



## Genetically modified microorganisms having improved tolerance towards l-serine.

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(54) **Title:** GENETICALLY MODIFIED MICROORGANISMS HAVING IMPROVED TOLERANCE TOWARDS L-SERINE

(57) **Abstract:** The present invention generally relates to the microbiological industry, and specifically to the production of L-serine or L-serine derivatives using genetically modified bacteria. The present invention provides genetically modified microorganisms, such as bacteria, wherein the expression of genes encoding for enzymes involved in the degradation of L-serine is attenuated, such as by inactivation, which makes them particularly suitable for the production of L-serine at higher yield. The present invention also provides means by which the microorganism, and more particularly a bacterium, can be made tolerant towards higher concentrations of serine. The present invention also provides methods for the production of L-serine or L-serine derivative using such genetically modified microorganisms.

## Genetically modified microorganisms having improved tolerance towards L-serine

### Technical field of the invention

5 The present invention generally relates to the microbiological industry, and specifically to the production of L-serine using genetically modified bacteria. The present invention provides genetically modified microorganisms, such as bacteria, wherein the expression of genes encoding for enzymes involved in the degradation of L-serine is attenuated, such as by inactivation, which makes them particularly suitable for the production of L-serine at higher yield. The present  
10 invention also provides means by which the microorganism, and more particularly a bacterium, can be made tolerant towards higher concentrations of serine. The present invention also provides methods for the production of L-serine or L-serine derivative using such genetically modified microorganisms.

### Background of the invention

15 L-serine is an amino acid that currently is used in the cosmetics, pharmaceutical and medical industry. The estimated annual production of serine is between 300-1000 tons (Leuchtenberger et al., 2005). The compound has also been identified as one of the top 30 most interesting biochemicals because of its potential use as a building block biochemical.

Serine is a key amino acid which is used as a precursor in the cell to produce many other amino  
20 acids such as glycine, cysteine, methionine and tryptophan (Sawers 1998). Apart from these amino acids serine has been also used to produce intermediates such as O-Acetyl serine (Maier 2003) or heterologous compounds such as ethylene glycol (Pereira et al. 2016). Ethanolamine can be produced from L-serine by expressing a serine decarboxylase as described previously (Pereira et al 2016). Ethylene glycol can furthermore be produced from ethanolamine by deamination  
25 (Pereira et al 2016). Glycine is derived from L-serine intracellularly. High flux of glycine is desirable for production a range of compounds such as thimine (Iwashima et al 1971).

The current production is based on conversion of glycine and methanol using resting cells (Hagishita et al., 1996), where methylotrophs convert methanol to formaldehyde and transfer the CH<sub>2</sub>-OH unit of the molecule to glycine using serine hydroxymethyltransferase (*glyA*). This

fermentation process is time consuming and glycine is an expensive starting material. Developing a method for producing serine at low cost directly from glucose is therefore attractive.

Serine has the potential to be made from glucose by fermentation with a very high theoretical yield (Burgard and Maranas, 2001). However, several challenges need to be addressed in order to  
5 increase the yield, the most crucial one being degradation of serine in the production organism. Serine has two key degradation pathways in *E. coli*. Serine to pyruvate catabolism is in *E. coli* catalyzed by three deaminases namely *sdaA*, *sdaB* and *tdcG*, while *C. glutamicum* only has one deaminase (*sdaA*) with activity towards serine. In both organisms, the conversion of serine to glycine is encoded by *glyA*. Serine production by knocking out only deaminases has been  
10 attempted in *E. coli* (Li et al., 2012) and *C. glutamicum* (Peters-Wendisch et al., 2005). In *E. coli* transient accumulation of 3.8mg/L from 1 g/L glucose was observed when only one of the pathway gene (*serA*) was overexpressed. Deletion of the deaminase on *C. glutamicum* lead to marginal and transient increase in the serine titer. In recent studies, *E. coli* was engineered to enhance the flux of 3-phosphoglycerate by perturbing the TCA-cycle and glyoxylate shunt (Gu et al., 2014). The resulting strain, where only one deaminase, was removed (*sdaA*) was reported to  
15 produce 8.45 g/L serine from 75 g/L glucose (11.2 % yield).

Down regulation of *glyA* (Peters-Wendisch et al., 2005) in *C. glutamicum* resulted in the production of 9 g/L serine from 40 g/L glucose but lead to an unstable strain. *glyA* is an important enzyme that converts serine to glycine and in this step transfers one carbon unit to tetra  
20 hydrofolate (THF), which is used as cofactor. Removal of the folic acid pathway and supplementation of folic acid lead to a stable *C. glutamicum*, and a production of 36 g/L serine, however with a relatively low yield (Stolz et al, 2007).

Deletion of both of the major serine degradation pathways (serine to pyruvate and serine to glycine) has not been previously been achieved. It is furthermore known that serine becomes  
25 toxic even at low concentrations in strains that lack the pyruvate degradation pathway (Zhang and Newman, 2008). It is expected that serine may inhibit the production of branched amino acids in *E. coli* (Hama et al., 1990), and the conversion of serine to hydroxypyruvate and acrylates, which is toxic to the cell (de Lorenzo, 2014). For efficient production of L-serine or a derivative thereof, it is therefore necessary to both remove the serine degradation pathways and address the problems  
30 associated with toxicity of serine.

### **Summary of the invention**

The object of the present invention is to provide means allowing a more efficient production of L-serine. More particularly, it is an object of the present invention to provide means allowing the production of L-serine at higher nominal yield and improved mass yield.

- 5 This is achieved by the finding that the production of L-serine can be enhanced by, e.g., inactivation of genes encoding enzymes involved in the degradation of L-serine, notably the genes *sdaA*, *sdaB*, *tdcG* and *glyA*.

The present invention thus provides in a first aspect a bacterium, especially a bacterium having an ability to produce L-serine, wherein said bacterium has been modified to attenuate expression of  
10 at least one gene coding for a polypeptide having serine deaminase activity and/or to attenuate expression of a gene coding for a polypeptide having serine hydroxymethyltransferase activity.

More particularly, the present invention provides a bacterium which has been modified to attenuate the expression of the genes *sdaA*, *sdaB*, *tdcG* and/or *glyA*, e.g., by inactivation of these genes.

- 15 The present invention provides in a second aspect a method for producing L-serine comprising: cultivating the bacterium as described above in a medium.

The present invention provides in a further aspect a (isolated) nucleic acid molecule, such a vector, comprising a nucleotide sequence encoding a polypeptide having an amino acid sequence which has at least about 90%, such as at least about 93%, at least about 95%, at least about 96%,  
20 at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position Y356, S357 and/or S359.

The present invention provides in a further aspect a (isolated) polypeptide having an amino acid sequence which has at least about 90%, such as at least about 93%, at least about 95%, at least  
25 about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position Y356, S357 and/or S359. The (isolated) polypeptide may be one expressed by (and isolated from) a bacterium of the invention.

The present invention provides in a further aspect a bacterium which expresses an aspartate kinase I / homoserine dehydrogenase I (ThrA) mutant having one or more amino acid substitutions which increase tolerance towards L-serine.

The present invention may be summarized by the following items:

- 5 1. A bacterium which has been modified to attenuate expression of at least one gene coding for a polypeptide having serine deaminase activity and/or to attenuate expression of a gene coding for a polypeptide having serine hydroxymethyltransferase activity.
2. The bacterium according to item 1, wherein the bacterium has been modified to attenuate the expression of at least one gene coding for a polypeptide having serine deaminase activity.
- 10 3. The bacterium according to item 1 or 2, wherein the bacterium has been modified to attenuate expression of at least two genes coding for polypeptides having serine deaminase activity.
4. The bacterium according to any one of items 1 to 3, wherein the bacterium has been modified to attenuate expression of at least three genes coding for polypeptides having serine deaminase activity.
- 15 5. The bacterium according to any one of items 1 to 4, wherein the at least one gene coding for a polypeptide having serine deaminase activity is selected from the group consisting of *sdaA*, *sdaB* and *tdcG*.
6. The bacterium according to any one of items 1 to 4, wherein the bacterium has been modified to attenuate expression of at least the gene *sdaA*.
- 20 7. The bacterium according to any one of items 1 to 6, wherein the bacterium has been modified to attenuate expression of at least the gene *sdaB*.
8. The bacterium according to any one of items 1 to 7, wherein the bacterium has been modified to attenuate expression of at least the gene *tdcG*.
- 25 9. The bacterium according to any one of claims 1 to 8, wherein the bacterium has been modified to attenuate the expression of a gene coding for a polypeptide having serine hydroxymethyltransferase activity.

10. The bacterium according to item 9, wherein the gene coding for a polypeptide having serine hydroxymethyltransferase activity is *glyA*.
11. The bacterium according to any one of items 1 to 10, wherein the bacterium has been modified to attenuate expression of the genes *sdaA*, *sdaB*, *tdcG* and *glyA*.
- 5 12. The bacterium according to any one of items 1 to 10, wherein the bacterium has been modified to attenuate expression of at most three of the genes *sdaA*, *sdaB*, *tdcG* and *glyA*.
13. The bacterium according to any one of items 1 to 12, wherein the expression of the gene or genes is attenuated by inactivation of the gene or genes.
14. The bacterium according to any one of items 1 to 13, wherein at least one gene coding for a  
10 polypeptide having serine deaminase activity is inactivated.
15. The bacterium according to any one of items 1 to 14, wherein at least two genes coding for polypeptides having serine deaminase activity are inactivated.
16. The bacterium according to any one of items 1 to 15, wherein at least three genes coding for polypeptides having serine deaminase activity are inactivated.
- 15 17. The bacterium according to any one of items 1 to 16, wherein at least one gene selected from the group consisting of *sdaA*, *sdaB* and *tdcG* is inactivated.
18. The bacterium according to any one of items 1 to 17, wherein the gene *sdaA* is inactivated.
19. The bacterium according to any one of items 1 to 18, wherein the gene *sdaB* is inactivated.
20. The bacterium according to any one of items 1 to 19, wherein the gene *tdcG* is inactivated.
- 20 21. The bacterium according to any one of claims 1 to 20, wherein a gene coding for a polypeptide having serine hydroxymethyltransferase activity is inactivated.
22. The bacterium according to item 9, wherein the gene coding for a polypeptide having serine hydroxymethyltransferase activity is *glyA*.
23. The bacterium according to any one of items 1 to 22, wherein the genes *sdaA*, *sdaB*, *tdcG*  
25 and *glyA* are inactivated.

24. The bacterium according to any one of items 1 to 22, wherein at most three of the genes *sdaA*, *sdaB*, *tdcG* and *glyA* are inactivated.
25. The bacterium according to any one of items 1 to 24, wherein said bacterium has been further modified to overexpress a 3-phosphoglycerate dehydrogenase, a phosphoserine  
5 phosphatase and a phosphoserine aminotransferase.
26. The bacterium according to any one of claims 1 to 25, wherein said bacterium comprises an exogenous nucleic acid molecule comprising a nucleotide sequence encoding a 3-phosphoglycerate dehydrogenase.
27. The bacterium according to any one of items 1 to 26, wherein said bacterium comprises an  
10 exogenous nucleic acid molecule comprising a nucleotide sequence encoding a 3-phosphoserine aminotransferase.
28. The bacterium according to any one of items 1 to 27, wherein said bacterium comprises an exogenous nucleic acid molecule comprising a nucleotide sequence encoding a phosphoserine phosphatase.
- 15 29. The bacterium according to any one of items 26 to 28, wherein the exogenous nucleic acid molecule(s) is an expression vector.
30. The bacterium according to any one of items 26 to 28, wherein the exogenous nucleic acid is stably integrated into the genome of the bacterium.
31. The bacterium according to any one of items 1 to 30, wherein said bacterium is capable of  
20 growing in a minimal culture medium comprising L-serine at a concentration of at least about 6.25 g/L.
32. The bacterium according to any one of items 1 to 31, wherein said bacterium is capable of growing in a minimal culture medium comprising L-serine at a concentration of at least about 6.25 g/L at a growth rate of at least 0.1 hr<sup>-1</sup> during exponential growth.
- 25 33. The bacterium according to any one of items 1 to 32, wherein said bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in one or more amino acid substitutions which increase tolerance towards L-serine.



34. The bacterium according to any one of items 1 to 33, wherein said bacterium expresses a aspartate kinase I / homoserine dehydrogenase I (ThrA) mutant having one or more amino acid substitutions which increase tolerance towards L-serine.

5 35. The bacterium according to any one of items 1 to 34, wherein said bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in one or more amino acid substitutions in the encoded polypeptide at a position selected from the group consisting of Y356, S357 and S359.

10 36. The bacterium according to any one of items 1 to 35, wherein said bacterium expresses a aspartate kinase I / homoserine dehydrogenase I (ThrA) mutant having one or more amino acid substitutions at a position selected from the group consisting of Y356, S357 and S359.

15 37. The bacterium according to any one of items 1 to 36, wherein said bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position Y356, one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position S357 and/or one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position S359; wherein the substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D, Y356E, Y356F, Y356A, Y356I, Y356P, Y356H, Y356R and Y356L; the substitution at position S357 is selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H, S357K, S357I and S357M; and the substitution at position S359 is selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L.

25 38. The bacterium according to any one of items 1 to 37, wherein said bacterium expresses a aspartate kinase I / homoserine dehydrogenase I (ThrA) mutant having an amino acid substitution at position Y356, wherein the substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D, Y356E, Y356F, Y356A, Y356I, Y356P, Y356H, Y356R and Y356L.

30 39. The bacterium according to any one of items 1 to 38, wherein said bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position S357, wherein the substitution at position S357 is selected

from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H, S357K, S357I and S357M.

40. The bacterium according to any one of items 1 to 29, wherein said bacterium expresses a aspartate kinase I / homoserine dehydrogenase I (ThrA) mutant having an amino acid substitution  
5 at position S357, wherein the substitution at position S357 is selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H, S357K, S357I and S357M.

41. The bacterium according to any one of items 1 to 40, wherein said bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in an amino acid substitution  
10 in the encoded polypeptide at position S359, wherein the substitution at position S359 is selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L.

42. The bacterium according to any one of items 1 to 42, wherein said bacterium expresses a aspartate kinase I / homoserine dehydrogenase I (ThrA) mutant having an amino acid substitution  
15 at position S359, wherein the substitution at position S359 is selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L.

43. The bacterium according to any one of items 1 to 42, wherein said bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in one or more amino acid  
20 substitutions selected from the group consisting of Y356C, S357R and S359R.

44. The bacterium according to any one of items 1 to 43, wherein said bacterium expresses a aspartate kinase I / homoserine dehydrogenase I (ThrA) mutant having one or more amino acid substitutions selected from the group consisting of Y356C, S357R and S359R.

45. The bacterium according to any one of items 1 to 44, wherein said bacterium has been  
25 further modified to overexpress the gene *ydeD*.

46. The bacterium according to any one of items 1 to 45, wherein said bacterium comprises an exogenous nucleic acid molecule comprising a nucleotide sequence encoding the protein product of the gene *ydeD*.

47. The bacterium according to item 46, wherein the exogenous nucleic acid molecule is an expression vector.
48. The bacterium according to item 46, wherein the exogenous nucleic acid is stably integrated into the genome of the bacterium.
- 5 49. The bacterium according to any one of items 1 to 48, wherein said bacterium comprises within the *lrp* gene one or more nucleotide substitutions resulting in an amino acid substitution, such as non-conservative amino acid substitution, at position D143, such as the amino acid substitution D143G, in the encoded polypeptide.
- 10 50. The bacterium according to any one of items 1 to 49, wherein said bacterium comprises within the *rho* gene one or more nucleotide substitutions resulting in an amino acid substitution, such as non-conservative amino acid substitution, at position R87, such as the amino acid substitution R87L, in the encoded polypeptide.
- 15 51. The bacterium according to any one of items 1 to 50, wherein said bacterium comprises within the *eno* gene one or more nucleotide substitutions resulting in an amino acid substitution, such as non-conservative amino acid substitution, at position V164, such as the amino acid substitution V164L, in the encoded polypeptide.
- 20 52. The bacterium according to any one of items 1 to 51 wherein said bacterium comprises within the *argP* gene one or more nucleotide substitutions resulting in an amino acid substitution, such as non-conservative amino acid substitution, at position Q132, such as the amino acid substitution Q132K, in the encoded polypeptide.
53. The bacterium according to any one of items 1 to 52, wherein said bacterium comprises within the *tufA* gene one or more nucleotide substitutions resulting in an amino acid substitution, such as non-conservative amino acid substitution, at position G19, such as the amino acid substitution G19V, in the encoded polypeptide.
- 25 54. The bacterium according to any one of items 1 to 53, wherein said bacterium comprises within the *cycA* gene one or more nucleotide substitutions resulting in an amino acid substitution, such as non-conservative amino acid substitution, at position I220, such as the amino acid substitution I220V, in the encoded polypeptide.

55. The bacterium according to any one of items 1 to 54, wherein said bacterium comprises within the *rpe* gene one or more nucleotide substitutions resulting in an amino acid substitution, such as non-conservative amino acid substitution, at position I202, such as the amino acid substitution I202T, in the encoded polypeptide.
- 5 56. The bacterium according to any one of items 1 to 55, wherein said bacterium comprises within the *yojI* gene one or more nucleotide substitutions resulting in an amino acid substitution, such as non-conservative amino acid substitution, at position D334, such as the amino acid substitution D334H, in the encoded polypeptide.
57. The bacterium according to any one of items 1 to 56, wherein said bacterium comprises  
10 within the *hyaF* gene one or more nucleotide substitutions resulting in an amino acid substitution, such as non-conservative amino acid substitution, at position V120, such as the amino acid substitution V120G, in the encoded polypeptide.
58. The bacterium according to any one of items 1 to 57, wherein said bacterium has been further modified to attenuate expression of the gene *pykF* (e.g., by inactivation of the gene).
- 15 59. The bacterium according to any one of items 1 to 58, wherein the gene *pykF* is inactivated.
60. The bacterium according to any one of items 1 to 59, wherein said bacterium has been further modified to attenuate expression of the gene *malt* (e.g., by inactivation of the gene).
61. The bacterium according to any one of items 1 to 60, wherein the gene *malt* is inactivated.
62. The bacterium according to any one of items 1 to 61, wherein said bacterium comprises  
20 within the *rpoB* gene one or more nucleotide substitutions resulting in an amino acid substitution, such as non-conservative amino acid substitution, at position P520, such as the amino acid substitution P520L, in the encoded polypeptide.
63. The bacterium according to any one of items 1 to 62, wherein said bacterium comprises  
25 within the *fumB* gene one or more nucleotide substitutions resulting in an amino acid substitution, such as non-conservative amino acid substitution, at position T218, such as the amino acid substitution T218P, in the encoded polypeptide.
64. The bacterium according to any one of items 1 to 63, wherein said bacterium comprises within the *gshA* gene one or more nucleotide substitutions resulting in an amino acid substitution,

such as non-conservative amino acid substitution, at position A178, such as the amino acid substitution A178V, in the encoded polypeptide.

65. The bacterium according to any one of items 1 to 64, wherein said bacterium has been further modified to attenuate expression of the gene *lamB* (e.g., by inactivation of the gene).
- 5 66. The bacterium according to any one of items 1 to 65, wherein the gene *lamB* is inactivated.
67. The bacterium according to any one of items 1 to 66, wherein said bacterium comprises within its genome a deletion of about 2854 bp from a location which corresponds to location 850092 in the *E. coli* K12 MG1655 reference genome deposited under NCBI accession number NC\_000913.2.
- 10 68. The bacterium according to any one of items 1 to 67, wherein the bacterium comprises within its genome an insertion of an 768 bp long insertion sequence element IS1 in the lagging strand at a location which corresponds to location 3966174 in the *E. coli* K12 MG1655 reference genome deposited under NCBI accession number NC\_000913.2.
69. The bacterium according to any one of items 1 to 68, wherein the bacterium comprises within  
15 its genome an insertion of 1bp at a location which corresponds to location 2942629 in the *E. coli* K12 MG1655 reference genome deposited under NCBI accession number NC\_000913.2.
70. The bacterium according to any one of items 1 to 69, wherein the bacterium comprises within its genome an insertion of a 1342 bp long insertion sequence element IS4 at a location which corresponds to location 2942878 in the *E. coli* K12 MG1655 reference genome deposited under  
20 NCBI accession number NC\_000913.2.
71. The bacterium according to any one of items 1 to 70, the bacterium comprises within its genome an insertion of 1bp at a location which corresponds to location 2599854 in the *E. coli* K12 MG1655 reference genome deposited under NCBI accession number NC\_000913.2.
72. The bacterium according to any one of items 1 to 71, wherein the bacterium comprises  
25 within its genome an insertion of an 768 bp long insertion sequence element IS1 in the lagging strand at a location which corresponds to location 2492323 in the *E. coli* K12 MG1655 reference genome deposited under NCBI accession number NC\_000913.2.

73. The bacterium according to any one of items 1 to 72, wherein the bacterium comprises within its genome an insertion of an 1195 bp long insertion sequence element IS5 at a location which corresponds to location 121518 in the *E. coli* K12 MG1655 reference genome deposited under NCBI accession number NC\_000913.2.
- 5 74. The bacterium according to any one of items 1 to 73, wherein the bacterium comprises within its genome an insertion of an 768 bp long insertion sequence element IS1 in the lagging strand at a location which corresponds to location 1673670 in the *E. coli* K12 MG1655 reference genome deposited under NCBI accession number NC\_000913.2.
75. The bacterium according to any one of items 1 to 74, wherein said bacterium has been  
10 modified to attenuate expression of a gene coding for a polypeptide having Glucose 6-phosphate-1-dehydrogenase (G6PDH) activity.
76. The bacterium according to item 75, wherein the expression of the *zwf* gene is attenuated.
77. The bacterium according to item 75 or 76, wherein the expression of the gene is attenuated by inactivation of the gene.
- 15 78. The bacterium according to any one of items 75 to 77, wherein the gene *zwf* is inactivated.
79. The bacterium according to any one of items 1 to 78, wherein said bacterium expresses a polypeptide encoded by the *brnQ* gene, wherein said polypeptide terminates after position 308 or any position upstream thereof.
80. The bacterium according to any one of items 1 to 79, wherein said bacterium has been  
20 further modified to attenuate expression of the gene *brnQ* (e.g., by inactivation of the gene).
81. The bacterium according to any one of items 1 to 80, wherein the gene *brnQ* is inactivated.
82. The bacterium according to any one of items 1 to 81, wherein said bacterium belongs to the *Enterobacteriaceae* family.
83. The bacterium according to any one of items 1 to 82, wherein said bacterium belongs to a  
25 genus selected from the group consisting of *Escherichia*, *Arsenophonus*, *Biostraticola*, *Brenneria*, *Buchnera*, *Budvicia*, *Buttiauxella*, *Cedecea*, *Citrobacter*, *Cosenzaea*, *Cronobacter*, *Dickeya*, *Edwardsiella*, *Enterobacter*, *Erwinia*, *Ewingella*, *Gibbsiella*, *Hafnia*, *Klebsiella*, *Leclercia*,

*Leminorella, Lonsdalea, Mangrovibacter, Moellerella, Morganella, Obesumbacterium, Pantoea, Pectobacterium, Phaseolibacter, Photorhabdus, Plesiomonas, Proteus, Rahnella, Raoultella, Saccharobacter, Salmonella, Samsonia, Serratia, Shimwellia, Sodalis, Tatumella, Thorsellia, Trabulsiella, Wigglesworthia, Yersinia and Yokenella.*

- 5 84. The bacterium according to any one of items 1 to 83, wherein said bacterium belongs to the genus *Escherichia*.
85. The bacterium according to any one of items 1 to 84, wherein said bacterium is *Escherichia coli*.
- 10 86. A method for producing L-serine or a L-serine derivative, the method comprises cultivating a bacterium according to any one of items 1 to 85 in a culture medium.
87. The method according to item 86, wherein the method is for producing L-serine.
88. The method according to item 86, wherein the method is for producing an L-serine derivative.
- 15 89. The method according to item 88, wherein the L-serine derivative is selected from the group consisting of L-cysteine, L-methionine, L-glycine, O-acetylserine, L-tryptophan, thiamine, ethanolamine and ethylene glycol.
90. The method according to item 86, wherein the method is for producing L-cysteine.
91. The method according to item 86, wherein the method is for producing L-methionine.
92. The method according to item 86, wherein the method is for producing L-glycine.
- 20 93. The method according to item 86, wherein the method is for producing O-acetylserine.
94. The method according to item 86, wherein the method is for producing L-tryptophan.
95. The method according to item 86, wherein the method is for producing thiamine.
96. The method according to item 86, wherein the method is for producing ethanolamine.
96. The method according to item 86, wherein the method is for producing ethylene glycol.
- 25 97. The method according to any one of items 86 to 96, the method further comprises collecting L-serine or the L-serine derivative from the culture medium.
98. A (isolated) nucleic acid molecule, such a vector, comprising a nucleotide sequence encoding a polypeptide having an amino acid sequence which has at least about 90%, such as at

least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position Y356, S357 and/or S359.

5 99. The (isolated) nucleic acid molecule according to item 98, wherein the polypeptide has the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position Y356, S357 and/or S359.

100. The (isolated) nucleic acid molecule according to item 98 or 99, wherein the amino acid substitution is at position Y356.

10 101. The (isolated) nucleic acid molecule according to any one of items 98 to 100 wherein the amino acid substitution is at position S357.

102. The (isolated) nucleic acid molecule according to any one of items 98 to 101, wherein the amino acid substitution is at position S359.

15 103. The (isolated) nucleic acid molecule according to any one of items 98 to 102, wherein the substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D, Y356E, Y356F, Y356A, Y356I, Y356P, Y356H, Y356R and Y356L; the substitution at position S357 is selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H, S357K, S357I and S357M; and the substitution at position S359 is selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L.

20 104. A (isolated) polypeptide having an amino acid sequence which has at least about 90%, such as at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position Y356, S357 and/or S359.

25 105. The (isolated) polypeptide according to item 104, where the polypeptide has the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position Y356, S357 and/or S359.

106. The (isolated) polypeptide according to any one of items 104 or 105, wherein the amino acid substitution is at position Y356.



107. The (isolated) polypeptide according to any one of items 104 to 106, wherein the amino acid substitution is at position S357.
108. The (isolated) polypeptide according to any one of items 104 to 107, wherein the amino acid substitution is at position S359.
- 5 109. The (isolated) polypeptide according to any one of items 104 to 108, wherein the substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D, Y356E, Y356F, Y356A, Y356I, Y356P, Y356H, Y356R and Y356L; the substitution at position S357 is selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H, S357K, S357I and S357M; and the  
10 substitution at position S359 is selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L.
110. A bacterium comprising the nucleic acid molecule according to any one of items 98 to 103.
111. A bacterium comprising the polypeptide according to any one of items 104 to 109.
112. A bacterium which expresses an aspartate kinase I / homoserine dehydrogenase I (*ThrA*)  
15 mutant having one or more amino acid substitutions which increase tolerance towards L-serine.
113. The bacterium according to item 112, wherein said bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in one or more amino acid substitutions in the encoded polypeptide at a position selected from the group consisting of Y356, S357 and S359.
114. The bacterium according to item 112 or 113, wherein said bacterium expresses an  
20 aspartate kinase I / homoserine dehydrogenase I (*ThrA*) mutant having one or more amino acid substitutions at a position selected from the group consisting of Y356, S357 and S359.
115. The bacterium according to any one of items 112 to 114, wherein said bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position Y356, one or more nucleotide substitutions resulting in an  
25 amino acid substitution in the encoded polypeptide at position S357 and/or one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position S359; wherein the substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D, Y356E, Y356F, Y356A, Y356I,

Y356P, Y356H, Y356R and Y356L; the substitution at position S357 is selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H, S357K, S357I and S357M; and the substitution at position S359 is selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and  
5 S359L.

116. The bacterium according to any one of items 112 to 115, wherein said bacterium expresses a aspartate kinase I / homoserine dehydrogenase I (ThrA) mutant having an amino acid substitution at position Y356, wherein the substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D, Y356E, Y356F,  
10 Y356A, Y356I, Y356P, Y356H, Y356R and Y356L.

117. The bacterium according to any one of items 112 to 116, wherein said bacterium comprises within the thrA gene one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position S357, wherein the substitution at position S357 is selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F,  
15 S357H, S357K, S357I and S357M.

118. The bacterium according to any one of items 112 to 117, wherein said bacterium expresses a aspartate kinase I / homoserine dehydrogenase I (ThrA) mutant having an amino acid substitution at position S357, wherein the substitution at position S357 is selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H, S357K,  
20 S357I and S357M.

119. The bacterium according to any one of items 112 to 118, wherein said bacterium comprises within the thrA gene one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position S359, wherein the substitution at position S359 is selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A,  
25 S359C, S359K, S359E and S359L.

120. The bacterium according to any one of items 112 to 119, wherein said bacterium expresses an aspartate kinase I / homoserine dehydrogenase I (ThrA) mutant having an amino acid substitution at position S359, wherein the substitution at position S359 is selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K,  
30 S359E and S359L.

121. The bacterium according to any one of items 112 to 120, wherein said bacterium comprises within the thrA gene one or more nucleotide substitutions resulting in one or more amino acid substitutions selected from the group consisting of Y356C, S357R and S359R.

5 122. The bacterium according to any one of items 112 to 121, wherein said bacterium comprises an exogenous nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide having an amino acid sequence which has at least about 90% sequence identity to the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position Y356, S357 and/or S359.

10 123. The bacterium according to claim 122, wherein the substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D, Y356E, Y356F, Y356A, Y356I, Y356P, Y356H, Y356R and Y356L; the substitution at position S357 is selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H, S357K, S357I and S357M; and the substitution at position S359 is selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C,  
15 S359K, S359E and S359L.

124. The bacterium according to any one of items 112 to 123, wherein said bacterium comprises an exogenous nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide having an amino acid sequence which has at least about 90% sequence identity to the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position Y356.

20 125. The bacterium according to item 124, wherein the substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D, Y356E, Y356F, Y356A, Y356I, Y356P, Y356H, Y356R, Y356L.

25 126. The bacterium according to any one of items 112 to 125, wherein said bacterium comprises an exogenous nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide having an amino acid sequence which has at least about 90% sequence identity to the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position S357.

127. The bacterium according to item 126, wherein the substitution at position S357 is selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H, S357K, S357I and S357M.

128. The bacterium according to any one of items 112 to 127, wherein said bacterium comprises an exogenous nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide having an amino acid sequence which has at least about 90% sequence identity to the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position S359.

- 5 129. The bacterium according to item 128, wherein the substitution at position S359 is selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L.

### **Brief description of the figures**

- 10 **Figure 1:** Deletion of the main genes involved in serine degradation, *sdaA*, *sdaB*, *tdcG* and *glyA* in *E. coli*. The removal of the four genes was demonstrated using PCR with primers specific to the relevant genes.

**Figure 2:** Vector maps of the constructs (Example 2)

**Figure 3:** Serine production during batch fermentation in shake flasks.

- 15 **Figure 4:** Increased tolerance towards serine can be achieved by overexpression of *ydeD*, a potential serine transporter. The figure shows the growth of the cells in the presence of various concentrations of serine.

**Figure 5:** Increased tolerance towards serine can be achieved by random mutagenesis. Growth rates of the parent strain (Q1) and evolved strains are shown at different serine concentrations.

- 20 **Figure 6:** Adaptive Laboratory Evolution (ALE) experiment for improving tolerance towards serine. (A) Growth rates during the evolution experiment. (B) Improved growth of evolved strains in the presence of high concentrations of serine.

- 25 **Figure 7:** The effect of mutations in *thrA* on tolerance towards serine. Three specific mutations of *thrA* (Y356C, S357R, S359R) were introduced into the Q1 background and the growth of the clones was compared to the growth of the Q1 strain in the presence of 6.25 g/L of serine.

**Figure 8:** Identification of mutations that cause increased tolerance towards serine. Amplicon sequencing analysis was used to analyze the effect of ALE mutations after introduction into the Q1 (DE3) strain by MAGE.

- 30 **Figure 9:** (A) Serine production and cell density (OD 600 nm) measured at different time points during fed batch fermentation of the Q1(DE3) and Q3(DE3) strains. (B) Production of serine from

the glucose fed to the fermentor. The slope of the curve indicates the mass yield during the fermentation.

**Figure 10:** (A) Cell density and serine titer of ALE 8-8 (DE3) strain. (B) Mass yield from glucose.

**Figure 11:** Growth rate of mutant *E. coli* strains having different amino acid substitutions observed at positions 356 (11A), 357 (11B) and 359 (11C) of ThrA, respectively (the amino acid substitution is denoted by the respective one-letter code). The growth rate of *E. coli* carrying the wild type *thrA* gene is denoted "wt".

**Figure 12:** Growth rate of mutant  $\Delta sdaA$  *E. coli* strains having different amino acid substitutions observed at positions 356 (11A), 357 (11B) and 359 (11C) of ThrA, respectively (the amino acid substitution is denoted by the respective one-letter code). The growth rate of *E. coli* carrying the wild type *thrA* gene is denoted "wt".

**Figure 13:** Growth profile of multiplex genome engineered strains.

**Figure 14:** Growth rate of *E. coli* MG1655 expressing wild type *thrA* or mutant variants of *thrA*.

15 The present invention is now described in more detail below.

### **Detailed description of the invention**

Unless specifically defined herein, all technical and scientific terms used have the same meaning as commonly understood by a skilled artisan in the fields of biochemistry, genetics, and microbiology.

All methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, with suitable methods and materials being described herein. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will prevail. Further, the materials, methods, and examples are illustrative only and are not intended to be limiting, unless otherwise specified.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, and recombinant DNA, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, Current Protocols in Molecular Biology (Frederick M. AUSUBEL, 2000, 5 Wiley and son Inc, Library of Congress, USA); Molecular Cloning: A Laboratory Manual, Third Edition, (Sambrook et al, 2001, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press); Oligonucleotide Synthesis (M. J. Gait ed., 1984); Mullis et al. U.S. Pat. No. 4,683,195; Nucleic Acid Hybridization (B. D. Harries & S. J. Higgins eds. 1984); Transcription And Translation (B. D. Hames & S. J. Higgins eds. 1984); Culture Of Animal Cells (R. I. Freshney, Alan R. Liss, Inc., 10 1987); Immobilized Cells And Enzymes (IRL Press, 1986); B. Perbal, A Practical Guide To Molecular Cloning (1984); the series, Methods In ENZYMOLOGY (J. Abelson and M. Simon, eds.-in-chief, Academic Press, Inc., New York), specifically, Vols.154 and 155 (Wu et al. eds.) and Vol. 185, "Gene Expression Technology" (D. Goeddel, ed.); Gene Transfer Vectors For Mammalian Cells (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); Immunochemical Methods In 15 Cell And Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); Handbook Of Experimental Immunology, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); and Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

## 20 *Bacterium of the invention*

As indicated above, the present invention is inter alia based on the finding that the production of L-serine can be enhanced by, e.g., inactivation of genes encoding enzymes involved in the degradation of L-serine, notably the genes *sdaA*, *sdaB*, *tdcG* and *glyA*.

25 Accordingly, the present invention provides a bacterium, especially a bacterium having an ability to produce L-serine, wherein said bacterium has been modified to attenuate expression of at least one gene coding for a polypeptide having serine deaminase activity and/or to attenuate expression of a gene coding for a polypeptide having serine hydroxymethyltransferase activity.

According to certain embodiments, a bacterium of the invention has been modified to attenuate the expression of at least one gene coding for a polypeptide having serine deaminase activity, 30 such as at least one gene selected from the group consisting of *sdaA*, *sdaB* and *tdcG*.

According to certain embodiments, a bacterium of the invention has been modified to attenuate expression of at least two genes coding for polypeptides having serine deaminase activity, such as at least two genes selected from the group consisting of *sdaA*, *sdaB* and *tdcG*.

5 According to certain embodiments, a bacterium of the invention has been modified to attenuate expression of at least three genes coding for polypeptides having serine deaminase activity, such as the genes *sdaA*, *sdaB* and *tdcG*.

According to certain embodiments, a bacterium of the invention has been modified to attenuate the expression of a gene coding for a polypeptide having serine hydroxymethyltransferase activity, such as the gene *glyA*.

10 According to certain embodiments, a bacterium of the invention has been modified to attenuate expression of genes coding for polypeptides having serine deaminase activity and to attenuate expression of a gene coding for a polypeptide having serine hydroxymethyltransferase activity.

15 According to certain embodiments, a bacterium of the invention has been modified to attenuate expression of at least one gene coding for polypeptides having serine deaminase activity, but has not been modified to attenuate expression of a gene coding for a polypeptide having serine hydroxymethyltransferase activity.

20 The genes *sdaA*, *sdaB* and *tdcG* encode L-serine deaminase I (SdaA), L-serine deaminase II (SdaB) and L-serine deaminase III (TdcG), respectively, which are the three enzymes carrying out the sole step in the pathway of L-serine degradation, converting serine into the basic cellular building block, pyruvate. Further information regarding *sdaA*, *sdaB* and *tdcG* of, e.g., *Escherichia coli* is available at EcoCyc ([www.biocyc.org](http://www.biocyc.org)) under Accession numbers EG10930, EG11623 and G7624, respectively. Representative nucleotide sequences of *sdaA*, *sdaB* and *tdcG* are set forth in SEQ ID NOs: 1 to 3, respectively.

25 The gene *glyA* encodes serine hydroxymethyltransferase (GlyA) which converts serine to glycine, transferring a methyl group to tetrahydrofolate, thus forming 5,10-methylene-tetrahydrofolate (5,10-mTHF). 5,10-mTHF is the major source of C1 units in the cell, making GlyA a key enzyme in the biosynthesis of purines, thymidine, methionine, choline and lipids. Further information regarding *glyA* of, e.g., *Escherichia coli* is available at EcoCyc ([www.biocyc.org](http://www.biocyc.org)) under Accession number EG10408. A representative nucleotide sequence of *glyA* is set forth in SEQ ID NO: 4.

According to certain embodiments, a bacterium of the invention has been modified to attenuate expression of the gene *sdaA*. According to certain embodiments, a bacterium of the invention has been modified to attenuate expression of the gene *sdaB*. According to certain embodiments, a bacterium of the invention has been modified to attenuate expression of the gene *tdcG*.

5 According to certain embodiments, a bacterium of the invention has been modified to attenuate expression of the gene *glyA*.

According to certain embodiments, a bacterium of the invention has been modified to attenuate expression of the genes *sdaA* and *sdaB*. According to certain embodiments, a bacterium of the invention has been modified to attenuate expression of the genes *sdaA* and *tdcG*. According to  
10 certain embodiments, a bacterium of the invention has been modified to attenuate expression of the genes *sdaA* and *glyA*. According to certain embodiments, a bacterium of the invention has been modified to attenuate expression of the genes *sdaB* and *tdcG*. According to certain  
15 embodiments, a bacterium of the invention has been modified to attenuate expression of the genes *sdaB* and *glyA*. According to certain embodiments, a bacterium of the invention has been modified to attenuate expression of the genes *tdcG* and *glyA*.

According to certain embodiments, a bacterium of the invention has been modified to attenuate expression of the genes *sdaA*, *sdaB* and *tdcG*. According to certain embodiments, a bacterium of the invention has been modified to attenuate expression of the genes *sdaA*, *sdaB* and *glyA*.  
20 According to certain embodiments, a bacterium of the invention has been modified to attenuate expression of the genes *sdaB*, *tdcG* and *glyA*.

According to certain embodiments, a bacterium of the invention has been modified to attenuate the expression of the genes *sdaA*, *sdaB*, *tdcG* and *glyA*.

According to certain embodiments, a bacterium of the invention has been modified to attenuate expression of at most three of the genes *sdaA*, *sdaB*, *tdcG* and *glyA*. More specifically, a bacterium  
25 of the invention may thus be a bacterium which has not been modified to attenuate expression of all the genes *sdaA*, *sdaB*, *tdcG* and *glyA*.

According to certain embodiments, a bacterium of the invention has been modified to attenuate expression of the genes *sdaA*, *sdaB* and *glyA*, but has not been modified to attenuate expression of the gene *tdcG*.



According to certain embodiments, a bacterium of the invention has been modified to attenuate expression of the genes *sdaA*, *tdcG* and *glyA*, but has not been modified to attenuate expression of the gene *sdaB*.

5 According to certain embodiments, a bacterium of the invention has been modified to attenuate expression of the genes *sdaB*, *tdcG* and *glyA*, but has not been modified to attenuate expression of the gene *sdaA*.

According to certain embodiments, a bacterium of the invention has been modified to attenuate the expression of at least one gene selected from *sdaA*, *sdaB* and *tdcG*, but has not been modified to attenuate expression of the gene *glyA*.

10 The expression of the gene or genes may be attenuated by inactivation of the gene or genes. Thus, a bacterium according to the invention may be a bacterium which has been modified to inactivate at least one gene coding for polypeptides having serine deaminase activity and/or to inactivate a gene coding for a polypeptide having serine hydroxymethyltransferase activity. Accordingly, a bacterium of the invention may be a bacterium wherein at least one gene coding  
15 for polypeptides having serine deaminase activity and/or a gene coding for a polypeptide having serine hydroxymethyltransferase activity is inactivated.

According to certain embodiments, the expression of at least one gene selected from the genes *sdaA*, *sdaB* and *tdcG* is attenuated by inactivation of the gene(s). Thus, a bacterium is provided wherein at least one gene selected from the genes *sdaA*, *sdaB* and *tdcG* is inactivated. According  
20 to certain embodiments, the expression of at least two genes selected from the genes *sdaA*, *sdaB* and *tdcG* is attenuated by inactivation of the genes. Thus, a bacterium is provided wherein at least two genes selected from the genes *sdaA*, *sdaB* and *tdcG* are inactivated. According to certain embodiments, the expression of the genes *sdaA*, *sdaB* and *tdcG* is attenuated by inactivation of the genes. Thus, a bacterium is provided wherein the genes *sdaA*, *sdaB* and *tdcG* are inactivated.

25 According to certain embodiments, the expression of *glyA* is attenuated by inactivation of the gene. Thus, a bacterium is provided wherein the gene *glyA* is inactivated. According to certain embodiments, the expression of at least one gene selected from the genes *sdaA*, *sdaB* and *tdcG*, and the expression of *glyA* is attenuated by inactivation of the genes. Thus, a bacterium is provided wherein at least one gene selected from the genes *sdaA*, *sdaB* and *tdcG*, and the gene  
30 *glyA* are inactivated. According to particular embodiments, the expression of the genes *sdaA*,

*sdaB*, *tdcG* and *glyA* is attenuated by inactivation of these genes. Thus, a bacterium is provided wherein the genes *sdaA*, *sdaB*, *tdcG* and *glyA* are inactivated.

According to certain embodiments, a bacterium of the invention has been modified to inactivate at least one gene selected from *sdaA*, *sdaB*, *tdcG* and *glyA*. Thus, a bacterium is provided wherein  
5 at least one gene selected from *sdaA*, *sdaB*, *tdcG* and *glyA* is inactivated. According to certain embodiments, a bacterium of the invention has been modified to inactivate at least two genes selected from *sdaA*, *sdaB*, *tdcG* and *glyA*. Thus, a bacterium is provided wherein at least two genes selected from *sdaA*, *sdaB*, *tdcG* and *glyA* are inactivated. According to certain  
10 embodiments, a bacterium of the invention has been modified to inactivate at least three genes selected from *sdaA*, *sdaB*, *tdcG* and *glyA*. Thus, a bacterium is provided wherein at least three genes selected from the *sdaA*, *sdaB*, *tdcG* and *glyA* are inactivated.

According to certain embodiments, a bacterium of the invention has been modified to inactivate at least one gene selected from *sdaA*, *sdaB* and *tdcG*. Thus, a bacterium is provided wherein at least one gene selected from *sdaA*, *sdaB* and *tdcG* is inactivated.

15 According to certain embodiments, a bacterium of the invention has been modified to inactivate at least two genes selected from *sdaA*, *sdaB* and *tdcG*. Thus, a bacterium is provided wherein at least two genes selected from *sdaA*, *sdaB* and *tdcG* are inactivated.

According to certain embodiments, a bacterium of the invention has been modified to inactivate at least the gene *sdaA*. Thus, a bacterium is provided wherein at least the gene *sdaA* is  
20 inactivated. According to certain embodiments, a bacterium of the invention has been modified to inactivate at least the gene *sdaB*. Thus, a bacterium is provided wherein at least the gene *sdaB* is inactivated. According to certain embodiments, a bacterium of the invention has been modified to inactivate at least the gene *tdcG*. Thus, a bacterium is provided wherein at least the gene *tdcG* is inactivated. According to certain embodiments, a bacterium of the invention has been modified  
25 to inactivate the gene *glyA*. Thus, a bacterium is provided wherein at least the gene *glyA* is inactivated.

According to certain embodiments, a bacterium of the invention has been modified to inactivate at least the genes *sdaA* and *sdaB*. Thus, a bacterium is provided wherein at least the genes *sdaA* and *sdaB* are inactivated. According to certain embodiments, a bacterium of the invention has  
30 been modified to inactivate at least the genes *sdaA* and *tdcG*. Thus, a bacterium is provided

wherein at least the genes *sdaA* and *tdcG* are inactivated. According to certain embodiments, a bacterium of the invention has been modified to inactivate at least the genes *sdaA* and *glyA*. Thus, a bacterium is provided wherein at least the genes *sdaA* and *glyA* are inactivated. According to certain embodiments, a bacterium of the invention has been modified to inactivate at least the genes *sdaB* and *tdcG*. Thus, a bacterium is provided wherein at least the genes *sdaB* and *tdcG* are inactivated. According to certain embodiments, a bacterium of the invention has been modified to inactivate at least the genes *sdaB* and *glyA*. Thus, a bacterium is provided wherein at least the genes *sdaB* and *glyA* are inactivated. According to certain embodiments, a bacterium of the invention has been modified to inactivate at least the genes *tdcG* and *glyA*. Thus, a bacterium is provided wherein at least the genes *tdcG* and *glyA* are inactivated.

According to certain embodiments, a bacterium of the invention has been modified to inactivate at least the genes *sdaA*, *sdaB* and *tdcG*. Thus, a bacterium is provided wherein at least the genes *sdaA*, *sdaB* and *tdcG* are inactivated. According to certain embodiments, a bacterium of the invention has been modified to inactivate at least the genes *sdaA*, *sdaB* and *glyA*. Thus, a bacterium is provided wherein at least the genes *sdaA*, *sdaB* and *glyA* are inactivated. According to certain embodiments, a bacterium of the invention has been modified to inactivate at least the genes *sdaB*, *tdcG* and *glyA*. Thus, a bacterium is provided wherein at least the genes *sdaB*, *tdcG* and *glyA* are inactivated.

According to certain embodiments, a bacterium of the invention has been modified to inactivate the genes *sdaA*, *sdaB*, *tdcG* and *glyA*. Thus, a bacterium is provided wherein the genes *sdaA*, *sdaB*, *tdcG* and *glyA* are inactivated.

According to certain embodiments, a bacterium of the invention has been modified to inactivate at most three of the genes *sdaA*, *sdaB*, *tdcG* and *glyA*. Thus, a bacterium is provided wherein at most three of the genes *sdaA*, *sdaB*, *tdcG* and *glyA* are inactivated. More specifically, a bacterium of the invention may thus be a bacterium wherein one, two or three, but not all, of the genes *sdaA*, *sdaB*, *tdcG* and *glyA* are inactivated.

According to certain embodiments, a bacterium of the invention has been modified to inactivate the genes *sdaA*, *sdaB* and *glyA*, but has not been modified to inactivate the gene *tdcG*. Thus, a bacterium is provided wherein the genes *sdaA*, *sdaB* and *glyA* are inactivated, but not the gene *tdcG*.

According to certain embodiments, a bacterium of the invention has been modified to inactivate the genes *sdaA*, *tdcG* and *glyA*, but has not been modified to inactivate the gene *sdaB*. Thus, a bacterium is provided wherein the genes *sdaA*, *tdcG* and *glyA* are inactivated, but not the gene *sdaB*.

5 According to certain embodiments, a bacterium of the invention has been modified to inactivate the genes *sdaB*, *tdcG* and *glyA*, but has not been modified to inactivate the gene *sdaA*. Thus, a bacterium is provided wherein the genes *sdaB*, *tdcG* and *glyA* are inactivated, but not the gene *sdaA*.

10 According to certain embodiments, a bacterium of the invention has been modified to inactivate at least one gene selected from *sdaA*, *sdaB* and *tdcG*, but has not been modified to inactivate the gene *glyA*. Thus, a bacterium is provided wherein the genes *sdaA*, *sdaB* and *tdcG* are inactivated, but not the gene *glyA*.

Expression of a gene can be attenuated by introducing a mutation into the gene on the chromosome so that the intracellular activity of the protein encoded by the gene is decreased as  
15 compared to an unmodified strain. Mutations which result in attenuation of expression of the gene include the replacement of one nucleotide or more to cause an amino acid substitution in the protein encoded by the gene (missense mutation), introduction of a stop codon (nonsense mutation), deletion or insertion of nucleotides to cause a frame shift, insertion of a drug-resistance gene, or deletion of a part of the gene or the entire gene (Qiu and Goodman, 1997;  
20 Kwon et al., 2000). Expression can also be attenuated by modifying an expression regulating sequence such as the promoter, the Shine-Dalgarno (SD) sequence, etc. (W095/34672).

For example, the following methods may be employed to introduce a mutation by gene recombination. A mutant gene encoding a mutant protein with decreased activity can be prepared, and the bacterium to be modified can be transformed with a DNA fragment containing  
25 the mutant gene. Then, the native gene on the chromosome is replaced with the mutant gene by homologous recombination, and the resulting strain can be selected. Gene replacement using homologous recombination can be conducted by employing a linear DNA, which is known as "lambda-red mediated gene replacement" (Datsenko and Wanner, 2000), or by employing a plasmid containing a temperature-sensitive replication origin (U.S. Patent 6,303,383 or JP 05-  
30 007491 A). Furthermore, site-specific mutation by gene substitution can also be incorporated

using homologous recombination such as set forth above using a plasmid which is unable to replicate in the host.

5 Expression of the gene can also be attenuated by inserting a transposon or an IS factor into the coding region of the gene (U.S. Patent No. 5,175,107), or by conventional methods, such as by mutagenesis with UV irradiation or nitrosoguanidine (N-methyl-N'-nitro-N-nitrosoguanidine), site-directed mutagenesis, gene disruption using homologous recombination, and/or gene replacement (Yu et al., 2000; and Datsenko and Wanner, 2000), such as the "lambda-red mediated gene replacement". The lambda-red mediated gene replacement is a particularly suitable method to inactivate one or more genes as described herein. Hence, according to particular  
10 embodiments, expression of genes is attenuated by inactivation of the genes using lambda-red mediated gene replacement.

The inactivation of at least one of *sdaA*, *sdaB*, *tdcG* and *glyA* results in an increase in specific productivity and yield of L-serine from glucose.

15 As shown in Figure 3, the inactivation of all four genes (*sdaA*, *sdaB*, *tdcG* and *glyA*) involved in serine degradation results in the highest specific productivity and the highest yield from glucose compared to inactivation of only the three genes involved in L-serine degradation via the serine to pyruvate pathway (*sdaA*, *sdaB*, and *tdcG*).

20 Serine is produced from glyceraldehyde-3-phosphate using three enzymes encoded by the genes *serA* (encoding a 3-phosphoglycerate dehydrogenase), *serB* (encoding a phosphoserine phosphatase) and *serC* (encoding a phosphoserine aminotransferase). In order to increase production of L-serine, these genes may be overexpressed. Relevant information regarding *serA*, *serB* and *serC* of, e.g., *Escherichia coli* is available at EcoCyc ([www.biocyc.org](http://www.biocyc.org)) under Accession numbers EG10944, EG10945 and EG10946, respectively.

25 Therefore, according to certain embodiments, the bacterium has been modified to overexpress a 3-phosphoglycerate dehydrogenase, a phosphoserine phosphatase and a phosphoserine aminotransferase. More particularly, the bacterium has been further modified to overexpress the genes *serA*, *serB* and *serC*. This may be achieved by introducing into the bacterium one or more (such as two or three) exogenous nucleic acid molecules, such as one or more vectors, comprising a nucleotide sequence encoding a 3-phosphoglycerate dehydrogenase, a nucleotide sequence

encoding a phosphoserine phosphatase and/or a nucleotide sequence encoding a phosphoserine aminotransferase.

5 The 3-phosphoglycerate dehydrogenase may be derived from the same species as the bacterium in which it is overexpressed or may be derived from a species different to the one in which it is overexpressed (i.e. it is heterologous). According to certain embodiments, the 3-phosphoglycerate dehydrogenase is derived from the same species as the bacterium in which it is overexpressed. According to certain other embodiments, the 3-phosphoglycerate dehydrogenase is derived from a species different to the one in which it is overexpressed (i.e. it is heterologous).

10 According to certain embodiments, the bacterium comprises an exogenous nucleic acid molecule comprising a nucleotide sequence encoding a 3-phosphoglycerate dehydrogenase. The exogenous nucleic acid molecule may comprise a nucleotide sequence encoding a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 5. The exogenous nucleic acid molecule may comprise a nucleotide sequence encoding a polypeptide comprising an amino acid sequence set  
15 which has at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 5. Preferably, the polypeptide has 3-phosphoglycerate dehydrogenase activity. More preferably, the polypeptide has 3-phosphoglycerate dehydrogenase activity similar to that of the polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 5. The exogenous nucleic acid molecule may comprise a nucleotide sequence encoding a  
20 polypeptide comprising an amino acid sequence set which has at least about 95%, such as at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 5. Preferably, the polypeptide has 3-phosphoglycerate dehydrogenase activity. More, preferably, the polypeptide has 3-phosphoglycerate dehydrogenase activity similar to that of the polypeptide comprising an amino  
25 acid sequence set forth in SEQ ID NO: 5. The exogenous nucleic acid molecule may comprise a nucleotide sequence encoding a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 5, wherein 1 or more, such as about 1 to about 50, about 1 to about 40, about 1 to about 35, about 1 to about 30, about 1 to about 25, about 1 to about 20, about 1 to about 15, about 1 to about 10, about 1 to about 5, or about 1 to about 3, amino acid residues are substituted, deleted,  
30 and/or inserted. Preferably, the polypeptide has 3-phosphoglycerate dehydrogenase activity. More preferably, the polypeptide has 3-phosphoglycerate dehydrogenase activity similar to that

of the polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 5. The exogenous nucleic acid molecule may comprise a nucleotide sequence encoding a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 5, wherein about 1 to about 5, such as about 1 to about 3, amino acid residues are substituted, deleted, and/or inserted. Preferably, the polypeptide has 3-phosphoglycerate dehydrogenase activity. More preferably, the polypeptide has 3-phosphoglycerate dehydrogenase activity similar to that of the polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 5.

It is further beneficial to overexpress a mutant *serA* gene which encodes a 3-phosphoglycerate dehydrogenase being resistant towards feedback inhibition of serine. This may, for example, be achieved by deleting the last four C-terminal residues of the wild type 3-phosphoglycerate dehydrogenase (SerA). Alternatively, feedback inhibition of SerA can be removed by mutating the three residues H344, N346 and N364 to alanine. A representative amino acid sequence of such 3-phosphoglycerate dehydrogenase mutant is set forth in SEQ ID NO: 6. Therefore, according to particular embodiments, the bacterium comprises an exogenous nucleic acid molecule comprising a nucleotide sequence encoding a 3-phosphoglycerate dehydrogenase being resistant towards feedback inhibition of serine. The exogenous nucleic acid molecule may comprise a nucleotide sequence encoding a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 6. The exogenous nucleic acid molecule may comprise a nucleotide sequence encoding a polypeptide comprising an amino acid sequence set which has at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 6. Preferably, the polypeptide has 3-phosphoglycerate dehydrogenase activity. More preferably, the polypeptide has 3-phosphoglycerate dehydrogenase activity similar to that of the polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 6. The exogenous nucleic acid molecule may comprise a nucleotide sequence encoding a polypeptide comprising an amino acid sequence set which has at least about 95%, such as at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 6. Preferably, the polypeptide has 3-phosphoglycerate dehydrogenase activity. More preferably, the polypeptide has 3-phosphoglycerate dehydrogenase activity similar to that of the polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 6. The exogenous nucleic acid molecule may comprise a nucleotide sequence encoding a polypeptide comprising an amino

acid sequence set forth in SEQ ID NO: 6, wherein 1 or more, such as about 1 to about 50, about 1 to about 40, about 1 to about 35, about 1 to about 30, about 1 to about 25, about 1 to about 20, about 1 to about 15, about 1 to about 10, about 1 to about 5, or about 1 to about 3, amino acid residues are substituted, deleted, and/or inserted. Preferably, the polypeptide has 3-phosphoglycerate dehydrogenase activity. More preferably, the polypeptide has 3-phosphoglycerate dehydrogenase activity similar to that of the polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 6. The exogenous nucleic acid molecule may comprise a nucleotide sequence encoding a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 6, wherein about 1 to about 5, such as about 1 to about 3, amino acid residues are substituted, deleted, and/or inserted. Preferably, the polypeptide has 3-phosphoglycerate dehydrogenase activity. More preferably, the polypeptide has 3-phosphoglycerate dehydrogenase activity similar to that of the polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 6.

The phosphoserine phosphatase may be derived from the same species as the bacterium in which it is overexpressed or may be derived from a species different to the one in which it is overexpressed (i.e. it is heterologous). According to certain embodiments, the phosphoserine phosphatase is derived from the same species as the bacterium in which it is overexpressed. According to certain other embodiments, the phosphoserine phosphatase is derived from a species different to the one in which it is overexpressed (i.e. it is heterologous).

According to certain embodiments, the bacterium comprises an exogenous nucleic acid molecule comprising a nucleotide sequence encoding a phosphoserine phosphatase. The exogenous nucleic acid molecule may comprise a nucleotide sequence encoding a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 7. The exogenous nucleic acid molecule may comprise a nucleotide sequence encoding a polypeptide comprising an amino acid sequence set which has at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 7. Preferably, the polypeptide has phosphoserine phosphatase activity. More preferably, the polypeptide has phosphoserine phosphatase activity similar to that of the polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 7. The exogenous nucleic acid molecule may comprise a nucleotide sequence encoding a polypeptide comprising an amino acid sequence set which has at least about 95%, such as at least about 96%, at least about 97%, at



least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 7. Preferably, the polypeptide has phosphoserine phosphatase activity. More preferably, the polypeptide has phosphoserine phosphatase activity similar to that of the polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 7. The exogenous nucleic acid molecule may comprise a nucleotide sequence encoding a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 7, wherein 1 or more, such as about 1 to about 50, about 1 to about 40, about 1 to about 35, about 1 to about 30, about 1 to about 25, about 1 to about 20, about 1 to about 15, about 1 to about 10, about 1 to about 5, or about 1 to about 3, amino acid residues are substituted, deleted, and/or inserted. Preferably, the polypeptide has phosphoserine phosphatase activity. More preferably, the polypeptide has phosphoserine phosphatase activity similar to that of the polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 7. The exogenous nucleic acid molecule may comprise a nucleotide sequence encoding a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 7, wherein about 1 to about 5, such as about 1 to about 3, amino acid residues are substituted, deleted, and/or inserted. Preferably, the polypeptide has phosphoserine phosphatase activity. More preferably, the polypeptide has phosphoserine phosphatase activity similar to that of the polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 7.

The phosphoserine aminotransferase may be derived from the same species as the bacterium in which it is overexpressed or may be derived from a species different to the one in which it is overexpressed (i.e. it is heterologous). According to certain embodiments, the phosphoserine aminotransferase is derived from the same species as the bacterium in which it is overexpressed. According to certain other embodiments, the phosphoserine aminotransferase is derived from a species different to the one in which it is overexpressed (i.e. it is heterologous).

According to certain embodiments, the bacterium comprises an exogenous nucleic acid molecule comprising a nucleotide sequence encoding a phosphoserine aminotransferase. The exogenous nucleic acid molecule may comprise a nucleotide sequence encoding a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 8. The exogenous nucleic acid molecule may comprise a nucleotide sequence encoding a polypeptide comprising an amino acid sequence set forth which has at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 8. Preferably the polypeptide has phosphoserine

aminotransferase activity. More preferably, the polypeptide has phosphoserine aminotransferase activity similar to that of the polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 8. The exogenous nucleic acid molecule may comprise a nucleotide sequence encoding a polypeptide comprising an amino acid sequence set which has at least about 95%, such as at least  
5 about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 8. Preferably, the polypeptide has phosphoserine aminotransferase activity. More preferably, the polypeptide has phosphoserine aminotransferase activity similar to that of the polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 8. The exogenous nucleic acid molecule may comprise a nucleotide sequence encoding a  
10 polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 8, wherein 1 or more, such as about 1 to about 50, about 1 to about 40, about 1 to about 35, about 1 to about 30, about 1 to about 25, about 1 to about 20, about 1 to about 15, about 1 to about 10, about 1 to about 5, or about 1 to about 3, amino acid residues are substituted, deleted, and/or inserted. Preferably, the polypeptide has phosphoserine aminotransferase activity. More preferably, the polypeptide  
15 has phosphoserine aminotransferase activity similar to that of the polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 8. The exogenous nucleic acid molecule may comprise a nucleotide sequence encoding a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 8, wherein about 1 to about 5, such as about 1 to about 3, amino acid residues are substituted, deleted, and/or inserted. Preferably, the polypeptide has phosphoserine  
20 aminotransferase activity. More preferably, the polypeptide has phosphoserine aminotransferase activity similar to that of the polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 8.

A bacterium, such as *Escherichia coli*, which has been modified to attenuate expression of genes coding for polypeptides having serine deaminase activity and/or to attenuate expression of a gene  
25 coding for a polypeptide having serine hydroxymethyltransferase (e.g., by inactivation of the genes), may show a low tolerance towards serine. Therefore, it would be desirable to provide a bacterium which shows increased tolerance towards serine.

In this respect, the present inventors have found that product toxicity can be reduced by overexpression of novel exporters, by evolving bacterial strains by random mutagenesis, and by  
30 adaptive evolution. As a result, bacteria having increased tolerance towards serine are provided. "Increased tolerance" as used herein means that a bacterium is capable of growing in a minimal

culture medium (such as M9 minimal medium) comprising L-serine at a concentration of at least about 6.25 g/L.

According to certain embodiments, a bacterium of the present invention is capable of growing in a minimal culture medium (such as M9 minimal medium) comprising L-serine at a concentration of at least about 6.25 g/L (such as at least about 12.5 g/L). According to particular embodiments, the bacterium is capable of growing in a minimal culture medium (such as M9 minimal medium) comprising L-serine at a concentration of at least about 12.5 g/L (such as at least about 25 g/L). According to particular embodiments, the bacterium is capable of growing in a minimal culture medium (such as M9 minimal medium) comprising L-serine at a concentration of at least about 25 g/L (such as at least about 40 g/L). According to particular embodiments, the bacterium is capable of growing in a minimal culture medium (such as M9 minimal medium) comprising L-serine at a concentration of at least about 40 g/L (such as at least about 50 g/L). According to particular embodiments, the bacterium is capable of growing in a minimal culture medium (such as M9 minimal medium) comprising L-serine at a concentration of at least about 50 g/L (such as at least about 75 g/L). According to particular embodiments, the bacterium is capable of growing in a minimal culture medium (such as M9 minimal medium) comprising L-serine at a concentration of at least about 75 g/L (such as at least about 100 g/L). According to particular embodiments, the bacterium is capable of growing in a minimal culture medium (such as M9 minimal medium) comprising L-serine at a concentration of at least about 100 g/L.

Preferably, the minimal culture medium, such as M9 minimal medium, is supplemented with 2mM glycine and 2g/L glucose. The bacterium is generally cultivated using adequate aeration at about 37°C for a period of about 24 to about 40 hours.

According to certain embodiments, a bacterium of the present invention is capable of growing in a minimal culture medium (such as M9 minimal medium) comprising L-serine at a concentration of at least about 6.25 g/L (such as at least about 12.5 g/L) at a growth rate of at least about 0.1 hr<sup>-1</sup> during exponential growth. According to particular embodiments, a bacterium of the invention is capable of growing in a minimal culture medium (such as M9 minimal medium) comprising L-serine at a concentration of at least about 12.5 g/L (such as at least about 25 g/L) at a growth rate of at least about 0.1 hr<sup>-1</sup> during exponential growth. According to particular embodiments, a bacterium of the invention is capable of growing in a minimal culture medium (such as M9

minimal medium) comprising L-serine at a concentration of at least about 25 g/L (such as at least about 50 g/L) at a growth rate of at least about 0.1 hr<sup>-1</sup> during exponential growth.

Preferably, the minimal culture medium, such as M9 minimal medium, is supplemented with 2mM glycine and 2g/L glucose. The bacterium is generally cultivated using adequate aeration at  
5 about 37°C for a period of about 24 to about 40 hours.

According to certain embodiments, a bacterium is provided which contains at least one mutation which leads to an at least 20% increase in growth rate compared to an otherwise identical bacterium not carrying said mutation.

One novel exporter which when overexpressed in a bacterium improved tolerance towards serine  
10 is the O-acetylserine/cysteine export protein encoded by the gene *ydeD*. Further information regarding *ydeD* of, e.g., Escherichia coli is available at EcoCyc ([www.biocyc.org](http://www.biocyc.org)) under Accession numbers EG11639. A representative amino acid sequence of such exporter protein is set forth in SEQ ID NO: 9. Therefore, the present invention provides a bacterium which has been modified to overexpress the gene *ydeD*.

15 According to certain embodiments, a bacterium of the invention comprises an exogenous nucleic acid molecule, such as an expression vector, comprising a nucleotide sequence encoding the protein product of the gene *ydeD*. According to particular embodiments, the bacterium comprises an exogenous nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 9. The exogenous nucleic acid  
20 molecule may comprise a nucleotide sequence encoding a polypeptide comprising an amino acid sequence set which has at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 9. Preferably, the polypeptide has O-acetylserine and/or cysteine transporter activity. More preferably, the polypeptide has O-acetylserine and/or  
25 cysteine transporter activity similar to that of the polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 9. The exogenous nucleic acid molecule may comprise a nucleotide sequence encoding a polypeptide comprising an amino acid sequence set which has at least about 95%, such as at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 9. Preferably, the

polypeptide has O-acetylserine and/or cysteine transporter activity. More preferably, the polypeptide has O-acetylserine and/or cysteine transporter activity similar to that of the polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 9. The exogenous nucleic acid molecule may comprise a nucleotide sequence encoding a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 9, wherein 1 or more, such as about 1 to about 50, about 1 to about 40, about 1 to about 35, about 1 to about 30, about 1 to about 25, about 1 to about 20, about 1 to about 15, about 1 to about 10, about 1 to about 5, or about 1 to about 3, amino acid residues are substituted, deleted, and/or inserted. Preferably, the polypeptide has O-acetylserine and/or cysteine transporter activity. More preferably, the polypeptide has O-acetylserine and/or cysteine transporter activity similar to that of the polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 9. The exogenous nucleic acid molecule may comprise a nucleotide sequence encoding a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 9, wherein about 1 to about 5, such as about 1 to about 3, amino acid residues are substituted, deleted, and/or inserted. Preferably, the polypeptide has O-acetylserine and/or cysteine transporter activity. More preferably, the polypeptide has O-acetylserine and/or cysteine transporter activity similar to that of the polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 9.

A modified YdeD polypeptide containing an additional stretch of 6 histidine residues at the C-terminus is set forth in SEQ ID NO: 10. According to particular embodiments, the bacterium comprises an exogenous nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 10. The exogenous nucleic acid molecule may comprise a nucleotide sequence encoding a polypeptide comprising an amino acid sequence set which has at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 10. Preferably, the polypeptide has O-acetylserine and/or cysteine transporter activity. More preferably, the polypeptide has O-acetylserine and/or cysteine transporter activity similar to that of the polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 10. The exogenous nucleic acid molecule may comprise a nucleotide sequence encoding a polypeptide comprising an amino acid sequence set which has at least about 95%, such as at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 10. Preferably, the polypeptide has O-acetylserine and/or cysteine transporter activity. More preferably, the

polypeptide has O-acetylserine and/or cysteine transporter activity similar to that of the polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 10. The exogenous nucleic acid molecule may comprise a nucleotide sequence encoding a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 10, wherein 1 or more, such as about 1 to about 50, about 1 to about 40, about 1 to about 35, about 1 to about 30, about 1 to about 25, about 1 to about 20, about 1 to about 15, about 1 to about 10, about 1 to about 5, or about 1 to about 3, amino acid residues are substituted, deleted, and/or inserted. Preferably, the polypeptide has O-acetylserine and/or cysteine transporter activity. More preferably, the polypeptide has O-acetylserine and/or cysteine transporter activity similar to that of the polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 10. The exogenous nucleic acid molecule may comprise a nucleotide sequence encoding a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 10, wherein about 1 to about 5, such as about 1 to about 3, amino acid residues are substituted, deleted, and/or inserted. Preferably, the polypeptide has O-acetylserine and/or cysteine transporter activity. More preferably, the polypeptide has O-acetylserine and/or cysteine transporter activity similar to that of the polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 10.

As shown in Figure 4, growth of a bacterium, such as *E. coli*, lacking the main serine degradation pathways is severely growth inhibited in the presence of even low concentrations of serine. Upon overexpression of *ydeD*, the tolerance towards serine is increased substantially, suggesting that YdeD may potentially transport serine out of the cell.

A bacterium of the invention having improved tolerance towards serine, such as one capable of growing in a minimal culture medium comprising L-serine at a concentration of at least about 6.25 g/L as mentioned above, can be obtained by random mutagenesis or by adaptive evolution. Respective details are provided in Examples 4 and 5, respectively.

Adaptive evolution may, for example, be achieved by carrying out the following method: Prior to the start of the experiment, suitable tubes are filled with 25 ml of culture media which are kept at 37 °C in a heat block. Controlled aeration is obtained using magnetic tumble stirrers placed inside the tubes and spinning at 1,800 rpm. At the start of the experiment, a single colony (of the starter strain) is grown overnight in one of the tubes, and 100 µL aliquots are used to inoculate a new tube containing 25 ml of fresh culture media. As the bacteria grow, multiple OD measurements at

600 nm are performed. Growth rates are calculated by taking the slope of a least-square linear regression line fit to the logarithm of the OD measurements. Once reaching a target OD of 0.4, 100 µl of culture are used to inoculate a new tube containing 25 ml of culture media. This way, cultures are serially passed (2-3 times per day) to tubes with fresh media after reaching the  
5 targeted cell density such that stationary phase is never reached. The experiment is initiated with an L-serine concentration of 3 g/L serine, followed by an increase to 6 g/L of L-Serine after the desired growth rate has been reached. Once the populations achieved a stable phenotype (i.e. growth rate), the L-Serine concentration is increased to 12 g/L. This process is repeated iteratively using 24, 50, 75, and 100 g/L of L-serine. The final population may then be plated on the LB-agar  
10 for further cultivation and selection of an L-serine tolerant strain. The foregoing method may be performed manually or by using an automated system enable the propagation of evolving populations over the course of many days while monitoring their growth rates.

As further demonstrate herein, the present inventors have identified beneficial mutations in a number of genes which confer tolerance towards L-serine. Respective genes and mutations are  
15 depicted in Table S5.

One such gene is *thrA* which encodes an aspartate kinase I / homoserine dehydrogenase I (ThrA). Further information regarding *thrA* of, e.g., Escherichia coli is available at EcoCyc ([www.biocyc.org](http://www.biocyc.org)) under Accession numbers EG10998. A representative amino acid sequence of a wild type Aspartate kinase I / homoserine dehydrogenase I (ThrA) is set forth in SEQ ID NO: 11. As  
20 demonstrated in Example 6 and 10, introducing certain mutation within the amino acid sequence of the aspartate kinase I / homoserine dehydrogenase results in a very significant increase in tolerance towards serine (Figures 7 and 11). Particularly, the following mutations have been shown to be beneficial: Y356C, S357R and S359R.

As further demonstrated in Example 14, expressing an aspartate kinase I / homoserine dehydrogenase I (ThrA) mutant as detailed herein also results in an increased tolerance towards  
25 L-serine of a bacterium wherein none of the genes *sdaA*, *sdaB*, *tdcG* and *glyA* have been inactivated. This means that the increased tolerance towards L-serine seen with such ThrA mutants is independent from whether or not genes encoding enzymes involved in the degradation of L-serine, notably the genes *sdaA*, *sdaB*, *tdcG* and *glyA*, are inactivated.

Therefore, a bacterium of the invention may be a bacterium which expresses an aspartate kinase I / homoserine dehydrogenase I (ThrA) mutant having one or more amino acid substitutions which increase tolerance towards L-serine.

5 According to certain embodiments, the present invention provides a bacterium which expresses an aspartate kinase I / homoserine dehydrogenase I (ThrA) mutant which is not inhibited by L-serine.

10 According to certain embodiments, the present invention provides a bacterium which comprises within the *thrA* gene one or more nucleotide substitutions resulting in one or more amino acid substitutions which increase tolerance towards L-serine. More particularly, a bacterium is provided which comprises within the *thrA* gene one or more nucleotide substitutions resulting in one or more amino acid substitutions in the encoded polypeptide at a position selected from the group consisting of Y356, S357 and S359. A bacterium of the invention may thus express an aspartate kinase I / homoserine dehydrogenase I (ThrA) having one or more amino acid substitutions at a position selected from the group consisting of Y356, S357 and S359. More particularly, a bacterium of the invention may express a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11 which comprises one or more amino acid substitutions at a position selected from the group consisting of Y356, S357 and S359. Preferably, the one or more amino acid substitutions are non-conservative substitutions.

20 According to certain embodiments, the present invention provides a bacterium which comprises within the *thrA* gene one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position Y356. A bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises an amino acid substitution at position Y356. According to particular embodiments, the amino acid substitution is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, 25 Y356D, Y356E, Y356F, Y356A, Y356I, Y356P, Y356H, Y356R, and Y356L. According to other particular embodiments, the amino acid substitution is selected from the group consisting of Y356C, Y356T, Y356V, Y356W, Y356Q, Y356G, Y356E, Y356F, Y356A, Y356I, Y356P, Y356H, Y356R, and Y356L. According to more particular embodiments, the amino acid substitution is selected from the group consisting of Y356C, Y356T, Y356W, Y356F, Y356A, Y356I, Y356P, Y356H, Y356R,



and Y356L. According to other more particular embodiments, the amino acid substitution is selected from the group consisting of Y356C, Y356W, Y356F, Y356I, Y356P, Y356R, and Y356L.

According to certain embodiments, the bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a Y356C substitution in the encoded polypeptide. A

5 bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a Y356C substitution. According to certain embodiments, the bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a Y356T substitution in the encoded polypeptide. A bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a Y356T substitution.

10 According to certain embodiments, the bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a Y356V substitution in the encoded polypeptide. A bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a Y356V substitution. According to certain embodiments, the bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a Y356S substitution in the encoded polypeptide. A bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a Y356S substitution.

15 According to certain embodiments, the bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a Y356W substitution in the encoded polypeptide. A bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a Y356W substitution. According to certain embodiments, the bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a Y356G substitution in the encoded polypeptide. A bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a Y356G substitution.

20 According to certain embodiments, the bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a Y356N substitution in the encoded polypeptide. A bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a Y356N substitution. According to certain embodiments, the bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a Y356D substitution in the encoded polypeptide. A bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a Y356D substitution.

25 According to certain embodiments, the bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a Y356E substitution in the encoded polypeptide. A

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bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a Y356E substitution. According to certain embodiments, the bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a Y356F substitution in the encoded polypeptide. A bacterium of the invention may thus express a

5 polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a Y356F substitution.

According to certain embodiments, the bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a Y356A substitution in the encoded polypeptide. A

bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a Y356I substitution. According to certain embodiments, the

10 bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a Y356A substitution in the encoded polypeptide. A bacterium of the invention may thus express a

polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a Y356I substitution.

According to certain embodiments, the bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a Y356P substitution in the encoded polypeptide. A

15 bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a Y356P substitution. According to certain embodiments, the

bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a Y356H substitution in the encoded polypeptide. A bacterium of the invention may thus express a

polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a Y356H substitution.

20 According to certain embodiments, the bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a Y356R substitution in the encoded polypeptide. A

bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a Y356R substitution. According to certain embodiments, the

bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a

25 Y356L substitution in the encoded polypeptide. A bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a Y356L substitution.

According to certain embodiments, the present invention provides a bacterium which comprises within the *thrA* gene one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position S357. A bacterium of the invention may thus express a

30 polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises an amino acid substitution at position S357. According to particular embodiments, the amino acid substitution is

selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N,

S357F, S357H, S357K, S357I and S357M. According to other particular embodiments, the amino acid substitution is selected from the group consisting of S357R, S357V, S357G, S357L, S357Y, S357A, S357N, S357F and S357H. According to more particular embodiments, the amino acid substitution is selected from the group consisting of S357R, S357A, S357N and S357F. According to other more particular embodiments, the amino acid substitution is selected from the group consisting of S357A and S357F.

According to certain embodiments, the bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a S357R substitution in the encoded polypeptide. A bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a S357R substitution. According to certain embodiments, the bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a S357V substitution in the encoded polypeptide. A bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a S357V substitution. According to certain embodiments, the bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a S357P substitution in the encoded polypeptide. A bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a S357P substitution. According to certain embodiments, the bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a S357G substitution in the encoded polypeptide. A bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a S357G substitution. According to certain embodiments, the bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a S357L substitution in the encoded polypeptide. A bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a S357L substitution. According to certain embodiments, the bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a S357Y substitution in the encoded polypeptide. A bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a S357Y substitution. According to certain embodiments, the bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a S357A substitution in the encoded polypeptide. A bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a S357A substitution. According to certain embodiments, the bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a

S357N substitution in the encoded polypeptide. A bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a S357N substitution. According to certain embodiments, the bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a S357F substitution in the encoded polypeptide. A bacterium

5 of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a S357F substitution. According to certain embodiments, the bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a S357H substitution in the encoded polypeptide. A bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a S357H substitution.

10 According to certain embodiments, the bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a S357K substitution in the encoded polypeptide. A bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a S357K substitution. According to certain embodiments, the bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a

15 S357I substitution in the encoded polypeptide. A bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a S357I substitution. According to certain embodiments, the bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a S357M substitution in the encoded polypeptide. A bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein

20 said polypeptide comprises a S357M substitution.

According to certain embodiments, the present invention provides a bacterium which comprises within the *thrA* gene one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position S359. A bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises an amino acid

25 substitution at position S359. According to particular embodiments, the amino acid substitution is selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L. According to other particular embodiments, the amino acid substitution is selected from the group consisting of S359R, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L. According to more particular embodiments, the

30 amino acid substitution is selected from the group consisting of S359R, S359T, S359P, S359V, S359Q, S359A, S359E and S359L. According to other more particular embodiments, the amino acid substitution is selected from the group consisting of S359R, S359T, S359P, S359Q, S359A,

S359E and S359L. According to other more particular embodiments, the amino acid substitution is selected from the group consisting of S359R, S359T, S359Q, S359A and S359E. According to other more particular embodiments, the amino acid substitution is selected from the group consisting of S359R, S359T and S359A. According to other more particular embodiments, the amino acid substitution is selected from the group consisting of S359R and S359A.

According to certain embodiments, the bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a S359R substitution in the encoded polypeptide. A bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a S359R substitution. According to certain embodiments, the bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a S359G substitution in the encoded polypeptide. A bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a S359G substitution. According to certain embodiments, the bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a S359M substitution in the encoded polypeptide. A bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a S359M substitution. According to certain embodiments, the bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a S359F substitution in the encoded polypeptide. A bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a S359F substitution. According to certain embodiments, the bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a S359T substitution in the encoded polypeptide. A bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a S359T substitution. According to certain embodiments, the bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a S359P substitution in the encoded polypeptide. A bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a S359P substitution. According to certain embodiments, the bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a S359V substitution in the encoded polypeptide. A bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a S359V substitution. According to certain embodiments, the bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a S359Q substitution in the encoded polypeptide. A bacterium of the invention may thus express a

polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a S359Q substitution. According to certain embodiments, the bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a S359A substitution in the encoded polypeptide. A bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein

5 said polypeptide comprises a S359A substitution. According to certain embodiments, the bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a S359C substitution in the encoded polypeptide. A bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a S359C substitution. According to certain embodiments, the bacterium comprises within the *thrA* gene one or more

10 nucleotide substitutions resulting in a S359K substitution in the encoded polypeptide. A bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a S359K substitution. According to certain embodiments, the bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a S359E substitution in the encoded polypeptide. A bacterium of the invention may thus express a

15 polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a S359E substitution. According to certain embodiments, the bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a S359L substitution in the encoded polypeptide. A bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a S359L substitution.

20 According to certain embodiments, the present invention provides a bacterium which comprises within the *thrA* gene one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position Y356, one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position S357 and/or one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at

25 position S359; wherein the substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D, Y356E, Y356F, Y356A, Y356I, Y356P, Y356H, Y356R and Y356L; the substitution at position S357 is selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H, S357K, S357I and S357M; and the substitution at position S359 is selected from the group consisting of

30 S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L.

According to certain embodiments, the present invention provides a bacterium which comprises within the *thrA* gene one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position Y356 and/or one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position S357; wherein the  
5 substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D, Y356E, Y356F, Y356A, Y356I, Y356P, Y356H, Y356R and Y356L; and the substitution at position S357 is selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H, S357K, S357I and S357M.

According to certain embodiments, the present invention provides a bacterium which comprises  
10 within the *thrA* gene one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position Y356 and/or one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position S359; wherein the substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D, Y356E, Y356F, Y356A, Y356I, Y356P, Y356H, Y356R and  
15 Y356L; and the substitution at position S359 is selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L.

According to certain embodiments, the present invention provides a bacterium which comprises within the *thrA* gene one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position S357 and/or one or more nucleotide substitutions  
20 resulting in an amino acid substitution in the encoded polypeptide at position S359; wherein the substitution at position S357 is selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H, S357K, S357I and S357M; and the substitution at position S359 is selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L.

According to certain embodiments, the present invention provides a bacterium which comprises within the *thrA* gene one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position Y356, one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position S357 and one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position S359;  
25 wherein the substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D, Y356E, Y356F, Y356A, Y356I, Y356P, Y356H,  
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Y356R and Y356L; the substitution at position S357 is selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H, S357K, S357I and S357M; and the substitution at position S359 is selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L.

5 According to certain embodiments, the present invention provides a bacterium which comprises within the *thrA* gene one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position Y356 and one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position S357; wherein the substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W,  
10 Y356Q, Y356G, Y356N, Y356D, Y356E, Y356F, Y356A, Y356I, Y356P, Y356H, Y356R and Y356L; and the substitution at position S357 is selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H, S357K, S357I and S357M.

According to certain embodiments, the present invention provides a bacterium which comprises within the *thrA* gene one or more nucleotide substitutions resulting in an amino acid substitution  
15 in the encoded polypeptide at position Y356 and one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position S359; wherein the substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D, Y356E, Y356F and Y356A; and the substitution at position S359 is selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q,  
20 S359A, S359C, S359K, S359E and S359L.

According to certain embodiments, the present invention provides a bacterium which comprises within the *thrA* gene one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position S357 and one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position S359; wherein the substitution  
25 at position S357 is selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H, S357K, S357I and S357M; and the substitution at position S359 is selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L.

According to certain embodiments, a bacterium of the invention express a polypeptide having the  
30 amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at



position Y356, S357 and/or S359, wherein the substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D, Y356E, Y356F, Y356A, Y356I, Y356P, Y356H, Y356R and Y356L; the substitution at position S357 is selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H, S357K, S357I and S357M; and the substitution at position S359 is selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L.

According to certain embodiments, a bacterium of the invention express a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position Y356 and/or S357, wherein the substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D, Y356E, Y356F, Y356A, Y356I, Y356P, Y356H, Y356R and Y356L; and the substitution at position S357 is selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H, S357K, S357I and S357M.

According to certain embodiments, a bacterium of the invention express a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position Y356 and/or S359, wherein the substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D, Y356E, Y356F, Y356A, Y356I, Y356P, Y356H, Y356R and Y356L; and the substitution at position S359 is selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L.

According to certain embodiments, a bacterium of the invention express a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position S357 and/or S359, wherein the substitution at position S357 is selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H, S357K, S357I and S357M; and the substitution at position S359 is selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L.

According to certain embodiments, a bacterium of the invention express a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at

position Y356, wherein the substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D, Y356E, Y356F, Y356A, Y356I, Y356P, Y356H, Y356R and Y356L.

5 According to certain embodiments, a bacterium of the invention express a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position S357, wherein the substitution at position S357 is selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H, S357K, S357I and S357M; and the substitution at position S359 is selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L.

10 According to certain embodiments, a bacterium of the invention express a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position S359, wherein the substitution at position S359 is selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L.

15 According to certain embodiments, a bacterium of the invention express a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position Y356 and S357, wherein the substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D, Y356E, Y356F and Y356A; and the substitution at position S357 is selected from the group consisting of S357R,  
20 S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H, S357K, S357I and S357M.

According to certain embodiments, a bacterium of the invention express a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position Y356 and S359, wherein the substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D, Y356E, Y356F,  
25 Y356A, Y356I, Y356P, Y356H, Y356R and Y356L; and the substitution at position S359 is selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L.

According to certain embodiments, a bacterium of the invention express a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at  
30 position S357 and S359, wherein the substitution at position S357 is selected from the group

consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H, S357K, S357I and S357M; and the substitution at position S359 is selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L.

- 5 According to certain embodiments, a bacterium of the invention express a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position Y356, S357 and S359, wherein the substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D, Y356E, Y356F, Y356A, Y356I, Y356P, Y356H, Y356R and Y356L; the substitution at position S357 is
- 10 selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H, S357K, S357I and S357M; and the substitution at position S359 is selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L.

- According to certain embodiments, a bacterium of the invention expresses a polypeptide having
- 15 the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 356 tyrosine is replaced by cysteine. According to certain embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 356 tyrosine is replaced by threonine. According to certain embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11,
- 20 wherein at position 356 tyrosine is replaced by valine. According to certain embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 356 tyrosine is replaced by serine. According to certain embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 356 tyrosine is replaced by tryptophan.
- 25 According to certain embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 356 tyrosine is replaced by glutamine. According to certain embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 356 tyrosine is replaced by glycine. According to certain embodiments, a bacterium of the invention
- 30 expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 356 tyrosine is replaced by asparagine. According to certain embodiments, a bacterium

of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 356 tyrosine is replaced by aspartic acid. According to certain embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 356 tyrosine is replaced by glutamic acid.

5 According to certain embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 356 tyrosine is replaced by phenylalanine. According to certain embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 356 tyrosine is replaced by alanine. According to certain embodiments, a bacterium of the invention  
10 expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 356 tyrosine is replaced by isoleucine. According to certain embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 356 tyrosine is replaced by proline. According to certain embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in  
15 SEQ ID NO: 11, wherein at position 356 tyrosine is replaced by histidine. According to certain embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 356 tyrosine is replaced by arginine. According to certain embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 356 tyrosine is replaced  
20 by leucine.

According to certain embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 357 serine is replaced by arginine. According to certain embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 357 serine is  
25 replaced by valine. According to certain embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 357 serine is replaced by proline. According to certain embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11, wherein at  
30 position 357 serine is replaced by glycine. According to certain embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 357 serine is replaced by leucine. According to certain embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in

SEQ ID NO: 11, wherein at position 357 serine is replaced by tyrosine. According to certain embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 357 serine is replaced by alanine. According to certain embodiments, a bacterium of the invention expresses a polypeptide having  
5 the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 357 serine is replaced by asparagine. According to certain embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 357 serine is replaced by phenylalanine. According to certain embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11,  
10 wherein at position 357 serine is replaced by histidine.

According to certain embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 357 serine is replaced by lysine. According to certain embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 357 serine is  
15 replaced by isoleucine. According to certain embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 357 serine is replaced by methionine.

According to certain embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 359 serine is replaced by  
20 arginine. According to certain embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 359 serine is replaced by glycine. According to certain embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 359 serine is replaced by methionine. According to certain embodiments, a bacterium of the invention  
25 expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 359 serine is replaced by phenylalanine. According to certain embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 359 serine is replaced by threonine. According to certain embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in  
30 SEQ ID NO: 11, wherein at position 359 serine is replaced by proline. According to certain embodiments, a bacterium of the invention expresses a polypeptide having the amino acid

sequence set forth in SEQ ID NO: 11, wherein at position 359 serine is replaced by valine. According to certain embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 359 serine is replaced by glutamine. According to certain embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 359 serine is replaced by alanine. According to certain embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 359 serine is replaced by cysteine. According to certain embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 359 serine is replaced by lysine. According to certain embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 359 serine is replaced by glutamic acid. According to certain embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 359 serine is replaced by leucine.

According to certain embodiments, a bacterium of the invention expresses a polypeptide having an amino acid sequence which has at least about 90%, such as at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position Y356, S357 and/or S359, wherein the substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D, Y356E, Y356F, Y356A, Y356I, Y356P, Y356H, Y356R and Y356L; the substitution at position S357 is selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H, S357K, S357I and S357M; and the substitution at position S359 is selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L.

According to certain embodiments, a bacterium of the invention expresses a polypeptide having an amino acid sequence which has at least about 90%, such as at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position Y356 and/or S357, wherein the substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D,

Y356E, Y356F, Y356A, Y356I, Y356P, Y356H, Y356R and Y356L; and the substitution at position S357 is selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H, S357K, S357I and S357M.

According to certain embodiments, a bacterium of the invention expresses a polypeptide having  
5 an amino acid sequence which has at least about 90%, such as at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position Y356 and/or S359, wherein the substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D,  
10 Y356E, Y356F, Y356A, Y356I, Y356P, Y356H, Y356R and Y356L; and the substitution at position S359 is selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L.

According to certain embodiments, a bacterium of the invention expresses a polypeptide having an amino acid sequence which has at least about 90%, such as at least about 93%, at least about  
15 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position S357 and/or S359, wherein the substitution at position S357 is selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H, S357K, S357I and S357M; and the substitution at position S359 is selected from the group  
20 consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L.

According to certain embodiments, a bacterium of the invention expresses a polypeptide having an amino acid sequence which has at least about 90%, such as at least about 93%, at least about  
25 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position Y356, wherein the substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D, Y356E, Y356F, Y356A, Y356I, Y356P, Y356H, Y356R and Y356L.

According to certain embodiments, a bacterium of the invention expresses a polypeptide having  
30 an amino acid sequence which has at least about 90%, such as at least about 93%, at least about

95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position S357, wherein the substitution at position S357 is selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H, S357K, S357I and S357M.

According to certain embodiments, a bacterium of the invention expresses a polypeptide having an amino acid sequence which has at least about 90%, such as at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position S359, wherein the substitution at position S359 is selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L.

According to certain embodiments, a bacterium of the invention expresses a polypeptide having an amino acid sequence which has at least about 93%, such as at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position Y356, wherein the substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D, Y356E, Y356F, Y356A, Y356I, Y356P, Y356H, Y356R and Y356L.

According to certain embodiments, a bacterium of the invention expresses a polypeptide having an amino acid sequence which has at least about 93%, such as at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position S357, wherein the substitution at position S357 is selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H, S357K, S357I and S357M.

According to certain embodiments, a bacterium of the invention expresses a polypeptide having an amino acid sequence which has at least about 93%, such as at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at



position S359, wherein the substitution at position S359 is selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L.

5 According to certain embodiments, a bacterium of the invention expresses a polypeptide having an amino acid sequence which has at least about 95%, such as at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position Y356, wherein the substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D, Y356E, Y356F, Y356A, Y356I, Y356P, Y356H, Y356R and  
10 Y356L.

According to certain embodiments, a bacterium of the invention expresses a polypeptide having an amino acid sequence which has at least about 95%, such as at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position S357, wherein the  
15 substitution at position S357 is selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H, S357K, S357I and S357M.

According to certain embodiments, a bacterium of the invention expresses a polypeptide having an amino acid sequence which has at least about 95%, such as at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set  
20 forth in SEQ ID NO: 11 which comprises an amino acid substitution at position S359, wherein the substitution at position S359 is selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L.

According to certain embodiments, a bacterium of the invention expresses a polypeptide having an amino acid sequence which has at least about 90%, such as at least about 93%, at least about  
25 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position Y356, S357 and S359, wherein the substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D, Y356E, Y356F, Y356A, Y356I, Y356P, Y356H, Y356R and Y356L; the substitution at position S357 is  
30 selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N,

S357F, S357H, S357K, S357I and S357M; and the substitution at position S359 is selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L.

5 According to certain embodiments, a bacterium of the invention expresses a polypeptide having an amino acid sequence which has at least about 90%, such as at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position Y356 and S357, wherein the substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D,  
10 Y356E, Y356F, Y356A, Y356I, Y356P, Y356H, Y356R and Y356L; and the substitution at position S357 is selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H, S357K, S357I and S357M.

According to certain embodiments, a bacterium of the invention expresses a polypeptide having an amino acid sequence which has at least about 90%, such as at least about 93%, at least about  
15 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position Y356 and S359, wherein the substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D, Y356E, Y356F, Y356A, Y356I, Y356P, Y356H, Y356R and Y356L; and the substitution at position  
20 S359 is selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L.

According to certain embodiments, a bacterium of the invention expresses a polypeptide having an amino acid sequence which has at least about 90%, such as at least about 93%, at least about  
25 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position S357 and S359, wherein the substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D, Y356E, Y356F, Y356A, Y356I, Y356P, Y356H, Y356R and Y356L; the substitution at position S357 is selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N,  
30 S357F, S357H, S357K, S357I and S357M; and the substitution at position S359 is selected from the

group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L.

According to certain embodiments, a bacterium of the invention expresses a polypeptide having an amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at  
5 position Y356, S357 and/or S359, wherein the substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D, Y356E, Y356F, Y356A, Y356I, Y356P, Y356H, Y356R and Y356L; the substitution at position S357 is selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H, S357K, S357I and S357M; and the substitution at position S359 is selected from the  
10 group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L; and wherein 1 or more, such as about 1 to about 50, about 1 to about 40, about 1 to about 35, about 1 to about 30, about 1 to about 25, about 1 to about 20, about 1 to about 15, about 1 to about 10, about 1 to about 5, or about 1 to about 3, further amino acid residues are substituted, deleted, and/or inserted.

15 The bacterium may express a polypeptide having an amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position Y356, S357 and/or S359, wherein the substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D, Y356E, Y356F, Y356A, Y356I, Y356P, Y356H, Y356R and Y356L; the substitution at position S357 is selected from the group consisting of S357R, S357V,  
20 S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H, S357K, S357I and S357M; and the substitution at position S359 is selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L; and wherein about 1 to about 5, such as about 1 to about 3, further amino acid residues are substituted, deleted, and/or inserted.

25 According to certain embodiments, the present invention provides a bacterium which comprises within the *thrA* gene one or more nucleotide substitutions resulting in one or more amino acid substitutions in the encoded polypeptide selected from the group consisting of Y356C, S357R and S359R. A bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises one or more (such as two or three) amino acid substitutions  
30 selected from the group consisting of Y356C, S357R and S359R. According to certain embodiments, a bacterium of the invention comprises within the *thrA* gene one or more

nucleotide substitutions resulting in one or more (such as two) amino acid substitutions in the encoded polypeptide selected from the group consisting of Y356C and S357R. A bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises one or more (such as two) amino acid substitutions in the encoded polypeptide selected from the group consisting of Y356C and S357R. According to certain embodiments, a bacterium of the invention comprises within the *thrA* gene one or more nucleotide substitutions resulting in one or more (such as two) amino acid substitutions in the encoded polypeptide selected from the group consisting of Y356C and S359R. A bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises one or more (such as two) amino acid substitutions in the encoded polypeptide selected from the group consisting of Y356C and S359R. According to certain embodiments, a bacterium of the invention comprises within the *thrA* gene one or more nucleotide substitutions resulting in one or more (such as two) amino acid substitutions in the encoded polypeptide selected from the group consisting of S357R and S359R. A bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises one or more (such as two) amino acid substitutions in the encoded polypeptide selected from the group consisting of S357R and S359R.

According to certain embodiments, the bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a Y356C substitution in the encoded polypeptide. A bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a Y356C substitution. According to certain embodiments, the bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a S357R substitution in the encoded polypeptide. A bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a S357R substitution. According to certain embodiments, the bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in a S359R substitution in the encoded polypeptide. A bacterium of the invention may thus express a polypeptide encoded by the *thrA* gene, wherein said polypeptide comprises a S359R substitution.

A bacterium of the invention may thus express an aspartate kinase I / homoserine dehydrogenase I (ThrA) having one or more amino acid substitutions selected from the group consisting of Y356C, S357R and S359R. More particularly, a bacterium of the invention may express a polypeptide

having the amino acid sequence set forth in SEQ ID NO: 11 which comprises one or more amino acid substitutions selected from the group consisting of Y356C, S357R and S359R. According to certain embodiments, the bacterium expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 356 tyrosine is replaced by cysteine. According to

5 certain embodiments, the bacterium expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 357 serine is replaced by arginine. According to certain embodiments, the bacterium expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 359 serine is replaced by arginine.

According to certain embodiments, the bacterium expresses a polypeptide having an amino acid

10 sequence set which has at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 11 which comprises one or more amino acid substitutions selected from the group consisting of Y356C, S357R and S359R.

According to certain embodiments, the bacterium expresses a polypeptide having an amino acid

15 sequence which has at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 356 tyrosine is replaced by cysteine. According to particular embodiments, the bacterium expresses a polypeptide having an amino acid sequence which has at least about 95%, such as at least about 96%, at least about

20 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 356 tyrosine is replaced by cysteine.

According to certain embodiments, the bacterium expresses a polypeptide having the amino acid sequence which has at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the

25 amino acid sequence set forth in SEQ ID NO: 11, wherein at position 357 serine is replaced by arginine. According to particular embodiments, the bacterium expresses a polypeptide having the amino acid sequence which has at least about 95%, such as at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 357 serine is replaced by arginine.

According to certain embodiments, the bacterium expresses a polypeptide having the amino acid sequence which has at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 359 serine is replaced by arginine. According to particular embodiments, the bacterium expresses a polypeptide having the amino acid sequence which has at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 11, wherein at position 359 serine is replaced by arginine.

According to certain embodiments, the bacterium expresses a polypeptide having an amino acid sequence set forth in SEQ ID NO: 11 which comprises one or more amino acid substitutions selected from the group consisting of Y356C, S357R and S359R, wherein 1 or more, such as about 1 to about 50, about 1 to about 40, about 1 to about 35, about 1 to about 30, about 1 to about 25, about 1 to about 20, about 1 to about 15, about 1 to about 10, about 1 to about 5, or about 1 to about 3, further amino acid residues are substituted, deleted, and/or inserted. The bacterium may express a polypeptide having an amino acid sequence set forth in SEQ ID NO: 11 which comprises one or more amino acid substitutions selected from the group consisting of Y356C, S357R and S359R, wherein about 1 to about 5, or about 1 to about 3, further amino acid residues are substituted, deleted, and/or inserted.

The ThrA polypeptide mutant(s) described above may be (over-)expressed by the bacterium by way of an exogenous nucleic acid molecule, such as an expression vector, which has been introduced into the bacterium. Therefore, according to certain embodiments, the bacterium of the invention comprises an exogenous nucleic acid molecule comprising a nucleotide sequence encoding a aspartate kinase I / homoserine dehydrogenase I (ThrA) polypeptide mutant as described above.

For example, a bacterium of the invention may comprise an exogenous nucleic acid molecule, such as an expression vector, comprising a nucleotide sequence encoding a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position Y356, S357 and/or S359. According to certain embodiments, a bacterium of the invention thus comprises an exogenous nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position Y356, S357 and/or S359, wherein the substitution at position Y356 is

selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D, Y356E, Y356F, Y356A, Y356I, Y356P, Y356H, Y356R and Y356L; the substitution at position S357 is selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H, S357K, S357I and S357M; and the substitution at position S359 is selected  
5 from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L.

According to certain embodiments, a bacterium of the invention comprises an exogenous nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide having an amino acid sequence which has at least about 90%, such at least about 93%, at least about 95%, at least  
10 about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position Y356, S357 and/or S359. According to certain embodiments, a bacterium of the invention comprises an exogenous nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide having an amino acid sequence which has at least about 90%, such at  
15 least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position Y356, S357 and/or S359, wherein the substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D, Y356E, Y356F, Y356A, Y356I, Y356P, Y356H, Y356R and  
20 Y356L; the substitution at position S357 is selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H, S357K, S357I and S357M; and the substitution at position S359 is selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L.

According to certain embodiments, a bacterium of the invention comprises an exogenous nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide having an amino acid sequence which has at least about 93%, at least about 95%, at least about 96%, at least about  
25 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position Y356, S357 and/or S359. According to certain embodiments, a bacterium of the invention comprises an exogenous  
30 nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide having an amino acid sequence which has at least about 93%, at least about 95%, at least about 96%, at least about

97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position Y356, S357 and/or S359, wherein the substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D, Y356E, Y356F, Y356A, Y356I, Y356P, Y356H, Y356R and Y356L; the substitution at position S357 is selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H, S357K, S357I and S357M; and the substitution at position S359 is selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L.

According to certain embodiments, a bacterium of the invention comprises an exogenous nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide having an amino acid sequence which has at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position Y356, S357 and/or S359. According to certain embodiments, a bacterium of the invention comprises an exogenous nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide having an amino acid sequence which has at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position Y356, S357 and/or S359, wherein the substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D, Y356E, Y356F, Y356A, Y356I, Y356P, Y356H, Y356R and Y356L; the substitution at position S357 is selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H, S357K, S357I and S357M; and the substitution at position S359 is selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L.

In this connection, the present invention further provides a (isolated) nucleic acid molecule, such an expression vector, comprising a nucleotide sequence encoding a ThrA mutant as described above. Such nucleic acid may be introduced into the bacterium of the invention. According to certain embodiments, the nucleic acid molecule comprises a nucleotide sequence encoding a polypeptide having an amino acid sequence which has at least about 90%, such as at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 11 which



comprises an amino acid substitution at position Y356, S357 and/or S359. According to certain embodiments, the nucleic acid molecule comprises a nucleotide sequence encoding a polypeptide having an amino acid sequence which has at least about 90%, such as at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%,  
5 sequence identity to the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position Y356, S357 and/or S359; wherein the substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D, Y356E, Y356F, Y356A, Y356I, Y356P, Y356H, Y356R and Y356L; the substitution at position S357 is selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y,  
10 S357A, S357N, S357F, S357H, S357K, S357I and S357M; and the substitution at position S359 is selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L.

According to certain embodiments, the nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11 which  
15 comprises an amino acid substitution at position Y356, S357 and/or S359. According to certain embodiments, the nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position Y356, S357 and/or S359, wherein the substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N,  
20 Y356D, Y356E, Y356F, Y356A, Y356I, Y356P, Y356H, Y356R and Y356L; the substitution at position S357 is selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H, S357K, S357I and S357M; and the substitution at position S359 is selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L.

25 The (isolated) nucleic acid molecule may be the exogenous nucleic acid as detailed above. The (isolated) nucleic acid molecule may comprise suitable regulatory elements such as a promoter that is functional in the bacterial cell to cause the production of an mRNA molecule and that is operably linked to the nucleotide sequence encoding said polypeptide. Further details on suitable regulatory elements are provided below with respect to an “*exogenous*” nucleic acid molecule,  
30 and apply *mutatis mutandis*.

The present invention also provides a bacterium which comprises within the *lrp* gene one or more nucleotide substitutions resulting in the amino acid substitution D143G in the encoded polypeptide. Further information regarding *lrp* of, e.g., *Escherichia coli* is available at EcoCyc ([www.biocyc.org](http://www.biocyc.org)) under Accession number EG10547. According to certain embodiments, a bacterium of the invention expresses a polypeptide encoded by the *lrp* gene, wherein in said polypeptide at position 143 D is replaced by G. A representative amino acid sequence of a polypeptide encoded by the *lrp* gene is set forth in SEQ ID NO: 12. According to particular embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 12 or a polypeptide having the amino acid sequence which has at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 12, wherein in said amino acid sequence at position 143 D is replaced by G.

According to certain embodiments, the present invention provides a bacterium which comprises within the *lrp* gene one or more nucleotide substitutions resulting in one or more amino acid substitutions which increase tolerance towards L-serine. More particularly, a bacterium is provided which comprises within the *lrp* gene one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position D143. According to particular embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 12 or a polypeptide having the amino acid sequence which has at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 12, wherein in said amino acid sequence at position 143 D is replaced by another amino acid. Preferably, the one or more amino acid substitutions are non-conservative substitutions.

The present invention also provides a bacterium which comprises within the *rho* gene one or more nucleotide substitutions resulting in the amino acid substitution R87L in the encoded polypeptide. Further information regarding *rho* of, e.g., *Escherichia coli* such as nucleotide sequence of the gene or amino acid sequence of the encoded polypeptide is available at EcoCyc ([www.biocyc.org](http://www.biocyc.org)) under Accession number EG10845. According to certain embodiments, a bacterium of the invention expresses a polypeptide encoded by the *rho* gene, wherein in said polypeptide at position 87 R is replaced by L. A representative amino acid sequence of a

polypeptide encoded by the *rho* gene is set forth in SEQ ID NO: 13. According to particular embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 13 or a polypeptide having the amino acid sequence which has at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%,  
5 at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 13, wherein in said amino acid sequence at position 87 R is replaced by L.

According to certain embodiments, the present invention provides a bacterium which comprises within the *rho* gene one or more nucleotide substitutions resulting in one or more amino acid substitutions which increase tolerance towards L-serine. More particularly, a bacterium is  
10 provided which comprises within the *rho* gene one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position R87. According to particular embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 13 or a polypeptide having the amino acid sequence which has at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%,  
15 at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 13, wherein in said amino acid sequence at position 87 R is replaced by another amino acid. Preferably, the one or more amino acid substitutions are non-conservative substitutions.

The present invention also provides a bacterium which comprises within the *eno* gene one or  
20 more nucleotide substitutions resulting in the amino acid substitution V164L in the encoded polypeptide. Further information regarding *eno* of, e.g., *Escherichia coli* is available at EcoCyc ([www.biocyc.org](http://www.biocyc.org)) under Accession number EG10258. According to certain embodiments, a bacterium of the invention expresses a polypeptide encoded by the *eno* gene, wherein in said polypeptide at position 164 V is replaced by L. A representative amino acid sequence of a  
25 polypeptide encoded by the *eno* gene is set forth in SEQ ID NO: 14. According to particular embodiments, a bacterium of the invention may thus express a polypeptide having the amino acid sequence set forth in SEQ ID NO: 14 or a polypeptide having the amino acid sequence which has at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth  
30 in SEQ ID NO: 14, wherein in said amino acid sequence at position at position 164 V is replaced by L.

According to certain embodiments, the present invention provides a bacterium which comprises within the *eno* gene one or more nucleotide substitutions resulting in one or more amino acid substitutions which increase tolerance towards L-serine. More particularly, a bacterium is provided which comprises within the *eno* gene one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position V164. According to particular  
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embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 14 or a polypeptide having the amino acid sequence which has at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth  
10  
in SEQ ID NO: 14, wherein in said amino acid sequence at position 164 V is replaced by another amino acid. Preferably, the one or more amino acid substitutions are non-conservative substitutions.

The present invention also provides a bacterium which comprises within the *argP* gene one or more nucleotide substitutions resulting in the amino acid substitution V164L in the encoded  
15  
polypeptide. Further information regarding *argP* of, e.g., *Escherichia coli* is available at EcoCyc ([www.biocyc.org](http://www.biocyc.org)) under Accession number EG10490. According to certain embodiments, a bacterium of the invention expresses a polypeptide encoded by the *argP* gene, wherein in said polypeptide at position 132 Q is replaced by K. A representative amino acid sequence of a polypeptide encoded by the *argP* gene is set forth in SEQ ID NO: 15. According to particular  
20  
embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 15 or a polypeptide having the amino acid sequence which has at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 15, wherein in said amino acid sequence at position at position 132 Q is replaced by  
25  
K.

According to certain embodiments, the present invention provides a bacterium which comprises within the *argP* gene one or more nucleotide substitutions resulting in one or more amino acid substitutions which increase tolerance towards L-serine. More particularly, a bacterium is provided which comprises within the *argP* gene one or more nucleotide substitutions resulting in  
30  
an amino acid substitution in the encoded polypeptide at position Q132. According to particular embodiments, a bacterium of the invention expresses a polypeptide having the amino acid

sequence set forth in SEQ ID NO: 15 or a polypeptide having the amino acid sequence which has at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 15, wherein in said amino acid sequence at position 132 Q is replaced by another  
5 amino acid. Preferably, the one or more amino acid substitutions are non-conservative substitutions.

The present invention also provides a bacterium which comprises within the *tufA* gene one or more nucleotide substitutions resulting in the amino acid substitution G19V in the encoded polypeptide. Further information regarding *tufA* of, e.g., *Escherichia coli* is available at EcoCyc  
10 ([www.biocyc.org](http://www.biocyc.org)) under Accession number EG11036. According to certain embodiments, a bacterium of the invention expresses a polypeptide encoded by the *tufA* gene, wherein in said polypeptide at position 19 G is replaced by V. A representative amino acid sequence of a polypeptide encoded by the *tufA* gene is set forth in SEQ ID NO: 16. According to particular  
15 embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 16 or a polypeptide having the amino acid sequence which has at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 16, wherein in said amino acid sequence at position at position 19 G is replaced by  
V.

20 According to certain embodiments, the present invention provides a bacterium which comprises within the *tufA* gene one or more nucleotide substitutions resulting in one or more amino acid substitutions which increase tolerance towards L-serine. More particularly, a bacterium is provided which comprises within the *tufA* gene one or more nucleotide substitutions resulting in  
25 an amino acid substitution in the encoded polypeptide at position G19. According to particular embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 16 or a polypeptide having the amino acid sequence which has at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth  
30 in SEQ ID NO: 16, wherein in said amino acid sequence at position 19 G is replaced by another amino acid. Preferably, the one or more amino acid substitutions are non-conservative substitutions.

The present invention also provides a bacterium which comprises within the *cycA* gene one or more nucleotide substitutions resulting in the amino acid substitution I220V in the encoded polypeptide. Further information regarding *cycA* of, e.g., *Escherichia coli* is available at EcoCyc ([www.biocyc.org](http://www.biocyc.org)) under Accession numbers EG12504. According to certain embodiments, a bacterium of the invention expresses a polypeptide encoded by the *cycA* gene, wherein in said polypeptide at position 220 I is replaced by V. A representative amino acid sequence of a polypeptide encoded by the *cycA* gene is set forth in SEQ ID NO: 17. According to particular embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 17 or a polypeptide having the amino acid sequence which has at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 17, wherein in said amino acid sequence at position at position 220 I is replaced by V.

According to certain embodiments, the present invention provides a bacterium which comprises within the *cycA* gene one or more nucleotide substitutions resulting in one or more amino acid substitutions which increase tolerance towards L-serine. More particularly, a bacterium is provided which comprises within the *cycA* gene one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position I220. According to particular embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 17 or a polypeptide having the amino acid sequence which has at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 17, wherein in said amino acid sequence at position 220 I is replaced by another amino acid. Preferably, the one or more amino acid substitutions are non-conservative substitutions.

The present invention also provides a bacterium which comprises within the *rpe* gene one or more nucleotide substitutions resulting in the amino acid substitution I202T in the encoded polypeptide. Further information regarding *rpe* of, e.g., *Escherichia coli* is available at EcoCyc ([www.biocyc.org](http://www.biocyc.org)) under Accession numbers M004. According to certain embodiments, a bacterium of the invention expresses a polypeptide encoded by the *rpe* gene, wherein in said polypeptide at position 202 I is replaced by T. A representative amino acid sequence of a

polypeptide encoded by the *rpe* gene is set forth in SEQ ID NO: 18. According to particular embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 18 or a polypeptide having the amino acid sequence which has at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%,  
5 at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 18, wherein in said amino acid sequence at position at position 202 I is replaced by T.

According to certain embodiments, the present invention provides a bacterium which comprises within the *rpe* gene one or more nucleotide substitutions resulting in one or more amino acid  
10 substitutions which increase tolerance towards L-serine. More particularly, a bacterium is provided which comprises within the *rpe* gene one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position 202I. According to particular embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 18 or a polypeptide having the amino acid sequence which has  
15 at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 18, wherein in said amino acid sequence at position 202 I is replaced by another amino acid. Preferably, the one or more amino acid substitutions are non-conservative substitutions.

20 The present invention also provides a bacterium which comprises within the *yojI* gene one or more nucleotide substitutions resulting in the amino acid substitution D334H in the encoded polypeptide. Further information regarding *yojI* of, e.g., *Escherichia coli* is available at EcoCyc ([www.biocyc.org](http://www.biocyc.org)) under Accession numbers EG12070. According to certain embodiments, a bacterium of the invention expresses a polypeptide encoded by the *yojI* gene, wherein in said  
25 polypeptide at position 334 D is replaced by H. A representative amino acid sequence of a polypeptide encoded by the *yojI* gene is set forth in SEQ ID NO: 19. According to particular embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 19 or a polypeptide having the amino acid sequence which has  
30 at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth

in SEQ ID NO: 19, wherein in said amino acid sequence at position at position 334 D is replaced by H.

5 According to certain embodiments, the present invention provides a bacterium which comprises within the *yojI* gene one or more nucleotide substitutions resulting in one or more amino acid substitutions which increase tolerance towards L-serine. More particularly, a bacterium is provided which comprises within the *yojI* gene one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position D334. According to particular  
10 embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 19 or a polypeptide having the amino acid sequence which has at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 19, wherein in said amino acid sequence at position 334 D is replaced by another amino acid. Preferably, the one or more amino acid substitutions are non-conservative substitutions.

15 The present invention also provides a bacterium which comprises within the *hyaF* gene one or more nucleotide substitutions resulting in the amino acid substitution V120G in the encoded polypeptide. Further information regarding *hyaF* of, e.g., *Escherichia coli* is available at EcoCyc ([www.biocyc.org](http://www.biocyc.org)) under Accession numbers EG10473. According to certain embodiments, a bacterium of the invention expresses a polypeptide encoded by the *hyaF* gene, wherein in said  
20 polypeptide at position 120 V is replaced by G. A representative amino acid sequence of a polypeptide encoded by the *hyaF* gene is set forth in SEQ ID NO: 20. According to particular embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 20 or a polypeptide having the amino acid sequence which has at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%,  
25 at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 20, wherein in said amino acid sequence at position at position 120 V is replaced by G.

According to certain embodiments, the present invention provides a bacterium which comprises within the *hyaF* gene one or more nucleotide substitutions resulting in one or more amino acid  
30 substitutions which increase tolerance towards L-serine. More particularly, a bacterium is provided which comprises within the *hyaF* gene one or more nucleotide substitutions resulting in



an amino acid substitution in the encoded polypeptide at position V120. According to particular embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 20 or a polypeptide having the amino acid sequence which has at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%,  
5 at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 20, wherein in said amino acid sequence at position 120 V is replaced by another amino acid. Preferably, the one or more amino acid substitutions are non-conservative substitutions.

The present invention also provides a bacterium which comprises within the *pykF* gene one or  
10 more nucleotide substitutions resulting in the amino acid substitution E250\* in the encoded polypeptide, where \* designates a stop codon. Alternatively, the *pykF* gene may comprise one or more nucleotide substitutions resulting in the termination of the encoded polypeptide at a position upstream of position 250. Further information regarding *pykF* of, e.g., *Escherichia coli* is available at EcoCyc ([www.biocyc.org](http://www.biocyc.org)) under Accession numbers EG10804. According to certain  
15 embodiments, a bacterium of the invention expresses a polypeptide encoded by the *pykF* gene, wherein said polypeptide terminates after position 249 or any position upstream thereof. A representative amino acid sequence of a polypeptide encoded by the *pykF* gene is set forth in SEQ ID NO: 21. According to particular embodiments, a bacterium of the invention expresses a  
20 polypeptide having the amino acid sequence set forth in SEQ ID NO: 22 or a polypeptide having the amino acid sequence which has at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 22.

According to other certain embodiments, a bacterium of the invention has been further modified to attenuate the expression of the *pykF* gene (e.g., by inactivation of the gene). Attenuation, and  
25 more particularly inactivation, of the gene expression can be achieved as described herein above. For example, lambda red mediated gene replacement may be used for inactivating gene expression.

The present invention also provides a bacterium which comprises within the *malT* gene one or  
30 more nucleotide substitutions resulting in the amino acid substitution Q420\* in the encoded polypeptide, where \* designates a stop codon. Alternatively, the *malT* gene may comprise one or more nucleotide substitutions resulting in the termination of the encoded polypeptide at a

position upstream of position 420. Further information regarding *malt* of, e.g., *Escherichia coli* is available at EcoCyc ([www.biocyc.org](http://www.biocyc.org)) under Accession numbers EG10562. According to certain embodiments, a bacterium of the invention expresses a polypeptide encoded by the *malt* gene, wherein said polypeptide terminates after position 419 or any position upstream thereof. A  
5 representative amino acid sequence of a polypeptide encoded by the *malt* gene is set forth in SEQ ID NO: 23. According to particular embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 24 or a polypeptide having the amino acid sequence which has at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity  
10 to the amino acid sequence set forth in SEQ ID NO: 24.

According to other certain embodiments, a bacterium of the invention has been further modified to attenuate the expression of the *malt* gene (e.g., by inactivation of the gene). Attenuation, and more particularly inactivation, of the gene expression can be achieved as described herein above. For example, lambda red mediated gene replacement may be used for inactivating gene  
15 expression.

The present invention also provides a bacterium which comprises within the *rpoB* gene one or more nucleotide substitutions resulting in the amino acid substitution P520L in the encoded polypeptide. Further information regarding *rpoB* of, e.g., *Escherichia coli* is available at EcoCyc ([www.biocyc.org](http://www.biocyc.org)) under Accession numbers EG10894. According to certain embodiments, a  
20 bacterium of the invention expresses a polypeptide encoded by the *rpoB* gene, wherein in said polypeptide at position 520 P is replaced by L. A representative amino acid sequence of a polypeptide encoded by the *rpoB* gene is set forth in SEQ ID NO: 25. According to particular embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 25 or a polypeptide having the amino acid sequence which has  
25 at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 25, wherein in said amino acid sequence at position at position 520 P is replaced by L.

According to certain embodiments, the present invention provides a bacterium which comprises  
30 within the *rpoB* gene one or more nucleotide substitutions resulting in one or more amino acid substitutions which increase tolerance towards L-serine. More particularly, a bacterium is

provided which comprises within the *rpoB* gene one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position P520. According to particular embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 25 or a polypeptide having the amino acid sequence which has

5 at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 25, wherein in said amino acid sequence at position 520 P is replaced by another amino acid. Preferably, the one or more amino acid substitutions are non-conservative substitutions.

10 The present invention also provides a bacterium which comprises within the *fumB* gene one or more nucleotide substitutions resulting in the amino acid substitution T218P in the encoded polypeptide. Further information regarding *fumB* of, e.g., *Escherichia coli* is available at EcoCyc ([www.biocyc.org](http://www.biocyc.org)) under Accession numbers EG10357. According to certain embodiments, a bacterium of the invention expresses a polypeptide encoded by the *fumB* gene, wherein in said

15 polypeptide at position 218 T is replaced by P. A representative amino acid sequence of a polypeptide encoded by the *fumB* gene is set forth in SEQ ID NO: 26. According to particular embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 26 or a polypeptide having the amino acid sequence which has

20 at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 26, wherein in said amino acid sequence at position at position 218 T is replaced by P.

According to certain embodiments, the present invention provides a bacterium which comprises within the *fumB* gene one or more nucleotide substitutions resulting in one or more amino acid

25 substitutions which increase tolerance towards L-serine. More particularly, a bacterium is provided which comprises within the *fumB* gene one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position T218. According to particular embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 26 or a polypeptide having the amino acid sequence which has

30 at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth

in SEQ ID NO: 26, wherein in said amino acid sequence at position 218 T is replaced by another amino acid. Preferably, the one or more amino acid substitutions are non-conservative substitutions.

5 The present invention also provides a bacterium which comprises within the *gshA* gene one or more nucleotide substitutions resulting in the amino acid substitution A178V in the encoded polypeptide. Further information regarding *gshA* of, e.g., *Escherichia coli* is available at EcoCyc ([www.biocyc.org](http://www.biocyc.org)) under Accession numbers EG10418. According to certain embodiments, a bacterium of the invention expresses a polypeptide encoded by the *gshA* gene, wherein in said polypeptide at position 178 A is replaced by V. A representative amino acid sequence of a  
10 polypeptide encoded by the *gshA* gene is set forth in SEQ ID NO: 27. According to particular embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 27 or a polypeptide having the amino acid sequence which has at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth  
15 in SEQ ID NO: 27, wherein in said amino acid sequence at position at position 178 A is replaced by V.

According to certain embodiments, the present invention provides a bacterium which comprises within the *gshA* gene one or more nucleotide substitutions resulting in one or more amino acid substitutions which increase tolerance towards L-serine. More particularly, a bacterium is  
20 provided which comprises within the *gshA* gene one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position A178. According to particular embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 27 or a polypeptide having the amino acid sequence which has at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%,  
25 at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 27, wherein in said amino acid sequence at position 178 A is replaced by another amino acid. Preferably, the one or more amino acid substitutions are non-conservative substitutions.

The present invention also provides a bacterium which comprises within the *lamB* gene one or  
30 more nucleotide substitutions resulting in the amino acid substitution Q112\* in the encoded polypeptide, where \* designates a stop codon. Alternatively, the *lamB* gene may comprise one or

more nucleotide substitutions resulting in the termination of the encoded polypeptide at a position upstream of position 112. Further information regarding *lamB* of, e.g., *Escherichia coli* such as nucleotide sequence of the gene or amino acid sequence of the encoded polypeptide is available at EcoCyc ([www.biocyc.org](http://www.biocyc.org)) under Accession numbers EG10528. According to certain

5   embodiments, a bacterium of the invention expresses a polypeptide encoded by the *lamB* gene, wherein said polypeptide terminates after position 111 or any position upstream thereof. A representative amino acid sequence of a polypeptide encoded by the *lamB* gene is set forth in SEQ ID NO: 28. According to particular embodiments, a bacterium of the invention expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 29 or a polypeptide having

10   the amino acid sequence which has at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 29.

According to other certain embodiments, a bacterium of the invention has been further modified to attenuate the expression of the *lamB* gene (e.g., by inactivation of the gene). Attenuation, and

15   more particularly inactivation, of the gene expression can be achieved as described herein above. For example, lambda red mediated gene replacement may be used for inactivating gene expression.

A bacterium of the present invention may comprise one or more, such as two or more, three or more, four or more, or five or more, gene mutations as mentioned.

20   For example, the bacterium may comprise one or more (such as two or more) gene mutations selected from the group consisting of: one or more nucleotide substitutions within the *Irp* gene resulting in an amino acid substitution at position D143 in the encoded polypeptide, one or more nucleotide substitutions within the *rho* gene resulting in an amino acid substitution at position R87 in the encoded polypeptide, one or more nucleotide substitutions within the *eno* gene

25   resulting in an amino acid substitution at position V164 in the encoded polypeptide, and one or more nucleotide substitutions within the *argP* gene resulting in an amino acid substitution at position V164 in the encoded polypeptide.

For example, the bacterium may comprise one or more (such as two or more) gene mutations selected from the group consisting of: one or more nucleotide substitutions within the *Irp* gene

resulting in the amino acid substitution D143G in the encoded polypeptide, one or more nucleotide substitutions within the *rho* gene resulting in the amino acid substitution R87L in the encoded polypeptide, one or more nucleotide substitutions within the *eno* gene resulting in the amino acid substitution V164L in the encoded polypeptide, and one or more nucleotide  
5 substitutions within the *argP* gene resulting in the amino acid substitution V164L in the encoded polypeptide.

According to certain embodiments, a bacterium of the invention comprises within the *thrA* gene one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position Y356, said substitution being selected from the group consisting of Y356C,  
10 Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D, Y356E, Y356F, Y356A, Y356I, Y356P, Y356H, Y356R and Y356L; one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position S357, said substitution being selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H,  
15 S357K, S357I and S357M; and/or one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position S359, said substitution being selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L; and one or more (such as two or more) gene mutations selected from the group consisting of: one or more nucleotide substitutions within the *lrp* gene resulting in an  
20 amino acid substitution at position D143 (such as D143G) in the encoded polypeptide, one or more nucleotide substitutions within the *rho* gene resulting in the amino acid substitution at position R87 (such as R87L) in the encoded polypeptide, one or more nucleotide substitutions within the *eno* gene resulting in the amino acid substitution at position V164 (such as V164L) in the encoded polypeptide, and one or more nucleotide substitutions within the *argP* gene resulting in an amino acid substitution at position V164 (such as V164L) in the encoded  
25 polypeptide.

According to certain embodiments, a bacterium of the present invention comprises one or more nucleotide substitutions within the *thrA* gene resulting in one or more amino acid substitution in the encoded polypeptide selected from the group consisting of Y356C, S357R and S359R; and one or more (such as two or more) gene mutations selected from the group consisting of: one or  
30 more nucleotide substitutions within the *lrp* gene resulting in the amino acid substitution D143G in the encoded polypeptide, one or more nucleotide substitutions within the *rho* gene resulting in

the amino acid substitution R87L in the encoded polypeptide, one or more nucleotide substitutions within the *eno* gene resulting in the amino acid substitution V164L in the encoded polypeptide, and one or more nucleotide substitutions within the *argP* gene resulting in the amino acid substitution V164L in the encoded polypeptide.

- 5 According to certain embodiments, a bacterium of the present invention comprises one or more nucleotide substitutions within the *thrA* gene resulting in the amino acid substitution Y356C in the encoded polypeptide; and one or more (such as two or more) gene mutations selected from the group consisting of: one or more nucleotide substitutions within the *lrp* gene resulting in the amino acid substitution D143G in the encoded polypeptide, one or more nucleotide substitutions  
10 within the *rho* gene resulting in the amino acid substitution R87L in the encoded polypeptide, one or more nucleotide substitutions within the *eno* gene resulting in the amino acid substitution V164L in the encoded polypeptide, and one or more nucleotide substitutions within the *argP* gene resulting in the amino acid substitution V164L in the encoded polypeptide.

- 15 According to certain embodiments, a bacterium of the present invention comprises one or more nucleotide substitutions within the *thrA* gene resulting in the amino acid substitution S357R in the encoded polypeptide; and one or more (such as two or more) gene mutations selected from the group consisting of: one or more nucleotide substitutions within the *lrp* gene resulting in the amino acid substitution D143G in the encoded polypeptide, one or more nucleotide substitutions within the *rho* gene resulting in the amino acid substitution R87L in the encoded polypeptide, one  
20 or more nucleotide substitutions within the *eno* gene resulting in the amino acid substitution V164L in the encoded polypeptide, and one or more nucleotide substitutions within the *argP* gene resulting in the amino acid substitution V164L in the encoded polypeptide.

- 25 According to certain embodiments, a bacterium of the present invention comprises one or more nucleotide substitutions within the *thrA* gene resulting in the substitution S359R in the encoded polypeptide; and one or more (such as two or more) gene mutations selected from the group consisting of: one or more nucleotide substitutions within the *lrp* gene resulting in the amino acid substitution D143G in the encoded polypeptide, one or more nucleotide substitutions within the *rho* gene resulting in the amino acid substitution R87L in the encoded polypeptide, one or more nucleotide substitutions within the *eno* gene resulting in the amino acid substitution V164L in the

encoded polypeptide, and one or more nucleotide substitutions within the *argP* gene resulting in the amino acid substitution V164L in the encoded polypeptide.

According to certain embodiments, a bacterium of the invention comprises within its genome a deletion of the first 5 bp of gene *rhtA* gene, a complete deletion of genes *ompX* and *ybiP*, a  
5 deletion of 239 bp of sRNA *rybA* and a deletion of 77 bp of gene *mntS*. According to certain  
embodiments, the bacterium comprises within its genome a deletion of about 2854 bp from a  
location which corresponds to location 850092 in the genome sequence NC\_000913. This deletion  
results in a deletion of the first 5 bp of gene *rhtA* gene, a complete deletion of genes *ompX* and  
*ybiP*, a deletion of 239 bp of sRNA *rybA* and a 77 bp deletion of gene *mntS*. Such deletion can be  
10 achieved by using the lambda-red or cam-sacB-system.

According to certain embodiments, a bacterium of the invention comprises within its genome an  
insertion of an insertion sequence element IS1 (e.g., having a length of about 768 bp) in the  
intergenic region between genes *trxA* and *rho*. According to certain embodiments, the bacterium  
comprises within its genome an insertion of an insertion sequence element IS1 (e.g., having a  
15 length of about 768 bp) in the lagging strand at a location which corresponds to location 3966174  
in the genome sequence NC\_000913. According to particular embodiments, the bacterium further  
comprises a duplication of around 9 bp upstream and downstream of insertion sequence.

According to certain embodiments, a bacterium of the invention comprises within its genome an  
insertion of 1bp in the intergenic region between genes *gcvA* and *ygdI*. According to certain  
20 embodiments, the bacterium comprises within its genome an insertion of 1bp at a location which  
corresponds to location 2942629 in the genome sequence NC\_000913.

According to certain embodiments, a bacterium of the invention comprises within its genome an  
insertion of an insertion sequence element IS4 (e.g., having a length of about 1342 bp) in the  
intergenic region between genes *gcvA* and *ygdI*. According to certain embodiments, the  
25 bacterium comprises within its genome an insertion of an insertion sequence element IS4 (e.g.,  
having a length of about 1342 bp) at a location which corresponds to location 2942878 in the  
genome sequence NC\_000913. According to particular embodiments, the bacterium further  
comprises a duplication of about 13 bp upstream and downstream of insertion sequence.



According to certain embodiments, a bacterium of the invention comprises within its genome an insertion of 1bp in the intergenic region between genes *dapA* and *gcvR*. According to certain embodiments, the bacterium comprises within its genome an insertion of 1bp at a location which corresponds to location 2599854 in the genome sequence NC\_000913.

- 5 According to certain embodiments, a bacterium of the invention comprises within its genome an insertion of an insertion sequence element IS1 (e.g., having a length of about 768 bp) which lead to a truncation of gene *frc*. According to certain embodiments, the bacterium comprises within its genome an insertion sequence element IS1 (e.g., having a length of about 768 bp) in the lagging strand at a location which corresponds to location 2492323 in the genome sequence NC\_000913.
- 10 According to particular embodiments, the bacterium further comprises a duplication of 9 bp upstream and downstream of insertion sequence.

According to other certain embodiments, a bacterium of the invention has been further modified to attenuate the expression of the *frc* gene (e.g., by inactivation of the gene). Attenuation, and more particularly inactivation, of the gene expression can be achieved as described herein above.

- 15 For example, lambda red mediated gene replacement may be used for inactivating gene expression.

- According to certain embodiments, a bacterium of the invention comprises within its genome an insertion of an insertion sequence element IS5 (e.g., having a length of about 1195 bp) which leads to deletion of the majority of gene *aroP*. According to certain embodiments, the bacterium
- 20 comprises within its genome an insertion sequence element IS5 (e.g., having a length of about 1195 bp) at a location which corresponds to location 121518 in the genome sequence NC\_000913. According to particular embodiments, the bacterium further comprises a duplication of 4 bp upstream and downstream of insertion sequence.

- According to other certain embodiments, a bacterium of the invention has been further modified
- 25 to attenuate the expression of the *aroP* gene (e.g., by inactivation of the gene). Attenuation, and more particularly inactivation, of the gene expression can be achieved as described herein above. For example, lambda red mediated gene replacement may be used for inactivating gene expression.

- According to certain embodiments, a bacterium of the invention comprises within its genome an
- 30 insertion of an insertion sequence element IS1 (e.g., having a length of about 768 bp) in the

intergenic region between genes *mdtJ* and *tqsA*. According to certain embodiments, the bacterium comprises within its genome an insertion sequence element IS1 (e.g., having a length of about 768 bp) in the lagging strand at a location which corresponds to location 1673670 in the genome sequence NC\_000913. According to particular embodiments, the bacterium further  
5 comprises a duplication of around 9 bp upstream and downstream of insertion sequence.

According to certain embodiments, a bacterium of the invention comprises within its genome a nucleotide substitution, such as a C->T substitution, within the intergenic region between genes *trxB* and *lrp*. According to certain embodiments, the bacterium comprises within its genome a nucleotide substitution, such as a C->T substitution, at a location which corresponds to location  
10 923321 in the genome sequence NC\_000913. Such mutation is 271 bp upstream of *trxB* and 274 bp upstream of *lrp*.

According to certain embodiments, a bacterium of the invention comprises within its genome a nucleotide substitution, such as a T->C substitution, within the intergenic region between genes *yftB* and *fkfB*. According to certain embodiments, the bacterium comprises within its genome a nucleotide substitution, such as a T->C substitution, at a location which corresponds to location  
15 4428871 in the genome sequence NC\_000913. Such mutation is 154 bp upstream of *yftB* and 64 bp upstream of *fkfB*.

As further demonstrate herein, attenuating (e.g., by inactivating of the gene) the expression of a gene coding for a polypeptide having Glucose 6-phosphate-1-dehydrogenase (G6PDH) activity in a reversed engineered strain resulted in a significantly increased production and yield of L-serine  
20 from glucose as shown in Table S9 (Example 8).

Therefore, according to certain embodiments, a bacterium of the invention has been modified to attenuate expression of a gene coding for a polypeptide having glucose 6-phosphate-1-dehydrogenase (G6PDH) activity. More particularly, the present invention provides a bacterium  
25 which has been modified to attenuate the expression of the gene *zwf*. Further information regarding *zwf* of, e.g., *Escherichia coli* is available at EcoCyc ([www.biocyc.org](http://www.biocyc.org)) under Accession numbers EG11221. A representative nucleotide sequence of *zwf* is set forth in SEQ ID NO: 30.

The gene expression may be attenuated by inactivation of the gene. Thus, a bacterium according to the invention can be one which has been modified to inactivate the gene coding for a

polypeptide having Glucose 6-phosphate-1-dehydrogenase (G6PDH) activity (e.g, by inactivation of the gene).

Attenuation, and more particularly inactivation, of the gene expression can be achieved as described herein above. For example, lambda red mediated gene replacement may be used for  
5 inactivating gene expression.

According to certain embodiments, a bacterium of the invention expresses a polypeptide encoded by the *brnQ* gene, wherein said polypeptide terminates after position 308 or any position upstream thereof. According to other certain embodiments, a bacterium of the invention has been further modified to attenuate the expression of the *brnQ* gene (e.g., by inactivation of the  
10 gene). Attenuation, and more particularly inactivation, of the gene expression can be achieved as described herein above. For example, lambda red mediated gene replacement may be used for inactivating gene expression.

According to certain embodiments, a bacterium of the invention has been further modified to attenuate expression of a gene coding for a polypeptide having glucose 6-phosphate-1-  
15 dehydrogenase (G6PDH) activity; express a polypeptide encoded by the *thrA* gene, wherein in said polypeptide at position 357 serine is replaced by arginine; expresses a polypeptide encoded by the *rho* gene, wherein in said polypeptide at position 87 R is replaced by L; and expresses a polypeptide encoded by the *brnQ* gene, wherein said polypeptide terminates after position 308 or any position upstream thereof. Alternatively, the bacterium may be modified to attenuate the  
20 expression of the *brnQ* gene (e.g., by inactivation of the gene). Suitable methods for attenuation of gene expression are described above.

Further information regarding *brnQ* of, e.g., *Escherichia coli* is available at EcoCyc ([www.biocyc.org](http://www.biocyc.org)) under Accession numbers EG12168. A representative amino acid sequence of *brnQ* is set forth in SEQ ID NO: 31.

25 According to particular embodiments, the bacterium has been further modified to attenuate expression of the gene *zwf* (e.g, by inactivation of the gene); expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11 or a polypeptide having at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 11,  
30 wherein in said amino acid sequence at position 357 serine is replaced by arginine; express a

polypeptide having the amino acid sequence set forth in SEQ ID NO: 13 or a polypeptide having the amino acid sequence which has at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 13, wherein in said amino acid sequence at  
5 position 87 R is replaced by L; and expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 32 or a polypeptide having the amino acid sequence which has at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 32.

10 According to particular embodiments, the bacterium has been further modified to attenuate expression of the gene *zwf* and *brnQ* (e.g., by inactivation of the genes); and expresses a polypeptide having the amino acid sequence set forth in SEQ ID NO: 11 or a polypeptide having at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in  
15 SEQ ID NO: 11, wherein in said amino acid sequence at position 357 serine is replaced by arginine; express a polypeptide having the amino acid sequence set forth in SEQ ID NO: 13 or a polypeptide having the amino acid sequence which has at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 13, wherein in said amino acid  
20 sequence at position 87 R is replaced by L.

As detailed above, a bacterium of the invention may have been modified to overexpress certain polypeptides as detailed herein, which means that an exogenous nucleic acid molecule, such as a DNA molecule, which comprises a nucleotide sequence encoding said polypeptide has been introduced in the bacterium. Techniques for introducing exogenous nucleic acid molecule, such as  
25 a DNA molecule, into a bacterial cells are well-known to those of skill in the art, and include transformation (e.g., heat shock or natural transformation) among others.

In order to facilitate overexpression of a polypeptide in the bacterium, the exogenous nucleic acid molecule may comprise suitable regulatory elements such as a promoter that is functional in the bacterial cell to cause the production of an mRNA molecule and that is operably linked to the  
30 nucleotide sequence encoding said polypeptide.

Promoters useful in accordance with the invention are any known promoters that are functional in a given host cell to cause the production of an mRNA molecule. Many such promoters are known to the skilled person. Such promoters include promoters normally associated with other genes, and/or promoters isolated from any bacteria. The use of promoters for protein expression is generally known to those of skilled in the art of molecular biology, for example, see Sambrook et al., *Molecular cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. , 1989. The promoter employed may be inducible, such as a temperature inducible promoter (e.g., a pL or pR phage lambda promoters, each of which can be controlled by the temperature-sensitive lambda repressor cI857). The term “inducible” used in the context of a promoter means that the promoter only directs transcription of an operably linked nucleotide sequence if a stimulus is present, such as a change in temperature or the presence of a chemical substance (“chemical inducer”). As used herein, “chemical induction” according to the present invention refers to the physical application of an exogenous or endogenous substance (incl. macromolecules, e.g., proteins or nucleic acids) to a host cell. This has the effect of causing the target promoter present in the host cell to increase the rate of transcription. Alternatively, the promoter employed may be constitutive. The term “constitutive” used in the context of a promoter means that the promoter is capable of directing transcription of an operably linked nucleotide sequence in the absence of stimulus (such as heat shock, chemicals etc.).

Temperature induction systems work, for example, by employing promoters that are repressed by thermolabile repressors. These repressors are active at lower temperatures for example at 30°C, while unable to fold correctly at 37 °C and are therefore inactive. Such circuits therefore can be used to directly regulate the genes of interest (St-Pierre et al. 2013) also by genome integration of the genes along with the repressors. Examples of such as a temperature inducible expression system are based on the pL and/or pR λ phage promoters which are regulated by the thermolabile cI857 repressor. Similar to the genome integrated DE3 system, the expression of the T7 RNA polymerase gene may also be controlled using a temperature controlled promoter system (Mertens et al. 1995), while the expression of the genes of interest can be controlled using a T7 promoter.

Non-limiting examples of promoters functional in bacteria, such as *Escherichia coli*, include both constitutive and inducible promoters such as T7 promoter, the beta-lactamase and lactose promoter systems; alkaline phosphatase (phoA) promoter, a tryptophan (trp) promoter system,

tetracycline promoter, lambda-phage promoter, ribosomal protein promoters; and hybrid promoters such as the tac promoter. Other bacterial and synthetic promoters are also suitable.

Besides a promoter, the exogenous nucleic acid molecule may further comprise at least one regulatory element selected from a 5' untranslated region (5'UTR) and 3' untranslated region (3' UTR). Many such 5' UTRs and 3' UTRs derived from prokaryotes and eukaryotes are well known to the skilled person. Such regulatory elements include 5' UTRs and 3' UTRs normally associated with other genes, and/or 5' UTRs and 3' UTRs isolated from any bacteria.

Usually, the 5' UTR contains a ribosome binding site (RBS), also known as the Shine Dalgarno sequence which is usually 3-10 base pairs upstream from the initiation codon.

The exogenous nucleic acid molecule may be a vector or part of a vector, such as an expression vector. Normally, such a vector remains extrachromosomal within the bacterial cell which means that it is found outside of the nucleus or nucleoid region of the bacterium.

It is also contemplated by the present invention that the exogenous nucleic acid molecule is stably integrated into the genome of the bacterium. Means for stable integration into the genome of a host cell, e.g., by homologous recombination, are well known to the skilled person.

A bacterium in accordance with the present invention can be produced from any suitable bacterium, such as a Gram-positive or Gram-negative bacterium.

Examples of bacteria which can be used to derive a bacterium of the invention belong to the *Enterobacteriaceae* family, such as bacteria belonging to a genus selected from the group consisting of *Escherichia*, *Arsenophonus*, *Biostraticola*, *Brenneria*, *Buchnera*, *Budvicia*, *Buttiauxella*, *Cedecea*, *Citrobacter*, *Cosenzaea*, *Cronobacter*, *Dickeya*, *Edwardsiella*, *Enterobacter*, *Erwinia*, *Ewingella*, *Gibbsiella*, *Hafnia*, *Klebsiella*, *Leclercia*, *Leminorella*, *Lonsdalea*, *Mangrovibacter*, *Moellerella*, *Morganella*, *Obesumbacterium*, *Pantoea*, *Pectobacterium*, *Phaseolibacter*, *Photorhabdus*, *Plesiomonas*, *Proteus*, *Rahnella*, *Raoultella*, *Saccharobacter*, *Salmonella*, *Samsonia*, *Serratia*, *Shimwellia*, *Sodalis*, *Tatumella*, *Thorsellia*, *Trabulsiella*, *Wigglesworthia*, *Yersinia* and *Yokenella*.

According to certain other embodiments, the bacterium belongs to a genus selected from the group selected from *Escherichia*, *Bacillus*, *Lactococcus*, *Lactobacillus*, *Clostridium*, *Corynebacterium*, *Geobacillus*, *Streptococcus*, *Pseudomonas*, *Streptomyces*, *Shigella*,

*Acinetobacter, Citrobacter, Salmonella, Klebsiella, Enterobacter, Erwinia, Kluyvera, Serratia, Cedecea, Morganella, Hafnia, Edwardsiella, Providencia, Proteus and Yersinia.*

According to particular embodiments, the bacterium belongs to the genus *Escherichia*. According to particular embodiments, the bacterium is *Escherichia coli*. Non-limiting examples of a  
5 bacterium belonging to the genus *Escherichia*, which can be used to derive a bacterium of the invention, are *Escherichia coli* K-12 (especially substrain MG1655 or W3110), BL21, W, or Crooks. According to more particular embodiments, the bacterium is *Escherichia coli* K-12.

According to other particular embodiments, the bacterium belongs to the genus *Corynebacterium*. A non-limiting example of a bacterium of the genus *Corynebacterium* is  
10 *Corynebacterium glutamicum*. According to more particular embodiments, the bacterium is *Corynebacterium glutamicum*.

According to other particular embodiments, the bacterium belongs to the genus *Bacillus*. Non-limiting examples of a bacterium of the genus *Bacillus* are *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, and *Bacillus mojavensis*. According to more particular embodiments, the  
15 bacterium is *Bacillus subtilis*. According to other more particular embodiments, the bacterium is *Bacillus licheniformis*.

According to other particular embodiments, the bacterium belongs to the genus *Lactococcus*. A non-limiting example of a bacterium of the genus *Lactococcus* is *Lactococcus lactis*. According to more particular embodiments, the bacterium is *Lactococcus lactis*.

20 According to other particular embodiments, the bacterium belongs to the genus *Streptomyces*. A non-limiting example of a bacterium of the genus *Streptomyces* are *Streptomyces lividans*, *Streptomyces coelicolor*, or *Streptomyces griseus*. According to more particular embodiments, the bacterium is *Streptomyces lividans*. According to other more particular embodiments, the bacterium is *Streptomyces coelicolor*. According to other more particular embodiments, the  
25 bacterium is *Streptomyces griseus*.

According to other particular embodiments, the bacterium belongs to the genus *Pseudomonas*. A non-limiting example of a bacterium of the genus *Pseudomonas* is *Pseudomonas putida*. According to more particular embodiments, the bacterium is *Pseudomonas putida*.

*Method of the invention*

The present invention also provides methods for producing L-serine or a L-serine derivative using a bacterium according to the present invention. Particularly, the present invention provides a method for producing L-serine or a L-serine derivative, said method comprises cultivating a bacterium as detailed herein in a culture medium.

According to certain embodiments, present invention provides a method for producing L-serine. Particularly, the present invention provides a method for producing L-serine, said method comprises cultivating a bacterium as detailed herein in a culture medium. The method may further comprise collecting L-serine from the culture medium.

According to certain embodiments, present invention provides a method for producing a L-serine derivative. Particularly, the present invention provides a method for producing a L-serine derivative, said method comprises cultivating a bacterium as detailed herein in a culture medium. The L-serine derivative may be selected from the group consisting of L-cysteine, L-methionine, L-glycine, O-acetylserine, L-tryptophan, thiamine, ethanolamine and ethylene glycol. The method may further comprise collecting the L-serine derivative from the culture medium.

According to certain embodiments, present invention provides a method for producing L-cysteine. Particularly, the present invention provides a method for producing L-cysteine, said method comprises cultivating a bacterium as detailed herein in a culture medium. The method may further comprise collecting L-cysteine from the culture medium.

According to certain embodiments, present invention provides a method for producing L-methionine. Particularly, the present invention provides a method for producing L-methionine; said method comprises cultivating a bacterium as detailed herein in a culture medium. The method may further comprise collecting L-methionine from the culture medium.

According to certain embodiments, present invention provides a method for producing L-glycine. Particularly, the present invention provides a method for producing L-glycine; said method comprises cultivating a bacterium as detailed herein in a culture medium. The method may further comprise collecting L-glycine from the culture medium.

According to certain embodiments, present invention provides a method for producing L-cysteine. Particularly, the present invention provides a method for producing O-acetylserine, said method



comprises cultivating a bacterium as detailed herein in a culture medium. The method may further comprise collecting O-acetylserine from the culture medium.

According to certain embodiments, present invention provides a method for producing L-glycine. Particularly, the present invention provides a method for producing L-tryptophan; said method  
5 comprises cultivating a bacterium as detailed herein in a culture medium. The method may further comprise collecting L-tryptophan from the culture medium.

According to certain embodiments, present invention provides a method for producing L-cysteine. Particularly, the present invention provides a method for producing thiamine, said method  
10 comprises cultivating a bacterium as detailed herein in a culture medium. The method may further comprise collecting thiamine from the culture medium.

According to certain embodiments, present invention provides a method for producing ethanolamine. Particularly, the present invention provides a method for producing ethanolamine; said method comprises cultivating a bacterium as detailed herein in a culture medium. The method may further comprise collecting ethanolamine from the culture medium.

15 According to certain embodiments, present invention provides a method for producing ethylene glycol. Particularly, the present invention provides a method for producing ethylene glycol; said method comprises cultivating a bacterium as detailed herein in a culture medium. The method may further comprise collecting ethylene glycol from the culture medium.

20 The culture medium employed may be any conventional medium suitable for culturing a bacterium cell in question, and may be composed according to the principles of the prior art. The medium will usually contain all nutrients necessary for the growth and survival of the respective bacterium, such as carbon and nitrogen sources and other inorganic salts. Suitable media, e.g. minimal or complex media, are available from commercial suppliers, or may be prepared according to published receipts, e.g. the American Type Culture Collection (ATCC) Catalogue of  
25 strains. Non-limiting standard medium well known to the skilled person include Luria Bertani (LB) broth, Sabouraud Dextrose (SD) broth, MS broth, Yeast Peptone Dextrose, BMMY, GMMY, or Yeast Malt Extract (YM) broth, which are all commercially available. A non-limiting example of suitable media for culturing bacterial cells, such as E. coli cells, including minimal media and rich media such as Luria Broth (LB), M9 media, M17 media, SA media, MOPS media, Terrific Broth, YT  
30 and others.

In order to further increase the yield of L-serine or L-serine derivative, the culture medium may further be supplemented with L-threonine. The culture medium may generally contain L-threonine at a concentration from about 0.05 to about 10 g/L, such as from about 0.05 to about 5 g/L, from about 0.05 to about 2.5 g/L, from about 0.05 to about 1 g/L or from about 0.05 to about 5 g/L. According to certain embodiments, the culture medium contains L-threonine at a concentration from about 0.05 to about 5 g/L. According to certain other embodiments, the culture medium contains L-threonine at a concentration from about 0.05 to about 2.5 g/L. According to certain other embodiments, the culture medium contains L-threonine at a concentration from about 0.05 to about 1 g/L. According to certain other embodiments, the culture medium contains L-threonine at a concentration from about 0.05 to about 0.5 g/L. According to certain other embodiments, the culture medium contains L-threonine at a concentration from about 0.1 to about 1 g/L. According to other embodiments, the culture medium contains L-threonine at a concentration from about 0.2 to about 1 g/L.

The carbon source may be any suitable carbon substrate known in the art, and in particular any carbon substrate commonly used in the cultivation of bacteria and/ or fermentation. Non-limiting examples of suitable fermentable carbon substrates are C5 sugars (such as arabinose or xylose), C6 sugars (such as glucose), acetate, glycerol, plant oils, sucrose, yeast extract, peptone, casaminoacids or mixtures thereof. A carbon source of particular interest is a C6 sugar such as glucose.

As the nitrogen source, various ammonium salts such as ammonia and ammonium sulfate, other nitrogen compounds such as amines, a natural nitrogen source such as peptone, soybean-hydrolysate, and digested fermentative microorganism can be used. As minerals, potassium monophosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, calcium chloride, and the like can be used.

The cultivation can be preferably performed under aerobic conditions, such as by a shaking culture, and by a stirring culture with aeration, at a temperature of about 20 to about 40 °C, such as about 30 to 38 °C, preferably about 37°C. The pH of the culture is usually from about 5 and about 9, such as from about 6.5 and 7.5. The pH of the culture can be adjusted with ammonia, calcium carbonate, various acids, various bases, and buffers. Usually, 1 to 5-day cultivation leads to accumulation of L-serine in the culture medium.

After cultivation, solids such as cells can be removed from the culture medium by centrifugation or membrane filtration. L-serine or the L-serine derivative can be collected by conventional method for isolation and purification chemical compounds from a medium. Well-known purification procedures include, but are not limited to, centrifugation or filtration, precipitation, ion exchange, chromatographic methods such as e.g. ion exchange chromatography or gel filtration chromatography, and crystallization methods.

The present invention thus provides L-serine or a L-serine derivative obtainable by a method as detailed herein.

## 10 **Certain other definitions**

The term "bacterium having ability to produce L-serine" as used herein means a bacterium which is able to produce and cause accumulation of L-serine in a culture medium, can mean that the bacterium is able to cause accumulation in an amount not less than 0.4 g/L, when cultured in minimal M9 media supplemented with 2 g/L glucose, 2 mM glycine and 1 mM threonine at 37°C with adequate aeration for 40 hours.

The phrase "bacterium which has been modified to attenuate expression of at least one gene encoding a polypeptide having serine deaminase activity" as used herein means that the bacterium has been modified in such a way that the modified bacterium contains a reduced amount of the at least one polypeptide having serine deaminase activity. More particularly, the phrase means that the bacterium is unable to synthesize the polypeptide(s) having serine deaminase activity. An attenuate expression can be determined by comparing the level of expression of the polypeptide having serine deaminase activity encoded by the at least one gene within the modified bacterium compared to that of an otherwise identical bacterium that does not carry said modification (reference bacterium).

The phrase "bacterium which has been modified to attenuate expression of the gene encoding a polypeptide having serine hydroxymethyltransferase activity" as used herein means that the bacterium has been modified in such a way that the modified bacterium contains a reduced amount of the polypeptide having serine hydroxymethyltransferase activity. More particularly, the phrase means that the bacterium is unable to synthesize a polypeptide having serine

hydroxymethyltransferase activity. An attenuate expression can be determined by comparing the level of expression of the polypeptide having serine hydroxymethyltransferase activity encoded by the gene within the modified bacterium compared to that of an otherwise identical bacterium that does not carry said modification (reference bacterium).

5 The phrase "bacterium which has been modified to attenuate expression of the gene encoding a polypeptide having glucose 6-phosphate-1-dehydrogenase (G6PDH) activity" as used herein means that the bacterium has been modified in such a way that the modified bacterium contains a reduced amount of the polypeptide having glucose 6-phosphate-1-dehydrogenase (G6PDH) activity. More particularly, the phrase means that the bacterium is unable to synthesize a  
10 polypeptide having glucose 6-phosphate-1-dehydrogenase (G6PDH) activity. An attenuate expression can be determined by comparing the level of expression of the polypeptide having glucose 6-phosphate-1-dehydrogenase (G6PDH) activity encoded by the gene within the modified bacterium compared to that of an otherwise identical bacterium that does not carry said modification (reference bacterium).

15 The phrase "bacterium of the invention has been further modified to attenuate the expression of the *pykF* gene" as used herein means that the bacterium has been modified in such a way that the modified bacterium contains a reduced amount of the polypeptide encoded by the *pykF* gene. More particularly, the phrase means that the bacterium is unable to synthesize a polypeptide encoded by the *pykF* gene. An attenuate expression can be determined by comparing the level of  
20 expression of the polypeptide encoded by the *pykF* gene within the modified bacterium compared to that of an otherwise identical bacterium that does not carry said modification (reference bacterium).

The phrase "bacterium of the invention has been further modified to attenuate the expression of the *malT* gene" as used herein means that the bacterium has been modified in such a way that  
25 the modified bacterium contains a reduced amount of the polypeptide encoded by the *malT* gene. More particularly, the phrase means that the bacterium is unable to synthesize a polypeptide encoded by the *malT* gene. An attenuate expression can be determined by comparing the level of expression of the polypeptide encoded by the *malT* gene within the modified bacterium compared to that of an otherwise identical bacterium that does not carry said  
30 modification (reference bacterium).

The phrase “bacterium of the invention has been further modified to attenuate the expression of the *lamB* gene” as used herein means that the bacterium has been modified in such a way that the modified bacterium contains a reduced amount of the polypeptide encoded by the *lamB* gene. More particularly, the phrase means that the bacterium is unable to synthesize a polypeptide encoded by the *lamB* gene. An attenuate expression can be determined by comparing the level of expression of the polypeptide encoded by the *lamB* gene within the modified bacterium compared to that of an otherwise identical bacterium that does not carry said modification (reference bacterium).

The phrase “bacterium of the invention has been further modified to attenuate the expression of the *frc* gene” as used herein means that the bacterium has been modified in such a way that the modified bacterium contains a reduced amount of the polypeptide encoded by the *frc* gene. More particularly, the phrase means that the bacterium is unable to synthesize a polypeptide encoded by the *frc* gene. An attenuate expression can be determined by comparing the level of expression of the polypeptide encoded by the *frc* gene within the modified bacterium compared to that of an otherwise identical bacterium that does not carry said modification (reference bacterium).

The phrase “bacterium of the invention has been further modified to attenuate the expression of the *aroP* gene” as used herein means that the bacterium has been modified in such a way that the modified bacterium contains a reduced amount of the polypeptide encoded by the *aroP* gene. More particularly, the phrase means that the bacterium is unable to synthesize a polypeptide encoded by the *aroP* gene. An attenuate expression can be determined by comparing the level of expression of the polypeptide encoded by the *aroP* gene within the modified bacterium compared to that of an otherwise identical bacterium that does not carry said modification (reference bacterium).

The phrase “bacterium of the invention has been further modified to attenuate the expression of the *brnQ* gene” as used herein means that the bacterium has been modified in such a way that the modified bacterium contains a reduced amount of the polypeptide encoded by the *brnQ* gene. More particularly, the phrase means that the bacterium is unable to synthesize a polypeptide encoded by the *brnQ* gene. An attenuate expression can be determined by comparing the level of expression of the polypeptide encoded by the *brnQ* gene within the modified bacterium compared to that of an otherwise identical bacterium that does not carry said modification (reference bacterium).

The phrase "inactivation of a gene" can mean that the modified gene encodes a completely non-functional protein. It is also possible that the modified DNA region is unable to naturally express the gene due to the deletion of a part of or the entire gene sequence, the shifting of the reading frame of the gene, the introduction of missense/nonsense mutation(s), or the modification of an adjacent region of the gene, including sequences controlling gene expression, such as a promoter, enhancer, attenuator, ribosome-binding site, etc. Preferably, a gene of interest is inactivated by deletion of a part of or the entire gene sequence, such as by gene replacement.

The presence or absence of a gene on the chromosome of a bacterium can be detected by well-known methods, including PCR, Southern blotting, and the like. In addition, the level of gene expression can be estimated by measuring the amount of mRNA transcribed from the gene using various well-known methods, including Northern blotting, quantitative RT-PCR, and the like. The amount of the protein encoded by the gene can be measured by well-known methods, including SDS-PAGE followed by an immunoblotting assay (Western blotting analysis), and the like.

"Polypeptide" and "protein" are used interchangeably herein to denote a polymer of at least two amino acids covalently linked by an amide bond, regardless of length or post-translational modification (e.g., glycosylation, phosphorylation, lipidation, myristilation, ubiquitination, etc.). Included within this definition are D- and L-amino acids, and mixtures of D- and L-amino acids.

"Nucleic acid" or "polynucleotide" are used interchangeably herein to denote a polymer of at least two nucleic acid monomer units or bases (e.g., adenine, cytosine, guanine, thymine) covalently linked by a phosphodiester bond, regardless of length or base modification.

"Recombinant" or "non-naturally occurring" when used with reference to, e.g., a host cell, nucleic acid, or polypeptide, refers to a material, or a material corresponding to the natural or native form of the material, that has been modified in a manner that would not otherwise exist in nature, or is identical thereto but produced or derived from synthetic materials and/or by manipulation using recombinant techniques. Non-limiting examples include, among others, recombinant host cells expressing genes that are not found within the native (non-recombinant) form of the cell or express native genes that are otherwise expressed at a different level.

"Heterologous" as used herein means that a polypeptide is normally not found in or made (i.e. expressed) by the host organism, but derived from a different species.

“Substitution” or “substituted” refers to modification of the polypeptide by replacing one amino acid residue with another, for instance the replacement of an Serine residue with a Glycine or Alanine residue in a polypeptide sequence is an amino acid substitution. When used with reference to a polynucleotide, “substitution” or “substituted” refers to modification of the polynucleotide by replacing one nucleotide with another, for instance the replacement of a cytosine with a thymine in a polynucleotide sequence is a nucleotide substitution.

“Conservative substitution”, when used with reference to a polypeptide, refers to a substitution of an amino acid residue with a different residue having a similar side chain, and thus typically involves substitution of the amino acid in the polypeptide with amino acids within the same or similar class of amino acids. By way of example and not limitation, an amino acid with an aliphatic side chain may be substituted with another aliphatic amino acid, e.g., alanine, valine, leucine, and isoleucine; an amino acid with hydroxyl side chain is substituted with another amino acid with a hydroxyl side chain, e.g., serine and threonine; an amino acid having an aromatic side chain is substituted with another amino acid having an aromatic side chain, e.g., phenylalanine, tyrosine, tryptophan, and histidine; an amino acid with a basic side chain is substituted with another amino acid with a basic side chain, e.g., lysine and arginine; an amino acid with an acidic side chain is substituted with another amino acid with an acidic side chain, e.g., aspartic acid or glutamic acid; and a hydrophobic or hydrophilic amino acid is replaced with another hydrophobic or hydrophilic amino acid, respectively.

“Non-conservative substitution”, when used with reference to a polypeptide, refers to a substitution of an amino acid in a polypeptide with an amino acid with significantly differing side chain properties. Non-conservative substitutions may use amino acids between, rather than within, the defined groups and affects (a) the structure of the peptide backbone in the area of the substitution (e.g., serine for glycine), (b) the charge or hydrophobicity, or (c) the bulk of the side chain. By way of example and not limitation, an exemplary non-conservative substitution can be an acidic amino acid substituted with a basic or aliphatic amino acid; an aromatic amino acid substituted with a small amino acid; and a hydrophilic amino acid substituted with a hydrophobic amino acid.

“Deletion” or “deleted”, when used with reference to a polypeptide, refers to modification of the polypeptide by removal of one or more amino acids in the reference polypeptide. Deletions can

comprise removal of 1 or more amino acids, 2 or more amino acids, 5 or more amino acids, 10 or more amino acids, 15 or more amino acids, or 20 or more amino acids, up to 10% of the total number of amino acids, or up to 20% of the total number of amino acids making up the polypeptide while retaining enzymatic activity and/or retaining the improved properties of an engineered enzyme. Deletions can be directed to the internal portions and/or terminal portions of the polypeptide, in various embodiments, the deletion can comprise a continuous segment or can be discontinuous.

"Insertion" or "inserted", when used with reference to a polypeptide, refers to modification of the polypeptide by addition of one or more amino acids to the reference polypeptide. Insertions can comprise addition of 1 or more amino acids, 2 or more amino acids, 5 or more amino acids, 10 or more amino acids, 15 or more amino acids, or 20 or more amino acids. Insertions can be in the internal portions of the polypeptide, or to the carboxy or amino terminus. The insertion can be a contiguous segment of amino acids or separated by one or more of the amino acids in the reference polypeptide.

"Expression" includes any step involved in the production of a polypeptide (e.g., encoded enzyme) including, but not limited to, transcription, post-transcriptional modification, translation, post-translational modification, and secretion.

As used herein, "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid molecule to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded nucleic acid loop into which additional nucleic acid segments can be ligated. Certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "expression vectors". Certain other vectors are capable of facilitating the insertion of an exogenous nucleic acid molecule into a genome of a bacterium. Such vectors are referred to herein as "transformation vectors". In general, vectors of utility in recombinant nucleic acid techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" can be used interchangeably as the plasmid is the most commonly used form of a vector. Large numbers of suitable vectors are known to those of skill in the art and commercially available.

As used herein, "promoter" refers to a sequence of DNA, usually upstream (5') of the coding



region of a structural gene, which controls the expression of the coding region by providing recognition and binding sites for RNA polymerase and other factors which may be required for initiation of transcription. The selection of the promoter will depend upon the nucleic acid sequence of interest. A suitable "promoter" is generally one which is capable of supporting the  
5 initiation of transcription in a bacterium of the invention, causing the production of an mRNA molecule.

As used herein, "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding  
10 sequence is achieved under conditions compatible with the control sequence. A promoter sequence is "operably-linked" to a gene when it is in sufficient proximity to the transcription start site of a gene to regulate transcription of the gene.

"Percentage of sequence identity," "% sequence identity" and "percent identity" are used herein to refer to comparisons between an amino acid sequence and a reference amino acid sequence.  
15 The "% sequence identify", as used herein, is calculated from the two amino acid sequences as follows: The sequences are aligned using Version 9 of the Genetic Computing Group's GAP (global alignment program), using the default BLOSUM62 matrix (see below) with a gap open penalty of -12 (for the first null of a gap) and a gap extension penalty of -4 (for each additional null in the gap). After alignment, percentage identity is calculated by expressing the number of matches as a  
20 percentage of the number of amino acids in the reference amino acid sequence.

The following BLOSUM62 matrix is used:



## Examples

For the first time, the present inventors show that a bacterium, such as *E. coli*, lacking all four serine degradation genes (*sdaA*, *sdaB*, *tdcG* and *glyA*) can be constructed (Example 1). Said strain shows higher serine production yield than single and triple deaminase knock outs, when serine pathway is upregulated (Example 2). However, the resulting strain had a low tolerance towards serine, which has also been reported in an *E. coli* triple deletion strain lacking *sdaA*, *sdaB* and *tdcG* (Zhang and Newman, 2008). The inventors furthermore demonstrate that product toxicity can be reduced by over-expression of novel exporters (Example 3), by evolving strains by random mutagenesis (Example 4), and by adaptive evolution (Example 5). The strain was furthermore reverse engineered in order to identify the causative mutations (Example 6). During fed batch fermentation, the tolerant strain shows improved serine production (12.6 g/L with a mass yield of 36.7 % from glucose) when compared to the parental quadruple deletion strain (Example 7). This is the highest serine mass yield reported so far from glucose in any production organism.

The inventors additionally demonstrate that inhibition of the pentose phosphate pathway by deletion of *zwf* in the presence of other causative mutations leads to a further increase in serine production yield (Example 8).

The inventors further have identified beneficial mutations in ThrA resulting in an increase tolerance of the bacterial strain, including wild type *E. coli* and strains carrying an *sdaA* deletion, towards L-serine (Examples 10, 12, 13 and 14).

### **20 Example 1 – Deletion of key degradation pathways**

Serine has two key degradation pathways in *E. coli*: Serine to pyruvate, which is encoded by three deaminases namely *sdaA*, *sdaB* and *tdcG*, and conversion of serine to glycine, which is encoded by *glyA*. The deletion of *glyA* renders *E. coli* auxotrophic for glycine. Deletion of *sdaA*, *sdaB* and *tdcG* was done sequentially by using the lambda red mediated gene replacement method (Datsenko and Wanner, 2000). The protocol applied for deleting these genes is similar to the protocol described by Sawitzke et al. (2013). Primers used for amplification of the kanamycin cassette are shown in Table S1. The PCR reaction contained 250 nM each of KF and KR primer of the given gene, 250 µM of dNTP mix, 4 Units of Phusion polymerase (Thermoscientific, Waltham, MA, USA), 40 µl of HF buffer and 10 ng of pKD4 plasmid. The following two-step PCR protocol was used for the PCR amplification: An initial denaturation step at 98°C for 40 seconds, followed by 5 cycles of

denaturation at 98°C for 10 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 90 seconds the cycle, followed by 20 cycles, where the annealing temperature was increased from 55°C to 65°C. The PCR products were column purified (Macherey Nagel, Durn, Germany) and concentration was measured using a Nanodrop instrument (Thermoscientific, Waltham, MA, USA) and subjected to overnight DpnI digestion. *E. coli* MG1655 was used as parent strain to make sequential knock outs. The parent strain was transformed with pKD46 were grown in 2YT-amp media at 30°C and 250 rpm. The expression of exo, beta and gamma proteins were induced by addition of 20 mM arabinose at mid log phase (O.D. 0.4 to 0.5) and the cells were harvested after additional 1 h incubation. Culture was then transferred to 50 ml ice cold falcon tubes and centrifuged at 6500 rpm for 5 min at 4°C. Supernatant was discarded and cells were washed twice with ice cold 10 % glycerol. These electro-competent cells were transformed with 200 ng of kanamycin cassette, and transformants were plated on LB-kan plates. The kanamycin cassette was removed using the plasmid pcp20 encoding flippase gene. Primers for checking the loop out are shown in Table S2. Serine hydroxymethyltransferase encoded by *glyA* was deleted using the P1 phage system protocol (Thomason et al., 2007). A strain from the Keio collection (Baba et al., 2006) harboring *glyA::kan* was grown in 5 ml of LB-kan supplemented with 0.2 % Glucose, 25 mM CaCl<sub>2</sub> and 1 mM Glycine media. At early log phase (O.D. 0.1), culture was transduced with P1 phage lysate. The culture was incubated for three hours at 37°C and 250 rpm for cell lysis. Lysate was filter sterilized by using 0.45 µm filter. 1 ml of overnight culture of the *E. coli* strain having *sdaA*, *sdaB* and *tdcG* deleted, was resuspended in 200 µL of P1 salt solution and was incubated with 100 µL of the above lysate for 1 h. Cells were then grown overnight in 2 mL LB media supplemented with 2mM glycine and 200 mM sodium citrate. Cells were plated on LB-kan plate supplemented with 2mM glycine and 10 mM sodium citrate. The clones were restreaked onto a new plate in order to remove any phage contamination, and isolated colonies were checked for cassette insertion. The loop out was done using pcp20 plasmid. The resulting quadruple deletion strain (Figure 1) is referred to as Q1. This example demonstrates that it is possible to delete *sdaA*, *sdaB*, *tdcG* and *glyA* in *E. coli*, something that has not previously been achieved, and is thus unexpected.

**Table S1:** Primers used for amplification of kanamycin cassette

Primer name	Sequence
sdaA_KF	GCGCTGTTATTAGTTCGTTACTGGAAGTCCAGTCACCTTGTCAGGAGTATTATCGTGGTGTAG

	GCTGGAGCTGCTTCG
sdaA_KR	CGCCCATCCGTTGCAGATGGGCGAGTAAGAAGTATTAGTCACACTGGACCATATGAATATCC TCCTTAGTTCC
sdaB_KF	CGCTTTCGGGCGGCGCTTCTCCGTTTTAACGCGATGTATTCCTATGGTGTAGGCTGGAGCT GCTTCG
sdaB_KR	GGCCTCGCAAAACGAGGCCTTTGGAGAGCGATTAATCGCAGGCAACCATATGAATATCCTCC TTAGTTCC
tdcG_KF	CGTTCGCTCCACTTCACTGAACGGCAATCCGAGGGTGTGGATATGGTGTAGGCTGGAGCTG CTTCG
tdcG_KR	GTGCACCCAAGGATGAAAGCTGACAGCAATGTCAGCCGCAGACCACCATATGAATATCCTCC TTAGTTCC
glyA_KF	GTTAGCTGAGTCAGGAGATGCGGATGTTAAAGCGTGAAATGAACATTGCCGTGTAGGCTGG AGCTGCTTCG
glyA_KR	CAACGAGCACATTGACAGCAAATCACCGTTTCGCTTATGCGTAAACCGGCATATGAATATCCT CCTTAGTTCC

**Table S2:** Primers used for checking the deletion of given genes

Primer name	Sequence
sdaA_cF	GCGCTGTTATTAGTTCGTTACTGGAAGTCC
sdaA_cR	CGCCCATCCGTTGCAGATGGGC
sdaB_cF	CGCTTTCGGGCGGCGCTTCTC
sdaB_cR	GGCCTCGCAAAACGAGGCCTTTGG
tdcG_cF	CGTTCGCTCCACTTCACTGAACGG
tdcG_cR	GTGCACCCAAGGATGAAAGCTGACAGC
glyA_cF	GTTAGCTGAGTCAGGAGATGCGGATGTT
glyA_cR	CAACGAGCACATTGACAGCAAATCACCG

### Example 2 – Overexpression of serine biosynthesis pathway for production of serine

- 5 Serine is produced in *E. coli* from three enzymes encoded by *serA*, *serB* and *serC*. All genes were isolated from *E. coli* MG1655 using primers with respective gene names (Table S3). The 100 µl PCR mixture contain 250 nM each of forward and reverse primer, 250 µM of dNTP, 2 U of Phusion polymerase, 1 X HF buffer, 1 µl of overnight culture. The following two-step PCR protocol was used for the PCR amplification: An initial denaturation step at 98°C for 40, followed by 5 cycles of
- 10 denaturation at 98°C for 10 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 90 seconds the cycle, followed by 20 cycles, where the annealing temperature was increased from 55°C to 65°C. After column purification, the gene products and plasmids were digested using Fast digest enzymes (Thermoscientific, Waltham, MA, USA). About 500 ng of PCR product or 1 µg

of plasmids were subjected to digestion by 1 µl each of the restriction enzymes in 1X fast digest buffer. The reaction was incubated for 3h and then column purified again. *serA* was subjected to double digestion with NcoI and NotI while *serC* was digested with NdeI and PaeI. pCDF-Duet was first digested with NcoI and NotI for cloning of *serA* leading to plasmid pCDF-Duet-*serA* and this plasmid was later used for cloning of *serC* thus making pCDF-Duet-*serA-serC*. The gene encoding *serB* was cloned in pACYC-Duet vector at NcoI and PaeI site leading to pACYC-*serB*. Typical ligation reaction include 1 X T4 ligase buffer 50 ng of plasmid DNA and 100 ng of insert and 0.3 µl/10 µl of T4DNA ligase (Thermoscientific, Waltham, MA, USA).

Feedback inhibition of *serA* was removed by mutating three residues H344, N346 and N364 to alanine (Al-Rabiee et al., 1996) by site directed mutagenesis (Table S4). The master mix was used as mentioned above with the only modification that the master mix was divided in two equal aliquots, after which forward and reverse primers were added to each aliquot. A total of 100 ng of pCDF-Duet-*serA-serC* plasmid was used as a template. The two step PCR program: initial denaturation at 98°C for 40 sec, denaturation at 98°C for 10 sec, annealing 60°C for 30 sec, extension 72°C for 4 min and 30 sec the cycle was repeated 5 times and then the two aliquots were mixed and redistributed for additional 15 cycles. To enable swapping of vector backbones, the NcoI site inside *serC* was removed by the same approach using the primers listed in Table S4.

Figure 2 shows the vector maps of the plasmid constructs.

**Table S3:** Primers used for amplification and cloning of serine production pathway

Primer name	Sequence
serA-NcoI	GGCCCATGGCAAAGGTATCGCTGGAG
serA NotI_R	ATTGCGGCCGCTTAGTACAGCAGACGGGCGCGA
serB_NcoI_F	GGCCCATGGCTAACATTACCTGGTGCG
serB_R_HindIII	GCCAAGCTT TTATTACTTCTGATTACAGGCTGCC
serB_PaeI_R	GCCTTAATTAATTACTTCTGATTACAGGCTGCC
serC_F_NdeI	GGCCATATGATGGCTCAAATCTTCAATTTTAGTTCTGG
serC_R_PaeI	GCCTTAATTAATCAT TAACCGTGACGGCGTTTGAAC
ydeD_F_NdeI	CCGCATATGTCGCGAAAAGATGGGGTG
ydeD_cHis_R-PaeI	GCCTTAATTAATGATGATGATGATGATGACTTC CCACCT TTACCGCTT TACGCC
ydeD_NcoI_F1	CCGCCATGGCGCGAAAAGATGGGGTG

20

**Table S4:** Primers used for site directed mutagenesis of serine production pathway

Primer name	Sequence
serA N364A_F	CGAGCAGGGCGTCGCTATCGCCGCGCAATA
serA N364A_R	TATTGCGCGGCGATAGCGACGCCCTGCTCG
serA H344AN346A_F	CTGAT CACATCGCTGAAGCT CGTCCGGGCGTGC
serA H344AN346A_R	GCACGCCCCGACGAGCTTCAGCGATGTGCATCAG
serC_Ncolc_F	CAAGGTATTATTCTGTCATGGCGGTGGTCGCG
serC_Ncolc_R	CGCGACCACCGCCATGACAGAATAATACCTTG

### DE3 integration and serine production during batch fermentation

The serine production was checked in M9 minimal media. Glucose M9 minimal media consisted of 2 g/L glucose, 0.1 mM CaCl<sub>2</sub>, 2.0 mM MgSO<sub>4</sub>, 1× trace element solution, and 1× M9 salts. The 4,000× trace element stock solution consisted of 27 g/L FeCl<sub>3</sub>\*6H<sub>2</sub>O, 2 g/L ZnCl<sub>2</sub>\*4H<sub>2</sub>O, 2 g/L CoCl<sub>2</sub>\*6H<sub>2</sub>O, 2 g/L NaMoO<sub>4</sub>\*2H<sub>2</sub>O, 1 g/L CaCl<sub>2</sub>\*H<sub>2</sub>O, 1.3 g/L CuCl<sub>2</sub>\*6H<sub>2</sub>O, 0.5 g/L H<sub>3</sub>BO<sub>3</sub>, and Concentrated HCl dissolved in ddH<sub>2</sub>O and sterile filtered. The 10× M9 salts stock solution consisted of 68 g/L Na<sub>2</sub>HPO<sub>4</sub> anhydrous, 30 g/L KH<sub>2</sub>PO<sub>4</sub>, 5 g/L NaCl, and 10 g/L NH<sub>4</sub>Cl dissolved in ddH<sub>2</sub>O and autoclaved.

To use pET vectors as expression system, a DE3 cassette containing T7 polymerase was integrated into the genome of each deletion mutant using a Lambda DE3 lysogenization kit (Millipore, Damstadt Germany). Subsequently, strains (MG1655 (DE3)) carrying single ( $\Delta$ *sdaA*), triple ( $\Delta$ *sdaA*,  $\Delta$ *tdcG*,  $\Delta$ *sdaB*), and quadruple deletions ( $\Delta$ *sdaA*,  $\Delta$ *tdcG*,  $\Delta$ *sdaB* and  $\Delta$ *glyA*) were transformed with pCDF-Duet1-serAmut-serC and pACYC-serB. The resulting glycerol stocks were grown overnight in 2YT medium containing 0.1 % glucose and supplemented with required antibiotics (Spectinomycin and chloramphenicol). Overnight cultures were inoculated in triplicates into flasks containing M9 medium supplemented with 0.2 % glucose, 1mM threonine, required antibiotics (For the quadruple deletion strain, 2 mM glycine was also supplemented). Flasks were incubated at 37°C at 250 rpm. Serine production was induced by addition of 40 μM IPTG after the cultures reached an optical density of 0.55 to 0.65. O.D. measurements and sampling were done at regular time intervals for following 60 hr. Samples were filtered and subjected to HPLC for glucose and byproduct analysis using a method described previously (Kildegaard et al., 2014) with the only exception that the column temperature was kept at 30°C. Serine concentrations were determined using LCMS. The LC-MS/MS system consisted of a CTC autosampler module, a high pressure mixing pump and a column module (Advance, Bruker, Fremont, CA, USA). The injection volume

was 1  $\mu$ l. The chromatography was performed on a ZIC-chILIC column, 150mm  $\times$  2.1mm, 3 $\mu$ m pore size, (SeQuant, Merck Millipore). In front of the separation column was a 0.5  $\mu$  depth filter and guard column, the filter (KrudKatcher Classic, phenomenex) and guard column ZIC-chILIC, 20  $\times$  2.1 mm (SeQuant, Merck). Eluent A: 20 mM ammonium acetate pH adjusted to 3.5 with formic acid in milliQ water. Eluent B: Acetonitrile. The total flow rate of eluent A and B was 0.4 ml/min. The isocratic elution 35%, and the total run time was 5 minutes. Retention time was 2.8 minutes for serine. The MS–MS detection was performed on a EVOQ triple quadrupole instrument (Bruker, California, USA) equipped with an atmospheric pressure ionization (API) interface. The mass spectrometer was operated with electrospray in the positive ion mode (ESI+). The spray voltage was set to 4500 V. The con gas flow was 20 l/h, and the cone temperature was set at 350  $^{\circ}$ C. The heated probe gas flow was set at 50 l/h with a temperature of 350  $^{\circ}$ C. Nebulizer flow was set at 50 l/h, and the exhaust gas was turned on. Argon was used as collision gas at a pressure of 1.5 mTorr. Detection was performed in multiple reacting monitoring (MRM) mode. The quantitative transition was 106  $\rightarrow$  60, and the qualitative transition was 106  $\rightarrow$  70. The collision energy was optimized to 7eV for both transitions. Calibration standards of serine were prepared in media used for serine production and diluted 50:50 with 0.2% Formic acid in Acetonitrile. The concentration of the calibration standards was in the range from 0.001 to 0.5 g/L.

The experiment demonstrates that the deletion of all four genes involved in serine degradation in *E. coli*, referred to as Q1(DE3), results in the highest specific productivity and the highest yield from glucose as shown on Figure 3.

### **Example 3: Reducing product toxicity by overexpression of transporter**

Deletion of the main serine degradation pathways in *E. coli* results in significant decreased tolerance towards serine. In order to increase the tolerance, a transporter with a potential specificity for serine (*ydeD*) was overexpressed and tested during growth in high concentrations of serine. *ydeD* was cloned in pCDF-1b at NcoI and PaeI site by amplification with primers mentioned in Table S3 and the protocol given in Example 1.

Q1(DE3) was transformed with the pCDF-1b empty vector and the pCDF-*ydeD*-c-His plasmid (Figure 4). Transformants were selected on LB-spectinomycin plates. Overnight cultures of these transformants were inoculated in M9 media supplemented with 2mM glycine, 2 g/L glucose and spectinomycin in 1:50 ratio. Cultures were incubated at 37 $^{\circ}$ C and 250 rpm at an optical density of



0.4 to 0.5, after which 800  $\mu$ l was added to a 48-well biolector plate (M2P labs, Baeswieler, Germany) containing 800  $\mu$ l M9 media with varying serine concentration (12.5, 25 and 50 g/L) and 2mM glycine. The expression of *ydeD* was induced with 100  $\mu$ M IPTG. The growth was then monitored in the Biolector instrument (M2P labs, Baeswieler, Germany) at 37°C and 70 %  
5 humidity with continuous shaking. Gain was set to 20 % and scattering intensity was measured every 5 min for next 40 h.

The experiment demonstrates that the growth of *E. coli* lacking the main serine degradation pathways are severely growth inhibited in the presence of even low concentrations of serine. Upon overexpression of *ydeD*, the tolerance towards serine is increased substantially (Figure 4),  
10 suggesting that YdeD may potentially transport serine out of the cell.

#### **Example 4 – Random mutagenesis for serine tolerant strain**

Inactivation of the main serine degradation pathways in *E. coli* results in significant decreased tolerance towards serine. In order to increase the tolerance, the Q1 strain (obtained in Example 1) was grown in M9 media supplemented with 2mM glycine and 3 g/L serine overnight. 1 ml of  
15 culture was spread in 6 well petri plate and exposed to UV irradiation (CBS Scientific, San Diego, CA, USA) for 30 min. The culture was then added to 5 ml M9 media supplemented with 2 mM glycine, 2 g/L glucose and 50 g/L serine for enrichment of the tolerant mutants. The culture was incubated at 37°C and 250 rpm for 3 days and then plated on M9 plate supplemented with 50 g/L serine for the selection of the tolerant clones. Colonies were isolated and the growth rates and  
20 tolerance was estimated using the following method:

Cultures were grown overnight in 2xYT media. They were then inoculated in 96 well flat bottom micro titer plates with 150  $\mu$ L of M9 media containing 0.2 % glucose and various concentration of Serine (in triplicates). For strains containing the *glyA* deletion, 2mM glycine was also supplemented to the media. A total of 1.5  $\mu$ L of cells (1:100) was used as inoculum. If required  
25 minor changes in volume were done to ensure equal amount of cells. Plates were sealed with Microamp clear adhesive film (Applied Biosystems, Warrington, UK) and were incubated in Microtiter plate reader (Biotek, Winooski VT, USA). The reader was set at 37°C with continuous shaking, and the O.D was monitored every 5 to 10 minutes at 630 nm for 32 hours.

The experiment demonstrates that random mutagenesis can be used to increase the tolerance  
30 towards serine significantly (Figure 5). The resulting strain is referred to as Q3 below.

### Example 5 – Adaptive evolution for L-serine tolerance

Apart from random mutagenesis, serine tolerance of a strain having the main serine degradation pathways inactivated was also increased by Adaptive evolution. Seven independent populations of the Q1 strain were adaptively evolved in M9 minimal media supplemented with 2 mM glycine and increasing concentrations of the amino acid L-Serine at 37 °C along 60 days.

An Adaptive Laboratory Evolution (ALE) experiment was achieved by using an automated system, which enables the propagation of evolving populations over the course of many days while monitoring their growth rates. Prior to the start of the experiment, the system filled the required tubes with 25 ml of culture media and kept them at 37 °C in a heat block. Controlled aeration was obtained using magnetic tumble stirrers placed inside the tubes and spinning at 1,800 rpm. At the start of the experiment, a single colony was grown overnight in one of the tubes placed inside the system, and 100 µL aliquots were used by the robotics platform to inoculate seven independent flasks. As the bacteria grew, the automated system performed multiple OD measurements at 600 nm for each flask. Growth rates were automatically calculated by taking the slope of a least-square linear regression line fit to the logarithm of the OD measurements (Figure 6). Once reaching a target OD of 0.4, 100 µL of culture was used to inoculate a new flask. This way, cultures were serially passed (~2-3 times per day) to flasks with fresh media after reaching a targeted cell density such that stationary phase was never reached. The populations were initially incubated with 3 g/L serine after desired growth rate was reached culture was supplemented with 6 g/L of L-Serine. When populations achieved a stable phenotype (i.e. growth rate), the L-Serine concentration was increased to 12 g/L. This process was repeated iteratively using 24, 50, 75, and 100 g/L of L-serine. The final population was plated on the LB-agar and 2 clones of each of the populations selected. The evolved strains had significantly increased tolerance when tested for growth in the presence of high concentrations of serine by using the MTP based assay described in Example 5, above. The results are shown in Figure 6.

### Genomic DNA extraction, library sequencing and analysis

Two clones of each evolved cultures as well as the strain evolved by random UV-mutagenesis (Example 5) were genome sequenced. Genomic DNA was extracted from 1.5 ml of overnight cultures of *E. coli* strains using QIAamp DNA Mini Kit (QIAGEN, Germany). The genomic libraries

were generated using the TruSeq<sup>®</sup> Nano DNA LT Sample Preparation Kit (Illumina Inc., San Diego CA, USA). Briefly, 100 ng of genomic DNA diluted in 52.5 ul TE buffer was fragmented in Covaris Crimp Cap microtubes on a Covaris E220 ultrasonicator (Covaris, Brighton, UK) with 5% duty factor, 175 W peak incident power, 200 cycles/burst, and 50-s duration under frequency sweeping mode at 5.5 to 6°C (Illumina recommendations for a 350-bp average fragment size). The ends of fragmented DNA were repaired by T4 DNA polymerase, Klenow DNA polymerase, and T4 polynucleotide kinase. The Klenow exo minus enzyme was then used to add an 'A' base to the 3' end of the DNA fragments. After the ligation of the adapters to the ends of the DNA fragments, DNA fragments ranging from 300-400 bp were recovered by beads purification. Finally, the adapter-modified DNA fragments were enriched by 3-cycle-PCR. Final concentration of each library was measured by Qubit<sup>®</sup> 2.0 Fluorometer and Qubit DNA Broad range assay (Life Technologies, Paisley, UK). Average dsDNA library size was determined using the Agilent DNA 7500 kit on an Agilent 2100 Bioanalyzer. Libraries were normalized and pooled in 10 mM Tris-Cl, pH 8.0, plus 0.05% Tween 20 to the final concentration of 10 nM. Denaturated in 0.2N NaOH, 10 pM pool of 20 libraries in 600 µl ice-cold HT1 buffer was loaded onto the flow cell provided in the MiSeq Reagent kit v2 300 cycles and sequenced on a MiSeq (Illumina Inc., San Diego, CA, USA) platform with a paired-end protocol and read lengths of 151 nt.

The breseq pipeline (Deatherage and Barrick, 2014) version 0.23 with bowtie2 (Langmead and Salzberg) was used to map sequencing reads and identify sequence variants relative to the *E. coli* K12 MG1655 reference genome (NCBI accession number NC\_000913.2). Gene deletions present in the strains were verified manually based on missing coverage regions in the genome. Common variants found in MG1655 stock cultures (Freddolino et al., 2012) were excluded from further analysis. All sequencing samples had an average mapped coverage of at least 25x. The experiment identifies mutations that cause increased tolerance towards high concentrations of serine as shown in Table S5.

**Table S5:** Mutations found in the strains evolved for increased serine tolerance. The reference number for the genome sequence is NC\_000913.

Gene Name	Change	Annotation	Q1	Q3	ALE5-4	ALE5-8	ALE6-1	ALE6-2
thrA	A->G	Y356C	0	0	0	0	0	0
thrA	A->C	S357R	0	0	1	1	0	0
thrA	T->A	S359R	0	0	0	0	1	1

trxA/rho	IS1 -1 9 bp	Insertion <sup>1</sup>	0	0	0	0	0	0
rho	G->T	R87L	0	0	0	0	1	1
gcvA/ygdI	1 bp	Insertion <sup>2</sup>	0	0	0	0	1	1
gcvA/ygdI	IS4 1 13 bp	Insertion <sup>3</sup>	0	0	1	1	0	0
dapA/gcvR	1 bp	Insertion <sup>4</sup>	0	0	1	0	1	1
lrp	A->G	D143G	0	0	0	0	0	0
trxB/lrp	C->T	Intergenic <sup>9</sup> (-271/-274)	0	0	0	0	1	1
frc	IS1 -1 9 bp	Insertion <sup>5</sup>	0	0	0	0	0	0
eno	C->G	V164L	0	0	0	0	0	0
argP	C->A	Q132K	0	0	0	0	0	0
tufA	C->A	G19V	0	0	0	0	0	0
cycA	A->G	I220V	0	0	0	0	0	0
rhtA/ompX/ybiP/mntS	2854 bp	Deletion <sup>6</sup>	0	1	0	0	0	0
rpe	A->G	I202T	0	0	0	0	1	1
ytfB/fklB	T->C	Intergenic <sup>10</sup> (-154/-64)	0	0	0	0	1	1
yojL	G->C	D334H	0	0	0	0	1	0
aroP	IS5 1 4 bp	Insertion <sup>7</sup>	0	0	1	1	0	0
hyaF	T->G	V120G	0	0	1	1	0	0
mdtJ/tqsA	IS1 -1 9 bp	Insertion <sup>8</sup>	0	0	0	0	0	0
pykF	G->T	E250*	0	0	1	1	0	0
malT	C->T	Q420*	0	0	1	1	0	0
rpoB	C->T	P520L	0	0	1	1	0	0
fumB	T->G	T218P	0	0	0	0	0	0
gshA	G->A	A178V	0	0	0	1	0	0
lamB	C->T	Q112*	0	0	0	1	0	0
<b>Gene Name</b>	<b>Change</b>	<b>Annotation</b>	<b>Q1</b>	<b>ALE8-3</b>	<b>ALE8-8</b>	<b>ALE9-3</b>	<b>ALE9-8</b>	
thrA	A->G	Y356C	0	1	1	1	1	
thrA	A->C	S357R	0	0	0	0	0	
thrA	T->A	S359R	0	0	0	0	0	
trxA/rho	IS1 -1 9 bp	Insertion <sup>1</sup>	0	1	1	1	1	
rho	G->T	R87L	0	0	0	0	0	
gcvA/ygdI	1 bp	Insertion <sup>2</sup>	0	0	0	0	0	
gcvA/ygdI	IS4 1 13 bp	Insertion <sup>3</sup>	0	0	0	0	0	
dapA/gcvR	1 bp	Insertion <sup>4</sup>	0	0	0	0	0	
lrp	A->G	D143G	0	1	1	1	1	
trxB/lrp	C->T	Intergenic <sup>9</sup> (-271/-274)	0	0	0	0	0	
frc	IS1 -1 9 bp	Insertion <sup>5</sup>	0	1	1	1	1	
eno	C->G	V164L	0	1	1	1	1	
argP	C->A	Q132K	0	1	1	1	1	
tufA	C->A	G19V	0	1	1	1	1	

cycA	A->G	I220V	0	1	1	1	1	
rhtA/ompX/ybiP/mntS	2854 bp	Deletion <sup>6</sup>	0	0	0	0	0	
rpe	A->G	I202T	0	0	0	0	0	
ytfB/fklB	T->C	Intergenic <sup>10</sup> (-154/-64)	0	0	0	0	0	
yojI	G->C	D334H	0	0	0	0	0	
aroP	IS5 1 4 bp	Insertion <sup>7</sup>	0	0	0	0	0	
hyaF	T->G	V120G	0	0	0	0	0	
mdtI/tqsA	IS1 -1 9 bp	Insertion <sup>8</sup>	0	0	0	0	1	
pykF	G->T	E250*	0	0	0	0	0	
malT	C->T	Q420*	0	0	0	0	0	
rpoB	C->T	P520L	0	0	0	0	0	
fumB	T->G	T218P	0	0	0	1	0	
gshA	G->A	A178V	0	0	0	0	0	
lamB	C->T	Q112*	0	0	0	0	0	

\*: designates a stop codon

5 Insertion<sup>1</sup>: Insertion of a 768 bp long insertion sequence element IS1 in the lagging strand at the location 3966174, which is an intergenic region between genes *trxA* and *rho*. Duplication of 9 bp upstream and downstream of insertion sequence is observed.

Insertion<sup>2</sup>: Insertion of 1bp at location 2942629, which is an intergenic region between genes *gcvA* and *ygdl*.

10 Insertion<sup>3</sup>: Insertion of 1342 bp long insertion sequence element IS4 at the location 2942878, which is an intergenic region between genes *gcvA* and *ygdl*. Duplication of around 13 bp upstream and downstream of insertion sequence is observed.

Insertion<sup>4</sup>: Insertion of 1bp at location 2599854, which is an intergenic region between genes *dapA* and *gcvR*.

15 Insertion<sup>5</sup>: Insertion of 768 bp long insertion sequence element IS1 in the lagging strand at location 2492323, which leads to a truncation of gene *frc*. Duplication of 9 bp upstream and downstream of insertion sequence is observed.

Deletion<sup>6</sup>: Deletion of 2854 bp from location 850092, resulting in deletion of the first 5 bp of *rhtA*, complete deletion of genes *ompX* and *ybiP*, deletion of 239 bp of sRNA *rybA* and 77 bp deletion of *mntS* gene.

Insertion<sup>7</sup>: Insertion of 1195 bp long insertion sequence element IS5 at location 121518 which leads to deletion of the majority of *aroP*. Duplication of 4 bp upstream and downstream of insertion sequence is observed.

5 Insertion<sup>8</sup>: Insertion of 768 bp long insertion sequence element IS1 in the lagging strand at location 1673670, which is an intergenic region between genes *mdtJ* and *tqsA*. Duplication of 9 bp upstream and downstream of insertion sequence is observed.

Intergenic<sup>9</sup>: C->T mutation at location 923321 which is an intergenic region between *trxB* and *lrp*. The mutation is 271 bp upstream of *trxB* and 274 bp upstream of *lrp*.

10 Intergenic<sup>10</sup>: T->C mutation at the location 4428871 which is an intergenic region between *yftB* and *fkIB*. The mutation is 154 bp upstream of *yftB* and 64 bp upstream of *fkIB*.

### **Example 6 – Reverse engineering of ALE mutations by cam-sacB system and multiplex genome engineering**

15 In order to identify mutations that cause increased tolerance towards serine, the mutations identified in Example 5 were introduced into the Q1(DE3) strain using two different methods as described below.

#### **A. Introduction of *thrA* mutations**

20 Mutations in *thrA* were introduced into the genome of the Q1(DE3) strain using a cam-sacB based selection system. Cam-sacB was inserted using pKD46 plasmid harboring *exo*, *beta* and *gamma* genes for recombination. Positive selection for cassette insertion was done by selecting clones for chloramphenicol resistance. The loss of cassette was selected by replica plating clones on LB-chloramphenicol and LB-sucrose plate containing 15 % sucrose (no NaCl). The Cam-sacB cassette was amplified using *thrA\_camsacB\_F* and *R* (Table S6) primers respectively. Apart from template and extension time, the reaction mixture and PCR program was the same as described in example

25 1. The extension time was 2 min and 30sec, while template was 1 µl of overnight culture of the *E. coli* culture containing cam-sacB cassette. Competent Q1(DE3) cells were then transformed with 200 ng of *thrA*-cam-sacB cassette, and were plated on LB-chloramphenicol-ampicillin plates after two hour and incubated overnight at 30°C. A single colony was picked and made

electrocompetent after 1 h of induction (Example 1) and transformed with *thrA* alleles. After 2 hours of recovery, cells were plated on LB-sucrose plates and incubated at 37°C to cure pKD46 plasmid. 24 clones of each experiment were replica plated on LB-chloramphenicol and LB plates. Clones that did not grow on chloramphenicol plates were checked for the loss of the cassette by colony PCR and were subsequently sequenced by Sanger sequencing.

All three strains containing *thrA* mutations (Y356C, S357R or S359R) were grown in M9 media supplemented with 2mM glycine, 2 g/L glucose and 6.25 g/L L-serine. In this experiment, the background was not subtracted from the measurements, resulting in apparent increase in the OD for the Q1 strain. However, under these conditions, the Q1 strain did not show significant growth. On the other hand, each of the *thrA* mutations resulted in a similar and very significant increase in tolerance towards L-serine (Figure7), allowing them to grow at a L-serine concentration of at least 6.25 g/L opposed to the parent Q1 strain. Since all *thrA* mutants had same growth profile, S357R mutant was chosen as the parent strain (referred to as strain Q1(DE3)-*thrA*S357R) for the multiplex genome engineering described below.

**Table S6:** Primers used for genome integration of *thrA* mutations in the genome.

Primer name	Sequence
<i>thrA_gF</i>	ATGCGAGTGTTGAAGTTCGGCG
<i>thrA_gR</i>	TCAGACTCCTAACTTCCATGAGAGGG
<i>thrA_camsacB_F</i>	ATGCGAGTGTTGAAGTTCGGCGGTACATCAGTGGCAAATGCAGAACGTTTAAAAT GAGACGTTGATCGGCACG
<i>thrA_camsacB_R</i>	TCAGACTCCTAACTTCCATGAGAGGGTACGTAGCAGATCAGCAAAGACACCAAAG GGAAAACGTCCATATGCAC

### B. Multiplex genome engineering, screening and amplicon sequencing of selected ALE mutations

In order to identify mutations that cause increased tolerance towards serine, selected mutations identified in Example 5 were introduced into the Q1(DE3)-*thrA*S357R strain using multiplex genome engineering (Wang et al., 2011). The protocol applied for multiplex genome engineering was similar to the method published by Wang and Church, 2011. Strain Q1(DE3)-*thrA*S357R was transformed with plasmid pMA1 (containing only beta protein under control by the arabinose inducible promoter). Clones were plated on LB-ampicillin plates and incubated at 37°C. Colonies

were cultured overnight in TY-amp-gly media at 37°C and re-inoculated in 25 ml fresh TY-gly media the following day. After reaching an O.D. of 0.4 at 37°C, arabinose was added to a final concentration of 0.2 %. Cells were re-incubated at 37°C and 250 rpm for additional 15 min and were then made electro-competent by washing twice with 10 % glycerol (ice cold). Final volume of electro competent cells was adjusted to 200 µl using 10 % glycerol. The primers targeting the loci (Irp D143G, eno V164L, argP Q132K, cycA 1220V, pykF E250\*, rpoB520L, gcvA\*, yojL D334H, rho R87L) are given in Table S7. All primers were pooled and adjusted to a final concentration of 10 pmol/µl. To 50 µl of cells, 1 µl of this mix was added and transformed by electroporation. The transformed cells were directly added to 25 ml TY-gly media and were incubated at 37°C, 250 rpm for two hours to reach O.D. of 0.4, followed by arabinose induction and transformation as above for second round of multiplex engineering. The process was repeated six times. After the 6th round of multiplex genome engineering, the cells were incubated overnight. The resulting library of mutants was subsequently grown in M9 minimal media with and without serine. This way, mutations resulting in increased growth rates in minimal media or increased tolerance towards serine were enriched: A total of 1.5 µl of overnight culture was added in triplicates to wells in micro titer plates containing 150 µl triplicates of M9 media supplemented with 2 mM glycine and 2 g/L glucose (minimal media) or M9 media with 2 mM glycine, 2 g/L glucose and 25 g/L serine (minimal media with serine). Growth profiles were monitored at 37°C with continuous fast shaking in a microtiter plate reader for 40 hr. Optical density was measured at 630 nm every 5 min. Triplicates were pooled and used as template for amplicon generation. The PCR program and reaction composition was as described in Example 1. Illumina protocol was followed for amplicon processing and next gen sequencing was as described in Example 5. Sequencing results were analyzed computationally, and the enrichment of the various mutations was calculated as the frequency of the mutations in either minimal media or minimal media with serine, divided by the frequency of the mutations in the unselected library (Figure 8). This experiment shows that the mutations in Irp, rho, argP and eno results in increased tolerance towards serine.

**Table S7:** Primers used for Multiplex genome engineering

Primer name	Sequence
IrpD143G	GCTTGACTTCTTCCATAACAACGTAAGTGCGCGTCCCGTTAACCCAGGCAGACGC AGCAGGGTTTC
enoV164L	CCAACCGGCTGAATCATGAATTCTTGA ATGTCGAGATTATTATCAGCGTGCTCACCACCGTTGATGATG



argPQ132K	CTCAACTTGCAGGTAGAAGATGAAACGAGGAAAGAGAGGCTCCGCCGCGGCGA AGTGGTCGGC
cycAI220V	CAGCTCAATCCCCACGAAAGCTAATACGGCAACTTGAAAACCGGCAAAGAAGCCA CTTAAACCTTTC
pykFE250***	GCATCATGGTTGCGCGTGGCGAC CTCGGCGTTTAGTGACCCTAAATCTTCGCCCAGAAGATGATGA
rpoBP520L	GATACGACGTTTGTGCGTAATTTCTGAGAGGAGATTATTTGGTCCATAAACTGA GACAGCTG
gcvA***	CTCGTAAGGCATTTAGCGGTGGTA ATGGTTATC ATTAGGCTATTAACCTTTGATGTTAAATG
yojLD334H	GGTGAGATTAATCGGACCAACGGAGAAGGCATTGTGTTGGTATGCAAACGTCAC GTTACGCAGCTCCAGC
rhoR87L	GAGATGGTATCACCAGTGCGGAGATTAATCTTAGTATCTGAGAAGGGGAAACG TAGATGTCATCAG

**Table S8:** Primers used for amplicon sequencing

Primer name	Sequence
lrp_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCGGTGATTCGACTACCTGTTG
lrp_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCGCGTCTTAATAACCAGACGAT
enoV164_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGCTGTACGAGCACATCGCTGAAC
enoV164_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCCATGCGGATGGCTTCTTTCAC
argP_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GACAGTCTGGCGACGTGGTTGCT
argP_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTATCGACAAGACAACCTCGGCAGCG
cycA_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGAAGCGTCATTCGCGCATTTG
cycA_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGTTAATCGCGCGTGGCAGTG
pyfK_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGCCTCAACAACTTCGACGA
pyfK_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGAATCCAGCATCTGGGTCGC
rpoB_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCAACGCCAAGCCGATTTCCG
rpoB_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGTCTCGAACTTCGAAGCCTGC
gcvA_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGCTGGTAGAAGCTCAACGGAC
gcvA_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGTGCGGCATCAAAAACCTCG
yojI_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGCCTTTCAAAGCAGAGTTCCGC
yojI_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTACCGTTGCCCAATCAG
rho_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGCAGGATGGATTTGGTTTCCTCC
rho_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCAGCGCAAATAGCGTTCACC

**Example 7 – Serine production by tolerant and susceptible *E. coli* during fed batch fermentation**

- 5 A new vector was constructed in order to upregulate expression of exporter along with serine pathway in the pACYC-Duet vector by following the same above strategy as mentioned in Example 2. *serB* was cloned using NcoI and HindIII double digestion followed by ligation. The resulting

vector (pACYC-Duet-*serB*) was then subjected to double digestion by NdeI and PacI for cloning of ydeD-c-His. Both strains Q1 (DE3) and Q3 (DE3) were made electrocompetent by growing cells in 30 ml 2xYT media supplemented with 0.2 % glucose (37°C, 250 rpm) to mid log phase. Cells were harvested by centrifugation at 6500 rpm for 5 min at 4°C, and were washed twice by ice cold 10 %  
5 glycerol. Cells were finally resuspended in 500 µL of 10 % glycerol and 50 µL of cells were transformed with plasmids pCDF-Duet1-*serAmut-serC* and pACYC-*serB-ydeD-c-His* for serine production.

Serine production was demonstrated during fed batch fermentation in 1L fermenters (Sartorius, Gottingen, Germany). The media contained 2g/L MgSO<sub>4</sub>\*7H<sub>2</sub>O, 2 g/L KH<sub>2</sub>PO<sub>4</sub>, 5 g/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>,  
10 7.5 g/L Glucose, 2 g/L Yeast extract, 0.6 g/L glycine, 0.12 g/L threonine, 4X trace element (as mentioned in Example 2), 50 mg/L spectinomycin and 25 mg/L chloramphenicol. For the *E. coli* Q1(DE3) strain, the initial glucose concentration was 10 g/L in order to enhance the growth before induction. The feed generally contained 140 g/L glucose, 24 g/L Ammonium sulfate, 2g/L glycine, 0.12 g/L threonine, 2.5 g/L each of MgSO<sub>4</sub>\*7H<sub>2</sub>O and KH<sub>2</sub>PO<sub>4</sub>, 1X trace elements 50 mg/L  
15 spectinomycin and 25 mg/L chloramphenicol, while the feed for the Q1(DE3) strain contained 120 g/L of glucose.

A log phase culture was used to inoculate 500 ml media by 1:50 inoculum ratio. The cultures were allowed to grow over night in the fermenter at 37°C, 1000 rpm baffle speed and 20 % air saturation. Feed at the rate of 8 g/h was started after glucose concentration was below 250 mg/L  
20 in the media. Production was induced at late log phase (O.D. 7.5 to 9.5) by the addition of 40 µM IPTG and feed rate was reduced to 6 g/h. Samples were taken at regular intervals and were subjected to HPLC and LCMS analysis as mentioned before.

The experiment demonstrates that serine can be produced at high titer and with a high yield in *E. coli* using fed batch fermentation (Figure 9). The titers were 6.8 g/L and 12.5 g/L for the Q1(DE3) and the Q3(DE3) strain, respectively. The fermentations resulted in a surprisingly high mass yield of 36.7 % from glucose in the Q3(DE3) strain when compared to 24.3 % in the Q1(DE3) strain (Figure 9B). This shows that a higher titer and yield can be achieved using the production strain that is tolerant towards serine.

#### **Example 8 – Reverse engineered strains showing increased serine production**

Strains reverse engineered in Example 6 were checked for serine tolerance as mentioned in Example 3. The best serine tolerant strain (F7) was checked for serine production and was also genome sequenced as described in Example 5. Plasmids for overexpression of the serine production pathway were introduced as described in Example 7. Shake flask experiments were carried out as described in Example 2, with the only difference being that the concentration of glucose was 2.5 g/L. The O.D. measurements and sampling were done at regular intervals, and the samples were analyzed using methods described in Example 2. Surprisingly, said strain resulted in a significantly increased production and yield of serine from glucose as shown in Table S9. The genome sequence revealed a deletion of *zwf*, which encodes the first enzyme in the pentose phosphate pathway. Additionally the strain carried the *thrAS357R* and *rhoR87L* mutations. Furthermore the strain also had a truncation of the branched chain amino acid exporter *brnQ* (105 bp was deleted starting at location 419986. The 439 amino acid protein was truncated after 308 amino acids).

**Table S9:** Effect of  $\Delta zwf$  on production of serine

	Serine concentration in g/L (+/- standard deviation)	Yield from glucose (g serine/ g glucose)
$\Delta sdaA \Delta sdaB \Delta tdcG \Delta glyA$ (Q1)	0.925 (+/- 0.048)	0.37
$\Delta sdaA \Delta sdaB \Delta tdcG \Delta glyA \Delta zwf$ $\Delta brnq \text{ thrA S357R rho R87L}$	<b>1.323 (+/- 0.06)</b>	<b>0.53</b>

15

#### Example 9 – Production by ALE 8

To use pET vectors as expression system, a DE3 cassette containing T7 polymerase was integrated into the genome of the ALE 8-8 mutant (Example 5) using a Lambda DE3 lysogenization kit (Millipore, Damstadt Germany) resulting in the strain ALE 8-8(DE3). Subsequently, the ALE 8-8(DE3) was made electrocompetent by growing cells in 30 ml 2xYT media supplemented with 0.2 % glucose (37°C, 250 rpm) to mid log phase. Cells were harvested by centrifugation at 6500 rpm for 5 min at 4°C, and were washed twice by ice cold 10 % glycerol. Cells were finally resuspended in 500  $\mu$ L of 10 % glycerol and 50  $\mu$ L of cells were transformed with plasmids pCDF-Duet1-*serAmut-serC* and pACYC-*serB* for serine production.

Serine production was demonstrated during fed batch fermentation in 1L fermenters (Sartorius, Gottingen, Germany) as mentioned in Example 7. The media contained 2g/L  $MGSO_4 \cdot 7H_2O$ , 2 g/L  $KH_2PO_4$ , 5 g/L  $(NH_4)_2SO_4$ , 10 g/L Glucose, 2 g/L Yeast extract, 0.6 g/L glycine, 4X trace elements

(as mentioned in Example 2), 50 mg/L spectinomycin and 25 mg/L chloramphenicol. The 375 g of feed contained 400 g/L glucose, 24 g/L Ammonium sulfate, 2g/L glycine, 2.5 g/L of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  and 5 g/L of  $\text{KH}_2\text{PO}_4$ , 1X trace elements 50 mg/L spectinomycin and 25 mg/L chloramphenicol. A log phase culture was used to inoculate 500 ml media by 1:50 inoculum ratio. The cultures were  
5 allowed to grow overnight in the fermenter at 37°C, 1000 rpm baffle speed and 20 % air saturation. Production was induced at late log phase (O.D. 8.5 to 9.5) by the addition of 80  $\mu\text{M}$  IPTG and feed at the rate of 8 g/h was started. Samples were taken at regular intervals and were subjected to HPLC and LCMS analysis as mentioned before.

The experiment shows that both a high cell density and a high serine titer (55.0 g/L) can be  
10 obtained in ALE 8-8 (DE3) strain (Figure 10A) with a mass yield from glucose of 36.0% (Figure 10B).

#### **Example 10 – Over expression and site saturation mutagenesis of *thrA* at site Y356, S357 and S359**

Using site saturation mutagenesis (SSM), the selected amino acid residue/s can be mutated to all other 20 amino acids. This makes it possible to investigate the effect of amino acid substitutions  
15 on the enzyme activity and in turn on phenotype. The *thrA* gene was amplified from MG-1655 wt strain using primers *thrA*\_NcoI\_F and *thrA*\_HindIII (primer sequences in Table S10) and cloned in pACYC-Duet1 plasmid using NcoI and HindIII enzyme. The protocol of cloning was same as for cloning *serB* in Example 2. The vector constructed was pACYC-*thrA*. This plasmid was used as a template for SSM. SSM for observed *thrA* mutations in ALE strains (Y356, S357 and S359) was  
20 performed using primers given in Table S10. The SSM protocol was similar to SDM protocol applied in Example 2 for mutagenesis of feedback insensitive *serA*. PCR product was DpnI digested and transformed in Q1 (DE3) strain, which carries the wild type *thrA* gene in its genome, and plated on LB-chloramphenicol plates supplemented with 0.1 % glucose and 4 mM glycine. Chloramphenicol was supplemented in all following media for plasmid stability.

25 Individual colonies were picked and inoculated into 96 well MTP plate containing 2x TY media supplemented with 0.1 % glucose and 2 mM glycine. 90 clones (1 microtiter plate (MTP)) from each SSM library was picked (in total 3 plates). The Q1(DE3) strain harboring pACYC-Duet empty vector and pACYC-*thrA*wt was inoculated as a control strain in each plate. The plate was incubated at 37 °C and 300 rpm overnight. This pre-culture plate was used as inoculum from  
30 which 1  $\mu\text{L}$  was added to a new 96 well MTP plate containing 100  $\mu\text{L}$  of M9 media supplemented

with 0.2% glucose, 2 mM glycine. The culture was incubated for 4 h after which 25  $\mu$ L of 1 mM IPTG was added to induce the expression. The plate was subsequently incubated for 2 h and then M9 media containing 0.2 % glucose, 2mM glycine and 12.5 g/L L-serine was added thus reaching final serine concentration of 6.25 g/L. The plate was then incubated with shaking in an MTP reader for growth analysis as described before. Selected clones showing tolerance from each plate were collected in 1 plate and the tolerance studies were repeated for these strains. The plasmids from the tolerant cloned were sequenced and the growth rates were estimated from the log phase of culture. This way, a number of amino acid substitutions that result in tolerance towards serine were identified (Figure 11). Figure 11 (A-C) shows the growth rate of mutant strains having different amino acid substitutions observed at positions 356, 357 and 359 of ThrA, respectively (denoted by the respective one-letter code). The growth rate of *E. coli* carrying the wild type *thrA* gene is denoted "wt".

The experiment demonstrates that amino acid substitutions at position 356, 357 and/or 359 in the native ThrA protein confer increased tolerance of modified strains towards L-serine. The data further demonstrates that overexpression of mutants of ThrA can increase the tolerance towards L-serine even in strains having a native *thrA* gene present in their genome.

**Table S10:** Primers used for cloning and site saturation mutagenesis (SSM) of *thrA*.

thrA_NcoI_F	GCGCCATGGGAGTGTTGAAGTTCGGCG
thrA_HindIII_R	GCCAAGCTTTCAGACTCCTAACTTCCATGAGAGGG
thrA_Y356NNK_F	CGCAGAAACTGATGCTMNNNTTCGGAAGATGATTGCG
thrA_Y356NNK_R	CGCAGAAACTGATGCTMNNNTTCGGAAGATGATTGCG
thrA_S357NNK_F	CAATCATCTTCCGAATACNNKATCAGTTTCTGCGTTCC
thrA_S357NNK_R	GGAACGCAGAAACTGATMNNGTATTCGGAAGATGATTG
thrA_S359NNK_F	CCGAATACAGCATCNNKTTCTGCGTTCCAC
thrA_S359NNK_R	GTGGAACGCAGAAAMNNGATGCTGTATTCGG

**Example 11: Deletion of serine deaminase encoded by *sdaA***

Deletion of *sdaA* was done using the lambda red mediated gene replacement method (Datsenko and Wanner, 2000). The protocol applied for deleting these genes is similar to the protocol

described by Sawitzke et al. (2013). Primers used for amplification of the kanamycin cassette are shown in Table 1. The PCR reaction contained 250 nM each of KF and KR primer of the given gene, 250  $\mu$ M of dNTP mix, 4 Units of Phusion polymerase (Thermoscientific, Waltham, MA, USA), 40  $\mu$ l of HF buffer and 10 ng of pKD4 plasmid. The following two-step PCR protocol was used for the

5 PCR amplification: An initial denaturation step at 98°C for 40 seconds, followed by 5 cycles of denaturation at 98°C for 10 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 90 seconds the cycle, followed by 20 cycles, where the annealing temperature was increased from 55°C to 65°C. The PCR products were column purified (Macherey Nagel, Durn, Germany) and concentration was measured using a Nanodrop instrument (Thermoscientific, Waltham, MA, USA)

10 and subjected to overnight DpnI digestion. *E. coli* MG1655 was used as parent strain to make sequential knock outs. The parent strain was transformed with pKD46 were grown in 2YT-amp media at 30°C and 250 rpm. The expression of exo, beta and gamma proteins were induced by addition of 20 mM arabinose at mid log phase (O.D. 0.4 to 0.5) and the cells were harvested after additional 1 h incubation. Culture was then transferred to 50 ml ice cold falcon tubes and

15 centrifuged at 6500 rpm for 5 min at 4°C. Supernatant was discarded and cells were washed twice with ice cold 10 % glycerol. These electro-competent cells were transformed with 200 ng of kanamycin cassette, and transformants were plated on LB-kan plates. The kanamycin cassette was removed using the plasmid pcp20 encoding flippase gene. Primers for checking the loop out are shown in Table 2. To use pET vectors as expression system, a DE3 cassette containing T7

20 polymerase was integrated into the genome of each deletion mutant using a Lambda DE3 lysogenization kit (Millipore, Damstadt Germany).

As a result, a strain is provided wherein the *sdaA* gene is deleted. This strain has been used in subsequent examples.

**Table S11:** Primers used for amplification of kanamycin cassette

Primer name	Sequence
sdaA_KF	GCGCTGTTATTAGTTCGTTACTGGAAGTCCAGTCACCTTGTCAGGAGTATTATCGTGGTGTAG GCTGGAGCTGCTTCG
sdaA_KR	CGCCCATCCGTTGCAGATGGGCGAGTAAGAAGTATTAGTCACACTGGACCATATGAATATCC TCCTTAGTTCC

25

**Table S12:** Primers used for checking the deletion of given genes

Primer name	Sequence
sdaA_cF	GCGCTGTTATTAGTTCGTTACTGGAAGTCC
sdaA_cR	CGCCCATCCGTTGCAGATGGGC

**Example 12 – Overexpression and site saturation mutagenesis of *thrA* at site Y356, S357 and S359**

Over expression of *thrA* was achieved using the plasmid pACYC-Duet. The *thrA* gene was amplified from MG-1655 wt strain using primers *thrA*\_NcoI\_F and *thrA*\_HindIII (primer sequences in Table S10) and cloned in pACYC-Duet1 plasmid using NcoI and HindIII enzyme. The 100 µl PCR mixture contained 250 nM each of forward and reverse primer, 250 µM of dNTP, 2 U of Phusion polymerase, 1 X HF buffer, 1 µl of overnight culture. The following two-step PCR protocol was used for the PCR amplification: An initial denaturation step at 98°C for 40, followed by 5 cycles of denaturation at 98°C for 10 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 120 seconds the cycle, followed by 20 cycles, where the annealing temperature was increased from 55°C to 65°C. After column purification, the gene products and plasmids were digested using Fast digest enzymes (Thermoscientific, Waltham, MA, USA). About 500 ng of PCR product or 1 µg of plasmids were subjected to digestion by 1 µl each of the restriction enzymes in 1X fast digest buffer. The reaction was incubated for 3h and then column purified again. Ligation reaction included 1 X T4 ligase buffer, 50 ng of plasmid DNA and 100 ng of insert and 0.3 µl per 10 µl of T4DNA ligase (Thermoscientific, Waltham, MA, USA). The ligation mixture was transformed in chemically competent DH5α cells and plated on Lb-chloramphenicol plates. The plasmid was isolated using plasmid prep kit (Macherey Nagel, Durn, Germany).

Using site saturation mutagenesis (SSM), the selected amino acid residue/s can be mutated to all other 20 amino acids. This makes it possible to investigate the effect of amino acid substitutions on the enzyme activity and in turn on phenotype. SSM for *thrA* sites Y356, S357 and S359 was performed using primers shown in Table S10. The master mix was used as mentioned above with the only modification that the master mix was divided in two equal aliquots, after which forward and reverse primers were added to each aliquot. A total of 100 ng of pACYC-Duet-*thrA* plasmid was used as a template. The two step PCR program: initial denaturation at 98°C for 40 sec, denaturation at 98°C for 10 sec, annealing 60°C for 30 sec, extension 72°C for 5 min the cycle was repeated 5 times and then the two aliquots were mixed and subjected to an additional 15 cycles.

PCR product was DpnI digested and transformed in the *sdaAΔ* (DE3) strain (Example 11), which carries the wild type *thrA* gene in its genome, and plated on LB-chloramphenicol plates supplemented with 0.1 % glucose. Chloramphenicol was supplemented in all following media for plasmid stability.

5 Individual colonies were picked and inoculated into 96 well micro titer plates (MTP) containing 2x TY media supplemented with 0.1 % glucose and 2 mM Glycine. 240 clones from each SSM library was picked (in total 3 plates). The *sdaAΔ* (DE3) strain harboring pACYC-Duet empty vector and pACYC-*thrA*wt was inoculated as a control strain in each plate. The plate was incubated at 37 °C and 300 rpm overnight. This pre-culture plate was used as inoculum from which 1 μL was added  
10 to a new 96 well MTP plate containing 100 μL of M9 media supplemented with 0.2% glucose. The culture was incubated for 4 h after which 25 uL of 1 mM IPTG was added to induce the expression. The plate was subsequently incubated for 2 h and then M9 media containing 0.2 % glucose and 25 g/L L-serine was added thus reaching final serine concentration of 12.5 g/L. The plates were incubated in Microtiter plate reader (Biotek, Winooski VT, USA). The reader was set at  
15 37°C with continuous shaking, and the O.D was monitored every 5 minutes at 630 nm for 18 hours. The plasmids from the tolerant clones were sequenced and the growth rates were estimated from the log phase of culture. This way, a number of amino acid substitutions that result in tolerance towards serine were identified. Figures 12 (A-C) shows the growth rate of mutant strains having different amino acid substitutions observed at positions 356 (12A), 357  
20 (12B) and 359 (12C) of *ThrA*, respectively (denoted by the respective one-letter code). The growth rate of *E. coli* carrying the wild type *thrA* gene is denoted "wt".

In conclusion, the experiment demonstrates that overexpression of mutants of *thrA* can increase the tolerance towards L-serine of strains having a native *thrA* gene present in its genome. This works for example in a strain carrying a single genomic deletion of *sdaA*. This way, the experiment  
25 identifies mutants that confer L-serine tolerance.

### **Example 13: Multiplex genome engineering of beneficial mutations enhancing serine tolerance**

In order to investigate if genome integration of beneficial mutations identified in the Q1 strain may also cause increased tolerance towards serine in other strains, the mutations were genome integrated into the *sdaAΔ* (DE3) strain (Example 11) using multiplex genome engineering (Wang et al., 2011). The protocol applied for multiplex genome engineering was similar to the method  
30



published by Wang and Church, 2011. Strain *sdaAΔ* (DE3) (Example 11) was transformed with plasmid pMA7-sacB plasmid (Lennen et al. 2015). Clones were plated on LB-ampicillin plates and incubated at 37°C. Colonies were cultured overnight in TY-amp-gly media at 37°C and re-inoculated in 25 ml fresh TY-gly media the following day. After reaching an O.D. of 0.4 at 37°C, 5 arabinose was added to a final concentration of 0.2 %. Cells were re-incubated at 37°C and 250 rpm for additional 15 min and were then made electro-competent by washing twice with 10 % glycerol (ice cold). Final volume of electro competent cells was adjusted to 200 µl using 10 % glycerol. The primers targeting the loci (*lrp* D143G, *thrA* S357R) are shown in Table S13. The primers were pooled and adjusted to a final concentration of 10 pmol/µl. A total of 1.5 µl of this 10 mix was added to 50 µl of cells and transformed by electroporation. The transformed cells were directly added to 25 ml TY-gly media and were incubated at 37°C, 250 rpm for two hours to reach O.D. of 0.4, followed by arabinose induction and transformation as above for second round of multiplex engineering. The process was repeated three times. After the 3<sup>rd</sup> round of multiplex genome engineering, the cells were resuspended in 2 ml TY media. 1 ml was centrifuged at 16000 15 rpm for 1 min to centrifuge the cells and remove the supernatant cells were then suspended in minimal M9 media and plated on M9-agar plate containing 12.5 g/L L-serine and was incubated for 40 h in 37°C incubator. A similar protocol was followed for the parental strain. More than 400 clones were observed on the plate containing genome engineered cells while only few spontaneous mutants were observed on the control plate. This shows that by employing such 20 genome engineering approach, beneficial mutations can be easily engineered and selected for in these cells.

To investigate if these clones were serine tolerant, they were pre-cultured in a 96 well plate as described in Example 12. After 8 h of incubation, 3 µL of cells were inoculated in 150 µL M9 media containing 15g/L L-Serine and subjected for growth profile studies in a micro titer plate reader as 25 described in Example 12. Figure 13 shows the difference between the control (wt) and the genome engineered strains. Thirty random clones from this plate were sequenced for targeted sites. In this small sample size, 5 *lrp* mutants and more than 15 *thrA* mutants were found. All *lrp* mutants were found to also contain *thrA* mutation. The growth rates were estimated from the logarithmic growth phase. Average growth rates and deviations were determined from at least 4 30 samples as shown in Figure 13.

In conclusion, it was demonstrated that the genomic mutation of *Irp*-D143G and *thrA*-S357R significantly increases the L-serine tolerance in a strain carrying for example a single deletion of *sdaA*.

**Table S13:** Primers used for Multiplex genome engineering

Primer name	Sequence (5' to 3')
IrpD143G	GCTTGACTTCTTCCATAACAACGTAAGTGCGCGTCCCGTTAACCCAGGCAGACGC AGCAGGGTTTC
thrAS357R	CACACAGTCGCTTTGTGGAACGCAGAACTAATCCGATACTCCGAAGATGATTGC GTAATCAGCACC

5

**Example 14: Over expression of *thrA* and site saturation mutagenesis of *thrA* at site Y356, S357 and S359 in a wild type *E. coli* strain.**

Wild type *E. coli* is naturally more tolerant towards L-serine due to presence of serine deaminases and serine hydroxymethyltransferase. At high serine concentrations, such as 50 g/L, serine does affect the growth rate of the wild type strain. In this example we show that the serine tolerance can be increased in the wild type strain by introduction of mutations that were found to confer serine tolerance in the Q1 strain (Example 5 and 6). As an example mutant variants of *thrA* were overexpressed in wild type *E. coli* MG1655. All the methods and materials were as described in Example 12, except that the strain used for transformation was *E. coli* MG1655 (DE3). Additionally, after IPTG induction of *thrA* expression, the cells were grown in M9 media containing 0.2 % glucose, and 100 g/L L-serine was added to make a final concentration of 50 g/L L-serine. Growth profiles were monitored using a microtiter plate reader, and growth rate of *E. coli* MG1655 expressing wild type *thrA* or mutant variants of *thrA* are shown in Figure 14.

In conclusion, the experiment demonstrates that overexpression of mutant variants of *thrA* can increase the tolerance towards L-serine of wild type *E. coli* having a native *thrA* gene present in its genome. This demonstrates the positive effect of mutants of *thrA* even in an *E. coli* wild type strain that does not have attenuated activity of serine deaminases or serine hydroxymethyltransferase.

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## Claims

1. A bacterium which has been modified to attenuate expression of at least one gene coding for a polypeptide having serine deaminase activity and/or to attenuate expression of a gene coding for a polypeptide having serine hydroxymethyltransferase activity.
- 5 2. The bacterium according to claim 1, wherein the at least one gene coding for a polypeptide having serine deaminase activity is selected from the group consisting of *sdaA*, *sdaB* and *tdcG*.
3. The bacterium according to claim 1 or 2, wherein the gene coding for a polypeptide having serine hydroxymethyltransferase activity is *glyA*.
4. The bacterium according to any one of claims 1 to 3, wherein the bacterium has been  
10 modified to attenuate expression of the genes *sdaA*, *sdaB*, *tdcG* and *glyA*.
5. The bacterium according to any one of claims 1 to 3, wherein the bacterium has been modified to attenuate expression of at most three of the genes *sdaA*, *sdaB*, *tdcG* and *glyA*.
6. The bacterium according to any one of claims 1 to 5, wherein the expression of the gene or genes is attenuated by inactivation of the gene or genes.
- 15 7. The bacterium according to any one of claims 1 to 6, wherein at least one gene coding for a polypeptide having serine deaminase activity is inactivated.
8. The bacterium according to any one of claims 1 to 7, wherein at least a gene coding for a polypeptide having serine hydroxymethyltransferase activity is inactivated.
9. The bacterium according to any one of claims 1 to 7, wherein at least one of the genes  
20 *sdaA*, *sdaB*, *tdcG* and *glyA* is inactivated.
10. The bacterium according to any one of claims 1 to 9, wherein the genes *sdaA*, *sdaB*, *tdcG* and *glyA* are inactivated.
11. The bacterium according to any one of claims 1 to 9, wherein at most three of the genes *sdaA*, *sdaB*, *tdcG* and *glyA* are inactivated.

12. The bacterium according to any one of claims 1 to 11, wherein said bacterium is capable of growing in a minimal culture medium comprising L-serine at a concentration of at least about 6.25 g/L.
13. The bacterium according to any one of claims 1 to 12, wherein said bacterium is capable of growing in a minimal culture medium comprising L-serine at a concentration of at least about 6.25 g/L at a growth rate of at least  $0.1 \text{ hr}^{-1}$  during exponential growth.
14. The bacterium according to any one of claims 1 to 13, wherein said bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in one or more amino acid substitutions which increase tolerance towards L-serine.
15. The bacterium according to any one of claims 1 to 14, wherein said bacterium expresses an aspartate kinase I / homoserine dehydrogenase I (ThrA) mutant having one or more amino acid substitutions which increase tolerance towards L-serine.
16. The bacterium according to any one of claims 1 to 15, wherein said bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in one or more amino acid substitutions in the encoded polypeptide at a position selected from the group consisting of Y356, S357 and S359.
17. The bacterium according to any one of claims 1 to 16, wherein said bacterium expresses an aspartate kinase I / homoserine dehydrogenase I (ThrA) mutant having one or more amino acid substitutions at a position selected from the group consisting of Y356, S357 and S359.
18. The bacterium according to any one of claims 1 to 17, wherein said bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position Y356, one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position S357 and/or one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position S359; wherein the substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D, Y356E, Y356F, Y356A, Y356I, Y356P, Y356H, Y356R and Y356L; the substitution at position S357 is selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H, S357K, S357I and S357M; and the substitution at position S359 is selected from the group consisting of

S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L.

19. The bacterium according to any one of claims 1 to 18, wherein said bacterium expresses a aspartate kinase I / homoserine dehydrogenase I (ThrA) mutant having an amino acid substitution  
5 at position Y356, wherein the substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D, Y356E, Y356F, Y356A, Y356I, Y356P, Y356H, Y356R and Y356L.

20. The bacterium according to any one of claims 1 to 19, wherein said bacterium comprises  
10 within the *thrA* gene one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position S357, wherein the substitution at position S357 is selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H, S357K, S357I and S357M.

21. The bacterium according to any one of claims 1 to 20, wherein said bacterium expresses a  
15 aspartate kinase I / homoserine dehydrogenase I (ThrA) mutant having an amino acid substitution at position S357, wherein the substitution at position S357 is selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H, S357K, S357I and S357M.

22. The bacterium according to any one of claims 1 to 21, wherein said bacterium comprises  
20 within the *thrA* gene one or more nucleotide substitutions resulting in an amino acid substitution in the encoded polypeptide at position S359, wherein the substitution at position S359 is selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L.

23. The bacterium according to any one of claims 1 to 22, wherein said bacterium expresses a  
25 aspartate kinase I / homoserine dehydrogenase I (ThrA) mutant having an amino acid substitution at position S359, wherein the substitution at position S359 is selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L.



24. The bacterium according to any one of claims 1 to 23, wherein said bacterium comprises within the *thrA* gene one or more nucleotide substitutions resulting in one or more amino acid substitutions selected from the group consisting of Y356C, S357R and S359R.

5 25. The bacterium according to any one of claims 1 to 24, wherein said bacterium expresses a aspartate kinase I / homoserine dehydrogenase I (ThrA) mutant having one or more amino acid substitutions selected from the group consisting of Y356C, S357R and S359R.

10 26. The bacterium according to any one of claims 1 to 25, wherein said bacterium expresses a polypeptide having an amino acid sequence which has at least about 90% sequence identity to the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position Y356, S357 and/or S359, wherein the substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D, Y356E, Y356F, Y356A, Y356I, Y356P, Y356H, Y356R and Y356L; the substitution at position S357 is selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H, S357K, S357I and S357M; and the substitution at position S359 is selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L.

20 27. The bacterium according to any one of claims 1 to 26, wherein said bacterium comprises an exogenous nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide having an amino acid sequence which has at least about 90% sequence identity to the amino acid sequence set forth in SEQ ID NO: 11 which comprises an amino acid substitution at position Y356, S357 and/or S359.

25 28. The bacterium according to claim 27, wherein the substitution at position Y356 is selected from the group consisting of Y356C, Y356T, Y356V, Y356S, Y356W, Y356Q, Y356G, Y356N, Y356D, Y356E, Y356F, Y356A, Y356I, Y356P, Y356H, Y356R and Y356L; the substitution at position S357 is selected from the group consisting of S357R, S357V, S357P, S357G, S357L, S357Y, S357A, S357N, S357F, S357H, S357K, S357I and S357M; and the substitution at position S359 is selected from the group consisting of S359R, S359G, S359M, S359F, S359T, S359P, S359V, S359Q, S359A, S359C, S359K, S359E and S359L.

30 29. The bacterium according to any one of claims 1 to 28, wherein said bacterium has been further modified to overexpress the gene *ydeD*.

30. The bacterium according to any one of claims 1 to 29, wherein said bacterium comprises one or more gene mutations selected from the group consisting of: one or more nucleotide substitutions within the *lrp* gene resulting in the amino acid substitution D143G in the encoded polypeptide, one or more nucleotide substitutions within the *rho* gene resulting in the amino acid substitution R87L in the encoded polypeptide, one or more nucleotide substitutions within the *eno* gene resulting in the amino acid substitution V164L in the encoded polypeptide, one or more nucleotide substitutions within the *argP* gene resulting in the amino acid substitution Q132K in the encoded polypeptide, one or more nucleotide substitutions within the *tufA* gene resulting in the amino acid substitution G19V in the encoded polypeptide, gene one or more nucleotide substitutions within the *cycA* resulting in the amino acid substitution I220V in the encoded polypeptide, one or more nucleotide substitutions within the *rpe* gene resulting in the amino acid substitution I202T in the encoded polypeptide, one or more nucleotide substitutions within the *yojI* gene resulting in the amino acid substitution D334H in the encoded polypeptide, one or more nucleotide substitutions within the *hyaF* gene resulting in the amino acid substitution V120G in the encoded polypeptide, one or more nucleotide substitutions within the *pykF* gene resulting in the amino acid substitution E250\* in the encoded polypeptide, where \* designates a stop codon, one or more nucleotide substitutions within the *maltT* gene resulting in the amino acid substitution Q420\* in the encoded polypeptide, where \* designates a stop codon, one or more nucleotide substitutions within the *rpoB* gene resulting in the amino acid substitution P520L in the encoded polypeptide, one or more nucleotide substitutions within the *fumB* gene resulting in the amino acid substitution T218P in the encoded polypeptide, one or more nucleotide substitutions within the *gshA* gene resulting in the amino acid substitution A178V in the encoded polypeptide, and one or more nucleotide substitutions within the *lamB* gene resulting in the amino acid substitution Q112\* in the encoded polypeptide, where \* designates a stop codon.
31. The bacterium according to any one of claims 1 to 30, wherein said bacterium belongs to the *Enterobacteriaceae* family.
32. The bacterium according to any one of claims 1 to 31, wherein said bacterium belongs to the genus *Escherichia*.
33. The bacterium according to any one of claims 1 to 32, wherein said bacterium is *Escherichia coli*.

34. A method for producing L-serine or a L-serine derivative, the method comprises cultivating a bacterium according to any one of claims 1 to 33 in a culture medium.

35. The method according to claim 34, wherein the L-serine derivative is selected from the group consisting of L-cysteine, L-methionine, L-glycine, O-acetylserine, L-tryptophan, thiamine ethanolamine and ethylene glycol.

5

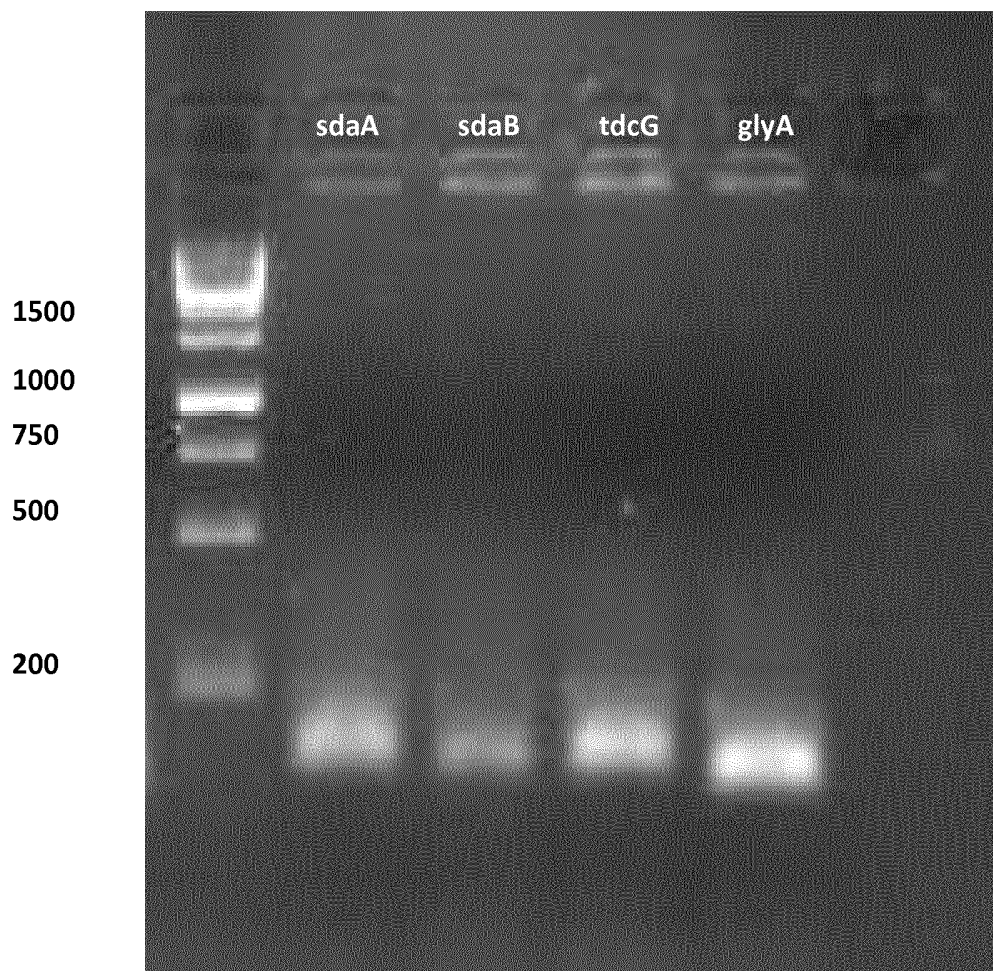


Figure 1

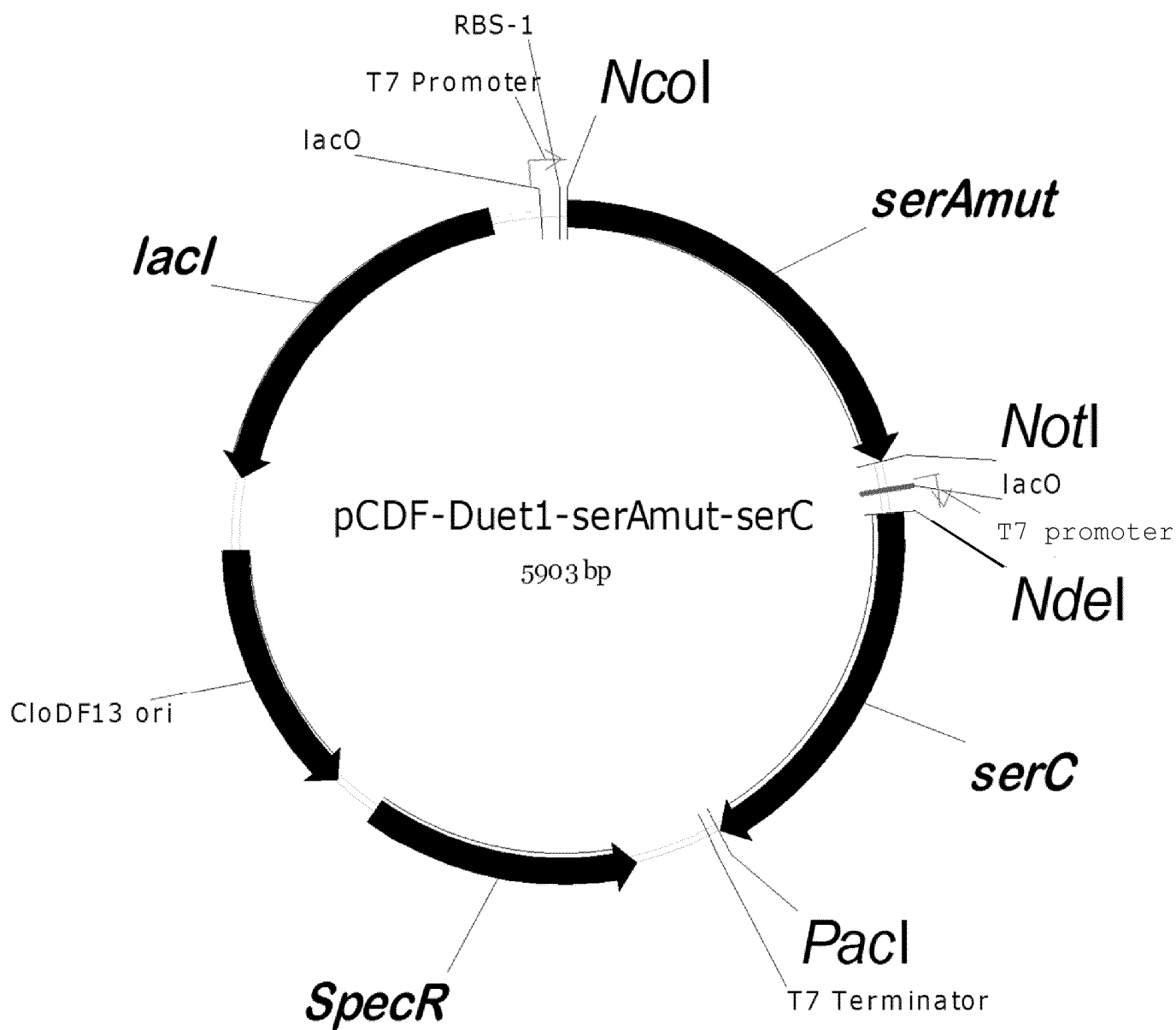


Figure 2

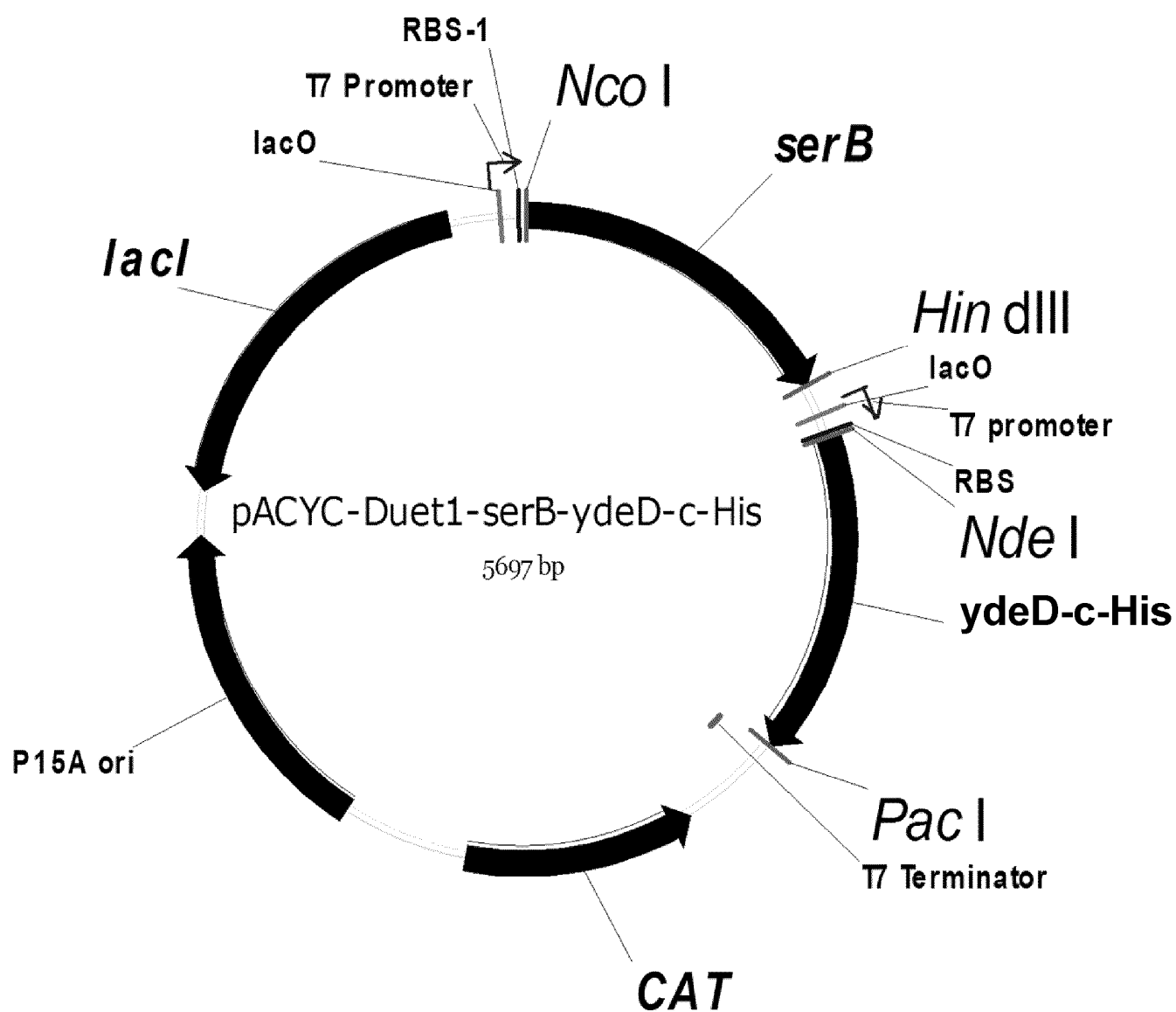


Figure 2 (cont.)

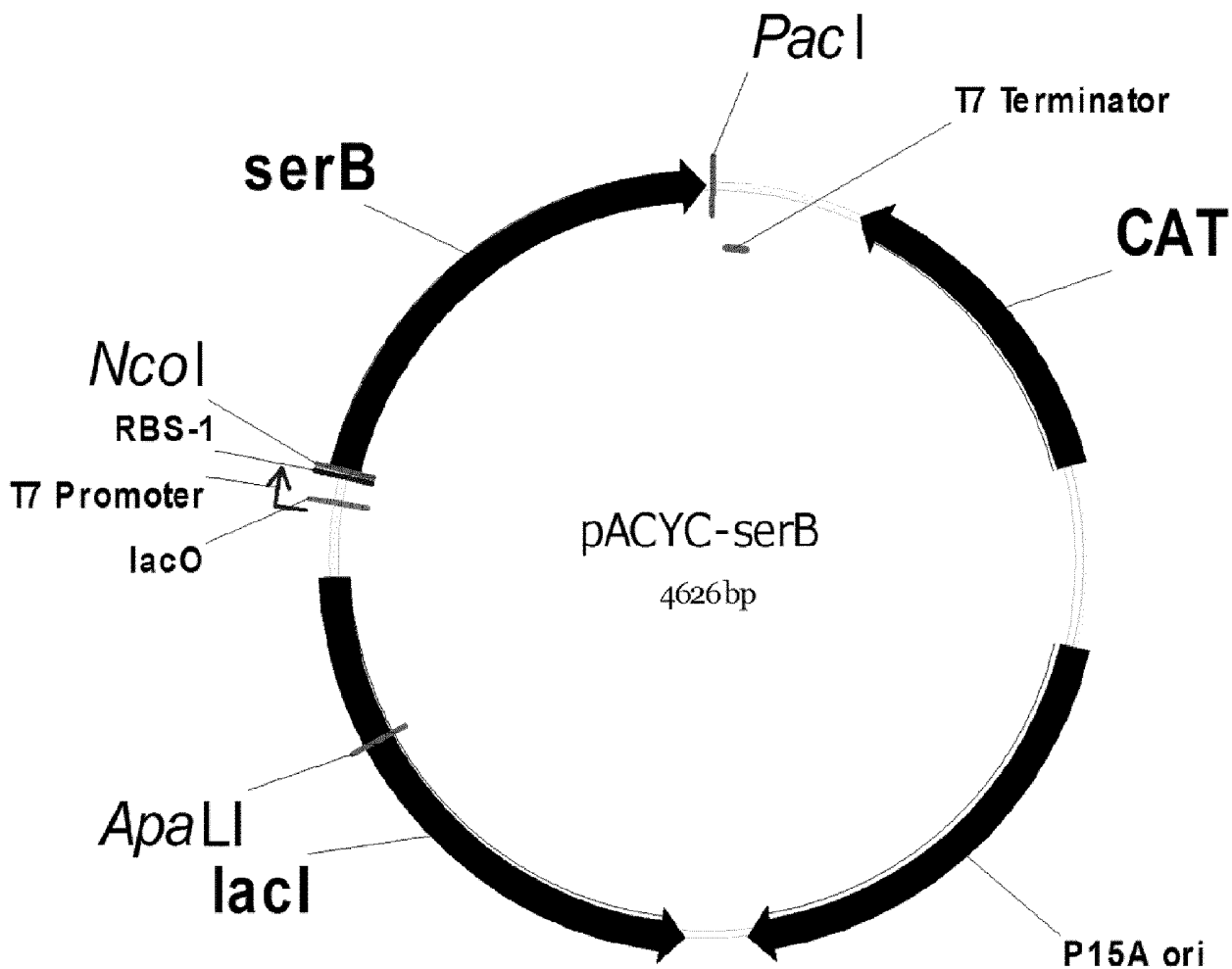


Figure 2 (cont.)

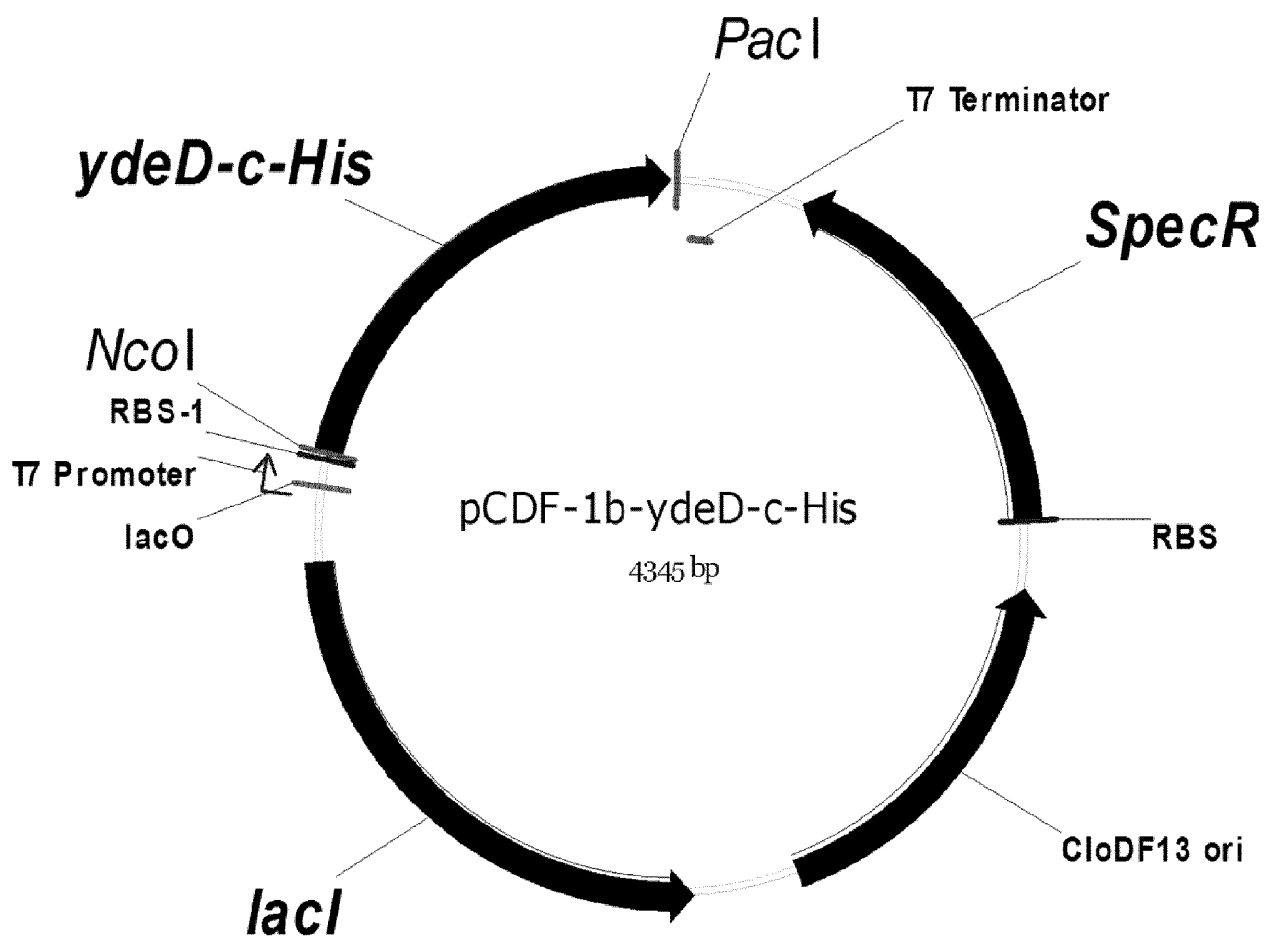


Figure 2 (cont.)



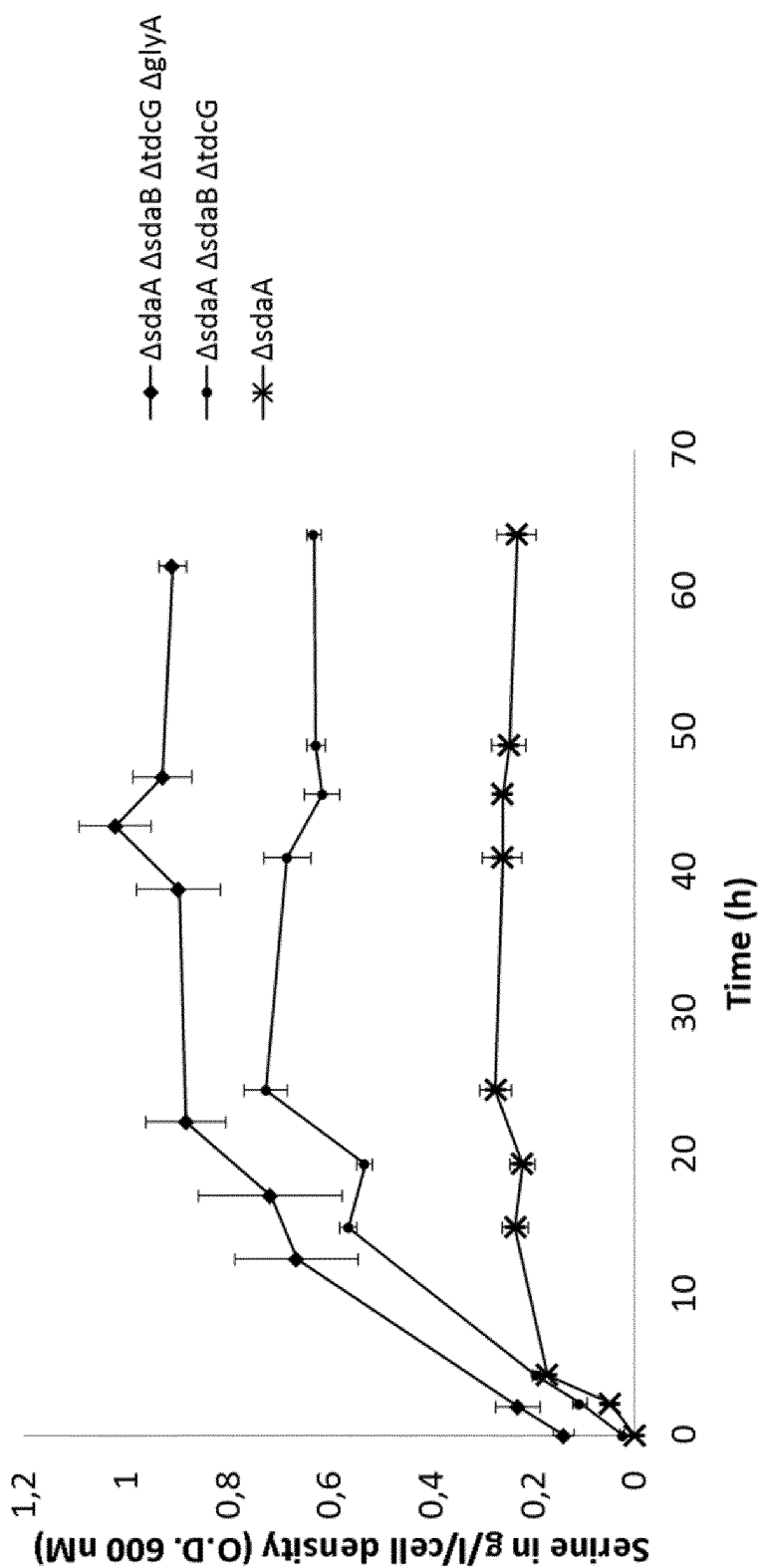


Figure 3

	Serine concentration in g/L (+/- standard deviation)	Yield from glucose (g serine / g glucose)
ΔsdaA ΔsdaB ΔtdcG ΔglyA (Q1(DE3))	0.889 (+/- 0.057)	0.445
sdaAΔ sdaBΔ tdcGΔ ::(DE3)	0.536 (+/- 0.019)	0.268
ΔsdaA ::(DE3)	0.493 (+/- 0.033)	0.247

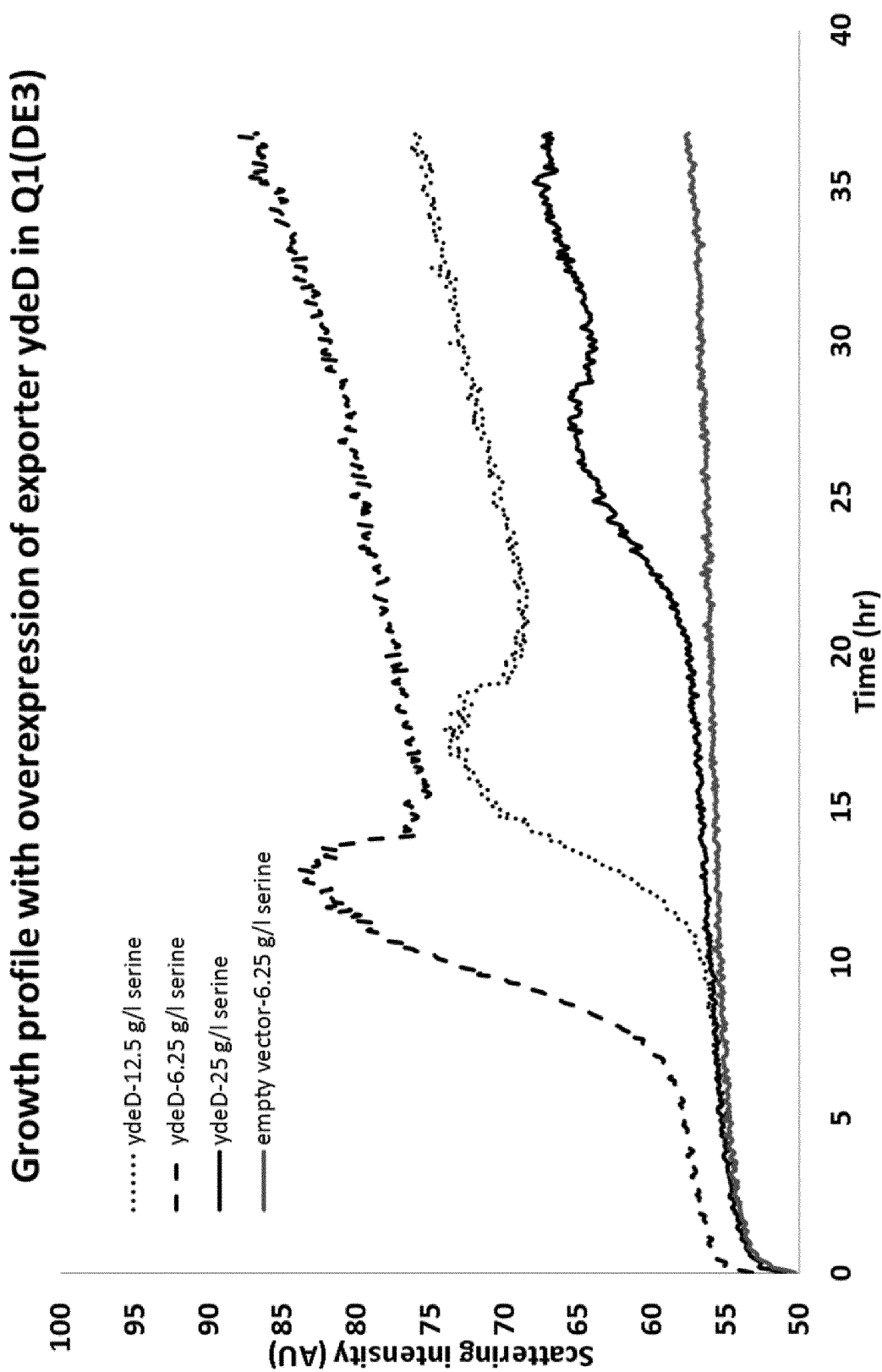


Figure 4

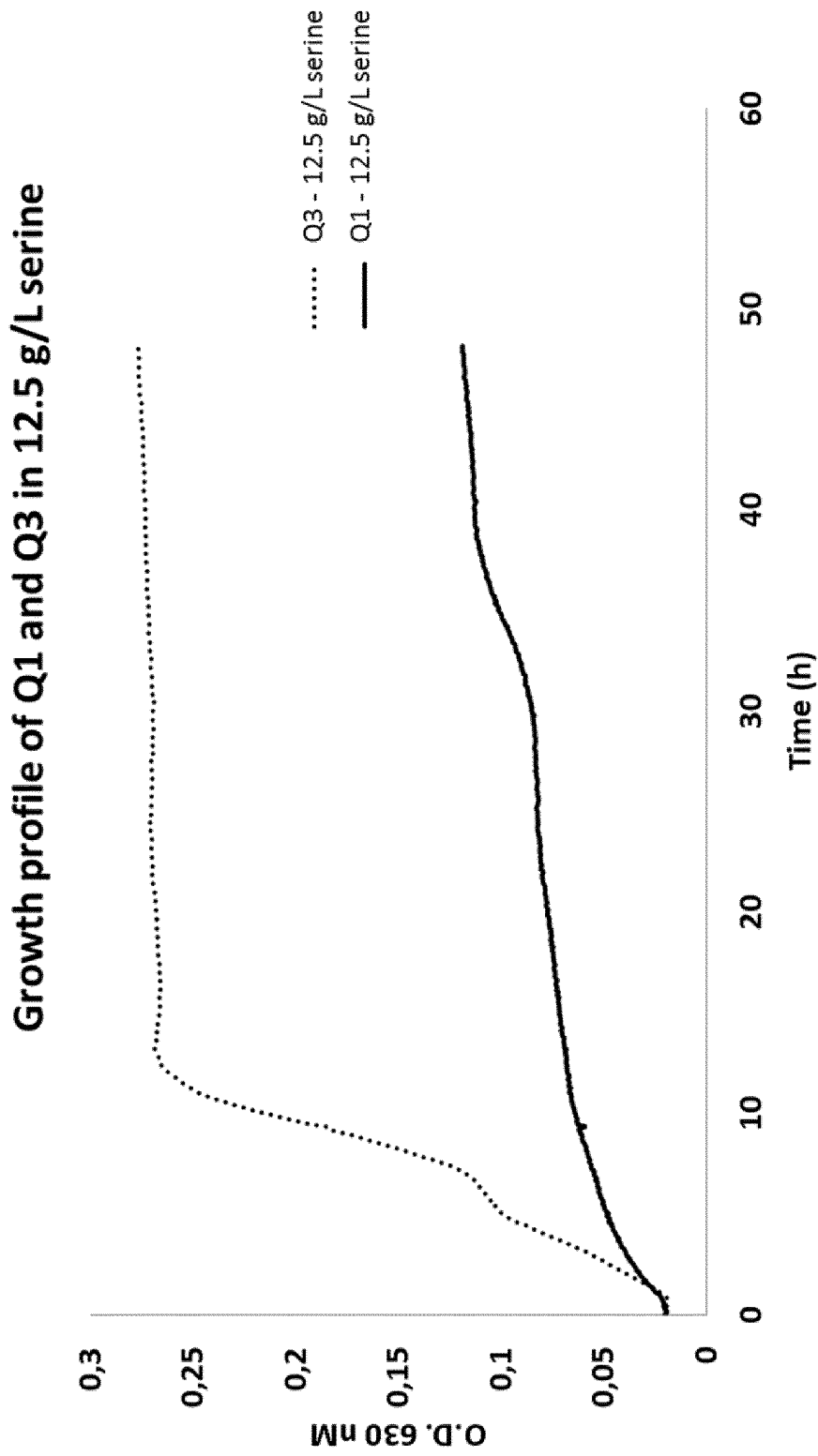


Figure 5

6A.

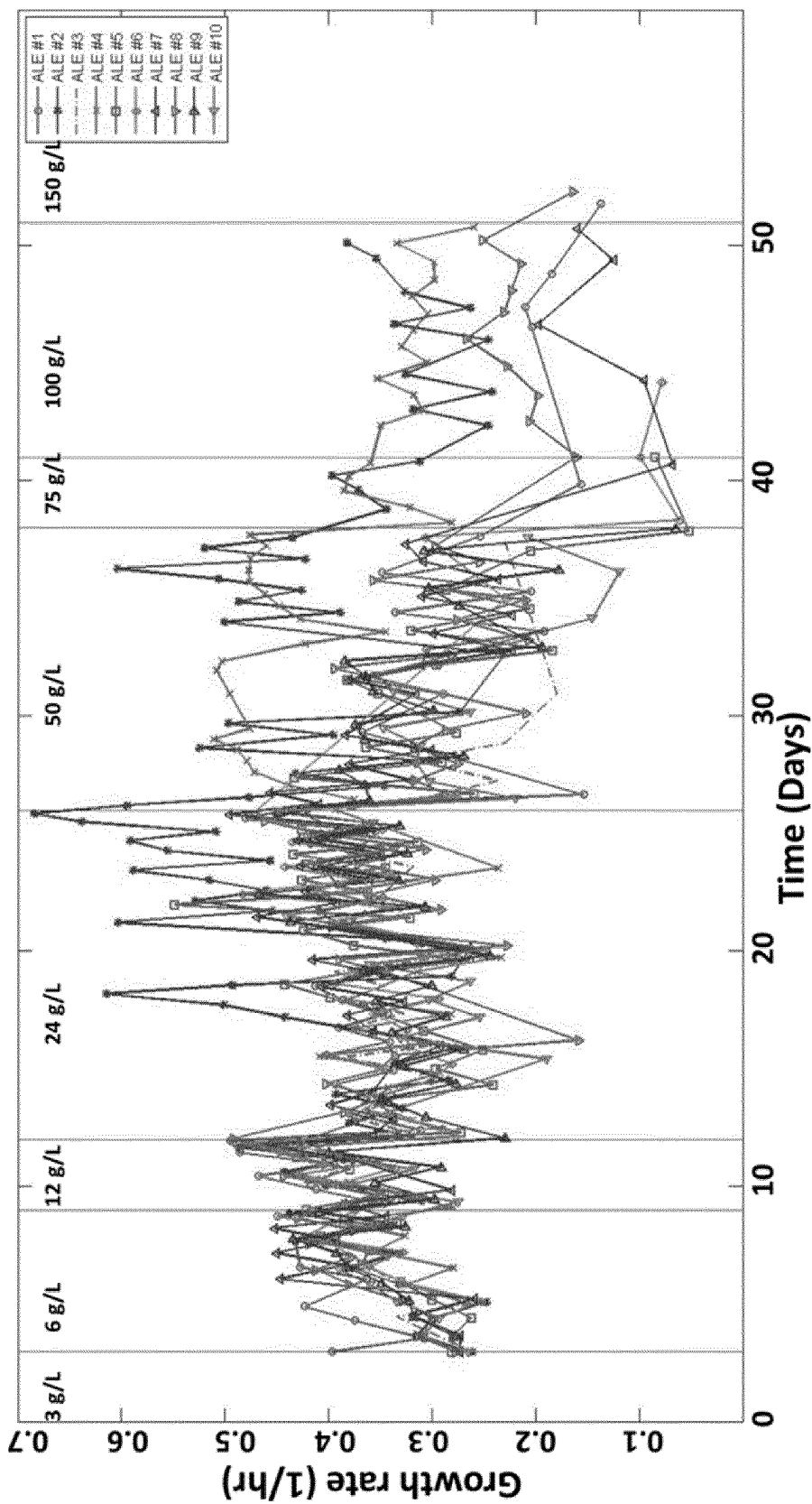


Figure 6

6B.

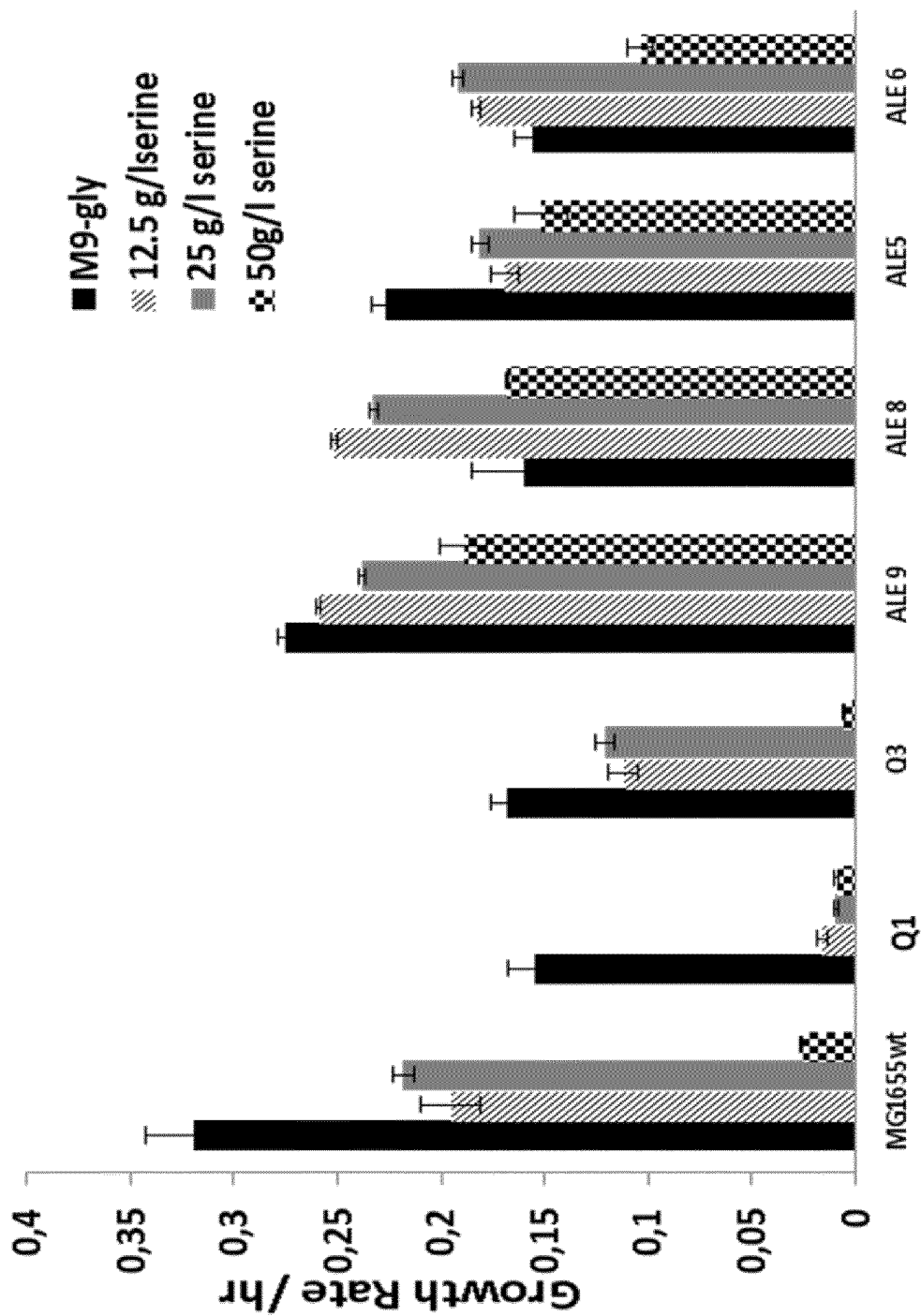


Figure 6

6C.

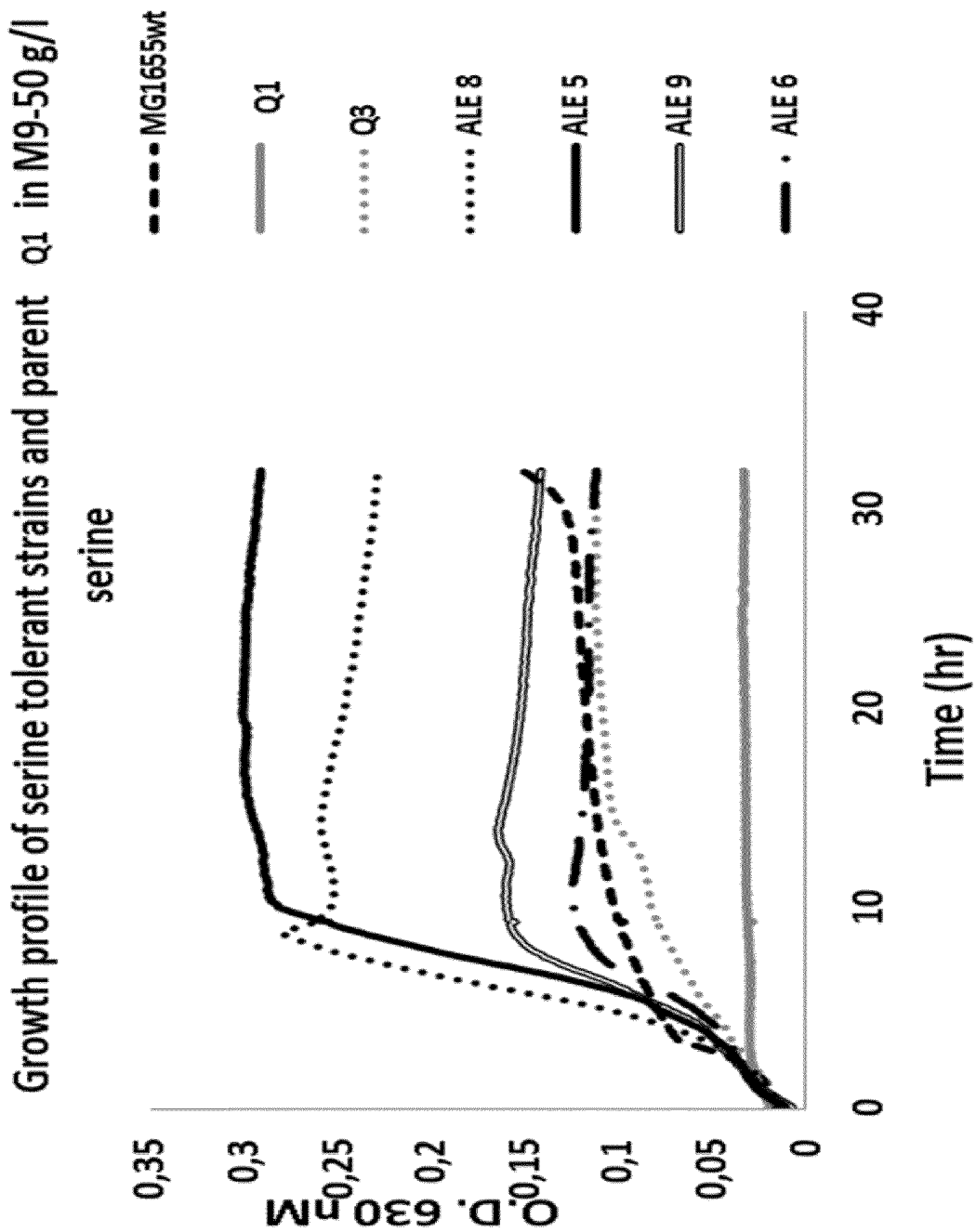


Figure 6

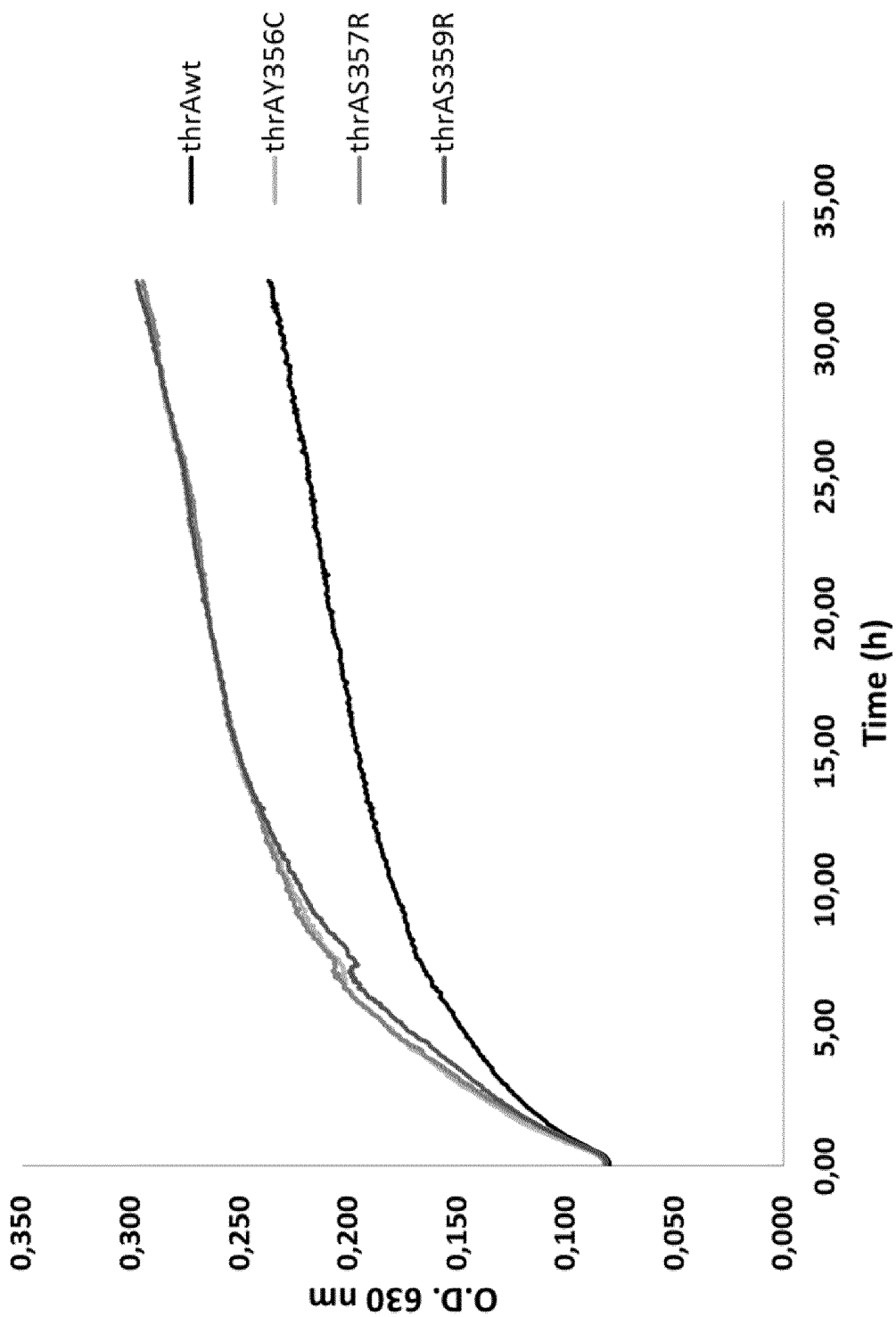


Figure 7

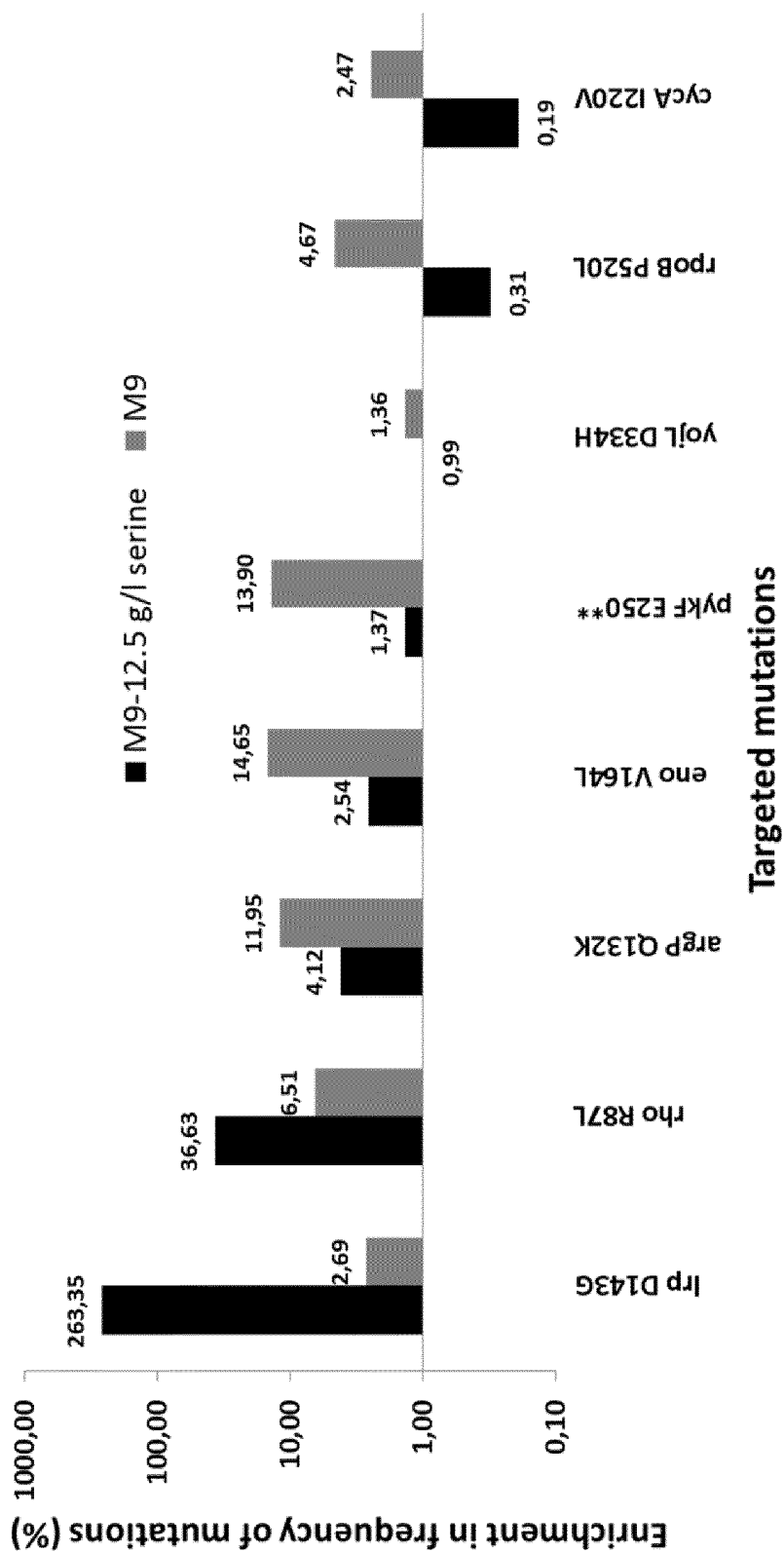


Figure 8



9A.

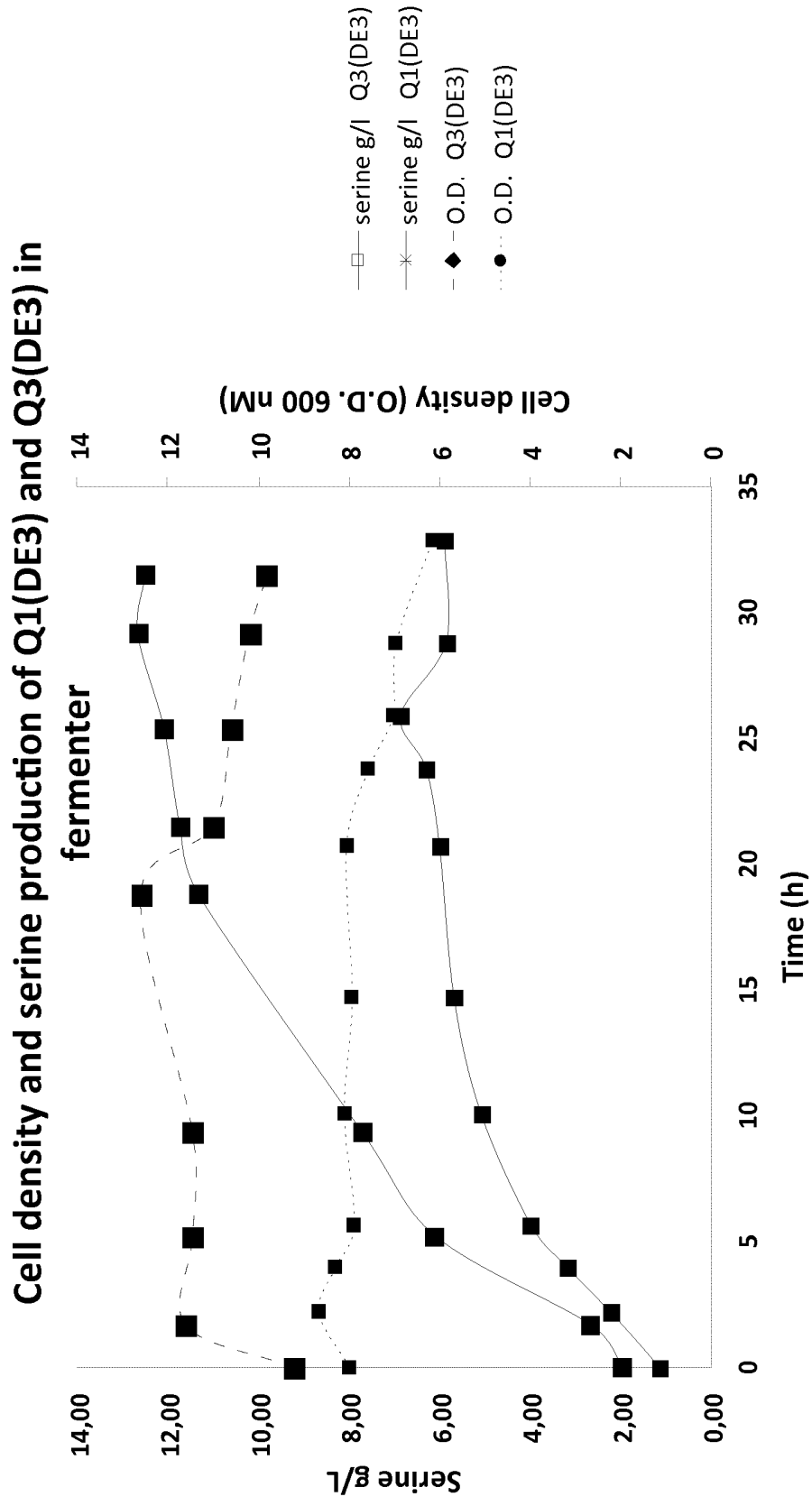
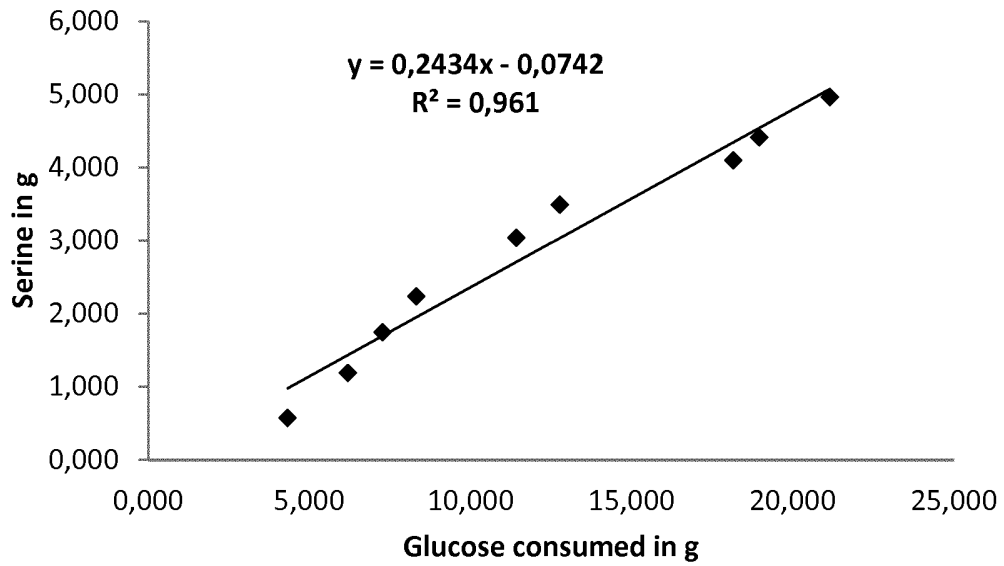


Figure 9

9B.

**Q1(DE3) efficiency in serine production**



**Q3(DE3) efficiency in serine production**

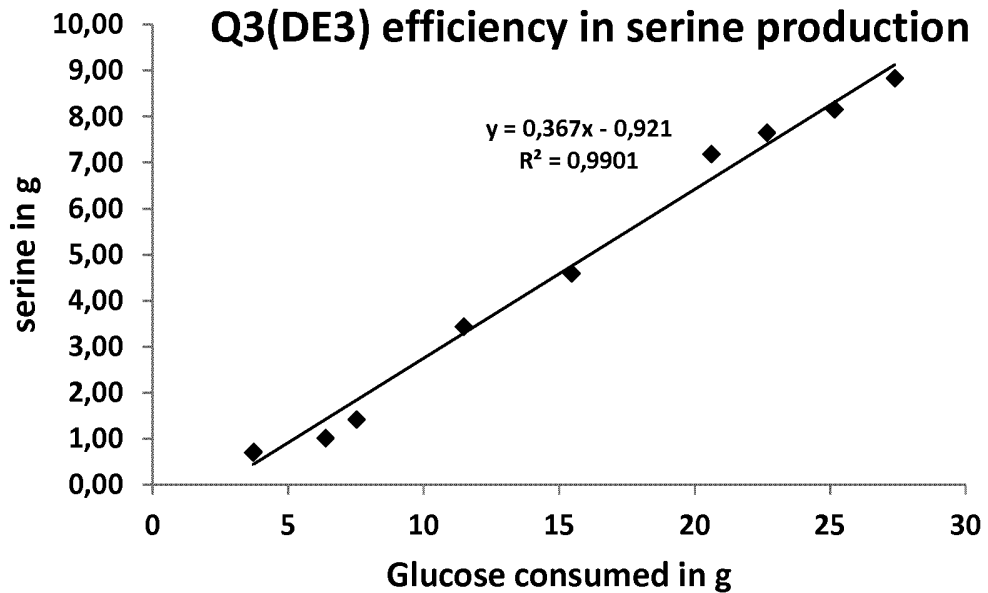
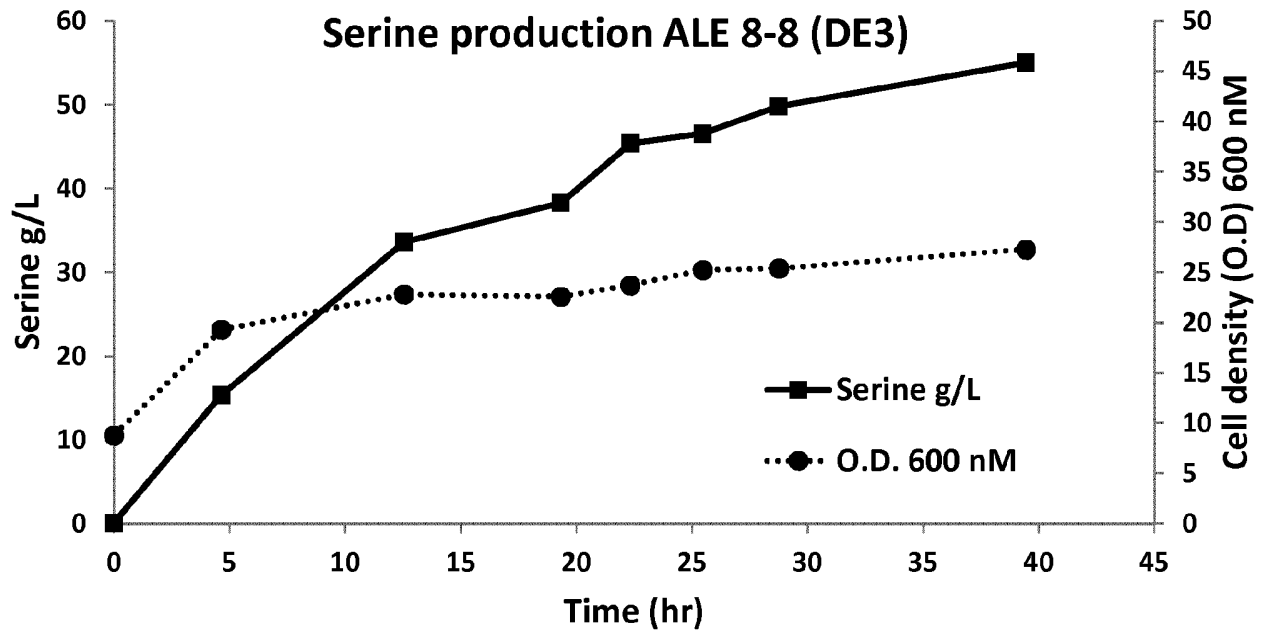


Figure 9

10A.



10B.

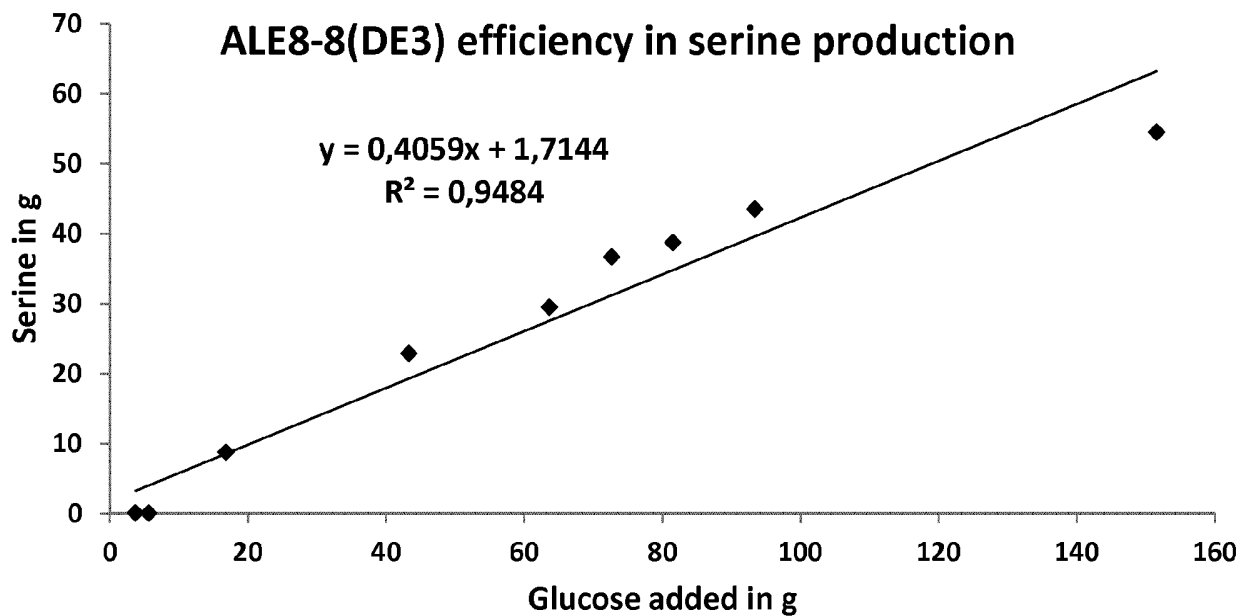
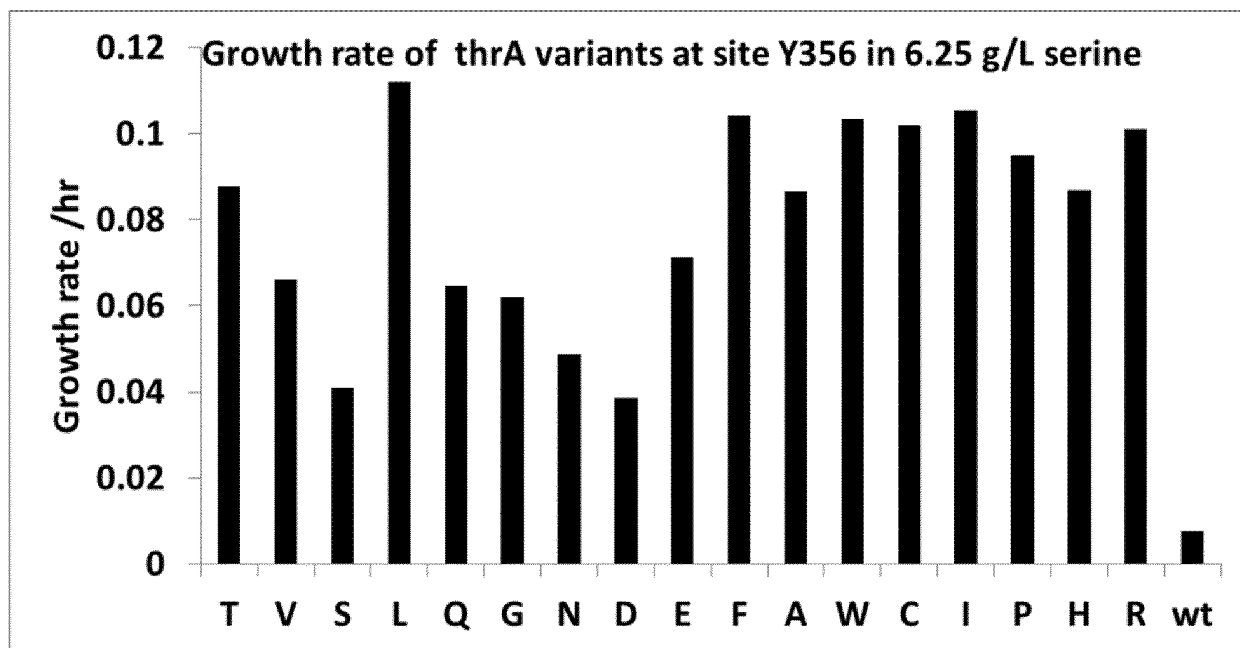


Figure 10

11A.



11B.

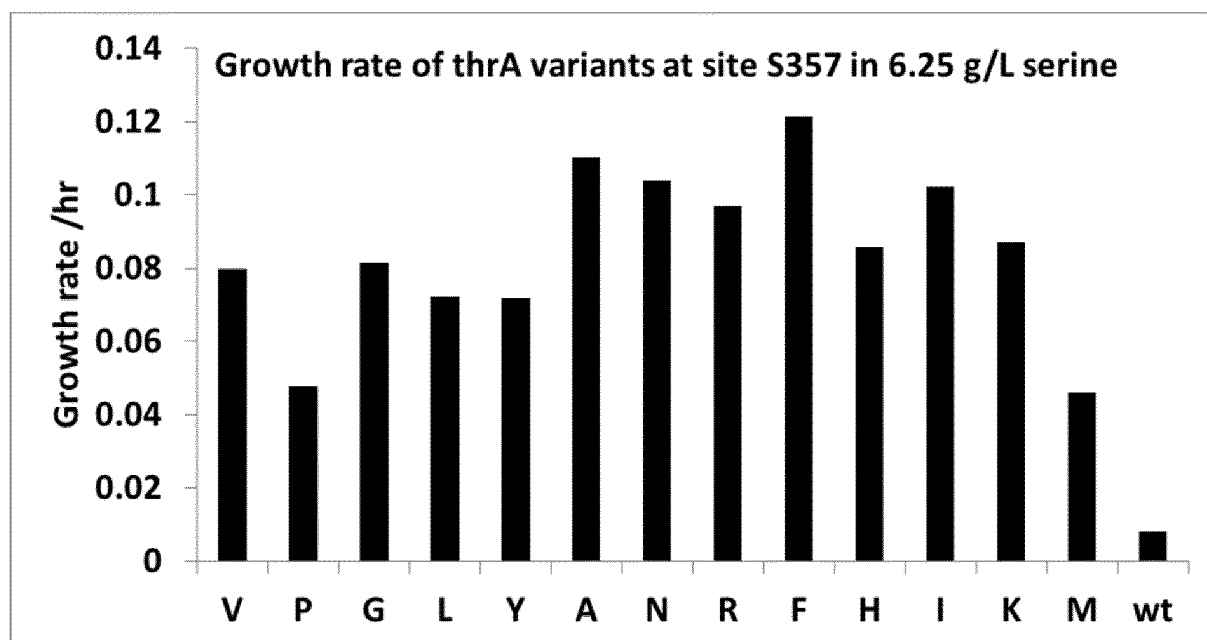


Figure 11

## 11C.

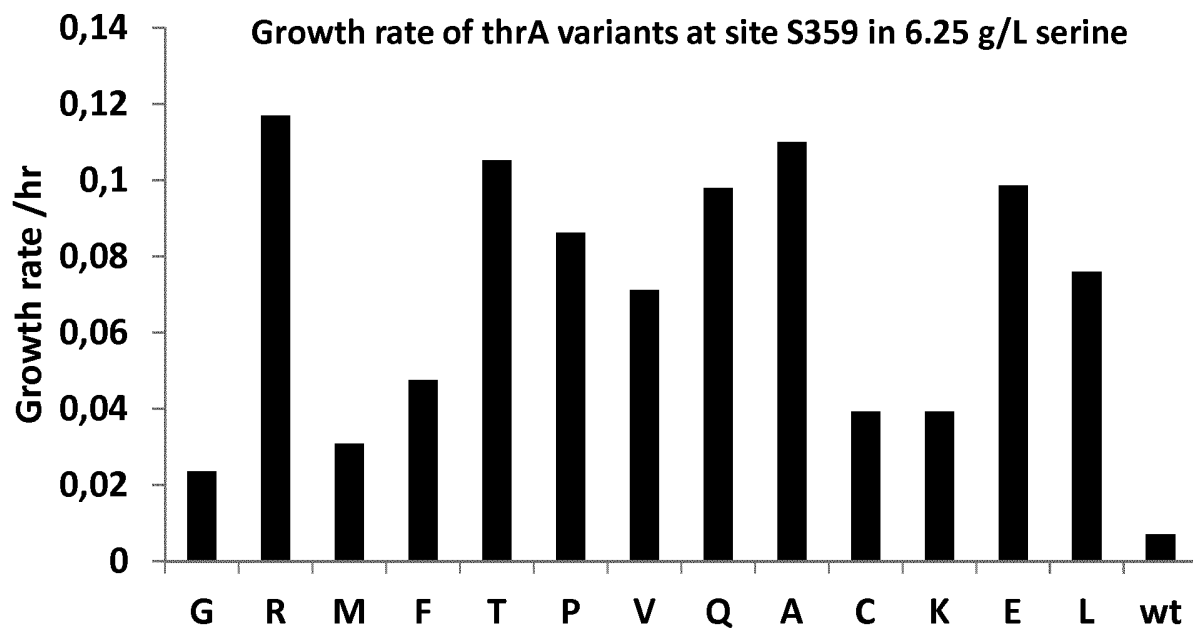
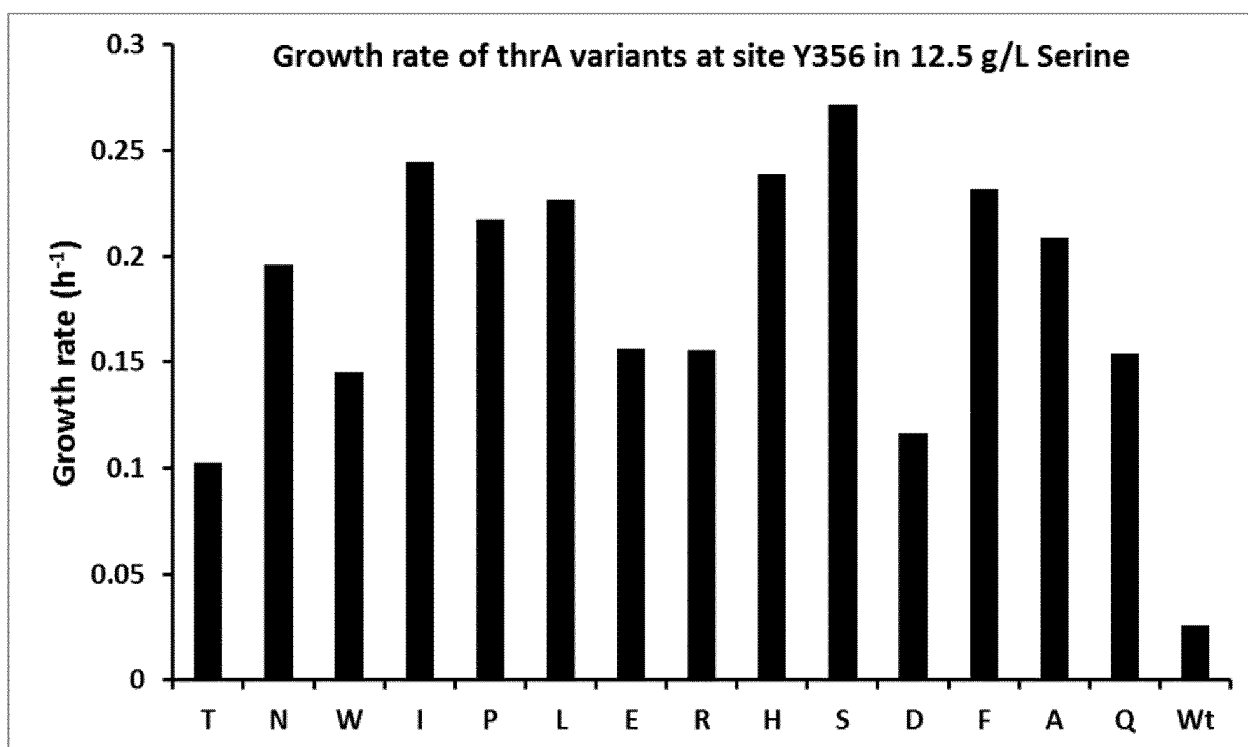
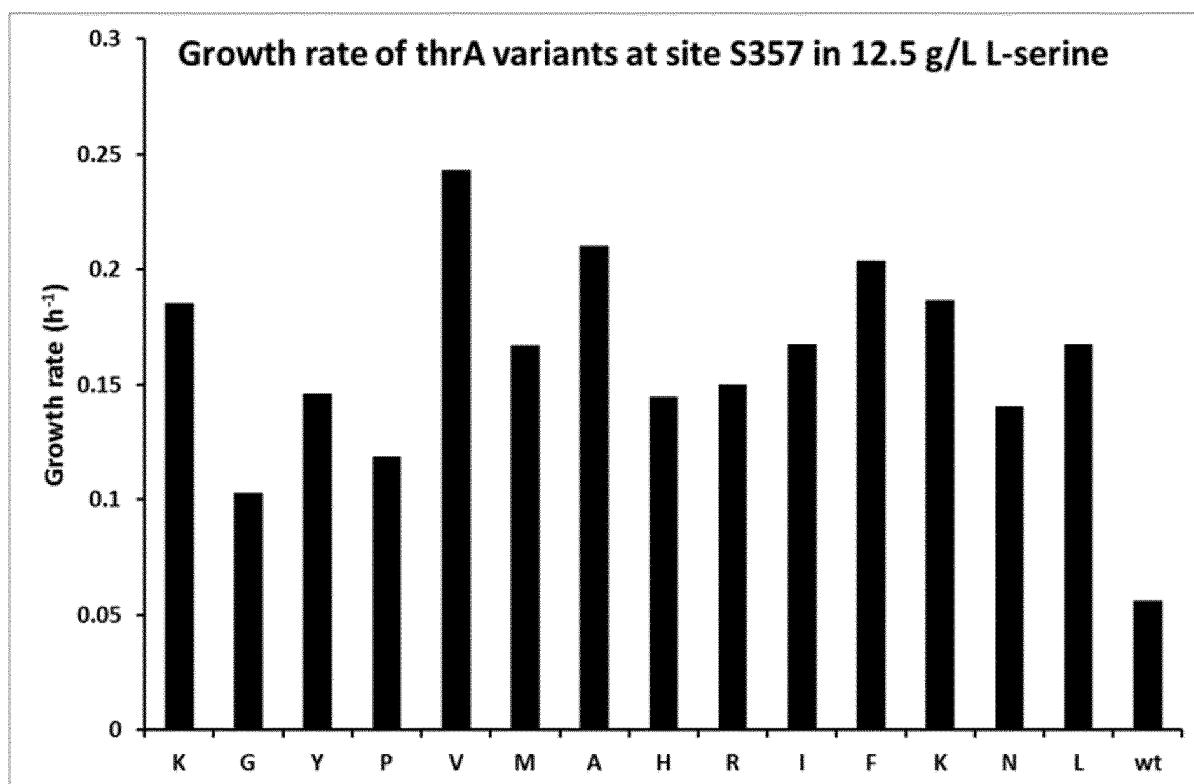


Figure 11

**12A.**



**12B.**



**Figure 12**

## 12C.

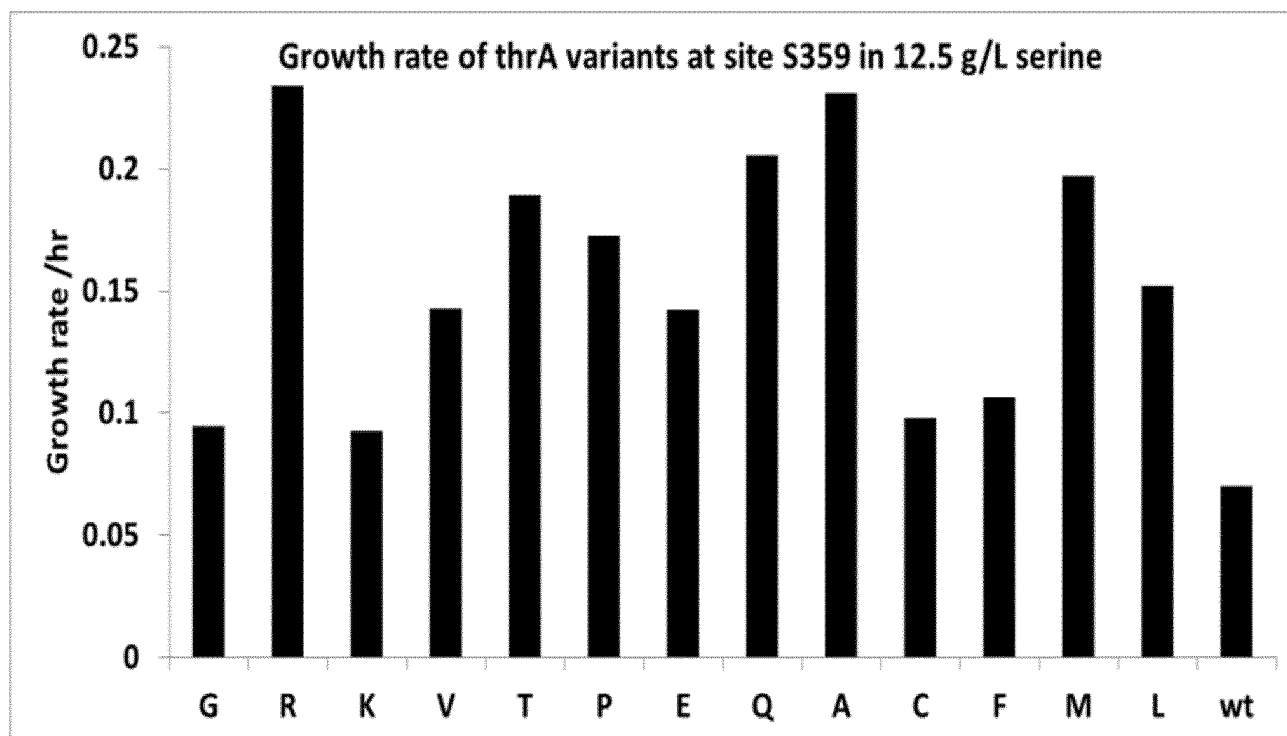


Figure 12

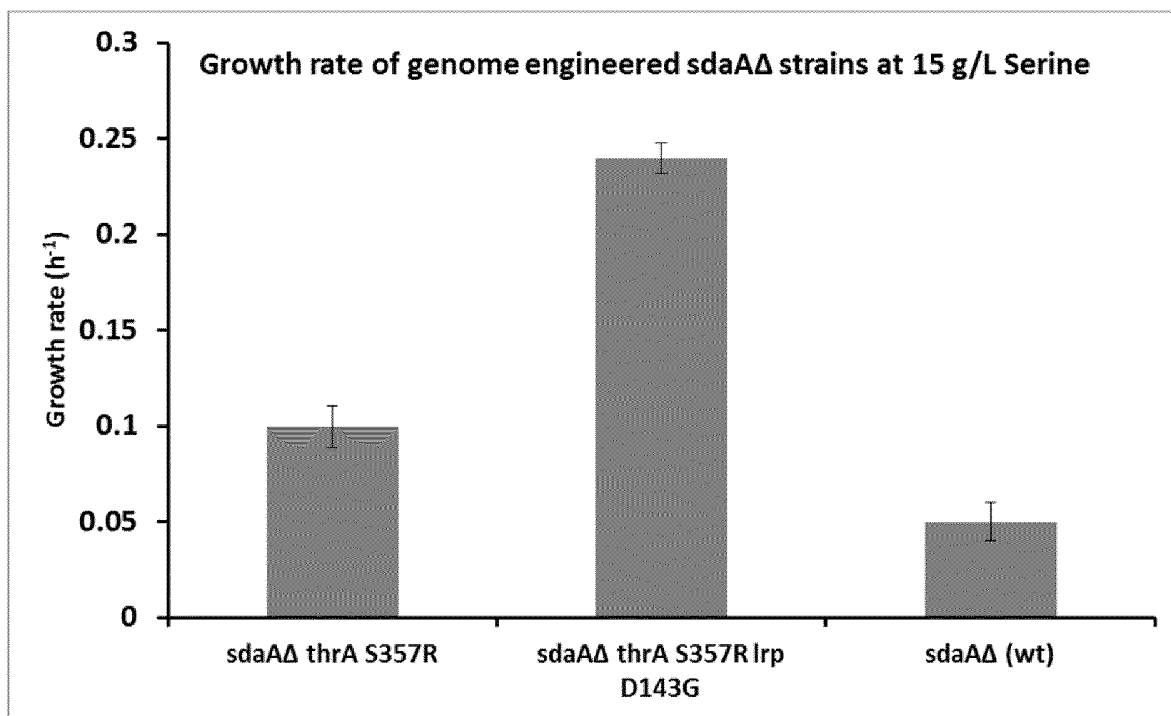
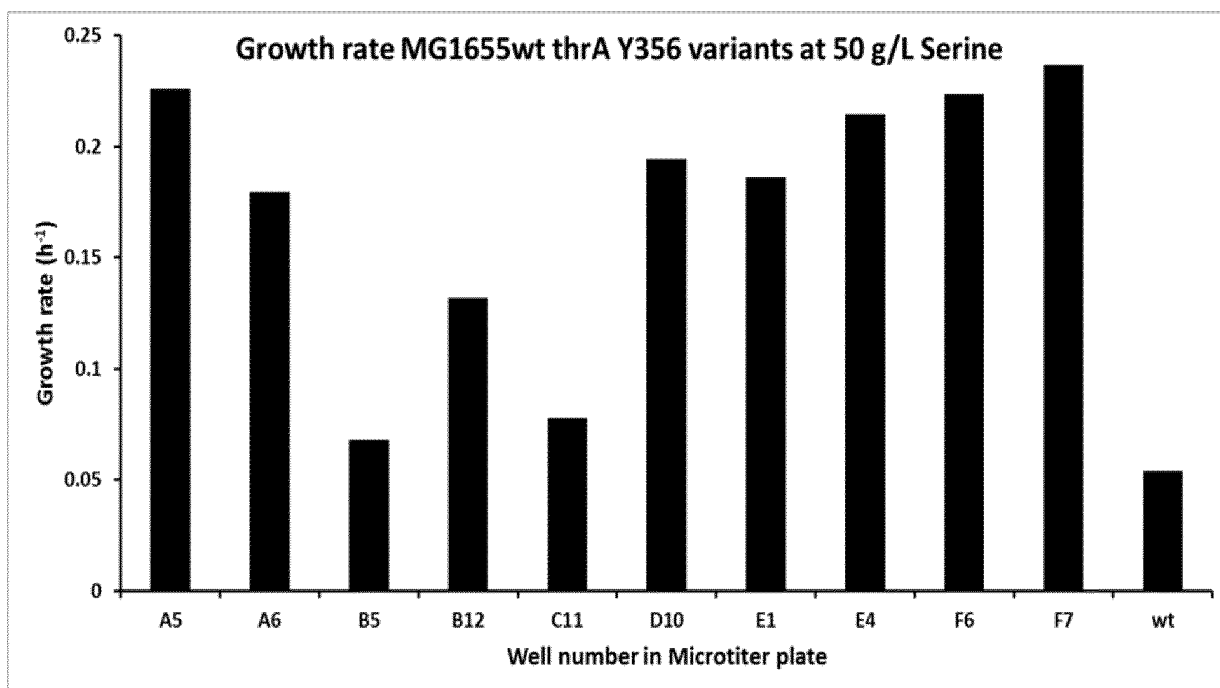
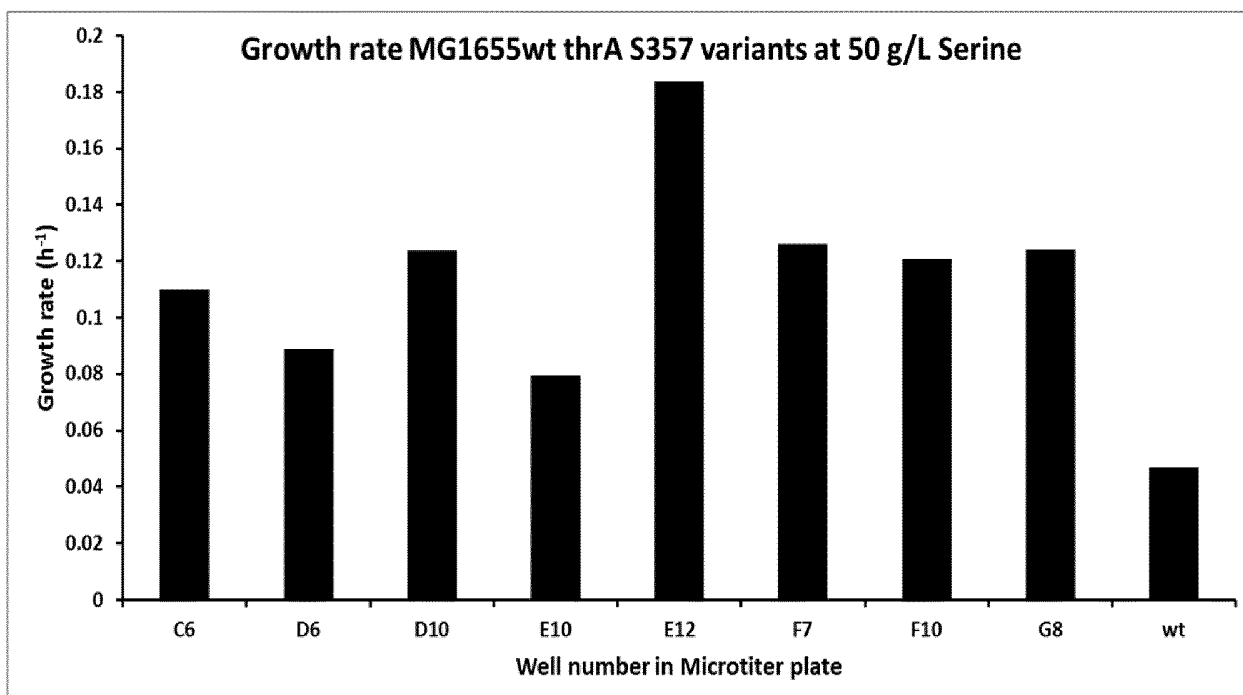


Figure 13



**14A.****14B.****Figure 14**

## 14C.

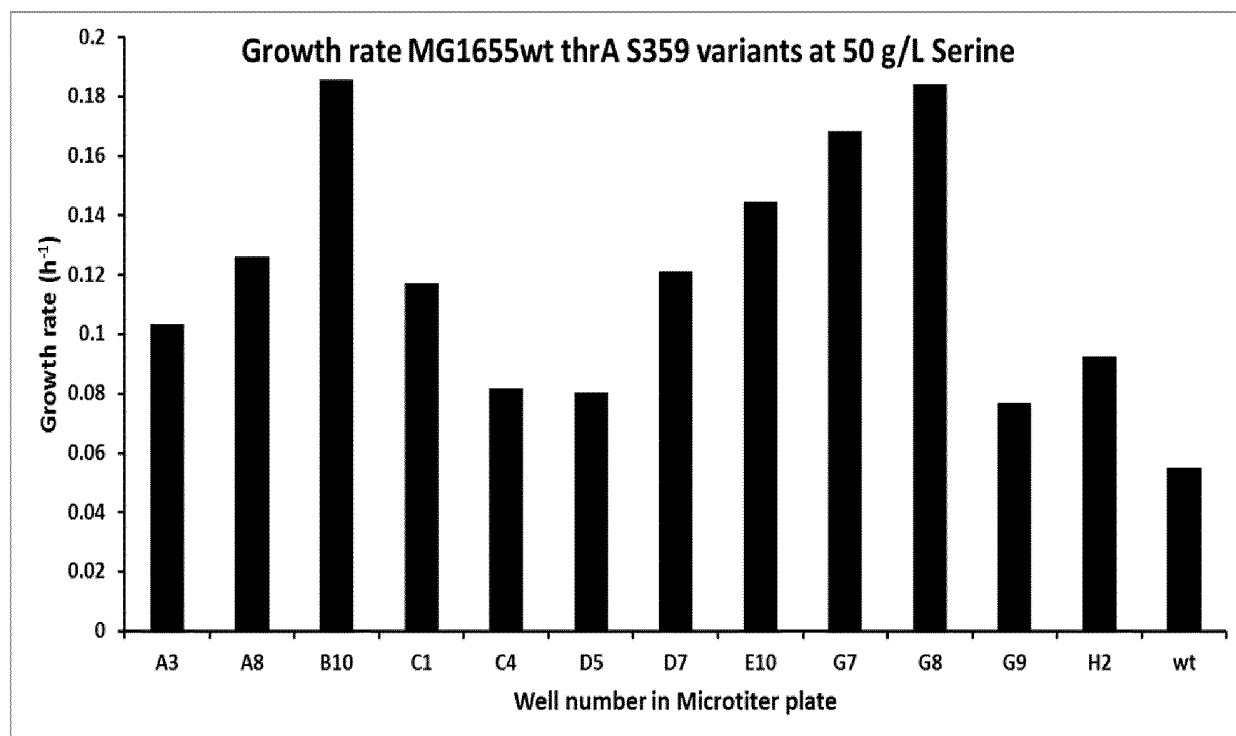


Figure 14

# INTERNATIONAL SEARCH REPORT

International application No PCT/EP2016/051728
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<b>A. CLASSIFICATION OF SUBJECT MATTER</b> INV. C12P13/06 C12N15/00 ADD.				
According to International Patent Classification (IPC) or to both national classification and IPC				
<b>B. FIELDS SEARCHED</b>				
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, BIOSIS, WPI Data				
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>				
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.		
X	P. PETERS-WENDISCH ET AL: "Metabolic Engineering of Corynebacterium glutamicum for L-Serine Production", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 71, no. 11, 1 November 2005 (2005-11-01), pages 7139-7144, XP055017302, ISSN: 0099-2240, DOI: 10.1128/AEM.71.11.7139-7144.2005	1-15, 29-35		
Y	abstract ----- -/--	16-28		
<table style="width: 100%; border: none;"> <tr> <td style="width: 50%; border: none;"><input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C.</td> <td style="width: 50%; border: none;"><input checked="" type="checkbox"/> See patent family annex.</td> </tr> </table>			<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C.	<input checked="" type="checkbox"/> See patent family annex.
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C.	<input checked="" type="checkbox"/> See patent family annex.			
* Special categories of cited documents :				
"A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "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 "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "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	Date of mailing of the international search report			
4 April 2016	15/04/2016			
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer  Herrmann, Klaus			

## INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2016/051728

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	YU LI ET AL: "Construction of Escherichia coli strains producing-serine from glucose", BIOTECHNOLOGY LETTERS, SPRINGER NETHERLANDS, DORDRECHT, vol. 34, no. 8, 1 May 2012 (2012-05-01), pages 1525-1530, XP035089780, ISSN: 1573-6776, DOI: 10.1007/S10529-012-0937-0 cited in the application abstract	1,2,5-7, 9,11-15, 29-35
Y	-----	16-28
A	SHUJUAN LAI ET AL: "Metabolic engineering and flux analysis of Corynebacterium glutamicum for L-serine production", SCIENCE CHINA LIFE SCIENCES, SP SCIENCE CHINA PRESS, HEIDELBERG, vol. 55, no. 4, 9 May 2012 (2012-05-09), pages 283-290, XP035052326, ISSN: 1869-1889, DOI: 10.1007/S11427-012-4304-0 abstract	1-35
A	-----	1-35
A	XIAO ZHANG ET AL: "Deficiency in l-serine deaminase results in abnormal growth and cell division of Escherichia coli K-12", MOLECULAR MICROBIOLOGY, 1 July 2008 (2008-07-01), pages ???-???, XP055199519, ISSN: 0950-382X, DOI: 10.1111/j.1365-2958.2008.06315.x cited in the application the whole document	1-35
A	-----	1-35
A	CN 102 703 371 A (UNIV TIANJIN SCIENCE & TECH) 3 October 2012 (2012-10-03) the whole document	1-35
A	-----	1-35
A	WO 2007/144018 A1 (METABOLIC EXPLORER SA [FR]; SOUCAILLE PHILIPPE [FR]) 21 December 2007 (2007-12-21) claim 13	1-35
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# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2016/051728

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
CN 102703371	A	03-10-2012	NONE
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WO 2007144018	A1	21-12-2007	US 2009325245 A1 31-12-2009
			WO 2007144018 A1 21-12-2007
			WO 2007144346 A1 21-12-2007
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