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(54) Title: METHODS FOR INCREASING PRODUCTION OF CARBAPENEM ANTIBIOTICS AND DERIVATIVES IN BAC-
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(57) Abstract: The present invention is in the field of production of carbapenem antibiotics by microorganisms, specifically bacteria. The present invention relates to an improved method for producing high levels of antibiotics, and specifically carbapenem antibiotics, a method for producing an engineered microorganism capable of producing the antibiotic and said microorganism, and a method for improving antibiotic production with an engineered microorganism.



Methods for increasing production of carbapenem antibiotics and derivatives in bacteria

FIELD OF THE INVENTION

5 The present invention is in the field of production of carbapenem antibiotics by microorganisms, specifically bacteria.

BACKGROUND OF THE INVENTION

The present invention is in the field of production of antibiotics by microorganisms, specifically bacteria.

10 An antibiotic relates to an agent that either kills or inhibits the growth of a microorganism. At present most modern antibacterials are naturally occurring compounds, or semisynthetic modifications thereof. Some compounds are still isolated from living organisms, such as aminoglycosides, whereas
15 many others are produced by chemical synthesis. Following screening of antibacterials against a wide range of bacteria, production of the active compounds or of their precursors is often carried out in strongly aerobic conditions.

Antibacterial compounds may be classified on the basis of
20 their origin into natural, semisynthetic, and synthetic. Another classification system is based on biological activity; in this classification, antibacterials are divided into two broad groups according to their biological effect on microorganisms: Bactericidal agents kill bacteria, and bacteriostatic agents
25 slow down or stall bacterial growth.

Antibiotics have many medical uses, such as treatment of a bacterial infection, of a protozoan infection, immunomodulation, and non-operative resource for patients who have non-complicated acute appendicitis. Also prevention of infection may be
30 considered, such as of a surgical wound, and dental antibiotic prophylaxis.

The use of antibiotics to treat and cure infectious disease has removed one of the major causes of death to the human population. Recently, the antibiotic arsenal is losing its
35 effectiveness, as resistant bacteria are beginning to spread, making infections that only a decade ago would be considered trivial often fatal. Furthermore, healthcare costs are rising worldwide. Any technology that might alleviate the upward pressure on healthcare costs would save lives.

Antibacterial antibiotics may also be classified based on their mechanism of action, chemical structure, or spectrum of activity; typically they target a bacterial function or growth process, such as a bacterial cell wall, and a cell membrane, and interference with essential bacterial enzymes. Bactericidal aminoglycosides may target protein synthesis (macrolides, lincosamides and tetracycline) but are typically not bacteriostatic. Further detailed categorization may be done.

In general a problem with present antibiotics is that microorganisms become resistant. In view thereof, and also in view of other treatments, novel antibiotics may need to be developed. While novel antibiotics have been discovered, the microbial producers are often species that are either difficult or impossible to culture in large-scale fermentations. This presents a severe hindrance to producing the antibiotics on an industrial scale, which is needed if the antibiotics are to find medical applications. Hence, alternative production methods may be required to develop and produce antibiotics at an increased speed and frequency.

Various chemical classes of antibiotics exist, such as the β -lactam class. Members of this β -lactam class are carbapenems, penicillins and cephalosporins. An example of a carbapenem is thienamycin, a naturally derived product of *Streptomyces cattleya*.

In general, production of antibiotics and precursors thereof by microorganisms normally not producing the antibiotic is inherently cumbersome, as increased levels of antibiotics inside the microorganism kill the microorganism. So despite successes in identifying metabolic pathways for antibiotic production, improvements have only been established towards non-toxic or slightly toxic intermediate products.

Antibiotics may be produced within engineered bacteria; however, some antibiotics cannot be made commercially by these engineered bacteria because the host is not amenable to genetic engineering or industrial cultivation processes. In an alternative natural producers can be mutated or otherwise manipulated, but alterations to natural producers may not be sufficient to achieve industrially-relevant titres or productivity. In these instances it would be advantageous to

make the antibiotic within a cultivation-friendly host such as *Escherichia coli* or *Bacillus subtilis*. However, these hosts are susceptible to antibiotics, limiting their application to antibiotic production.

5 Antibiotics are therefore not normally produced by susceptible hosts because they are toxic to the hosts, and this toxicity would impede production to high titres. It is therefore a problem to make antibiotics in bacterial species that are highly susceptible to the antibiotic. This problem has not been
10 addressed yet to the knowledge of the inventors. Typically production of such antibiotic compounds is achieved in native producers, which have natural mechanisms to resist antibiotics. Even these native hosts do not produce large amounts as they are rarely totally resistant to their own products. In principle
15 these natural mechanisms could be replicated in engineered strains. However, there is no guarantee these mechanisms would work in alternative production hosts such as *E. coli*. Recently advances have been made in identifying potential production routes, such as for kanamycin.

20 Carbapenems exemplify a class of antibiotics that cannot be economically produced via microbial synthesis due to low titers. Carbapenems show powerful broad-spectrum activity and resist inactivation by β -lactamases far better than other β -lactam antibiotics, making carbapenems valuable last-resort treatments
25 against multi-drug resistant infections.

Some documents recite carbapenem production routes. For instance US2013/0065878 A1 recites cell-free systems for generating carbapenems.

US 5,871,922 A (or WO 95/32294 A) recites genes involved in
30 the biosynthetic pathway of carbapenem, comprising: a) at least one of the genes *carA*, *carB*, *carC*, *carD*, *carE*, *carF*, *carG*, *carH*, b) DNA capable of hybridizing to any of the genes defined in a) and capable of functioning as such genes in the biosynthetic pathway of a carbapenem, c) DNA which is a) or b) above by
35 virtue of the degeneracy of the genetic code. Polypeptides encoded by such DNA.

Coulthurst et al. in "Regulation and biosynthesis of Carbapenem antibiotics in Bacteria", Nature Reviews, Microbiology, Vol. 3, No. 4, p. 295-306 (2005) recites some

biosynthetic principles of carbapenem and carbapenem production in bacteria, which may involve genetic engineering. On p. 296-297 it is clearly indicated that only low titres are obtainable, which is supposed to relate to < 0.1 mg/l.

5 McGowan et al. in "Analysis of bacterial carbapenem antibiotic production genes reveals a novel β -lactam biosynthesis pathway", *Molecular Microbiology*, 22(3), p. 415-426 (1998) recites a method for preparation of an engineered microorganism, such as *E.coli*, capable of carbapenam production, such as
10 expression of CarA and CarB.

Núñez et al. in "The Biosynthetic Gene Cluster for the β -Lactam Carbapenem Thienamycin in *Streptomyces cattleya*", *Chemistry & Biology*, Vol. 10, p. 301-311, (2003) recites
15 identification of genes involved in biosynthesis of thienamycin and insertion thereof in bacterial hosts thereby producing thienamycin.

Bodner et al. in "Definition of the Common and Divergent Steps in Carbapenem β -Lactam Antibiotic Biosynthesis", *ChemBioChem*, Vol. 12, p. 2159-2165 (2011), and McGowan et al. in
20 "Bacterial production of carbapenems and clavams: evolution of β -lactam antibiotic pathways", *Trends in Microbiology*, Vol. 6, No. 5, p. 203-208 (1998) recite preparation of an engineered microorganism capable of carbapenem production comprising expression of CarA and CarB in *E. coli*.

25 US 2015/0353939 A1 recites preparation of an engineered microorganism capable of carbapenem production comprising expression of CarA and CarB in a host cell, wherein the sequence used originates from *P. carotovora*.

The present invention therefore relates to an improved method
30 for producing high levels of antibiotics, and specifically carbapenem antibiotics, which solves one or more of the above problems and drawbacks of the prior art, providing reliable results, without jeopardizing functionality and advantages.

SUMMARY OF THE INVENTION

35 The present invention relates to an improved method of production of an engineered microorganism according to claim 1, wherein the microorganism is selected from fungi and bacteria, wherein the microorganism is capable of producing 1-pyrroline-5-carboxylate (P5C) and comprises genes encoding a glutamate-5-

semialdehyde dehydrogenase ProA) and glutamate 5-kinase (ProB). In principle one could even start with a naturally or modified microorganism having the *proA* and *proB* genes, removing at least one of these genes, and continue according to the invention;

5 these modified microorganisms are considered to fall under the scope of the microorganisms as claimed. Therewith amongst others high titres (from a few hundred to about ten thousand LC/MS counts, or likewise 1-100 mg/l, such as 2-20 mg/l) are obtained; put different a 50-100-fold improvement in productivity is

10 obtained. In an example *E. coli* is used as a host to engineer a synthesis pathway for carbapenem (5R)-carbapen-2-em-3-carboxylic acid (known as Car). By additionally identifying and

overexpressing a cofactor of a key enzyme, and by increasing supply of native metabolites that serve as Car precursors, a

15 productivity enhancement of 35-60-fold was obtained. As all known carbapenem pathways share initial steps in their biosynthetic pathways, other carbapenems can be produced likewise. The present invention enables production of

antibiotics that are considered immediately toxic to a

20 microorganism's cell, such as carbapenem antibiotics, which are derived from a common metabolite (3S,5S)-carbapenam. The present class of antibiotics (carbapenems) cannot yet be industrially produced via a biological process, and is produced using chemical synthesis, which greatly raises its cost to medical

25 systems worldwide. A benefit of the present methods is the development of a process for producing an expensive class of antibiotics via inexpensive microbial synthesis. The development of such processes has caused antibiotic costs to dramatically plummet for other β -lactam antibiotics. A further benefit is

30 that the ease of genetic modifications enables the antibiotic production pathway to be readily modifiable by the addition or removal of other genes, enabling the production of derivatives the present antibiotics that evade resistance mechanisms. The

term "gene" also refers to homologues thereof, and derivatives thereof. The term "derivative" is used in its chemical meaning, being a compound that is derived from a similar compound by a chemical reaction. The term "analogue" or "structural analogue" is used in its chemical meaning as being a compound having a structure similar to that of another one, but differing from it

in respect of a certain component. The term "precursor" is used to refer to a first compound that participates in chemical reaction that produces a further compound, and the first compound therefore precedes the further compound. It is noted
5 that this would not overcome the problem of resistance; it would however expand the repertoire of available drugs.

The present method improves the production of precursors of the carbapenem class of antibiotics, which are used extensively in the clinic; in an example *Escherichia coli* is manipulated
10 such that production of β -lactam antibiotics are made possible while avoiding cell lysis, which would normally cease antibiotic production.

Inventors have engineered in an example the metabolism of *Escherichia coli* to produce a precursor to clinically-relevant
15 antibiotics known as carbapenems. In an example inventors cloned genes from *Pectobacterium carotovora* species that produces a simple carbapenem, known as "Car" (1-carbapen-2-em-3-carboxylic acid). A codon-optimized Car pathway was cloned into a high-copy number plasmid (pCarCBA) which was transformed into *E. coli*
20 BL21. In addition inventors implemented several modifications to the strain that together have resulted in a 60-fold increase in total Car productivity, as defined by amount of antibiotic produced per cell per hour. Several of these improvements are applicable for production of other carbapenem compounds, such as
25 thienamycin and their derivatives, such as N-acetylthienamycin, and N-acetyldehydrothienamycin.

Inventors also used several new approaches for generating antibiotics while preserving the metabolism of the production host, without which, productivity is considered to be severely
30 limited by the toxicity of the antibiotic compound. These are approaches that are found generally useful for production of antibiotics in vulnerable species.

Inventors have found a way to produce sufficient Car to lyse the producing cells, thus providing a platform to test
35 approaches to circumvent β -lactam toxicity to cells that enable production of antibiotics to continue over time. In an example they have combined feedback-resistant ProB enzyme complex within a living system, resulting in increases in carboxymethylproline and antibiotic production. In addition inventors used a method

for increasing malonyl-CoA concentrations to improve carboxymethylproline production. They also used timed iron feeding to delay Car production until a sufficient amount of biomass had been accumulated. Withholding iron (Fe) is found to render iron-dependent CarC enzyme inactive. Iron is added only once sufficient numbers of cells have accumulated in the growth medium: this leads to a 3.5-fold increase in final Car levels. Inventors inhibit lysis caused by β -lactam antibiotics by inhibiting fatty acid synthesis. In addition, CarE is used specifically to improve CarC (for (5R)-carbapenem-3-carboxylate synthase) activity.

In a first step of the present engineering method at least one nucleotide sequence comprising at least one of genes *carB* (for Carboxymethylproline synthase), *carA* (for Carbapenam-3-carboxylate synthase), and *proB*, wherein the enzyme product encoded by *proB** is relieved from inhibition by proline, and optionally *carE* (ferredoxin) and/or *carC*, encoded thereon, wherein genes *carB*, *carA*, and *proB*, are provided at least once, is provided. Subsequently a step of expressing the genes encoded on the at least one nucleotide sequence in the microorganism is performed, and finally a step of culturing the microorganism in a medium. Therewith at least one nucleotide sequence comprising genetic codes is provided to the microorganism, wherein the microorganism is selected from fungi and bacteria, which enables the microorganism to produce (3S,5S)-carbapenam.

In an exemplary biochemical production route of a microorganism a precursor molecule 1-pyrroline-5-carboxylate (P5C) is converted into carboxymethyl proline. Thereto the *carB* gene is provided into the present microorganism. Typically as a co-enzyme malonyl-CoA is required for this conversion. Next the carboxymethyl proline is converted into the precursor (3S,5S)-carbapenam. Thereto the *carA* gene is provided into the present microorganism. Typically ATP is required for this conversion. In an example the carbapenem antibiotics are derived from a common precursor, (3S,5S)-carbapenam. Examples thereof are mentioned below. For instance, for thienamycin further genes *thnL*, *thnP*, or *thnK* (methyl transferase), and remaining genes required for thienamycin production obtained from *Streptomyces cattleya* are introduced.

In a further step (3S,5S)-carbapenam may be converted into carbapenam. Thereto the *carC* and/or *carE* gene is/are introduced into the present microorganism. It is noted that in an aqueous environment carbapenam is typically unstable.

5 The present invention now enables the production of antibiotics in bacterial species that are highly susceptible to the antibiotic, and thus are not natively produced by these susceptible hosts like *E. coli*, because they are toxic to the hosts, and this toxicity would impede production to high titres.

10 The present method avoids exposure to the antibiotic to some extent while the cell remains vulnerable (during growth, and expression of antibiotic production enzymes). The present method helps surmount this barrier, which enables in principle production of several classes of antibiotics within hosts rather
15 than native producers. This not only leads to lower production costs for antibiotics, but more importantly, it enables rapid development of production methods for novel antibiotics.

It is noted that according to the knowledge of the inventor the present problem has not even been addressed. As mentioned,
20 typically production of antibiotic compounds is achieved in native producers, which have natural mechanisms to resist antibiotics. These natural mechanisms could in principle be replicated in engineered strains. However, these natural mechanisms may be overwhelmed (even in native producers) at high
25 concentrations achieved of antibiotics or toxic precursors during production in an industrial fermentation. Furthermore there is no guarantee these mechanisms would work in alternative susceptible production hosts such as *E. coli*.

The present inventor has found that production of antibiotics
30 in genetically-tractable and fast-growing species can be much faster and much cheaper than production in native strains, as genetic manipulation tools have been established. Growth is very rapid (enabling the quick production of large quantities of biomass), and there is much experience with using e.g. *E. coli*
35 in industrial fermentation, indicating that boundary conditions per se for growth of *E. coli* are well known. In another aspect the production of antibiotics in *E. coli* enables a much quicker development of antibiotic production pathways after the discovery of the genes responsible for their production. This

also enables a rapid diversification of antibiotics using biochemical diverse synthesis.

It is noted that antibiotic production in susceptible cells is expected to limit achievable titres by inhibiting biomass production (growth), and by disrupting cell metabolism. OD₆₀₀ measurements clearly indicate growth inhibition occurring very early (2 h) after induction of high-production pathways.

For better understanding of the biosynthesis routes, engineering of microorganisms, and details thereof, reference can be made to the presentation by H. Shomar et al, 3rd Synthetic Biology Congress, London, United Kingdom. October 20, 2016, and a to be published paper by G. Bokinsky on the same topic, as well as a paper entitled "Metabolic engineering of a carbapenem antibiotic pathway in *Escherichia coli*" of amongst others G. Bokinsky, of which the contents are incorporated by reference.

In a second aspect the present invention relates to a microorganism, wherein the microorganism is selected from Enterobacteriales, Actinobacteria, Bacillaceae, Streptomyces, Ascomycota and Basidiomycota, such as *E.coli*, *S.cerevisiae* and *Bacillus subtilis*, obtainable by the engineering method according to the invention.

In a third aspect the present invention relates to a method of producing a carbapenem compound, comprising the step of providing the microorganism of the present invention.

In a fourth aspect the present invention relates to a method for improving antibiotic production with an engineered microorganism, such as the microorganism of the present invention. Typically microorganisms, such as *E. coli*, rely on Fe (or Fe ions) when growing. It has now been found, which is unexpected in view of the Fe dependency, that if Fe is withheld for a period of time, starting when growth of the culture is initiated by inoculation, until sufficient cell biomass has been generated, such as during at least 0.5 hour in an initial biomass production stage, preferably during at least 1 hour, such as at least two hours, cell lysis can be prevented and production of the antibiotic is increased significantly. The amount of cell biomass may be considered sufficient at $5 \cdot 10^8$ – $5 \cdot 10^9$ cells per ml, which can be verified with spectrophotometry by measuring the optical density such as at 600 nm. A concentration

of Fe in a medium (such as for activating CarC) is preferably 10^{-6} – 10^{-4} mole/l Fe, more preferably $5 \cdot 10^{-6}$ – $5 \cdot 10^{-5}$ mole/l, e.g. 10^{-5} – $3 \cdot 10^{-5}$ mole/l. Fe is typically present as Fe^{2+} and/or Fe^{3+} .

Likewise production of antibiotic is increased if cell growth is inhibited.

In an example thereof the present antibiotic is produced in so-called growth-arrested cells; the cells are put in circumstances where amino acid starvation occurs. It is considered that non-growing cells show increased resistance to antibiotics. A method for creating non-growing cells that are still capable of producing antibiotics is to withhold a nutrient needed for growth, which nutrient is not critical for antibiotic production, such as amino acids. The removal of ProC (for Pyrroline-5-carboxylate reductase) is an example of this approach. proC knockout cells are unable to synthesize proline, and thus cannot grow without exogenously-provided proline. When a culture runs out of proline, growth will arrest as a consequence, and the cells will cease to grow; these cells are found to become more resistant to antibiotics (especially β -lactam antibiotics, such as carbapenems). Thus, cells can be grown in the presence of limiting proline, such that the cells will cease to grow, but continue to consume other nutrients available (e.g. glutamate, glucose, ammonia) which will be used to produce the carbapenem antibiotics (or other antibiotics). The proline concentration may be tuned to attain a desired amount of biomass (as biomass production is considered impossible without proline). The time at which cells are triggered to produce carbapenem antibiotics (as expressing the Car pathway requires proline, but activity does not) may also be tuned. This is also supported by timing Fe supplementation (i.e. by withholding Fe until proline is exhausted, ensuring no Car is produced until the cell is protected against Car by proline starvation). Thus production may be split into two phases: a growth phase, during which the cells use exogenous proline to produce biomass and express the Car pathway, and an antibiotic production phase, during which the cells have consumed all the available proline and are producing the Car antibiotic in a growth-arrested state that renders them resistant to the

toxicity of the antibiotic.

Proline may be supplemented during the production phase. A phenomenon that has been observed so far is that the productivity (rate of amino acid production) of the growth-arrested cells during amino acid starvation decays over several hours, due to unknown factors. Supplementation of the limiting amino acid can restore productivity once again. Addition of very small amounts of proline during production phase is considered sufficient to restore productivity. Next to the present approach with proline (wherein the *proC* knockout is present, limitation of other amino acids or nutrients whose lack causes immunity to β -lactam antibiotics, is envisaged. This includes, but is not limited to, phenylalanine, tryptophan, tyrosine, leucine, isoleucine, valine, serine, glycine, alanine, and glutamine. This approach is considered better than other triggers for growth arrest, as amino acid limitation can be readily engineered by proper formulation of growth medium used in fermentation, and does not rely on the expression of growth-arresting proteins (e.g. HipA (for Serine/threonine-protein kinase toxin HipA), RelA), which depend upon the addition of expensive chemical inducers, and the maintenance of plasmids encoding the growth-arresting proteins.

In a fifth aspect the present invention relates to an antibiotic obtainable by the present invention. In an exemplary embodiment the antibiotic product may further comprises residual products of the production method. These residual products may provide further advantages, such as inherently a mixture of antibiotics may be produced, making the mixture (or cocktail) more effective.

Thereby the present invention provides a solution to one or more of the above mentioned problems and drawbacks.

Advantages of the present description are detailed throughout the description.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates in a first aspect to an engineering method according to claim 1.

In an exemplary embodiment of the present engineering method the microorganism is capable of producing 1-pyrroline-5-carboxylate (P5C), wherein the microorganism is preferably

selected from fungi and bacteria, such as from Enterobacteriales, such as *Escherichia*, such as *Escherichia coli*, Actinobacteria, Streptomyces, such as *Streptomyces cattleya*, *Streptomyces argenteolus*, *Streptomyces flavogriseus*,
5 Bacillaceae, such as a *Bacillus*, such as *Bacillus subtilis*, and Fungi, such as Ascomycota and Basidiomycota, such as Saccharomycetes, such as *S. cerevisiae*. These genera and species have been found especially suited for engineering genes thereof.

In an exemplary embodiment of the present engineering method
10 the at least one nucleotide sequence additionally comprises *proA* (for Gamma-glutamyl phosphate reductase). In an initial step of the present synthesis glutamate (Glu) may be converted into glutamyl-5-phosphate (G5P). Thereto the *proB* gene (for Glutamate 5-kinase) is provided into the present microorganism in order to
15 support said conversion. In a further step of the present synthesis glutamyl-5-phosphate (G5P) may be converted into L-glutamate-5-semialdehyde (GSH). Thereto the *proA* gene is provided into the present microorganism in order to support said conversion. GSH may than release a water molecule in order to
20 convert to P5C in an equilibrium reaction.

In an exemplary embodiment of the present engineering method a glutamate kinase enzyme is co-expressed, and the glutamate kinase has been mutated to relieve feedback inhibition by proline, wherein the *proB* gene has been mutated to *proB**,
25 wherein the enzyme product ProB encoded by *proB** is relieved from inhibition by proline, such as feedback resistant ProB, such as at least one of *E. coli* E143A (glutamate 143 mutated to alanine), K145A (lysine 145 mutated to alanine), I69E (isoleucine 69 mutated to glutamate, and N134D. As such it has
30 been found that yields of antibiotics can be increased. One *proB* mutation that has been shown to relieve feedback inhibition by proline is I69E, though other mutations that relieve feedback inhibition, such as N134D, K145A, may also increase yields of antibiotic.

35 In an exemplary embodiment of the present engineering method the genes *carB*, *carA*, and optionally at least one of *carE*, *carC*, *proA*, and feedback resistant *proB**, are arranged in at least one operon, preferably an operon in a plasmid, preferably in the same plasmid. Likewise the genes and operon may be incorporated

in a chromosome of the microorganism, or provided on a separate plasmid, especially for production on industrial scale. Genes can be introduced in the genetic material of a host, they can be added as a plasmid comprising the genes, wherein typically the plasmid further comprises a gene for producing a toxic resistance. As such it has been found easier to incorporate said genes into the microorganism. The optional genes may support the yield of the present antibiotic. It was found that co-expressing the Car pathway with ProA and feedback-resistant mutants of ProB improved the yield. Co-expression of mutant ProB I69E with the enzymes CarCBAE (BL21pCarCBAE_ProABmut and BL21 pCarCBADE_ProABmut) improved Car productivity by 5-fold compared to the strain pCarCBAE. In contrast, co-expression of wild-type ProAB enzymes did not significantly increase Car productivity or CMP titres. Co-expressing CarE and feedback-resistant ProBA improved Car titres measured early after induction by nearly 40-fold compared to the minimal pathway.

In an exemplary embodiment of the present engineering method the at least one nucleotide sequence further comprises at least one of genes *carD*, *carF*, *carG*, *carH*, and modified *FabD*, optionally arranged in the present operon. These further genes can typically be found in microorganisms, such as *P. carotovorum* or *Streptomyces cattleya*, and may be associated with production of carbapenem or precursors thereof. It was found that co-expression of both CarD and CarE with CarCBA (BL21pCarCBADE) increased Car productivity (defined as hCar per cell per unit time) by 11-fold compared to the minimal pathway pCarCBA. Further research identified that CarD itself did not significantly increase Car productivity, whereas expression of CarE alone was sufficient to recover the 11-fold productivity increase observed with CarDE expression. *FabB*/*FabF*/*FabD* were not expressed with the Car enzymes. These Fab enzymes are typically expressed by the bacteria to make fats for membranes. In an embodiment inventors modified *FabD* (for Malonyl CoA-acyl carrier protein transacylase) so that it would be degraded at a certain point during growth, which was found to channel more precursors into the Car pathway, and also arrested growth.

In an exemplary embodiment of the present engineering method the *proC* gene has been removed. As such conversion of P5C to

proline is significantly reduced and thereby antibiotic yield is increased.

In an exemplary embodiment of the present engineering method the at least one nucleotide sequence is extracted from *P.*

5 *carotovorum* or *Streptomyces cattleya*. That is it is identified, isolated and extracted, as well as treated further, such as by PCR. Likewise the nucleotide sequence is produced by chemical synthesis.

10 In general for the engineering of microorganisms, as described above, the DNA of the microbial species, or likewise a combination of species, can be changed. After changing the DNA in an initial stage the microorganism is cultured in order to obtain a population. Said population may be used for further purposes, such as producing an antibiotic.

15 In a second aspect the present invention relates to a microorganism, wherein the microorganism is selected from Enterobacteriales, Actinobacteria, Bacillaceae, Streptomyces, Ascomycota and Basidiomycota, *Escherichia*, such as *Escherichia coli*, *Bacillus*, such as *Bacillus subtilis*, *Streptomyces*
20 *cattleya*, *Streptomyces argenteolus*, *Streptomyces flavogriseus*, and Saccharomycetes, such as *S. cerevisiae*, obtainable by an engineering method according to the invention.

In a third aspect the present invention relates to a method of producing a carbapenem compound, comprising the step of
25 providing the microorganism of claim 13, culturing the microorganism, and thereby producing at least one of a carbapenem (1-carbapen-2-em-3-carboxylic acid), (3S,5S) carbapenam, and carbapenem.

In an exemplary embodiment of the present production method
30 the carbapenem compound is a carbapenem antibiotic, such as azabicyclo[3.2.0]hept-2-ene-2-carboxylic acids, such as 7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acids, such as thienamycin ((5R,6S)-3-[(2-Aminoethyl)thio]-6-[(1R)-1-hydroxyethyl]-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic
35 acid), imipenem (5R,6S)-6-[(1R)-1-hydroxyethyl]-3-((2-[(iminomethyl)amino]ethyl)thio)-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid, meropenem 4R,5S,6S)-3-(((3S,5S)-5-(Dimethylcarbamoyl)pyrrolidin-3-yl)thio)-6-((R)-1-hydroxyethyl)-4-methyl-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid,

ertapenem (4R,5S,6S)-3-[(3S,5S)-5-[(3-carboxyphenyl)carbamoyl]pyrrolidin-3-yl]sulfanyl-6-(1-hydroxyethyl)-4-methyl-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid, doripenem (4R,5S,6S)-6-(1-Hydroxyethyl)-4-methyl-7-oxo-3-(((5S)-5-((sulfamoylamino)methyl)pyrrolidin-3-yl)thio)-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid, panipenem/betamipron (5R,6S)-3-{[(3S)-1-ethanimidoylpyrrolidin-3-yl]sulfanyl}-6-[(1R)-1-hydroxyethyl]-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid, biapenem (4R,5S,6S)-3-(6,7-dihydro-5H-pyrazolo[1,2-a][1,2,4]triazol-8-ium-6-ylsulfanyl)-6-(1-hydroxyethyl)-4-methyl-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylate, razupenem (4R,5S,6S)-6-((R)-1-hydroxyethyl)-4-methyl-3-((4-((S)-5-methyl-2,5-dihydro-1H-pyrrol-3-yl)thiazol-2-yl)thio)-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid, tebipenem (4R,5S,6S)-(Pivaloyloxy)methyl 3-((1-(4,5-dihydrothiazol-2-yl)azetidin-3-yl)thio)-6-((R)-1-hydroxyethyl)-4-methyl-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylate, lenapenem, and tomopenem ((4R,5S,6S)-3-[(3S,5S)-5-[(3S)-3-[[2-(diaminomethylideneamino)acetyl]amino]pyrrolidine-1-carbonyl]-1-methylpyrrolidin-3-yl]sulfanyl-6-[(1R)-1-hydroxyethyl]-4-methyl-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid), or a derivative thereof, or an analogue thereof. Hence a large variety of antibiotics can be produced.

In an exemplary embodiment of the present production method iron is withheld for a period of time, starting with withholding iron when growth of the culture is initiated by inoculation, generating sufficient cell biomass and then stopping with withholding iron, such as during at least 0.5 hour during bacterial growth, preferably during at least 1 hour, such as at least two hours, therewith preventing cell lysis, wherein the medium comprises 10^{-6} - 10^{-4} mole/l Fe, more preferably $5 \cdot 10^{-6}$ - $5 \cdot 10^{-5}$ mole/l, such as 10^{-5} - $3 \cdot 10^{-5}$ mole/l. The amount of cell biomass may be considered sufficient at $5 \cdot 10^8$ - $5 \cdot 10^9$ cells per ml, which can be verified with spectrophotometry by measuring optical density such as at 600 nm. The OD₆₀₀ is preferably > 1, more preferably > 2, or even >5 or > 10. Surprisingly and unexpectedly, as typically microorganisms as *E. coli* rely on Fe for their biosynthesis, reducing an amount of Fe in e.g. the growth medium

in an initial stadium of antibiotic production, significantly increases the amount of carbapenem produced by about a factor 3-5. Likewise, when Fe is withheld fully hardly any carbapenem is produced, showing the dependency of the production pathway activity within the microorganism on Fe during antibiotic production.

In an exemplary embodiment of the present production method the carbapenem compound is produced in at least one stage, and cell growth is in at least one antibiotic production stage inhibited, thereby delaying expression of antibiotic synthesis to the next stage. It has been found that such inhibition enables antibiotic production to continue for longer, such as about 50-200% longer. In an exemplary embodiment expression of at least one gene of *carB*, *carA*, *carC*, *carD*, *carE*, *carF*, *carG*, *carH*, *proA*, and *proB**, is delayed, such as by overexpressing *HipA*.

In an exemplary embodiment of the present production method a 10-fold improvement in production of Car is found by co-expressing the gene *carE* (from the original operon).

In an example a several-fold improvement in production of Car is found by increasing precursor supply by co-expression of feedback-resistant mutants of ProB, which are not inhibited by proline and thus are found to supply the Car pathway with a higher concentration of precursor molecule P5C.

In an exemplary embodiment of the present production method an improvement is achieved in the precursor pool carboxymethylproline by increasing the precursor malonyl-CoA.

In an exemplary embodiment of the present production method an improvement is achieved by preventing cell lysis when the antibiotic is produced; this is considered to prevent the antibiotic from halting production, thereby improving carbapenam production.

In an exemplary embodiment of the present production method an improvement is achieved by a method for preventing cell lysis before sufficient biomass is generated: by withholding iron (Fe), CarC is considered to be kept inactive until a sufficient amount of cells have accumulated. This may prevent antibiotic accumulation, which may lead to cell lysis and prevent the accumulation of sufficient biomass that could enable high-titre

production. Once enough biomass has accumulated, addition of iron is found to trigger Car production. Inventors have identified an optimum time for iron addition for maximum benefit.

5 In an exemplary embodiment the present production method comprises at least one further biological or chemical synthesis step of producing an antibiotic, such as addition or removal of a moiety, such as methylation and thiolation, oxidation, reduction, epimerization, reacting with a further compound,
10 etc.; i.e. one may start with the present biosynthesis of carbapenam and complete a full synthesis, towards a desired molecule, with at least one further biological or chemical synthesis step, preferably only (a) biological synthesis step(s) as production can then be carried out by a microorganism. When
15 using only microorganism production is typically limited to natural occurring carbapenems. The present method may be considered to deliver an intermediate product in such a case.

By splitting the present method in various steps also alternative biosynthesis routes become directly available,
20 starting from more common steps. Such makes the present method very versatile.

The above characteristics of the host cell provide specific advantages for the production of specific antibiotics, such as carbapenems.

25 In a fifth aspect the present invention relates to an antibiotic product obtained by the present method. In an exemplary embodiment the product further comprises residual products of the production method. These residual products may provide further advantages, such as inherently a
30 mixture of antibiotics may be produced, making the mixture (or cocktail) more effective.

In a sixth aspect the present invention relates to a method for improving antibiotic production with an engineered microorganism, such as the microorganism according to the
35 invention, wherein iron is withheld for a period of time, starting with withholding iron when growth of a culture is initiated by inoculation, generating sufficient cell biomass and then stopping with withholding iron, therewith preventing cell lysis, wherein the medium comprises 10^{-6} – 10^{-4} mole/l iron, and/or

wherein the antibiotic is produced in at least one stage and wherein cell growth is in at least one antibiotic production stage inhibited thereby delaying expression of antibiotic synthesis to the next stage. For sufficient cell biomass the
5 OD_{500} is preferably > 1 , more preferably > 2 , or even > 5 or > 10 .

In an exemplary embodiment of the method for improving antibiotic production the medium comprises $5 \cdot 10^{-6}$ – $5 \cdot 10^{-5}$ mole iron/l, such as 10^{-5} – $3 \cdot 10^{-5}$ mole iron/l.

In an exemplary embodiment of the method for improving
10 antibiotic production lysis is inhibited by fatty acid synthesis inhibition. For example, once sufficient biomass has been reached, in a similar manner as with iron limitation, i.e. grow cells, and then inhibit cell growth at the same time as when antibiotic production is started.

15 The one or more of the above examples and embodiments may be combined, falling within the scope of the invention.

FIGURES

Fig. 1: Car biosynthesis pathway.

Fig. 2: diagram of examples of the present invention
20 superimposed on the Car pathway.

Fig. 3: production of Car.

Fig. 4: Effect of Fe delay on Car production.

Fig. 5a,b: Operon construct.

Fig. 6a,b shows the chemical structures of carboxymethyl-proline
25 (CMP) and hCar, respectively.

Figs. 7-10 show experimental results.

DETAILED DESCRIPTION OF THE FIGURES

Fig. 1: Integration of the Car synthesis pathway from *P. carotovorum* with *E. coli* proline synthesis pathway. The
30 production of the carbapenam intermediate is considered relevant to synthetic pathways of other carbapenam antibiotics (side paths). The figure has been detailed through the description. Encircled is the β -lactam part in (3S,5S)-carbapenam. Also indicated are exemplary antibiotics thienamycin (B), imipenem
35 (C), meropenem (D), ertapenem (E), and doripenem (F).

Fig. 2 shows an overview of steps modified that result in increases of Car or carboxymethylproline production. In section 1 a relief of inhibition of ProBA by proline is achieved. In section 2 intracellular malonyl-CoA concentrations are

increased. Such can e.g. be done by inhibiting a fatty acid pathway, such as by timed starvation of the microorganism and/or by removing fatty acid synthesis enzymes from the microorganism.

In section 3 CarC activity is regenerated with CarE. This is

5 found to be Fe dependent. In section 4 ProC is removed to decrease competition for P5C. Therewith genes are expressed, a high cell mass is obtained, CarE/CarC are/can be activated, and production is increased (Fe). Also indicated are exemplary antibiotics panipenem/betamipron (G), biapenem (H), razupenem
10 (I), tebipenem (J), lenapenem (K), and tomopenem (L).

Fig. 3 shows the production of Car per cell after 24 hours. Influences of various boundary conditions are studied. With Carc, CarA, and CarB (CAB) and tricine added small amounts of Car were produced. With CarC, CarB, CarA, CarD and CarE (CBADE)
15 and with CarC, CarB, CarA, and CarE (CBAE), i.e. with CarE, the production of Car significantly increased. Mutant ProBA* further increased the production of Car. Exemplary nucleotide sequence are provided as separate documents.

Figure 4: Effect of Fe delay on Car production. When no Fe
20 was supplied hardly or no Car was produced (bottom line). Under conditions when Fe was supplied production gradually picked up over time (middle line). Unexpectedly, when supply of Fe was delayed for some time, initial production of Car stayed low, but when Fe was supplied production of Car increased rapidly. The
25 vertical axis shows total Car production, whereas the horizontal axis shows time (hours).

Fig. 5a shows the naturally-occurring operon encoding enzymes for Car production as found in *P. carotovorum*. Therein three enzymes, CarA, CarB, and CarC, are found necessary for the
30 biosynthesis of Car. The enzymes CarD and CarE are found to increase the production of Car.

Fig. 5b shows an exemplary embodiment of the present artificial, engineered, operon for *E.coli*. It is preferably encoded on a plasmid, preferably on the same plasmid; an
35 alternative to the plasmid is a chromosome. Therein the genes *proB**, *proA*, *carA*, *carB*, *carC*, and *carE*, are shown. ProB* is a mutant of *E. coli* glutamate kinase enzyme ProB in which feedback inhibition has been removed. ProA is *E. coli* glutamate semi-aldehyde dehydrogenase. The genes are turned on c.q. off at the

same time.

Figure 7 shows the Car biosynthesis pathway and LC/MS detection of metabolites (details of the LC-MS method can be found in the above articles of Bokinsky). (a) The enzyme CarB joins P5C, an intermediate of proline synthesis, with the fatty acid precursor malonyl-CoA to yield carboxymethylproline (CMP). CarA catalyses the ATP-dependent formation of the β -lactam ring to generate carbapenam, the precursor to all known naturally-occurring carbapenems. CarC catalyses two enzymatic steps: C5 epimerisation and C2-C3 desaturation to produce the bioactive carbapenam nucleus of Car. As with all carbapenems, the β -lactam ring of Car is susceptible to spontaneous hydrolysis. Highlighted are the strategies employed to increase Car production. (I) increasing P5C by relieving allosteric inhibition by proline; (II) eliminating consumption of malonyl-CoA by FabD; (III) regenerating CarC activity by expressing the ferredoxin CarE. (b) Chromatograms of Car pathway metabolites and by-products detected in cultures of *E. coli* pCarB, pCarAB, pCarCBA, and BL21: CMP (3), carbapenam (4) and hCar (6). Fragmentation spectra are included as well.

Figure 8 shows Metabolic engineering of the Car biosynthesis pathway in *E. coli*. (a) Successive metabolic engineering interventions increased productivity by 60-fold over the minimal pathway (*E. coli* pCarCBA). Productivity is calculated from hCar LC/MS counts in culture supernatants divided by the cell density (OD600) recorded 1.5 h after induction. (b) hCar titers in culture supernatants of engineered strains 1.5 h after induction. Error bars represent the standard deviation of 5 mL culture triplicates derived from a single colony. (c) (Top) Gene schematic of plasmid pCarCBA_E encoding CarCBA and CarE under the control of orthogonal promoters PLacUV5 (IPTG-inducible) and PTet (aTc-inducible), respectively. (Bottom) Effect of CarE induction on hCar productivity (hCar counts/OD600) recorded 1.5 h after induction. Solid line represents average productivity in relation to aTc inducer concentration. Dots represent values of biological duplicates from 25 mL cultures. (d) Average pathway metabolite levels (LC/MS counts) of duplicate 25 mL cultures 4 h after induction. Circle areas are proportional to the peak area of CMP, carbapenam, and hCar.

Figure 9 shows Car production limits growth, causes lysis, and decreases achievable antibiotic titres. (a) Growth curves of engineered strains after induction of Car production. Dots represent 25 mL-culture biological replicates. (b) Photographs of cultures 24 h after induction exhibiting significant cell debris and poor growth in Car-producing strains. (c) Cell permeability measurements in Car-producing cultures 24 h after induction. As optical density is not an accurate measure of total biomass produced in lysed cultures, propidium iodide (PI) fluorescence was normalized instead to total protein precipitated by methanol-chloroform extraction from whole cultures. Error bars represent the standard deviation of analytical triplicates. (d) LC/MS analysis of hCar titres after induction. Dots represent 25 mL-culture biological replicates. (e) Productivity comparison at 2 h after induction with final titres achieved at 24 h. Productivity of lysed cultures was estimated by hCar titres per total protein extracted in culture samples collected 2 h after induction (dark bars). In addition it is found that mutations of cysteine residues (C43S and C46S) that participate in the FeS cluster of CarE (BL21 pCarCBAEmut) eliminate the high-production phenotype. Productivity is calculated from hCar LC/MS counts divided by the cell density (OD600) recorded in culture supernatants 1.5 h after induction.

Figure 10 shows Engineering antibiotic tolerance and improved Car pathway flux. (a) Growth curves of pCarCBAE_ProABmut cultures after induction indicating lysis (45% OD600 decrease) in BL21 strains, which is reduced in fabD-pdt#3 strain (17% OD600 decrease). (b) Cell permeability measurements 24 h after induction indicates that FAS inhibition in fabD-pdt#3 reduces Car-induced lysis. (c) Titres of 3, 4, and 6 (left, centre, and right, respectively) in culture supernatants 24 h after induction. FAS inhibition and malonyl-CoA accumulation increases Car pathway flux. Error bars represent the standard deviation of biological triplicates grown from 3 separate colonies.

The invention is further detailed by the accompanying example, which is exemplary and explanatory of nature and are not limiting the scope of the invention. To the person skilled in the art it may be clear that many variants, being obvious or

not, may be conceivable falling within the scope of protection, defined by the present claims.

EXAMPLE

Example of an experiment for carbapenem production.

Strains and growth medium.

E. coli BL21(DE3) was used for Car production experiments. For all Car production experiments, cells freshly-transformed with plasmids encoding the Car pathway were used. Transformants were grown overnight on selective media agar plates at 37°C. Colonies grown for more than 48 h on agar plates showed poor growth when inoculated into liquid medium. Car production cultures were grown in MOPS-based minimal medium (8.372 g L⁻¹ MOPS, 0.717 g L⁻¹ Tricine, 2.92 g L⁻¹ NaCl, 11 mg L⁻¹ MgCl₂•7H₂O, 0.56 µg L⁻¹ CaCl₂, 200 µL micronutrient stock⁴¹) supplemented with 4 g L⁻¹ D-glucose, 28.5 mM NH₄Cl, 10 µM FeSO₄ and 5 g L⁻¹ potassium glutamate. The antibiotics kanamycin (25 µg/mL), ampicillin (50 µg/mL) and chloramphenicol (17.5 µg/mL) were added when appropriate.

Plasmid construction.

All plasmids were constructed using Gibson assembly in *E. coli* DH5α. Genes encoding the Car enzymes from *P. carotovorum* (*carABCDE*) were codon-optimized for expression in *E. coli*. The *proAB* genes were obtained by PCR amplification from *E. coli* BL21(DE3) genomic DNA. The mutant variants I69E of ProB, and C43S/C46S of CarE were constructed by PCR site-directed mutagenesis using the primers P1 and P2 in Supplementary Table 2. Biosynthetic operons were assembled and cloned into the pBbE5k BglBrick backbone. The plasmid pCarCBA_E was constructed from pCarCBA, by addition of the CarE expression unit under control of the inducible PTet promoter. The reverse sequence of the CarE inducible expression unit was placed at the 3' end of the bi-directional terminator present on the pBbE5k BglBrick backbone. *hipA* was cloned from *E. coli* MG1655 genomic DNA and inserted into the pBbS2c BglBrick backbone to make pHipA. To construct the mf-Lon protease expression vector, we introduced the codon optimised mf-Lon gene and strong ribosome binding site from pECL275 into the pBbA2c BglBrick backbone. The resulting plasmid pmf-Lon contains a chloramphenicol resistance cassette. The derivative plasmid pmf-Lon-bis contains an ampicillin

resistance cassette instead. All the strains and plasmids constructed in this work are listed in Supplementary Table 1.

Construction of strains with tuneable protein degradation.

The degradation tag pdt#339 at the C terminus of the genes of interest in *E. coli* BL21(DE3) chromosomal DNA. For each targeted gene (*fabB*, *fabD* and *fabF*), PCR products were generated that contained the pdt#3 tag amplified from pECT3 and 37-42 bp 5' extensions with homology to the C terminus, and 3' extensions with homology to the immediate 3' untranslated region of the gene of interest. The P1 and P2 primer sequences and full-length primers used to target *fabB*, *fabD* and *fabF*. Genomic pdt#3 insertions were performed using homologous recombination by transforming the PCR products into *E. coli* BL21(DE3) containing pKD46. Successful insertions were verified by PCR. The kanamycin resistance cassette was subsequently removed using the plasmid pCP20. The resulting strains, *fabB*-pdt#3, *fabD*-pdt#3 and *fabF*-pdt#3 were screened by PCR and verified by DNA sequencing.

Genetic manipulations.

The specific nucleotide sequences as used are provided in the appendices to this application.

Growth conditions.

Production of Car in *E. coli* from the present synthetic operon, is achieved using the following growth conditions.

Medium composition

Car production is carried out in a MOPS minimal medium, which composition was based on the standard recipe described in <http://www.genome.wisc.edu/resources/protocols/mopsminimal.html>. A 10X MOPS salts solution without iron was prepared according to the recipe above, except for the addition of FeSO₄ salts. The Car production medium was prepared using the iron-depleted MOPS salts, supplemented with 0.4% glucose, 28.5 mM NH₄Cl and 0.5% potassium glutamate.

10x MOPS-Fe Salts (1 L)

A one litre solution was prepared having 83.72 g MOPS, 7.17 g Tricine, 29.2 g NaCl, 0.11 g MgCl₂·7H₂O, and 28 µL of 20 g CaCl₂.

MCM-Fe (500 mL)

A 500 ml solution was prepared having 50 mL 10X MOPS-Fe salts, 20 mL 10% glucose, 7.5 mL 1.9 M NH₄Cl, 5 mL K₂HPO₄, 1 mL K₂SO₄, 50 mL 5% potassium glutamate, and 366.5 mL millipore H₂O.

Culture conditions and iron supplementation

Freshly transformed Car producing strains are incubated overnight in a minimal medium without iron (supplemented with the required antibiotics) at 37°C and 250 rpm, the day prior the experiment.

Car production is carried out by inoculating 25 mL of medium from the overnight cultures, and with a normalized initial cell density (monitored as optical density measured at 600 nm) of 0.06. Cultures are incubated at 37°C and 250 rpm in 250 mL Erlenmeyer flasks to an OD₆₀₀ of 0.5-0.6 followed by induction of the Car pathway with IPTG (0.25 mM final concentration). This high density was not found if the cultures are grown in the presence of iron, due to the leaky expression of the Car pathway. Simultaneously, a sterile solution of 28 mg/mL FeSO₄ (~0.18 M/l) is freshly prepared and diluted 10-fold (~0.018 M/l); 25µL of the diluted iron sulfate solution is added at time of induction to each culture. The presence of Fe²⁺ ions (~1.8 *10⁻⁵ M/l) initiates the catalytic activity of the enzyme CarC.

CarE titration experiment.

As the enzyme CarC is found to be iron-dependent, an iron-deficient MOPS-based production medium (without FeSO₄ supplementation) was used to limit Car production from leaky expression of the Car pathway. Individual colonies of BL21(DE3) pCarCBA_E were grown overnight in 5 mL of iron-deficient production medium. Overnight cultures were diluted into 250 mL Erlenmeyer flasks containing 25 mL selective iron-deficient production medium to an initial OD₆₀₀ of 0.06. Cultures were grown at 37 °C with shaking (250 rpm) until reaching an OD₆₀₀ of 0.25. At this point, CarE expression was induced by addition of anhydrous tetracycline (aTc). The cultures were further incubated for 2 h, after which CarCBA expression was induced with 0.25 ml 5 mM IPTG and the medium supplemented with 10 µM FeSO₄. Cultures were then incubated at 37 °C with shaking and samples were collected 1.5 h after induction. Supernatant aliquots were collected and stored at -80 °C for LC/MS.

Validation

To detect and quantify the levels of production of the intermediate CMP (carboxymethyl-proline) and carbapenem, an LC-MS analytical method was developed, based on HILIC

chromatography. It is considered that as the end product carbapenem is chemically unstable, its hydrolysed form hCar is detected.

Metabolite concentrations in the culture supernatant were measured using liquid chromatography/mass spectrometry (LC/MS). Samples of the producing cultures were taken at different time points and immediately centrifuged at maximum speed for 3 minutes. For each sample, the supernatant was collected and stored at -20°C until measurement. Prior analysis, 5µL of supernatant was transferred into a clean Eppendorf containing 195 µL ACN with 0.1% formic acid. This is to ensure that the total amount of carbapenem present in samples would be hydrolysed and converted to hCar, and to ensure optimal conditions for the chromatography. For measurement of CMP and hCar, liquid chromatography separation was conducted at 30°C with an Agilent ZORBAX HILIC Plus column (100-mm length, 2.1-mm internal diameter, 3.5 µm particle size) using a LC-MS system (Agilent) consisting of a binary pump (G1312B), an autosampler (G7167A), a temperature-controlled column compartment (G1316A), and a triple quadrupole mass spectrometer (G6460C) equipped with a standard ESI source. For each measurement 2 µL of injection volume was used. The mobile phase was composed of 25 mM ammonium formate (solvent A) and 100% acetonitrile (solvent B). The metabolites were separated with a gradient from 95% to 60% of solvent B for 5 minutes at a flow rate of 0.5 ml/min, a subsequent gradient from 60% to 50% for 2 minutes at a flow rate from 0.5 mL/min to 0.6 mL/min was carried, 50% to 95% solvent B for 2 minutes at 0.7 mL/min, followed by a hold at 95% solvent B for 2 min at a flow of 0.5 mL/min.

Peaks were analysed by mass spectrometry using ESI ionization in MRM mode. The precursor ions analysed for each compound was determined by mass calculation based on the chemical formula. Biological controls were used to confirm that the peaks obtained (and the resulting product ions used for quantification and qualitative analysis) were exclusively present in the presence of the exogenous enzymes responsible for their synthesis and corresponding substrates.

Compound	Formula	Mass	Pre-cursor ion	Product ions	Dwell	Frag-mentor	Collision Energy (V)	Polarity	Retention Time
hCar	C ₇ H ₉ NO ₄	171.05	170	126 100	60	90	8 8	Negative	2.75 min
CMP	C ₇ H ₁₁ NO ₄	173.07	174.1	128 114	60	90	12 12	Positive	4.946 min

Table 1: analytical results.

The analytical results show that Car was produced in detectable amounts.

Circumventing antibiotic toxicity to improve Car production and Car pathway flux

Toxicity of Car limits achievable cell density of production cultures and severely limits antibiotic titres. Inventors improved tolerance to antibiotic products without compromising productivity. An approach to mitigate biomass limitation caused by Car toxicity was to induce expression of the Car pathway once a sufficient amount of biomass is produced, rather than at an early point during exponential phase. Induction of BL21 pProABmut_CBAE at a higher cell density (OD₆₀₀ 1 rather than OD₆₀₀ 0.35) increased both maximum biomass and hCar titre by nearly 2-fold. However, lysis was still observed in late-induced cultures as decreasing OD₆₀₀ from 3 to 24 h. A phenotype known as persistence, in which cells are temporarily immune to antibiotic exposure, is artificially induced by expression of growth-arresting toxin proteins. Overexpression of e.g. toxin HipA causes growth-arrest and confers β -lactam tolerance. HipA-arrested cultures survive β -lactam exposure while remaining metabolically active, and are able to sustain production of the isoprenoid precursor mevalonate from a heterologous pathway, while resisting phage-induced lysis.

β -lactam tolerance was, in an alternative approach, achieved by direct inhibition of fatty acid synthesis (FAS). Inhibition of FAS using mycotoxin cerulenin inhibits phospholipid synthesis and confers β -lactam tolerance. Cerulenin treatment was found to cause accumulation of malonyl-CoA, a substrate of CarB.

Inhibition of FAS by cerulenin thus benefits carbapenem

production in two ways: by decreasing lysis, and by increasing availability of a precursor metabolite. Treatment of Car-producing cultures with 20 µg/mL cerulenin decreased Car-induced lysis. Cerulenin was found to increase titres of CMP by nearly 5-fold. Although malonyl-CoA accumulation did not significantly improve hCar titres, FAS inhibition greatly improved flux into the Car pathway while alleviating Car-induced lysis. In an alternative to cerulenin a synthetic protein degradation system to target FAS enzymes which consume malonyl-CoA was used. pdt#3 was appended to chromosomally-encoded fatty acid synthesis enzymes which use malonyl-CoA as a substrate (FabB, FabF, and FabD), and induced expression of the *mf*-Lon protease. Similar to cerulenin treatment, induction of the *mf*-Lon protease caused both growth arrest and malonyl-CoA accumulation in BL21 FabD-pdt#3 and increased CMP production in BL21 FabD-pdt#3_pCarCBAE_ProABmut. Simultaneous induction of the Car pathway and FabD degradation reduced both cell lysis and membrane permeability. While hCar titers were not significantly increased by FabD degradation, malonyl-CoA accumulation substantially improved flux into the Car pathway, increasing titres of carbapenam 4 by 5-fold.

Supplementary Table 2. List of primers used

Primer name Full sequence

P1-I69E TGAGCACCTGGGTTACCCGGAAGTCCAGCGACTGAAGCCTCGAAACAAGTCTGGC

P2-I69E TTGTTTCGAGGCTTCAGTCGCTGGCAGTTCCGGGTAAACCCAGGTGCTCACG

P1-CarE CCGGTGTATCCAAGGTGCGCCTGACGTCCGGCAACGTCAACATGGATCATTCTGGTGGG

P2-CarE ACGTTGCCGGACGTCAGGCGCACCTTGGATACACCGGAGTAACCAGAAGCACAGCGATAA

P1-fabB GCGCGCACCAACGCCACGCTGGTAATGCGCAAGCTGAAAGATGCGGCGAACAAAAACGAA

P2-fabB GATGCGACGCTGGCGCGCCTTACCCGACCTACGGCGAATTATGTAGGCTGGAGCTGCTT

P1-fabD TGAACGAACCTTCAGCGATGGCAGCGGCGCTCGAGCTTGCGGCGAACAAAAACGAA

P2-fabD CAGTGCGATTTTTCTTCAAAATTCATGATTTTCCTCTTTTATGTAGGCTGGAGCTGCTT

P1-fabF GCTTCGGTGGCACTAATGGTCTTTTGATCTTTAAAAAGATCGCGCGAACAAAAACGAA

P2-fabF CGCAAGCGGACCTTTTATATGGGTGGGAAATGACAACTTATGTAGGCTGGAGCTGCTT

Supplementary Table 1. List of strains and plasmids used

Plasmid name	Description	Reference or source	Used in
<u>Plasmids</u>			
pCarAB	Codon optimized CarAB enzymes in pBbaA5k backbone	This work	F2, F3
pCarCBA	Codon optimized CarCBA enzymes in pBbaA5k backbone	This work	F2, F3, S3
pCarCBAE	Codon optimized CarCBAE enzymes in pBbaA5k backbone	This work	F2, F3, S3, S4
pCarCBAE_ProABmut	ProABmut and codon optimized CarCBAE enzymes (in that order) in pBbaA5k backbone	This work	F2, F3, F4, S4, S5, S7
pCarCBADE	Codon optimized CarCBADE enzymes in pBbaA5k backbone	This work	F2 a,b
pCarCBADE_ProABmut	ProABmut and codon optimized CarCBADE enzymes (in that order) in pBbaA5k backbone	This work	F2 a,b , S4
pCarCBADE_ProABwt	ProABwt and codon optimized CarCBADE enzymes (in that order) in pBbaA5k backbone	This work	F2 a,b , S4
pCarCBA_E	Codon optimized CarCBA enzymes under the control of p_{lacUV5} , and CarE under control of p_{tet}	This work	F2c
pCarE	Codon optimized CarE enzyme in pBbaA5k backbone	This work	S2
pCarE_mutant	Mutant variant of CarE (C43S/C46S) in pBbaA5k backbone	This work	S2
pCarCBAEmut	Codon optimized CarCBA enzymes and mutant variant of CarE (C43S/C46S) in pBbaA5k backbone	This work	S3
pHipA	HipA toxin in pBbS2c	This work	S5
pmf-Lon	Codon optimized mf-Lon protease in pBbA2c	This work	F4, S7
pmf-Lon-bis	Codon optimized mf-Lon protease in pBbA2a	This work	S6
pECL275	Source of codon optimized mf-Lon protease	Cameron, MIT	
pECT3	Source of pdt#3 tag	Cameron, MIT	
pCFR	Malonyl-CoA biosensor	V. Libis, Evry	S6
<u>Strains</u>			
<i>Escherichia coli</i> DH5 α	<i>E. coli</i> strain for cloning and plasmid amplification	Invitrogen	
<i>Escherichia coli</i> BL21(DE3)	<i>E. coli</i> strain for antibiotic production and HipA overexpression. Source of <i>proAB</i> .	Invitrogen	
<i>Escherichia coli</i> MG1655	Source of <i>hipA</i>	ATCC	
<i>Escherichia coli fabD</i> -pdt#3	<i>E. coli</i> strain BL21(DE3) containing FabD-pdt#3 fusion	This work	F4, S6
<i>Escherichia coli fabB</i> -pdt#3	<i>E. coli</i> strain BL21(DE3) containing FabB-pdt#3 fusion	This work	S6
<i>Escherichia coli fabF</i> -pdt#3	<i>E. coli</i> strain BL21(DE3) containing FabF-pdt#3 fusion	This work	S6

CLAIMS

1. Method for the production of an engineered microorganism, capable of (3S,5S)-carbapenam production, comprising the steps of

providing a microorganism, wherein the microorganism is selected from fungi and bacteria, wherein the microorganism is capable of producing 1-pyrroline-5-carboxylate (P5C) and comprises genes encoding a glutamate-5-semialdehyde dehydrogenase (*proA*) and glutamate 5-kinase (*proB*),

providing at least one nucleotide sequence comprising at least one of genes *carB*, *carA*, and *proB**, and optionally *carE* and/or *carC*, encoded thereon, wherein genes *carB*, *carA*, and *proB**, are provided at least once,

expressing the genes encoded on the at least one nucleotide sequence in the microorganism, and

culturing the microorganism in a medium.

2. Method according to claim 1, wherein the microorganism is preferably selected from Enterobacteriales, Actinobacteria, Bacillaceae, Streptomyces, Ascomycota and Basidiomycota.

3. Method according to claim 2, wherein the microorganism is selected from *Escherichia*, such as *Escherichia coli*, *Bacillus*, such as *Bacillus subtilis*, *Streptomyces cattleya*, *Streptomyces argenteolus*, *Streptomyces flavogriseus*, and Saccharomycetes, such as *S.cerevisiae*.

4. Method according to any of the preceding claims, wherein the at least one nucleotide sequence additionally comprises *proA*.

5. Method according to claim 4, wherein a glutamate kinase enzyme is co-expressed, and the glutamate kinase has been mutated to relieve feedback inhibition by proline.

6. Method according to any of the preceding claims, wherein the *proB** is feedback resistant ProB, such as at least one of *E. coli* ProB I69E, E143A, K145A, and N134D.

7. Method according to any of the preceding claims, wherein the genes *carB*, and *carA*, are arranged in at least one operon, preferably simultaneously expressing the genes.

8. Method according to claim 7, wherein the operon is a plasmid operon.

9. Method according to any of the preceding claims, wherein the

at least one nucleotide sequence further comprises at least one of genes *carD*, *carF*, *carG*, *carH*, and modified *FabD*.

10. Method according to claim 9, wherein at least one of *carE*, *carC*, *proA*, *proB**, and *FabD*, are arranged in the operon of claim 7 or claim 8.

11. Method according to any of the preceding claims, wherein the *proC* gene has been removed.

12. Method according to any of the preceding claims, wherein the at least one nucleotide sequence of any of the preceding claims is obtained from *P. carotovorum* or *Streptomyces cattleya*.

13. Microorganism obtainable by a method according to any of the preceding claims, wherein the microorganism is selected from Enterobacteriales, Actinobacteria, Bacillaceae, Streptomyces, Ascomycota and Basidiomycota.

14. Method of producing a carbapenem compound, comprising the step of providing the microorganism of claim 13, culturing the microorganism, and thereby producing at least one of (1-carbapen-2-em-3-carboxylic acid), (3S,5S) carbapenam, and carbapenem.

15. Method according to claim 14, wherein the carbapenem compound is a carbapenem antibiotic.

16. Method according to claim 15, wherein the carbapenem compound is selected from azabicyclo[3.2.0]hept-2-ene-2-carboxylic acids, such as 7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acids, such as thienamycin ((5R,6S)-3-[(2-Aminoethyl)thio]-6-[(1R)-1-hydroxyethyl]-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid), imipenem (5R,6S)-6-[(1R)-1-hydroxyethyl]-3-[(2-[(iminomethyl)amino]ethyl)thio]-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid, meropenem 4R,5S,6S)-3-(((3S,5S)-5-(Dimethylcarbamoyl)pyrrolidin-3-yl)thio)-6-((R)-1-hydroxyethyl)-4-methyl-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid, ertapenem (4R,5S,6S)-3-[(3S,5S)-5-[(3-carboxyphenyl)carbamoyl]pyrrolidin-3-yl]sulfanyl-6-(1-hydroxyethyl)-4-methyl-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid, doripenem (4R,5S,6S)-6-(1-Hydroxyethyl)-4-methyl-7-oxo-3-(((5S)-5-((sulfamoylamino)methyl)pyrrolidin-3-yl)thio)-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid, panipenem/betamipron (5R,6S)-3-[(3S)-1-ethanimidoylpyrrolidin-

3-yl)sulfanyl)- 6-[(1R)-1-hydroxyethyl]-7-oxo-1-
 azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid, biapenem
 (4R,5S,6S)-3-(6,7-dihydro-5H-pyrazolo[1,2-a][1,2,4]triazol-8-
 ium-6-ylsulfanyl)- 6-(1-hydroxyethyl)- 4-methyl-7-oxo-1-
 5 azabicyclo[3.2.0]hept-2-ene-2-carboxylate, razupenem
 (4R,5S,6S)-6-((R)-1-hydroxyethyl)-4-methyl-3-((4-((S)-5-methyl-
 2,5-dihydro-1H-pyrrol-3-yl)thiazol-2-yl)thio)-7-oxo-1-
 azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid, tebipenem
 (4R,5S,6S)-(Pivaloyloxy)methyl 3-((1-(4,5-dihydrothiazol-2-
 10 yl)azetidin-3-yl)thio)-6-((R)-1-hydroxyethyl)-4-methyl-7-oxo-1-
 azabicyclo[3.2.0]hept-2-ene-2-carboxylate, lenapenem, and
 tomopenem ((4R,5S,6S)-3-[(3S,5S)-5-[(3S)-3-[[2-
 (diaminomethylideneamino)acetyl]amino]pyrrolidine-1-carbonyl]-1-
 methylpyrrolidin-3-yl)sulfanyl-6-[(1R)-1-hydroxyethyl]-4-methyl-
 15 7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid), a
 derivative thereof, and an analogue thereof.

17. Method according to any of claims 14-16,
 wherein iron is withheld for a period of time, the period of
 time starting with withholding iron when growth of the culture
 20 is initiated by inoculation, generating cell biomass with $OD_{600} > 1$
 and then stopping with withholding iron, therewith preventing
 cell lysis, wherein the medium comprises 10^{-6} - 10^{-4} mole/l iron.

18. Method according to claim 17, wherein the medium comprises
 $5 \cdot 10^{-6}$ - $5 \cdot 10^{-5}$ mole iron/l, such as 10^{-5} - $3 \cdot 10^{-5}$ mole iron/l.

25 19. Method of producing a carbapenem compound according to any
 of claims 14-18, wherein the carbapenem compound is produced in
 at least one stage, wherein cell growth is in at least one
 antibiotic production stage inhibited thereby delaying
 expression of antibiotic synthesis to the next stage.

30 20. Method according to any of claims 14-19, wherein expression
 of at least one gene of *carB*, *carA*, *carC*, *carD*, *carE*, *carF*,
carG, *carH*, *proA*, *proB**, and *proB*, is delayed, such as by
 overexpressing *HipA*.

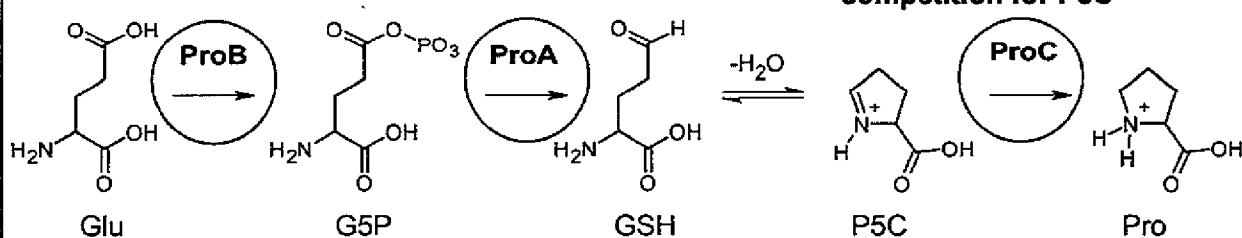
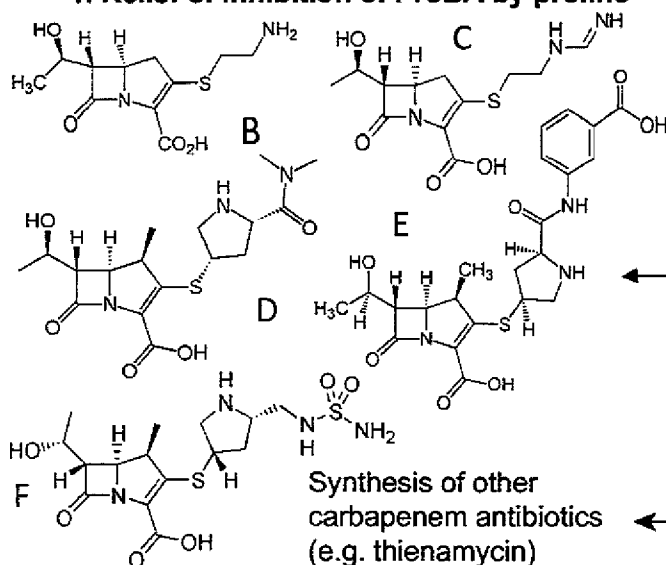
21. Method according to any of claims 14-20, comprising at least
 35 one further biological or chemical synthesis step of producing
 an antibiotic carbapenem compound, preferably only (a)
 biological synthesis step(s).

22. Method for improving antibiotic production with an
 engineered microorganism, such as the microorganism of claim 13,

wherein iron is withheld for a period of time, starting with withholding iron when growth of a culture is initiated by inoculation, generating sufficient cell biomass and then stopping with withholding iron, therewith preventing cell lysis, wherein the medium comprises 10^{-6} - 10^{-4} mole/l iron, and/or wherein the antibiotic is produced in at least one stage and wherein cell growth is in at least one antibiotic production stage inhibited thereby delaying expression of antibiotic synthesis to the next stage.

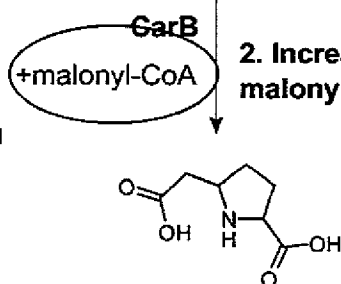
23. Method according to claim 22, wherein the medium comprises $5 \cdot 10^{-6}$ - $5 \cdot 10^{-5}$ mole iron/l, such as 10^{-5} - $3 \cdot 10^{-5}$ mole iron/l.
24. Method according to claim 22 or 23, wherein lysis is inhibited by fatty acid synthesis inhibition.

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Proline biosynthesis (native to *E. coli*)**1. Relief of inhibition of ProBA by proline**

Synthesis of other carbapenem antibiotics (e.g. thienamycin)

A

2. Increasing intracellular malonyl-CoA concentrations

Carboxymethyl proline

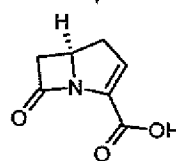
CarA

+ATP

(3S,5S)-carbapenam

Car biosynthesis**3. Regenerating CarC activity with CarE**

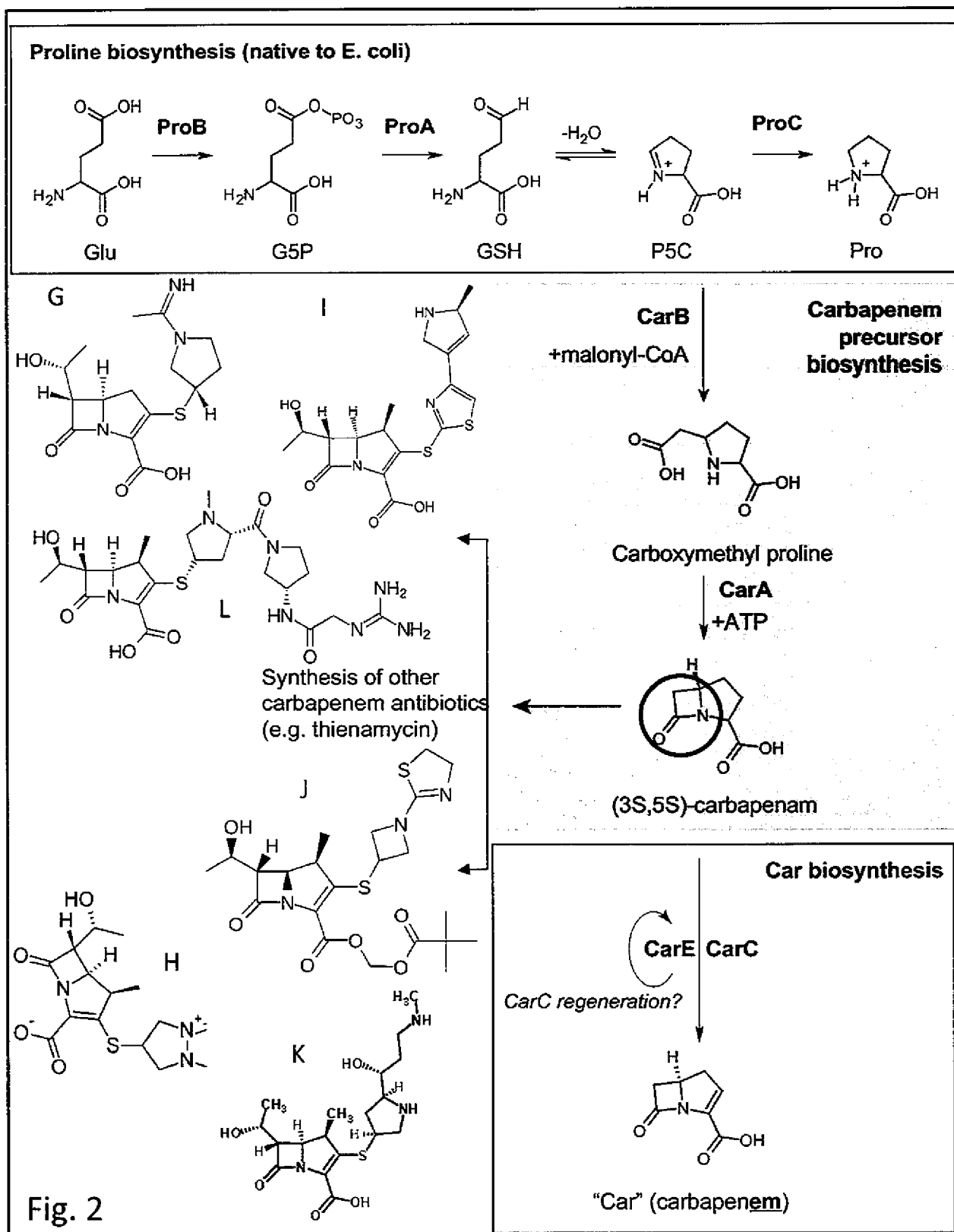
CarC regeneration?



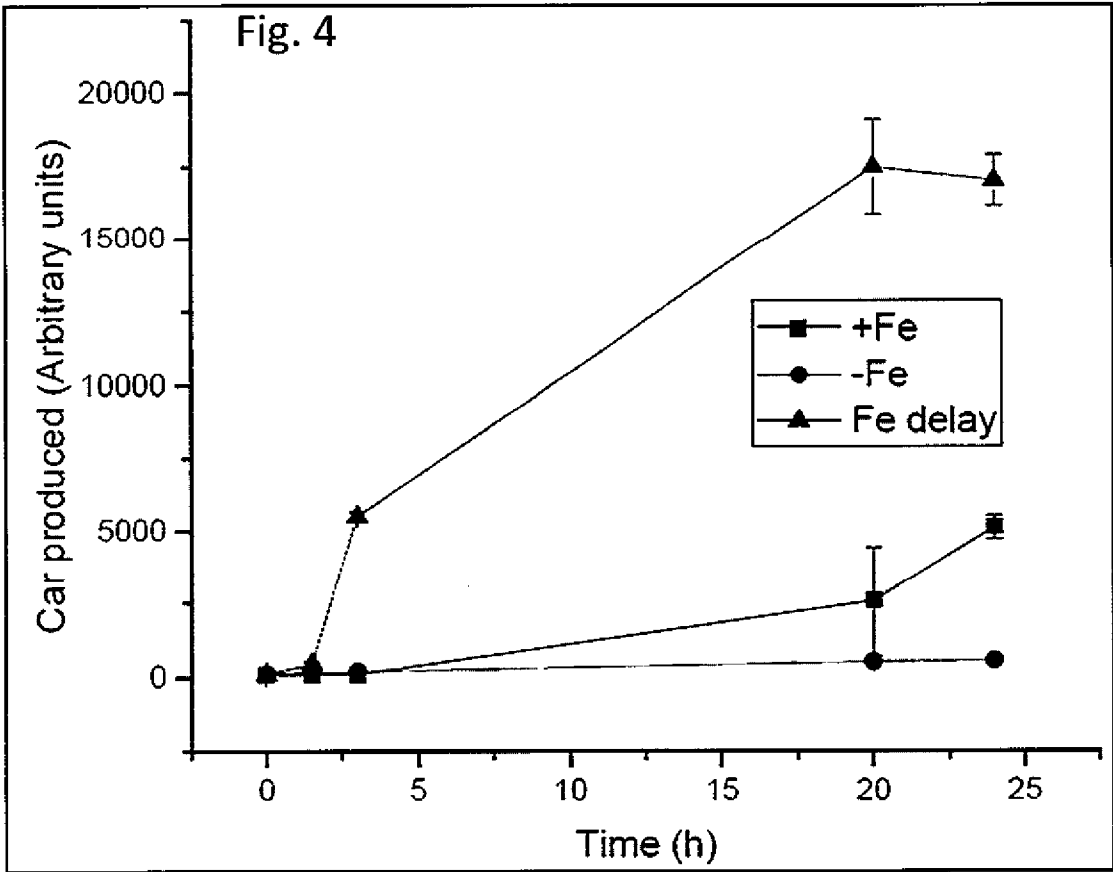
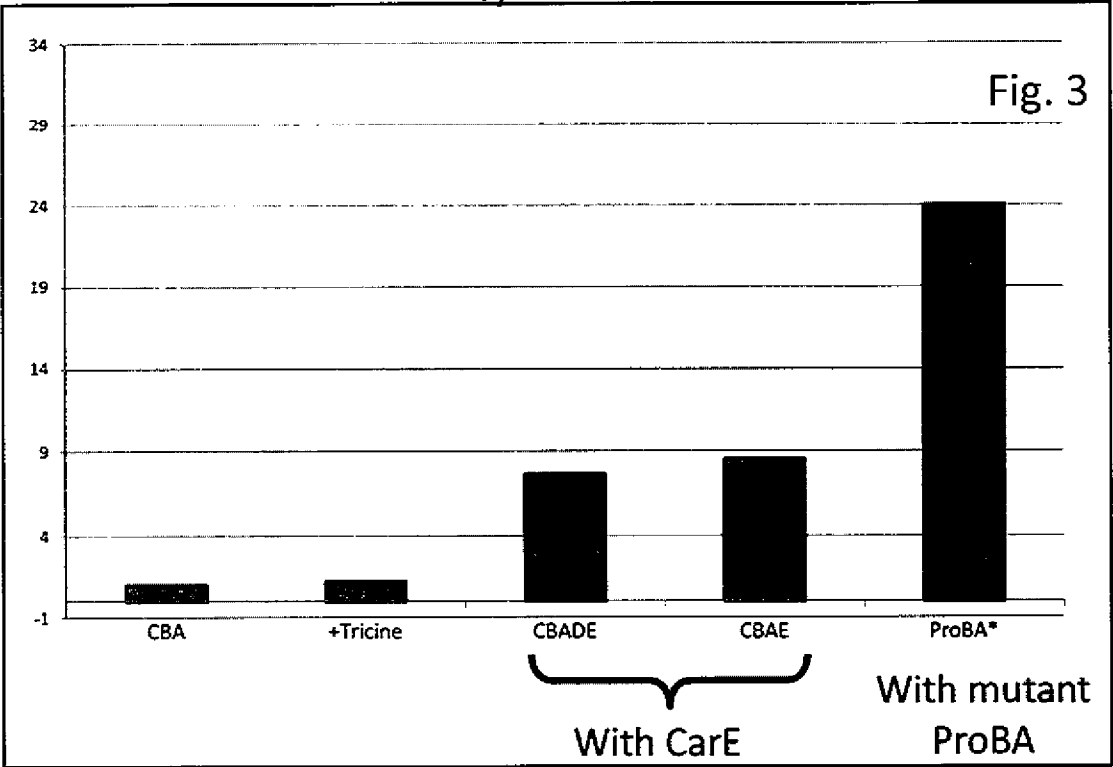
"Car" (carbapenem)

Fig. 1

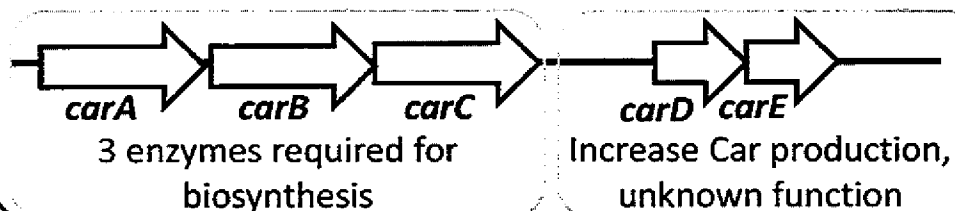
2/8



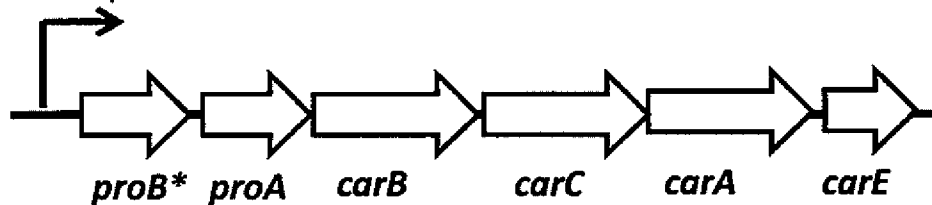
3/8



4/8

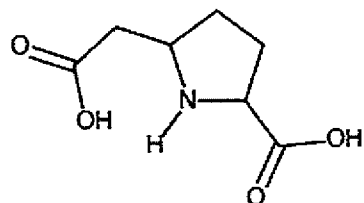
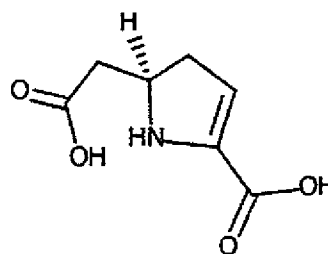
Figure 5a*Operon found in Pectobacterium carotovorum:***Figure 5b**

lacUV5 promoter



ProB* = mutant of *E. coli* glutamate kinase enzyme ProB in which feedback inhibition by proline is attenuated

ProA = *E. coli* glutamate semialdehyde dehydrogenase

**Fig. 6a****Fig. 6b**

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Fig. 7

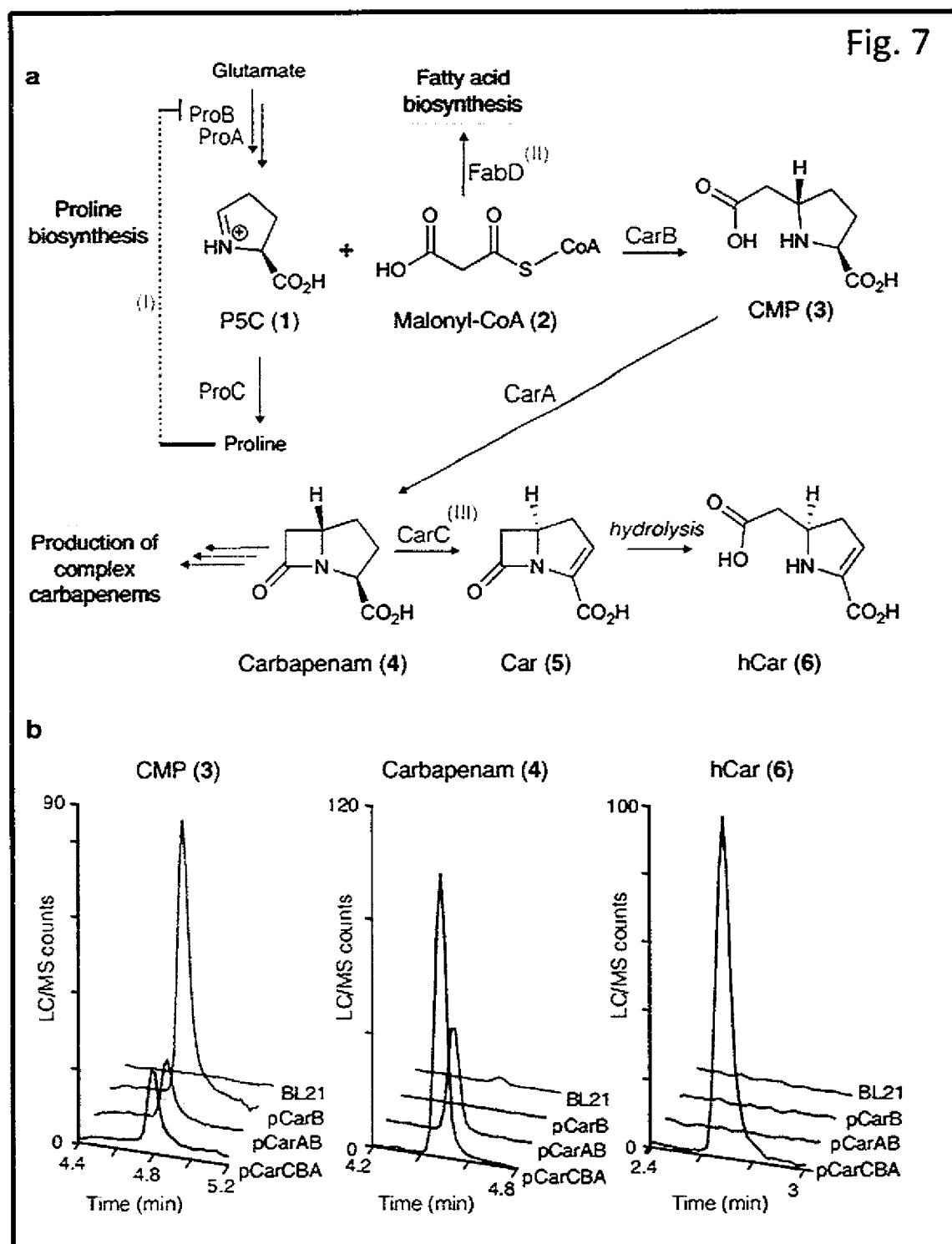


Fig. 8

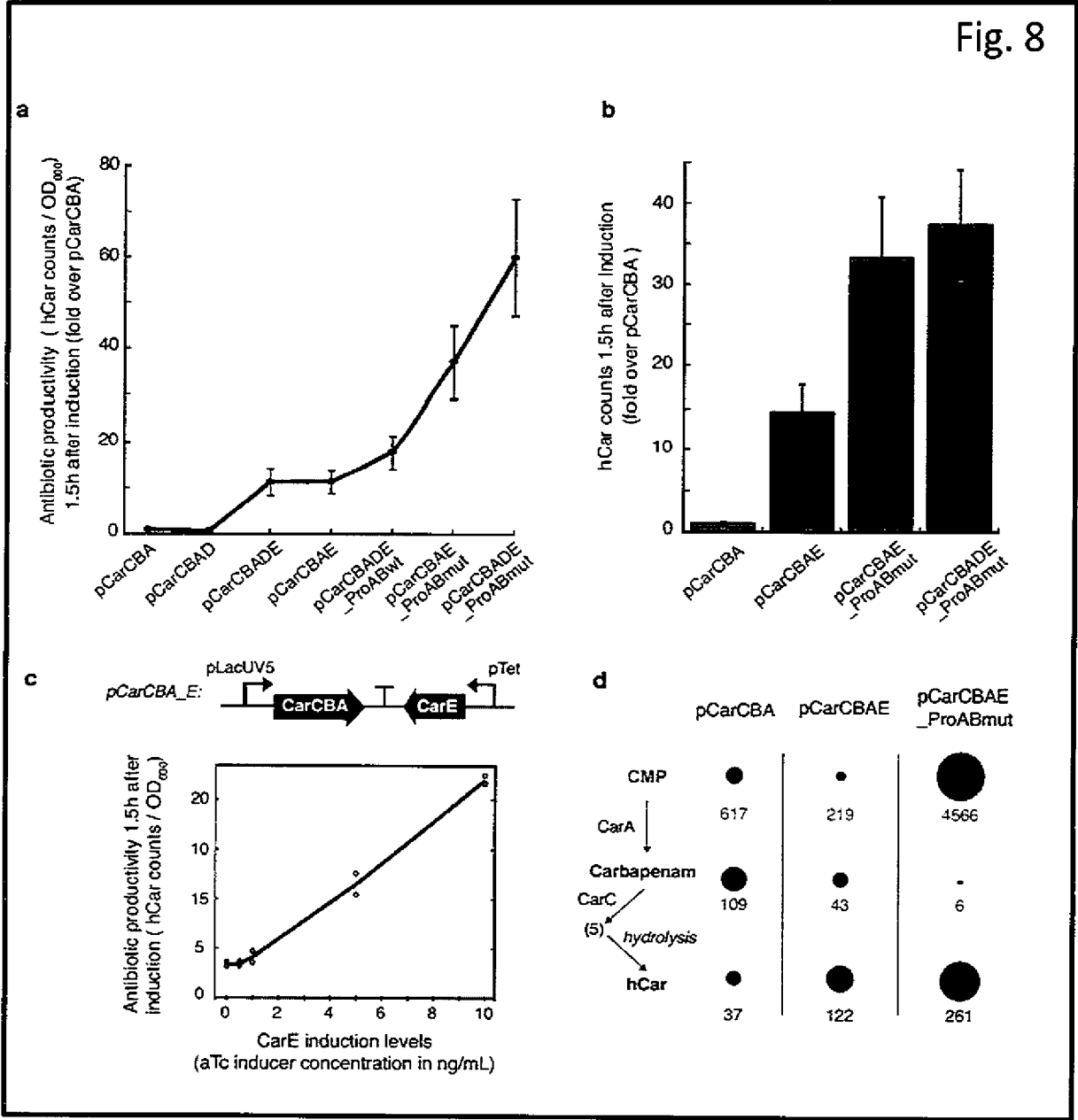


Fig. 9

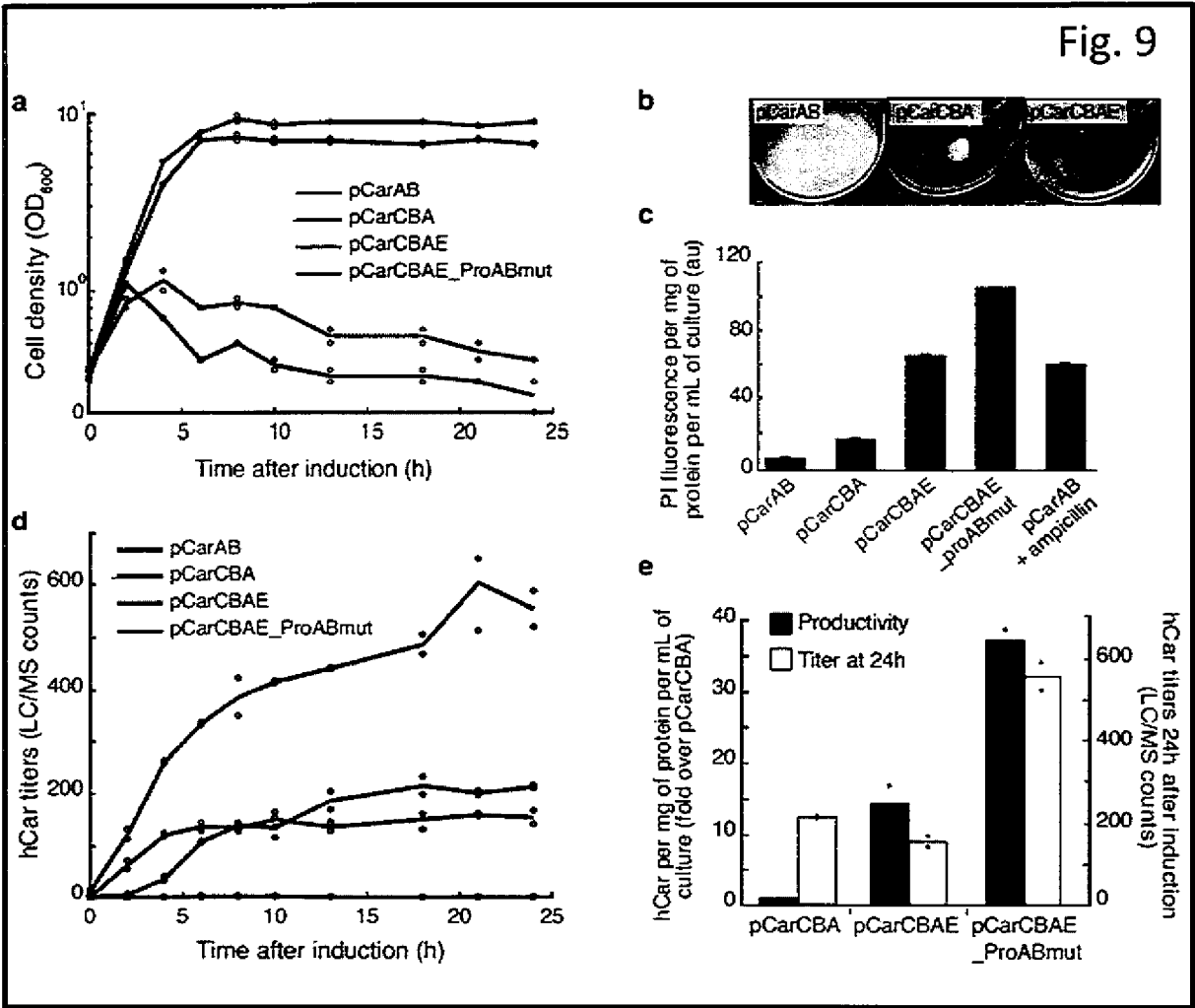
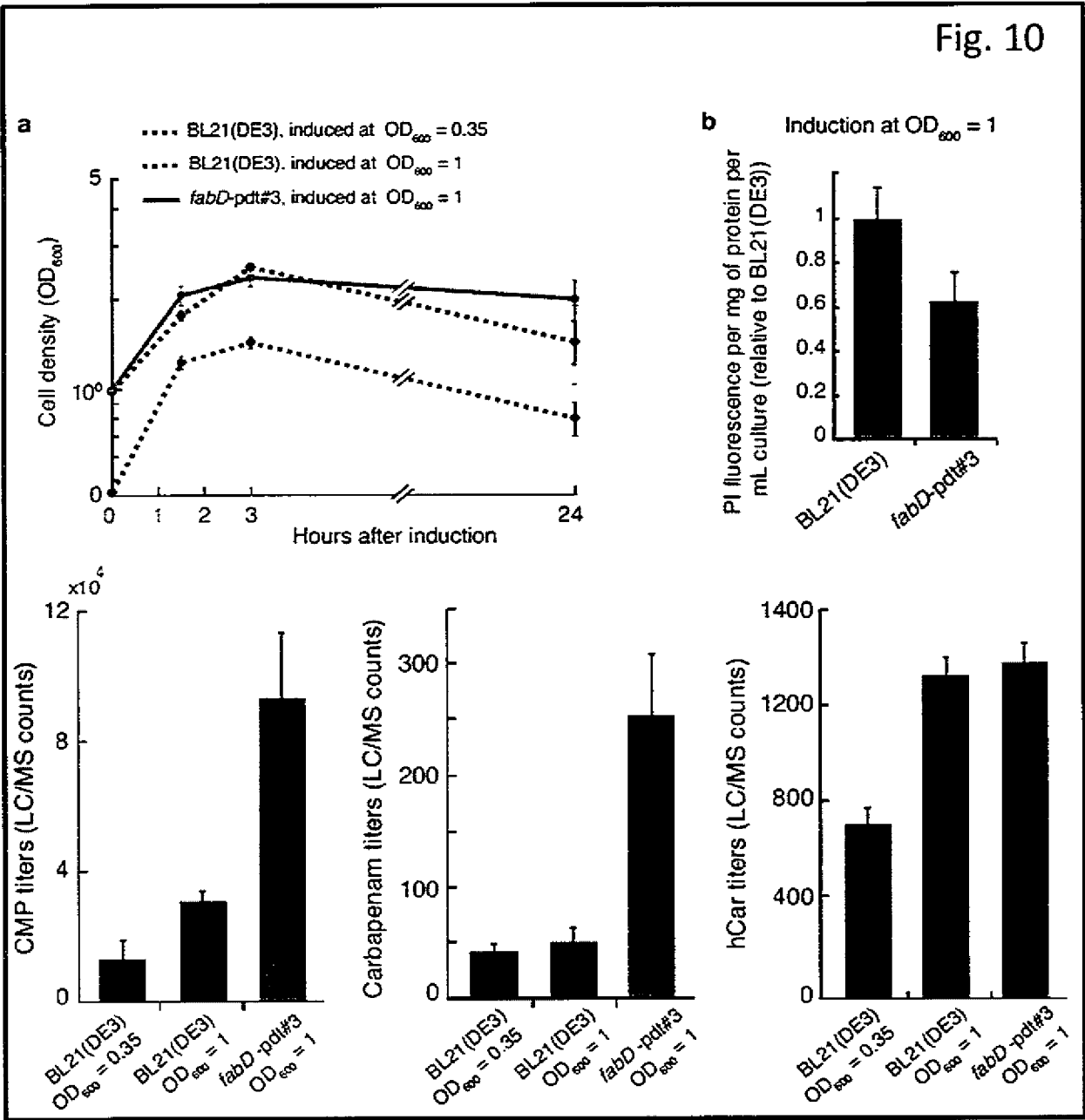


Fig. 10



INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2017/050564

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P17/18 C12N15/52
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12P C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, CHEM ABS Data, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	COULTHURST SARAH J ET AL: "Regulation and biosynthesis of carbapenem antibiotics in bacteria", NATURE REVIEWS. MICROBIO, NATURE PUBLISHING GROUP, GB, vol. 3, no. 4, April 2005 (2005-04), pages 295-306, XP008159336, ISSN: 1740-1526, DOI: 10.1038/NRMICRO1128 [retrieved on 2005-03-10] cited in the application page 205 - page 207; figures 4,5 ----- -/--	1-24



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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"E" earlier application or patent but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

6 December 2017

Date of mailing of the international search report

13/12/2017

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Authorized officer

Boeker, Ruth

INTERNATIONAL SEARCH REPORT

International application No

PCT/NL2017/050564

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	S. J. MCGOWAN ET AL: "Analysis of bacterial carbapenem antibiotic production genes reveals a novel beta-lactam biosynthesis pathway", MOLECULAR MICROBIOLOGY, vol. 22, no. 3, November 1996 (1996-11), pages 415-426, XP055049792, DOI: 10.1046/j.1365-2958.1996.00125.x cited in the application the whole document	1-24
A	LUZ ELENA NÚÑEZ ET AL: "The Biosynthetic Gene Cluster for the [beta]-Lactam Carbapenem Thienamycin in Streptomyces cattleya", CHEMISTRY & BIOLOGY, vol. 10, no. 4, April 2003 (2003-04), pages 301-311, XP055215330, ISSN: 1074-5521, DOI: 10.1016/S1074-5521(03)00069-3 cited in the application the whole document	1-24
Y	MICAH J. BODNER ET AL: "Definition of the Common and Divergent Steps in Carbapenem [beta]-Lactam Antibiotic Biosynthesis", CHEMBIOCHEM, vol. 12, no. 14, 19 September 2011 (2011-09-19), pages 2159-2165, XP055049873, ISSN: 1439-4227, DOI: 10.1002/cbic.201100366 cited in the application the whole document	22-24
A		1-21
A	SIMON J MCGOWAN ET AL: "Bacterial production of carbapenems and clavams: evolution of b-lactam antibiotic pathways", TRENDS IN MICROBIOLOGY, vol. 6, no. 5, May 1998 (1998-05), pages 203-208, XP055378971, cited in the application the whole document	1-24
Y	US 2015/353939 A1 (BOKINSKY GREGORY E [NL] ET AL) 10 December 2015 (2015-12-10) cited in the application	22-24
A	paragraphs [0180] - [0189]; claims; figures; example 2	1-21
	-/--	

INTERNATIONAL SEARCH REPORT

International application No

PCT/NL2017/050564

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 95/32294 A1 (UNIV WARWICK [GB]; UNIV NOTTINGHAM [GB]; SALMOND GEORGE PEACOCK COPLAN) 30 November 1995 (1995-11-30) page 4, paragraph 2 - page 5, paragraph 3; claims page 11, paragraph 4 -----	1-24

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/NL2017/050564

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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