

Novel compounds

The present invention relates to novel bacterial genes and processes for improving the manufacture of clavams e.g. clavulanic acid. The present invention also provides novel organisms capable of producing increased amounts of clavulanic acid.

5 Microorganisms, in particular *Streptomyces* *sp.* produce a number of antibiotics including clavulanic acid and other clavams, cephalosporins, polyketides, cephamycins, tunicamycin, holomycin and penicillins. There is considerable interest in being able to manipulate the absolute and relative amounts of these antibiotics produced by the microorganism and accordingly there have been a large number of
10 studies investigating the metabolic and genetic mechanisms of the biosynthetic pathways [Domain, A.L. (1990) "Biosynthesis and regulation of beta-lactam antibiotics." In : 50 years of Penicillin applications, history and trends]. Many of the enzymes which carry out the various steps in the metabolic pathways and the genes which code for these enzymes are known.

15 Clavams can be arbitrarily divided into two groups dependent on their ring stereochemistry (5S and 5R clavams). The biochemical pathways for the biosynthesis of 5R and 5S clavams have not yet been fully elucidated but it has been suggested that they are derived from the same starter units (an as yet unidentified 3 carbon compound [Townsend, C.A. and Ho, M.F. (1985) J. Am. Chem. Soc. 107 (4), 1066-1068 and
20 Elson, S.W. and Oliver, R.S. (1978) J. Antibiotics XXXI No.6, 568] and arginine [Valentine, B.P. *et al* (1993) J. Am Chem. Soc. 115, 1210-1211] and share some common intermediates [Iwata-Reuyl, D. and C.A. Townsend (1992) J. Am. Chem. Soc. 114: 2762-63, and Janc, J.W. *et al* (1993) Bioorg. Med. Chem. Lett. 3:2313-16].

Examples of 5S clavams include clavam-2-carboxylate (C2C), 2-
25 hydroxymethylclavam (2HMC), 2-(3-alanyl)clavam, valclavam and clavaminic acid [GB 1585661, Rohl, F. *et al.* Arch. Microbiol. 147:315-320, US 4,202,819] There are, however, few examples of 5R clavams and by far the most well known is the beta lactamase inhibitor clavulanic acid which is produced by the fermentation of *Streptomyces clavuligerus*. Clavulanic acid, in the form of potassium clavulanate is
30 combined with the beta-lactam amoxycillin in the antibiotic AUGMENTIN (Trade Mark SmithKline Beecham). Because of this commercial interest, investigations into the understanding of clavam biosynthesis have concentrated on the biosynthesis of the

5R clavam, clavulanic acid, by *S.clavuligerus*. A number of enzymes and their genes associated with the biosynthesis of clavulanic acid have been identified and published. Examples of such publications include Hodgson, J.E. *et al.*, Gene 166, 49-55 (1995), Aidoo, K.A. *et al.*, Gene 147, 41-46 (1994), Paradkar, A.S. *et al.*, J. Bact. 177(5), 1307-14 (1995). In contrast nothing is known about the biosynthesis and genetics of 5S clavams other than clavaminic acid which is a clavulanic acid precursor produced by the action of clavaminic acid synthase in the clavulanic acid biosynthetic pathway in *S. clavuligerus*.

Gene cloning experiments have identified that *S.clavuligerus* contains two clavaminic acid synthase isoenzymes, cas1 and cas2 [Marsh, E.N. *et al* Biochemistry 31, 12648-657, (1992)] both of which can contribute to clavulanic acid production under certain nutritional conditions [Paradkar, A.S. *et al.*, J. Bact. 177(5), 1307-14 (1995)]. Clavaminic acid synthase activity has also been detected in other clavulanic acid producing micro-organisms, ie. *S. jumonjinensis* [Vidal, C.M., ES 550549, (1987)] and *S .katsurahamamus* [Kitano, K. *et al.*, JP 53-104796, (1978)] as well as *S. antibioticos*, a producer of the 5S clavam, valclavam [Baldwin, J.E. *et al.*, Tetrahedron Letts. 35(17), 2783-86, (1994)] . The latter paper also reported *S. antibioticos* to have proclavaminic acid amidino hydrolase activity, another enzyme known to be involved in clavulanic acid biosynthesis. All other genes identified in *S.clavuligerus* as involved in clavam biosynthesis have been reported to be required for clavulanic acid biosynthesis [Hodgson, J.E. *et al.*, Gene 166, 49-55 (1995), Aidoo, K.A. *et al.*, Gene 147, 41-46 (1994)] and as yet none have been reported which are specific for the biosynthesis of 5S clavams.

We have now identified certain genes which are specific for the biosynthesis of 5S clavams as exemplified by C2C and 2HMC in *S. clavuligerus*. Accordingly the present invention provides DNA comprising one or more genes which are specific for 5S clavam biosynthesis in *S. clavuligerus* and which are not essential for 5R clavam (e.g. clavulanic acid) biosynthesis.

By "gene" as used herein we also include any regulatory region required for gene function or expression. In a preferred aspect the DNA is as identified as Figure 1. Preferably the DNA comprises the nucleotide sequences indicated in Figure 1 designated as orfup3, orfup2, orfup1, orfdwn1, orfdwn2 and orfdwn3. The present

invention also provides proteins coded by said DNA. The present invention also provides vectors comprising the DNA of the invention and hosts containing such vectors.

Surprisingly we have found that when at least one of the genes according to the invention is defective the amount of clavulanic acid produced by the organism is increased. Accordingly the present invention also provides processes for increasing the amount of clavulanic acid produced by a suitable microorganism. In one aspect of the invention the genes identified can be manipulated to produce an organism capable of producing increased amounts of clavam, suitably clavulanic acid. The findings of the present work also allow an improved process for the identification of organisms with higher clavulanic acid production comprising a preliminary screening for organisms with low or no 5S clavam production (for example by hplc and/or clavam bioassay as described in the examples herein).

Suitably the 5S clavam genes of the present invention can be obtained by conventional cloning methods (such as PCR) based on the sequences provided herein. The function of the gene can be interfered with or eliminated/deleted by genetic techniques such as gene disruption [Aidoo, K.A. et al., (1994), Gene, 147, 41-46]., random mutagenesis, site directed mutagenesis and antisense RNA.

In a further aspect of the invention there are provided plasmids containing one or more defective genes, preferably the plasmids *pCEC060*, *pCEC061*, *pDES3*, *pCEC056* and *pCEC057*, described below. The genes can be made defective in a variety of ways. For example by insertion of a fragment of DNA encoding for an antibiotic resistance gene which completely abolishes the activity of that gene. Alternatively, other strategies may be employed to produce defective genes, including insertion of DNA not encoding for an antibiotic resistance gene, deletion of part of the gene, deletion of all of the gene or alteration of the nucleotide sequence of the gene by addition and/or substitution of one or more nucleotides. Defective genes according to the invention may be defective to different extents. They may be defective in that their activity is completely abolished or a proportion of the original activity may be retained.

Suitably, the plasmids of the invention are used to transform an organism such as *S. clavuligerus*, e.g. strain ATCC 27064 (which corresponds to *S. clavuligerus* NRRL 3585). Suitable transformation methods can be found in relevant sources

including : Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989), *Molecular cloning: a laboratory manual, 2nd Ed.*, ColdSpring Harbor Laboratory, Cold Spring Harbor, N.Y ; Hopwood, D.A. *et al.* (1985), *Genetic Manipulation of Streptomyces. A Cloning Manual*, and Paradkar, A.S. and Jensen, S.E. (1995), J. Bacteriol. 177 (5): 1307-1314.

Strains of the species *S. clavuligerus* are used industrially to produce clavulanic acid (potassium clavulanate). Within the British and United States Pharmacopoeias for potassium clavulanate (British Pharmacopoeia 1993, Addendum 1994, p1362-3 and U.S. Pharmacopeia Official Monographs 1995, USP 23 NF18 p384-5) the amounts of the toxic 5S clavam, clavam-2-carboxylate, are specifically controlled.

Therefore in a further aspect of the invention there is provided an organism capable of producing high amounts of clavulanic acid but has been made unable to make C2C or capable of producing high amounts of clavulanic acid but able to make only low levels of C2C. Suitably the clavulanic acid producing organism contains one or more defective clavam genes, and is preferably the *S. clavuligerus* strain 56-1A, 56-3A, 57-2B, 57-1C, 60-1A, 60-2A, 60-3A, 61-1A, 61-2A, 61-3A, and 61-4A, described below. Such organisms are suitable for the production of clavulanic acid without the production of the 5S clavam, clavam-2-carboxylate or with significantly reduced production of clavam-2-carboxylate.

EXAMPLES

In the examples all methods are as in Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning A Laboratory Manual* (2nd Edition), or Hopwood, D.A. *et al.* (1985) *Genetic Manipulation of Streptomyces. A Cloning Manual*, and Paradkar, A.S. and Jensen, S.E. (1995) J. Bacteriol. 177 (5): 1307-1314 unless otherwise stated.

I. DNA sequencing of the *Streptomyces clavuligerus* chromosome upstream and downstream of the clavamate synthase gene *casI*.

A. Isolation of *casI*.

To isolate chromosomal DNA fragments from *Streptomyces clavuligerus* NRRL 3585 encoding the gene for clavamate synthase isozyme 1 (*casI*) an oligonucleotide probe RMO1 was synthesised based on nucleotides 9-44 of the

previously sequenced *casI* gene (Marsh, E.N., Chang, M.D.T. and Townsend, C.A. (1992) *Biochemistry* 31: 12648-12657). Oligonucleotides were constructed using standard methods on an Applied Biosystems 391 DNA Synthesiser. The sequence of RMO1, a 36-mer, was synthesised in the antiparallel sense to that published by Marsh et al (1992, *ibid*) RMO1 was radiolabelled with ^{32}P using standard techniques for end-labelling DNA oligonucleotides (Sambrook *et al.*, 1989 *ibid*), and was used to screen a cosmid bank of *Streptomyces clavuligerus* genomic DNA by Southern hybridization as described by Stahl and Amann (In : *Nucleic acid techniques in bacterial systematics*. Ed. E. Stackebrandt and M. Goodfellow. Toronto : John Wiley and Sons, p. 205-248, 1991). The genomic bank of *S. clavuligerus* DNA, prepared in cosmid pLAFR3, was as described by Doran, J.L *et al.*, (1990), *J. Bacteriol.* 172 (9), 4909-4918.

Colony blots of the *S. clavuligerus* cosmid bank were incubated overnight with radiolabelled RMO1 at 60°C in a solution consisting of 5 x SSC, 5 x Denhardt's solution, and 0.5% SDS (1 x SDS: 0.15 M NaCl + 0.015 M Na₃citrate; 1 x Denhardt's solution: 0.02% BSA, 0.02% Ficoll, and 0.02% PVP). The blots were then washed at 68°C for 30 minutes in a solution of 0.5 x SSC + 0.1% SDS. One cosmid clone, 10D7, was isolated that hybridised strongly to RMO1 and gave hybridization signals upon digestion with restriction endonucleases *SacI* and *EcoRI* that were consistent with hybridization signals detected in similar experiments with digests of *S. clavuligerus* genomic DNA.

B. DNA sequencing of the *S. clavuligerus* chromosome flanking *casI*.

A partial restriction map of cosmid 10D7 was generated using restriction endonucleases *SacI*, *NcoI*, and *KpnI*. Southern hybridization experiments between RMO1 and various digests of 10D7 DNA indicated that *casI* was most likely located at one end of a 7-kb *SacI*-*SacI* DNA subfragment. This fragment consisted of the *casI* open reading frame and approximately 6 kb of upstream DNA. The 7-kb fragment was then subcloned from a *SacI* digest of 10D7 in the phagemid vector pBluescriptII SK+ (2.96 kb; Stratagene), thus generating the recombinant plasmid pCEC007.

To facilitate the process of sequencing the chromosome upstream of *casI*, a 3-kb *NcoI*-*NcoI* subfragment of the 7-kb *SacI*-*SacI* fragment was subcloned in pUC120 (3.2 kb; Vieira and Messing, *Methods Enzymol.* 153, 3-11, 1987)) in both

orientations, generating the recombinant plasmids pCEC026 and pCEC027. The 3-kb subfragment consisted of the amino-terminal-encoding portion of *casI* and approximately 2.6 kb of upstream DNA.

Nested, overlapping deletions were created in both pCEC026 and pCEC027 using exonuclease III and S1 nuclease digestion (Sambrook *et al.*, 1989 *ibid*) and the DNA sequence of the 3-kb *NcoI-NcoI* fragment was determined on both strands by the dideoxy chain termination method (Sanger, F., Nicklen, S. and Coulson, A.R. (1977), Proc. Natl. Acad. Sci. U.S.A. 74: 5463-5467) using a Taq dye-deoxy³ terminator kit and an Applied Biosystems 373A Sequencer.

To determine the DNA sequence of the chromosome immediately downstream of *casI* a 4.3-kb *KpnI-EcoRI* DNA fragment was subcloned from cosmid clone 10D7 in pBluescriptII SK+, generating pCEC018. From pCEC018 a 3.7-kb *SacI-SacI* subfragment was cloned in pSL1180 (3.422 kb, Pharmacia); one of the *SacI* termini of this fragment partially overlapped the TGA stop codon of *casI*, the other was vector encoded. Both orientations of the 3.7-kb fragment were obtained during subcloning and the resulting recombinant plasmids were designated pCEC023 and pCEC024. Nested, overlapping deletions were created in both plasmids and the DNA sequence of the 3.7-kb fragment was determined on both strands. The nucleotide sequence of the *S. clavuligerus* chromosome generated in these experiments, including and flanking *casI* sequence is shown in Fig. 1.

II. Functional analysis of the open reading frames flanking *casI*.

Computer analysis of the DNA sequence upstream of *casI* predicted the presence of two complete orfs and one incomplete orf. All three orfs were located on the opposite DNA strand to *casI* and were thus oriented in the opposite direction. The first open reading frame, *orfup1*, was located 579 bp upstream of *casI* and encoded a polypeptide of 344 amino acids (aa). The second open reading frame, *orfup2*, was located at 437 bp beyond the 3'-end of *orfup1* and encoded a 151 aa polypeptide. Beyond *orfup2* is *orfup3*. The start codon of *orfup3* overlaps the translational stop codon of *orfup2*, suggesting that the two orfs are translationally coupled. No translational stop codon for *orfup3* was located on the 3-kb *NcoI-NcoI* fragment.

A similar analysis of the DNA sequence downstream of *casI* predicted the presence of two complete orfs and one incomplete orf. Two of the orfs were located on the opposite DNA strand to *casI* and were thus oriented towards *casI*. The third orf was located on the same strand as *casI* and was thus oriented away from it. The first downstream open reading frame, *orfdown1*, was located 373 bp downstream of *casI* and encoded a 328 aa polypeptide. The second open reading frame, *orfdown2*, was located 55 bp upstream of *orfdown1* and encoded a 394 aa polypeptide. At 315 bp upstream of *orfdown2* and on the opposite strand was *orfdown3*. Because no stop codon was observed for *orfdown3* on the 3.7-kb fragment, it encoded an incomplete polypeptide of 219 aa.

Gene Disruption of the *orfup* and *orfdown* open reading frames

To assess the possible roles of the open reading frames flanking *casI* in the biosynthesis of clavulanic acid and the other clavams produced by *S. clavuligerus*, insertional inactivation or deletion mutants were created by gene replacement. The method used for gene disruption and replacement was essentially as described by Paradkar and Jensen (1995 *ibid*).

A. *orfup1*

A 1.5-kb *NcoI*-*NcoI* fragment carrying the apramycin resistance gene (*apr^r*), constructed as described in Paradkar and Jensen (1995 *ibid*), was treated with Klenow fragment to generate blunted termini (Sambrook *et al.*, 1989 *ibid*) and was ligated to pCEC026 that had been digested with *BsaBI* and likewise treated with Klenow fragment. pCEC026 possesses a *BsaBI* site located within *orfup1* at 636 bp from the translational start codon. The ligation mixture was used to transform competent cells of *E. coli* GM 2163 (available from New England Biolabs, USA., Marinus, M.G. *et al* M G G (1983) vol 122, p288-9) to apramycin resistance. From the resulting transformants two clones containing plasmids pCEC054 and pCEC055 were isolated; by restriction analysis pCEC054 was found to possess the *apr^r*-fragment inserted in the same orientation as *orfup1*, while pCEC055 possessed it in the opposite orientation.

To introduce pCEC054 into *S. clavuligerus*, plasmid DNA was digested with *Bam*HI and *Hind*III and ligated to the high-copy number *Streptomyces* vector pIJ486 (6.2 kb; Ward *et al.*, (1986) Mol. Gen. Genet. 203: 468-478). The ligation mixture was then used to transform *E. coli* GM2163 competent cells to apramycin resistance.

5 From the resulting transformants one clone, possessing the shuttle plasmid pCEC061, was isolated. This plasmid was then used to transform *S. clavuligerus* NRRL 3585. The resulting transformants were put through two successive rounds of sporulation on non-selective media and then replica plated to antibiotic containing media to identify apramycin-resistant and thiostrepton-sensitive transformants. From this process four

10 putative mutants (61-1A, -2A, -3A and -4A) were chosen for further analysis.

To confirm that these putative mutants were disrupted in *orfup1* genomic DNA was prepared from isolates 61-1A and 61-2A, digested with *Sac*I and subjected to Southern blot analysis. The results of the Southern blot were consistent with a double cross-over having occurred and demonstrated that these mutants are true disruption

15 replacement mutants in *orfup1*.

The mutants 61-1A, -2A, -3A and -4A were grown in Soya-Flour medium and their culture supernatants were assayed by HPLC for clavulanic acid and clavam production. The composition of the Soya-Flour medium and the method for assaying clavams by HPLC were as previously reported (Paradkar and Jensen, 1995 *ibid*) except

20 that the running buffer for the HPLC assay consisted of 0.1 M NaH₂PO₄ + 6% methanol, pH 3.68 (adjusted with glacial acetic acid). The HPLC analysis indicated that none of the mutants produced detectable levels of clavam-2-carboxylate or 2-hydroxymethylclavam. Furthermore, when culture supernatants were bioassayed against *Bacillus* sp. ATCC 27860, using the method of Pruess and Kellett (1983, J.

25 Antibiot. 36: 208-212), none of the mutants produced detectable levels of alanylclavam. In contrast, HPLC assays of the culture supernatants showed that the mutants appeared to produce superior levels of clavulanic acid when compared to the wild-type (Table 1).

Table 1

Clavulanic acid titre (CA) of orfup1 mutants in shake flask tests

STRAIN	70 HOURS	70 HOURS	93 HOURS	93 HOURS
	CA ug /ml	CA ug/mg DNA	CA ug /ml	CA ug/mg DNA
NRRL 3585 #1	87	915	166	1963
NRRL 3585 #2	66	790	159	1842
61-1A	272	2894	439	6113
61-2A	199	2148	225	2928
61-3A	54	692	221	2585
61-4A	0	0	226	2422

5 orfup1 Deletion

A cloning experiment was undertaken to create a gene deletion of 654 nucleotides between the *AatII* sites of *orfup1*. PCR products were generated using the oligonucleotide primers listed below and the above described pCEC061 as the
 10 template. The original nucleotide sequence was altered to incorporate a *PstI* into oligo 11 and a *SphI* site in oligo 14.

Oligonucleotide Pair 1 used to generate PCR product 1

15 Primer 11 5' dCTGACGCTGCAGGAGGAAGTCCCGC 3'
 Primer 12 5' dCGGGGCGAGGACGTCGTCCCGATCC 3'

Oligonucleotide Pair 2 used to generate PCR product 2

20 Primer 13 5' dGAGCCCCTGGACGTCGGCGGTGTCC 3'
 Primer 14 5' dGACGGTGCATGCTCAGCAGGGAGCG 3'

Standard PCR reactions were carried out using the PTC-200 Peltier thermal cycler from GRI (Felsted, Dunmow, Essex, CM6 3LD).

PCR product 1 was generated using primers 11 and 12. This product is approximately 1Kb and contains the carboxy terminus of *orfup1* from the second *AatII* site and downstream regions.

5 PCR product 2 was generated using primers 13 and 14. This product is approximately 1.1Kb and contains the amino terminus of *orfUp1* from the first *AatII* site and upstream regions.

10 PCR product 2 was ligated to *SrfI* digested pCR-Script Amp SK(+) as per instruction manual (Stratagene Ltd, Cambridge Science Park, Milton Road, Cambridge CB4 4GF). The ligation mixture was used to transform Epicurian *E.coli* XL1- Blue MRF' Kan supercompetent cells (available from Stratagene) to ampicillin resistance (as per manufacturers instructions). Plasmid DNA was isolated from the resulting transformants and DNA restriction analysis revealed that 7 clones containing plasmid into which PCR product 2 had been ligated. One of these plasmids was designated
15 pDES1.

20 PCR product 1 was digested with *PstI* and *AatII* and the resultant DNA fractionated by agarose gel electrophoresis. The 1Kb fragment was excised and eluted from the gel using the Sephaglas band prep kit. (Pharmacia, St Albans, Herts, ALI 3AW). The isolated fragment was then ligated to *AatII* and *PstI* digested pDES1. The ligation mixture was used to transform competent cells of *E.coli* XL1-Blue (available from Stratagene) to ampicillin resistance (as per manufacturers instructions). Plasmid DNA was isolated from the resulting transformants and restriction analysis revealed that 1 clone contained plasmid into which PCR product 1 had been ligated. This plasmid was
25 designated pDES2.

To introduce pDES2 into *S.clavuligerus* the plasmid was further modified to contain an origin of replication which could function in streptomyces. To achieve this the plasmid DNA of pDES2 was digested with *EcoRI* and *HindIII* and ligated into the high
30 copy number *Streptomyces* vector pIJ486 (6.2Kb: Ward et al.,(1986) Mol. Gen. Genet. 203:468-478) also digested with *EcoRI* and *HindIII*. The ligation mixture was used to transform *E.coli* (JM109) competent cells (Stratagene Ltd) to ampicillin resistance.

Plasmid DNA was isolated from the resulting transformants and restriction analysis revealed 6 clones possessed pDES2 containing pIJ486. One of these plasmids was designated pDES3. The plasmid pDES3 was used to transform an *S.clavuligerus* strain in which the *orfup1* gene had already been disrupted by insertion of the apramycin resistance gene (as described above). Thiostrepton resistant transformants were selected and these transformants were then put through 3 rounds of sporulation on non-selective media and screened for the loss of apramycin resistance. From this process 45 mutants were identified which had lost apramycin resistance. These were then analysed by HPLC which confirmed that these strains, like the *orfup1* disruptants 61-1A, 61-2A, 61-3A and 61-4A were unable to produce clavam 2 carboxylate and 2 hydroxymethyl clavam when fermented in conditions where these clavams are normally produced.

B. *orfdown1* and *orfdown2*

A deletion/replacement mutant in *orfdown1* and *orfdown2* was created by first digesting pCEC018 (7.3 kb) with *NcoI* and liberating a 1-kb subfragment containing most of *orfdown1* and a portion of *orfdown2*. The digest was fractionated by agarose-gel electrophoresis and the 6.3-kb fragment was excised and eluted from the gel. This fragment was then ligated to an *NcoI-NcoI* DNA fragment carrying *apr^r* and used to transform *E. coli* XL1-Blue to apramycin resistance. One clone was obtained from this experiment but restriction analysis of the resulting recombinant plasmid revealed that two copies of the apramycin resistance fragment had been ligated into the deletion plasmid. To eliminate the extra copy of the *apr^r*-fragment, the plasmid was digested with *NcoI* and self-ligated. The ligation mixture was used to transform *E. coli* GM2163 to apramycin resistance. From the transformants, two clones were isolated that contained plasmids pCEC052 and pCEC053 both of which possessed only one copy of the *apr^r*-fragment; pCEC052 possessed the *apr^r*-fragment inversely oriented with respect to *orfdown1* and 2, while pCEC053 possessed the *apr^r*-fragment inserted in the same orientation as *orfdown1* and 2.

A shuttle plasmid of pCEC052 was constructed by ligating *Bam*HI-digested pCEC052 with similarly digested pIJ486 and transforming *E. coli* GM2163 to apramycin resistance. From this experiment one clone was isolated that contained the

shuttle plasmid pCEC060. This plasmid was used to transform wild-type *S. clavuligerus* 3585 to apramycin and thiostrepton resistance. The resulting transformants were put through two rounds of sporulation under non-selective conditions and then replica plated to antibiotic containing media to identify apramycin resistant, thiostrepton sensitive colonies. Three putative mutants (60-1A, -2A and -3A) were chosen for further analysis.

To establish the identity of these putative mutants genomic DNA was isolated from strains 60-1A and 60-2A and digested with either SacI or BstEII and subjected to southern blot analysis. The hybridisation bands generated from this experiment were consistent with both strains having undergone a double cross-over event demonstrating that these mutants are true disruption replacement mutants in *orfdwn1/2*.

When these were cultured in Soya-Flour medium and their culture supernatants assayed by HPLC, none of the mutants produced detectable levels of clavam-2-carboxylate or 2-hydroxymethylclavam. A bioassay of the culture supernatants showed that the mutants also failed to produce detectable levels of alanylclavam. As with the *orfup1* mutants, the *orfdwn1/2* mutants are capable of producing superior to wild-type levels of clavulanic acid (Table2).

Table 2

Clavulanic acid titre (CA) of *orfdwn1/2* mutants in shake flask tests

STRAIN	70 HOURS	70 HOURS	93 HOURS	93 HOURS
	CA ug/ml	CA ug/mg DNA	CA ug/ml	CA ug/mg DNA
NRRL 3585 #1	87	915	166	1963
NRRL 3585 #2	66	790	159	1842
60-1A	164	1872	260	2911
60-2A	187	2013	108	1320
60-3A	79	994	214	2161

orfdwn3

To disrupt *orfdwn3* pCEC023 (consisting of a 3.7-kb fragment of *cas1* downstream DNA subcloned into pSL1180) was digested with NcoI and then self ligated. After transforming *E.coli* with the ligation mixture a clone was isolated that possessed the plasmid pCEC031. This plasmid retained only the 1.9kb NcoI-EcoRI

fragment encoding a portion of orfdwn2 and the incomplete orfdwn3. An examination of the DNA sequence revealed that pCEC031 possessed a unique BstEII site at 158bp from the translational start site of orfdwn3. Therefore, pCEC031 was digested with BstEII, treated with Klenow fragment to create blunt ends and then ligated to a blunted apramycin resistance cassette. The ligation mixture was used to transform *E. coli* GM2163 to apramycin resistance and ampicillin resistance. Two transformants were selected that contained respectively pCEC050 and pCEC051. restriction analysis revealed that the apramycin resistance cassette was orientated in the same orientation as orfdwn3 in pCEC050 and in the opposite orientation in pCEC051. Both of these plasmids were then digested with HindIII and ligated to similarly digested pIJ486. The ligation mixtures were then used separately to transform *E. coli* GM2163 to apramycin and ampicillin resistance. The shuttle plasmids pCEC056 (pCEC050 + pIJ486) and pCEC057 (pCEC051+ pIJ486) were isolated from the resultant transformants. Both plasmids were then used to transform *S. clavuligerus* NRRL 3585.

One transformant was selected from each transformant experiment and put through two successive rounds of sporulation on non-selective media and then replica plated to antibiotic containing media to identify apramycin-resistant and thiostrepton-sensitive transformants. From this process two putative mutants were isolated from the progeny of each primary transformant. (56-1A and 56-3A for pCEC056, and 57-1C and 57-2B for pCEC057).

To establish the identity of these putative mutants genomic DNA was isolated from these strains and digested with either SacI or Acc65I and subjected to Southern blot analysis. The hybridisation bands generated from this experiment were consistent with both strains having undergone a double cross-over event demonstrating that these mutants are true disruption replacement mutants in orfdwn3.

When these strains were cultured in Soya-Flour medium and their culture supernatants assayed by HPLC, the mutants produced greatly reduced levels of clavam-2-carboxylate or 2-hydroxymethylclavam. A bioassay of the culture supernatants showed that the mutants also failed to produce detectable levels of alanylclavam. As with the orfup1 and orfdwn1/2 mutants, the orfdwn3 mutants were capable of producing superior to wild-type levels of clavulanic acid (Table 3).

Table 3

Clavulanic acid titre (CA) of orfdwn3 mutants in shake flask tests

STRAIN	71 HOURS	71 HOURS	93 HOURS	93 HOURS
	CA ug/ml	CA ug/mg DNA	CA ug/ml	CA ug/mg DNA
NRRL 3585 #1A	180	1580	193	1790
NRRL 3585 #1B	179	1640	266	2310
56-1A	34	110	235	2160
56-3A	225	2140	274	2740
57-1C	253	2910	277	2920
57-2B	242	2240	193	1860

5 The application discloses the following nucleotide sequences:

- SEQ ID No. 1 : DNA sequence of Figure 1
- SEQ ID No. 2 : orfup3 sequence
- SEQ ID No. 3 : orfup2 sequence
- 10 SEQ ID No. 4 : orfup1 sequence
- SEQ ID No. 5 : orfdwn1 sequence
- SEQ ID No. 6 : ofrdwn2 sequence
- SEQ ID No. 7 : orfdwn3 sequence
- SEQ ID No. 8 : oligonucleotide primer 11 sequence
- 15 SEQ ID No. 9 : oligonucleotied primer 12 sequence
- SEQ ID No. 10: oligonucleotide primer 13 sequence
- SEQ ID No. 11: oligonucleotide primer 14 sequence

5 NcoI

1 GGTACCGCCCCGCCGCGACGGGGCCTCGGAGCCGGCCTGGCCACTGGTCCTGGTGGGGCC 60
M A P P P Q G P A E A P G T V L V V G

10 61 ACCCTATCACCGGGCGGTGGGCGCGCTCGTCTGAGGGCCTGTGCCTGGGCACCCACACGC 120
T P Y H G A V R R L L S G S V S G H T H

15 121 GCCTTTCCGGGCCTCCGGCCCCAGTGTGGTGCCCATTTGCGCGCCACAGGAACGGGCGCAT 180
* L W P Y R A T D K G A Y
A S L G P P R T M

20 181 TAGCCCCAGGTCTATCTGCTTCCGGGCCACCTGCTCCTTCAGGGCGTGGAGCATCTGGCA 240
D P D L Y V F A R H V L F D R V E Y V T

25 241 CGTGGTCGCGGGCCGCCGGGTGAGCCCCAGTGGGCGGGCGGTGCCGGGCAGGGCCACGAG 300
C W R G A A W E P D G A R W P G D R H E

30 301 TGGCACCCACCACGGGAGGCGCCGCTCCTCAAGCCAGGGCCAGTCTTAGGTCAACTGCCT 360
G H T T G E A A L L E T G T L I W N V S

35 361 GGTGTCTACCACCCACTAGCTCGCCTACCACGGGGGCTCCAGCAGCTTCTCGGCCCGCTA 420
W L H H T I S R I T G G L D D F L R A I

421 GAGCCTGAACGGGGCCCCGGTCTGGGGTGAACCCCTTCTTCTTCTGGCGCAGGAGCCGCTT 480
E S K G R A L G W K P F F F V A D E A F

481 CATCAGCTAGCGCCCCCACGGCAGCGACGGCTGCGGCGGCAACAGCTTGCGGAACCTTCAT 540
Y D I A P T G D S G V G G N D F A K F Y

55 541 GCGCCACTACTGGCGGAACGCGACGAGCAGGCAGTATGGCCGGCTACGGTGCCTGTACTT 600
A T I V A K R Q E D T M G A S A V S M

601 TGCTGGAGGTCTCTAAGGCCCCACCGACACGACCCCGACGCCTTCCCCACAGGGGGCGCTT 660
<orfup2

661 CCTGCCGCCTGCGGGCGCTGCGGGCGCCGGCAGAGGGGGCCGCTGCCCAGGGTTCGCAGGAC 720

721 CTCTCCCGAACC GCCCGCCGAAC TGGGCACGACAGGGCGCCGAACGCCTTGCGCTTCATG 780

781 GCCGGTCGCATGCCCCGAACGTGGCCTGCACATGCGGGCCAGCCCTGGGGAGCATGGGGGC 840

841 CTCGGCCGGCTGGGGCCGCCGAGGCCCCCATGCCTGCGCGGCCTGGCCGGGCTCGCTCGG 900

901 CCTGCCCAGCCTGCCACGCGCACCAAGGCCACACAGCCTGTGAGCCTGCCTGGCCTGCC 960

961 ACGCGCACCAAGGCCACACAGCCTGTGAGCCTGCCAGCCTGCCACGCGCACCAAGGCC 1020

1021 GTGCGGCCTGCCCAGTCAACGGCTAGTACCGCTCGTTACGGCCCCACATGGCGAGGGGCC 1080

* N G I M A L L A P T Y R E G

1081 TGTGGCCCAACCCTCTAGCGCCGGCAGTGGAGGCGCTCCCTGGCCAGCAGGTTCGGCCTAGC 1140

S V P H S I A A T V E A L S R D D L R I

1141 TCCGCCGCCGCTCTAACAGGCGCTCTACCGGGCCCAAGCGCCACGGGCCCTAGCCCTGCT 1200

S A A A L N D A L H A P N A T G P I P V

1201 GCAGGAGCGGGGCCACCACGTTCGGTCCGCTCGCGCTCGACACGGTCCCAGTCGGGGTCTG 1260

V D E G R H H L W A L A L Q A L T L G L

1261 GCAGGCGCTGGCCCGCGTCGGCCACGTTCGTTGCTCGCCAACGCGCGCTCCCGGCCTCGCG 1320

G D A V P R L R H L L S R N R A L A P A

1321 ACTTGGCCCCGACCGGGGCCGCTTCAGGAGCAGGGGGTCTAGCAGCCACCACGCCTACC 1380

S F R P Q G R R F D E D G L D D T T R I

1381 ACGGCCACTCTTTTGGGGCAGGGTCTCCCCGCATTGCTGCTAGGGCTAGGGGTTCGAGGG 1440

T G T L F G R G L P A Y A V I G I G L E

1441 CCGTCTGCCCCTGGTGGAGCAGGAGCTAGGGCGCGCTGGTGTCCGAGGTGAGCGAGACGT 1500

R C V P V V E D E I G R S W L S W E S Q

1501 GGCGGCAGTGGCCACGTGGCGCAGGCGGGCCGCGTCGCACCGGCGCCTCCCGAGCCTCT 1560

V A T V P H V A D A R R L T A A S P E S

1561 CTGGCTCGGACGCCTGGAACGGGAGCGCGTGGTTCGAGCCGGTGGCGTGGGTGCCAGAGGA 1620
 L G L R R V K G E R V L E A V A G V T E
 1621 GCTAGCCGTGGCGGCCCCAGGCAGGTCACGACCATCATGTCCAGCTACGCCAGCCACGGCT 1680
 E I P V A P D T W H Q Y Y L D I R D T G
 1681 CTGCTGCGTCCCTGGCAAGCGTCCGGCGCGCCTGCATCCTGCCGAGCGGCGTGTTCGGGA 1740
 L R R L S R E C A A R V Y S P E G C L G
 1741 CCCTCCGCGGCAGCCTGCTCGCGTGGTACGGCTTGAACCACCGCTAGTCGTGGAGCAGGG 1800
 Q S A G D S S R V M G F K T A I L V E D
 1801 CCGCCGGGCGCTGGCGGGCAGGCTCGTCGAGGAGTGGCCGCGGCTCGGGGACCTGCAGCC 1860
 R R G A V A R G L L E E G A G L G Q V D
 1861 GCCACAGGTCGTCCCACTGGGGCCGCAGCTGCCGCCGCGCCTACCACCGGCAGCGGGCCC 1920
 A T D L L T V G A D V A A R I T A T A R
 1921 GCGCCAGGCCCCGCAGGCATCTTCAGCCACCAGCCGTCCGTCGGCTCGGGGACCCGTGACT 1980
 A R D P R G Y F D T T P L C G L G Q A S
 1981 GGCCTTCCAGGGCGTCCCGCGCCTGGCCGCCTGCGCCTTGGCGCCGCCTGTGCCTTGGCC 2040
 V P L D R L A R V P P R P V A A S V S G
 <orfup1
 2041 CCGGGGACTCGGGCGGAGAGCGGGACATACGGAACCTCCACAGGCGGAGCCGGGAACGGG 2100
 GGCCCCCTGAGCCCGCCTCTCGCCCTGTATGCCTTGGAGGTGTCCGCCTCGGCCCTTGCCC
 A P S E P P S R S M
 2101 ACGAGGGCGAGGACGGGACGGAACGAAGGAGGACGGGACGGACAGCACGGACGGGACG 2160
 TGCTCCCGCTCCTGCCCTGCCTTGCTTCCTCTCCTGCCCTGCCTGTGCTGCCTGCCCTGC
 2161 GACGGAACGGAGTCGGGAACCGGGGGGGGTGACCGGAACCGGGCCGTCTTGGCCCTCCC 2220
 CTGCCTTGCTCAGCCCTTGGCCCCCCCCCACTGGCCTTGGCCCCGCAGGAACCGGGAGGG
 2221 CCGTCCTCCCCGCCATCCGCCGTTCTCCCCGTTCCCTCTCCCGTCCTCCAGCCAACACC 2280
 GGCAGGAGGGGCGGTAGGCGGCAAGAGGGGGCAAGGGAGAGGGCAGGAGGTGCGTTGTGG

2281 GCCGCCCTTTCCAAGCGCTTGACACGGCACCGACAGCCGCCCGGGCGCCGATGGGGA 2340
CGGCGGGAAAGGTTTCGCGAACTGTGCCGTGGCTGTCCGGCGGCGGCCCGGGCTACCCCT

2341 CCCGTGCCCCGCCGGTGAGCGGCGGTGAGCGCCGGTACGGGACCCACGCGCCGCCGCCG 2400
GGGCACGGGCGGCCACTCGCCGCCACTCGCGGCCATGCCCTGGGGTGCGCGGCGGCGGGC

2401 GGCGCCCCGCCAGGGCCCGCGCGGCCACCCCGGCCCGCCCGGCCGGAGCGGCGATCCGGG 2460
CCGCGGGCGGTCCCGGGCGCGCCGGTGGGGCCGGGCGGGCCGGCCTCGCCGCTAGGCCC

2461 CCGCTCGCTGCAAGAGGAACATCCACAGCCGCACAAGGAGCGCTCCGCACAGTGGGCACC 2520
GGCGAGCGACGTTCTCCTTGTAGGTGTCCGGCGTGTTCCTCGCGAGGCGTGTACCCGTGG

2521 ACGTCCGCCCCGTCCCCACACCGTGGCCGGTCCCCACCGGACAGCACAGCACCGCACAG 2580
TGCAGGCGGGGCAGGGGGTGTGGCACCGGCCAGGGGTGGCCTGTCTGTCTGTGGCGTGTCTC

2581 CACCACATCGCACGGCACAGCACAGCACCGGCCACGAGGAACCAAGGAAAGGAACCAC 2640
GTGGTGTAGCGTGCCGTGTCTGTCTGTGGTGGCCGTGCTCCTTGGTTCCTTTCCTTGGTG

cas1>

M T S V D C T A Y G P E L R A L A A

2641 ACCACCATGACCTCAGTGGACTGCACCGCGTACGGCCCCGAGCTGCGCGCGCTCGCCGCC 2700
TGGTGGTACTGGAGTCACCTGACGTGGCGCATGCCGGGGCTCGACGCGCGCGAGCGGCGG

2701 CGGCTGCCCCGGACCCCCCGGGCCGACCTGTACGCCTTCCTGGACGCCGCGCACACAGCC 2760
R L P R T P R A D L Y A F L D A A H T A

2761 GCCGCCTCGCTCCCCGGCGCCCTCGCCACCGCGCTGGACACCTTCAACGCCGAGGGCAGC 2820
A A S L P G A L A T A L D T F N A E G S

2821 GAGGACGGCCATCTGCTGCTGCGCGGCCTCCCGGTGGAGGCCGACGCCGACCTCCCCACC 2880
E D G H L L L R G L P V E A D A D L P T

NcoI

2881 ACCCCGAGCAGCACCCCGGCGCCCGAGGACCGCTCCCTGCTGACCATGGAGGCCATGCTC 2940
T P S S T P A P E D R S L L T M E A M L

KpnI

2941 GGA CTGGTGGGCGCGCGGCTCGGTCTGCACACGGGGTACCGGGAGCTGCGCTCGGGCACG 3000

G L V G R R L G L H T G Y R E L R S G T

 3001 GTCTACCACGACGTGTACCCGTCGCCCCGGCGCGCACCACCTGTCTCTCGGAGACCTCCGAG 3060
 V Y H D V Y P S P G A H H L S S E T S E

 3061 ACGCTGCTGGAGTTCCACACGGAGATGGCCTACCACCGGCTCCAGCCGAACCTACGTCATG 3120
 T L L E F H T E M A Y H R L Q P N Y V M

 3121 CTGGCCTGCTCCCCGGGCGGACACGAGCGCACGGCGGCCACACTCGTCGCCTCGGTCCGC 3180
 L A C S R A D H E R T A A T L V A S V R

 3181 AAGGCGCTGCCCCCTGCTGGACGAGAGGACCCGGGCCCCGGCTCCTCGACCGGAGGATGCCC 3240
 K A L P L L D E R T R A R L L D R R M P

 3241 TGCTGCGTGGATGTGGCCTTCCGCGGCGGGGTGGACGACCCGGGCGCCATCGCCCAGGTC 3300
 C C V D V A F R G G V D D P G A I A Q V

 3301 AAACCGCTCTACGGGGACGCGGACGATCCCTTCCTCGGGTACGACCGCGAGCTGCTGGCG 3360
 K P L Y G D A D D P F L G Y D R E L L A

 3361 CCGGAGGACCCGCGGACAAGGAGGCCGTCGCCCCCTGTCCAAGGCGCTCGACGAGGTC 3420
 P E D P A D K E A V A A L S K A L D E V

 3421 ACGGAGGCGGTGTATCTGGAGCCCGGCGATCTGCTGATCGTCGACAACTTCCGCACCACG 3480
 T E A V Y L E P G D L L I V D N F R T T

 3481 CACGCGCGGACGCCGTTCTCGCCCCGCTGGGACGGGAAGGACCGCTGGCTGCACCGCGTC 3540
 H A R T P F S P R W D G K D R W L H R V

 3541 TACATCCGCACCGACCGCAATGGACAGCTCTCCGGCGGCGAGCGCGGGCGACGTCGTC 3600
 Y I R T D R N G Q L S G G E R A G D V V

 A F T P R G * *SacI*
 3601 GCCTTCACACCGCGCGGCTGAGCTCCCGGGTCCGACACCGCGCGGCTGAACCCACGGTCC 3660
 CGGAAGTGTGGCGCGCCGACTCGAGGGGCCAGGCTGTGGCGCGCCGACTTGGGTGCCAGG

 3661 GGGGCCCACGGTCCGGCACCGCGCGGCTGAGCCCCCGGGTCCGGCAGCGGGCGGCTGAAC 3720
 CCCCGGGTGCCAGGCCGTGGCGCGCCGACTCGGGGGCCCAGGCCGTGCCCCGCGGACTTG

3721 CCCCCCCCCGGGCCACCGCCCGACCGCCCCCGCGCACCGGACGCGCCCGCCTGTACGGCG 3780
 GGGGCGGGGCCCCGGTGGCGGGCTGGCGGGGGCGCGTGGCCTGCGCGGGCGGACATGCCGC

 3781 GTCCCGCCCCGGGCCCCGTACACCTGAAGCGCCCGGCGGACCGCCGCCCGCCGGGGGACGG 3840
 CAGGGCGGGCCCCGGGCATGTGGACTTCGCGGGCCGCGCTGGCGGGCGGGGCGGCCCCCTGCC

 -----> <-----
 3841 ACAGAGCCCGGTGCGGGAGGACGTCCTCCCGCACCCGGCTCCACCGTTCCGCACCGACC 3900
 TGTCTCGGCCCCACGCCCTCCTGCAGGAGGGCGTGGGCCGAGGGTGGCAAGGCGTGGCTGG

 3901 GCACCCGACCGTGCCGCGAGGCGCCACCGGCACCGCACCGCCCGCGCCGGCAGCCACCACA 3960
 CGTGGGCTGGCACGGCGTCCGCGGTGGCCGTGGCGTGGCGGGCGCGGCCGTGCGGTGGTGT

 3961 GGCGCCACGCGCCCGCCGACGGTGCCCGCGCTGCTCAGCCCCCGTCCACCGGGCTGTCCAG 4020
 CCGCGGTGCGGCGGGCGTGCCACGGGCGCGACGAGTCGGGGGCAGGTGGCCCCGACAGGTC

 * G G D V P S D L

 4021 GTCGGCGGCGTTCGCGCGGGGGCTACTTGAGGGCCAGCCGCGGCTGGGGGGCTGGGGCG 4080
 L R R L A G G I F E R D A A S G G S G A

 4081 CTCTACGGGGGTGTGAGGGCCCTAGTGGAGGTGCTCCGTATGCCGTGCTCTAGCCGGTG 4140
 L H G W V G P I V E L S A Y P L L D A V

 4141 GGCGAAGAGCAGGAGCTGCCGCTTTGTGTGCAGGTCCCGCGGGCCGTCGTGGTGCCGGGC 4200
 R K E D E V A F C V D L A G P L V V A R

 4201 GCGGCACTGCCTCCGGTCGCGGCGGAGCTGCGAGGGGGGCGGGGGCCACAGCGGGGGTG 4260
 A T V S A L A A E V S G G A G P T A G V

 NcoI
 4261 TAGGCACAAGAGGGTCCACGCGTGGTACCACTCGTCTAGGCGCCGCGGCCCGGGCCTCTC 4320
 D T N E W T R V M T L L D A A G P G S L

 4321 CTTCTGGACGAGGGTCTTCGGCCACTCCATGAGGAGCGCCACCGCTTTGGGTCGAGGGC 4380
 F V Q E W F G T L Y E E R T A F G L E R

 4381 CACCCGTGCCGCCCCGGGTCTTCCTTGCGCTCCAGGGGGTGGGCCGCTTGTGGGCCGGGCG 4440
 H A R R A W F S R S T G W G A F V R G A

<orfdwn1

5221 CCAGAACATCAGGCTCGGCTAGCCTGGGCAGAGCGGCCAGCGCGCTCGCGGAGCCACTT 5280
 T K Y D S G I P G T E G T A R L A E T F

5 NcoI
 5281 CGGGTACCCCGGCTTGGTCAAGAGCTTCTACTTCGGCGGCGGCGCCCTGCGGGTCACCAC 5340
 G M P G F W N E F I F G G G R S A W H H

5341 CCGGAGCGGCCTCAGGGCCCTCTGGTCCTGCAGGAAGTAGTGGGGCTGGGCGAGCGGGGC 5400
 A E G S D R S V L V D K M V G V R E G R

5401 GCGTCCACGGCACCGGGCGGCGGAGCCGGAGGAGGGCCATCTACAGGTAGTCGGCCCCG 5460
 R L T G H G A A E A E E R Y I D M L R A

5 5461 CTGCTAGACCAGCAGCCACAAGTAGTCCTAGCCGTGGTGCGGGAGGGCCCGTGTCTTGGC 5520
 V I Q D D T N M L I P V V G E R A C F R

5521 CTTGCACAGGAGTGACTTCGACTTGCCGACCTTCTGCCCCGCCACCCCGCGACCATCCC 5580
 F T D E S F S F P Q F V P P H P R Q Y P

5581 GAACCCGCGCTACGGGTGGAGCGCCTACTGCGGCAAGAGCAGCTCCGGGGCCGGCATCGC 5640
 K P A I G V E R I V G N E D L G R G Y R

5641 CGCGTGGCGGAGCATCCCCCTTGAGGTCCAGGCCGTGGCCCTAGCAGGTGACGAGGGGCCT 5700
 R V A E Y P F E L D P V P I T W Q E G S

5701 CACCCACTTGACAGAGCCAGCAGGTGCGGAAGAACTACTAGAGGGTCACGAGGAGCTTCTC 5760
 H T F T E T T W A K K I I E W H E E F L

5761 CCGTGCTAACGCGGCCAGGGCGAGGGGCCGAGCCTGTCCCACGGCGGCTGGGGCATGTG 5820
 A R N R R D R E G A D S L T G G V G Y V

5821 GACGGGGTACTACAGCCGGGTGCGGAAGACCTTGGGCGCGCGCTAGGGCTGCTTCCGCGC 5880
 Q G M I D A W R K Q F G R A I G V F A R

5881 CGGGGCCCCAGTACACCAGCTCGTAGCGGTCTAGGAGCCGGTCGGCGTCGCCTAACACGTC 5940
 G R T M H D L M A L D E A L R L P N H L

5941 GCCGTCTGCAACCGGTAGACCGGCTGGGCCTACACGGCCCAGACGTACGGCTCCATCTC 6000

P L V N A M Q G V R I H R T Q M G L Y L

6001 GGGGTCGTACTAGCCCAACAACCTCTGGAGCTTTGGGAGCCACACCTTCACCACGAGCCA 6060
G L M I P N N S V E F G E T H F H H E T

6061 CTTCTGTCTAGGGGTCATCGGCTCAAGCAGCCGGCGGACGCGGACGGCCCACTCGACGGC 6120
F S L G W Y G L E D A A Q A Q R T L Q R

6121 CTCGTACAAGACCATCAAGACGCCTAACTGGGGGCGGTATGGGGCGACCTGGACGCGTAC 6180
L M N Q Y N Q P N V G A M G R Q V Q A H

<orfdown2

6181 ACTGCCGACCGTTGGCAGATAGAAGAGAATGGACTTCACCCTGGCTCCTCCGGTTCGCGG 6240
TGACGGCTGGCAACCGTCTATCTTCTTACCTGAAGTGGGACCGAGGAGGCCAAGCGCC
S G V T P L Y F L I S K M

6241 CGCCCTCCATTGACGTGCGCCGAAAGCGGCTCGACCGTCCCACTCCGCCCTTGAGTTCCG 6300
GCGGGAGGTAAGTGCACGCGGCTTTCGCCGAGCTGGCAGGGTGAGGCGGGAAGTCAAGGC

6301 TCTGACGCCGCGCCAGTTCGGCGGGCCGTCCGCCGGGGTGGCCGCCGGGGTCCGCACCCGC 6360
AGACTGCGGCGCGGTTCAGCCGCCCGGCGAGCGGCCCCACGGGCGGCCCCAGGCGTGGGCG

6361 CGGACGGCACGGCGCGCACCGCGCGCGCGGCGCTTCGGGGCACCGGGCTCGACGGGGTGC 6420
GCCTGCCGTGCCGCGCGTGGCGCGCGCGCCGGAAGCCCCGTGGCCCGAGCTGCCCCACG

6421 TCAGCGGGACGTCCAACGGAAGGCAAGCCCCGTACCCAGCCTGGTCAAGGCGCTCATCG 6480
AGTCGCCCTGCAGGTTGCCTTCCGTTCCGGGGCATGGGTTCGGACCAAGTTCGCGAGTAGC

orfdown3>

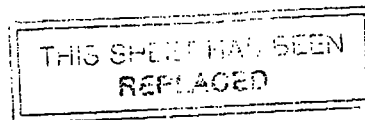
6481 CCATTCCCTGAGGAGGTCCCGCCTTGACCACAGCAATCTCCGCGCTCCCGACCGTGCCCG 6540
GGTAAGGGACTCCTCCAGGGCGGAAGTGGTGTCTGTTAGAGGCGCGAGGGCTGGCACGGGC

6541 GCTCCGGACTCGAAGCACTGGACCGTGCCACCCTCATCCACCCACCCTCTCCGGAACA 6600
S G L E A L D R A T L I H P T L S G N T

6601 CCGCGGAACGGATCGTGCTGACCTCGGGGTCCGGCAGCCGGGTCCGCGACACCGACGGCC 6660
A E R I V L T S G S G S R V R D T D G R

6661 GGGAGTACCTGGACGCGAGCGCCGTCCTCGGGGTGACCCAGGTGGGCCACGGCCGGGCCG 6720
E Y L D A S A V L G V T Q V G H G R A E
.
6721 AGCTGGCCCCGGGTCGCGGCCGAGCAGATGGCCCCGGCTGGAGTACTTCCACACCTGGGGGA 6780
L A R V A A E Q M A R L E Y F H T W G T
.
6781 CGATCAGCAACGACCGGGCGGTGGAGCTGGCGGCACGGCTGGTGGGGCTGAGCCCCGGAGC 6840
I S N D R A V E L A A R L V G L S P E P
.
6841 CGCTGACCCGCGTCTACTTCACCAGCGGCGGGGCCGAGGGCAACGAGATCGCCCTGCGGA 6900
L T R V Y F T S G G A E G N E I A L R M
.
6901 TGGCCCGGCTCTACCACCACCGGCGCGGGGAGTCCGCCCGTACCTGGATACTCTCCCGCC 6960
A R L Y H H R R G E S A R T W I L S R R
.
6961 GGTCGGCCTACCACGGCGTCGGATACGGCAGCGGCGGCGTCACCGGCTTCCCCGCCTACC 7020
S A Y H G V G Y G S G G V T G F P A Y H
.
7021 ACCAGGGCTTCGGCCCCCTCCCTCCCGGACGTCGACTTCCTGACCCCGCCGAGCCCTACC 7080
Q G F G P S L P D V D F L T P P Q P Y R
.
7081 GCCGGGAGCTGTTGCGCCGTTCCGACGTCACCGACTTCTGCCTCGCCGAACCTGCGCGAGA 7140
R E L F A G S D V T D F C L A E L R E T
.
Sau
7141 CCATCGACCGGATCGGCCCCGAGCGGATCGCGGCGATGATCGGCGAGCCGATC
I D R I G P E R I A A M I G E P I

CLAIMS



1. DNA comprising one or more genes specific for 5S clavam biosynthesis in *S. clavuligerus* and which is not essential for 5R clavam biosynthesis.
5
2. DNA according to claim 1 as identified in Figure 1 (SEQ ID No. 1).
3. DNA according to claim 1 having the sequence or substantially the sequence shown in Figure 1 as orfup3, orfup2, orfup1, orfdwn1, orfdwn2 or orfdwn3 (SEQ ID
10 Nos. 2-7).
4. DNA according to claim 1 having the sequence or substantially the sequence shown in Figure 1 as orfup1 (SEQ ID No. 4).
- 15 5. DNA which hybridises under conditions of high stringency with the DNA of any one of claims 1 to 4.
6. A vector comprising the DNA of any one of claims 1 to 5 in which one or more of the genes specific for 5S clavam biosynthesis has been disrupted or otherwise made
20 defective.
7. A vector according to claim 6 containing one or more defective genes which is pCEC060, pCEC061, pCEC056, pCEC057 or pDES3.
- 25 8. A vector according to claim 7 which is pCEC061 or pDES3.
9. A host containing the vector of any one of claims 6 to 8.
10. A host according to claim 9 which is capable of producing raised levels of
30 clavulanic acid.



11. A host according to claim 9 or 10 which is capable of producing low or no levels of 5S clavam.
12. A host according to any one of claims 9 to 11 which is *S. clavuligerus*.
- 5 13. *S. clavuligerus* comprising DNA corresponding to an open reading frame flanking cas1 which DNA has been disrupted or otherwise made defective.
14. *S. clavuligerus* according to claim 13 wherein the open reading frame is orfup3,
10 orfup2, orfup1, orfdwn1, orfdwn2 or orfdwn3.
15. A process for improving 5R clavam production in a suitable microorganism comprising manipulation of DNA as defined in any one of claims 1 to 5 and its inclusion in the said microorganism.
- 15 16. A process according to claim 15 wherein said suitable microorganism is *S. clavuligerus*.
17. A process for improving 5R clavam production in *S. clavuligerus* comprising
20 disrupting or otherwise making defective DNA regions flanking cas1.
18. A process according to claim 15, 16 or 17 wherein said DNA corresponds to open reading frames orfup3, orfup2, orfup1, orfdwn 1, orfdwn2 or orfdwn3.
- 25 19. A process according to any of claims 15 to 18 wherein said DNA corresponds to open reading frame orfup1.
20. A process according to any one of claims 15 to 19 wherein said 5R clavam is clavulanic acid.
- 30

This Sheet Has Been Replaced



21. A process for the identification of a microorganism suitable for high 5R clavam production comprising a preliminary screening for microorganisms with low or no 5S clavam production.
- 5 22. A process according to claim 21 wherein the microorganism is *S. clavuligerus*.
23. A process according to claim 21 or 22 wherein the 5R clavam is clavulanic acid.
- 10 24. A process according to any one of claims 21 to 23 wherein one or more genes specific for the production of 5S clavams is defective.
25. A microorganism which is capable of 5R clavam production and low or no 5S clavam production obtainable by the process of any one of claims 15 to 24.
- 15 26. A microorganism obtainable by the process of claim 25 which is capable of producing clavulanic acid but which does not produce clavam-2-carboxylate and / or 2-hydroxymethylclavam.
- 20 27. A microorganism obtained by the process of claim 15 which is strain 56-1A, 56-3A, 57-2B, 57-1C, 60-1A, 60-2A, 60-3A, 61-1A, 61-2A, 61-3A or 61-4A.
28. Clavulanic acid obtainable by the fermentation of a microorganism as defined in claim 25 or 26.
- 25 29. Clavulanic acid according to claim 28 which is free of clavam-2-carboxylate.
- 30 31. Clavulanic acid according to claim 28 in the form of its potassium salt.
- 30 32. Clavulanic acid which is free of any 5S clavam.
32. Clavulanic acid which is free of any clavam-2-carboxylate.

This Sheet Has Been Replaced



THIS SHEET HAS BEEN
REPLACED

33. A composition comprising potassium clavulanate according to claim 30 in combination with a beta-lactam antibiotic.
- 5 34. A composition according to claim 33 in which the beta-lactam antibiotic is amoxycillin.
35. A process for the preparation of a composition comprising potassium clavulanate and amoxycillin which process comprises producing clavulanic acid from a
10 microorganism according to claim 25 and thereafter converting it to the potassium salt and combining the potassium salt with amoxycillin.

This Sheet Has Been Replaced

