

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
18 January 2007 (18.01.2007)

PCT

(10) International Publication Number
WO 2007/006593 A2

(51) International Patent Classification:
C12P 37/00 (2006.01) *C12N 9/02* (2006.01)

(21) International Application Number:
PCT/EP2006/061479

(22) International Filing Date: 10 April 2006 (10.04.2006)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
05106347.7 12 July 2005 (12.07.2005) EP

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

- without international search report and to be republished upon receipt of that report
- with sequence listing part of description published separately in electronic form and available upon request from the International Bureau

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: MUTANT EXPANDASES

(57) Abstract: The present invention relates to a mutant expandase that is a variant of a model polypeptide with expandase activity whereby the mutant expandase has an at least 2.5-fold improved *in vitro* expandase activity towards adipyl-6-APA in comparison with a model polypeptide with expandase activity.



WO 2007/006593 A2

MUTANT EXPANDASES

The present invention relates to mutant expandase enzymes, polynucleotides
5 encoding such enzymes, to microorganisms transformed with said polynucleotides
encoding said mutant expandase enzymes and with the use of such mutant expandase
enzymes or such microorganisms in the ring expansion of 5-carboxypentanoyl-6-
aminopenicillanic acid (adipyl-6-APA = ad-6-APA).

Beta-lactam antibiotics constitute the most important group of antibiotic
10 compounds with a long history of clinical use. Among this group, the prominent ones
are the penicillins and cephalosporins. Penicillins are naturally produced by various
filamentous fungi such as *Penicillium* (e.g. *P. chrysogenum*). Cephalosporins are
naturally produced by various microorganisms such as *Acremonium* (e.g. *A.*
chrysogenum) and *Streptomyces* (e.g. *Streptomyces clavuligerus*)

15 As a result of classical strain improvement techniques, the production levels of
the antibiotics in *P. chrysogenum* and *A. chrysogenum* have increased remarkably over
the past decades. With the increasing knowledge of the biosynthetic pathways leading
to penicillins and cephalosporins, and the advent of recombinant DNA technology, new
tools for the improvement of production strains have become available.

20 Most enzymes involved in β -lactam biosynthesis have been identified and their
corresponding genes have been cloned, as can be found in Ingolia and Queener, Med
Res Rev (1989) 9:245-264 (biosynthesis route and enzymes), and Aharonowitz,
Cohen, and Martin, Ann Rev Microbiol (1992) 46:461-495 (gene cloning).

The first two steps in the biosynthesis of penicillin in *P. chrysogenum* are the
25 condensation of the three amino acids L-5-amino-5-carboxypentanoic acid (L- α -
aminoadipic acid) (A), L-cysteine (C) and L-valine (V) into the tripeptide LLD-ACV,
followed by cyclization of this tripeptide to form isopenicillin N. This compound contains
the typical β -lactam structure.

The third step involves the replacement of the hydrophilic side chain of L-5-
30 amino-5-carboxypentanoic acid by a hydrophobic side chain by the action of the
enzyme acyltransferase (AT).

In EP-A-0448180 has been described that the enzymatic exchange reaction
mediated by AT takes place inside a cellular organelle, the microbody. The observation
that substantial quantities of deacetoxycephalosporin C (DAOC) can be formed by non-
35 precursed *P. chrysogenum* transformants expressing deacetoxycephalosporin C
synthase (EC 1.14.20.1 - DAOCS, further indicated herein as expandase) implies the

presence of significant amounts of penicillin N, the natural substrate for expandase, in *P. chrysogenum* (Alvi et al., J Antibiot (1995) 48:338-340). However, the D- α -amino-adipyl side chains of DAOC cannot be easily removed.

Cephalosporins are much more expensive than penicillins. One reason is that some cephalosporins (e.g., cephalexin) are made from penicillins by a number of chemical conversions. Another reason is that, so far, only cephalosporins with a D- α -amino-adipyl side chain could be fermented. Cephalosporin C, by far the most important starting material in this respect, is very soluble in water at any pH, thus implying lengthy and costly isolation processes using cumbersome and expensive column technology. Cephalosporin C obtained in this way has to be converted into therapeutically used cephalosporins by a number of chemical and enzymatic conversions.

The methods currently favored in industry to prepare the intermediate 7-amino-deacetoxycephaloporan acid (7-ADCA) involve complex chemical steps leading to the expansion and derivatization of penicillin G. One of the necessary chemical steps to produce 7-ADCA involves the expansion of the 5-membered penicillin ring structure to a 6-membered cephalosporin ring structure (see for instance US 4,003,894). This complex chemical processing is both expensive and noxious to the environment.

Consequently, there is a great desire to replace such chemical processes with enzymatic reactions such as enzymatic catalysis, preferably during fermentation. A key to the replacement of the chemical expansion process by a biological process is the central enzyme in the cephalosporin biosynthetic pathway, expandase.

The expandase enzyme from the bacterium *Streptomyces clavuligerus* (*S. clavuligerus*) was found to carry out, in some cases, penicillin ring expansions. When introduced into *P. chrysogenum*, it can convert the penicillin ring structure into the cephalosporin ring structure, as described in Cantwell et al., Proc R Soc Lond B (1992) 248:283-289. The expandase enzyme has been well characterized (EP-A-0366354) both biochemical and functional, as has its corresponding gene. Both physical maps of the *cefE* gene (the gene encoding the expandase enzyme of *S. clavuligerus* - EP-A-0341892), DNA sequence and transformation studies in *P. chrysogenum* with *cefE* have been described. The DNA and amino acid sequence of the *S. clavuligerus* expandase enzyme are represented in SEQ ID NO 1.

Another source for an expandase enzyme is the bacterium *Nocardia lactamdurans* (*N. lactamdurans*, formerly *S. lactamdurans*). Both the biochemical

properties of the enzyme and the DNA sequence of the gene encoding the enzyme have been described – see Cortes et al., J Gen Microbiol (1987) 133:3165-3174 and Coque et al., Mol Gen Genet (1993) 236:453-458, respectively

Recently, novel expandase (*cefE*) genes and enzymes have been found in
 5 *Streptomyces jumonjinensis*, *Streptomyces ambofaciens* and *Streptomyces chartreuses* – see Hsu et al. (2004), Appl. and Environm. Microbiol. 70, 6257-6263. Table 1 summarizes the amino acid sequence identities from several expandases and shows that the sequence identities range from 67 to 85%, depending on the source of the enzyme.

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Table 1: Amino acid sequence identities of several expandases (data taken from Hsu et al).

Source	<i>S. clavuligerus</i>	<i>S. jumonjinensis</i>	<i>S. ambofaciens</i>	<i>S. chartreuses</i>	<i>N. lactamdurans</i>
<i>S. clavuligerus</i>	100				
<i>S. jumonjinensis</i>	81	100			
<i>S. ambofaciens</i>	81	85	100		
<i>S. chartreuses</i>	76	77	79	100	
<i>N. lactamdurans</i>	70	68	70	67	100

In the biosynthesis of cephalosporins in prokaryotic cells, the
 15 deacetoxycephalosporin-C is subsequently converted to deacetyl-cephalosporin-C by the enzyme deacetylcephalosporin C synthase also named deacetoxycephalosporin-C hydroxylase or hydroxylase (EC 1.14.11.26 - DACS). Genes encoding such hydroxylases are named *cefF*-genes (e.g. see Hsu et al.) The expandase found in eukaryotic cells, e.g. *Acremonium chrysogenum* can catalyze the direct conversion of
 20 penicillin N to deacetoxycephalosporin-C due to possession of both expandase and hydrolyase activity, hence the encoded gene is termed *cefEF* (see Hsu et al.).

As defined herein, the term expandase relates to expandase enzymes (EC 1.14.20.1, encoded by *cefE* genes) as well as expandases also possessing in addition the hydroxylase activity (EC 1.14.20.1 + EC 1.14.11.26 encoded by *cefEF* genes).

25 Since the expandase enzyme catalyses the expansion of the 5-membered thiazolidine ring of penicillin N to the 6-membered dihydrothiazine ring of DAOC, this

enzyme would be of course a logical candidate to replace the ring expansion steps of the chemical process. Unfortunately, the enzyme works on the penicillin N intermediate of the cephalosporin biosynthetic pathway, but not or very inefficiently on the readily available inexpensive penicillins as produced by *P. chrysogenum*, like penicillin V or
5 penicillin G. Penicillin N is commercially not available and even when expanded, its D- α -amino-adipyl side chain cannot be easily removed by penicillin acylases.

It has been reported that the expandase enzyme is capable of expanding penicillins with particular side chains to the corresponding 7-ADCA derivative. This feature of the expandase has been exploited in the technology as disclosed in EP-A-
10 0532341, WO95/04148 and WO95/04149. In these disclosures the conventional chemical *in vitro* conversion of penicillin G to 7-ADCA has been replaced by the *in vivo* conversion of certain 6-aminopenicillanic acid (6-APA) derivatives in recombinant *Penicillium chrysogenum* strains transformed with an expandase gene.

More particularly, EP-A-0532341 teaches the *in vivo* use of the expandase
15 enzyme in *P. chrysogenum*, in combination with a adipyl side chain (further referred to as adipyl) as a feedstock, which is a substrate for the acyltransferase enzyme in *P. chrysogenum*. This leads to the formation of adipyl-6-APA, which is converted by an expandase enzyme introduced into the *P. chrysogenum* strain to yield adipyl-7-ADCA. Finally, the removal of the adipyl side chain is described, yielding 7-ADCA as a final
20 product. Furthermore, EP-A-540210 teaches the similar production of adipyl-7-ADAC and adipyl-7-ACA.

As an alternative approach, expansion of penicillin G using *P. chrysogenum* transformed with *cefE* has been proposed in EP0828850. Furthermore, in order to increase the expansion of penicillin G the use of mutant *cefE* gene of *S. clavuligerus*
25 has been described. US 5,919,680, WO 98/02551, WO 99/33994, WO 01/85951 and EP 1348759 all disclose mutant *cefE* genes allegedly coding for enzymes having a higher expandase activity on penicillin G.

Nevertheless, these mutant expandases still do not provide for a commercially attractive process for the production of cephalosporins.

30 Therefore, there is a need for further improving the process wherein cephalosporins are prepared from expansion of adipyl-6-APA. This process is characterized by an efficient removal of the side chain material from the cephalosporin core material. One step to further improve this process is improvement of the expandase activity on adipyl-6-APA.

35 In a first aspect, the invention provides a mutant expandase that is a variant of

a model polypeptide with expandase activity. Preferably, the invention provides a mutant expandase, whereby the mutant expandase has an at least 2.5-fold improved *in vitro* expandase activity towards adipyl-6-APA in comparison with a model polypeptide with expandase activity. The determination of the *in vitro* expandase activity towards adipyl-6-APA is described in detail in the Materials and Methods. More preferably the *in vitro* expandase activity towards adipyl-6-APA of the mutant expandase is improved at least 3-fold, more preferably at least 4-fold, more preferably at least 5-fold, more preferably at least 6-fold, more preferably at least 7-fold, more preferably at least 8-fold, more preferably at least 9-fold, more preferably at least 10-fold, more preferably at least 11-fold more preferably at least 12-fold, more preferably at least 13-fold, more preferably at least 14-fold, more preferably at least 15-fold more preferably at least 16-fold more preferably at least 17-fold more preferably at least 18-fold more preferably at least 19-fold, more preferably at least 20-fold. The invention also provides a mutant expandase which is preferably being modified at least at an amino acid position selected from the group consisting of positions 2, 59, 73, 89, 90, 99, 101, 105, 113, 155, 170, 177, 209, 213, 217, 244, 249, 251, 277, 278, 280, 281, 293, 300, 306, 307 and 311 using the amino acid position numbering of the amino acid sequence of the expandase enzyme encoded by the *cefE* gene of *Streptomyces clavuligerus*. The nucleotide sequence of the *cefE* gene of *Streptomyces clavuligerus* as well as the amino acid sequence encoded by said *cefE* gene are depicted in SEQ ID NO: 1. More preferably, the mutant expandase is modified at least at an amino acid position selected from the group consisting of positions 2, 59, 89, 90, 99, 105, 113, 170, 177, 209, 213, 217, 249, 251, 278, 280 and 293. Most preferably, the mutant expandase is modified at least at an amino acid position selected from the group consisting of positions 2, 89, 90, 99, 105, 177, 251, 278, 280 and 293.

With "altered or mutant expandase" in the context of the present invention is meant any enzyme having expandase activity, which has not been obtained from a natural source and for which the amino acid sequence differs from the complete amino acid sequence of the natural expandase enzyme.

Most preferably, the invention provides a mutant expandase whereby the mutant expandase has an at least 2.5-fold improved *in vitro* expandase activity towards adipyl-6-APA in comparison with a model polypeptide with expandase activity, more preferably at least 3-fold, more preferably at least 4-fold, more preferably at least 5-fold, more preferably at least 6-fold, more preferably at least 7-fold, more preferably at least 8-fold, more preferably at least 9-fold, more preferably at least 10-fold, more

preferably at least 11-fold more preferably at least 12-fold, more preferably at least 13-fold, more preferably at least 14-fold, more preferably at least 15-fold more preferably at least 16-fold more preferably at least 17-fold more preferably at least 18-fold more preferably at least 19-fold, more preferably at least 20-fold and whereby the mutant expandase is preferably being modified at at least an amino acid position selected from the group consisting of positions 2, 59, 73, 89, 90, 99, 101, 105, 113, 155, 170, 177, 209, 213, 217, 244, 249, 251, 277, 278, 280, 281, 293, 300, 306, 307 and 311 using the amino acid position numbering of the amino acid sequence of the expandase enzyme encoded by the *cefE* gene of *Streptomyces clavuligerus*. More preferably, the mutant expandase is modified at least at an amino acid position selected from the group consisting of positions 2, 59, 89, 90, 99, 105, 113, 170, 177, 209, 213, 217, 249, 251, 278, 280 and 293. Most preferably, the mutant expandase is modified at least at an amino acid position selected from the group consisting of positions 2, 89, 90, 99, 105, 177, 251, 278, 280 and 293.

The activity of expandase can be measured under various conditions. The concentration of the substrate (e.g. adipyl-6-APA) in the activity assay determines whether or not the expandase activity is measured under conditions wherein the $V_{\max}$ determines the activity (in case the substrate concentration is much higher, e.g. 5-20 fold than the K_M of the expandase for its substrate) or under conditions wherein in addition also the K_M is determining the activity of the expandase (in case the substrate concentration is roughly equal to or (much) lower - e.g. <0,05 - 0.2-fold - than the K_M of the expandase for its substrate). The improvement factor of the mutant expandases may be measured under these various conditions.

The modification at an amino acid position may comprise a substitution by another amino acid, selected from the group of 20 L-amino acids that occur in Nature – see Table 2. Alternatively, the modification at an amino acid position may comprise a deletion of the amino acid at said position. Furthermore, the modification at an amino acid position may comprise a substitution of one or more amino acids at the C-terminal or N-terminal side of said amino acid.

Table 2

Amino Acid	3-letter code	1-letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cystein	Cys	C

Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophane	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

The model polypeptide with expandase activity as used in the present invention is selected from the group consisting of a polypeptide with expandase activity, preferably having an amino acid sequence according to SEQ ID NO: 1 and polypeptides with expandase activity having an amino acid sequence with a percentage identity with SEQ ID NO: 1 of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90%, most preferably at least 95%, such as the expandase enzymes that are summarized in Table 1.

The present invention preferably provides mutant expandases that have modifications at least at

- 1 or more amino acid positions selected from the group consisting of 2, 59, 73, 89, 90, 99, 101, 105, 113, 155, 170, 177, 209, 213, 217, 244, 249, 251, 277, 278, 280, 281, 293, 300, 307 and 311
- 1 or more amino acid positions selected from the group consisting of 2, 59, 89, 90, 99, 105, 113, 170, 177, 209, 213, 217, 249, 251, 278, 280 and 293
- 1 or more amino acid positions selected from the group consisting 2, 89, 90, 99, 105, 177, 251, 278, 280 and 293.
- 2 or more amino acid positions selected from the group consisting of 2, 89, 277, 281 and 300, more preferably at positions 2+281 or 89+281 or 277+300.
- 3 or more amino acid positions selected from the group consisting of 2, 89, 281, 293 and 311, more preferably at positions 2+89+281 or 89+281+311 or 89+281+293.
- 4 or more amino acid positions selected from the group consisting of 2, 73, 89, 90, 217, 244, 277, 280, 281, 306, 307 and 311, more preferably at positions

2+277+280+281 or 2+281+307+311 or 73+89+281+311 or 89+217+281+311 or 89+244+281+311 or 89+281+307+311 or 277+281+306+311 or 89+281+306+311 or 90+281+306+311.

- 5 ○ 5 or more amino acid positions selected from the group consisting of 2, 73, 89, 90, 155, 213, 249, 278, 281, 293, 300, 306, 307 and 311, more preferably at positions 2+89+281+306+311 or 2+155+281+306+311 or 73+89+213+281+311 or 89+78+218+307+311, 89+281+293+300+311 or 89+249+281+307+311.
- 10 ○ 6 amino acid positions selected from the group consisting of 90+105+113+281+306+311.
- 10 ○ 7 or more amino acid positions selected from the group consisting of 2, 73, 89, 105, 113, 155, 170, 177, 251, 277, 280, 281, 293, 300, 306, 307 and 311, more preferably 73+89+281+293+300+307+311 or 105+113+155+177+281+306+311 or 155+177+277+280+281+306+ 311.
- 15 ○ 8 or more amino acid positions selected from the group consisting of 2, 89, 90, 99, 105, 113, 155, 177, 277, 281, 307 and 311 more preferably at 2+90+99+105+113+281+306+311 or 2+90+105+113+155+177+277+281
- 15 ○ 9 amino acid positions selected from the group consisting of 2+90+105+113+155+177+281+306+311.
- 20 ○ 10 amino acid positions selected from the group consisting of 2, 59, 90, 101, 105, 113, 155, 177, 209, 277, 281, 307 and 311 more preferably at 2+90+105+113+155+177+277+281+306+311.
- 20 ○ 11 amino acid positions selected from the group consisting of 2, 90, 105, 113, 155, 177, 277, 281, 293, 307, 311.

The invention furthermore provides mutant expandases as defined hereinbefore that are variants of the expandase of *Streptomyces clavuligerus*, the mutant expandase being modified at an amino acid position selected from the group consisting of positions D2, S59, M73, T89, N90, M99, Y101, T105, G113, C155, H170, P177, G209, T213, Y217, H244, R249, L277, E280, C281, T293, G300, R306, R307 and A311 whereby the letter preceding the number denotes the respective amino acid (one letter code) and the subsequent number the amino acid position in the amino acid sequence of the expandase enzyme of *Streptomyces clavuligerus* which is depicted in SEQ ID NO: 1.

Preferred mutant expandases that are variants of the expandase of *Streptomyces clavuligerus* have modifications at least at

- 35 ○ 1 or more amino acid positions selected from the group consisting of D2, S59,

M73, T89, N90, M99, T105, G113, C155, H170, P177, G209, T213, Y217, H244, R249, D251, L277, A278, E280, C281, T293, G300, R306, R307 and A311

- 5 ○ 1 or more amino acid positions selected from the group consisting of D2, S59, T89, N90, M99, T105, G113, H170, P177, G209, T213, Y217, R249, D251, A278, E280 and T293
- 1 or more amino acid positions selected from the group consisting D2, T89, N90, M99, T105, P177, D251, A278, E280 and T293.
- 10 ○ 2 or more amino acid positions selected from the group consisting of D2, T89, L277, C281 and G300.
- 3 or more amino acid positions selected from the group consisting of D2, T89, C281, T293 and A311
- 4 or more amino acid positions selected from the group consisting of D2, M73, T89, N90, Y217, H244, L277, E280, C281, R306, R307 and A311
- 15 ○ 5 or more amino acid positions selected from the group consisting of D2, M73, T89, N90, C155, T213, R249, A278, C281, T293, R306, R307 and A311
- 6 amino acid positions selected from the group consisting of N90+T105+G113+C281+R306+A311.
- 7 or more amino acid positions selected from the group consisting of D2, M73, 20 T89, T105, G113, C155, H170, P177, D251, L277, E280, C281, T293, G300, R306, R307 and A311
- 8 or more amino acid positions selected from the group consisting of D2, T89, N90, M99, T105, G113, C155, P177, L277, C281, R306, R307 and A311.
- 9 amino acid positions selected from the group consisting of 25 D2+N90+T105+G113+C155+P177+C281+R306+A311.
- 10 amino acid positions selected from the group consisting of D2, S59, N90, T105, G113, T105, G113, C155, P177, G209, L277, C281, R306, R307, A311.
- 11 amino acid positions selected from the group consisting of D2, N90, T105, G113, C155, P177, L277, C281, R306, R307, A311.

30

Preferred modifications at the respective positions in the expandase of *Streptomyces clavuligerus* are the following (using the one-letter code for amino acids)

- D at position 2 according to SEQ ID NO 1 replaced by hydrophilic amino acids Y, N, H, D, Q, E, K, R, S or T, preferably by Y, N, Q or H. Most preferred are 35 replacements D2Y, D2N and D2H.

- M at position 73 according to SEQ ID NO 1 replaced by A, V, I, L, N, Q, H, K or R, preferably N, Q, H, L or I. Most preferred are replacements M73H and M73I.
- T at position 89 according to SEQ ID NO 1 replaced by K, R, A, C, P, V, I, L, or M, preferably A, V, I, L, R or K, more preferably A, V or K. Most preferred is replacement T89K.
- N at position 90 according to SEQ ID NO 1 replaced by H, R, K, N, Q, W, S, C, T or Y, preferably K, R, N, S, W. Most preferred are replacements N90W and N90S.
- M at position 99 according to SEQ ID NO 1 replaced by A, R, K, Q, E, N, D, S or T. more preferably K, Q, E or T. Most preferred is replacement M99T.
- T at position 105 according to SEQ ID NO 1 replaced by A, V, I, L, R, K, H, Q or N, preferably by V, I, L, R or K. Most preferred are replacements T105I and T105K.
- G at position 113 according to SEQ ID NO 1 replaced by A, P, Q, K, R, E, D or N, more preferably A, D, E, Q, K or R. Most preferred is replacement G113D.
- C at position 155 according to SEQ ID NO 1 replaced by A, N, S, T, V, W, more preferably A, S, T, V or W. Most preferred is replacement C155W.
- P at position 177 according to SEQ ID NO 1 replaced by K, E, L, A, V, T, I, more preferably L, A, V, T, I. Most preferred are replacements P177L and P177I.
- T at position 213 according to SEQ ID NO 1 replaced by Q, G, A, V, I, R, S, more preferably G, A, V, I, S. Most preferred is replacement T213A.
- Y at position 217 according to SEQ ID NO 1 replaced by A, V, H, P, T, N or Q, more preferably A, P, N, Q or H. Most preferred is replacement Y217H.
- H at position 244 according to SEQ ID NO 1 replaced by N, Q, K or R, more preferably K or R. Most preferred is replacement H244R.
- R at position 249 according to SEQ ID NO 1 replaced by G, S, T, N, D, Q, E, K, R, H or C, more preferably G, S, D, N or C. Most preferred is replacement R249C.
- D at position 251 according to SEQ ID NO 1 replaced by G.
- L at position 277 according to SEQ ID NO 1 replaced by E, A, Q, K, R, H or T more preferably Q, E, K, R, H, T. Most preferred are replacements L277Q, L277T and L277K.
- E at position 280 according to SEQ ID NO 1 replaced by G, A, Q, K, R or H, more preferably G, Q or R. Most preferred is replacement E280G.
- C at position 281 according to SEQ ID NO 1 replaced by Y, S, L, W, A, S, T or H, more preferably A, S, T, W, H or Y. Most preferred is replacement C281Y.
- T at position 293 according to SEQ ID NO 1 replaced by S, D, N, K or R, more preferably S, K or R. Most preferred is replacement T293K.

- G at position 300 according to SEQ ID NO 1 replaced by A, S, T, N, D, Q, E, K, R, H or L, more preferably A, S, T, K, R, H or L. Most preferred are replacements G300S, G300K and G300L.
- R at position 306 according to SEQ ID NO 1 replaced by H or a deletion.
- 5 • R at position 307 according to SEQ ID NO 1 replaced by T, K, A, S, V, G, D, N, Q, E, R, H, I or a deletion, more preferably S, T, K, R, H, I or a deletion. Most preferred are replacements R307T, R307I and R307deletion.
- A at position 311 according to SEQ ID NO 1 replaced by D.

Highly preferred mutant expandases are the mutant expandases that are summarized in Table 3 and 4. Preferably, the mutant expandases provided by the present invention have an at least 2.5-fold improved expandase activity on adipyl-6-APA as defined before. Alternatively, the mutant expandases provided by the present invention have an improved expandase activity on Pen-G of at least 1.5-fold in comparison with a model polypeptide with expandase activity, more preferably at least 3-fold, more preferably at least 4-fold, more preferably at least 5-fold, more preferably at least 6-fold, more preferably at least 7-fold, more preferably at least 8-fold. Mutant expandases with an improved activity on Pen-G can be used advantageously in a process for the production of phenylacetyl-7-ADCA as described further below. Preferably, the mutant expandases provided by the present invention have an improved expandase on both adipyl-6-APA as well as Pen-G as defined hereinbefore.

The present invention also provides mutant expandases with a decreased or even absent expandase activity with iso-penicillin N (iPN). Preferably the expandase activity with either adipyl-6-APA or Pen-G is not affected, but more preferably the expandase activity with either adipyl-6-APA or Pen-G or both is improved as defined hereinbefore. The advantage of these more preferred mutant expandases is that the decreased or even absent expandase activity with iso-penicillin N (iPN) results in less byproduct in a fermentation process producing adipyl-7-ADCA or phenylacetyl-7-ADCA.

Preferred mutant expandases have a decreased or even absent expandase activity with iso-penicillin N (iPN) as a substrate optionally combined with an improved expandase activity on ad-6-APA as a substrate and have been modified at position 89 according to the amino acid numbering of SEQ ID No 1. whereby the naturally occurring amino acid has been replaced, preferably by a positively charged amino acid such as lysine, arginine or histidine; most preferred being lysine.

A highly preferred mutant expandase is selected from the group consisting of

H401, H402, H403, H501, H502, H503, H504, H505, H506, H507, H508, H601, H602, H603, H604, H605, H606, H607, H608, H609, H650, H651, H652, H653, H654, H655, H656, H657, H658, H659, H660, H661, H662, G601, G602, G603, G604, G605, G606, G607, G608, G609, G610, G611, G613, G614, H701, H702, H703, H704, H705, H706

5 (see Table 3 and 4).

In a second aspect, the invention provides a polynucleotide encoding the mutant expandase of the present invention. The polynucleotide encoding the mutant expandase according to the present invention can be any polynucleotide that encodes the proper amino acid sequence according to the invention. Alternatively, the polynucleotide of the invention may comprise a coding sequence in which the codon usage for the various amino acids deviates from the codon usage in *S. clavuligerus*. For example, the codon usage may be adapted to the codon usage of a particular host cell, which will or has been transformed with the DNA fragment encoding the altered expandase.

15 In a third aspect, the invention provides an expression vector or expression cassette comprising the polynucleotide of the invention as defined hereinbefore.

In a fourth aspect, the invention provides a transformed host cell, transformed with the polynucleotide of the invention or the expression vector or expression cassette of the invention. The transformed host cell may be used for the production of the mutant expandase of the invention or the host cell may be used for the production of a beta-lactam compound of interest.

20 Host cells for the production of the mutant expandase of the invention are preferably host cells which are known in the art for their efficient protein or enzyme production, either extracellular or intracellularly, for example microorganisms such as fungi, yeast and bacteria. Examples of preferred host cells comprise, but are not limited to, the following genera: *Aspergillus* (e.g. *A. niger*, *A. oryzae*), *Penicillium* (e.g. *P. emersonii*, *P. chrysogenum*), *Saccharomyces* (e.g. *S. cerevisiae*), *Kluyveromyces* (e.g. *K. lactis*), *Bacillus* (e.g. *B. subtilis*, *B. licheniformis*, *B. amyloliquefaciens*), *Escherichia*, (*E. coli*), *Streptomyces* (e.g. *S. clavuligerus*).

30 Host cells for the production of a beta-lactam compound of interest are preferably host cells that are known in the art for their efficient beta-lactam compound production. Examples of preferred host cells comprise, but are not limited to, the following genera: *Penicillium* (e.g. *P. chrysogenum*), *Acremonium* (e.g. *A. chrysogenum*), *Streptomyces* (e.g. *S. clavuligerus*), *Nocardia* (e.g. *N. lactamdurans*),
35 *Lysobacter* (e.g. *L. lactamgenus*) and *Flavobacterium* species.

In a fifth aspect, the invention provides a process for the production of the mutant expandase of the invention comprising cultivating the transformed host cell according to the invention under conditions conducive to the production of the mutant expandase and, optionally, recovering the mutant expandase. The recovered mutant expandase may be used advantageously in an in vitro process to produce a desired cephalosporin from a corresponding penicillin, for instance the recovered mutant expandase may be used in a process to produce phenylacetyl-7-ADCA from Pen-G or adipyl-7-ADCA from ad-6-APA.

In a sixth aspect, the invention provides a process for the production of a beta-lactam compound of interest comprising cultivating the transformed host cell according to the invention under conditions conducive to the production of the beta-lactam compound of interest and, optionally, recovering the beta-lactam compound. Preferred beta-lactam compounds belong to the group of cephalosporins such as phenylacetyl-7-ADCA, adipyl-7-ADCA, adipyl-7-ADAC and adipyl-7-ACA. In a preferred embodiment, the invention provides a process for the production of phenylacetyl-7-ADCA or adipyl-7-ADCA by cultivating a selected strain of *Penicillium chrysogenum*, that has been transformed with a selected polynucleotide of the invention that encodes a mutant expandase of the invention. A highly preferred mutant expandase is selected from the group consisting of H401, H402, H403, H501, H502, H503, H504, H505, H506, H507, H508, H601, H602, H603, H604, H605, H606, H607, H608, H609, H650, H651, H652, H653, H654, H655, H656, H657, H658, H659, H660, H661, H662, G601, G602, G603, G604, G605, G606, G607, G608, G609, G610, G611, G613, G614, H701, H702, H703, H704, H705, H706 (see Table 3 and 4). For the production of phenylacetyl-7-ADCA, a mutant expandase is selected that has a high improvement factor on Pen-G as a substrate. For the production of adipyl-7-ADCA, a mutant expandase is selected that has a high improvement factor on ad-6-APA as a substrate.

In another preferred embodiment, the invention provides a process for the production of 7-ADCA comprising the process for the production of phenylacetyl-7-ADCA or adipyl-7-ADCA as described herein before, followed by, after optional purification of said 7-ADCA-derivatives, a process step in which the phenylacetyl or adipyl side chain of phenylacetyl-7-ADCA and adipyl-7-ADCA respectively is cleaved off thereby generating 7-ADCA and the liberated side chains acid. Said cleavage can be obtained by chemical means or, more preferably, enzymatically using an acylase enzyme. Suitable acylases for the cleavage of the adipyl side chain are obtainable from various *Pseudomonas* species such as *Pseudomonas* SY-77 or *Pseudomonas* SE-83.

Suitable acylases for the cleavage of the phenylacetyl side chains are the penicillin acylases from *Escherichia coli* or *Alcaligenes faecalis*.

In a seventh aspect, the invention provides a process for the production of a cephalorin from a corresponding penicillin whereby the expansion of the 5-membered thiazolidine ring of the penicillin to the 6-membered dihydrothiazine ring of the cephalorin occurs in an in vitro process, catalyzed by a mutant expandase of the invention. In a preferred process, adipyl-6-APA is expanded to the corresponding adipyl-7-ADCA by a mutant expandase of the invention, preferably a mutant expandase that has a high improvement factor adipyl-6-APA as a substrate. In another preferred process, Pen-G is expanded to the corresponding phenylacetyl-7-ADCA by a mutant expandase of the invention, preferably a mutant expandase that has a high improvement factor on Pen-G as a substrate. The process may be followed by, after optional purification of said 7-ADCA-derivatives, a process step in which the side chains of adipyl-7-ADCA and phenylacetyl-7-ADCA are cleaved off thereby generating 7-ADCA and the liberated side chains acid – supra vide. The desired end product 7-ADCA can be recovered according to methods known in the art involving chemical or enzymatic cleavage of the side chain of adipyl-7-ADCA or phenylacetyl-7-ADCA and optionally the resulting 7-ADCA can be further purified and/or crystallized.

In an eight aspect, the invention provides a method to obtain the mutant expandases of the invention whereby the process comprises the following steps:

1. Mutagenesis of a cloned gene encoding a model polypeptide with expandase activity thus obtaining a collection of mutagenised genes encoding the mutant expandases;
2. Expression of the collection of mutagenised genes encoding the mutant expandases in a suitable host and screening the collection of mutant expandases for an improved activity with a suitable substrate;
3. Optionally repeating steps 1 and 2 one or several times using either the gene encoding the model polypeptide with expandase activity or one or more of the mutagenised genes encoding mutant expandases with an improved activity on the suitable substrate.

Cloning of the gene encoding a model polypeptide with expandase activity can be carried out according to methods known in the art.

Preferred model polypeptides with expandase activity are selected from the group consisting of a polypeptide with expandase activity obtainable from *Streptomyces clavuligerus*, preferably having an amino acid sequence according to

SEQ ID NO: 1 and polypeptides with expandase activity having an amino acid sequence with a percentage identity with SEQ ID NO: 1 of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90%, most preferably at least 95%, such as the expandase enzymes that are summarized in Table 1.

Any mutagenesis technique can be employed which results in mutations over the entire gene. Suitable techniques are error prone (EP) PCR (Polymerase Chain Reaction) and/or by using saturated Mutation Primer PCR (sMPP) exactly according to WO 03/010183.

Materials and Methods

1. General

- Oligonucleotides were synthesized by Invitrogen (Carlsbad CA, US).
- DNA sequencing was carried out by SEQLAB (Göttingen, Germany) or by Baseclear (Leiden, The Netherlands).
- Restriction enzymes were purchased from Invitrogen.
- The protein assays were carried out according to the method described by Bradford, M.M. (1976) Anal Biochem. 72:248-54
- *Escherichia coli* DH10B electromax competent cells were obtained from Invitrogen.
- The protocol was delivered by the manufacturer.
- SDS-PAGE electrophoresis was carried out in the system supplied by Invitrogen. NuPAGE Novex Bis-Tris Gels (Invitrogen). SimplyBlue SafeStain Microwave protocol
- To create the CefE expression vector, the ampicillin selection marker in pBAD/Myc-His (commercially available from Invitrogen™) is replaced by the zeocin selection marker. Additionally, the maltose binding protein is fused to the wild type *S. clavuligerus* expandase gene and ligated behind the pBAD promoter resulting in the vector pBAD-MH-Zeo-MBP-ScEwt. The wild type CefE is exchanged by the library of error prone mutants.

2. Primary screening in microtitre plates (MTP)

Growth and induction

The expandase *E. coli* library is plated on Luria-Bertani (LB) medium plates low salt agar + 25 µg/ml Zeocine and incubated overnight at 37°C. Microtitre plates (MTP) with 150 µl LB low salt medium and 25 µg/ml Zeocine are inoculated from the plates

and incubated 36 hrs at 25°C and 550 rpm.

From the MTP, 5 µl is inoculated in deep well plate with 1 ml 2*TY (tryptone and yeast extract) medium + 50 µg/ml Zeocine + arabinose. To the remaining culture in the MTP, 100 µl 50% glycerol is added and frozen at -80°C as glycerol stock.

- 5 The deep well plate is incubated for 30 hrs at 25°C and 550 rpm. The deep well plate cultures are centrifuged at 2750 rpm (Heraeus multifuge 4 kr.) for 10 min. The supernatant is discarded and the pellet is frozen at -20°C.

Cell free extract (CFE)

- 10 The frozen pellets obtained in the previous section are thawed in 200µl extraction buffer (50 mM Tris/HCl pH=7.5; 5 mM DTT; 0.1 mg/ml DNase; 5 mM MgSO₄·H₂O and 0.5 mg/ml lysozyme). To resuspend pellets, the plates are shaken. The plates are incubated for 30 minutes at room temperature and centrifuged for 10 minutes at 2750 rpm.

15

3 Secondary screening in shake flasks

Growth and induction

- 20 Selected colonies are inoculated from plate in 10 ml LB low salt + Zeocin 25 µg/ml and incubated overnight at 37°C, 280 rpm. The grown culture is inoculated in 100 ml LB low salt with 25 µg/ml Zeocine at an optical density (600 nm) between 0.010 and 0.050 (Biochrom Ultraspec 2000). After growth at 37°C, 280 rpm, the cells are harvested at an optical density at 600 nm between 0.4-0.6. Then, arabinose is added (final concentration 0.2%) and induced overnight at 27°C at 220 rpm. The cultures are centrifuged, the supernatant is discarded and the pellet is frozen at -20°C.

25

Cloning of the *E. coli* expression vectors

- 30 The fusion product is ligated in the pBAD/MH MBP-ScEwt Dest zeo-zeo vector by an *EcoRI/SalI* digestion. This replaces the wild type expandase gene by the library genes. To prevent significant numbers of wild type constructs, the ligation constructs were digested with *SmaI*.

Construction of the libraries

- 35 Transformation of *E. coli* with the ligation mix yielded libraries of roughly 12,000-13,000 colonies. To test the quality of the libraries, a random set of colonies was selected and the constructs were digested with restriction enzymes. This showed

that approximately 26% of the library revealed an aberrant pattern. Next, sequence analysis was performed on 10 regular clones to determine mutation frequencies. Sequences showed that both saturation and error prone mutations were present at a satisfactory level.

5

4 Expandase assay

A total of 300 μ l of a reaction mixture consisting of 50 mM Tris/HCl pH 7.5; 1 mM DTT; 2.7 mM Ascorbate; 0.05 mM ATP; 24 mM α -ketoglutarate; 0.06 mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; 4 mM adipyl-6-amino-penicillanic acid (ad-6-APA) was added to 75 μ l CFE and incubated at 29°C for the desired time. Adding 60 μ l maleic acid with 10 g/l EDTA stopped the reaction. The formation of adipyl-7-amino-desacetoxy-cephalosporinic acid (ad-7-ADCA) was detected using $^1\text{H-NMR}$. Under these conditions, the expandase activity is measured close to V_{max} conditions since the K_M of the *S. clavuligeris* wild type enzyme for ad-6-APA is ca 0.4 mM. Alternatively, the assay may be carried out using 0.4 mM ad-6-APA ($\sim K_M$ of the *S. clavuligeris* wild type enzyme for ad-6-APA).

10

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The adipyl-6-APA was obtained from 6-APA and adipic acid catalyzed by glutaryl acylase (EC 3.5.1.11). Alternatively, adipyl-6-APA can be obtained by culturing for instance a suitable *Penicillium chrysogenum* strain in the presence of adipic acid as precursor.

EXAMPLES

Example 1

1st generation improved expandases (H400 series).

25

The first generation expandase library was constructed by performing error prone (EP) PCR (Polymerase Chain Reaction) on wild type *Streptomyces clavuligerus* CefE gene (SEQ ID No.1). After screening this library for improved conversion of ad-6-APA to ad-7-ADCA, 3 different mutant genes were selected. The mutants exhibited an improved ad-6-APA expansion activity up to 2.5-fold (H401, H402, H403: see Table 3).

30

Example 2

2nd generation improved expandases (H500 series)

The second-generation expandase library was constructed by using saturated Mutation Primer PCR (sMPP) exactly according to WO 03/010183. The selected

35

mutant genes from the first generation (H401, H402 and H403) were used as templates for the construction of the 2nd generation library.

The sMPP was performed by using Taq polymerase, thereby introducing additional mutations (random) and increasing the variation of the library. Designed
5 primers annealed at the mutation positions and were saturated at the mutations found in the 1st generation expandase hits. Additionally, a universal forward and reverse primer was designed in which an *Nde*I and *Nsi*I site were introduced respectively. This facilitated cloning into *Penicillium* expression vector for testing of the mutant expandases *in vivo*.

10 The library was grown and expression of the expandase was induced as described in the materials and methods section. The formation of ad-7-ADCA was determined using NMR as described in the materials and methods section.

Approximately 150 improved mutant expandases were selected and retested for their ad-6-APA expandase activity. Based upon the results of this retest, 17 mutants
15 were selected for final tests in shake flasks.

In total, 15 of the 17 selected hits showed a significant improved expandase activity with ad-6-APA as a substrate. To exclude the false positive selection of expression mutants, protein levels were quantified on SDS-PAGE. This confirmed that the improved activity was not due to stronger expression of expandase in *E. coli*, but
20 that the improvement could be attributed to an improved specific activity of the expandase mutant.

In total, 8 mutant expandases were identified which exhibited an improvement factor of their ad-6-APA expandase up to 4,5-fold (compared to the wild type expandase of *S. clavuligerus* – SEQ ID No 1.). These mutants are designated H501 –
25 H508 (see Table 3).

Example 3

3rd generation of improved expandases (H600 series)

The 8 mutants obtained in the 2nd generation Example 2 (H501 - H508) were
30 used as templates for the construction of the 3rd generation expandase library. This library was constructed in the same way as the 2nd generation expandase library. Additionally, the SKA signaling sequence at the C-terminus of the expandases was changed to SKD, causing expression of expandase solely in the cytosol when expressed in *Penicillium*.

35 The screening of this library yielded 22 different expandase mutant genes that

were significantly improved between 5-fold and 11-fold compared to *S. clavuligerus* expandase (Table 3, H600 series).

Screening of the same library for a second time using Pen-G at 7 mM as a substrate yielded 13 additional mutant expandases (G601-G611 and G613-G614; see Table 3)

Example 4

4rd generation of improved expandases (H700 series)

Ten mutants obtained in the 3rd generation Example 3 (G606, G612, H602, H606, H650, H651, H653, H655, H658, H661) were used as templates for the construction of the 4th generation expandase library. This library was constructed in the same way as the 3rd generation expandase library.

The screening of this library yielded 6 different expandase mutant genes that were significantly improved (up to 20-fold) when tested for their expandase activity on ad-6-APA at 0.4 mM concentration (Table 4, H701-H706).

Example 5

Activity of improved mutant expandases with iso-penicillin N (iPN) and Penicillin-G (Pen-G) as a substrate.

The activity of several mutant expandases was measured using iPN and Pen-G as a substrate. The assay with iPN as a substrate was carried out as described in the materials and methods section except that ad-6-APA was replaced by the same concentration of iPN (4 mM). The assay with Pen-G as a substrate was carried out as described in the materials and methods section except that ad-6-APA was replaced by 7 mM Pen-G. Table 3 summarizes the activities of the various expandase mutants for iPN and Pen-G.

Whereas most of the expandase mutants tested exhibited a 5-fold improvement of their expandase activity with iPN, mutants that carry the T89K mutation lost virtually all activity towards iPN.

The activity towards Pen-G of the various expandase mutants tested was either the same as the wild type expandase (improvement factor is 1) or improved up to 8-fold. The data further show that there is no correlation whatsoever between the improvement factors obtained for a single mutant with ad-6-APA and Pen-G as a substrate. The ratio between the respective improvement factors for ad-6-APA and Pen-G vary in a range from 0.1 (e.g. H654) to 1.2 (e.g. H503).

Table 3. Mutant expandases and their expandase activity toward ad-6-APA, Pen-G and

- iPN. Each mutant is identified by its code; the mutations are introduced in the model expandase from *S. clavuligerus* (SEQ ID NO 1). The expandase activity is expressed as improvement factor compared to the model expandase from *S. clavuligerus* (expandase activity = 1 by definition) according to the in vitro expandase assays described in the Materials and Methods and Example 4.

Mutant		Mutations	Expandase Activity (Improvement factor)		
	Code		ad-6-APA	Pen-G	iPN
1.	H401	L277Q	1.5		
2.	H402	D2N+C281Y	2.5		
3.	H403	T89A	1.5		
4.	H501	T89A+C281Y	3.9	3.0	2.6
5.	H502	D2Y+C281Y+R306Δ+A311D	4.5	4.0	5.3
6.	H503	D2N+T89V+C281Y	3.5	4.0	3.2
7.	H504	T89K+C281Y+T293K	3.9	1.0	0.0
8.	H505	T89K+C281Y	3.3	1.0	0.0
9.	H506	L277Q+G300S	2.9	3.0	4.6
10.	H507	D2Y+N90S+T105I+G113D+C155W+P177L+L277T+C281Y	3.7	3.0	3.0
11.	H508	D2H+L277Q+E280G+C281Y	4.6	4.0	5.1
12.	H601	M73H+T89K+C281Y+A311D	7.5	1.2	0.0
13.	H602	T89K+Y217H+C281Y+A311D	7.6	1.1	0.0
14.	H603	T89K+H244R+C281Y+A311D	6.8	1.7	0.0
15.	H604	T89K+C281Y+R307T+A311D	6.9	1.4	0.0
16.	H605	T89K+C281Y+R307T+A311D	7.2	1.5	0.0
17.	H606	M73I+T89K+C281Y+T293K+G300S+R307T+A311D	8.8	1.9	0.0
18.	H607	T89K+C281Y+T293K+G300S+A311D	7.0	1.3	0.0
19.	H608	T89K+C281Y+A311D	5.8	1.0	0.0
20.	H609	M73H+T89K+T213A+C281Y+A311D	6.2	1.3	0.0
21.	H650	T105I+G113D+C155W+P177L+C281Y+R306Δ+A311D	8.2	7.1	11,0
22.	H651	C155W+P177L+L277Q+E280G+C281Y+R306Δ+A311D	10.6	8.9	11,0
23.	H652	L277Q+C281Y+R306Δ+A311D		5.0	10,0
24.	H653	T89V+C281Y+R306Δ+A311D	9.0	5.8	11,0
25.	H654	T89K+R249C+C281Y+R306Δ+A311D	6.3	0.8	0,0
26.	H655	D2Y+N90S+T105I+G113D+C155W+P177L+C281Y+R306Δ+A311D	8.2	7.1	10,0
27.	H656	D2N+N90S+M99T+T105I+G113D+C281Y+R306Δ+A311D	5.0	3.7	5,0
28.	H657	N90S+T105I+G113D+C281Y+R306Δ+A311D	6.9	4.6	8,0
29.	H658	D2N+T89V+C281Y+R306Δ+A311D	8.4	6.3	10,0
30.	H659	T89K+C281Y+R306Δ+A311D	6.4	0.8	0,0
31.	H660	N90W+C281Y+R306Δ+A311D	5.3	4.4	5,0
32.	H661	D2Y+C155W+C281Y+R306Δ+A311D	9.7	6.4	
33.	H662	D2Y+N90S+T105I+G113D+C155W+P177L+L277T+C281Y+R306Δ+A311D	5.6	5.2	6,0
34.	G601	T89V+A278V+C281L+307deletion+A311D	7.4	6.8	
35.	G602	D2N+T89V+C155W+P177L+L277T+C281Y+A311D	3.9	4.4	

36.	G603	S59G+N90S+T105I+G113D+C155W+P177L+G209S+C281Y+307deletion+A311D	6.3	6.4	
37.	G604	D2N+N90W+C281Y+307deletion+A311D	4.6	5.7	
38.	G605	D2Y+C155W+P177I+C281Y+307deletion+A311D+SGRS	5.1	4.6	
39.	G606	D2Y+N90S+Y101F+T105I+G113D+C155W+P177I+C281Y+307deletion+A311D	8.2	8.1	
40.	G607	N90S+T105I+G113D+C155W+P177I+C281Y+R307T+A311D	7.2	6.7	
41.	G608	D2Y+N90S+T105I+G113D+C155W+P177I+L277T+C281Y+R307I+A311D	6.0	4.6	
42.	G609	D2Y+T89V+C281Y+307deletion+A311D	6.6	4.5	
43.	G610	D2N+T89V+T105I+G13D+C155W+P177L+C281Y+A311D	6.4	7.7	
44.	G611	T89V+T105I+G13D+C155W+P177L+C281Q+307deletion+A311D	7.0	7.9	
45.	G613	T89V+H170Y+D251G+L277Q+C281Y+307deletion+A311D	4.3	4.4	
46.	G614	D2Y+N90S+T105I+G113D+C155w+P177L+L277T+C281Y+T293K+307deletion+A311D	7.2	8.9	

Table 3 shows that 46 mutant expandases have been obtained which have improvement factors in the range of 1.5 to 10.6-fold.

5 Table 4

Mutant	Code	Mutations	Expandase Activity with adipyl-6-APA (Improvement factor)	
			4 mM	0.4 mM
47.	H701	T105I+G113D+C281Y+G300K+307deletion+A311D	13	20
48.	H702	N90S+T105I+G113D+C155W+L277K+C281Y+G300L+307deletion+A311D	12	16
49.	H703	D2Y+M73I+T89K+ T105K+G113D+ C281Y+307deletion+A311D	8	12
50.	H704	M73I+T89K+L277K+C281Y+307deletion+A311D	8	11
51.	H705	D2N+M73I+T89K+L277Q+E280G+C281Y+307deletion+A311D	9	13
52.	H706	C155W+C281Y+G300K+307deletion+A311D	11	16

CLAIMS

1. A mutant expandase that is a variant of a model polypeptide with expandase activity whereby the mutant expandase has an at least 2.5-fold improved *in vitro* expandase activity towards adipyl-6-APA in comparison with a model polypeptide with expandase activity.
5
2. A mutant expandase that is a variant of a model polypeptide with expandase activity whereby the mutant expandase has been modified at at least an amino acid position selected from the group consisting of positions 2, 59, 73, 89, 90, 99, 101, 105, 113, 155, 170, 177, 209, 213, 217, 244, 249, 251, 277, 278, 280, 281, 293, 300, 306, 307 and 311 using the amino acid position numbering of the amino acid sequence of the expandase enzyme encoded by the *cefE* gene of *Streptomyces clavuligerus* (SEQ ID NO: 1).
10
3. A mutant expandase according to claim 2 whereby the mutant expandase has been modified at at least an amino acid position selected from the group consisting of positions 2, 59, 89, 90, 99, 105, 113, 170, 177, 209, 213, 217, 249, 251, 278, 280 and 293, more preferably from the group consisting of positions 2, 89, 90, 99, 105, 177, 251, 278, 280 and 293.
15
4. A mutant expandase according to claim 1 which has been modified at at least an amino acid position selected from the group as defined in claim 2 or which has been modified at an amino acid position selected from any of the groups as defined in claim 3.
20
5. A mutant expandase according to anyone of claims 1-4 that comprises at least 2 or more amino acid positions selected from the group consisting of positions 2, 89, 277, 281 and 300, more preferably at positions 2+281 or 89+281 or 277+300.
25
6. A mutant expandase according to anyone of claims 1-5 that comprises at least 3 or more amino acid positions selected from the group consisting of positions 2, 89, 281, 293 and 311, more preferably at positions 2+89+281 or 89+281+311 or 89+281+293.
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7. A mutant expandase according to anyone of claims 1-6 that comprises at least 4 or more amino acid positions selected from the group consisting of positions 2, 73, 89, 90, 217, 244, 277, 281, 306, 307 and 311, more preferably at positions 2+277+280+281 or 2+281+306+311 or 73+89+281+311 or 89+217+281+311 or 89+244+281+311 or 89+281+307+311 or 277+281+306+311 or 89+281+306+311 or 90+281+306+311.
8. A mutant expandase according to anyone of claims 1-7 that comprises at least 5 or more amino acid positions selected from the group consisting of positions 2, 73, 89, 155, 213, 249, 281, 293, 300, 306 and 311, more preferably at positions 2+89+281+306+311 or 2+155+281+306+311 or 73+89+213+281+311 or 89+281+293+300+311 or 89+249+281+306+311.
9. A mutant expandase according to anyone of claims 1-8 that comprises at least 6 amino acid positions selected from the group consisting of positions 90+105+113+281+306+311.
10. A mutant expandase according to anyone of claims 1-9 that comprises at least 7 or more amino acid positions selected from the group consisting of positions 73, 89, 105, 113, 155, 177, 277, 280, 281, 293, 300, 306, 307 and 311, more preferably 73+89+281+293+300+307+311 or 105+113+155+177+281+306+311 or 155+177+277+280+281+306+ 311.
11. A mutant expandase according to anyone of claims 1-10 that comprises at least 8 or more amino acid positions selected from the group consisting of positions 2, 90, 99, 105, 113, 155, 177, 277, 281, 306 and 311 more preferably at 2+90+99+105+113+281+306+311 or 2+90+105+113+155+177+277+281.
12. A mutant expandase according to anyone of claims 1-11 that comprises at least 9 amino acid positions selected from the group consisting of positions 2+90+105+113+155+177+281+306+311.
13. A mutant expandase according to claim 1 that comprises at least 10 amino acid positions selected from the group consisting of positions 2+90+105+113+155+177+281+306+311.

14. A mutant expandase according to anyone of claims 2, 3 and 4-13 that has at least a 2.5-fold improved *in vitro* expandase activity towards adipyl-6-APA in comparison with a model polypeptide with expandase activity.
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15. A mutant expandase according to anyone of the preceding claims wherein the mutant expandase is a variant of the expandase of *S. clavuligerus* encoded by the gene *cefE* as depicted in SEQ ID No. 1.
- 10
16. A mutant expandase according to claim 15, wherein the mutant expandase has been modified at at least an amino acid position selected from the group consisting of positions D2, S59, M73, T89, N90, M99, Y101, T105, G113, C155, H170, P177, G209, T213, Y217, H244, R249, D251, L277, A278, E280, C281, T293, G300, R306, R307 and A311, more preferably at at least an amino acid
- 15
- position selected from the group consisting of positions 2, 59, 89, 90, 99, 105, 113, 170, 177, 209, 213, 217, 249, 251, 278, 280 and 293, more preferably from the group consisting of positions 2, 89, 90, 99, 105, 177, 251, 278, 280 and 293.
17. A polynucleotide encoding the mutant expandase of anyone of the preceding
- 20
- claims.
18. An expression vector or cassette comprising the polynucleotide of claim 17.
19. A host cell transformed with the polynucleotide of claim 17 or the vector or
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- cassette of claim 18.
20. A method of producing the mutant expandase of claims 1-16 comprising cultivating a host cell according to claim 19 under conditions conducive to the production of the mutant expandase and, optionally, recovering the polypeptide.
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21. A method of producing a β -lactam compound of interest comprising cultivating a host cell according to claim 20 under conditions conducive to the production of the β -lactam compound and, optionally, recovering the β -lactam compound.
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22. A method according to claim 21, further comprising N-deacylating the β -lactam

compound to produce an N-deacylated β -lactam compound.

23. A method to obtain the mutant expandases according to anyone of claims 1-16, whereby the method comprises the following steps:
- 5 ○ Mutagenesis of a cloned gene encoding a model polypeptide with expandase activity, preferably *cefE* encoding the expandase from *S. clavuligerus*, thus obtaining a collection of mutagenised genes encoding the mutant expandases;
 - 10 ○ Expression of the collection of mutagenised genes encoding the mutant expandases in a suitable host and screening the collection of mutant expandases for an improved activity with a suitable substrate, preferably ad-6-APA;
 - 15 ○ Optionally repeating steps 1 and 2 one or several times using thereby mutagenising the gene encoding the model polypeptide with expandase activity and/or one or more of the mutagenised genes encoding mutant expandases with an improved activity on the suitable substrate.