

Streptomyces clavuligerus

FIELD OF THE INVENTION

The invention generally relates to the improvement of clavulanic acid production in *Streptomyces clavuligerus* (*S. clavuligerus*). In particular, the invention provides a *S. clavuligerus* comprising point mutations in the *ccaR* promoter region and gene, a vector comprising a *ccaR* promoter region and gene comprising said mutations for making said *S. clavuligerus* and a method for producing clavulanic acid using said *S. clavuligerus*, and pharmaceutical formulations prepared using clavulanic acid produced using said *S. clavuligerus*.

BACKGROUND TO THE INVENTION

β -lactam antibiotics, such as penicillins, are the most widely used class of antibiotics (Bush & Bradford (2016) *Cold Spring Harb Perspect Med*). However, certain pathogens have developed resistance to β -lactam antibiotics by producing β -lactamase, thereby reducing the effectiveness of β -lactam antibiotics.

Clavulanic acid is a β -lactam compound structurally related to the penicillins that is produced as a fermentation product by *S. clavuligerus*. Clavulanic acid was isolated from *S. clavuligerus* and characterised as a novel β -lactamase inhibitor by Reading and Cole in 1976 (Reading & Cole, (1977) *Antimicrob. Agents Chemother.*). The addition of clavulanic acid was shown to enhance the antibacterial activity of β -lactamase-labile antibiotics.

Since then, clavulanic acid has been widely used in the pharmaceutical industry in combination with β -lactam antibiotics to treat infections caused by β -lactamase producing pathogens that would otherwise be resistant to β -lactam antibiotics. It is understood that the β -lactam structure of clavulanic acid binds to the active site of β -lactamase produced by the pathogen, in place of the β -lactam antibiotic, causing irreversible inhibition of the enzyme (Labia and Peduzzi, (1985) *Drugs Exp. Clin. Res*; Georgopadkou (2004) *Exp. Opin. Investig. Drugs*).

The improvement of *S. clavuligerus* strains plays an important role in reducing production costs during industrial fermentation of clavulanic acid. Strain development strategies to obtain higher yields of clavulanic acid in industrial fermentations have traditionally depended largely on random mutagenesis and selection techniques. However, with increased understanding of key biosynthetic pathways and regulatory mechanisms involved in clavulanic acid production by *S. clavuligerus*, strain development strategies have also incorporated a knowledge-based rational approach (Paradkar (2013) *The Journal of Antibiotics*).

Thus, there is an ongoing need to develop additional strains of *Streptomyces clavuligerus* that produce higher yields of clavulanic acid and improved processes using such strains for producing a higher yield of clavulanic acid at a lower cost.

SUMMARY OF THE INVENTION

Surprisingly, the inventors have found that *S. clavuligerus* strains comprising mutations in the *ccaR* promoter region and the *ccaR* gene produces a higher titre of clavulanic acid and at a faster rate as compared to a wild-type (WT) *S. clavuligerus*. The present disclosure will allow an improved process for clavulanic acid production, particularly at an industrial scale, wherein higher titres of clavulanic acid are produced at lower costs as compared to WT *S. clavuligerus*.

According to a first aspect of the present invention, there is provided a *S. clavuligerus* comprising two point mutations in a *ccaR* promoter region and a substitution mutation in a *ccaR* gene, wherein the point mutations in the *ccaR* promoter region are a C (cytosine) to T (thymine) mutation at a site corresponding to position 48 of SEQ ID NO:1 and a G (guanine) to A (adenine) point mutation at a site corresponding to position 143 of SEQ ID NO:1; and wherein the mutation in the *ccaR* gene is an arginine to tryptophan substitution at a site corresponding to position 32 of SEQ ID NO: 2.

According to a further aspect of the invention, there is provided a *S. clavuligerus* of the invention for use in the production of clavulanic acid.

According to another aspect of the invention, there is provided a vector comprising nucleic acid sequences comprising a *ccaR* promoter region comprising two point mutations and a *ccaR* gene comprising a substitution mutation, wherein the point mutations in the *ccaR* promoter region are a C to T mutation at a site corresponding to position 48 of SEQ ID NO:1 and a G to A mutation at a site corresponding to position 143 of SEQ ID NO:1; and wherein the substitution mutation in the *ccaR* gene is an arginine to tryptophan substitution at a site corresponding to position 32 of SEQ ID NO: 2.

According to yet another aspect of the invention, there is provided a method for producing clavulanic acid comprising the steps of growing a *S. clavuligerus* of the invention and recovering clavulanic acid produced by said *S. clavuligerus*, or progeny thereof.

According to a further aspect of the invention, there is provided a method for producing a pharmaceutical formulation comprising a β -lactam antibiotic and clavulanic acid, the method comprising the steps of (a) growing a *S. clavuligerus* of the invention; (b) recovering clavulanic acid produced by said *S. clavuligerus*, or progeny thereof; and (c) preparing the pharmaceutical formulation by combining the clavulanic acid recovered in step (b) with the β -lactam antibiotic.

DESCRIPTION OF DRAWINGS/FIGURES

FIG. 1: Effect on fermentation of *ccaR* mutations M1, M2 and M3 combined: Clavulanic acid titre profiles

FIG: 2: Effect of *ccaR* mutation: Fermentation titre at 65 hours

FIG. 3: Effect of *ccaR* mutations on fermentation: Clavulanic acid titre profiles

FIG. 4: Effect of combined *ccaR* mutations M1, M2 and M3 on fermentation viscosity profiles

FIG. 5: Effect of *ccaR* mutations on fermentation viscosity profiles

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides significant improvements to the process of clavulanic acid production using *S. clavuligerus* by introducing mutations in the *ccaR* promoter region and gene.

5 Clavulanic acid produced by *S. clavuligerus* strains of the present invention may be combined with a β -lactam antibiotic, such as amoxicillin to produce AUGMENTIN.

Biosynthesis of clavulanic acid can be divided into early and late stage synthesis and, accordingly, depending on their function, genes involved in clavulanic acid synthesis may also be categorised into early and late stage genes.

10 The *S. clavuligerus ccaR* gene is located within the cephamycin C gene cluster and is upstream of the *blp* gene (Perez-Llarena *et al.* (1997) *J. Bacteriol.*). The nucleotide sequence of the *ccaR* promoter region, *ccaR* gene and the linked *blp* gene is available on GenBank (accession no. Z81324).

The *ccaR* gene encodes a positive-acting transcriptional regulator, CcaR, that controls the production of both clavulanic acid and cephamycin C (Alexander & Jensen (1998) *J. Bacteriol.*; 15 Santamarta *et al.* (2011) *Mol. Microbiol.*). Clavulanic acid and cephamycin C production was abolished in *S. clavuligerus ccaR* knock-out mutants and restored on re-introduction of wild-type *ccaR*, showing that CcaR positively regulates the production of both proteins.

CcaR regulates clavulanic acid production both directly and indirectly. Directly by regulating the expression of the *ceaS2-bls-pah-cas2* polycistronic transcript, which products are involved in the 20 'early' reaction pathway leading to the formation of clavaminic acid. Indirectly by regulating expression of *clar*, which in turn regulates expression of genes involved in the 'late' reaction pathway of converting clavaminic acid to clavulanic acid. Furthermore, CcaR was shown to bind upstream of *ccaR* and autoregulate its own expression.

The inventors have identified three mutations within the *ccaR* promoter region and gene, 25 outlined below, for developing an improved *S. clavuligerus* strain that produces a higher titre of clavulanic acid over a shorter fermentation period as compared to a WT *S. clavuligerus* strain.

Mutation 1 (M1) is a point mutation from C to T at a site corresponding position 48 of SEQ ID NO: 1 (48C>T).

30 Mutation 2 (M2) is a point mutation from G to A at a site corresponding to position 143 of SEQ ID NO: 1 (143G>A).

Mutation 3 (M3) is an arginine to tryptophan amino acid substitution at a site corresponding to position 32 of SEQ ID NO:2 (R32W).

According one aspect of the invention, there is provided a *Streptomyces clavuligerus* comprising two point mutations in a *ccaR* promoter region and a substitution mutation in a *ccaR* gene, 35 wherein the point mutations in the *ccaR* promoter region are a C to T mutation at a site corresponding to 48 of SEQ ID NO:1 and a G to A mutation at a site corresponding to 143 of SEQ ID NO:1; and

wherein the mutation in the *ccaR* gene is an arginine to tryptophan substitution at a site corresponding to 32 of SEQ ID NO: 2.

In one embodiment, the arginine to tryptophan amino acid substitution is a result of a point mutation from C to T at a site corresponding to position 344 of SEQ ID NO: 1 (344C>T).

5 In one embodiment, the *S. clavuligerus* comprises a nucleic acid sequence of SEQ ID NO: 3. There is disclosed a *S. clavuligerus* comprising a nucleic acid sequence having at least 80%, 85%, 90%, 95% or more identity with SEQ ID NO: 3 wherein said nucleic acid sequence comprises, M1; M2; M3; M1 and M3; M1 and M2; M2 and M3; or M1, M2 and M3.

10 In one embodiment, the nucleic acid sequences of the *ccaR* promoter region and the nucleic acid sequences encoding the *ccaR* gene comprising one or more of M1, M2 and M3 are integrated into the chromosomal DNA of the *S. clavuligerus* strain. In another embodiment, the nucleic acid sequences of the *ccaR* promoter region and the nucleic acid sequences encoding the *ccaR* gene comprising one or more of M1, M2 and M3 are located extrachromosomally in the *S. clavuligerus* strain.

15 Multiple sequences of different strains of *S. clavuligerus* comprising the *ccaR* promoter region and gene sequences are available. The skilled person would readily recognise the respective positions corresponding to M1, M2 and M3 in the context of such different sequences. For example, *S. clavuligerus* F613-1 is an industrial strain. The genome of F613-1 was sequenced in 2016, providing
20 a complete genome sequence of *S. clavuligerus* (GenBank Accession no. [CP016559.1](#)). The skilled person would readily identify that within this sequence, the locations of M1, M2 and M3 correspond to positions 2,011,673, 2,011,768 and 2,011,969, respectively. As a further example, within the *S. clavuligerus* F1D-5 strain chromosome (GenBank Accession no. [CP032052.1](#)), the locations of M1, M2 and M3 correspond to positions 2,013,392, 2,013,487 and 2,013,688, respectively. Within GenBank
25 accession no. AH006362 (*S. clavuligerus* ATCC 27064), the locations of M1, M2 and M3 correspond to positions 14,042, 14,137 and 14,338, respectively.

A significant advantage seen in the strains after introduction of the *ccaR* promoter region and gene mutations was the speed of accretion of clavulanic acid. In other words, the *S. clavuligerus*
30 strains of the invention produce higher titres of clavulanic acid as compared to WT *S. clavuligerus* strain and the higher titre is reached within a reduced amount of fermentation time. This increased productivity of clavulanic acid by the mutant *S. clavuligerus* strains of the invention is key in enabling more efficient downstream processes in extraction and purification of clavulanic acid as the ratio of product to impurity is improved. Further, the capacity and speed of an industrial plant for producing
35 pharmaceutical formulations comprising clavulanic acid is increased, thereby reducing cost of production.

A *S. clavuligerus* strain of the present invention produces a higher titre of clavulanic acid as compared to a WT *S. clavuligerus* strain. In one embodiment, the *S. clavuligerus* is capable of producing a titre of about 0.5 g/L or more, about 0.75 g/L or more, about 1 g/L or more, about 1.25 g/L or more, about 1.5 g/L or more, about 1.75 g/L or more, about 2 g/L or more or about 2.5 g/L or more of clavulanic acid. In a further embodiment, the *S. clavuligerus* is capable of producing a titre of about 2.1 g/L or more of clavulanic acid.

In one embodiment, the *S. clavuligerus* is capable of producing a titre of about 0.5 g/L or more, about 0.75 g/L or more, about 1 g/L or more, about 1.25 g/L or more, about 1.5 g/L or more, about 1.75 g/L or more, about 2 g/L or more, about 2.1 g/L or more, or about 2.5 g/L of clavulanic acid is produced after 65 hours of fermentation. In a further embodiment, the *S. clavuligerus* is capable of producing a titre of about 2.1 mg/mL or more of clavulanic acid is produced after 65 hours of fermentation. In one embodiment, said titres are produced after about 40 hours, 45, hours, 50 hours, 55 hours, 60 hours, 65 hours, 70 hours, 72 hours, 75 hours, 80 hours, 85 hours, 90 hours, 95 hours, 100 hours, 105 hours, 110 hours, 115 hours, 120 hours, 125 hours, 130 hours, 135 hours or 140 hours of fermentation.

In one embodiment, the *S. clavuligerus* is capable of producing a titre of about 50% or more, about 75% or more, about 100% or more, about 125% or more, about 150% or more, about 175% or more, about 200% or more, about 225% or more, about 250% or more, about 275% or more, about 300% or more, about 325% or more, about 350% or more or about 375% or more, about 400% or more, about 425% or more, about 450% or more, about 475% or more, about 500% or more, about 525% or more, about 550% or more, about 575% or more, about 600% or more, about 625% or more, about 650% or more, about 675% or more, about 700% or more, about 725% or more, about 750% or more, about 775% or more, about 800% or more, about 825% or more, about 850% or more, about 875% or more, about 900% or more, about 925% or more, about 950% or more, about 975% or more, or about 1000% or more of clavulanic acid as compared to wild-type (WT) *S. clavuligerus*. In some embodiments, said percentage increases in titres of clavulanic acid as compared to WT *S. clavuligerus* are produced after 65 hours of fermentation. In some embodiments, said percentage increases in titres of clavulanic acid as compared to WT *S. clavuligerus* are produced after about 40, 45, 50, 55, 60, 65, 70, 72, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135 or 140 hours of fermentation. Preferably, in one embodiment, the *S. clavuligerus* of the invention is capable of producing a titre of about 1000% or more clavulanic acid after 65 hours of fermentation as compared to wild-type *S. clavuligerus*.

A further significant advantage conferred by the strains comprising the *ccaR* promoter region and gene mutations was a reduction in viscosity of the fermentation culture. *S. clavuligerus* is a filamentous organism and the morphology of the fermented culture is an important parameter affecting the efficiency of gas and heat transfer in a fermentation. Viscosity measurements of cultured

samples are used to indicate the difficulty and energy involved in controlling dissolved oxygen and maintaining temperature in a large-scale fermentation. A desirable feature of a culture is high titre without very high viscosity.

Therefore, in some embodiments, the *Streptomyces clavuligerus* of the present invention is capable of reducing viscosity of a fermentation broth, of which it is comprised, by about 10% or more, about 15% or more, about 20% or more, about 25% or more, about 30% or more, about 35% or more, or about 40% or more as compared to WT *Streptomyces clavuligerus*.

In one embodiment, the *Streptomyces clavuligerus* of the present invention is capable of reducing viscosity of a fermentation broth by about 40% or more after 65 hours of fermentation as compared to WT *Streptomyces clavuligerus*. In these embodiments, the fermentation broth comprises the *Streptomyces clavuligerus* of the present invention

In one embodiment, the *Streptomyces clavuligerus* of the present invention is capable of a viscosity of a fermentation broth, of which it is comprised, of about 35 centipoise or less, about 30 centipoise or less, 25 centipoise or less or 20 centipoise or less. In one embodiment, the *Streptomyces clavuligerus* of the present invention is capable of a reduction in viscosity of a fermentation broth of which it is comprised, relative to the viscosity of WT *Streptomyces clavuligerus*, of about 5 centipoise or more, about 7 or more, about 10 centipoise or more, 13 centipoise or more, 15 centipoise or more, 17 centipoise or more, or 20 centipoise or more. In one embodiment, the *Streptomyces clavuligerus* of the present invention is capable of a reduction in viscosity of a fermentation broth of which it is comprised, relative to the viscosity of WT *Streptomyces clavuligerus*, of about 6 centipoise or more, 13 centipoise or more, or 17 centipoise or more. In one embodiment, said viscosity, or reduction in viscosity is after 65 hours of fermentation. In one embodiment, the *Streptomyces clavuligerus* of the present invention is capable of a viscosity of a fermentation broth, of which it is comprised, of about 28 centipoise or less after 65 hours of fermentation. In one embodiment, the *Streptomyces clavuligerus* of the present invention is capable of a viscosity of a fermentation broth, of which it is comprised, of about 25 centipoise or less after 65 hours of fermentation.

Accordingly, an improved process of clavulanic acid production involving the mutant *S. clavuligerus* strains is provided.

S. clavuligerus may comprise one or more mutations selected from M1, M2 and M3. The *S. clavuligerus* may comprise two mutations selected from M1, M2 and M3. The *S. clavuligerus* may comprise two mutations selected from M1, M2 and M3, wherein one of the two mutations is M2. For example, a *S. clavuligerus* comprising M2 and M1 or a *S. clavuligerus* comprising M2 and M3. The *S. clavuligerus* may comprise a single mutation selected from M1, M2 and M3.

According to a further aspect of the invention, there is provided a *S. clavuligerus* of the invention for use in the production of clavulanic acid.

In a further aspect of the invention, there is provided a vector comprising nucleic acid sequences comprising a *ccaR* promoter region comprising two point mutations and a *ccaR* gene comprising a substitution mutation, wherein the mutations in the *ccaR* promoter region are a C to T point mutation at a site corresponding to position 48 of SEQ ID NO:1 and a G to A point mutation at a site corresponding to position 143 of SEQ ID NO:1; and wherein the mutation in the *ccaR* gene is an arginine to tryptophan substitution at a site corresponding to position 32 of SEQ ID NO: 2.

In one embodiment, the arginine to tryptophan amino acid substitution is a result of a point mutation from C to T at a site corresponding to position 344 of SEQ ID NO: 1.

In one embodiment, the vector is a plasmid. In another embodiment, the vector is linear DNA. In yet another embodiment, the vector is a bacteriophage. Other vectors able to artificially carry genetic material into another cell, where it can be replicated and/or expressed are well known in the art and may be used.

The *ccaR* promoter region is operably linked to the *ccaR* gene. However, in one embodiment, the *ccaR* promoter region is not operably linked to the *ccaR* gene.

In one embodiment, the vector comprises a nucleic acid sequence of SEQ ID NO: 3.

There is disclosed a *S. clavuligerus* comprising a nucleic acid sequence having at least 80, 85, 90, 95% or more identity with SEQ ID NO: 3, wherein said nucleic acid sequence comprises M1 and M2, M2 and M3, or M1, M2 and M3.

In a further embodiment, the vector of the invention is used for producing a *S. clavuligerus* of the invention.

Suitably, the mutated *ccaR* promoter region and gene of the present invention may be obtained by conventional cloning methods (such as PCR) based on the sequences provided herein.

Further suitably, the vectors of the invention are used to prepare a *S. clavuligerus* of the present invention by way of transformation into an organism capable of subsequent conjugation with *S. clavuligerus* using methods known in the art. For example, techniques and base vectors used in *Streptomyces* biology are described in Practical Streptomyces Genetics (T. Keiser, M.J. Bibb, M.J. Buttner, K.F. Chater, D.A. Hopwood (2000)), a manual published by the John Innes Centre.

To date, knowledge-based *S. clavuligerus* strain improvement strategies can be broadly classified into three approaches: (1) increasing the flow of precursors into the pathway, (2) increasing the gene dosage of key biosynthetic and/or regulatory genes and (3) eliminating competing reactions that divert the flow of pathways into non-clavulanic acid products.

By way of example, glyceraldehyde-3-phosphate (G3P) and arginine are two essential precursors in the clavulanic acid pathway. In addition to clavulanic acid production pathway, G3P may

alternatively enter the glycolytic pathway and then the Krebs cycle, channelled by glyceraldehyde-3-phosphate dehydrogenase (*gap*). A *gap-1* mutant *S. clavuligerus* strain showed 80-110% increase in clavulanic acid production over a strain with WT *gap-1* (Li & Townsend (2006) *Metab. Eng.*).

Furthermore, increasing the gene dosage of *cas2* and *ccaR* resulted in increased production levels of clavulanic acid (Hung *et al.* (2007) *J. Microbiol. Biotechnol.*).

cvm1 encodes an enzyme involved in the conversion of clavaminic acid to 3S, 5S clavams, which, contrary to the 3R, 5R stereochemistry of clavulanic acid, do not possess β -lactamase inhibitory property. Inactivation of *cvm1* led to increased levels of clavulanic acid production (Paradkar *et al.* (2001) *Appl. Environ. Microbiol.*).

In some embodiments, the *S. clavuligerus* strains of the invention further comprises genetic modifications known in the art. In one embodiment, a vector of the invention is used to introduce the *ccaR* promoter region and gene mutations to an *S. clavuligerus* strain comprising further genetic mutations (as compared to WT *S. clavuligerus*).

Genetic modifications of the art may be targeted mutations based on a knowledge-based approach or they may be mutations resulting from a random mutagenesis and selection approach. It is envisaged that such genetic modifications known in the art in combination with *ccaR* promoter region and gene mutations of the invention will provide further improvements to the *S. clavuligerus* strain, such as further increased clavulanic acid titre as compared to the respective genetic modification known in the art alone or the *ccaR* promoter and gene mutations of the invention alone.

The mutations within the *ccaR* promoter region and gene disclosed herein, may be introduced into a *S. clavuligerus* strain comprising further mutations but comprising WT *ccaR* promoter region and gene. This may be performed using the vectors carrying *ccaR* promoter region and gene mutations disclosed herein.

Alternatively, mutations known in the art may be introduced into *S. clavuligerus* strains of the invention. Such strains may be produced using routine genetic engineering methods known in the art.

Mutant strains of *S. clavuligerus* suitable for introducing the *ccaR* promoter region and gene mutations of the present invention have been described in WO98/33896 (SmithKline Beecham Plc; Governors of the University of Alberta).

In some embodiments, the *S. clavuligerus* strains of the present invention comprises a copy of the WT *ccaR* promoter region and gene. In other words, *ccaR* promoter region and gene comprising the mutations described herein does not replace the WT *ccaR* promoter region and gene, but is added to it. In some embodiments, the *S. clavuligerus* strains of the invention comprising mutations in the *ccaR* promoter region and gene comprise one or more copies of the WT *ccaR* promoter region and gene.

In yet a further aspect of the invention, there is provided a method for producing clavulanic acid comprising the steps of growing a *Streptomyces clavuligerus* of the invention and recovering clavulanic acid produced by said modified *Streptomyces clavuligerus*, or progeny thereof.

Methods for growing *S. clavuligerus* and recovering clavulanic acid are known in the art. For example, suitable conditions for fermentation of *S. clavuligerus* and extraction of clavulanic acid have been described in WO01/87891 (SmithKline Beecham P.L.C.) and by Ser *et al.* (Ser *et al.* (2016) *Front. Microbio.*).

5 In one embodiment, the method produces a titre of a titre of about 0.5 g/L or more, about 1 g/L or more, about 1.5 g/L or more, about 2 g/L or more or about 2.5 g/L or more of clavulanic acid.

Preferably, in one embodiment, said titres of clavulanic acid are produced by the method after 65 hours of fermentation. In some embodiments, said titres of clavulanic acid are produced by the method after about 50, 55, 60, 65, 70, 72, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135
10 or 140 hours of fermentation.

In one embodiment, the method produces about 50% or more, about 75% or more, about 100% or more, about 125% or more, about 150% or more, about 175% or more, about 200% or more, about 225% or more, about 250% or more, about 275% or more, about 300% or more, about 325% or more, about 350% or more or about 375% or more, about 400% or more, about 425% or
15 more, about 450% or more, about 475% or more, about 500% or more, about 525% or more, about 550% or more, about 575% or more, about 600% or more, about 625% or more, about 650% or more, about 675% or more, about 700% or more, about 725% or more, about 750% or more, about 775% or more, about 800% or more, about 825% or more, about 850% or more, about 875% or more, about 900% or more, about 925% or more, about 950% or more, about 975% or more, or
20 about 1000% or more of clavulanic acid as compared to wild-type (WT) *S. clavuligerus*. In some embodiments, said percentage increases in titres of clavulanic acid as compared to WT *S. clavuligerus* are produced after 65 hours of fermentation. In some embodiments, said percentage increases in titres of clavulanic acid as compared to WT *S. clavuligerus* are produced after about 40, 45, 50, 55, 60, 65, 70, 72, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135 or 140 hours of fermentation.
25 Preferably, in one embodiment, the *S. clavuligerus* of the invention is capable of producing a titre of about 1000% or more clavulanic acid after 65 hours of fermentation as compared to wild-type *S. clavuligerus*.

In some embodiments, the viscosity of the fermentation broth comprising a mutant *Streptomyces clavuligerus* strain of the present invention is reduced by about 10% or more, about
30 15% or more, about 20% or more, about 25% or more, about 30% or more, about 35% or more, or about 40% or more as compared to WT *Streptomyces clavuligerus*. In one embodiment, said reduction in viscosity is obtained after 65 hours of fermentation. In one embodiment, said reduction in viscosity is obtained after about 40, 45, 50, 55, 60, 65, 70, 72, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, or 140 hours of fermentation.

35 In one embodiment, the viscosity of the fermentation broth comprising a mutant *Streptomyces clavuligerus* strain of the present invention is reduced by about 40% or more after 65 hours of fermentation as compared to WT *Streptomyces clavuligerus*.

In one embodiment, the viscosity of the fermentation broth comprising a mutant *Streptomyces clavuligerus* strain of the present invention is about 35 centipoise or less, about 30 centipoise or less, 25 centipoise or less or 20 centipoise or less. In one embodiment, said viscosity is after 65 hours of fermentation. In one embodiment, the viscosity of the fermentation broth comprising a mutant
5 *Streptomyces clavuligerus* strain of the present invention is about 28 centipoise or less after 65 hours of fermentation. In one embodiment, the viscosity of the fermentation broth comprising a mutant *Streptomyces clavuligerus* strain of the present invention is about 25 centipoise or less after 65 hours of fermentation.

10 In yet another aspect of the invention, there is provided a method for producing a pharmaceutical formulation comprising a β -lactam antibiotic and clavulanic acid comprising the steps of

- (a) growing a modified *Streptomyces clavuligerus* of the invention;
- (b) recovering clavulanic acid produced by said modified *Streptomyces clavuligerus*,
15 or progeny thereof; and
- (c) preparing the pharmaceutical formulation by combining the clavulanic acid recovered in step (b) with the β -lactam antibiotic.

In a preferred embodiment, the β -lactam antibiotic is amoxicillin.

The product co-amoxiclav is marketed by GlaxoSmithKline as AUGMENTIN for treating
20 bacterial infections. It comprises a combination of the β -lactam antibacterial agent amoxycillin and clavulanic acid. The product is provided in various pharmaceutical formulations, for instance tablets, capsule powders and sachets containing free flowing granules. The pharmaceutical formulation may comprise different ratios of β -lactam antibiotic, such as amoxicillin or ticarcillin, and clavulanic acid. In one embodiment, clavulanic acid is combined with a β -lactam antibiotic at ratios ranging from 3:1
25 to 30:1. The specific ratio may be dependent on type of formulation, dosing regime, route of administration and/or target indication.

In some embodiments, the pharmaceutical formulations of the invention comprise amoxycillin trihydrate and potassium clavulanate.

Suitable pharmaceutical formulations comprising amoxicillin and clavulanic acid for use in the
30 present invention have been described in WO94/27557 (SmithKline Beecham Corporation) and WO98/35672 (SmithKline Beecham Laboratoires Pharmaceutiques).

Also disclosed herein is a pharmaceutical formulation comprising clavulanic acid produced by the method described herein, further comprising a β -lactam antibiotic. Preferably, the β -lactam
35 antibiotic is amoxicillin. In one embodiment, the pharmaceutical formulation is AUGMENTIN.

In yet a further aspect, there is provided a genetically engineered microorganism that produces clavulanic acid comprising two point mutations in a *ccaR* promoter region and a substitution mutation in a *ccaR* gene, wherein the point mutations in the *ccaR* promoter region are a C to T point mutation at a site corresponding to position 48 of SEQ ID NO:1 and a G to A point mutation at a site corresponding to position 143 of SEQ ID NO:1; and wherein the substitution mutation in the *ccaR* gene is an arginine to tryptophan substitution at a site corresponding to position 32 of SEQ ID NO: 2.

In one embodiment, the genetically engineered microorganism is *Streptomyces clavuligerus*. In a further embodiment, the *ccaR* gene is integrated into the chromosomal DNA of said microorganism.

In one embodiment, the arginine to tryptophan substitution is a result of a C to T point mutation at a site corresponding to position 344 of SEQ ID NO: 1. In another embodiment, the genetically engineered microorganism comprises a nucleic acid sequence of SEQ ID NO:3. In one embodiment, the genetically engineered microorganism is capable of producing a titre of about 0.5 g/L or more, about 1 g/L or more, about 1.5 g/L or more, about 2 g/L or more or about 2.5 g/L or more of clavulanic acid. In one embodiment, the genetically engineered microorganism is capable of producing a titre of about 2.1 g/L or more of clavulanic acid. In one embodiment, said titre of clavulanic acid is produced after 65 hours of fermentation. In one embodiment, the genetically engineered microorganism is capable of producing a titre of about 100% or more, about 200% or more, about 300% or more, about 400% or more, about 500% or more, about 600% or more, about 700% or more, about 800% or more, about 900% or more, or about 1000% or more of clavulanic acid after 65 hours of fermentation as compared to wild-type (WT) microorganism. It will be understood that references to WT microorganism refer to the same species of microorganism as the genetically engineered microorganism, that is to say a non-genetically engineered microorganism of the same species. In one embodiment, the genetically engineered microorganism is capable of producing a titre of about 1000% or more of clavulanic acid after 65 hours of fermentation as compared to WT microorganism. In one embodiment, the genetically engineered microorganism is capable of reducing viscosity of a fermentation broth by about 10% or more, about 15% or more, about 20% or more, about 25% or more, about 30% or more, about 35% or more, or about 40% or more after 65 hours of fermentation as compared to wild-type (WT) microorganism. In another embodiment, the microorganism is capable of reducing viscosity of a fermentation broth by about 40% or more after 65 hours of fermentation as compared to WT microorganism.

In one aspect, there is provided a genetically engineered microorganism described herein for use in the production of clavulanic acid.

In a further aspect, there is provided a method for producing clavulanic acid comprising the steps of growing a genetically engineered microorganism described herein; and recovering clavulanic acid produced by the genetically engineered microorganism, or progeny thereof.

5 In one embodiment of the method, a titre of about 0.5 g/L or more, about 1 g/L or more, about 1.5 g/L or more, about 2 g/L or more or about 2.5 g/L or more of clavulanic acid is produced. In a further embodiment, a titre of about 2.1 g/L or more of clavulanic acid is produced. In yet a further embodiment, said titre of clavulanic acid is produced after 65 hours of fermentation. In one embodiment of the method, a titre of about 100% or more, about 200% or more, about 300% or more, about 400% or more, about 500% or more, about 600% or more, about 700% or more, about 10 800% or more, about 900% or more, or about 1000% or more of clavulanic acid is produced after 65 hours of fermentation as compared to wild-type (WT) microorganism. In one embodiment, a titre of 1000% or more of clavulanic acid is produced after 65 hours of fermentation as compared to WT microorganism. In one embodiment, viscosity of a fermentation broth comprising the genetically engineered microorganism is reduced by about 10% or more, about 15% or more, about 20% or more, about 25% or more, about 30% or more, about 35% or more, or about 40% or more after 65 15 hours of fermentation as compared to WT microorganism. In one embodiment, viscosity of the fermentation broth comprising the genetically engineered microorganism is reduced by about 40% or more after 65 hours of fermentation as compared to WT microorganism.

20 In another aspect, there is provided a method for producing a pharmaceutical formulation comprising β -lactam antibiotic and clavulanic acid comprising the steps of

- (a) growing a genetically engineered microorganism described herein,
- (b) recovering clavulanic acid produced by the genetically engineered microorganism, or progeny thereof; and
- 25 (c) preparing the pharmaceutical formulation by combining the clavulanic acid recovered in step (b) with the β -lactam antibiotic.

In one embodiment, the β -lactam antibiotic is amoxicillin. In one embodiment, the pharmaceutical formulation is AUGMENTIN.

30 All aspects and embodiments of the *S. clavuligerus* strains described hereinbefore also apply to the genetically engineered microorganism that produces clavulanic acid. For avoid of doubt, such aspects and embodiments also include methods for producing clavulanic acid using the *S. clavuligerus* strains, method for producing a pharmaceutical formulation using the *S. clavuligerus* strains.

DEFINITIONS

35 Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which this invention belongs. All patents and publications referred to herein are incorporated by reference in their entirety.

The term "comprising" encompasses "including" or "consisting" *e.g.* a composition "comprising" X may consist exclusively of X or may include something additional *e.g.* X + Y.

The term "consisting essentially of" limits the scope of the feature to the specified materials or steps and those that do not materially affect the basic characteristic(s) of the claimed feature.

5 The term "consisting of" excludes the presence of any additional component(s).

The term "about" in relation to a numerical value x means, for example, $x \pm 10\%$, 5%, 2% or 1%.

The term "promoter region" as used herein refers to nucleic acid sequence comprising any regulatory region required for gene function or expression.

10 The term "*Streptomyces clavuligerus*", "*S. clavuligerus*", "*S. clavuligerus* strain" are used interchangeably and refers to *Streptomyces clavuligerus* and strains thereof.

The term "wild-type" or "WT" as used herein in the context of "*Streptomyces clavuligerus*", "*S. clavuligerus*", "*S. clavuligerus* strain" refers to a *S. clavuligerus* strain found in its natural, non-mutated form (*i.e.* *S. clavuligerus* – culture collection ATCC 27064). WT as used herein in the context
15 of *S. clavuligerus* in particular refers to a strain that does not comprise mutations in the *ccaR* promoter region and *ccaR* gene, namely, M1, M2 or M3.

The term "*ccaR*" and "*ccaR* gene" are used interchangeably and as used herein refers to a *ccaR* gene.

The term "CcaR" as used herein refers to the protein encoded by the *ccaR* gene.

20 The term "point mutation" as used herein refers to alteration of a single base pair in a nucleotide sequence. For example, one base may be substituted for another. Such a point mutation may have one of three effects. First, the base substitution can be a silent mutation where the altered codon corresponds to the same amino acid. Second, the base substitution can be a missense mutation where the altered codon corresponds to a different amino acid. Third, the base substitution can be a
25 nonsense mutation where the altered codon corresponds to a stop signal.

The term "substitution mutation" as used herein refers to the replacement of one amino acid protein with a different amino acid. It may also refer to the replacement of one base pair in a nucleotide sequence for another base pair.

30 The terms "hours of fermentation" and "log hour" in the context of fermentation as used herein, refer to the number of hours of fermentation since point of inoculation of the production media with a seed culture.

The term "fermentation broth" as used herein refers to a mixture of fermentation media and cells fermented therein, in particular *S. clavuligerus*.

35 The term "vector" or "nucleic acid vector" refers to a vehicle which is able to artificially carry genetic material into another cell, where it can be replicated and/or expressed.

The invention will now be described in further detail with reference to the following, non-limiting Examples.

EXAMPLES

5

Example 1

***ccaR* mutant plasmid construction**

Three mutations within the *ccaR* promoter region and *ccaR* gene were evaluated, either individually or in combination, for their effect on clavulanic acid production. The mutations were:

- 10 Mutation 1 (M1) – a point mutation from C to T at position 48 of SEQ ID NO: 1;
 Mutation 2 (M2) – a point mutation from G to A at position 143 of SEQ ID NO: 1; and
 Mutation 3 (M3) – an arginine to tryptophan amino acid substitution at position 32 of SEQ ID NO:2.

 The position of M1 and M2 are in the *ccaR* promoter region. The position of M3 is within the
15 *ccaR* gene.

Making the initial constructs

- Primers NC_18_22 and NC_18_23 were used to amplify the *ccaR* gene and surrounding 4Kb DNA (2Kb each side) by PCR using Q5™ polymerase. These primers contain a HindIII and XbaI site, respectively. Three PCRs were carried out using different genomic DNA as a
20 template – either from *Streptomyces clavuligerus* WT strain, strain 3 (SC3) or strain 4 (SC4). Using these different genomic preps will yield products containing no mutations, M1+M2 and all three mutations (M1+M2+M3), respectively.
- PCR products were cloned into pKC1132 containing a *codA* gene, which may be used as a selection tool, at its HindIII and XbaI site and transformed into *E. coli* NEB10. Colonies were screened by colony PCR using primers NC_18_22 and NC_18_23. Plasmids were extracted from colonies yielding a correct size PCR product and these named pCLV23 (WT sequence), pCLV24 (SC3 sequence/M1+M2) and pCLV25 (SC4 sequence/M1+M2+M3). Plasmids were
25 sequenced to check that the cloned DNA was correct.
30

Making plasmids containing one mutation

- Three 'mutagenesis' PCRs were set up, using primer sets *ccaR_QCM1_F* and *ccaR_QCM1_R*; *ccaR_QCM2_F* and *ccaR_QCM2_R*; *ccaR_QCM3_F* and *ccaR_QCM3_R* to make mutations M1, M2 and M3 respectively, using pCLV23 as a template. These differ from a standard PCR in that a high concentration of template DNA is used (1µl standard plasmid prep in a 25µl PCR
35

reaction), a low concentration of primers is used (1µl of a 1mM primer stock in a 25µl PCR reaction), the annealing temperature is always 60°C, and the PCR is only cycled 12 times.

- 5
- After PCR, each mix was digested with DpnI to remove template DNA and transformed into *E. coli* NEB10. Plasmids were prepped from 6 colonies of each mutagenesis and sequenced. Of each, a plasmid was picked which contained the correct mutation and these plasmids names pCLV26 (M1), pCLV27 (M2) and pCLV28 (M3).

Sequencing results and subsequent mutagenesis

- 10
- Of the original plasmids constructed, pCLV25 contained the correct sequence. pCLV23 was found to have a point mutation upstream of *ccaR* in the *orf10* gene. As a result, pCLV26, pCLV27 and pCLV28 also contained this mutation. pCLV24 was found to have an unwanted mutation in the *ccaR* gene.
- 15
- Mutagenesis PCRs were carried out on pCLV23, pCLV26, pCLV27 and pCLV28 using primers QC_correct_orf10_F and QC_correct_orf10_R. Resulting plasmids were sent for sequencing and of these a plasmid selected which contained the correct sequence in the *orf10* gene and the final plasmids designated pCLV29 (WT seq), pCLV31 (M1), pCLV32 (M2) and pCLV33 (M3).
- 20
- A mutagenesis PCR was carried out on pCLV24 using primers QC_correct_ccaRmut_F and QC_correct_ccaRmut_R. Resulting plasmids were sent for sequencing and of these a plasmid selected which contained the correct sequence in the *ccaR* gene. The final plasmid was designated pCLV30 (M1+M2).
- 25
- To obtain mutants which contain all iterations of the mutations two further plasmids were required to cover mutations M1+M3 and M2+M3. In order to add the M1 or M2 to plasmids already containing M3 mutagenesis PCRs were performed using pCLV33 (M3) as a template using primer sets ccaR_QCM1_F and ccaR_QCM1_R; ccaR_QCM2_F and ccaR_QCM2_R respectively. Resulting plasmids were sequenced and of these plasmids selected which
- 30
- contained the desired mutation. Final plasmids were designated pCLV34 (M1+M3) and pCLV35 (M2+M3).

Primer Table:

Primer name	Sequence
NC_18_22	ATATAAGCTTTGGCCCGCATCGGTCATTGAG
NC_18_23	ATATTCTAGACACATCTACATGGCGCTGGGC

ccaR_QCM1_F	GAACCCGGGGCTTCACGGCAAGTCGGGACGGGTGGGTG
ccaR_QCM1_R	CACCCACCCGTCCCGACTTGCCGTGAAGCCCCGGGTTC
ccaR_QCM2_F	GCTTAATCCACAGAAATTTCAAGGGTTACGGTTCGATC
ccaR_QCM2_R	GATCGAACCCTAACCCTTGAAATTTCTGTGGATTAAGC
ccaR_QCM3_F	AATGAGGCGAGGAATCGCCACTGTCGCTGCCCGCGGATG
ccaR_QCM3_R	CATCCGCGGGCAGCGACAGTGGCGATTCTCGCCTCATT
QC_correct_ccaRmut_F	CTTTCACGAGTGTACCCGGCCCCAGGAGCCGGATCGTCAC
QC_correct_ccaRmut_R	GTGACGATCCGGCTCCTGGGGCCGGTGACACTCGTGAAAG
QC_correct_orf10_F	GTAGCCGTCGAAGCGCAGATACTGCCGCCCTTCACCTGG
QC_correct_orf10_R	CCAGGTGAAGGGCGGCGAGTATCTGCGCTTCGACGGCTAC

Final Constructs:

Plasmid	Mutation		
	M1	M2	M3
pCLV25	X	X	X
pCLV30	X	X	
pCLV31	X		
pCLV32		X	
pCLV33			X
pCLV34	X		X
pCLV35		X	X

5

Example 2

ccaR* mutant *Streptomyces clavuligerus

10 *ccaR* mutant plasmids prepared as outlined in Example 1 were used to prepare *ccaR* mutant strains of *S. clavuligerus*.

1. Preparation of plasmid DNA

- 15
- Each plasmid was transformed into electro-competent *E. coli* ET12567 [pUZ8002]. This strain of *E. coli* is *dam*- *dcm*- thus yielding unmethylated plasmid DNA and harbours the pUZ8002 plasmid containing *oriT* (an origin of transfer gene) enabling conjugation to take place. ET12567 is resistant to chloramphenicol; pUZ8002 is resistant to kanamycin.

2. Conjugation of plasmid DNA

20 Day 1

- Each plasmid in *E. coli* ET12567 [pUZ8002] was inoculated into 5 mL Lysogeny Broth (LB) broth containing 50 µg/mL apramycin, 50 µg/mL kanamycin and 10 µg/mL chloramphenicol. This was incubated at 35°C at 250 rpm overnight.

5 Day 2

- 2 mL of each overnight *E. coli* culture was inoculated into a 250 mL straight-edged flask containing 30 mL Lysogeny broth (LB) + apramycin, kanamycin and chloramphenicol. This was incubated at 35°C at 250 rpm until the $OD_{600} = \sim 0.6$.

- 10
- 10 mL of each *E. coli* culture was poured into a 50 mL centrifuge tube and centrifuged at 4000 rpm for 5-10 minutes. The resulting pellet was then washed in 10 mL LB three times. The final *E. coli* pellet was resuspended in 500 µL LB.

- 15
- Two plates of *S. clavuligerus* spores per plasmid to be conjugated were harvested by pipetting 3 mL LB onto the plate, dislodging the spores using a loop then pipetting the LB from the plate and into a 2 mL microcentrifuge tube. Spores were centrifuged at 7000 rpm for 10 minutes and the resulting pellet washed once in LB. The final pellet was resuspended in 250 µL LB. Spores were incubated at 50°C for 10 minutes and then placed immediately on ice.

- 20
- 250 µL of the *E. coli* suspension was mixed with each aliquot of spores. This mix was then spread on to a L3M9 agar plate and incubated overnight at 26°C.

L3M9:

<u>Component</u>	g/L
MilliQ H ₂ O	1 litre
Thingum dextrin	3
Trehalose dihydrate	10
Di-potassium hydrogen orthophosphate anhydrous	0.5
Sodium chloride	1
Magnesium sulphate	1
Calcium chloride	0.5
Casamino acids	2
MOPS buffer	10.5

Zinc sulphate	0.001
Manganese sulphate monohydrate	0.00076
Iron II sulphate	0.001
Roko agar	30

Day 3

- 5
- Each plate was overlaid with apramycin and phosphomycin – 15 μ L 100 mg/mL apramycin stock and 45 μ L of 50 mg/mL phosphomycin stock in 700 μ L was spread evenly onto each plate, and the plates incubated at 26°C for 7-10 days.
3. Obtaining double-crossovers
- 10
- Apramycin-resistant colonies achieved by conjugation are single-crossovers – these colonies will have a copy of the mutated *ccaR* in the whole vector integrated into the chromosome of *S. clavuligerus* and will still contain the original copy of *ccaR* (WT). A secondary homologous recombination event is required to remove the inserted plasmid DNA along with the original copy of *ccaR*.
- 15
- Colonies were picked using a sterile wooden cocktail sticks and patched onto L3M9 plates which have been overlaid with apramycin and phosphomycin. These were incubated at 26°C for 7 – 10 days.
- 20
- Once patches have grown and begun to sporulate, strains were re-patched onto L3M9 plates containing apramycin alone and incubated at 26°C for 7 – 10 days.
 - Patches were subsequently patched onto L3M9 plates containing no antibiotic two times.
- 25
- Spores were harvested from the final L3M9 plates by pipetting 3 mL 10% sucrose onto the plate, dislodging the spores using a loop then pipetting this spore suspension into a sterile syringe containing cotton wool. Spores were pushed through the syringe and the resulting spore suspension diluted up to 10^{-8} . Dilutions were plated on L3M9 plates which had been overlaid with 300 μ L 10 mg/mL 5-fluorocytosine (the plasmid used in this work expresses CodA which converts 5-fluorocytosine to 5-fluorouracil, which is toxic. Thus, colonies able to grow on 5-fluorocytosine will have lost this plasmid from their chromosome). Plates were incubated at 26°C for 7 – 10 days.
- 30

- Colonies were picked, and replica plated (as patches) onto L3M9 plates containing apramycin and no apramycin. After 5 days, patches which had not grown on plates containing apramycin were deemed to have lost the plasmid which had been integrated into the *S. clavuligerus* chromosome (have undergone a secondary recombination event).

All constructs used contained the mutated *ccaR* region flanked by 2Kb of DNA sequences identical to that found upstream and downstream of *ccaR* as found on the *S. clavuligerus* genome, known as the homologous arms, which were required to facilitate homologous recombination between the plasmid and *S. clavuligerus* genome. After conjugation of plasmid DNA into *S. clavuligerus* mutants had undergone a single-crossover event between the plasmid and *S. clavuligerus* genome – yielding strains which contained both the original copy of *ccaR* and the mutated *ccaR* and plasmid DNA. Mutant strains subsequently undertook a second homologous recombination event during DNA replication in which the plasmid DNA is lost, and the mutated copy of *ccaR* is exchanged for the original copy of *ccaR*. These mutants are referred to as double-crossovers. This second recombination event may also result in the excision of the plasmid and mutated copy of *ccaR*, giving rise to strains which had reverted to the wild-type strain.

One of the genes located in the homologous arms in the plasmids used to mutate each strain was *orf10*. Although the function of this gene with regards to the clavulanic acid synthesis remains unknown, knocking-out this gene in *S. clavuligerus* gives rise to mutants which are unable to make clavulanic acid (Jensen *et al.*, (2000) *Antimicrob. Agents Chemother.*). A point mutation arose in this gene during the cloning steps to make the *ccaR* mutation constructs – this required repair such that the regions flanking the *ccaR* promoter region and gene have 100% homology to that found in the same location on the *S. clavuligerus* genome (see Example 1).

4. Sequencing double-crossovers

- Sequencing was required in order to confirm whether strains have retained the mutated copy of *ccaR* or original WT copy of *ccaR*.
- Apramycin sensitive patches were scraped using a sterile loop and this placed into 50 µL DMSO. This was frozen at -70°C, thawed and vortexed, and then frozen again two further times.
- The resulting mix was used as a template in a PCR reaction using PHUSION polymerase and primer set NC_20 and NC_21 yielding a ~1.5Kb PCR product which encompasses the mutations that have been introduced to the strains. PCR products were cleaned up and

Sanger sequenced using the sequencing primer ccaR_seq or ccaR_seq_rev which anneal within the PCR product.

Primer	Sequence
NC_20	ATATCCCGGGATGGCAATTAAAGAAATGCC
NC_21	ATATACTAGTTTACCCAGCCCACACGTCTT
ccaR_seq	AGGCCGACGTCTGCAACTGG
ccaR_seq_rev	CATCGGATCCGCCAGGTC

5

- The sequence obtained was aligned against the *S. clavuligerus* ccaR gene and promoter sequence to assess whether the correct mutations were present.

5. Preparation of working spore stocks

- S. clavuligerus* mutants were patched onto three L3M9 plates and incubated at 26°C for 2 weeks giving a lawn of growth on each plate. Spores were subsequently harvested by placing 3 mL 10% sucrose onto each plate and, using a sterile 10 µL loop, scraping off spores and placing them into a sterile 25 mL tube. The total volume of each spore suspension was made to 15 mL with 10% sucrose, then using a sterile sonication probe was sonicated at an amplitude of 14 microns for 30 seconds. Sonicated spore suspensions were subsequently aliquoted into cryovials and stored at -70°C until required for fermentation.
- To calculate number of colony forming units per mL (cfu/mL) in each spore suspension, one cryovial was thawed and a dilution series made to 10⁻⁸ in polysorbate 80 (0.1% v/v). 100 µL from 10⁻⁷ and 10⁻⁸ dilutions was plated onto Tryptic soy agar plates and incubated at 26°C for five days. Colonies were subsequently counted, and the cfl/mL calculated.

25 Example 3

Fermenter assay for clavulanic acid

Fermentation

ccaR mutant strains of *S. clavuligerus* generated as outlined in Example 2 were tested at 2 L micro-fermenter scale for clavulanic acid accretion and growth profiles against WT (SC2) *S. clavuligerus* working stock controls. (Testing was carried out across five fermenter runs comprising ten vessels per run. Each run included WT (SC2) controls to account for any run to run variation).

- 500 mL Erlenmeyer flasks containing 100 mL S13A seed media (20 g/L SOFARINE defatted soy flour, 10 g/L dextrin, 0.6 g/L potassium phosphate monobasic, 5 g/L rapeseed oil, pH adjusted to 7.0 with 10M sodium hydroxide) were inoculated from frozen spore stock to give 1.5×10^5 spores per mL. Flasks were incubated at 26°C and 220 rpm.
- After 52 hours a 3% volume was inoculated into 2L microfermenters containing 1.4 L S8A2 final stage media (38.5 g/L SOFARINE defatted soy flour, 10 g/L dextrin, 0.175 g/L potassium phosphate monobasic, 23 g/L rapeseed oil, 1 g/L Foamdoctor™ antifoam, 0.1 g/L magnesium chloride hexahydrate, 0.03 g/L ferrous sulphate heptahydrate, 0.005 g/L zinc chloride, 0.005 g/L cupric chloride, 0.005 g/L manganese sulphate monohydrate). Pre-inoculation media pH was adjusted to 7.0 using 17% v/v ammonium hydroxide solution and controlled at pH 6.8 throughout with this solution. Temperature was maintained at 26°C, agitation at 1400 rpm and airflow at 0.8 vvm (volume of air/volume of liquid/ minute). A 32% w/v maltodextrin feed was introduced from 24 hours at a rate of 1.4 mls/hr and maintained throughout. A 5.67% w/v potassium phosphate monobasic feed solution was introduced from 0 hours at a rate of 0.3 mls/hr until 24 hours, then increased to 1.1 mls/hr until 48 hours at which time it was terminated. Fermentations were sampled throughout for clavulanic acid titre and terminated at approximately 140 hours as described below.

Determining concentration of clavulanic acid

- 1.5 mL of shaken broth was poured into a disposable Eppendorf tube and centrifuged at 13,000 rpm at 4°C for 10 minutes.
- Supernatants were diluted 1 in 20 into COBAS cups using a Hamilton™ diluter. Duplicate dilutions were performed on each sample.
- Diluted samples were run on a MIRA S PLUS auto analyser using the parameters as outlined below. The assay involves a chemical reaction between clavulanic acid and imidazole detecting the change in absorbance when the two are mixed is measured at 313 nm.

General

Measurement Mode: ABSORB
 Reaction Mode: R-S
 Calibration Mode: SLOPE AVG
 Reagent Blank: REAG / DIL
 Cleaner: NO
 Wavelength: 813nm on Lab 101 Mira S Plus (Serial No: 35-8663)
 413nm on MFU Mira S Plus (Serial No: 36-3139)

	Decimal Position:	0	
	Unit:	µg/mL	
5	<u>Analysis</u>		
	Post Dil Factor:	NO	
	Conc. Factor:	NO	
10	Sample Cycle:	1	
	Volume:	8.0 µL	
	Diluent Name:	H ₂ O	
	Volume:	40.0 µL	
15	Reagent Cycle:	1	
	Volume:	280 µL	
	<u>Calculation</u>		
	Sample Limit:	NO	
20	Reaction Direction:	Increase	
	Check:	ON	
	Convers. Factor:	1.00000	
25	Offset:	0.00000	
	Test Range Low:	NO	
	High:	80000 µg/mL	
	Normal Range Low:	NO	
30	High:	80000 µg/mL	
	Number of Steps:	1	
	Calc. Step A:	Endpoint	
	Readings First:	T1 Last: 3	
35	<u>Calibration</u>		
	Calibration Interval:	Each Run	
	Blank		
40	Reagent Range Low:	-0.0500 A	
	High:	0.0500 A	
	Blank Range Low:	-0.0500 ΔA	
	High:	0.0500 ΔA	
45	Standard Pos.:	1	
	STD-1:	17460 µg/mL (MFU)	4300 µg/mL (Lab 101)
	STD-2:	17460 µg/mL	4300 µg/mL
	STD-3:	17460 µg/mL	4300 µg/mL
50	Replicate:	Single	
	Deviation:	3.0%	
	<u>Control</u>		
	CS1 Pos:	No	
	CS2 Pos:	No	
55	CS3 Pos:	No	

Note: The assay should be performed at 30°C.

Determining viscosity of the fermentation broth

Viscosity was measured using a Brookfield DV-II+ viscometer set to 20 rpm. 1 mL fermentation broth was placed into the centre of the viscometer sample cup and the motor switched on – the torque required to turn the spindle submerged in the fermentation broth giving a measurement of viscosity. Measurements were taken after a few seconds once the readings had stabilised.

Results

The biggest advantage seen in the mutants after introduction of the *ccaR* mutations was the speed of accretion of clavulanic acid. In the case of mutants containing all three mutations (M1+M2+M3) clavulanic acid titre was found to peak at 65 log hours into the fermentation and then level off thereafter (see FIG 1 and FIG 3). Thus, the 65 log hour time point will be used to compare productivities of clavulanic acid across all strains.

The presence of all three mutations (M1+M2+M3) in the *ccaR* promoter region and gene yielded the highest titre of clavulanic acid, giving a titre improvement at 65 log hours of 1031% over the control (WT *S. clavuligerus*) (see Table 1, FIG 1, FIG2 and FIG 3). The next highest titres were achieved by strains carrying the M2 mutation alone (808% of control at 65 log hours), highlighting that although this appears to be the most influential of the three mutations the other two are required in combination with this to fully maximise the effect on titre. The presence of M1 and M3, either alone or in combination gave an increase in titre but not to the same extent as with combining these with M2, giving titre increases over the WT control at 65 log hours for M1, M3 and M1+M3 of 163%, 145% and 133% respectively.

Table 1

Strain	No. of samples	Titre @65LH (mg/L)	Viscosity @65LH % of control
Wild Type	10	204	100
M1	6	332	163
M2	2	1,649	808
M3	5	297	145
M1+M3	4	272	133
M1+M2+M3	15	2,105	1031

The data for the viscosity at 65 log hours, average peak viscosity, percentage viscosity at 65 log hours and percentage peak viscosity relative to WT control as observed by the mutant strains are provided in Table 2.

5

Table 2

Strain	No of samples	Viscosity @65LH (centipoise)	Peak viscosity (centipoise)	Viscosity @65LH % of WT control	Peak viscosity % of WT control
Wild Type	10	41.6	58.8	100	100
M1	6	35.8	49.6	86	84
M2	2	28.5	41.5	68	71
M3	5	40.0	55.2	96	94
M1+M3	4	35.0	54.8	84	93
M1+M2+M3	15	24.6	37.9	59	64

The *ccaR* mutations have reduced both the viscosity at time of peak production and also the peak viscosity observed (see Table 2, FIG. 4 and FIG. 5). At 65 log hours, the greatest reduction in viscosity was produced during fermentation of strains carrying all three mutations (M1+M2+M3), followed by strains carrying M2 only, with 41% and 32% reduction in viscosity as compared to the WT control.

Low viscosity combined with high productivity is highly desirable attribute in *Streptomyces clavuligerus* fermentation.

15

In FIGS 1, 2, 3 and 4, error bars indicate $\pm$ one standard deviation for data acquired from multiple fermentation runs.

20 SEQUENCE LISTINGS

SEQ ID NO: 1: Nucleic acid sequence of a WT *ccaR* promoter region and gene.

SEQ ID NO: 2: Amino acid sequence of a WT *ccaR* gene.

SEQ ID NO: 3: Nucleic acid sequence of a *ccaR* promoter region and *ccaR* gene comprising mutations described herein.

25 SEQ ID NO: 4: Amino acid sequence of a *ccaR* gene comprising a substitution mutation described herein.

SEQ ID NO: 1

cccgtcgacgtccctcccacagcctcccacccacccgtcccgactcgccgtgaagccccgggttctccgggttcaccgaggctgtccaaat
cgtccatgccttgagggtcccgtgctgatcgaaccgtaacccttggaattctgtggattaagcgtaaacaatgggtgccgacaccaaggatt
acgccgaagccatgtccacccctctcggcgagggcggtgttcttcacaagggggaccgccatgaacacctggaatgatgtgacgatccggc
5 tctgggggccggtgacactcgtgaaaggtccgtaccgatacccatccgcgggcagcgacagcgggattcctcgctcattagcgctgcgac
cggggccaggtcatctcaaggaagcgatcatcgaagactcctgggacggggagccaccactgaccgtttcggggcagttgcagacgtcggc
ctggatgatccggaccgcgtggcgaggcggggtgccccgcgacgccccgggtcccacgaccgcggctacgaactgcgcgtcctgccg
gactccatcgacctcttcttccgggagggccgtgcgcgctgcgggacctgcacgcacgcggtcagcaccaggaggcgctccgaacggct
cgacacggcgctgcctgtggaagggggcccttcgcggatgtgacctccagtcggctgcggctgcggggcgagacctggaggagga
10 gcggaccgccgcggtcgagctgcgcgcctgatcgtatgcggcctcggctactacggggacgcgatccccggctgtcggagctcgtcgc
acgaccggttcgtgaggacctgtatgtgagcctgatgaaggcctactacgcggaggggccgagccgacgcgatccaggtcttcaccgc
gcgaaggacatcctgcgggagcagatcgcatcagccccggcgagcggtgacaagggtcatgcaggccatcctgcgtcaggacgagca
ggtcctgcgggtcggtaccccggcctga

15 **SEQ ID NO: 2**

MNTWNDVTIRLLGPVTLVKGSVPIPIRGQRQRFLASLALRPGQVISKEAIIEDSWDGEPLTVSGQLQTSAWMI
RTALAEAGLPRDALGSHDRGYELRVLPDSIDLFFREAVRAVRDLHARGQHQEASERLDTALALWKGPAFADVT
SSRLRLRGETLEEERTAAVELRALIDVGLGYYGDAITRLSELVDHDPFREDLYVSLMKAYYAEGRQADAIQVFHRA
KDILREQIGISPGERMTRVMQAILRQDEQVLRVGTGA

20

SEQ ID NO: 3

cccgtcgacgtccctcccacagcctcccacccacccgtcccgact**tg**ccgtgaagccccgggttctccgggttcaccgaggctgtccaaat
cgtccatgccttgagggtcccgtgctgatcgaaccgtaaccct**ga**aatttctgtggattaagcgtaaacaatgggtgccgacaccaaggatt
acgccgaagccatgtccacccctctcggcgagggcggtgttcttcacaagggggaccgccatgaacacctggaatgatgtgacgatccggc
25 tctgggggccggtgacactcgtgaaaggtccgtaccgatacccatccgcgggcagcgacag**tg**ggcgattcctcgctcattagcgctgcgac
cggggccaggtcatctcaaggaagcgatcatcgaagactcctgggacggggagccaccactgaccgtttcggggcagttgcagacgtcggc
ctggatgatccggaccgcgtggcgaggcggggtgccccgcgacgccccgggtcccacgaccgcggctacgaactgcgcgtcctgccg
gactccatcgacctcttcttccgggagggccgtgcgcgctgcgggacctgcacgcacgcggtcagcaccaggaggcgctccgaacggct
cgacacggcgctgcctgtggaagggggcccttcgcggatgtgacctccagtcggctgcggctgcggggcgagacctggaggagga
30 gcggaccgccgcggtcgagctgcgcgcctgatcgtatgcggcctcggctactacggggacgcgatccccggctgtcggagctcgtcgc
acgaccggttcgtgaggacctgtatgtgagcctgatgaaggcctactacgcggaggggccgagccgacgcgatccaggtcttcaccgc
gcgaaggacatcctgcgggagcagatcgcatcagccccggcgagcggtgacaagggtcatgcaggccatcctgcgtcaggacgagca
ggtcctgcgggtcggtaccccggcctga

35 (Positions of mutations (M1=48, M2=143, M3=344) are in bold and underlined)

SEQ ID NO: 4

MNTWNDVTIRLLGPVTLVKGSVPIPIRGQRQ**W**RFLASLALRPGQVISKEAIIEDSWDGEPPLTVSGQLQTSAWM
IRTALAEAGLPRDALGSHDRGYELRVLPDSIDLFVFREAVRAVRDLHARGQHQEASERLDTALALWKGPADFADVT
SSRLRLRGETLEEERTAAVELRALIDVGLGYYGDAITRLSELVDHDPFREDLYVSLMKAYYAEGRQADAIQVFHRA
5 KDILREQIGISPGERMTRVMQAILRQDEQVLRVGTPA

(Position of M3 mutation (R32W) is in bold and underlined)

CLAIMS

1. A *Streptomyces clavuligerus* comprising two point mutations in a *ccaR* promoter region and a substitution mutation in a *ccaR* gene, wherein the point mutations in the *ccaR* promoter region are a C to T point mutation at a site corresponding to position 48 of SEQ ID NO:1 and a G to A point mutation at a site corresponding to position 143 of SEQ ID NO:1; and wherein the substitution mutation in the *ccaR* gene is an arginine to tryptophan substitution at a site corresponding to position 32 of SEQ ID NO: 2.
2. The *Streptomyces clavuligerus* according to claim 1, wherein the arginine to tryptophan substitution is a result of a C to T point mutation at a site corresponding to position 344 of SEQ ID NO: 1.
3. The *Streptomyces clavuligerus* according to claim 1 or 2, comprising a nucleic acid sequence of SEQ ID NO:3.
4. The *Streptomyces clavuligerus* according to any preceding claim, capable of producing a titre of 0.5 g/L or more, 1 g/L or more, 1.5 g/L or more, 2 g/L or more or 2.5 g/L or more of clavulanic acid.
5. The *Streptomyces clavuligerus* according to any preceding claim, capable of producing a titre of 2.1 g/L or more of clavulanic acid.
6. The *Streptomyces clavuligerus* according to claim 4 or 5, wherein said titre of clavulanic acid is produced after 65 hours of fermentation.
7. The *Streptomyces clavuligerus* according to any one of claims 1 to 6, capable of producing a titre of 100% or more, 200% or more, 300% or more, 400% or more, 500% or more, 600% or more, 700% or more, 800% or more, 900% or more, or 1000% or more of clavulanic acid after 65 hours of fermentation as compared to wild-type (WT) *Streptomyces clavuligerus*.
8. The *Streptomyces clavuligerus* according to any one of claims 1 to 6, capable of producing a titre of 1000% or more of clavulanic acid after 65 hours of fermentation as compared to WT *Streptomyces clavuligerus*.

9. The *Streptomyces clavuligerus* according to any preceding claim, capable of reducing viscosity of a fermentation broth by 10% or more, 15% or more, 20% or more, 25% or more, 30% or more, 35% or more, or 40% or more after 65 hours of fermentation as compared to wild-type (WT) *Streptomyces clavuligerus*.
10. The *Streptomyces clavuligerus* according to claim 9, capable of reducing viscosity of a fermentation broth by 40% or more after 65 hours of fermentation as compared to WT *Streptomyces clavuligerus*.
11. A *Streptomyces clavuligerus* according to any preceding claim for use in the production of clavulanic acid.
12. A vector comprising nucleic acid sequences comprising a *ccaR* promoter region comprising two point mutations and a *ccaR* gene comprising a substitution mutation, wherein the point mutations in the *ccaR* gene promoter region are a C to T mutation at a site corresponding to position 48 of SEQ ID NO:1 and a G to A mutation at a site corresponding to position 143 of SEQ ID NO:1; and wherein the substitution mutation in the *ccaR* gene is an arginine to tryptophan substitution at a site corresponding to 32 of SEQ ID NO: 2.
13. The vector according to claim 12, wherein the arginine to tryptophan substitution is a result of a C to T point mutation at a site corresponding to position 344 of SEQ ID NO: 1.
14. The vector according to claim 12 or 13 comprising a nucleic acid sequence of SEQ ID NO: 3.
15. The vector of any one of claims 12 to 14 for use in producing a *Streptomyces clavuligerus* of any one of claims 1 to 10.
16. A method for producing clavulanic acid comprising the steps of
growing a *Streptomyces clavuligerus* of any one of claims 1 to 10; and
recovering clavulanic acid produced by said *Streptomyces clavuligerus*, or progeny thereof.
17. The method according to claim 16, wherein a titre of 0.5 g/L or more, 1 g/L or more, 1.5 g/L or more, 2 g/L or more or 2.5 g/L or more of clavulanic acid is produced.

18. The method according to claim 16, wherein a titre of 2.1 g/L or more of clavulanic acid is produced.
19. The method according to claim 17 or 18, wherein said titre of clavulanic acid is produced after 65 hours of fermentation.
20. The method according to any one of claims 16 to 19, wherein a titre of 100% or more, 200% or more, 300% or more, 400% or more, 500% or more, 600% or more, 700% or more, 800% or more, 900% or more, or 1000% or more of clavulanic acid is produced after 65 hours of fermentation as compared to wild-type (WT) *Streptomyces clavuligerus*.
21. The method according to any one of claims 16 to 20, wherein a titre of 1000% or more of clavulanic acid is produced after 65 hours of fermentation as compared to WT *Streptomyces clavuligerus*.
22. The method according to any one of claims 16 to 21, wherein viscosity of a fermentation broth comprising said *Streptomyces clavuligerus* is reduced by 10% or more, 15% or more, 20% or more, 25% or more, 30% or more, 35% or more, or 40% or more after 65 hours of fermentation as compared to WT *Streptomyces clavuligerus*.
23. The method according to claim 22, wherein viscosity of the fermentation broth comprising said *Streptomyces clavuligerus* is reduced by 40% or more after 65 hours of fermentation as compared to WT *Streptomyces clavuligerus*.
24. A method for producing a pharmaceutical formulation comprising a β -lactam antibiotic and clavulanic acid comprising the steps of
- (a) growing a *Streptomyces clavuligerus* of any one of claims 1 to 10;
 - (b) recovering clavulanic acid produced by said *Streptomyces clavuligerus*, or progeny thereof; and
 - (c) preparing the pharmaceutical formulation by combining the clavulanic acid recovered in step (b) with the β -lactam antibiotic.
25. The method according to claim 24, wherein the β -lactam antibiotic is amoxicillin.