherein said enzyme comprises selective activity consistent across hexanes, decanes, heptanes, octanes, and nonanes.

18. The isolated mutant P450 enzyme of Claim 1, wherein said enzyme comprises alkane hydroxylation activity that results in the same regiospecific product for hexane, heptane, octane, nonane, and decane for at least 40% of the total products produced from the alkane hydroxylation activity.

19. The isolated mutant P450 enzyme of Claim 1, wherein the selective activity comprises a total turnover for octane of more than about 1000.

20. The isolated mutant P450 enzyme of Claim 1, wherein the selective activity comprises a maximum rate of catalysis of octane of at least about 2001 (min^{-1}).

21. The isolated mutant P450 enzyme of Claim 1, further comprising alkane hydroxylation activity against ethane.

22. The isolated mutant P450 enzyme of Claim 21, wherein said selective hydroxylation mutation is selected from the group consisting of: A328V, A82L, R47C, K94I, P142S, F205C, S226R, A290V, and conservative variants of the aforementioned mutations.

23. The isolated mutant P450 enzyme of Claim 21, wherein said selective hydroxylation mutation comprises the following mutations: A328V, A82L, R47C, K94I,

P142S, F205C, S226R, A290V and conservative variants of the aforementioned mutations.

24. The isolated mutant P450 enzyme of Claim 23, comprising a V78A core mutation.

25. The isolated mutant P450 enzyme of Claim 24, further comprising T175I, A184V, H236Q, E252G, R255S, and L353V core mutations.

26. A method of making a mutant P450 enzyme with altered selective hydroxylation abilities, said method comprising:

providing a first mutant P450 that is capable of alkane hydroxylation of a substrate to produce a product with a first hydroxylation profile;

modifying at least one amino acid in said first mutant P450 to produce a second mutant P450, wherein said second mutant P450 is capable of alkane hydroxylation of the substrate to produce a product with a second hydroxylation profile.

27. The method of Claim 26, wherein the modification of the at least one amino acid is achieved through directed evolution.

28. The method of Claim 26, wherein the modification of the at least one amino acid is achieved through selected point mutations.

29. The method of Claim 28, wherein the point mutations are selected from an active site of the mutant P450.

30. A method of making a P450 enzyme with regioselective alkane hydroxylation activity, said method comprising:

selecting residues to alter to reduce the volume of the active site of a P450 enzyme;

replacing small hydrophobic residues in the active site with larger hydrophobic residues to create a mutant P450;

testing the resulting mutant P450 for regioselectivity; and

repeating the steps if no such P450 mutant is made, thereby making a P450 enzyme with regioselective alkane hydroxylation activity.

31. A method of making a P450 enzyme with enantioselective alkane hydroxylation activity, said method comprising:

selecting residues to alter to reduce the volume of the active site of a P450 enzyme;

replacing small hydrophobic residues in the active site with larger hydrophobic residues to create a mutant P450;

testing the resulting mutant P450 for enantioselectivity; and

repeating the steps if no such P450 mutant is made, thereby making a P450 enzyme with enantioselective alkane hydroxylation activity.

32. A method of making a P450 enzyme with alkane hydroxylation activity towards ethane, said method comprising:

selecting residues to alter to reduce the volume of the active site of a P450 enzyme;

replacing small hydrophobic residues in the active site with larger hydrophobic residues to create a mutant P450;

testing the resulting mutant P450 for ethane hydroxylation activity; and

repeating the steps if no such P450 mutant is made, thereby making a P450 enzyme with ethane hydroxylation activity.

33. An isolated nucleic acid encoding a cytochrome P450 mutant that has a higher capability than the corresponding wild-type cytochrome P450 to oxidize at least one substrate selected from an alkane comprising a carbon-chain of no more than 8 carbons, wherein the wild-type cytochrome P450 comprises an amino acid sequence identical to **SEQ ID NO: 2**, and the cytochrome P450 mutant is at least 80% identical to the sequence in **SEQ ID NO: 2** and has an amino acid substitution at a residue corresponding to a core residue selected from V78, H236, and E252, and wherein said mutant also has an amino acid substitution at a residue corresponding to a selective hydrolysis residue of **SEQ ID NO: 2** selected from R47C, A82L, K94I, P142S, C205V, S226R, A290V, and A328V.

34. A method of creating a regio- and enantioselective hydroxylation P450, said method comprising:

performing directed evolution on a P450 to obtain a mutant P450; and

modifying an active site of said mutant P450 so as to reduce the size of the active site of the mutant P450, thereby creating a regio- and enantioselective hydroxylation P450.

35. An isolated mutant P450 enzyme with regioselective alkane hydroxylation activity that has a higher degree of regiospecificity for the hydroxylation of octane than the wild-type and 139-3 mutant.

36. The isolated mutant P450 of Claim 35, wherein the regiospecificity of the mutant is at least 10% more than the 139-3 mutant.

37. The isolated mutant P450 of Claim 35, wherein the regiospecificity of the mutant is at least 70%.

38. The isolated mutant P450 of Claim 35, wherein the regiospecificity of the mutant is at least 80%.

39. The isolated mutant P450 of Claim 35, wherein the mutant P450 is a P450 BM-3.

40. An isolated mutant P450 enzyme, which predominantly hydroxylates a substrate at a first position, having an altered enantiospecificity for alkanes, said mutant P450 enzyme comprising a selective hydroxylation mutation, wherein the mutant P450 enzyme comprising the selective hydroxylation mutation predominantly hydroxylates the substrate at a second position.

41. The isolated mutant P450 of Claim 40, wherein the enantiospecificity allows for hydroxylation of a substrate to occur in a R configuration.

42. The isolated mutant P450 of claim 41, wherein the substrate is octane.

43. The isolated mutant P450 of claim 42, wherein the P450 is a BM-3.

44. A method of hydroxylating an alkane in a selective manner, said method comprising:

providing an isolated mutant P450 enzyme having selective hydroxylation activity towards an alkane; and

contacting said isolated mutant P450 with said alkane, whereby the isolated mutant P450 hydroxylates said alkane in a selective manner.

45. The method of Claim 44, wherein the selective manner is a regioselective manner.

46. The method of Claim 44, wherein the selective manner is an enantioselective manner.

47. The method of Claim 44, wherein the isolated mutant P450 hydroxylates said alkane with a turnover number of more than 100.

48. The method of Claim 44, wherein the alkane is an octane and wherein the isolated mutant P450 hydroxylates said octane with a turnover number of more than 1000.

49. The method of Claim 44, wherein said alkane is selected from the group consisting of decane, nonane, octane, heptane, hexane, pentane, propane, and ethane.

50. The method of Claim 44, wherein said isolated mutant P450 is an isolated mutant 1-12G P450.

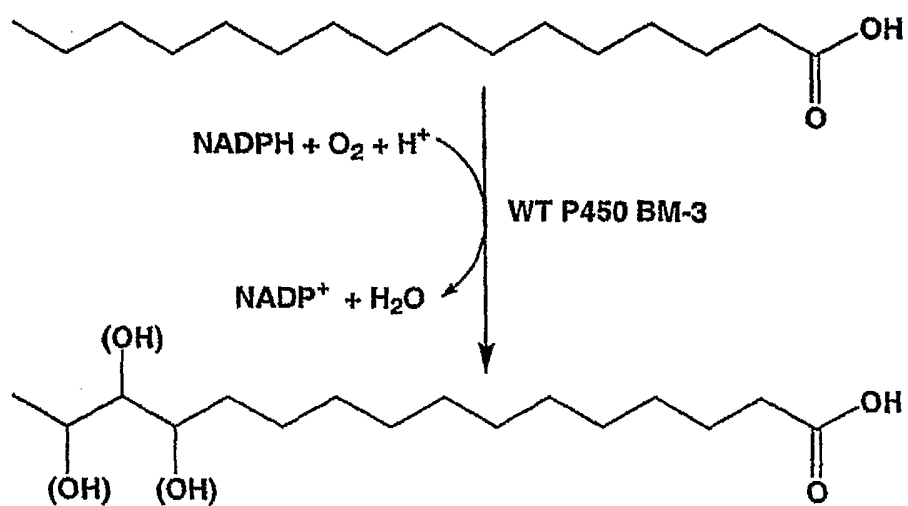


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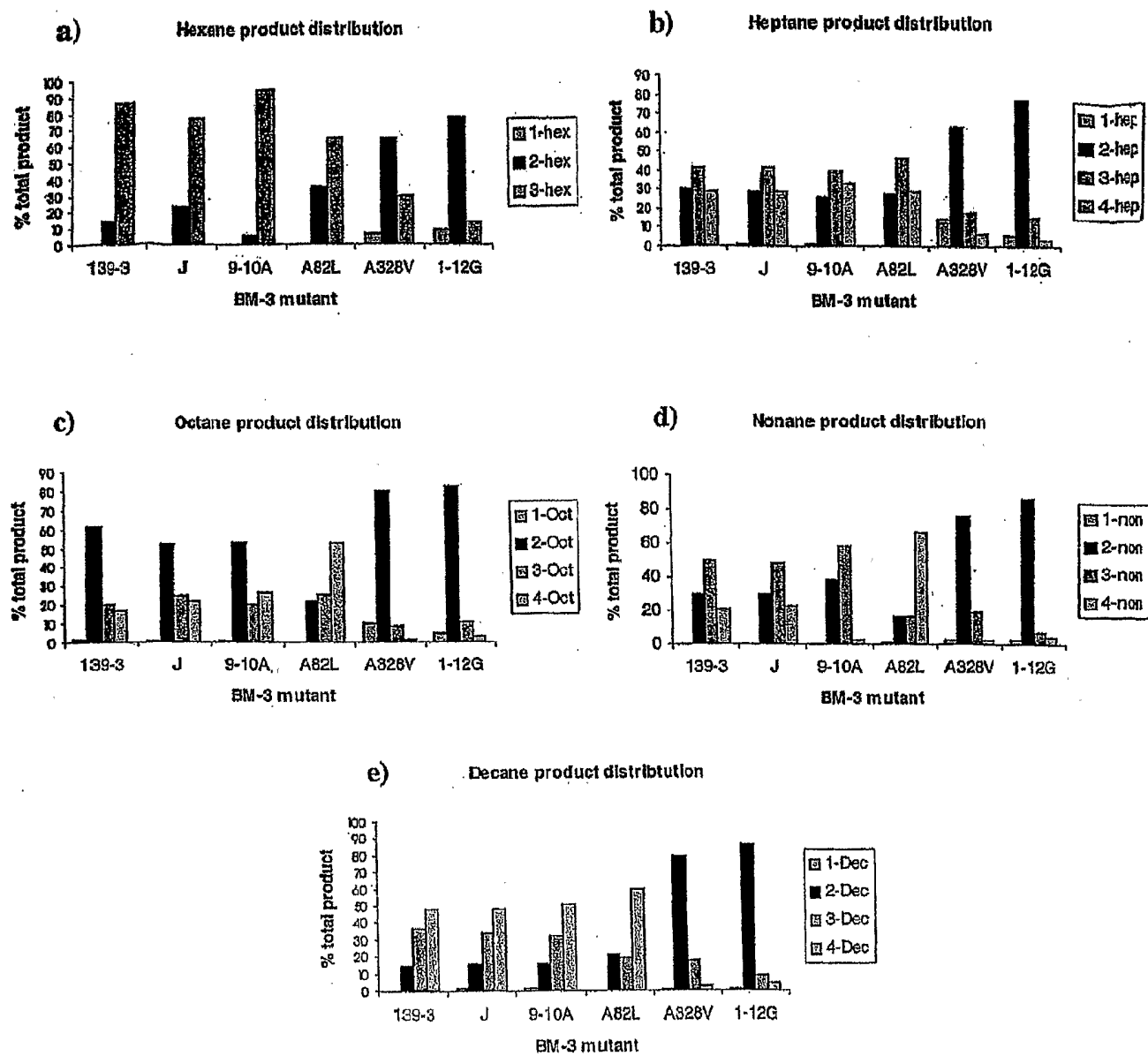
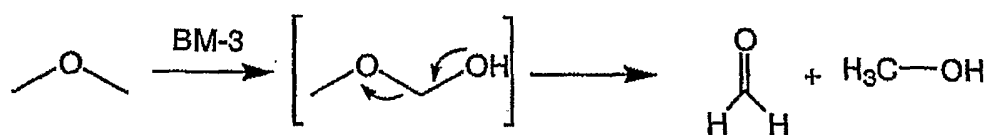


FIG.2

a)



b)

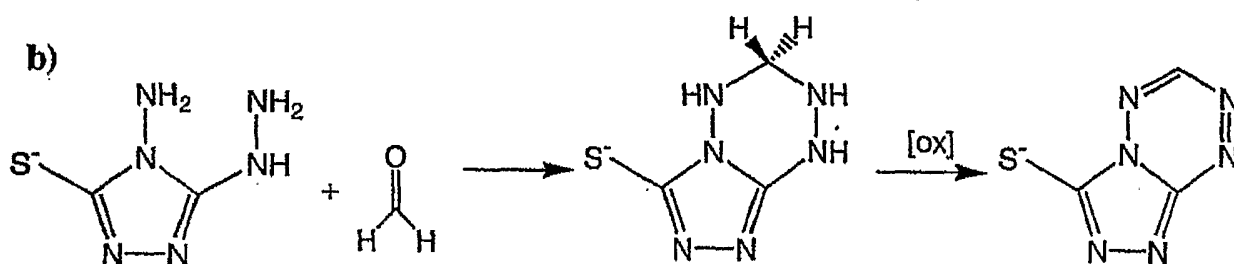


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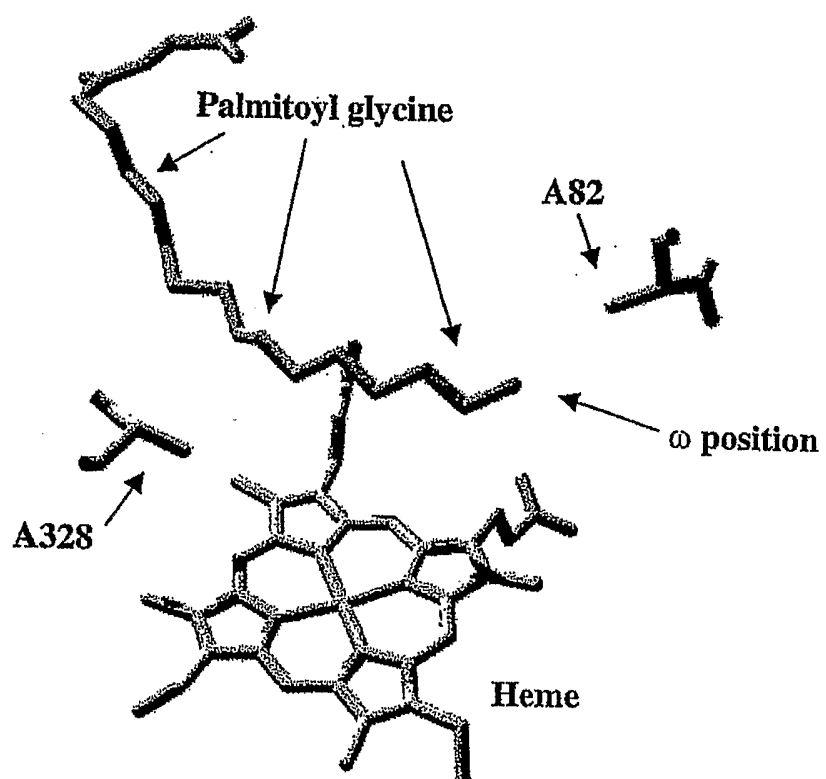
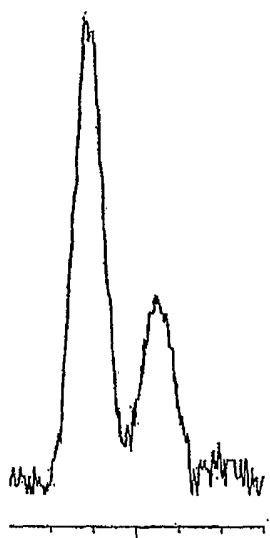


FIG.4

a)



b)

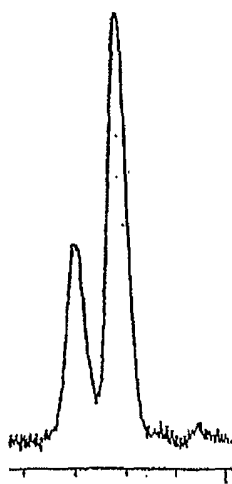


FIG.5

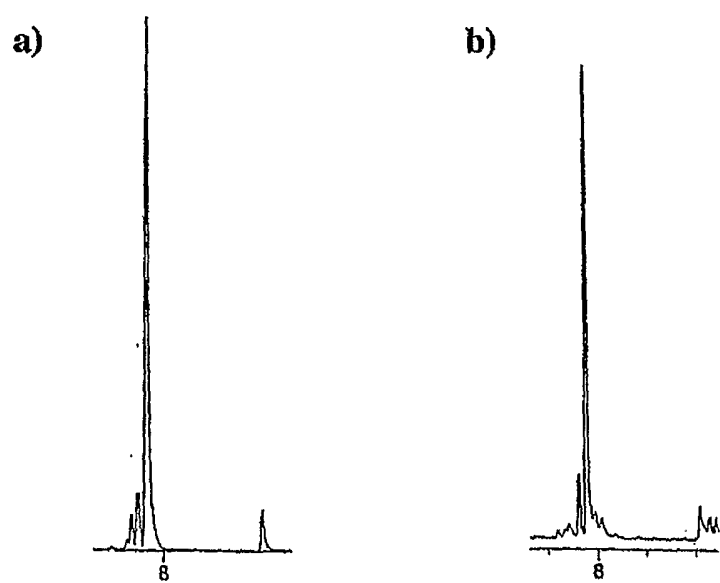


FIG.6

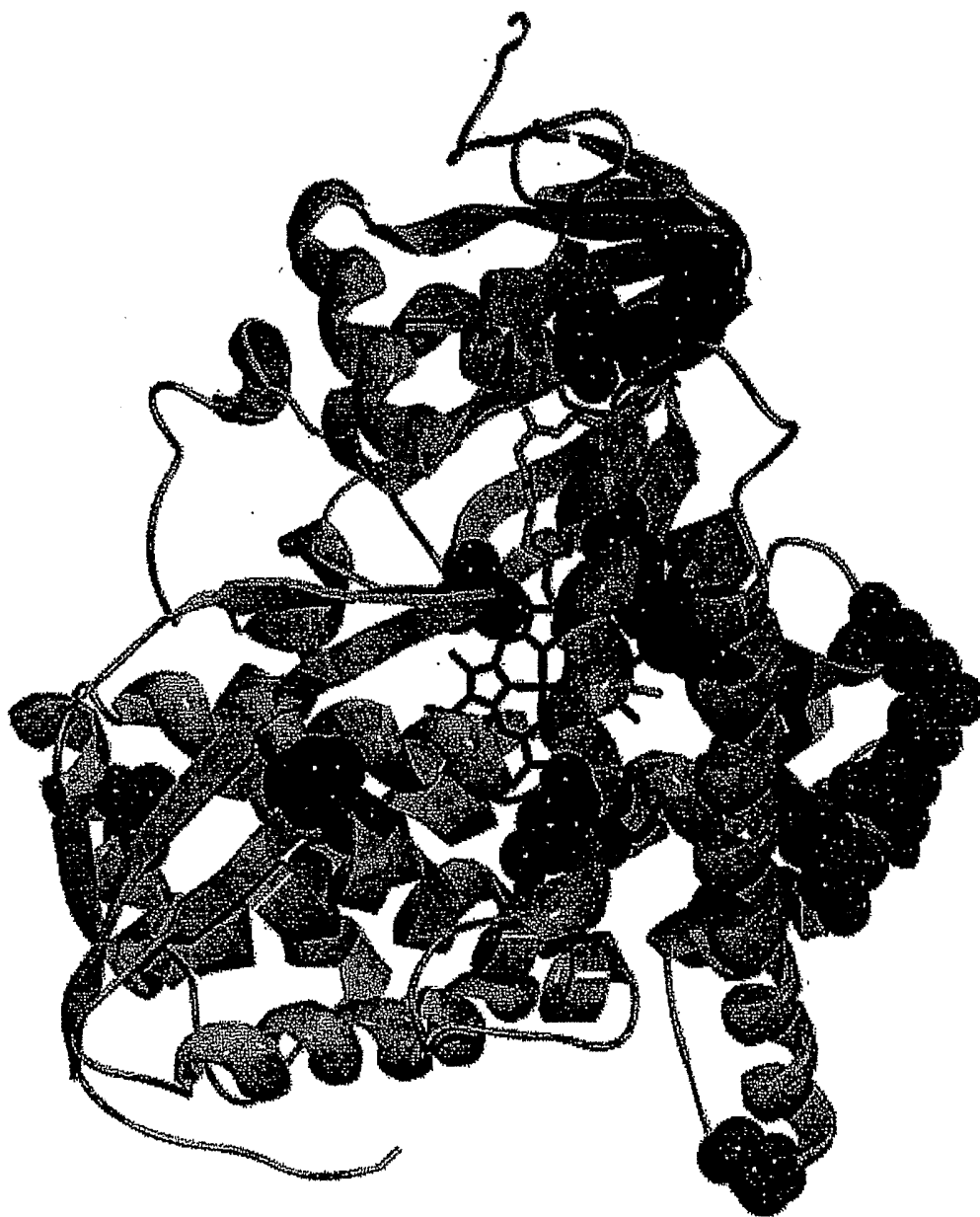


FIG.7

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Meinhold, Peter

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Asp	Ala	Ser	Gln	Gln	Ile	Arg	Leu	Glu	Ala	Glu	Glu	Glu	Lys	Leu	Ala
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His	Leu	Pro	Leu	Ala	Lys	Thr	Val	Ser	Val	Glu	Glu	Leu	Leu	Gln	Tyr
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<212> DNA

<213> Bacillus megaterium

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<211> 1048

<212> PRT

<213> Bacillus megaterium

<400> 4

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Ser Arg Phe Asp Lys Asn Leu Ser Gln Ala Leu Lys Phe Ala Arg Asp
 65           70           75           80
Phe Leu Gly Asp Gly Leu Phe Thr Ser Trp Thr His Glu Ile Asn Trp
      85           90           95
Lys Lys Ala His Asn Ile Leu Leu Pro Ser Phe Ser Gln Gln Ala Met
      100          105          110
Lys Gly Tyr His Ala Met Met Val Asp Ile Ala Val Gln Leu Val Gln
      115          120          125
Lys Trp Glu Arg Leu Asn Ala Asp Glu His Ile Glu Val Ser Glu Asp
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Arg Phe Asn Ser Phe Tyr Arg Asp Gln Pro His Pro Phe Ile Ile Ser
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      180          185          190
Pro Asp Asp Pro Ala Tyr Asp Glu Asn Lys Arg Gln Cys Gln Glu Asp
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Ile Lys Val Met Asn Asp Leu Val Asp Lys Ile Ile Ala Asp Arg Lys
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 Gln Ile Ile Thr Phe Leu Ile Ala Gly His Glu Thr Thr Ser Gly Leu
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 Leu Ser Phe Ala Leu Tyr Phe Leu Val Lys Asn Pro His Val Leu Gln
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 Lys Val Ala Glu Glu Ala Ala Arg Val Leu Val Asp Pro Val Pro Ser
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 Tyr Lys Gln Val Lys Gln Leu Lys Tyr Val Gly Met Val Leu Asn Glu
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 Gln Gln Pro Gly Ser Ala Arg Ser Thr Arg His Leu Glu Ile Glu Leu

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Pro  Lys  Glu  Ala  Ser  Tyr  Gln  Glu  Gly  Asp  His  Leu  Gly  Val  Ile  Pro
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Arg  Asn  Tyr  Glu  Gly  Ile  Val  Asn  Arg  Val  Thr  Ala  Arg  Phe  Gly  Leu
   705                710                715                720
Asp  Ala  Ser  Gln  Gln  Ile  Arg  Leu  Glu  Ala  Glu  Glu  Lys  Leu  Ala
        725                730                735
His  Leu  Pro  Leu  Ala  Lys  Thr  Val  Ser  Val  Glu  Glu  Leu  Leu  Gln  Tyr
        740                745                750
Val  Glu  Leu  Gln  Asp  Pro  Val  Thr  Arg  Thr  Gln  Leu  Arg  Ala  Met  Ala
        755                760                765
Ala  Lys  Thr  Val  Cys  Pro  Pro  His  Lys  Val  Glu  Leu  Glu  Ala  Leu  Leu
   770                775                780
Glu  Lys  Gln  Ala  Tyr  Lys  Glu  Gln  Val  Leu  Ala  Lys  Arg  Leu  Thr  Met
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Leu  Glu  Leu  Leu  Glu  Lys  Tyr  Pro  Ala  Cys  Glu  Met  Lys  Phe  Ser  Glu
        805                810                815
Phe  Ile  Ala  Leu  Leu  Pro  Ser  Ile  Arg  Pro  Arg  Tyr  Tyr  Ser  Ile  Ser
        820                825                830
Ser  Ser  Pro  Arg  Val  Asp  Glu  Lys  Gln  Ala  Ser  Ile  Thr  Val  Ser  Val
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Ser  Asn  Tyr  Leu  Ala  Glu  Leu  Gln  Glu  Gly  Asp  Thr  Ile  Thr  Cys  Phe
   865                870                875                880
Ile  Ser  Thr  Pro  Gln  Ser  Glu  Phe  Thr  Leu  Pro  Lys  Asp  Pro  Glu  Thr
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Glu  Ala  His  Leu  Tyr  Phe  Gly  Cys  Arg  Ser  Pro  His  Glu  Asp  Tyr  Leu
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Tyr  Gln  Glu  Glu  Leu  Glu  Asn  Ala  Gln  Ser  Glu  Gly  Ile  Ile  Thr  Leu
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His  Thr  Ala  Phe  Ser  Arg  Met  Pro  Asn  Gln  Pro  Lys  Thr  Tyr  Val  Gln
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His  Val  Met  Glu  Gln  Asp  Gly  Lys  Lys  Leu  Ile  Glu  Leu  Leu  Asp  Gln
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Gly  Ala  His  Phe  Tyr  Ile  Cys  Gly  Asp  Gly  Ser  Gln  Met  Ala  Pro  Ala
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Val  Glu  Ala  Thr  Leu  Met  Lys  Ser  Tyr  Ala  Asp  Val  His  Gln  Val  Ser
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Glu  Ala  Asp  Ala  Arg  Leu  Trp  Leu  Gln  Gln  Leu  Glu  Glu  Lys  Gly  Arg
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<211> 3144

<212> DNA

<213> Bacillus megaterium

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<211> 1048

<212> PRT

<213> Bacillus megaterium

<400> 6

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      35           40           45
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Ser Arg Phe Asp Lys Asn Leu Ser Gln Ala Leu Lys Phe Ala Arg Asp
      65           70           75           80
Phe Ala Gly Asp Gly Leu Phe Thr Ser Trp Thr His Glu Ile Asn Trp
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Lys Lys Ala His Asn Ile Leu Leu Pro Ser Phe Ser Gln Gln Ala Met
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      115           120           125
Lys Trp Glu Arg Leu Asn Ala Asp Glu His Ile Glu Val Ser Glu Asp
      130           135           140
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Arg Phe Asn Ser Phe Tyr Arg Asp Gln Pro His Pro Phe Ile Ile Ser
      165           170           175
Met Val Arg Ala Leu Asp Glu Val Met Asn Lys Leu Gln Arg Ala Asn
      180           185           190
Pro Asp Asp Pro Ala Tyr Asp Glu Asn Lys Arg Gln Cys Gln Glu Asp
      195           200           205
Ile Lys Val Met Asn Asp Leu Val Asp Lys Ile Ile Ala Asp Arg Lys
      210           215           220
Ala Arg Gly Glu Gln Ser Asp Asp Leu Leu Thr Gln Met Leu Asn Gly
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Lys Asp Pro Glu Thr Gly Glu Pro Leu Asp Asp Gly Asn Ile Ser Tyr
      245           250           255
Gln Ile Ile Thr Phe Leu Ile Ala Gly His Glu Thr Thr Ser Gly Leu
      260           265           270
Leu Ser Phe Ala Leu Tyr Phe Leu Val Lys Asn Pro His Val Leu Gln
      275           280           285
Lys Val Ala Glu Glu Ala Ala Arg Val Leu Val Asp Pro Val Pro Ser
      290           295           300
Tyr Lys Gln Val Lys Gln Leu Lys Tyr Val Gly Met Val Leu Asn Glu
      305           310           315           320
Ala Leu Arg Leu Trp Pro Thr Ala Pro Ala Phe Ser Leu Tyr Ala Lys
      325           330           335
Glu Asp Thr Val Leu Gly Gly Glu Tyr Pro Leu Glu Lys Gly Asp Glu
      340           345           350
Val Met Val Leu Ile Pro Gln Leu His Arg Asp Lys Thr Ile Trp Gly
      355           360           365
Asp Asp Val Glu Glu Phe Arg Pro Glu Arg Phe Glu Asn Pro Ser Ala
      370           375           380
Ile Pro Gln His Ala Phe Lys Pro Phe Gly Asn Gly Gln Arg Ala Cys
      385           390           395           400
Ile Gly Gln Gln Phe Ala Leu His Glu Ala Thr Leu Val Leu Gly Met
      405           410           415
Met Leu Lys His Phe Asp Phe Glu Asp His Thr Asn Tyr Glu Leu Asp
      420           425           430
Ile Lys Glu Thr Leu Thr Leu Lys Pro Glu Gly Phe Val Val Lys Ala
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 675 680 685
 Pro Lys Glu Ala Ser Tyr Gln Glu Gly Asp His Leu Gly Val Ile Pro
 690 695 700
 Arg Asn Tyr Glu Gly Ile Val Asn Arg Val Thr Ala Arg Phe Gly Leu
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 His Leu Pro Leu Ala Lys Thr Val Ser Val Glu Glu Leu Leu Gln Tyr
 740 745 750
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 755 760 765
 Ala Lys Thr Val Cys Pro Pro His Lys Val Glu Leu Glu Ala Leu Leu
 770 775 780
 Glu Lys Gln Ala Tyr Lys Glu Gln Val Leu Ala Lys Arg Leu Thr Met
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 865 870 875 880
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<212> DNA
<213> Bacillus megaterium
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<210> 8

<211> 1048

<212> PRT

<213> Bacillus megaterium

<400> 8

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Ala Asp Glu Leu Gly Glu Ile Phe Lys Phe Glu Ala Pro Gly Cys Val
35          40          45
Thr Arg Tyr Leu Ser Ser Gln Arg Leu Ile Lys Glu Ala Cys Asp Glu
50          55          60
Ser Arg Phe Asp Lys Asn Leu Ser Gln Ala Leu Lys Phe Ala Arg Asp
65          70          75          80
Phe Ala Gly Asp Gly Leu Phe Thr Ser Trp Thr His Glu Ile Asn Trp
85          90          95
Lys Lys Ala His Asn Ile Leu Leu Pro Ser Phe Ser Gln Gln Ala Met
100         105         110
Lys Gly Tyr His Ala Met Met Val Asp Ile Ala Val Gln Leu Val Gln
115        120        125
Lys Trp Glu Arg Leu Asn Ala Asp Glu His Ile Glu Val Ser Glu Asp
130        135        140
Met Thr Arg Leu Thr Leu Asp Thr Ile Gly Leu Cys Gly Phe Asn Tyr
145        150        155        160
Arg Phe Asn Ser Phe Tyr Arg Asp Gln Pro His Pro Phe Ile Ile Ser
165        170        175
Met Val Arg Ala Leu Asp Glu Val Met Asn Lys Leu Gln Arg Ala Asn
180        185        190
Pro Asp Asp Pro Ala Tyr Asp Glu Asn Lys Arg Gln Cys Gln Glu Asp
195        200        205
Ile Lys Val Met Asn Asp Leu Val Asp Lys Ile Ile Ala Asp Arg Lys

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210	215	220
Ala Arg Gly Glu Gln Ser Asp Asp Leu Leu Thr Gln Met Leu Asn Gly		
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Leu Ser Phe Ala Leu Tyr Phe Leu Val Lys Asn Pro His Val Leu Gln		270
	275	280
Lys Val Ala Glu Glu Ala Ala Arg Val Leu Val Asp Pro Val Pro Ser		285
	290	295
Tyr Lys Gln Val Lys Gln Leu Lys Tyr Val Gly Met Val Leu Asn Glu		300
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Ala Leu Arg Leu Trp Pro Thr Phe Pro Ala Phe Ser Leu Tyr Ala Lys		320
	325	330
Glu Asp Thr Val Leu Gly Gly Glu Tyr Pro Leu Glu Lys Gly Asp Glu		335
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Val Met Val Leu Ile Pro Gln Leu His Arg Asp Lys Thr Ile Trp Gly		350
	355	360
Asp Asp Val Glu Glu Phe Arg Pro Glu Arg Phe Glu Asn Pro Ser Ala		365
	370	375
Ile Pro Gln His Ala Phe Lys Pro Phe Gly Asn Gly Gln Arg Ala Cys		380
385	390	395
Ile Gly Gln Gln Phe Ala Leu His Glu Ala Thr Leu Val Leu Gly Met		400
	405	410
Met Leu Lys His Phe Asp Phe Glu Asp His Thr Asn Tyr Glu Leu Asp		415
	420	425
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	435	440
Lys Ser Lys Lys Ile Pro Leu Gly Gly Ile Pro Ser Pro Ser Thr Glu		445
	450	455
Gln Ser Ala Lys Lys Val Arg Lys Lys Ala Glu Asn Ala His Asn Thr		460
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Pro Leu Leu Val Leu Tyr Gly Ser Asn Met Gly Thr Ala Glu Gly Thr		480
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Ala Arg Asp Leu Ala Asp Ile Ala Met Ser Lys Gly Phe Ala Pro Gln		495
	500	505
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	515	520
Val Leu Ile Val Thr Ala Ser Tyr Asn Gly His Pro Pro Asp Asn Ala		525
	530	535
Lys Gln Phe Val Asp Trp Leu Asp Gln Ala Ser Ala Asp Glu Val Lys		540
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Gly Val Arg Tyr Ser Val Phe Gly Cys Gly Asp Lys Asn Trp Ala Thr		560
	565	570
Thr Tyr Gln Lys Val Pro Ala Phe Ile Asp Glu Thr Leu Ala Ala Lys		575
	580	585
Gly Ala Glu Asn Ile Ala Asp Arg Gly Glu Ala Asp Ala Ser Asp Asp		590
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Phe Glu Gly Thr Tyr Glu Glu Trp Arg Glu His Met Trp Ser Asp Val		605
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Ala Ala Tyr Phe Asn Leu Asp Ile Glu Asn Ser Glu Asp Asn Lys Ser		620
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Thr Leu Ser Leu Gln Phe Val Asp Ser Ala Ala Asp Met Pro Leu Ala		640
	645	650
Lys Met His Gly Ala Phe Ser Thr Asn Val Val Ala Ser Lys Glu Leu		655
	660	665
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Gln Gln Pro Gly Ser Ala Arg Ser Thr Arg His Leu Glu Ile Glu Leu
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 Pro Lys Glu Ala Ser Tyr Gln Glu Gly Asp His Leu Gly Val Ile Pro
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 Arg Asn Tyr Glu Gly Ile Val Asn Arg Val Thr Ala Arg Phe Gly Leu
 705 710 715 720
 Asp Ala Ser Gln Gln Ile Arg Leu Glu Ala Glu Glu Lys Leu Ala
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 His Leu Pro Leu Ala Lys Thr Val Ser Val Glu Glu Leu Leu Gln Tyr
 740 745 750
 Val Glu Leu Gln Asp Pro Val Thr Arg Thr Gln Leu Arg Ala Met Ala
 755 760 765
 Ala Lys Thr Val Cys Pro Pro His Lys Val Glu Leu Glu Ala Leu Leu
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 Glu Lys Gln Ala Tyr Lys Glu Gln Val Leu Ala Lys Arg Leu Thr Met
 785 790 795 800
 Leu Glu Leu Leu Glu Lys Tyr Pro Ala Cys Glu Met Lys Phe Ser Glu
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 Ser Ser Pro Arg Val Asp Glu Lys Gln Ala Ser Ile Thr Val Ser Val
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 Val Ser Gly Glu Ala Trp Ser Gly Tyr Gly Glu Tyr Lys Gly Ile Ala
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 Ser Asn Tyr Leu Ala Glu Leu Gln Glu Gly Asp Thr Ile Thr Cys Phe
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 Pro Leu Ile Met Val Gly Pro Gly Thr Gly Val Ala Pro Phe Arg Gly
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 Glu Ala His Leu Tyr Phe Gly Cys Arg Ser Pro His Glu Asp Tyr Leu
 930 935 940
 Tyr Gln Glu Glu Leu Glu Asn Ala Gln Ser Glu Gly Ile Ile Thr Leu
 945 950 955 960
 His Thr Ala Phe Ser Arg Met Pro Asn Gln Pro Lys Thr Tyr Val Gln
 965 970 975
 His Val Met Glu Gln Asp Gly Lys Lys Leu Ile Glu Leu Leu Asp Gln
 980 985 990
 Gly Ala His Phe Tyr Ile Cys Gly Asp Gly Ser Gln Met Ala Pro Ala
 995 1000 1005
 Val Glu Ala Thr Leu Met Lys Ser Tyr Ala Asp Val His Gln Val Ser
 1010 1015 1020
 Glu Ala Asp Ala Arg Leu Trp Leu Gln Gln Leu Glu Glu Lys Gly Arg
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 Tyr Ala Lys Asp Val Trp Ala Gly
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<211> 3144

<212> DNA

<213> Bacillus megaterium

<400> 9

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<211> 1048

<212> PRT

<213> Bacillus megaterium

<400> 10

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Ala Asp Glu Leu Gly Glu Ile Phe Lys Phe Glu Ala Pro Gly Cys Val
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Thr Arg Tyr Leu Ser Ser Gln Arg Leu Ile Lys Glu Ala Cys Asp Glu
 50           55           60
Ser Arg Phe Asp Lys Asn Leu Ser Gln Ala Leu Lys Phe Ala Arg Asp
65           70           75           80
Phe Ala Gly Asp Gly Leu Phe Thr Ser Trp Thr His Glu Ile Asn Trp
           85           90           95
Lys Lys Ala His Asn Ile Leu Leu Pro Ser Phe Ser Gln Gln Ala Met
           100          105          110
Lys Gly Tyr His Ala Met Met Val Asp Ile Ala Val Gln Leu Val Gln
           115          120          125
Lys Trp Glu Arg Leu Asn Ala Asp Glu His Ile Glu Val Ser Glu Asp
130          135          140
Met Thr Arg Leu Thr Leu Asp Thr Ile Gly Leu Cys Gly Phe Asn Tyr
145          150          155          160
Arg Phe Asn Ser Phe Tyr Arg Asp Gln Pro His Pro Phe Ile Ile Ser
           165          170          175
Met Val Arg Ala Leu Asp Glu Val Met Asn Lys Leu Gln Arg Ala Asn
           180          185          190
Pro Asp Asp Pro Ala Tyr Asp Glu Asn Lys Arg Gln Cys Gln Glu Asp
           195          200          205
Ile Lys Val Met Asn Asp Leu Val Asp Lys Ile Ile Ala Asp Arg Lys
210          215          220
Ala Arg Gly Glu Gln Ser Asp Asp Leu Leu Thr Gln Met Leu Asn Gly
225          230          235          240
Lys Asp Pro Glu Thr Gly Glu Pro Leu Asp Asp Gly Asn Ile Ser Tyr
           245          250          255
Gln Ile Ile Thr Phe Leu Ile Ala Gly His Glu Thr Thr Ser Gly Leu
           260          265          270
Leu Ser Phe Ala Leu Tyr Phe Leu Val Lys Asn Pro His Val Leu Gln
           275          280          285
Lys Val Ala Glu Glu Ala Ala Arg Val Leu Val Asp Pro Val Pro Ser
290          295          300
Tyr Lys Gln Val Lys Gln Leu Lys Tyr Val Gly Met Val Leu Asn Glu
305          310          315          320
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           325          330          335
Glu Asp Thr Val Leu Gly Gly Glu Tyr Pro Leu Glu Lys Gly Asp Glu
           340          345          350
Val Met Val Leu Ile Pro Gln Leu His Arg Asp Lys Thr Ile Trp Gly
           355          360          365
Asp Asp Val Glu Glu Phe Arg Pro Glu Arg Phe Glu Asn Pro Ser Ala
370          375          380
Ile Pro Gln His Ala Phe Lys Pro Phe Gly Asn Gly Gln Arg Ala Cys
385          390          395          400
Ile Gly Gln Gln Phe Ala Leu His Glu Ala Thr Leu Val Leu Gly Met
           405          410          415
Met Leu Lys His Phe Asp Phe Glu Asp His Thr Asn Tyr Glu Leu Asp
           420          425          430
Ile Lys Glu Thr Leu Thr Leu Lys Pro Glu Gly Phe Val Val Lys Ala

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Glu	Lys	Gln	Ala	Tyr	Lys	Glu	Gln	Val	Leu	Ala	Lys	Arg	Leu	Thr	Met	
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 Glu Ala His Leu Tyr Phe Gly Cys Arg Ser Pro His Glu Asp Tyr Leu
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 Tyr Gln Glu Glu Leu Glu Asn Ala Gln Ser Glu Gly Ile Ile Thr Leu
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 His Thr Ala Phe Ser Arg Met Pro Asn Gln Pro Lys Thr Tyr Val Gln
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 His Val Met Glu Gln Asp Gly Lys Lys Leu Ile Glu Leu Leu Asp Gln
 980 985 990
 Gly Ala His Phe Tyr Ile Cys Gly Asp Gly Ser Gln Met Ala Pro Ala
 995 1000 1005
 Val Glu Ala Thr Leu Met Lys Ser Tyr Ala Asp Val His Gln Val Ser
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 Glu Ala Asp Ala Arg Leu Trp Leu Gln Gln Leu Glu Glu Lys Gly Arg
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 Tyr Ala Lys Asp Val Trp Ala Gly
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<211> 3144

<212> DNA

<213> Bacillus megaterium

<400> 11

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<210> 12

<211> 1048

<212> PRT

<213> Bacillus megaterium

<400> 12

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           35           40           45
Thr Arg Tyr Leu Ser Ser Gln Arg Leu Ile Lys Glu Ala Cys Asp Glu
50           55           60
Ser Arg Phe Asp Lys Asn Leu Ser Gln Ala Leu Lys Phe Ala Arg Asp
65           70           75           80
Phe Ala Gly Asp Gly Leu Phe Thr Ser Trp Thr His Glu Ile Asn Trp
           85           90           95
Lys Lys Ala His Asn Ile Leu Leu Pro Ser Phe Ser Gln Gln Ala Met
           100          105          110
Lys Gly Tyr His Ala Met Met Val Asp Ile Ala Val Gln Leu Val Gln
           115          120          125
Lys Trp Glu Arg Leu Asn Ala Asp Glu His Ile Glu Val Ser Glu Asp
130          135          140
Met Thr Arg Leu Thr Leu Asp Thr Ile Gly Leu Cys Gly Phe Asn Tyr
145          150          155          160
Arg Phe Asn Ser Phe Tyr Arg Asp Gln Pro His Pro Phe Ile Ile Ser
           165          170          175
Met Val Arg Ala Leu Asp Glu Val Met Asn Lys Leu Gln Arg Ala Asn
           180          185          190
Pro Asp Asp Pro Ala Tyr Asp Glu Asn Lys Arg Gln Cys Gln Glu Asp
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                245                250                255
Gln Ile Ile Thr Phe Leu Ile Ala Gly His Glu Thr Thr Ser Gly Leu
                260                265                270
Leu Ser Phe Ala Leu Tyr Phe Leu Val Lys Asn Pro His Val Leu Gln
                275                280                285
Lys Val Ala Glu Glu Ala Ala Arg Val Leu Val Asp Pro Val Pro Ser
290                295                300
Tyr Lys Gln Val Lys Gln Leu Lys Tyr Val Gly Met Val Leu Asn Glu
305                310                315                320
Ala Leu Arg Leu Trp Pro Thr Met Pro Ala Phe Ser Leu Tyr Ala Lys
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                405                410                415
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465                470                475                480
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Lys Gln Phe Val Asp Trp Leu Asp Gln Ala Ser Ala Asp Glu Val Lys
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595                600                605
Phe Glu Gly Thr Tyr Glu Glu Trp Arg Glu His Met Trp Ser Asp Val
610                615                620
Ala Ala Tyr Phe Asn Leu Asp Ile Glu Asn Ser Glu Asp Asn Lys Ser
625                630                635                640
Thr Leu Ser Leu Gln Phe Val Asp Ser Ala Ala Asp Met Pro Leu Ala
                645                650                655
Lys Met His Gly Ala Phe Ser Thr Asn Val Val Ala Ser Lys Glu Leu

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 Gln Gln Pro Gly Ser Ala Arg Ser Thr Arg His Leu Glu Ile Glu Leu
 675 680 685
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 690 695 700
 Arg Asn Tyr Glu Gly Ile Val Asn Arg Val Thr Ala Arg Phe Gly Leu
 705 710 715 720
 Asp Ala Ser Gln Gln Ile Arg Leu Glu Ala Glu Glu Lys Leu Ala
 725 730 735
 His Leu Pro Leu Ala Lys Thr Val Ser Val Glu Glu Leu Leu Gln Tyr
 740 745 750
 Val Glu Leu Gln Asp Pro Val Thr Arg Thr Gln Leu Arg Ala Met Ala
 755 760 765
 Ala Lys Thr Val Cys Pro Pro His Lys Val Glu Leu Glu Ala Leu Leu
 770 775 780
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 Phe Ile Ala Leu Leu Pro Ser Ile Arg Pro Arg Tyr Tyr Ser Ile Ser
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 Ser Ser Pro Arg Val Asp Glu Lys Gln Ala Ser Ile Thr Val Ser Val
 835 840 845
 Val Ser Gly Glu Ala Trp Ser Gly Tyr Gly Glu Tyr Lys Gly Ile Ala
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 945 950 955 960
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 Gly Ala His Phe Tyr Ile Cys Gly Asp Gly Ser Gln Met Ala Pro Ala
 995 1000 1005
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 Tyr Ala Lys Asp Val Trp Ala Gly
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<210> 13

<211> 3144

<212> DNA

<213> Bacillus megaterium

<400> 13

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<211> 1048

<212> PRT

<213> Bacillus megaterium

<400> 14

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Thr Arg Tyr Leu Ser Ser Gln Arg Leu Ile Lys Glu Ala Cys Asp Glu
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Ser Arg Phe Asp Lys Asn Leu Ser Gln Ala Leu Lys Phe Ala Arg Asp
 65           70           75           80
Phe Ala Gly Asp Gly Leu Phe Thr Ser Trp Thr His Glu Ile Asn Trp
      85           90           95
Lys Lys Ala His Asn Ile Leu Leu Pro Ser Phe Ser Gln Gln Ala Met
      100           105           110
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      115           120           125
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 130           135           140
Met Thr Arg Leu Thr Leu Asp Thr Ile Gly Leu Cys Gly Phe Asn Tyr
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Arg Phe Asn Ser Phe Tyr Arg Asp Gln Pro His Pro Phe Ile Ile Ser
      165           170           175
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      180           185           190
Pro Asp Asp Pro Ala Tyr Asp Glu Asn Lys Arg Gln Cys Gln Glu Asp
      195           200           205
Ile Lys Val Met Asn Asp Leu Val Asp Lys Ile Ile Ala Asp Arg Lys
 210           215           220
Ala Arg Gly Glu Gln Ser Asp Asp Leu Leu Thr Gln Met Leu Asn Gly
 225           230           235           240
Lys Asp Pro Glu Thr Gly Glu Pro Leu Asp Asp Gly Asn Ile Ser Tyr
      245           250           255
Gln Ile Ile Thr Phe Leu Ile Ala Gly His Glu Thr Thr Ser Gly Leu
      260           265           270
Leu Ser Phe Ala Leu Tyr Phe Leu Val Lys Asn Pro His Val Leu Gln
      275           280           285
Lys Val Ala Glu Glu Ala Ala Arg Val Leu Val Asp Pro Val Pro Ser
      290           295           300
Tyr Lys Gln Val Lys Gln Leu Lys Tyr Val Gly Met Val Leu Asn Glu
 305           310           315           320
Ala Leu Arg Leu Trp Pro Thr Val Pro Ala Phe Ser Leu Tyr Ala Lys
      325           330           335
Glu Asp Thr Val Leu Gly Gly Glu Tyr Pro Leu Glu Lys Gly Asp Glu
      340           345           350
Val Met Val Leu Ile Pro Gln Leu His Arg Asp Lys Thr Ile Trp Gly
      355           360           365
Asp Asp Val Glu Glu Phe Arg Pro Glu Arg Phe Glu Asn Pro Ser Ala
      370           375           380
Ile Pro Gln His Ala Phe Lys Pro Phe Gly Asn Gly Gln Arg Ala Cys
 385           390           395           400
Ile Gly Gln Gln Phe Ala Leu His Glu Ala Thr Leu Val Leu Gly Met
      405           410           415
Met Leu Lys His Phe Asp Phe Glu Asp His Thr Asn Tyr Glu Leu Asp
      420           425           430

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Ile Lys Glu Thr Leu Thr Leu Lys Pro Glu Gly Phe Val Val Lys Ala
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Lys Ser Lys Lys Ile Pro Leu Gly Gly Ile Pro Ser Pro Ser Thr Glu
    450                455                460
Gln Ser Ala Lys Lys Val Arg Lys Lys Ala Glu Asn Ala His Asn Thr
    465                470                475                480
Pro Leu Leu Val Leu Tyr Gly Ser Asn Met Gly Thr Ala Glu Gly Thr
    485                490                495
Ala Arg Asp Leu Ala Asp Ile Ala Met Ser Lys Gly Phe Ala Pro Gln
    500                505                510
Val Ala Thr Leu Asp Ser His Ala Gly Asn Leu Pro Arg Glu Gly Ala
    515                520                525
Val Leu Ile Val Thr Ala Ser Tyr Asn Gly His Pro Pro Asp Asn Ala
    530                535                540
Lys Gln Phe Val Asp Trp Leu Asp Gln Ala Ser Ala Asp Glu Val Lys
    545                550                555                560
Gly Val Arg Tyr Ser Val Phe Gly Cys Gly Asp Lys Asn Trp Ala Thr
    565                570                575
Thr Tyr Gln Lys Val Pro Ala Phe Ile Asp Glu Thr Leu Ala Ala Lys
    580                585                590
Gly Ala Glu Asn Ile Ala Asp Arg Gly Glu Ala Asp Ala Ser Asp Asp
    595                600                605
Phe Glu Gly Thr Tyr Glu Glu Trp Arg Glu His Met Trp Ser Asp Val
    610                615                620
Ala Ala Tyr Phe Asn Leu Asp Ile Glu Asn Ser Glu Asp Asn Lys Ser
    625                630                635                640
Thr Leu Ser Leu Gln Phe Val Asp Ser Ala Ala Asp Met Pro Leu Ala
    645                650                655
Lys Met His Gly Ala Phe Ser Thr Asn Val Val Ala Ser Lys Glu Leu
    660                665                670
Gln Gln Pro Gly Ser Ala Arg Ser Thr Arg His Leu Glu Ile Glu Leu
    675                680                685
Pro Lys Glu Ala Ser Tyr Gln Glu Gly Asp His Leu Gly Val Ile Pro
    690                695                700
Arg Asn Tyr Glu Gly Ile Val Asn Arg Val Thr Ala Arg Phe Gly Leu
    705                710                715                720
Asp Ala Ser Gln Gln Ile Arg Leu Glu Ala Glu Glu Lys Leu Ala
    725                730                735
His Leu Pro Leu Ala Lys Thr Val Ser Val Glu Glu Leu Leu Gln Tyr
    740                745                750
Val Glu Leu Gln Asp Pro Val Thr Arg Thr Gln Leu Arg Ala Met Ala
    755                760                765
Ala Lys Thr Val Cys Pro Pro His Lys Val Glu Leu Glu Ala Leu Leu
    770                775                780
Glu Lys Gln Ala Tyr Lys Glu Gln Val Leu Ala Lys Arg Leu Thr Met
    785                790                795                800
Leu Glu Leu Leu Glu Lys Tyr Pro Ala Cys Glu Met Lys Phe Ser Glu
    805                810                815
Phe Ile Ala Leu Leu Pro Ser Ile Arg Pro Arg Tyr Tyr Ser Ile Ser
    820                825                830
Ser Ser Pro Arg Val Asp Glu Lys Gln Ala Ser Ile Thr Val Ser Val
    835                840                845
Val Ser Gly Glu Ala Trp Ser Gly Tyr Gly Glu Tyr Lys Gly Ile Ala
    850                855                860
Ser Asn Tyr Leu Ala Glu Leu Gln Glu Gly Asp Thr Ile Thr Cys Phe
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<210> 15
<211> 3144
<212> DNA
<213> Bacillus megaterium
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27

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gacggaagcc aaatggcacc tgccgttgaa gcaacgctta tgaaaagcta tgctgacgtt 3060
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<210> 16

<211> 1048

<212> PRT

<213> Bacillus megaterium

<400> 16

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 20           25           30
Ala Asp Glu Leu Gly Glu Ile Phe Lys Phe Glu Ala Pro Gly Cys Val
 35           40           45
Thr Arg Tyr Leu Ser Ser Gln Arg Leu Ile Lys Glu Ala Cys Asp Glu
 50           55           60
Ser Arg Phe Asp Lys Asn Leu Ser Gln Ala Leu Lys Phe Phe Arg Asp
 65           70           75           80
Phe Ala Gly Asp Gly Leu Phe Thr Ser Trp Thr His Glu Ile Asn Trp
 85           90           95
Lys Lys Ala His Asn Ile Leu Leu Pro Ser Phe Ser Gln Gln Ala Met
 100          105          110
Lys Gly Tyr His Ala Met Met Val Asp Ile Ala Val Gln Leu Val Gln
 115          120          125
Lys Trp Glu Arg Leu Asn Ala Asp Glu His Ile Glu Val Ser Glu Asp
 130          135          140
Met Thr Arg Leu Thr Leu Asp Thr Ile Gly Leu Cys Gly Phe Asn Tyr
 145          150          155          160
Arg Phe Asn Ser Phe Tyr Arg Asp Gln Pro His Pro Phe Ile Ile Ser
 165          170          175
Met Val Arg Ala Leu Asp Glu Val Met Asn Lys Leu Gln Arg Ala Asn
 180          185          190
Pro Asp Asp Pro Ala Tyr Asp Glu Asn Lys Arg Gln Cys Gln Glu Asp

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Ile	Lys	Val	Met	Asn	Asp	Leu	Val	Asp	Lys	Ile	Ile	Ala	Asp	Arg	Lys	
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Ala	Arg	Gly	Glu	Gln	Ser	Asp	Asp	Leu	Leu	Thr	Gln	Met	Leu	Asn	Gly	
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Lys	Asp	Pro	Glu	Thr	Gly	Glu	Pro	Leu	Asp	Asp	Gly	Asn	Ile	Ser	Tyr	
				245					250					255		
Gln	Ile	Ile	Thr	Phe	Leu	Ile	Ala	Gly	His	Glu	Thr	Thr	Ser	Gly	Leu	
			260					265					270			
Leu	Ser	Phe	Ala	Leu	Tyr	Phe	Leu	Val	Lys	Asn	Pro	His	Val	Leu	Gln	
		275					280					285				
Lys	Val	Ala	Glu	Glu	Ala	Ala	Arg	Val	Leu	Val	Asp	Pro	Val	Pro	Ser	
	290					295					300					
Tyr	Lys	Gln	Val	Lys	Gln	Leu	Lys	Tyr	Val	Gly	Met	Val	Leu	Asn	Glu	
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Ala	Leu	Arg	Leu	Trp	Pro	Thr	Ala	Pro	Ala	Phe	Ser	Leu	Tyr	Ala	Lys	
				325					330					335		
Glu	Asp	Thr	Val	Leu	Gly	Gly	Glu	Tyr	Pro	Leu	Glu	Lys	Gly	Asp	Glu	
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Val	Met	Val	Leu	Ile	Pro	Gln	Leu	His	Arg	Asp	Lys	Thr	Ile	Trp	Gly	
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Asp	Asp	Val	Glu	Glu	Phe	Arg	Pro	Glu	Arg	Phe	Glu	Asn	Pro	Ser	Ala	
	370					375					380					
Ile	Pro	Gln	His	Ala	Phe	Lys	Pro	Phe	Gly	Asn	Gly	Gln	Arg	Ala	Cys	
385					390					395					400	
Ile	Gly	Gln	Gln	Phe	Ala	Leu	His	Glu	Ala	Thr	Leu	Val	Leu	Gly	Met	
				405					410					415		
Met	Leu	Lys	His	Phe	Asp	Phe	Glu	Asp	His	Thr	Asn	Tyr	Glu	Leu	Asp	
			420					425					430			
Ile	Lys	Glu	Thr	Leu	Thr	Leu	Lys	Pro	Glu	Gly	Phe	Val	Val	Lys	Ala	
		435					440					445				
Lys	Ser	Lys	Lys	Ile	Pro	Leu	Gly	Gly	Ile	Pro	Ser	Pro	Ser	Thr	Glu	
	450					455					460					
Gln	Ser	Ala	Lys	Lys	Val	Arg	Lys	Lys	Ala	Glu	Asn	Ala	His	Asn	Thr	
465					470					475					480	
Pro	Leu	Leu	Val	Leu	Tyr	Gly	Ser	Asn	Met	Gly	Thr	Ala	Glu	Gly	Thr	
				485					490					495		
Ala	Arg	Asp	Leu	Ala	Asp	Ile	Ala	Met	Ser	Lys	Gly	Phe	Ala	Pro	Gln	
			500					505					510			
Val	Ala	Thr	Leu	Asp	Ser	His	Ala	Gly	Asn	Leu	Pro	Arg	Glu	Gly	Ala	
		515					520					525				
Val	Leu	Ile	Val	Thr	Ala	Ser	Tyr	Asn	Gly	His	Pro	Pro	Asp	Asn	Ala	
	530					535					540					
Lys	Gln	Phe	Val	Asp	Trp	Leu	Asp	Gln	Ala	Ser	Ala	Asp	Glu	Val	Lys	
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Lys Met His Gly Ala Phe Ser Thr Asn Val Val Ala Ser Lys Glu Leu
 660 665 670
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 675 680 685
 Pro Lys Glu Ala Ser Tyr Gln Glu Gly Asp His Leu Gly Val Ile Pro
 690 695 700
 Arg Asn Tyr Glu Gly Ile Val Asn Arg Val Thr Ala Arg Phe Gly Leu
 705 710 715 720
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 725 730 735
 His Leu Pro Leu Ala Lys Thr Val Ser Val Glu Glu Leu Leu Gln Tyr
 740 745 750
 Val Glu Leu Gln Asp Pro Val Thr Arg Thr Gln Leu Arg Ala Met Ala
 755 760 765
 Ala Lys Thr Val Cys Pro Pro His Lys Val Glu Leu Glu Ala Leu Leu
 770 775 780
 Glu Lys Gln Ala Tyr Lys Glu Gln Val Leu Ala Lys Arg Leu Thr Met
 785 790 795 800
 Leu Glu Leu Leu Glu Lys Tyr Pro Ala Cys Glu Met Lys Phe Ser Glu
 805 810 815
 Phe Ile Ala Leu Leu Pro Ser Ile Arg Pro Arg Tyr Tyr Ser Ile Ser
 820 825 830
 Ser Ser Pro Arg Val Asp Glu Lys Gln Ala Ser Ile Thr Val Ser Val
 835 840 845
 Val Ser Gly Glu Ala Trp Ser Gly Tyr Gly Glu Tyr Lys Gly Ile Ala
 850 855 860
 Ser Asn Tyr Leu Ala Glu Leu Gln Glu Gly Asp Thr Ile Thr Cys Phe
 865 870 875 880
 Ile Ser Thr Pro Gln Ser Glu Phe Thr Leu Pro Lys Asp Pro Glu Thr
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 Pro Leu Ile Met Val Gly Pro Gly Thr Gly Val Ala Pro Phe Arg Gly
 900 905 910
 Phe Val Gln Ala Arg Lys Gln Leu Lys Glu Gln Gly Gln Ser Leu Gly
 915 920 925
 Glu Ala His Leu Tyr Phe Gly Cys Arg Ser Pro His Glu Asp Tyr Leu
 930 935 940
 Tyr Gln Glu Glu Leu Glu Asn Ala Gln Ser Glu Gly Ile Ile Thr Leu
 945 950 955 960
 His Thr Ala Phe Ser Arg Met Pro Asn Gln Pro Lys Thr Tyr Val Gln
 965 970 975
 His Val Met Glu Gln Asp Gly Lys Lys Leu Ile Glu Leu Leu Asp Gln
 980 985 990
 Gly Ala His Phe Tyr Ile Cys Gly Asp Gly Ser Gln Met Ala Pro Ala
 995 1000 1005
 Val Glu Ala Thr Leu Met Lys Ser Tyr Ala Asp Val His Gln Val Ser
 1010 1015 1020
 Glu Ala Asp Ala Arg Leu Trp Leu Gln Gln Leu Glu Glu Lys Gly Arg
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 Tyr Ala Lys Asp Val Trp Ala Gly
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<210> 17

<211> 3144

<212> DNA

<213> Bacillus megaterium

<400> 17

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<210> 18

<211> 1048

<212> PRT

<213> Bacillus megaterium

<400> 18

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Ala Asp Glu Leu Gly Glu Ile Phe Lys Phe Glu Ala Pro Gly Cys Val
      35           40           45
Thr Arg Tyr Leu Ser Ser Gln Arg Leu Ile Lys Glu Ala Cys Asp Glu
      50           55           60
Ser Arg Phe Asp Lys Asn Leu Ser Gln Ala Leu Lys Phe Ser Arg Asp
      65           70           75           80
Phe Ala Gly Asp Gly Leu Phe Thr Ser Trp Thr His Glu Ile Asn Trp
      85           90           95
Lys Lys Ala His Asn Ile Leu Leu Pro Ser Phe Ser Gln Gln Ala Met
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Lys Gly Tyr His Ala Met Met Val Asp Ile Ala Val Gln Leu Val Gln
      115          120          125
Lys Trp Glu Arg Leu Asn Ala Asp Glu His Ile Glu Val Ser Glu Asp
      130          135          140
Met Thr Arg Leu Thr Leu Asp Thr Ile Gly Leu Cys Gly Phe Asn Tyr
      145          150          155          160
Arg Phe Asn Ser Phe Tyr Arg Asp Gln Pro His Pro Phe Ile Ile Ser
      165          170          175
Met Val Arg Ala Leu Asp Glu Val Met Asn Lys Leu Gln Arg Ala Asn
      180          185          190
Pro Asp Asp Pro Ala Tyr Asp Glu Asn Lys Arg Gln Cys Gln Glu Asp
      195          200          205
Ile Lys Val Met Asn Asp Leu Val Asp Lys Ile Ile Ala Asp Arg Lys
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Ala Arg Gly Glu Gln Ser Asp Asp Leu Leu Thr Gln Met Leu Asn Gly
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Lys Asp Pro Glu Thr Gly Glu Pro Leu Asp Asp Gly Asn Ile Ser Tyr
      245          250          255
Gln Ile Ile Thr Phe Leu Ile Ala Gly His Glu Thr Thr Ser Gly Leu
      260          265          270
Leu Ser Phe Ala Leu Tyr Phe Leu Val Lys Asn Pro His Val Leu Gln
      275          280          285
Lys Val Ala Glu Glu Ala Ala Arg Val Leu Val Asp Pro Val Pro Ser
      290          295          300
Tyr Lys Gln Val Lys Gln Leu Lys Tyr Val Gly Met Val Leu Asn Glu
      305          310          315          320
Ala Leu Arg Leu Trp Pro Thr Ala Pro Ala Phe Ser Leu Tyr Ala Lys
      325          330          335
Glu Asp Thr Val Leu Gly Gly Glu Tyr Pro Leu Glu Lys Gly Asp Glu
      340          345          350
Val Met Val Leu Ile Pro Gln Leu His Arg Asp Lys Thr Ile Trp Gly
      355          360          365
Asp Asp Val Glu Glu Phe Arg Pro Glu Arg Phe Glu Asn Pro Ser Ala
      370          375          380
Ile Pro Gln His Ala Phe Lys Pro Phe Gly Asn Gly Gln Arg Ala Cys
      385          390          395          400
Ile Gly Gln Gln Phe Ala Leu His Glu Ala Thr Leu Val Leu Gly Met
      405          410          415
Met Leu Lys His Phe Asp Phe Glu Asp His Thr Asn Tyr Glu Leu Asp

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			420							425							430						
Ile	Lys		Glu	Thr	Leu	Thr	Leu	Lys	Pro	Glu	Gly	Phe	Val	Val	Lys	Ala							
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Lys	Ser	Lys	Lys	Ile	Pro	Leu	Leu	Gly	Gly	Ile	Pro	Ser	Pro	Ser	Thr	Glu							
			450							455							460						
Gln	Ser	Ala	Lys	Lys	Val	Arg	Lys	Lys	Ala	Glu	Asn	Ala	His	Asn	Thr								
465					470							475							480				
Pro	Leu	Leu	Val	Leu	Tyr	Gly	Ser	Asn	Met	Gly	Thr	Ala	Glu	Gly	Thr								
					485							490							495				
Ala	Arg	Asp	Leu	Ala	Asp	Ile	Ala	Met	Ser	Lys	Gly	Phe	Ala	Pro	Gln								
			500							505							510						
Val	Ala	Thr	Leu	Asp	Ser	His	Ala	Gly	Asn	Leu	Pro	Arg	Glu	Gly	Ala								
			515							520							525						
Val	Leu	Ile	Val	Thr	Ala	Ser	Tyr	Asn	Gly	His	Pro	Pro	Asp	Asn	Ala								
			530							535							540						
Lys	Gln	Phe	Val	Asp	Trp	Leu	Asp	Gln	Ala	Ser	Ala	Asp	Glu	Val	Lys								
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Gly	Val	Arg	Tyr	Ser	Val	Phe	Gly	Cys	Gly	Asp	Lys	Asn	Trp	Ala	Thr								
					565							570							575				
Thr	Tyr	Gln	Lys	Val	Pro	Ala	Phe	Ile	Asp	Glu	Thr	Leu	Ala	Ala	Lys								
			580							585							590						
Gly	Ala	Glu	Asn	Ile	Ala	Asp	Arg	Gly	Glu	Ala	Asp	Ala	Ser	Asp	Asp								
			595							600							605						
Phe	Glu	Gly	Thr	Tyr	Glu	Glu	Trp	Arg	Glu	His	Met	Trp	Ser	Asp	Val								
			610							615							620						
Ala	Ala	Tyr	Phe	Asn	Leu	Asp	Ile	Glu	Asn	Ser	Glu	Asp	Asn	Lys	Ser								
625					630							635							640				
Thr	Leu	Ser	Leu	Gln	Phe	Val	Asp	Ser	Ala	Ala	Asp	Met	Pro	Leu	Ala								
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Lys	Met	His	Gly	Ala	Phe	Ser	Thr	Asn	Val	Val	Ala	Ser	Lys	Glu	Leu								
			660							665							670						
Gln	Gln	Pro	Gly	Ser	Ala	Arg	Ser	Thr	Arg	His	Leu	Glu	Ile	Glu	Leu								
			675							680							685						
Pro	Lys	Glu	Ala	Ser	Tyr	Gln	Glu	Gly	Asp	His	Leu	Gly	Val	Ile	Pro								
			690							695							700						
Arg	Asn	Tyr	Glu	Gly	Ile	Val	Asn	Arg	Val	Thr	Ala	Arg	Phe	Gly	Leu								
705					710							715							720				
Asp	Ala	Ser	Gln	Gln	Ile	Arg	Leu	Glu	Ala	Glu	Glu	Glu	Lys	Leu	Ala								
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His	Leu	Pro	Leu	Ala	Lys	Thr	Val	Ser	Val	Glu	Glu	Leu	Leu	Gln	Tyr								
			740							745							750						
Val	Glu	Leu	Gln	Asp	Pro	Val	Thr	Arg	Thr	Gln	Leu	Arg	Ala	Met	Ala								
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Ala	Lys	Thr	Val	Cys	Pro	Pro	His	Lys	Val	Glu	Leu	Ala	Leu	Leu	Leu								

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Ile Ser Thr Pro Gln Ser Glu Phe Thr Leu Pro Lys Asp Pro Glu Thr
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Pro Leu Ile Met Val Gly Pro Gly Thr Gly Val Ala Pro Phe Arg Gly
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Phe Val Gln Ala Arg Lys Gln Leu Lys Glu Gln Gly Gln Ser Leu Gly
      915                      920                      925
Glu Ala His Leu Tyr Phe Gly Cys Arg Ser Pro His Glu Asp Tyr Leu
      930                      935                      940
Tyr Gln Glu Glu Leu Glu Asn Ala Gln Ser Glu Gly Ile Ile Thr Leu
      945                      950                      955                      960
His Thr Ala Phe Ser Arg Met Pro Asn Gln Pro Lys Thr Tyr Val Gln
      965                      970                      975
His Val Met Glu Gln Asp Gly Lys Lys Leu Ile Glu Leu Leu Asp Gln
      980                      985                      990
Gly Ala His Phe Tyr Ile Cys Gly Asp Gly Ser Gln Met Ala Pro Ala
      995                      1000                      1005
Val Glu Ala Thr Leu Met Lys Ser Tyr Ala Asp Val His Gln Val Ser
      1010                      1015                      1020
Glu Ala Asp Ala Arg Leu Trp Leu Gln Gln Leu Glu Glu Lys Gly Arg
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Tyr Ala Lys Asp Val Trp Ala Gly
      1045

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<210> 19

<211> 3144

<212> DNA

<213> Bacillus megaterium

<400> 19

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gatatcgccg tgcagcttgt tcaaaagtgg gacgctctaa atgcagatga gcatattgaa 420
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<210> 20

<211> 1048

<212> PRT

<213> Bacillus megaterium

<400> 20

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      20           25           30
Ala Asp Glu Leu Gly Glu Ile Phe Lys Phe Glu Ala Pro Gly Cys Val
      35           40           45
Thr Arg Tyr Leu Ser Ser Gln Arg Leu Ile Lys Glu Ala Cys Asp Glu
      50           55           60
Ser Arg Phe Asp Lys Asn Leu Ser Gln Ala Leu Lys Phe Thr Arg Asp
      65           70           75           80
Phe Ala Gly Asp Gly Leu Phe Thr Ser Trp Thr His Glu Ile Asn Trp
      85           90           95
Lys Lys Ala His Asn Ile Leu Leu Pro Ser Phe Ser Gln Gln Ala Met
      100          105          110
Lys Gly Tyr His Ala Met Met Val Asp Ile Ala Val Gln Leu Val Gln
      115          120          125
Lys Trp Glu Arg Leu Asn Ala Asp Glu His Ile Glu Val Ser Glu Asp
      130          135          140
Met Thr Arg Leu Thr Leu Asp Thr Ile Gly Leu Cys Gly Phe Asn Tyr
      145          150          155          160
Arg Phe Asn Ser Phe Tyr Arg Asp Gln Pro His Pro Phe Ile Ile Ser
      165          170          175
Met Val Arg Ala Leu Asp Glu Val Met Asn Lys Leu Gln Arg Ala Asn
      180          185          190

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Pro Asp Asp Pro Ala Tyr Asp Glu Asn Lys Arg Gln Cys Gln Glu Asp
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Ile Lys Val Met Asn Asp Leu Val Asp Lys Ile Ile Ala Asp Arg Lys
    210      215      220
Ala Arg Gly Glu Gln Ser Asp Asp Leu Leu Thr Gln Met Leu Asn Gly
    225      230      235      240
Lys Asp Pro Glu Thr Gly Glu Pro Leu Asp Asp Gly Asn Ile Ser Tyr
    245      250      255
Gln Ile Ile Thr Phe Leu Ile Ala Gly His Glu Thr Thr Ser Gly Leu
    260      265      270
Leu Ser Phe Ala Leu Tyr Phe Leu Val Lys Asn Pro His Val Leu Gln
    275      280      285
Lys Val Ala Glu Glu Ala Ala Arg Val Leu Val Asp Pro Val Pro Ser
    290      295      300
Tyr Lys Gln Val Lys Gln Leu Lys Tyr Val Gly Met Val Leu Asn Glu
    305      310      315      320
Ala Leu Arg Leu Trp Pro Thr Ala Pro Ala Phe Ser Leu Tyr Ala Lys
    325      330      335
Glu Asp Thr Val Leu Gly Gly Glu Tyr Pro Leu Glu Lys Gly Asp Glu
    340      345      350
Val Met Val Leu Ile Pro Gln Leu His Arg Asp Lys Thr Ile Trp Gly
    355      360      365
Asp Asp Val Glu Glu Phe Arg Pro Glu Arg Phe Glu Asn Pro Ser Ala
    370      375      380
Ile Pro Gln His Ala Phe Lys Pro Phe Gly Asn Gly Gln Arg Ala Cys
    385      390      395      400
Ile Gly Gln Gln Phe Ala Leu His Glu Ala Thr Leu Val Leu Gly Met
    405      410      415
Met Leu Lys His Phe Asp Phe Glu Asp His Thr Asn Tyr Glu Leu Asp
    420      425      430
Ile Lys Glu Thr Leu Thr Leu Lys Pro Glu Gly Phe Val Val Lys Ala
    435      440      445
Lys Ser Lys Lys Ile Pro Leu Gly Gly Ile Pro Ser Pro Ser Thr Glu
    450      455      460
Gln Ser Ala Lys Lys Val Arg Lys Lys Ala Glu Asn Ala His Asn Thr
    465      470      475      480
Pro Leu Leu Val Leu Tyr Gly Ser Asn Met Gly Thr Ala Glu Gly Thr
    485      490      495
Ala Arg Asp Leu Ala Asp Ile Ala Met Ser Lys Gly Phe Ala Pro Gln
    500      505      510
Val Ala Thr Leu Asp Ser His Ala Gly Asn Leu Pro Arg Glu Gly Ala
    515      520      525
Val Leu Ile Val Thr Ala Ser Tyr Asn Gly His Pro Pro Asp Asn Ala
    530      535      540
Lys Gln Phe Val Asp Trp Leu Asp Gln Ala Ser Ala Asp Glu Val Lys
    545      550      555      560
Gly Val Arg Tyr Ser Val Phe Gly Cys Gly Asp Lys Asn Trp Ala Thr
    565      570      575
Thr Tyr Gln Lys Val Pro Ala Phe Ile Asp Glu Thr Leu Ala Ala Lys
    580      585      590
Gly Ala Glu Asn Ile Ala Asp Arg Gly Glu Ala Asp Ala Ser Asp Asp
    595      600      605
Phe Glu Gly Thr Tyr Glu Glu Trp Arg Glu His Met Trp Ser Asp Val
    610      615      620
Ala Ala Tyr Phe Asn Leu Asp Ile Glu Asn Ser Glu Asp Asn Lys Ser
    625      630      635      640
Thr Leu Ser Leu Gln Phe Val Asp Ser Ala Ala Asp Met Pro Leu Ala

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 Lys Met His Gly Ala Phe Ser Thr Asn Val Val Ala Ser Lys Glu Leu
 660 665 670
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 675 680 685
 Pro Lys Glu Ala Ser Tyr Gln Glu Gly Asp His Leu Gly Val Ile Pro
 690 695 700
 Arg Asn Tyr Glu Gly Ile Val Asn Arg Val Thr Ala Arg Phe Gly Leu
 705 710 715 720
 Asp Ala Ser Gln Gln Ile Arg Leu Glu Ala Glu Glu Glu Lys Leu Ala
 725 730 735
 His Leu Pro Leu Ala Lys Thr Val Ser Val Glu Glu Leu Leu Gln Tyr
 740 745 750
 Val Glu Leu Gln Asp Pro Val Thr Arg Thr Gln Leu Arg Ala Met Ala
 755 760 765
 Ala Lys Thr Val Cys Pro Pro His Lys Val Glu Leu Glu Ala Leu Leu
 770 775 780
 Glu Lys Gln Ala Tyr Lys Glu Gln Val Leu Ala Lys Arg Leu Thr Met
 785 790 795 800
 Leu Glu Leu Leu Glu Lys Tyr Pro Ala Cys Glu Met Lys Phe Ser Glu
 805 810 815
 Phe Ile Ala Leu Leu Pro Ser Ile Arg Pro Arg Tyr Tyr Ser Ile Ser
 820 825 830
 Ser Ser Pro Arg Val Asp Glu Lys Gln Ala Ser Ile Thr Val Ser Val
 835 840 845
 Val Ser Gly Glu Ala Trp Ser Gly Tyr Gly Glu Tyr Lys Gly Ile Ala
 850 855 860
 Ser Asn Tyr Leu Ala Glu Leu Gln Glu Gly Asp Thr Ile Thr Cys Phe
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 Ile Ser Thr Pro Gln Ser Glu Phe Thr Leu Pro Lys Asp Pro Glu Thr
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 Glu Ala His Leu Tyr Phe Gly Cys Arg Ser Pro His Glu Asp Tyr Leu
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 Tyr Gln Glu Glu Leu Glu Asn Ala Gln Ser Glu Gly Ile Ile Thr Leu
 945 950 955 960
 His Thr Ala Phe Ser Arg Met Pro Asn Gln Pro Lys Thr Tyr Val Gln
 965 970 975
 His Val Met Glu Gln Asp Gly Lys Lys Leu Ile Glu Leu Leu Asp Gln
 980 985 990
 Gly Ala His Phe Tyr Ile Cys Gly Asp Gly Ser Gln Met Ala Pro Ala
 995 1000 1005
 Val Glu Ala Thr Leu Met Lys Ser Tyr Ala Asp Val His Gln Val Ser
 1010 1015 1020
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 Tyr Ala Lys Asp Val Trp Ala Gly
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<210> 21

<211> 3144

<212> DNA

<213> Bacillus megaterium

<400> 21

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<210> 22

<211> 1048

<212> PRT

<213> Bacillus megaterium

<400> 22

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Ala Asp Glu Leu Gly Glu Ile Phe Lys Phe Glu Ala Pro Gly Cys Val
35      40      45
Thr Arg Tyr Leu Ser Ser Gln Arg Leu Ile Lys Glu Ala Cys Asp Glu
50      55      60
Ser Arg Phe Asp Lys Asn Leu Ser Gln Ala Leu Lys Phe Ala Arg Asp
65      70      75      80
Phe Cys Gly Asp Gly Leu Phe Thr Ser Trp Thr His Glu Ile Asn Trp
85      90      95
Lys Lys Ala His Asn Ile Leu Leu Pro Ser Phe Ser Gln Gln Ala Met
100     105     110
Lys Gly Tyr His Ala Met Met Val Asp Ile Ala Val Gln Leu Val Gln
115     120     125
Lys Trp Glu Arg Leu Asn Ala Asp Glu His Ile Glu Val Ser Glu Asp
130     135     140
Met Thr Arg Leu Thr Leu Asp Thr Ile Gly Leu Cys Gly Phe Asn Tyr
145     150     155     160
Arg Phe Asn Ser Phe Tyr Arg Asp Gln Pro His Pro Phe Ile Ile Ser
165     170     175
Met Val Arg Ala Leu Asp Glu Val Met Asn Lys Leu Gln Arg Ala Asn
180     185     190
Pro Asp Asp Pro Ala Tyr Asp Glu Asn Lys Arg Gln Cys Gln Glu Asp
195     200     205
Ile Lys Val Met Asn Asp Leu Val Asp Lys Ile Ile Ala Asp Arg Lys
210     215     220
Ala Arg Gly Glu Gln Ser Asp Asp Leu Leu Thr Gln Met Leu Asn Gly
225     230     235     240
Lys Asp Pro Glu Thr Gly Glu Pro Leu Asp Asp Gly Asn Ile Ser Tyr
245     250     255
Gln Ile Ile Thr Phe Leu Ile Ala Gly His Glu Thr Thr Ser Gly Leu
260     265     270
Leu Ser Phe Ala Leu Tyr Phe Leu Val Lys Asn Pro His Val Leu Gln
275     280     285
Lys Val Ala Glu Glu Ala Ala Arg Val Leu Val Asp Pro Val Pro Ser
290     295     300
Tyr Lys Gln Val Lys Gln Leu Lys Tyr Val Gly Met Val Leu Asn Glu
305     310     315     320
Ala Leu Arg Leu Trp Pro Thr Ala Pro Ala Phe Ser Leu Tyr Ala Lys
325     330     335
Glu Asp Thr Val Leu Gly Gly Glu Tyr Pro Leu Glu Lys Gly Asp Glu
340     345     350
Val Met Val Leu Ile Pro Gln Leu His Arg Asp Lys Thr Ile Trp Gly
355     360     365
Asp Asp Val Glu Glu Phe Arg Pro Glu Arg Phe Glu Asn Pro Ser Ala
370     375     380
Ile Pro Gln His Ala Phe Lys Pro Phe Gly Asn Gly Gln Arg Ala Cys
385     390     395     400
Ile Gly Gln Gln Phe Ala Leu His Glu Ala Thr Leu Val Leu Gly Met
405     410     415

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Ile	Lys	Glu	Thr	Leu	Thr	Leu	Lys	Pro	Glu	Gly	Phe	Val	Val	Lys	Ala
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Lys	Ser	Lys	Lys	Ile	Pro	Leu	Gly	Gly	Ile	Pro	Ser	Pro	Ser	Thr	Glu
	450					455				460					
Gln	Ser	Ala	Lys	Lys	Val	Arg	Lys	Lys	Ala	Glu	Asn	Ala	His	Asn	Thr
465					470					475					480
Pro	Leu	Leu	Val	Leu	Tyr	Gly	Ser	Asn	Met	Gly	Thr	Ala	Glu	Gly	Thr
			485					490						495	
Ala	Arg	Asp	Leu	Ala	Asp	Ile	Ala	Met	Ser	Lys	Gly	Phe	Ala	Pro	Gln
		500					505						510		
Val	Ala	Thr	Leu	Asp	Ser	His	Ala	Gly	Asn	Leu	Pro	Arg	Glu	Gly	Ala
	515						520					525			
Val	Leu	Ile	Val	Thr	Ala	Ser	Tyr	Asn	Gly	His	Pro	Pro	Asp	Asn	Ala
	530					535				540					
Lys	Gln	Phe	Val	Asp	Trp	Leu	Asp	Gln	Ala	Ser	Ala	Asp	Glu	Val	Lys
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Gly	Val	Arg	Tyr	Ser	Val	Phe	Gly	Cys	Gly	Asp	Lys	Asn	Trp	Ala	Thr
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Thr	Tyr	Gln	Lys	Val	Pro	Ala	Phe	Ile	Asp	Glu	Thr	Leu	Ala	Ala	Lys
		580					585						590		
Gly	Ala	Glu	Asn	Ile	Ala	Asp	Arg	Gly	Glu	Ala	Asp	Ala	Ser	Asp	Asp
	595					600						605			
Phe	Glu	Gly	Thr	Tyr	Glu	Glu	Trp	Arg	Glu	His	Met	Trp	Ser	Asp	Val
610					615					620					
Ala	Ala	Tyr	Phe	Asn	Leu	Asp	Ile	Glu	Asn	Ser	Glu	Asp	Asn	Lys	Ser
625					630					635					640
Thr	Leu	Ser	Leu	Gln	Phe	Val	Asp	Ser	Ala	Ala	Asp	Met	Pro	Leu	Ala
			645					650					655		
Lys	Met	His	Gly	Ala	Phe	Ser	Thr	Asn	Val	Val	Ala	Ser	Lys	Glu	Leu
		660						665					670		
Gln	Gln	Pro	Gly	Ser	Ala	Arg	Ser	Thr	Arg	His	Leu	Glu	Ile	Glu	Leu
		675						680				685			
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Arg	Asn	Tyr	Glu	Gly	Ile	Val	Asn	Arg	Val	Thr	Ala	Arg	Phe	Gly	Leu
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Asp	Ala	Ser	Gln	Gln	Ile	Arg	Leu	Glu	Ala	Glu	Glu	Glu	Lys	Leu	Ala
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His	Leu	Pro	Leu	Ala	Lys	Thr	Val	Ser	Val	Glu	Glu	Leu	Leu	Gln	Tyr
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Leu	Glu	Leu	Leu	Glu	Lys	Tyr	Pro	Ala	Cys	Glu	Met	Lys	Phe	Ser	Glu
			805					810					815		
Phe	Ile	Ala	Leu	Leu	Pro	Ser	Ile	Arg	Pro	Arg	Tyr	Tyr	Ser	Ile	Ser
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Ser	Ser	Pro	Arg	Val	Asp	Glu	Lys	Gln	Ala	Ser	Ile	Thr	Val	Ser	Val
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Pro Leu Ile Met Val Gly Pro Gly Thr Gly Val Ala Pro Phe Arg Gly
          900          905          910
Phe Val Gln Ala Arg Lys Gln Leu Lys Glu Gln Gly Gln Ser Leu Gly
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Glu Ala His Leu Tyr Phe Gly Cys Arg Ser Pro His Glu Asp Tyr Leu
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Tyr Gln Glu Glu Leu Glu Asn Ala Gln Ser Glu Gly Ile Ile Thr Leu
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His Thr Ala Phe Ser Arg Met Pro Asn Gln Pro Lys Thr Tyr Val Gln
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His Val Met Glu Gln Asp Gly Lys Lys Leu Ile Glu Leu Leu Asp Gln
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Gly Ala His Phe Tyr Ile Cys Gly Asp Gly Ser Gln Met Ala Pro Ala
          995          1000          1005
Val Glu Ala Thr Leu Met Lys Ser Tyr Ala Asp Val His Gln Val Ser
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<210> 23

<211> 3144

<212> DNA

<213> *Bacillus megaterium*

<400> 23

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<210> 24

<211> 1048

<212> PRT

<213> *Bacillus megaterium*

<400> 24

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Ala Asp Glu Leu Gly Glu Ile Phe Lys Phe Glu Ala Pro Gly Cys Val
        35           40           45
Thr Arg Tyr Leu Ser Ser Gln Arg Leu Ile Lys Glu Ala Cys Asp Glu
       50           55           60
Ser Arg Phe Asp Lys Asn Leu Ser Gln Ala Leu Lys Phe Ala Arg Asp
      65           70           75           80
Phe Phe Gly Asp Gly Leu Phe Thr Ser Trp Thr His Glu Ile Asn Trp
          85           90           95
Lys Lys Ala His Asn Ile Leu Leu Pro Ser Phe Ser Gln Gln Ala Met
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Lys Gly Tyr His Ala Met Met Val Asp Ile Ala Val Gln Leu Val Gln
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     145          150          155          160
Arg Phe Asn Ser Phe Tyr Arg Asp Gln Pro His Pro Phe Ile Ile Ser
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Met Val Arg Ala Leu Asp Glu Val Met Asn Lys Leu Gln Arg Ala Asn

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Lys	Asp	Pro	Glu	Thr	Gly	Glu	Pro	Leu	Asp	Asp	Gly	Asn	Ile	Ser	Tyr					
			245					250					255							
Gln	Ile	Ile	Thr	Phe	Leu	Ile	Ala	Gly	His	Glu	Thr	Thr	Ser	Gly	Leu					
			260					265					270							
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Lys	Val	Ala	Glu	Glu	Ala	Ala	Arg	Val	Leu	Val	Asp	Pro	Val	Pro	Ser					
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Val	Met	Val	Leu	Ile	Pro	Gln	Leu	His	Arg	Asp	Lys	Thr	Ile	Trp	Gly					
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Asp	Asp	Val	Glu	Glu	Phe	Arg	Pro	Glu	Arg	Phe	Glu	Asn	Pro	Ser	Ala					
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Met	Leu	Lys	His	Phe	Asp	Phe	Glu	Asp	His	Thr	Asn	Tyr	Glu	Leu	Asp					
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Lys	Ser	Lys	Lys	Ile	Pro	Leu	Gly	Gly	Ile	Pro	Ser	Pro	Ser	Thr	Glu					
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 675 680 685
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 690 695 700
 Arg Asn Tyr Glu Gly Ile Val Asn Arg Val Thr Ala Arg Phe Gly Leu
 705 710 715 720
 Asp Ala Ser Gln Gln Ile Arg Leu Glu Ala Glu Glu Glu Lys Leu Ala
 725 730 735
 His Leu Pro Leu Ala Lys Thr Val Ser Val Glu Glu Leu Leu Gln Tyr
 740 745 750
 Val Glu Leu Gln Asp Pro Val Thr Arg Thr Gln Leu Arg Ala Met Ala
 755 760 765
 Ala Lys Thr Val Cys Pro Pro His Lys Val Glu Leu Glu Ala Leu Leu
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<210> 25

<211> 3144

<212> DNA

<213> Bacillus megaterium

<400> 25

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<210> 26
 <211> 1048
 <212> PRT
 <213> Bacillus megaterium

<400> 26

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85      90      95
Lys Lys Ala His Asn Ile Leu Leu Pro Ser Phe Ser Gln Gln Ala Met
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115     120     125
Lys Trp Glu Arg Leu Asn Ala Asp Glu His Ile Glu Val Ser Glu Asp
130     135     140
Met Thr Arg Leu Thr Leu Asp Thr Ile Gly Leu Cys Gly Phe Asn Tyr
145     150     155     160
Arg Phe Asn Ser Phe Tyr Arg Asp Gln Pro His Pro Phe Ile Ile Ser
165     170     175
Met Val Arg Ala Leu Asp Glu Val Met Asn Lys Leu Gln Arg Ala Asn
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Pro Asp Asp Pro Ala Tyr Asp Glu Asn Lys Arg Gln Cys Gln Glu Asp
195     200     205
Ile Lys Val Met Asn Asp Leu Val Asp Lys Ile Ile Ala Asp Arg Lys
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Gln Ile Ile Thr Phe Leu Ile Ala Gly His Glu Thr Thr Ser Gly Leu
260     265     270
Leu Ser Phe Ala Leu Tyr Phe Leu Val Lys Asn Pro His Val Leu Gln
275     280     285
Lys Val Ala Glu Glu Ala Ala Arg Val Leu Val Asp Pro Val Pro Ser
290     295     300
Tyr Lys Gln Val Lys Gln Leu Lys Tyr Val Gly Met Val Leu Asn Glu
305     310     315     320
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Glu Asp Thr Val Leu Gly Gly Glu Tyr Pro Leu Glu Lys Gly Asp Glu
340     345     350
Val Met Val Leu Ile Pro Gln Leu His Arg Asp Lys Thr Ile Trp Gly
355     360     365
Asp Asp Val Glu Glu Phe Arg Pro Glu Arg Phe Glu Asn Pro Ser Ala
370     375     380
Ile Pro Gln His Ala Phe Lys Pro Phe Gly Asn Gly Gln Arg Ala Cys
385     390     395     400
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Lys	Ser	Lys	Lys	Ile	Pro	Leu	Gly	Gly	Ile	Pro	Ser	Pro	Ser	Thr	Glu		
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Gln	Ser	Ala	Lys	Lys	Val	Arg	Lys	Lys	Ala	Glu	Asn	Ala	His	Asn	Thr		
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Pro	Leu	Leu	Val	Leu	Tyr	Gly	Ser	Asn	Met	Gly	Thr	Ala	Glu	Gly	Thr		
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 Glu Ala His Leu Tyr Phe Gly Cys Arg Ser Pro His Glu Asp Tyr Leu
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 His Val Met Glu Gln Asp Gly Lys Lys Leu Ile Glu Leu Leu Asp Gln
 980 985 990
 Gly Ala His Phe Tyr Ile Cys Gly Asp Gly Ser Gln Met Ala Pro Ala
 995 1000 1005
 Val Glu Ala Thr Leu Met Lys Ser Tyr Ala Asp Val His Gln Val Ser
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<211> 3144

<212> DNA

<213> Bacillus megaterium

<400> 27

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<210> 28

<211> 1048

<212> PRT

<213> Bacillus megaterium

<400> 28

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Ala Asp Glu Leu Gly Glu Ile Phe Lys Phe Glu Ala Pro Gly Cys Val
35           40           45
Thr Arg Tyr Leu Ser Ser Gln Arg Leu Ile Lys Glu Ala Cys Asp Glu
50           55           60
Ser Arg Phe Asp Lys Asn Leu Ser Gln Ala Leu Lys Phe Ala Arg Asp
65           70           75           80
Phe Ile Gly Asp Gly Leu Phe Thr Ser Trp Thr His Glu Ile Asn Trp
85           90           95
Lys Lys Ala His Asn Ile Leu Leu Pro Ser Phe Ser Gln Gln Ala Met
100          105          110
Lys Gly Tyr His Ala Met Met Val Asp Ile Ala Val Gln Leu Val Gln
115          120          125
Lys Trp Glu Arg Leu Asn Ala Asp Glu His Ile Glu Val Ser Glu Asp
130          135          140
Met Thr Arg Leu Thr Leu Asp Thr Ile Gly Leu Cys Gly Phe Asn Tyr
145          150          155          160
Arg Phe Asn Ser Phe Tyr Arg Asp Gln Pro His Pro Phe Ile Ile Ser
165          170          175

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Pro Asp Asp Pro Ala Tyr Asp Glu Asn Lys Arg Gln Cys Gln Glu Asp
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Ile Lys Val Met Asn Asp Leu Val Asp Lys Ile Ile Ala Asp Arg Lys
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Ala Arg Gly Glu Gln Ser Asp Asp Leu Leu Thr Gln Met Leu Asn Gly
      225      230      235      240
Lys Asp Pro Glu Thr Gly Glu Pro Leu Asp Asp Gly Asn Ile Ser Tyr
      245      250      255
Gln Ile Ile Thr Phe Leu Ile Ala Gly His Glu Thr Thr Ser Gly Leu
      260      265      270
Leu Ser Phe Ala Leu Tyr Phe Leu Val Lys Asn Pro His Val Leu Gln
      275      280      285
Lys Val Ala Glu Glu Ala Ala Arg Val Leu Val Asp Pro Val Pro Ser
      290      295      300
Tyr Lys Gln Val Lys Gln Leu Lys Tyr Val Gly Met Val Leu Asn Glu
      305      310      315      320
Ala Leu Arg Leu Trp Pro Thr Ala Pro Ala Phe Ser Leu Tyr Ala Lys
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Glu Asp Thr Val Leu Gly Gly Glu Tyr Pro Leu Glu Lys Gly Asp Glu
      340      345      350
Val Met Val Leu Ile Pro Gln Leu His Arg Asp Lys Thr Ile Trp Gly
      355      360      365
Asp Asp Val Glu Glu Phe Arg Pro Glu Arg Phe Glu Asn Pro Ser Ala
      370      375      380
Ile Pro Gln His Ala Phe Lys Pro Phe Gly Asn Gly Gln Arg Ala Cys
      385      390      395      400
Ile Gly Gln Gln Phe Ala Leu His Glu Ala Thr Leu Val Leu Gly Met
      405      410      415
Met Leu Lys His Phe Asp Phe Glu Asp His Thr Asn Tyr Glu Leu Asp
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Ile Lys Glu Thr Leu Thr Leu Lys Pro Glu Gly Phe Val Val Lys Ala
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Lys Ser Lys Lys Ile Pro Leu Gly Gly Ile Pro Ser Pro Ser Thr Glu
      450      455      460
Gln Ser Ala Lys Lys Val Arg Lys Lys Ala Glu Asn Ala His Asn Thr
      465      470      475      480
Pro Leu Leu Val Leu Tyr Gly Ser Asn Met Gly Thr Ala Glu Gly Thr
      485      490      495
Ala Arg Asp Leu Ala Asp Ile Ala Met Ser Lys Gly Phe Ala Pro Gln
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Val Ala Thr Leu Asp Ser His Ala Gly Asn Leu Pro Arg Glu Gly Ala
      515      520      525
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Lys Gln Phe Val Asp Trp Leu Asp Gln Ala Ser Ala Asp Glu Val Lys
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Gly Val Arg Tyr Ser Val Phe Gly Cys Gly Asp Lys Asn Trp Ala Thr
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Thr Tyr Gln Lys Val Pro Ala Phe Ile Asp Glu Thr Leu Ala Ala Lys
      580      585      590
Gly Ala Glu Asn Ile Ala Asp Arg Gly Glu Ala Asp Ala Ser Asp Asp
      595      600      605
Phe Glu Gly Thr Tyr Glu Glu Trp Arg Glu His Met Trp Ser Asp Val
      610      615      620
Ala Ala Tyr Phe Asn Leu Asp Ile Glu Asn Ser Glu Asp Asn Lys Ser

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          660          665          670
Gln Gln Pro Gly Ser Ala Arg Ser Thr Arg His Leu Glu Ile Glu Leu
          675          680          685
Pro Lys Glu Ala Ser Tyr Gln Glu Gly Asp His Leu Gly Val Ile Pro
          690          695          700
Arg Asn Tyr Glu Gly Ile Val Asn Arg Val Thr Ala Arg Phe Gly Leu
705          710          715          720
Asp Ala Ser Gln Gln Ile Arg Leu Glu Ala Glu Glu Glu Lys Leu Ala
          725          730          735
His Leu Pro Leu Ala Lys Thr Val Ser Val Glu Glu Leu Leu Gln Tyr
          740          745          750
Val Glu Leu Gln Asp Pro Val Thr Arg Thr Gln Leu Arg Ala Met Ala
          755          760          765
Ala Lys Thr Val Cys Pro Pro His Lys Val Glu Leu Glu Ala Leu Leu
          770          775          780
Glu Lys Gln Ala Tyr Lys Glu Gln Val Leu Ala Lys Arg Leu Thr Met
785          790          795          800
Leu Glu Leu Leu Glu Lys Tyr Pro Ala Cys Glu Met Lys Phe Ser Glu
          805          810          815
Phe Ile Ala Leu Leu Pro Ser Ile Arg Pro Arg Tyr Tyr Ser Ile Ser
          820          825          830
Ser Ser Pro Arg Val Asp Glu Lys Gln Ala Ser Ile Thr Val Ser Val
          835          840          845
Val Ser Gly Glu Ala Trp Ser Gly Tyr Gly Glu Tyr Lys Gly Ile Ala
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Ser Asn Tyr Leu Ala Glu Leu Gln Glu Gly Asp Thr Ile Thr Cys Phe
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Ile Ser Thr Pro Gln Ser Glu Phe Thr Leu Pro Lys Asp Pro Glu Thr
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Glu Ala His Leu Tyr Phe Gly Cys Arg Ser Pro His Glu Asp Tyr Leu
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Tyr Gln Glu Glu Leu Glu Asn Ala Gln Ser Glu Gly Ile Ile Thr Leu
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His Thr Ala Phe Ser Arg Met Pro Asn Gln Pro Lys Thr Tyr Val Gln
          965          970          975
His Val Met Glu Gln Asp Gly Lys Lys Leu Ile Glu Leu Leu Asp Gln
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Gly Ala His Phe Tyr Ile Cys Gly Asp Gly Ser Gln Met Ala Pro Ala
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Val Glu Ala Thr Leu Met Lys Ser Tyr Ala Asp Val His Gln Val Ser
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Tyr Ala Lys Asp Val Trp Ala Gly
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<210> 29

<211> 3144

<212> DNA

<213> *Bacillus megaterium*

<400> 29

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<210> 30
 <211> 1048
 <212> PRT
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<400> 30

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 35           40           45
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Ser Arg Phe Asp Lys Asn Leu Ser Gln Ala Leu Lys Phe Ala Arg Asp
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Phe Leu Gly Asp Gly Leu Phe Thr Ser Trp Thr His Glu Ile Asn Trp
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Lys Lys Ala His Asn Ile Leu Leu Pro Ser Phe Ser Gln Gln Ala Met
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Lys Gly Tyr His Ala Met Met Val Asp Ile Ala Val Gln Leu Val Gln
 115          120          125
Lys Trp Glu Arg Leu Asn Ala Asp Glu His Ile Glu Val Ser Glu Asp
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Met Thr Arg Leu Thr Leu Asp Thr Ile Gly Leu Cys Gly Phe Asn Tyr
 145          150          155          160
Arg Phe Asn Ser Phe Tyr Arg Asp Gln Pro His Pro Phe Ile Ile Ser
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Pro Asp Asp Pro Ala Tyr Asp Glu Asn Lys Arg Gln Cys Gln Glu Asp
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Ile Lys Val Met Asn Asp Leu Val Asp Lys Ile Ile Ala Asp Arg Lys
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Ala Arg Gly Glu Gln Ser Asp Asp Leu Leu Thr Gln Met Leu Asn Gly
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Lys Asp Pro Glu Thr Gly Glu Pro Leu Asp Asp Gly Asn Ile Ser Tyr
 245          250          255
Gln Ile Ile Thr Phe Leu Ile Ala Gly His Glu Thr Thr Ser Gly Leu
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Leu Ser Phe Ala Leu Tyr Phe Leu Val Lys Asn Pro His Val Leu Gln
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Lys Val Ala Glu Glu Ala Ala Arg Val Leu Val Asp Pro Val Pro Ser
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Tyr Lys Gln Val Lys Gln Leu Lys Tyr Val Gly Met Val Leu Asn Glu
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Ala Leu Arg Leu Trp Pro Thr Ala Pro Ala Phe Ser Leu Tyr Ala Lys
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Glu Asp Thr Val Leu Gly Gly Glu Tyr Pro Leu Glu Lys Gly Asp Glu
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Val Met Val Leu Ile Pro Gln Leu His Arg Asp Lys Thr Ile Trp Gly
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Ile Pro Gln His Ala Phe Lys Pro Phe Gly Asn Gly Gln Arg Ala Cys
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Phe Val Gln Ala Arg Lys Gln Leu Lys Glu Gln Gly Gln Ser Leu Gly
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Glu Ala His Leu Tyr Phe Gly Cys Arg Ser Pro His Glu Asp Tyr Leu
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Tyr Gln Glu Glu Leu Glu Asn Ala Gln Ser Glu Gly Ile Ile Thr Leu
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His Thr Ala Phe Ser Arg Met Pro Asn Gln Pro Lys Thr Tyr Val Gln
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Gly Ala His Phe Tyr Ile Cys Gly Asp Gly Ser Gln Met Ala Pro Ala
      995              1000              1005
Val Glu Ala Thr Leu Met Lys Ser Tyr Ala Asp Val His Gln Val Ser
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<211> 3144

<212> DNA

<213> Bacillus megaterium

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<211> 1048

<212> PRT

<213> Bacillus megaterium

<400> 32

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Ser Arg Phe Asp Lys Asn Leu Ser Gln Ala Leu Lys Phe Ala Arg Asp
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Phe Ser Gly Asp Gly Leu Phe Thr Ser Trp Thr His Glu Ile Asn Trp
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Lys	Asp	Pro	Glu	Thr	Gly	Glu	Pro	Leu	Asp	Asp	Gly	Asn	Ile	Ser	Tyr		
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<211> 3144

<212> DNA

<213> *Bacillus megaterium*

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gacggaagcc aaatggcacc tgccgttgaa gcaacgctta tgaaaagcta tgctgacgtt 3120
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3144

<210> 34

<211> 1048

<212> PRT

<213> Bacillus megaterium

<400> 34

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Leu Pro Leu Leu Asn Thr Asp Lys Pro Val Gln Ala Leu Met Lys Ile
      20           25           30
Ala Asp Glu Leu Gly Glu Ile Phe Lys Phe Glu Ala Pro Gly Cys Val
      35           40           45
Thr Arg Tyr Leu Ser Ser Gln Arg Leu Ile Lys Glu Ala Cys Asp Glu
 50           55           60
Ser Arg Phe Asp Lys Asn Leu Ser Gln Ala Leu Lys Phe Ala Arg Asp
65           70           75           80
Phe Thr Gly Asp Gly Leu Phe Thr Ser Trp Thr His Glu Ile Asn Trp
      85           90           95
Lys Lys Ala His Asn Ile Leu Leu Pro Ser Phe Ser Gln Gln Ala Met
      100          105          110
Lys Gly Tyr His Ala Met Met Val Asp Ile Ala Val Gln Leu Val Gln
      115          120          125
Lys Trp Glu Arg Leu Asn Ala Asp Glu His Ile Glu Val Ser Glu Asp
130          135          140
Met Thr Arg Leu Thr Leu Asp Thr Ile Gly Leu Cys Gly Phe Asn Tyr
145          150          155          160
Arg Phe Asn Ser Phe Tyr Arg Asp Gln Pro His Pro Phe Ile Ile Ser
      165          170          175
Met Val Arg Ala Leu Asp Glu Val Met Asn Lys Leu Gln Arg Ala Asn
      180          185          190
Pro Asp Asp Pro Ala Tyr Asp Glu Asn Lys Arg Gln Cys Gln Glu Asp
      195          200          205
Ile Lys Val Met Asn Asp Leu Val Asp Lys Ile Ile Ala Asp Arg Lys
210          215          220
Ala Arg Gly Glu Gln Ser Asp Asp Leu Leu Thr Gln Met Leu Asn Gly
225          230          235          240
Lys Asp Pro Glu Thr Gly Glu Pro Leu Asp Asp Gly Asn Ile Ser Tyr
      245          250          255
Gln Ile Ile Thr Phe Leu Ile Ala Gly His Glu Thr Thr Ser Gly Leu
      260          265          270
Leu Ser Phe Ala Leu Tyr Phe Leu Val Lys Asn Pro His Val Leu Gln
      275          280          285
Lys Val Ala Glu Glu Ala Ala Arg Val Leu Val Asp Pro Val Pro Ser
290          295          300
Tyr Lys Gln Val Lys Gln Leu Lys Tyr Val Gly Met Val Leu Asn Glu
305          310          315          320
Ala Leu Arg Leu Trp Pro Thr Ala Pro Ala Phe Ser Leu Tyr Ala Lys
      325          330          335
Glu Asp Thr Val Leu Gly Gly Glu Tyr Pro Leu Glu Lys Gly Asp Glu
      340          345          350
Val Met Val Leu Ile Pro Gln Leu His Arg Asp Lys Thr Ile Trp Gly
      355          360          365
Asp Asp Val Glu Glu Phe Arg Pro Glu Arg Phe Glu Asn Pro Ser Ala
370          375          380
Ile Pro Gln His Ala Phe Lys Pro Phe Gly Asn Gly Gln Arg Ala Cys

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385          390          395          400
Ile Gly Gln Gln Phe Ala Leu His Glu Ala Thr Leu Val Leu Gly Met
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Met Leu Lys His Phe Asp Phe Glu Asp His Thr Asn Tyr Glu Leu Asp
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Ile Lys Glu Thr Leu Thr Leu Lys Pro Glu Gly Phe Val Val Lys Ala
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Pro Leu Leu Val Leu Tyr Gly Ser Asn Met Gly Thr Ala Glu Gly Thr
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Ala Arg Asp Leu Ala Asp Ile Ala Met Ser Lys Gly Phe Ala Pro Gln
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Val Ala Thr Leu Asp Ser His Ala Gly Asn Leu Pro Arg Glu Gly Ala
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Val Leu Ile Val Thr Ala Ser Tyr Asn Gly His Pro Pro Asp Asn Ala
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Lys Gln Phe Val Asp Trp Leu Asp Gln Ala Ser Ala Asp Glu Val Lys
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Gly Val Arg Tyr Ser Val Phe Gly Cys Gly Asp Lys Asn Trp Ala Thr
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Phe Glu Gly Thr Tyr Glu Glu Trp Arg Glu His Met Trp Ser Asp Val
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Lys Met His Gly Ala Phe Ser Thr Asn Val Val Ala Ser Lys Glu Leu
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Gln Gln Pro Gly Ser Ala Arg Ser Thr Arg His Leu Glu Ile Glu Leu
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Pro Lys Glu Ala Ser Tyr Gln Glu Gly Asp His Leu Gly Val Ile Pro
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Arg Asn Tyr Glu Gly Ile Val Asn Arg Val Thr Ala Arg Phe Gly Leu
705          710          715          720
Asp Ala Ser Gln Gln Ile Arg Leu Glu Ala Glu Glu Glu Lys Leu Ala
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His Leu Pro Leu Ala Lys Thr Val Ser Val Glu Glu Leu Leu Gln Tyr
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Glu Lys Gln Ala Tyr Lys Glu Gln Val Leu Ala Lys Arg Leu Thr Met
785          790          795          800
Leu Glu Leu Leu Glu Lys Tyr Pro Ala Cys Glu Met Lys Phe Ser Glu
          805          810          815
Phe Ile Ala Leu Leu Pro Ser Ile Arg Pro Arg Tyr Tyr Ser Ile Ser
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Ser Ser Pro Arg Val Asp Glu Lys Gln Ala Ser Ile Thr Val Ser Val
          835          840          845

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Val Ser Gly Glu Ala Trp Ser Gly Tyr Gly Glu Tyr Lys Gly Ile Ala
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 Ile Ser Thr Pro Gln Ser Glu Phe Thr Leu Pro Lys Asp Pro Glu Thr
 885 890 895
 Pro Leu Ile Met Val Gly Pro Gly Thr Gly Val Ala Pro Phe Arg Gly
 900 905 910
 Phe Val Gln Ala Arg Lys Gln Leu Lys Glu Gln Gly Gln Ser Leu Gly
 915 920 925
 Glu Ala His Leu Tyr Phe Gly Cys Arg Ser Pro His Glu Asp Tyr Leu
 930 935 940
 Tyr Gln Glu Glu Leu Glu Asn Ala Gln Ser Glu Gly Ile Ile Thr Leu
 945 950 955 960
 His Thr Ala Phe Ser Arg Met Pro Asn Gln Pro Lys Thr Tyr Val Gln
 965 970 975
 His Val Met Glu Gln Asp Gly Lys Lys Leu Ile Glu Leu Leu Asp Gln
 980 985 990
 Gly Ala His Phe Tyr Ile Cys Gly Asp Gly Ser Gln Met Ala Pro Ala
 995 1000 1005
 Val Glu Ala Thr Leu Met Lys Ser Tyr Ala Asp Val His Gln Val Ser
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 Tyr Ala Lys Asp Val Trp Ala Gly
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<210> 35

<211> 3144

<212> DNA

<213> Bacillus megaterium

<400> 35

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<210> 36

<211> 1048

<212> PRT

<213> Bacillus megaterium

<400> 36

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20          25          30
Ala Asp Glu Leu Gly Glu Ile Phe Lys Phe Glu Ala Pro Gly Cys Val
35          40          45
Thr Arg Tyr Leu Ser Ser Gln Arg Leu Ile Lys Glu Ala Cys Asp Glu
50          55          60
Ser Arg Phe Asp Lys Asn Leu Ser Gln Ala Leu Lys Phe Ala Arg Asp
65          70          75          80
Phe Ala Gly Asp Gly Leu Ile Thr Ser Trp Thr His Glu Ile Asn Trp
85          90          95
Lys Lys Ala His Asn Ile Leu Leu Pro Ser Phe Ser Gln Gln Ala Met
100         105         110
Lys Gly Tyr His Ala Met Met Val Asp Ile Ala Val Gln Leu Val Gln
115         120         125
Lys Trp Glu Arg Leu Asn Ala Asp Glu His Ile Glu Val Ser Glu Asp
130         135         140
Met Thr Arg Leu Thr Leu Asp Thr Ile Gly Leu Cys Gly Phe Asn Tyr
145         150         155         160

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Arg Phe Asn Ser Phe Tyr Arg Asp Gln Pro His Pro Phe Ile Ile Ser
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 180 185 190
 Pro Asp Asp Pro Ala Tyr Asp Glu Asn Lys Arg Gln Cys Gln Glu Asp
 195 200 205
 Ile Lys Val Met Asn Asp Leu Val Asp Lys Ile Ile Ala Asp Arg Lys
 210 215 220
 Ala Arg Gly Glu Gln Ser Asp Asp Leu Leu Thr Gln Met Leu Asn Gly
 225 230 235 240
 Lys Asp Pro Glu Thr Gly Glu Pro Leu Asp Asp Gly Asn Ile Ser Tyr
 245 250 255
 Gln Ile Ile Thr Phe Leu Ile Ala Gly His Glu Thr Thr Ser Gly Leu
 260 265 270
 Leu Ser Phe Ala Leu Tyr Phe Leu Val Lys Asn Pro His Val Leu Gln
 275 280 285
 Lys Val Ala Glu Glu Ala Ala Arg Val Leu Val Asp Pro Val Pro Ser
 290 295 300
 Tyr Lys Gln Val Lys Gln Leu Lys Tyr Val Gly Met Val Leu Asn Glu
 305 310 315 320
 Ala Leu Arg Leu Trp Pro Thr Ala Pro Ala Phe Ser Leu Tyr Ala Lys
 325 330 335
 Glu Asp Thr Val Leu Gly Gly Glu Tyr Pro Leu Glu Lys Gly Asp Glu
 340 345 350
 Val Met Val Leu Ile Pro Gln Leu His Arg Asp Lys Thr Ile Trp Gly
 355 360 365
 Asp Asp Val Glu Glu Phe Arg Pro Glu Arg Phe Glu Asn Pro Ser Ala
 370 375 380
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 385 390 395 400
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 405 410 415
 Met Leu Lys His Phe Asp Phe Glu Asp His Thr Asn Tyr Glu Leu Asp
 420 425 430
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 435 440 445
 Lys Ser Lys Lys Ile Pro Leu Gly Gly Ile Pro Ser Pro Ser Thr Glu
 450 455 460
 Gln Ser Ala Lys Lys Val Arg Lys Lys Ala Glu Asn Ala His Asn Thr
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 485 490 495
 Ala Arg Asp Leu Ala Asp Ile Ala Met Ser Lys Gly Phe Ala Pro Gln
 500 505 510
 Val Ala Thr Leu Asp Ser His Ala Gly Asn Leu Pro Arg Glu Gly Ala
 515 520 525
 Val Leu Ile Val Thr Ala Ser Tyr Asn Gly His Pro Pro Asp Asn Ala
 530 535 540
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 545 550 555 560
 Gly Val Arg Tyr Ser Val Phe Gly Cys Gly Asp Lys Asn Trp Ala Thr
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 Thr Tyr Gln Lys Val Pro Ala Phe Ile Asp Glu Thr Leu Ala Ala Lys
 580 585 590
 Gly Ala Glu Asn Ile Ala Asp Arg Gly Glu Ala Asp Ala Ser Asp Asp
 595 600 605
 Phe Glu Gly Thr Tyr Glu Glu Trp Arg Glu His Met Trp Ser Asp Val

610	615	620
Ala Ala Tyr Phe Asn Leu Asp Ile Glu Asn Ser Glu Asp Asn Lys Ser		
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Thr Leu Ser Leu Gln Phe Val Asp Ser Ala Ala Asp Met Pro Leu Ala		640
	645	650
Lys Met His Gly Ala Phe Ser Thr Asn Val Val Ala Ser Lys Glu Leu		655
	660	665
Gln Gln Pro Gly Ser Ala Arg Ser Thr Arg His Leu Glu Ile Glu Leu		670
	675	680
Pro Lys Glu Ala Ser Tyr Gln Glu Gly Asp His Leu Gly Val Ile Pro		685
	690	695
Arg Asn Tyr Glu Gly Ile Val Asn Arg Val Thr Ala Arg Phe Gly Leu		700
705	710	715
Asp Ala Ser Gln Gln Ile Arg Leu Glu Ala Glu Glu Glu Lys Leu Ala		720
	725	730
His Leu Pro Leu Ala Lys Thr Val Ser Val Glu Glu Leu Leu Gln Tyr		735
	740	745
Val Glu Leu Gln Asp Pro Val Thr Arg Thr Gln Leu Arg Ala Met Ala		750
	755	760
Ala Lys Thr Val Cys Pro Pro His Lys Val Glu Leu Glu Ala Leu Leu		765
	770	775
Glu Lys Gln Ala Tyr Lys Glu Gln Val Leu Ala Lys Arg Leu Thr Met		780
785	790	795
Leu Glu Leu Leu Glu Lys Tyr Pro Ala Cys Glu Met Lys Phe Ser Glu		800
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Phe Ile Ala Leu Leu Pro Ser Ile Arg Pro Arg Tyr Tyr Ser Ile Ser		815
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Ser Ser Pro Arg Val Asp Glu Lys Gln Ala Ser Ile Thr Val Ser Val		830
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Val Ser Gly Glu Ala Trp Ser Gly Tyr Gly Glu Tyr Lys Gly Ile Ala		845
	850	855
Ser Asn Tyr Leu Ala Glu Leu Gln Glu Gly Asp Thr Ile Thr Cys Phe		860
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Ile Ser Thr Pro Gln Ser Glu Phe Thr Leu Pro Lys Asp Pro Glu Thr		880
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Pro Leu Ile Met Val Gly Pro Gly Thr Gly Val Ala Pro Phe Arg Gly		895
	900	905
Phe Val Gln Ala Arg Lys Gln Leu Lys Glu Gln Gly Gln Ser Leu Gly		910
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	930	935
Tyr Gln Glu Glu Leu Glu Asn Ala Gln Ser Glu Gly Ile Ile Thr Leu		940
945	950	955
His Thr Ala Phe Ser Arg Met Pro Asn Gln Pro Lys Thr Tyr Val Gln		960
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His Val Met Glu Gln Asp Gly Lys Lys Leu Ile Glu Leu Leu Asp Gln		975
	980	985
Gly Ala His Phe Tyr Ile Cys Gly Asp Gly Ser Gln Met Ala Pro Ala		990
	995	1000
Val Glu Ala Thr Leu Met Lys Ser Tyr Ala Asp Val His Gln Val Ser		1005
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Glu Ala Asp Ala Arg Leu Trp Leu Gln Gln Leu Glu Glu Lys Gly Arg		1020
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<210> 37
 <211> 3144
 <212> DNA
 <213> *Bacillus megaterium*

<400> 37

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Lys	Lys	Ala	His	Asn	Ile	Leu	Leu	Pro	Ser	Phe	Ser	Gln	Gln	Ala	Met
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Gln	Ile	Ile	Thr	Phe	Leu	Ile	Ala	Gly	His	Glu	Thr	Thr	Ser	Gly	Leu
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Glu	Asp	Thr	Val	Leu	Gly	Gly	Glu	Tyr	Pro	Leu	Glu	Lys	Gly	Asp	Glu
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Phe Val Gln Ala Arg Lys	Gln Leu Lys Glu Gln	Gly Gln Ser Leu Gly
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Glu Ala His Leu Tyr Phe	Gly Cys Arg Ser Pro	His Glu Asp Tyr Leu
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<212> DNA

<213> Bacillus megaterium

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<211> 1048

<212> PRT

<213> Bacillus megaterium

<400> 40

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      35             40             45
Thr Arg Tyr Leu Ser Ser Gln Arg Leu Ile Lys Glu Ala Cys Asp Glu
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Ser Arg Phe Asp Lys Asn Leu Ser Gln Ala Leu Lys Phe Ala Arg Asp
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Phe Ala Gly Asp Gly Leu Val Thr Ser Trp Thr His Glu Ile Asn Trp
      85             90             95
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 <213> *Bacillus megaterium*

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<212> PRT
<213> Bacillus megaterium
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Lys	Asp	Pro	Glu	Thr	Gly	Glu	Pro	Leu	Asp	Asp	Gly	Asn	Ile	Ser	Tyr
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Gln	Ile	Ile	Thr	Phe	Leu	Ile	Ala	Gly	His	Glu	Thr	Thr	Ser	Gly	Leu
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Leu	Ser	Phe	Ala	Leu	Tyr	Phe	Leu	Val	Lys	Asn	Pro	His	Val	Leu	Gln
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Ala	Leu	Arg	Leu	Trp	Pro	Thr	Ala	Pro	Ala	Phe	Ser	Leu	Tyr	Ala	Lys
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Glu	Asp	Thr	Val	Leu	Gly	Gly	Glu	Tyr	Pro	Leu	Glu	Lys	Gly	Asp	Glu
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Val	Met	Val	Leu	Ile	Pro	Gln	Leu	His	Arg	As					

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Ile Lys Glu Thr Leu Thr Leu Lys Pro Glu Gly Phe Val Val Lys Ala				430
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Pro Leu Leu Val Leu Tyr Gly Ser Asn Met Gly Thr Ala Glu Gly Thr				480
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Ala Arg Asp Leu Ala Asp Ile Ala Met Ser Lys Gly Phe Ala Pro Gln				495
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		515		520
Val Leu Ile Val Thr Ala Ser Tyr Asn Gly His Pro Pro Asp Asn Ala				525
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Pro Lys Glu Ala Ser Tyr Gln Glu Gly Asp His Leu Gly Val Ile Pro				685
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Arg Asn Tyr Glu Gly Ile Val Asn Arg Val Thr Ala Arg Phe Gly Leu				700
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His Leu Pro Leu Ala Lys Thr Val Ser Val Glu Glu Leu Leu Gln Tyr				735
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Val Glu Leu Gln Asp Pro Val Thr Arg Thr Gln Leu Arg Ala Met Ala				750
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Ala Lys Thr Val Cys Pro Pro His Lys Val Glu Leu Glu Ala Leu Leu				765
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Glu Lys Gln Ala Tyr Lys Glu Gln Val Leu Ala Lys Arg Leu Thr Met				780
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<211> 3144

<212> DNA

<213> Bacillus megaterium

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<211> 1048

<212> PRT

<213> Bacillus megaterium

<400> 44

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 225 230 235 240
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 290 295 300
 Tyr Lys Gln Val Lys Gln Leu Lys Tyr Val Gly Met Val Leu Asn Glu
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 Thr Tyr Gln Lys Val Pro Ala Phe Ile Asp Glu Thr Leu Ala Ala Lys
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 Gly Ala Glu Asn Ile Ala Asp Arg Gly Glu Ala Asp Ala Ser Asp Asp

595															600															605														
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Glu	Ala	His	Leu	Tyr	Phe	Gly	Cys	Arg	Ser	Pro	His	Glu	Asp	Tyr	Leu																													
Tyr 945	Gln	Glu	Glu	Leu	Glu	Asn	Ala	Gln	Ser	Glu	Gly	Ile	Ile	Thr	Leu																													
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His	Val	Met	Glu	Gln	Asp	Gly	Lys	Lys	Leu	Ile	Glu	Leu	Leu	Asp	Gln																													
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Val	Glu	Ala	Thr	Leu	Met	Lys	Ser	Tyr	Ala	Asp	Val	His	Gln	Val	Ser																													
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 <211> 3144
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 <213> *Bacillus megaterium*

<400> 45

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<210> 46
<211> 1048
<212> PRT
<213> Bacillus megaterium
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Tyr	Lys	Gln	Val	Lys	Gln	Leu	Lys	Tyr	Val	Gly	Met	Val	Leu	Asn	Glu
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Ala	Leu	Arg	Leu	Trp	Pro	Thr	Ala	Pro	Ala	Phe	Ser	Leu	Tyr	Ala	Lys
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Glu	Asp	Thr	Val	Leu	Gly	Gly	Glu	Tyr	Pro	Leu	Glu	Lys	Gly	Asp	Glu
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 405 410 415
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 Gln Ser Ala Lys Lys Val Arg Lys Lys Ala Glu Asn Ala His Asn Thr
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<210> 47
<211> 3144
<212> DNA
<213> Bacillus megaterium
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<210> 48

<211> 1048

<212> PRT

<213> Bacillus megaterium

<400> 48

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Ala Asp Glu Leu Gly Glu Ile Phe Lys Phe Glu Ala Pro Gly Cys Val
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Thr Arg Tyr Leu Ser Ser Gln Arg Leu Ile Lys Glu Ala Cys Asp Glu
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Ser Arg Phe Asp Lys Asn Leu Ser Gln Ala Leu Lys Phe Ala Arg Asp
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Phe Ala Gly Asp Gly Leu Phe Thr Ser Trp Thr His Glu Ile Asn Trp
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Lys Gly Tyr His Ala Met Met Val Asp Ile Ala Val Gln Leu Val Gln
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Lys Trp Glu Arg Leu Asn Ala Asp Glu His Ile Glu Val Ser Glu Asp

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	180	185
Pro Asp Asp Pro Ala Tyr Asp Glu Asn Lys Arg Gln Cys Gln Glu Asp		190
	195	200
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Ala Arg Gly Glu Gln Ser Asp Asp Leu Leu Thr Gln Met Leu Asn Gly		220
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Lys Asp Pro Glu Thr Gly Glu Pro Leu Asp Asp Gly Asn Ile Ser Tyr		235
	245	250
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Leu Ser Phe Ala Leu Tyr Phe Leu Val Lys Asn Pro His Val Leu Gln		270
	275	280
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Asp Asp Val Glu Glu Phe Arg Pro Glu Arg Phe Glu Asn Pro Ser Ala		365
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Pro Leu Leu Val Leu Tyr Gly Ser Asn Met Gly Thr Ala Glu Gly Thr		475
	485	490
Ala Arg Asp Leu Ala Asp Ile Ala Met Ser Lys Gly Phe Ala Pro Gln		495
	500	505
Val Ala Thr Leu Asp Ser His Ala Gly Asn Leu Pro Arg Glu Gly Ala		510
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Val Leu Ile Val Thr Ala Ser Tyr Asn Gly His Pro Pro Asp Asn Ala		525
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Thr Tyr Gln Lys Val Pro Ala Phe Ile Asp Glu Thr Leu Ala Ala Lys		575
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		590

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 945 950 955 960
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 His Val Met Glu Gln Asp Gly Lys Lys Leu Ile Glu Leu Leu Asp Gln
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 Val Glu Ala Thr Leu Met Lys Ser Tyr Ala Asp Val His Gln Val Ser
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1045

<210> 49
 <211> 3144
 <212> DNA
 <213> *Bacillus megaterium*

<400> 49

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<211> 1048

<212> PRT

<213> Bacillus megaterium

<400> 50

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Phe Ala Gly Asp Gly Leu Phe Thr Ser Trp Thr His Glu Ile Asn Trp
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Lys Lys Ala His Asn Ile Leu Leu Pro Ser Phe Ser Gln Gln Ala Met
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Pro Asp Asp Pro Ala Tyr Asp Glu Asn Lys Arg Gln Cys Gln Glu Asp
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Gln Ile Ile Ser Phe Leu Ile Ala Gly His Glu Thr Thr Ser Gly Leu
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Leu Ser Phe Ala Leu Tyr Phe Leu Val Lys Asn Pro His Val Leu Gln
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<212> DNA

<213> Bacillus megaterium

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<211> 1048

<212> PRT

<213> Bacillus megaterium

<400> 52

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Ser Arg Phe Asp Lys Asn Leu Ser Gln Ala Leu Lys Phe Ala Arg Asp
      65           70           75           80
Phe Ala Gly Asp Gly Leu Phe Cys Ser Trp Thr His Glu Ile Asn Trp
      85           90           95
Lys Lys Ala His Asn Ile Leu Leu Pro Ser Phe Ser Gln Gln Ala Met
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Lys Gly Tyr His Ala Met Met Val Asp Ile Ala Val Gln Leu Val Gln
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Tyr Ala Lys Asp Val Trp Ala Gly
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<210> 53

<211> 3144

<212> DNA

<213> Bacillus megaterium

<400> 53

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<212> PRT

<213> Bacillus megaterium

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Ala Asp Glu Leu Gly Glu Ile Phe Lys Phe Glu Ala Pro Gly Arg Val
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Thr Arg Tyr Leu Ser Ser Gln Arg Leu Ile Lys Glu Ala Cys Asp Glu
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Ser Arg Phe Asp Lys Asn Leu Ser Gln Ala Leu Lys Phe Ala Arg Asp
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Phe Ala Gly Asp Gly Leu Phe Thr Ser Trp Thr His Glu Lys Asn Trp
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Lys Lys Ala His Asn Ile Leu Leu Pro Ser Phe Ser Gln Gln Ala Met
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Lys Gly Tyr His Ala Met Met Val Asp Ile Ala Val Gln Leu Val Gln
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Lys Trp Glu Arg Leu Asn Ala Asp Glu His Ile Glu Val Pro Glu Asp
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Arg Phe Asn Ser Phe Tyr Arg Asp Gln Pro His Pro Phe Ile Ile Ser
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Met Val Arg Ala Leu Asp Glu Val Met Asn Lys Leu Gln Arg Ala Asn
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Pro Asp Asp Pro Ala Tyr Asp Glu Asn Lys Arg Gln Cys Gln Glu Asp
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Leu Ser Phe Ala Leu Tyr Phe Leu Val Lys Asn Pro His Val Leu Gln
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Lys Val Ala Glu Glu Ala Ala Arg Val Leu Val Asp Pro Val Pro Ser
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Tyr Lys Gln Val Lys Gln Leu Lys Tyr Val Gly Met Val Leu Asn Glu
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Lys	Gln	Phe	Val	Asp	Trp	Leu	Asp	Gln	Ala	Ser	Ala	Asp	Glu	Val	Lys		
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Phe	Glu	Gly	Thr	Tyr	Glu	Glu	Trp	Arg	Glu	His	Met	Trp	Ser	Asp	Val		
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