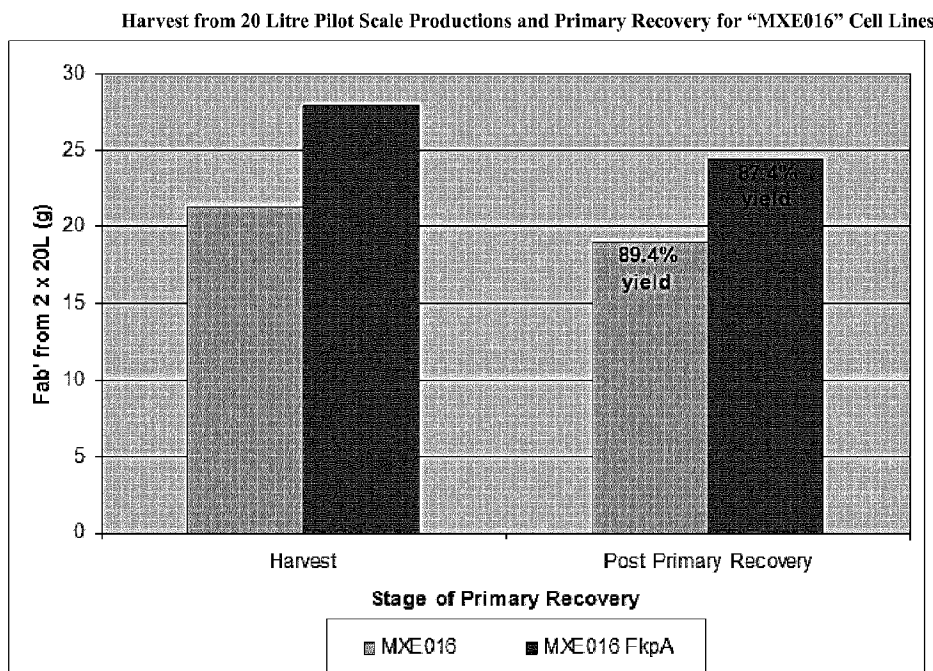




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(72) Inventeurs/Inventors:  
BASSETT, PHILIP JONATHAN, GB;  
HUMPHREYS, DAVID PAUL, GB;  
PATEL, PARESHKUMAR MANJIBHAI, GB  
(73) Propriétaire/Owner:  
UCB PHARMA S.A., BE  
(74) Agent: SMART & BIGGAR IP AGENCY CO.

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(57) **Abrégé/Abstract:**

The present disclosure relates to a recombinant gram-negative bacterial cell comprising: a.) a mutant spr gene encoding a spr protein having a mutation at one or more amino acids selected from D133, H145, H157, N31, R62, I70, Q73, C94, S95, V98, Q99, R100, L108, Y115, V135, L136, G140, R144 and G147 and b.) a gene capable of expressing or overexpressing one or more proteins capable of facilitating protein folding, such as FkpA, Skp, SurA, PPIA and PPiD, wherein the cell has reduced Tsp protein activity compared to a wild-type cell, methods employing the cells, use of the cells in the expression of proteins in particular antibodies, such as anti Fc Rn antibodies and proteins made by the methods described herein.

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- (71) **Applicant:** UCB PHARMA S.A. [BE/BE]; 60 Allée de la Recherche, B-1070 Brussels (BE).
- (72) **Inventors:** BASSETT, Philip Jonathan; c/o IPD, UCB Celltech, 208 Bath Road, Slough Berkshire SL1 3WE (GB). HUMPHREYS, David Paul; c/o IPD, UCB Celltech, 208 Bath Road, Slough Berkshire SL1 3WE (GB). PATEL, Pareshkumar Manjibhai; c/o IPD, UCB Celltech, 208 Bath Road, Slough Berkshire SL1 3WE (GB).
- (74) **Agent:** THOMPSON, John; UCB Celltech, 208 Bath Road, Slough Berkshire SL1 3WE (GB).
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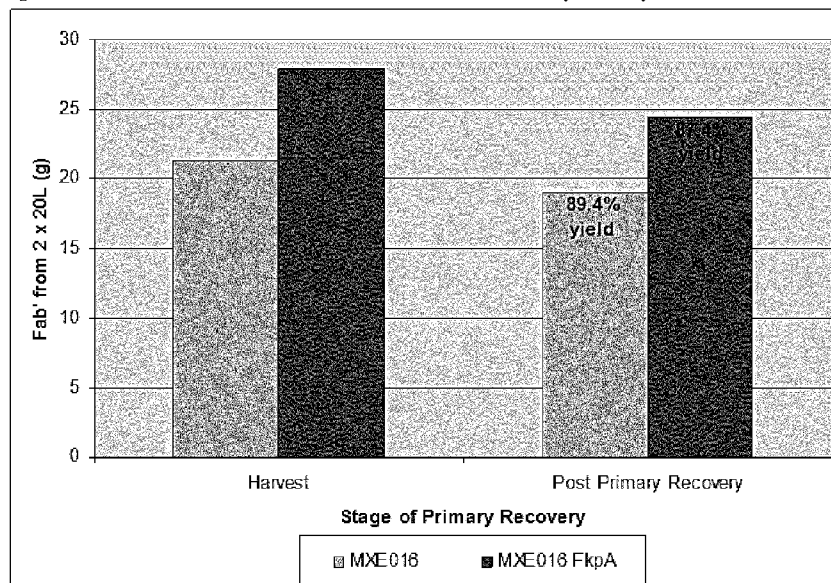
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(54) **Title:** RECOMBINANT BACTERIAL HOST CELL FOR PROTEIN EXPRESSION

Figure 4 Harvest from 20 Litre Pilot Scale Productions and Primary Recovery for "MXE016" Cell Lines



(57) **Abstract:** The present disclosure relates to a recombinant gram-negative bacterial cell comprising: a.) a mutant spr gene encoding a spr protein having a mutation at one or more amino acids selected from D133, H145, H157, N31, R62, I70, Q73, C94, S95, V98, Q99, R100, L108, Y115, V135, L136, G140, R144 and G147 and b.) a gene capable of expressing or overexpressing one or more proteins capable of facilitating protein folding, such as FkpA, Skp, SurA, PPIA and PPiD, wherein the cell has reduced Tsp protein activity compared to a wild-type cell, methods employing the cells, use of the cells in the expression of proteins in particular antibodies, such as anti Fc Rn antibodies and proteins made by the methods described herein.

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## RECOMBINANT BACTERIAL HOST CELL FOR PROTEIN EXPRESSION

The invention relates to a recombinant bacterial host strain, particularly *E. coli*. The invention also relates to a method for producing a protein of interest in such a cell.

### 5 Background of the Invention

Bacterial cells, such as *E. coli*, are commonly used for producing recombinant proteins. There are many advantages to using bacterial cells, such as *E. coli*, for producing recombinant proteins particularly due to the versatile nature of bacterial cells as host cells allowing the gene insertion via plasmids. *E. coli* have been used to produce many recombinant proteins including  
10 human insulin.

Despite the many advantages to using bacterial cells to produce recombinant proteins, there are still significant limitations including the difficulty of producing protease sensitive proteins. Proteases play an important role in turning over old, damaged or mis-folded proteins in the *E. coli* periplasm and cytoplasm. Bacterial proteases act to degrade the recombinant  
15 protein of interest, thereby often significantly reducing the yield of active protein.

A number of bacterial proteases have been identified. In *E. coli* proteases including Protease III (ptr), DegP, OmpT, Tsp, prlC, ptrA, ptrB, pepA-T, tsh, espc, eatA, clpP and lon have been identified.

Tsp (also known as Prc) is a 60kDa periplasmic protease. The first known substrate of  
20 Tsp was Penicillin-binding protein-3 (PBP3) (Determination of the cleavage site involved in C-terminal processing of penicillin-binding protein 3 of *Escherichia coli*; Nagasawa H, Sakagami Y, Suzuki A, Suzuki H, Hara H, Hirota Y. *J Bacteriol.* 1989 Nov;171(11):5890-3 and Cloning, mapping and characterization of the *Escherichia coli* Tsp gene which is involved in C-terminal processing of penicillin-binding protein 3; Hara H, Yamamoto Y, Higashitani A, Suzuki H,  
25 Nishimura Y. *J Bacteriol.* 1991 Aug;173 (15):4799-813) but it was later discovered that the Tsp was also able to cleave phage tail proteins and, therefore, it was renamed as Tail Specific Protease (Tsp) (Silber et al., *Proc. Natl. Acad. Sci. USA*, 89: 295-299 (1992)). Silber et al. (Deletion of the *prc(tsp)* gene provides evidence for additional tail-specific proteolytic activity in *Escherichia coli* K-12; Silber, K.R., Sauer, R.T.; *Mol Gen Genet* 1994 242:237-240)  
30 describes a *prc* deletion strain (KS1000) wherein the mutation was created by replacing a segment of the *prc* gene with a fragment comprising a Kan<sup>r</sup> marker.

The reduction of Tsp (*prc*) activity is desirable to reduce the proteolysis of proteins of interest. However, it was found that cells lacking protease *prc* show thermosensitive growth at low osmolarity. Hara et al isolated thermoresistant revertants containing extragenic suppressor  
35 (*spr*) mutations (Hara et al., *Microbial Drug Resistance*, 2: 63-72 (1996)). *Spr* is an 18kDa membrane bound periplasmic protease and the substrates of *spr* are Tsp and peptidoglycans in the outer membrane involved in cell wall hydrolysis during cell division. The *spr* gene is designated as UniProtKB/Swiss-Prot P0AFV4 (SPR\_ECOLI).

Improved protease deficient strains comprising a mutant *spr* gene have been described.  
40 Chen et al describes the construction of *E. coli* strains carrying different combinations of

mutations in *prc* (Tsp) and another protease, DegP, created by amplifying the upstream and downstream regions of the gene and ligating these together on a vector comprising selection markers and a *sprW174R* mutation (High-level accumulation of a recombinant antibody fragment in the periplasm of *Escherichia coli* requires a triple-mutant ( $\Delta$ DegP  $\Delta$ *prc* *sprW174R*) host strain (Chen C, Snedecor B, Nishihara JC, Joly JC, McFarland N, Andersen DC, Battersby JE, Champion KM. *Biotechnol Bioeng.* 2004 Mar 5;85(5):463-74). The combination of the  $\Delta$ DegP,  $\Delta$ *prc* and *sprW174R* mutations were found to provide the highest levels of antibody light chain, antibody heavy chain and F(ab')<sub>2</sub>-LZ. EP1341899 discloses an *E. coli* strain that is deficient in chromosomal DegP and *prc* encoding proteases DegP and Prc, respectively, and harbors a mutant *spr* gene that encodes a protein that suppresses growth phenotypes exhibited by strains harboring *prc* mutants.

Other improved protease deficient strains containing mutations in both Tsp and *spr* have been described in WO2011/086136.

Strains disclosed in WO02/48376 are *lac*<sup>-</sup> and cannot grow in cultures where thymidine, fucose or maltose are employed as the carbon source. This can be a severe disadvantage for strains intended for use on a commercial scale. There may be further disadvantages associated with the strains, for example the lack of production of alkaline phosphatase. The latter is a periplasmic protein involved in phosphate utilization from culture media.

Certain proteins exhibit peptidyl-prolyl isomerase and/or isomerase activity and/or chaperone activity and have been found to provide advantageous properties when employed in cell lines employed for recombinant protein expression.

The present invention provides new bacterial strains carrying both Tsp and *spr* mutations and at least one gene encoding a protein or proteins capable of facilitating protein folding which provide advantageous means for producing recombinant proteins.

## Summary of the Invention

In a first aspect of the present invention there is provided a recombinant gram-negative bacterial cell comprising:

- a. a mutant *spr* gene encoding a *spr* protein having a mutation at one or more amino acids selected from D133, H145, H157, N31, R62, I70, Q73, C94, S95, V98, Q99, R100, L108, Y115, V135, L136, G140, R144 and G147 and
- b. a gene or genes capable of expressing or overexpressing one or more proteins capable of facilitating protein folding, such as FkpA, Skp, SurA, PPiA and PPiD wherein the cell has reduced Tsp protein activity compared to a wild-type cell.

In one embodiment of the present invention there is provided a recombinant gram-negative bacterial cell encoding:

- a. a mutant *spr* gene encoding a *spr* protein having a mutation at one or more amino acids selected from D133, H145, H157, N31, R62, I70, Q73, C94, S95, V98, Q99, R100, L108, Y115, V135, L136, G140, R144 and G147,
- b. a gene or genes capable of expressing or overexpressing one or more proteins capable of facilitating protein folding, such as FkpA, Skp, SurA, PPiA and PPiD,

c. a gene capable of expressing a protein of interest, for example an antibody or binding fragment thereof

wherein the cell has reduced Tsp protein activity compared to a wild-type cell and the remainder of cell genomic DNA is isogenic with the wild-type cell from which the  
5 recombinant cell was derived.

In one embodiment, the cell's genome is isogenic to a wild-type bacterial cell except for the mutated spr gene, the modification required to reduce Tsp protein activity compared to a wild-type cell and the gene or genes expressing a protein capable of facilitating protein folding.

10 In a second aspect the present invention provides a recombinant gram-negative bacterial cell having reduced Tsp protein activity compared to a wild-type cell and comprising a mutant spr gene encoding a spr protein, wherein the cell's genome is isogenic to a wild-type bacterial cell except for the modification required to reduce Tsp protein activity compared to a wild-type cell, the mutated spr gene and the gene or genes introduced for expressing a protein  
15 capable of facilitating protein folding.

The present invention as claimed relates to a recombinant gram-negative bacterial cell comprising: a. a mutant spr gene encoding a spr protein having a mutation at one or more amino acids selected from D133, H145, H157, N31, R62, I70, Q73, C94, S95, V98, Q99, R100, L108, Y115, V135, L136, G140, R144 and G147 and b. an expression vector  
20 comprising a gene(s) expressing or overexpressing one or more proteins capable of facilitating protein folding selected from FkpA, Skp or a combination of FkpA and Skp, wherein the cell: has reduced Tsp protein activity compared to a wild-type cell, does not comprise a recombinant polynucleotide encoding DsbC, and is isogenic to a wild-type bacterial cell except for the mutant spr gene and except for the reduced Tsp protein activity as compared to  
25 the wild-type cell.

The cells provided by these aspects of the present invention show advantageous growth and protein production phenotypes.

In a third aspect, the present invention provides a method for producing a protein of interest comprising expressing the protein of interest in a recombinant gram-negative bacterial cell as defined above.

5 In a fourth aspect, the present invention also extends to proteins expressed from the process described herein.

### Brief Description of the Drawings

10 **Figure 1** shows the results of fermentations on the 5L scale performed with various combinations of host cells and “chaperone”. W3110 is a wild-type E coli strain. The various combinations were: wild type with no chaperone; wild-type with FkpA and Skp; MXE016 mutant spr and  $\Delta$  Tsp as published in WO2011/086136; MXE016 and FkpA; MXE016 and Skp; MXE016 and FkpA and Skp; MXE017 disclosed in WO2011/086136; MXE017 and FkpA and Skp

15 **Figure 2** shows the results of feed rate variation experiments at post induction feed rates 5.4, 6.0 and 7.0g/h, for MXE016 the majority of the additional Fab' made at higher feed rates was lost in the supernatant.

**Figures 3A,B** show cell viability (3A) and Fab' titres (3B) for MXE016 +/- FkpA.

**Figure 4** shows the primary recovery data for 20L pilot scale production.

20 **Figure 5** shows the primary recovery on SDS-PAGE stained gel under non-reducing conditions of a 20L pilot scale production. Other than the FkpA related bands the protein profile appears very similar between the two strains.

**Figure 6** shows a His-tag western blot under non-reducing conditions for a 20L pilot scale process. Full length FkpA detected and corresponds to the ~30kDa band and no signal was detected in MXE016 alone as expected.

**Figure 7A-C** shows various mutations in various genes

**Figure 7D** shows a diagrammatic representation of the creation of a vector comprising a polynucleotide sequence encoding a light chain of an antibody (LC), a heavy chain of an antibody (HC), a FkpA polynucleotide sequence and/or Skp polynucleotide

5 **Figure 8** shows various polynucleotide and amino acid sequences

#### **Brief Description of the Sequences**

SEQ ID NO:1 is the DNA sequence of the wild-type Tsp gene including the 6 nucleotides ATGAAC upstream of the start codon.

SEQ ID NO:2 is the amino acid sequence of the wild-type Tsp protein.

10 SEQ ID NO:3 is the DNA sequence of a mutated knockout Tsp gene including the 6 nucleotides ATGAAT upstream of the start codon.

SEQ ID NO:4 is the DNA sequence of the wild-type Protease III gene.

SEQ ID NO:5 is the amino acid sequence of the wild-type Protease III protein.

SEQ ID NO:6 is the DNA sequence of a mutated knockout Protease III gene.

15 SEQ ID NO:7 is the DNA sequence of the wild-type DegP gene.

SEQ ID NO:8 is the amino acid sequence of the wild-type DegP protein.

SEQ ID NO:9 is the DNA sequence of a mutated DegP gene.

SEQ ID NO:10 is the amino acid sequence of a mutated DegP protein.

20 SEQ ID NO: 11 is the sequence of the 5' oligonucleotide primer for the region of the mutated DegP gene comprising the Ase I restriction site.

SEQ ID NO: 12 is the sequence of the 3' oligonucleotide primer for the region of the mutated DegP gene comprising the Ase I restriction site.

SEQ ID NO: 13 is the sequence of the 5' oligonucleotide primer for the region of the mutated Tsp gene comprising the Ase I restriction site.

25 SEQ ID NO: 14 is the sequence of the 3' oligonucleotide primer for the region of the mutated Protease III gene comprising the Ase I restriction site.

SEQ ID NO: 15 is the sequence of the 5' oligonucleotide primer for the region of the mutated Protease III gene comprising the Ase I restriction site.

30 SEQ ID NO: 16 is the sequence of the 3' oligonucleotide primer for the region of the mutated Tsp gene comprising the Ase I restriction site.

SEQ ID NO: 17 is the DNA sequence of the wild-type spr gene.

SEQ ID NO: 18 is the sequence of the wild-type spr gene including the signal sequence which is the first 26 amino acid residues.

SEQ ID NO: 19 is the sequence of the non-mutated spr gene without the signal sequence.

35 SEQ ID NO: 20 is the nucleotide sequence of a mutated OmpT sequence comprising D210A and H212A mutations.

SEQ ID NO: 21 is the amino acid sequence of a mutated OmpT sequence comprising D210A and H212A mutations.

SEQ ID NO: 22 is the nucleotide sequence of a mutated knockout OmpT sequence.

40 SEQ ID NO: 23 shows the sequence of the OmpA oligonucleotide adapter.

SEQ ID NO: 24 shows the oligonucleotide cassette encoding intergenic sequence 1 (IGS1) for E. coli Fab expression.

SEQ ID NO: 25 shows the oligonucleotide cassette encoding intergenic sequence 2 (IGS2) for E. coli Fab expression.

5 SEQ ID NO: 26 shows the oligonucleotide cassette encoding intergenic sequence 3 (IGS3) for E. coli Fab expression.

SEQ ID NO: 27 shows the oligonucleotide cassette encoding intergenic sequence 4 (IGS4) for E. coli Fab expression.

SEQ ID NO: 28 is the DNA sequence of the wild-type FkpA gene.

10 SEQ ID NO: 29 is the protein sequence of the wild-type FkpA gene.

SEQ ID NO: 30 is the DNA sequence of the FkpA his tagged gene.

SEQ ID NO: 31 is the protein sequence of the FkpA his tagged gene.

SEQ ID NO: 32 is the DNA sequence of the wild-type skp gene.

SEQ ID NO: 33 is the protein sequence of the wild-type skp gene.

15 SEQ ID NO: 34 is the DNA sequence of the skp his tagged gene.

SEQ ID NO: 35 is the protein sequence of the skp his tagged gene.

SEQ ID NO: 36 to 74 show various amino acid and DNA sequences of FcRn antibodies or fragments thereof, which are suitable for expression in the cell line of the present invention. In particular SEQ ID NO: 50 is the amino acid sequence of the light chain variable region of an anti-FcRn antibody light chain 1519gH20 and SEQ ID NO:58 is the amino acid sequence of the heavy chain variable region of an anti-FcRn antibody heavy chain 1519gH20.

### **Detailed Description of the Preferred Embodiments of the Invention**

The present inventors have provided improved recombinant gram-negative bacterial cells suitable for expressing a recombinant protein of interest.

25 In one embodiment the protein is an antibody or binding fragment thereof, in particular a therapeutic antibody.

In particular, the inventors have provided improved recombinant gram-negative bacterial cells suitable for expressing a recombinant protein of interest by incorporating one or more gene or genes, encoding a protein for facilitating protein folding, into gram-negative bacterial cells carrying a mutated Tsp gene and a mutated spr gene

30 In one embodiment the gene or genes, encoding the protein for facilitating protein folding, are integrated into the cells genome, for example to provide a stable cell line. In one embodiment a recombinant protein for expression (such as a therapeutic protein) is transfected into a stable cell line to provide expression of the desired recombinant protein.

35 In one embodiment the gene or genes, encoding a protein for facilitating protein folding, are provided on one or more plasmids, for example the plasmids are transiently transfected into a cell to provide a cell line of the present disclosure.

In one embodiment the gene or genes, encoding a protein for facilitating protein folding, are provided on a plasmid also containing the coding sequence for a recombinant protein of interest.

40

In one embodiment the gene or genes, encoding a protein for facilitating protein folding, are provided on a plasmid that does not contain the coding sequence for a recombinant protein of interest.

5 In one embodiment the invention provides new strains having improved cell growth phenotype compared to wild-type bacterial cells and cells carrying just a mutated Tsp gene or a mutated Tsp and a mutated spr gene.

The cells of the present invention possess many advantages. The inventors have surprisingly found that cells according to the present disclosure may exhibit increased cell viability compared to a wild-type cell or a cell comprising a mutated Tsp gene and mutated spr gene.  
10

Cell viability is of particular importance in practical terms because cells that are not viable tend to lyse and create DNA debris in the culture. This DNA debris increases the, difficulty, cost and expense of purifying the desired protein. Therefore minimizing the DNA debris from lysed non-viable cells is a significant issue in manufacturing recombinant proteins efficiently, see for example US 6,258,560.  
15

Cell viability may be measured by any one of a number of routine techniques, for example employing a fluorescent dye and FACS analysis or similar.

Specifically, the cells according to the disclosure generally exhibit reduced cell lysis phenotype compared to cells carrying a mutated Tsp gene and a mutated spr gene.

20 Furthermore, the new strains may reduce leakage of protein from the cells and allow prolonged periplasmic accumulation compared to cells carrying a mutated Tsp gene and mutated spr gene. This is particularly important because, for example where total expression levels of target protein are similar but more protein is accumulated into the periplasm or less protein is accumulated in the supernatant because the strain is less leaky then cells with these less leaky  
25 properties will generally be more suitable for plant scale production because they facilitate protein recovery.

Further, the cells according to the present disclosure may exhibit increased yield of a protein of interest compared to a wild-type bacterial cell or a cell comprising a mutated Tsp gene and a mutated spr gene in the absence of a genes or genes encoding a protein such as FkpA.  
30 The improved protein yield may be the periplasmic protein yield and/or the supernatant protein yield. In one embodiment the cells of the present invention show improved periplasmic protein yield compared to a cell carrying a mutated Tsp gene and mutated spr gene due to reduced leakage from the cell.

The recombinant bacterial cells may be capable of faster rate of production of a protein  
35 of interest and, therefore, the same quantity of a protein of interest may be produced in a shorter time compared to a wild-type bacterial cell or a cell comprising a mutated Tsp gene and a mutated spr gene. The faster rate of production of a protein of interest may be especially significant over the initial period of growth of the cell, for example over the first 5, 10, 20 or 30 hours post induction of protein expression.

The cells according to the present invention preferably express a maximum yield in the periplasm and/or media of approximately 1.0g/L, 1.5g/L, 1.8g/L, 2.0g/L, 2.4g/L, 2.5g/L, 3.0g/L, 3.5g/L or 4.0g/L of a protein of interest.

5 Additionally the expression of a protein or proteins that facilitates folding further optimizes the expression by maximizing the protein provided with proper folding. The skilled person is well aware that appropriate folding is essential for biological function and thus isolating protein with the desired folding is vitally important. This is particularly important when the protein is expressed in a gram-negative cell because the protein expressed will not be natural to cell and thus the cell may not automatically express the protein with the appropriate  
10 folding. Inappropriate folding may express itself as aggregation or other impurities. The isolation of the desired protein may require extensive purification, which has cost implications and may also result in a low yield of the desired protein. Maximizing the amount of properly folded protein expressed minimizes the amount of purification required and may optimize the useable yield and thus is advantageous.

15 Advantages associated with the expression of a protein that facilitates folding include one or more of the following: Higher titre (e.g. increased to about 1.05g/L in comparison to wild-type 0.5g/L); Higher viability at harvest (e.g. >95%); Increased titre with increasing feed rate (better prospects for process development); Higher titre at commercial production scales such as 20L scale and higher viability at harvest; and Easier clarification of extract, in particular  
20 at 20L scale.

The cells provided by the present invention have reduced protease activity of Tsp compared to a wild-type cell, which may reduce proteolysis of a protein of interest, particularly proteins of interest which are proteolytically sensitive to Tsp. Therefore, the cells provided by the present invention may provide higher yield of the intact proteins, preferably of the protein of  
25 interest and a lower yield, or preferably no proteolytic fragments of proteins, preferably of the protein of interest, compared to a wild-type bacterial cell.

In one embodiment of the invention, the cells carry only the minimal mutations to the genome required to introduce the modifications according to the present disclosure. The bacterial cell may only differ from a wild-type bacterial cell by the one or more mutations to the spr gene and the modification required to reduce Tsp protein activity compared to a wild-type  
30 cell because for example the gene or genes encoding a protein for facilitating protein folding may be introduced transiently into the cell, such as on a plasmid. In one embodiment the cells do not carry any other mutations which may have deleterious effects on the cell's growth and/or ability to express a protein of interest.

35 Accordingly, one or more of the recombinant host cells of the present invention may exhibit improved protein expression and/or improved growth characteristics compared to cells comprising further genetically engineered mutations to the genomic sequence. The cells provided by the present invention are also more suitable for use to produce therapeutic proteins compared to cells comprising further disruptions to the cell genome.

The skilled person would easily be able to test a candidate cell clone to see if it has the desired yield of a protein of interest using methods well known in the art including a fermentation method, ELISA and protein G HPLC. Suitable fermentation methods are described in Humphreys D P, et al. (1997). Formation of dimeric Fabs in E. coli: effect of hinge size and isotype, presence of interchain disulphide bond, Fab' expression levels, tail piece sequences and growth conditions. J. IMMUNOL. METH. 209: 193-202; Backlund E. Reeks D. Markland K. Weir N. Bowering L. Larsson G. Fedbatch design for periplasmic product retention in Escherichia coli, Journal Article. Research Support, Non-U.S. Gov't Journal of Biotechnology. 135(4):358-65, 2008 Jul 31; Champion KM. Nishihara JC. Joly JC. Arnott D. Similarity of the Escherichia coli proteome upon completion of different biopharmaceutical fermentation processes. [Journal Article] Proteomics. 1(9):1133-48, 2001 Sep; and Horn U. Strittmatter W. Krebber A. Knupfer U. Kujau M. Wenderoth R. Muller K. Matzku S. Pluckthun A. Riesenberg D. High volumetric yields of functional dimeric miniantibodies in Escherichia coli, using an optimized expression vector and high-cell-density fermentation under non-limited growth conditions, Journal Article. Research Support, Non-U.S. Gov't Applied Microbiology & Biotechnology. 46(5-6):524-32, 1996 Dec. The skilled person would also easily be able to test secreted protein to see if the protein is correctly folded using methods well known in the art, such as protein G HPLC, circular dichroism, NMR, X-Ray crystallography and epitope affinity measurement methods.

The present invention will now be described in more detail.

The terms "protein" and "polypeptide" are used interchangeably herein, unless the context indicates otherwise. "Peptide" is intended to refer to 10 or less amino acids.

The terms "polynucleotide" includes a gene, DNA, cDNA, RNA, mRNA etc unless the context indicates otherwise.

'Reduced activity' as employed herein refers to 'lower levels of enzymatic activity, such as Tsp enzymic activity in comparison to the corresponding enzymic activity in a wild type strain when measured under comparable conditions in a suitable assay. In one embodiment the reduced activity is 50% or less, 40% or less, 30% or less, 20% or less, 10% or less or 5% or less of the enzymic activity of a wild-type comparator. In one embodiment the analysis to determine the levels of enzymic activity are performed concomitantly when the results are to be employed in a direct comparison.

Direct comparison as employed herein refers to where the numerical value of two or more results are compared for the purpose of evaluating if there is reduced activity as defined herein or ranking the activity results obtained from an assay.

As used herein, the term "comprising" in context of the present specification should be interpreted as "including".

Wild-type cell as employed herein employed interchangeably with non-mutated cell or control cell.

The non-mutated cell or control cell in the context of the present invention means a cell of the same type as the recombinant gram-negative cell of the invention wherein the cell has not

been modified to carry the above reduce Tsp protein activity and to carry the mutant spr gene. For example, a non-mutated cell may be a wild-type cell and may be derived from the same population of host cells as the cells of the invention before modification to introduce any mutations.

5 The expressions “cell”, “cell line”, “cell culture” and “strain” are used interchangeably.

The expression “phenotype of a cell comprising a mutated Tsp gene” in the context of the present invention means the phenotype exhibited by a cell having a mutant Tsp gene. Typically cells comprising a mutant Tsp gene may lyse, especially at high cell densities. The lysis of these cells causes any recombinant protein to leak into the supernatant. Cells carrying  
10 mutated Tsp gene may also show thermosensitive growth at low osmolarity. For example, the cells exhibit no or reduced growth rate or the cells die in hypotonic media at a high temperature, such as at 40°C or more.

The term “isogenic” in the context of the present invention means that the genome of the cell of the present invention has substantially the same or the same genomic sequence compared  
15 to the wild-type cell from which the cell is derived except for a mutated spr gene and the modification required to reduce Tsp protein activity compared to a wild-type cell. In this embodiment the genome of the cell comprises no further non-naturally occurring or genetically engineered mutations. In one embodiment the cell according to the present invention may have substantially the same genomic sequence compared to the wild-type cell except for the mutated  
20 spr gene and the modification required to reduce Tsp protein activity compared to a wild-type cell taking into account any naturally occurring mutations which may occur. In one embodiment, the cell according to the present invention may have exactly the same genomic sequence compared to the wild-type cell except for the mutated spr gene and the modification required to reduce Tsp protein activity compared to a wild-type cell.

25 The term “wild-type” in the context of the present invention means a strain of a gram-negative bacterial cell as it may occur in nature or may be isolated from the environment, which does not carry any genetically engineered mutations. An example of a wild-type strain of *E. coli* is W3110, such as W3110 K-12 strain.

Any suitable gram-negative bacterium may be used as the parental cell for producing the  
30 recombinant cell of the present invention. Suitable gram-negative bacterium include *Salmonella typhimurium*, *Pseudomonas fluorescens*, *Erwinia carotovora*, *Shigella*, *Klebsiella pneumoniae*, *Legionella pneumophila*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *E. coli*. Preferably the parental cell is *E. coli*. Any suitable strain of *E. coli* may be used in the present invention but preferably a wild-type W3110 strain, such as K-12 W3110, is used.

35 A drawback associated with the protease deficient bacterial strains previously created and used to express recombinant proteins is that they comprise additional mutations of genes involved in cell metabolism and DNA replication such as, for example *phoA*, *thiA*, *lac*, *rec*, *gal*, *ara*, *arg*, *thi* and *pro* in *E. coli* strains. These mutations may have many deleterious effects on the host cell including effects on cell growth, stability, recombinant protein expression yield and  
40 toxicity. Strains having one or more of these genomic mutations, particularly strains having a

high number of these mutations, may exhibit a loss of fitness which reduces bacterial growth rate to a level which is not suitable for industrial protein production. Further, any of the above genomic mutations may affect other genes in cis and/or in trans in unpredictable harmful ways thereby altering the strain's phenotype, fitness and protein profile. Further, the use of heavily  
5 mutated cells is not generally suitable for producing recombinant proteins for commercial use, particularly therapeutics, because such strains generally have defective metabolic pathways and hence may grow poorly or not at all in minimal or chemically defined media.

In one embodiment a cell according to the present invention is isogenic to a wild-type bacterial cell except for the mutated spr gene and the modification required to reduce Tsp  
10 protein activity compared to a wild-type cell. Only minimal mutations are made to the cell's genome to introduce the mutations. The cells do not carry any other mutations which may have deleterious effects on the cell's growth and/or ability to express a protein of interest. Accordingly, one or more of the recombinant host cells of the present invention may exhibit improved protein expression and/or improved growth characteristics compared to cells  
15 comprising further genetically engineered mutations to the genomic sequence. The cells provided by the present invention are also more suitable for use in the production of therapeutic proteins compared to cells comprising further disruptions to the cell genome.

In a preferred embodiment, the cell is isogenic to a wild-type *E. coli* cell, such as strain W3110, except for the mutated spr gene and the modification required to reduce Tsp protein  
20 activity compared to a wild-type cell.

The cell of the present invention may further differ from a wild-type cell by comprising a polynucleotide encoding the protein of interest. The polynucleotide sequence encoding the protein of interest may be exogenous or endogenous. The polynucleotide encoding the protein of interest may be contained within a suitable expression vector transformed into the cell and/or  
25 integrated into the host cell's genome. In the embodiment where the polynucleotide encoding the protein of interest is inserted into the host's genome, the cell of the present invention will also differ from a wild-type cell due to the inserted polynucleotide sequence encoding the protein of interest. Preferably the polynucleotide is in an expression vector in the cell thereby causing minimal disruption to the host cell's genome.

30 The spr protein is an *E. coli* membrane bound periplasmic protease.

The wild-type amino acid sequence of the spr protein is shown in SEQ ID NO:21 with the signal sequence at the N-terminus and in SEQ ID NO:22 without the signal sequence of 26 amino acids (according to UniProt Accession Number P0AFV4). The amino acid numbering of the spr protein sequence in the present invention includes the signal sequence. Accordingly, the  
35 amino acid 1 of the spr protein is the first amino acid (Met) shown in SEQ ID NO: 21.

The mutated spr gene is preferably the cell's chromosomal spr gene.

The mutated spr gene encodes a spr protein capable of suppressing the phenotype of a cell further comprising a mutated Tsp gene. Cells carrying a mutated Tsp gene may have a good cell growth rate but one limitation of these cells is their tendency to lyse, especially at high cell  
40 densities. Accordingly the phenotype of a cell comprising a mutated Tsp gene is a tendency to

lyse, especially at high cell densities. Cells carrying a mutated Tsp gene also show thermosensitive growth at low osmolarity. However, the spr mutations carried by the cells of the present invention, when introduced into a cell having reduced Tsp activity suppress the reduced Tsp phenotype and, therefore, the cell exhibits reduced lysis, particularly at a high cell density. The growth phenotype of a cell may be easily measured by a person skilled in the art during shake flask or high cell density fermentation technique. The suppression of the cell lysis phenotype may be seen from the improved growth rate and/or recombinant protein production, particularly in the periplasm, exhibited by a cell carrying spr mutant and having reduced Tsp activity compared to a cell carrying the Tsp mutant and a wild-type spr.

The cells according to the present invention comprise a mutant spr gene encoding a spr protein having a mutation at one or more amino acids selected from N31, R62, I70, Q73, C94, S95, V98, Q99, R100, L108, Y115, D133, V135, L136, G140, R144, H145, G147 and H157, preferably a mutation at one or more amino acids selected from C94, S95, V98, Y115, D133, V135, H145, G147 and H157. In this embodiment, the spr protein preferably does not have any further mutations.

The mutation of one or more of the above amino acids may be any suitable missense mutation to one, two or three of the nucleotides encoding the amino acid. The mutation changes the amino acid residue to any suitable amino acid which results in a mutated spr protein capable of suppressing the phenotype of a cell comprising a mutated Tsp gene. The missense mutation may change the amino acid to one which is a different size and/or has different chemical properties compared to the wild-type amino acid.

In one embodiment the mutant spr gene encodes an spr protein having one or more mutations selected from C94A, S95F, V98E, Y115F, D133A, V135D or G, H145A, G147C and H157A.

In one embodiment the mutation is to one, two or three of the catalytic triad of amino acid residues of C94, H145, and H157 (Solution NMR Structure of the NlpC/P60 Domain of Lipoprotein Spr from *Escherichia coli* Structural Evidence for a Novel Cysteine Peptidase Catalytic Triad, *Biochemistry*, 2008, 47, 9715-9717).

Accordingly, the mutated spr gene may comprise: a mutation to C94; or a mutation to H145; or a mutation to H157; or a mutation to C94 and H145; or a mutation to C94 and H157; or a mutation to H145 and H157; or a mutation to C94, H145 and H157.

In this embodiment, the spr protein preferably does not have any further mutations.

One, two or three of C94, H145 and H157 may be mutated to any suitable amino acid which results in a spr protein capable of suppressing the phenotype of a cell comprising a mutated Tsp gene. For example, one, two or three of C94, H145, and H157 may be mutated to a small amino acid such as Gly or Ala. Accordingly, the spr protein may have one, two or three of the mutations C94A, H145A and H157A. In one embodiment, the spr gene comprises the missense mutation C94A, which has been found to produce a spr protein capable of suppressing the phenotype of a cell comprising a mutated Tsp gene. In another embodiment, the spr gene

comprises the missense mutation H145A, which has been found to produce a spr protein capable of suppressing the phenotype of a cell comprising a mutated Tsp gene.

The designation for a substitution mutant herein consists of a letter followed by a number followed by a letter. The first letter designates the amino acid in the wild-type protein.

5 The number refers to the amino acid position where the amino acid substitution is being made, and the second letter designates the amino acid that is used to replace the wild-type amino acid.

In one embodiment the mutant spr protein comprises a mutation at one or more amino acids selected from N31, R62, I70, Q73, S95, V98, Q99, R100, L108, Y115, D133, V135, L136, G140, R144 and G147, preferably a mutation at one or more amino acids selected from S95, V98, Y115, D133, V135 and G147. In this embodiment, the spr protein preferably does not have any further mutations. Accordingly, the mutated spr gene may comprise: a mutation to N31; or a mutation to R62; or a mutation to I70; or a mutation to Q73; or a mutation to S95; or a mutation to V98; or a mutation to Q99; or a mutation to R100; or a mutation to L108; or a mutation to Y115; or a mutation to D133; or a mutation to V135; or a mutation to L136; or a mutation to G140; or a mutation to R144; or a mutation to G147.

15 In one embodiment the mutant spr protein comprises multiple mutations to amino acids: S95 and Y115; or N31, Q73, R100 and G140; or Q73, R100 and G140; or R100 and G140; or Q73 and G140; or Q73 and R100; or R62, Q99 and R144; or Q99 and R144.

20 One or more of the amino acids N31, R62, I70, Q73, S95, V98, Q99, R100, L108, Y115, D133, V135, L136, G140, R144 and G147 may be mutated to any suitable amino acid which results in a spr protein capable of suppressing the phenotype of a cell comprising a mutated Tsp gene. For example, one or more of N31, R62, I70, Q73, S95, V98, Q99, R100, L108, Y115, D133, V135, L136, G140 and R144 may be mutated to a small amino acid such as Gly or Ala.

In one embodiment the spr protein comprises one or more of the following mutations: N31Y, R62C, I70T, Q73R, S95F, V98E, Q99P, R100G, L108S, Y115F, D133A, V135D or V135G, L136P, G140C, R144C and G147C. In one embodiment the spr protein comprises one or more of the following mutations: S95F, V98E, Y115F, D133A, V135D or V135G and G147C. In this embodiment, the spr protein preferably does not have any further mutations.

30 In one embodiment the spr protein has one mutation selected from N31Y, R62C, I70T, Q73R, S95F, V98E, Q99P, R100G, L108S, Y115F, D133A, V135D or V135G, L136P, G140C, R144C and G147C. In this embodiment, the spr protein preferably does not have any further mutations.

In a further embodiment the spr protein has multiple mutations selected from: S95F and Y115F; N31Y, Q73R, R100G and G140C; Q73R, R100G and G140C; R100G and G140C; Q73R and G140C; Q73R and R100G; R62C, Q99P and R144C; or Q99P and R144C.

35 In one embodiment the mutated spr gene encodes a spr protein having a mutation C94A.

In one embodiment the mutated spr gene encodes a spr protein having a mutation V103E.

40 In one embodiment the mutated spr gene encodes a spr protein having a mutation D133A.

In one embodiment the mutated spr gene encodes a spr protein having a mutation V135D.

In one embodiment the mutated spr gene encodes a spr protein having a mutation V135A.

5 In one embodiment the mutated spr gene encodes a spr protein having a mutation H145A.

In one embodiment the mutated spr gene encodes a spr protein having a mutation G147C.

10 In one embodiment the mutated spr gene encodes a spr protein having a mutation H157A.

In one embodiment the mutant spr gene encodes a spr protein having a mutation selected from H145A, H157A and D133A.

15 In one embodiment of the present invention, any suitable mutation or mutations may be made to the spr gene which results in a spr protein capable of suppressing the phenotype of a cell comprising a mutated Tsp gene. Preferably, the spr protein may have one or more of the following mutations: N31Y, R62C, I70T, Q73R, C94A, S95F, V98E, Q99P, R100G, L108S, Y115F, D133A, V135D, V135G, L136P, G140C, R144C, H145A, G147C, H157A and W174R. In one embodiment the spr protein does not comprise the mutation W174R. Preferably, the spr gene comprises one or more mutations discussed above.

20 The cells according to the present invention have reduced Tsp protein activity compared to a wild-type cell. The expression "reduced Tsp protein activity compared to a wild-type cell" means that the Tsp activity of the cell is reduced compared to the Tsp activity of a wild-type cell. The cell may be modified by any suitable means to reduce the activity of Tsp.

25 In one embodiment the reduced Tsp activity is from modification of the endogenous polynucleotide encoding Tsp and/or associated regulatory expression sequences. The modification may reduce or stop Tsp gene transcription and translation or may provide an expressed Tsp protein having reduced protease activity compared to the wild-type Tsp protein.

30 In one embodiment an associated regulatory expression sequence is modified to reduce Tsp expression. For example, the promoter for the Tsp gene may be mutated to prevent expression of the gene.

In a preferred embodiment the cells according to the present invention carry a mutated Tsp gene encoding a Tsp protein having reduced protease activity or a knockout mutated Tsp gene.

Preferably the chromosomal Tsp gene is mutated.

35 As used herein, "Tsp gene" means a gene encoding protease Tsp (also known as Pre) which is a periplasmic protease capable of acting on Penicillin-binding protein-3 (PBP3) and phage tail proteins. The sequence of the wild-type Tsp gene is shown in SEQ ID NO: 1 and the sequence of the wild-type Tsp protein is shown in SEQ ID NO: 2.

Reference to the mutated Tsp gene or mutated Tsp gene encoding Tsp, refers to either a mutated Tsp gene encoding a Tsp protein having reduced protease activity or a knockout mutated Tsp gene, unless otherwise indicated.

5 The expression “mutated Tsp gene encoding a Tsp protein having reduced protease activity” in the context of the present invention means that the mutated Tsp gene does not have the full protease activity compared to the wild-type non-mutated Tsp gene.

Preferably, the mutated Tsp gene encodes a Tsp protein having 50% or less, 40% or less, 30% or less, 20% or less, 10% or less or 5% or less of the protease activity of a wild-type non-mutated Tsp protein. More preferably, the mutated Tsp gene encodes a Tsp protein having no  
10 protease activity. In this embodiment the cell is not deficient in chromosomal Tsp i.e. the Tsp gene sequence has not been deleted or mutated to prevent expression of any form of Tsp protein.

Any suitable mutation may be introduced into the Tsp gene in order to produce a protein having reduced protease activity. The protease activity of a Tsp protein expressed from a gram-negative bacterium may be easily tested by a person skilled in the art by any suitable method in  
15 the art, such as the method described in Keiler et al (Identification of Active Site Residues of the Tsp Protease\* THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 270, No. 48, Issue of December 1, pp. 28864–28868, 1995 Kenneth C. Keiler and Robert T. Sauer) wherein the protease activity of Tsp was tested.

Tsp has been reported in Keiler et al (supra) as having an active site comprising residues  
20 S430, D441 and K455 and residues G375, G376, E433 and T452 are important for maintaining the structure of Tsp. Keiler et al (supra) reports findings that the mutated Tsp genes S430A, D441A, K455A, K455H, K455R, G375A, G376A, E433A and T452A had no detectable protease activity. It is further reported that the mutated Tsp gene S430C displayed about 5-10% wild-type activity. Accordingly, the Tsp mutation to produce a protein having reduced protease  
25 activity may comprise a mutation, such as a missense mutation to one or more of residues S430, D441, K455, G375, G376, E433 and T452. Preferably the Tsp mutation to produce a protein having reduced protease activity may comprise a mutation, such as a missense mutation to one, two or all three of the active site residues S430, D441 and K455.

Accordingly the mutated Tsp gene may comprise: a mutation to S430; or a mutation to  
30 D441; or a mutation to K455; or a mutation to S430 and D441; or a mutation to S430 and K455; or a mutation to D441 and K455; or a mutation to S430, D441 and K455.

One or more of S430, D441, K455, G375, G376, E433 and T452 may be mutated to any suitable amino acid which results in a protein having reduced protease activity. Examples of suitable mutations are S430A, S430C, D441A, K455A, K455H, K455R, G375A, G376A,  
35 E433A and T452A. The mutated Tsp gene may comprise one, two or three mutations to the active site residues, for example the gene may comprise: S430A or S430C; and/or D441A; and/or K455A or K455H or K455R.

Preferably, the Tsp gene has the point mutation S430A or S430C.

The expression “knockout mutated Tsp gene” in the context of the present invention  
40 means that the gene comprises one or more mutations which prevents expression of the Tsp

protein encoded by the wild-type gene to provide a cell deficient in Tsp protein. The knockout gene may be partially or completely transcribed but not translated into the encoded protein. The knockout mutated Tsp gene may be mutated in any suitable way, for example by one or more deletion, insertion, point, missense, nonsense and frameshift mutations, to cause no expression of the protein. For example, the gene may be knocked out by insertion of a foreign DNA sequence, such as an antibiotic resistance marker, into the gene coding sequence.

In a preferred embodiment the Tsp gene is not mutated by insertion of a foreign DNA sequence, such as an antibiotic resistance marker, into the gene coding sequence. In this embodiment the Tsp gene may comprise a mutation to the gene start codon and/or one or more stop codons positioned downstream of the gene start codon and upstream of the gene stop codon thereby preventing expression of the Tsp protein. The mutation to the start codon may be a missense mutation of one, two or all three of the nucleotides of the start codon. Alternatively or additionally the start codon may be mutated by an insertion or deletion frameshift mutation. The Tsp gene comprises two ATG codons at the 5' end of the coding sequence, one or both of the ATG codons may be mutated by a missense mutation. The Tsp gene may be mutated at the second ATG codon (codon 3) to TCG. The Tsp gene may alternatively or additionally comprise one or more stop codons positioned downstream of the gene start codon and upstream of the gene stop codon. Preferably the knockout mutated Tsp gene comprises both a missense mutation to the start codon and one or more inserted stop codons. In a preferred embodiment the Tsp gene is mutated to delete "T" from the fifth codon thereby causing a frameshift resulting in stop codons at codons 11 and 16. In a preferred embodiment the Tsp gene is mutated to insert an Ase I restriction site to create a third in-frame stop codon at codon 21.

In a preferred embodiment the knockout mutated Tsp gene has the DNA sequence of SEQ ID NO: 3, which includes the 6 nucleotides ATGAAT upstream of the start codon. In one embodiment the mutated Tsp gene has the DNA sequence of nucleotides 7 to 2048 of SEQ ID NO:3.

In the present invention the cells also carry one or more genes capable of expressing or overexpressing one or more proteins capable of facilitating protein folding. Examples include proteins such as FkpA, Skp, SurA, PPIA and PPID.

In one embodiment the protein for facilitating protein folding is FkpA, Skp or a combination thereof.

In one embodiment the protein for facilitating protein folding is selected from FkpA or a combination of FkpA and Skp.

FkpA is a peptidyl-prolyl cis-trans isomerase with the Swiss-Prot number P45523.

Skp is chaperon protein with the Swiss-Prot number P0AEU7.

The protein for facilitating protein folding may be encoded by a gene in the cells genome or transiently transfected therein, for example on a plasmid or a combination of the same, as appropriate.

In one embodiment the recombinant gram-negative bacterial cell does not comprises a recombinant polynucleotide encoding DsbC.

In one embodiment of the present invention the recombinant gram-negative bacterial cell further comprises a mutated DegP gene encoding a DegP protein having chaperone activity and reduced protease activity and/or a mutated ptr gene, wherein the mutated ptr gene encodes a Protease III protein having reduced protease activity or is a knockout mutated ptr gene and/or a mutated OmpT gene, wherein the mutated OmpT gene encodes an OmpT protein having reduced protease activity or is a knockout mutated OmpT gene.

Preferably in this embodiment the cell's genome is isogenic to a wild-type bacterial cell except for the above mutations.

As used herein, "DegP" means a gene encoding DegP protein (also known as HtrA), which has dual function as a chaperone and a protease (Families of serine peptidases; Rawlings ND, Barrett AJ. *Methods Enzymol.* 1994;244:19-61). The sequence of the non-mutated DegP gene is shown in SEQ ID NO: 7 and the sequence of the non-mutated DegP protein is shown in SEQ ID NO: 8.

At low temperatures DegP functions as a chaperone and at high temperatures DegP has a preference to function as a protease (A Temperature-Dependent Switch from Chaperone to Protease in a Widely Conserved Heat Shock Protein. *Cell*, Volume 97, Issue 3, Pages 339 – 347. Spiess C, Beil A, Ehrmann M) and The proteolytic activity of the HtrA (DegP) protein from *Escherichia coli* at low temperatures, Skorko-Glonek J et al *Microbiology* 2008, 154, 3649-3658).

In the embodiments where the cell comprises the DegP mutation the DegP mutation in the cell provides a mutated DegP gene encoding a DegP protein having chaperone activity but not full protease activity.

The expression "having chaperone activity" in the context of the present invention means that the mutated DegP protein has the same or substantially the same chaperone activity compared to the wild-type non-mutated DegP protein. Preferably, the mutated DegP gene encodes a DegP protein having 50% or more, 60% or more, 70% or more, 80% or more, 90% or more or 95% or more of the chaperone activity of a wild-type non-mutated DegP protein. More preferably, the mutated DegP gene encodes a DegP protein having the same chaperone activity compared to wild-type DegP.

The expression "having reduced protease activity" in the context of the present invention means that the mutated DegP protein does not have the full protease activity compared to the wild-type non-mutated DegP protein. Preferably, the mutated DegP gene encodes a DegP protein having 50% or less, 40% or less, 30% or less, 20% or less, 10% or less or 5% or less of the protease activity of a wild-type non-mutated DegP protein. More preferably, the mutated DegP gene encodes a DegP protein having no protease activity. The cell is not deficient in chromosomal DegP i.e. the DegP gene sequences has not been deleted or mutated to prevent expression of any form of DegP protein.

Any suitable mutation may be introduced into the DegP gene in order to produce a protein having chaperone activity and reduced protease activity. The protease and chaperone activity of a DegP protein expressed from a gram-negative bacterium may be easily tested by a

person skilled in the art by any suitable method such as the method described in Spiess et al wherein the protease and chaperone activities of DegP were tested on MalS, a natural substrate of DegP (A Temperature-Dependent Switch from Chaperone to Protease in a Widely Conserved Heat Shock Protein. Cell, Volume 97, Issue 3, Pages 339 – 347. Spiess C, Beil A, Ehrmann M) and also the method described in The proteolytic activity of the HtrA (DegP) protein from Escherichia coli at low temperatures, Skorko-Glonck J et al Microbiology 2008, 154, 3649-3658.

DegP is a serine protease and has an active center consisting of a catalytic triad of amino acid residues of His105, Asp135 and Ser210 (Families of serine peptidases, Methods Enzymol., 1994, 244:19-61 Rawlings N and Barrett A). The DegP mutation to produce a protein having chaperone activity and reduced protease activity may comprise a mutation, such as a missense mutation to one, two or three of His105, Asp135 and Ser210.

Accordingly, the mutated DegP gene may comprise: a mutation to His105; or a mutation to Asp135; or a mutation to Ser210; or a mutation to His105 and Asp135; or a mutation to His105 and Ser210; or a mutation to Asp135 and Ser210; or a mutation to His105, Asp135 and Ser210.

One, two or three of His105, Asp135 and Ser210 may be mutated to any suitable amino acid which results in a protein having chaperone activity and reduced protease activity. For example, one, two or three of His105, Asp135 and Ser210 may be mutated to a small amino acid such as Gly or Ala. A further suitable mutation is to change one, two or three of His105, Asp135 and Ser210 to an amino acid having opposite properties such as Asp135 being mutated to Lys or Arg, polar His105 being mutated to a non-polar amino acid such as Gly, Ala, Val or Leu and small hydrophilic Ser210 being mutated to a large or hydrophobic residue such as Val, Leu, Phe or Tyr. Preferably, the DegP gene comprises the point mutation S210A, as shown in Figure 7c, which has been found to produce a protein having chaperone activity but not protease activity (A Temperature-Dependent Switch from Chaperone to Protease in a Widely Conserved Heat Shock Protein. Cell, Volume 97, Issue 3, Pages 339 – 347. Spiess C, Beil A, Ehrmann M).

DegP has two PDZ domains, PDZ1 (residues 260-358) and PDZ2 (residues 359-448), which mediate protein-protein interaction (A Temperature-Dependent Switch from Chaperone to Protease in a Widely Conserved Heat Shock Protein. Cell, Volume 97, Issue 3, Pages 339 – 347. Spiess C, Beil A, Ehrmann M). In one embodiment of the present invention the degP gene is mutated to delete PDZ1 domain and/or PDZ2 domain. The deletion of PDZ1 and PDZ2 results in complete loss of protease activity of the DegP protein and lowered chaperone activity compared to wild-type DegP protein whilst deletion of either PDZ1 or PDZ2 results in 5% protease activity and similar chaperone activity compared to wild-type DegP protein (A Temperature-Dependent Switch from Chaperone to Protease in a Widely Conserved Heat Shock Protein. Cell, Volume 97, Issue 3, Pages 339 – 347. Spiess C, Beil A, Ehrmann M).

The mutated DegP gene may also comprise a silent non-naturally occurring restriction site, such as Ase I in order to aid in identification and screening methods, for example as shown in Figure 7C.

5 The preferred sequence of the mutated DegP gene comprising the point mutation S210A and an Ase I restriction marker site is provided in SEQ ID NO: 9 and the encoded protein sequence is shown in SEQ ID NO: 10.

10 In the embodiments of the present invention wherein the cell comprises a mutated DegP gene encoding a DegP protein having chaperone activity and reduced protease activity, one or more of the cells provided by the present invention may provide improved yield of correctly folded proteins from the cell relative to mutated cells wherein the DegP gene has been mutated to knockout DegP preventing DegP expression, such as chromosomal deficient DegP. In a cell comprising a knockout mutated DegP gene preventing DegP expression, the chaperone activity of DegP is lost completely whereas in the cell according to the present invention the chaperone activity of DegP is retained whilst the full protease activity is lost. In these embodiments, one  
15 or more cells according to the present invention have a lower protease activity to prevent proteolysis of the protein whilst maintaining the chaperone activity to allow correct folding and transportation of the protein in the host cell.

In these embodiments, one or more cells according to the present invention may have improved cell growth compared to cells carrying a mutated knockout DegP gene preventing  
20 DegP expression. Without wishing to be bound by theory improved cell growth maybe exhibited due to the DegP protease retaining chaperone activity which may increase capacity of the cell to process all proteins which require chaperone activity. Accordingly, the production of correctly folded proteins necessary for the cell's growth and reproduction may be increased in one or more of the cells of the present invention compared to cells carrying a DegP knockout  
25 mutation thereby improving the cellular pathways regulating growth. Further, known DegP protease deficient strains are generally temperature-sensitive and do not typically grow at temperatures higher than about 28°C. However, the cells according to the present invention are not temperature-sensitive and may be grown at temperatures of 28°C or higher, including temperatures of approximately 30°C to approximately 37°C, which are typically used for  
30 industrial scale production of proteins from bacteria.

In one embodiment of the present invention the cell carries a mutated ptr gene. As used herein, "ptr gene" means a gene encoding Protease III, a protease which degrades high molecular weight proteins. The sequence of the non-mutated ptr gene is shown in SEQ ID NO: 4 and the sequence of the non-mutated Protease III protein is shown in SEQ ID NO: 5.

35 Reference to the mutated ptr gene or mutated ptr gene encoding Protease III, refers to either a mutated ptr gene encoding a Protease III protein having reduced protease activity or a knockout mutated ptr gene, unless otherwise indicated.

The expression "mutated ptr gene encoding a Protease III protein having reduced protease activity" in the context of the present invention means that the mutated ptr gene does  
40 not have the full protease activity compared to the wild-type non-mutated ptr gene.

Preferably, the mutated ptr gene encodes a Protease III having 50% or less, 40% or less, 30% or less, 20% or less, 10% or less or 5% or less of the protease activity of a wild-type non-mutated Protease III protein. More preferably, the mutated ptr gene encodes a Protease III protein having no protease activity. In this embodiment the cell is not deficient in chromosomal ptr i.e. the ptr gene sequence has not been deleted or mutated to prevent expression of any form of Protease III protein.

Any suitable mutation may be introduced into the ptr gene in order to produce a Protease III protein having reduced protease activity. The protease activity of a Protease III protein expressed from a gram-negative bacterium may be easily tested by a person skilled in the art by any suitable method in the art.

The expression "knockout mutated ptr gene" in the context of the present invention means that the gene comprises one or more mutations thereby causing no expression of the protein encoded by the gene to provide a cell deficient in the protein encoded by the knockout mutated gene. The knockout gene may be partially or completely transcribed but not translated into the encoded protein. The knockout mutated ptr gene may be mutated in any suitable way, for example by one or more deletion, insertion, point, missense, nonsense and frameshift mutations, to cause no expression of the protein. For example, the gene may be knocked out by insertion of a foreign DNA sequence, such as an antibiotic resistance marker, into the gene coding sequence.

In a preferred embodiment the gene is not mutated by insertion of a foreign DNA sequence, such as an antibiotic resistance marker, into the gene coding sequence. Preferably the Protease III gene comprise a mutation to the gene start codon and/or one or more stop codons positioned downstream of the gene start codon and upstream of the gene stop codon thereby preventing expression of the Protease III protein.

A mutation to the target knockout gene start codon causes loss of function of the start codon and thereby ensures that the target gene does not comprise a suitable start codon at the start of the coding sequence. The mutation to the start codon may be a missense mutation of one, two or all three of the nucleotides of the start codon. Alternatively or additionally the start codon may be mutated by an insertion or deletion frameshift mutation.

In a preferred embodiment the ptr gene is mutated to change the ATG start codon to ATT.

The knockout mutated ptr gene may alternatively or additionally comprise one or more stop codons positioned downstream of the gene start codon and upstream of the gene stop codon. Preferably the knockout mutated ptr gene comprises both a missense mutation to the start codon and one or more inserted stop codons.

The one or more inserted stop codons are preferably in-frame stop codons. However the one or more inserted stop codons may alternatively or additionally be out-of-frame stop codons. One or more out-of-frame stop codons may be required to stop translation where an out-of-frame start codon is changed to an in-frame start codon by an insertion or deletion frameshift mutation. The one or more stop codons may be introduced by any suitable mutation including a

nonsense point mutation and a frameshift mutation. The one or more stop codons are preferably introduced by a frameshift mutation and/or an insertion mutation, preferably by replacement of a segment of the gene sequence with a sequence comprising a stop codon. For example an Ase I restriction site may be inserted, which comprises the stop codon TAA.

5 In a preferred embodiment the ptr gene is mutated to insert an in-frame stop codon by insertion of an Ase I restriction site, as shown in Figure 7A. In a preferred embodiment the knockout mutated ptr gene has the DNA sequence of SEQ ID NO: 6.

The above described knockout mutations are advantageous because they cause minimal or no disruption to the chromosomal DNA upstream or downstream of the target knockout gene site and do not require the insertion and retention of foreign DNA, such as antibiotic resistance markers, which may affect the cell's suitability for expressing a protein of interest, particularly therapeutic proteins. Accordingly, one or more of the cells according to the present invention may exhibit improved growth characteristics and/or protein expression compared to cells wherein the protease gene has been knocked out by insertion of foreign DNA into the gene coding sequence.

15 In one embodiment the cells according to the present invention carry a mutated OmpT gene. As used herein, "OmpT gene" means a gene encoding protease OmpT (outer membrane protease T) which is an outer membrane protease. The sequence of the wild-type non-mutated OmpT gene is SWISS-PROT P09169.

20 Reference to a mutated OmpT gene or mutated OmpT gene encoding OmpT, refers to either a mutated OmpT gene encoding a OmpT protein having reduced protease activity or a knockout mutated OmpT gene, unless otherwise indicated.

The expression "mutated OmpT gene encoding an OmpT protein having reduced protease activity" in the context of the present invention means that the mutated OmpT gene does not have the full protease activity compared to the wild-type non-mutated OmpT gene. The mutated OmpT gene may encode an OmpT protein having 50% or less, 40% or less, 30% or less, 20% or less, 10% or less or 5% or less of the protease activity of a wild-type non-mutated OmpT protein. The mutated OmpT gene may encode an OmpT protein having no protease activity. In this embodiment the cell is not deficient in chromosomal OmpT i.e. the OmpT gene sequence has not been deleted or mutated to prevent expression of any form of OmpT protein.

30 Any suitable mutation may be introduced into the OmpT gene in order to produce a protein having reduced protease activity. The protease activity of a OmpT protein expressed from a gram-negative bacterium may be easily tested by a person skilled in the art by any suitable method in the art, such as the method described in Kramer et al (Identification of essential acidic residues of outer membrane protease OmpT supports a novel active site, FEBS Letters 505 (2001) 426-430) and Dekker et al (Substrate Specificity of the Integral Membrane Protease OmpT Determined by Spatially Addressed Peptide Libraries, Biochemistry 2001, 40, 1694-1701).

35 OmpT has been reported in Kramer et al (Identification of active site serine and histidine residues in Escherichia coli outer membrane protease OmpT FEBS Letters 2000 468, 220-224)

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discloses that substitution of serines, histidines and acidic residues by alanines results in ~10-fold reduced activity for Glu27, Asp97, Asp208 or His101, ~500-fold reduced activity for Ser99 and ~10000-fold reduced activity for Asp83, Asp85, Asp210 or His212. Vandeputte-Rutten et al (Crystal Structure of the Outer Membrane Protease OmpT from Escherichia coli suggests a novel catalytic site, The EMBO Journal 2001, Vol 20 No 18 5033-5039) as having an active site comprising a Asp83-Asp85 pair and a His212-Asp210 pair. Further Kramer et al (Lipopolysaccharide regions involved in the activation of Escherichia coli outer membrane protease OmpT, Eur. J. Biochem. FEBS 2002, 269, 1746-1752) discloses that mutations D208A, D210A, H212A, H212N, H212Q, G216K/K217G, K217T and R218L in loop L4 all resulted in partial or virtually complete loss of enzymatic activity.

Accordingly, the OmpT mutation to produce a protein having reduced protease activity may comprise a mutation, such as a missense mutation to one or more of residues E27, D43, D83, D85, D97, S99, H101 E111, E136, E193, D206, D208, D210, H212 G216, K217, R218 & E250.

One or more of E27, D43, D83, D85, D97, S99, H101 E111, E136, E193, D206, D208, D210, H212 G216, K217, R218 & E250 may be mutated to any suitable amino acid which results in a protein having reduced protease activity. For example, one or more of E27, D43, D83, D85, D97, S99, H101 E111, E136, E193, D206, D208, D210, H212 G216, K217, R218 & E250 may be mutated to alanine. Examples of suitable mutations are E27A, D43A, D83A, D85A, D97A, S99A, H101A E111A, E136A, E193A, D206A, D208A, D210A, H212A, H212N, H212Q, G216K, K217G, K217T, R218L & E250A. In one embodiment the mutated OmpT gene comprises D210A and H212A mutations. A suitable mutated OmpT sequence comprising D210A and H212A mutations is shown in SEQ ID NO: 23.

The expression "knockout mutated OmpT gene" in the context of the present invention means that the gene comprises one or more mutations thereby causing no expression of the protein encoded by the gene to provide a cell deficient in the protein encoded by the knockout mutated gene. The knockout gene may be partially or completely transcribed but not translated into the encoded protein. The knockout mutated OmpT gene may be mutated in any suitable way, for example by one or more deletion, insertion, point, missense, nonsense and frameshift mutations, to cause no expression of the protein. For example, the gene may be knocked out by insertion of a foreign DNA sequence, such as an antibiotic resistance marker, into the gene coding sequence.

In one embodiment the OmpT gene comprises a mutation to the gene start codon and/or one or more stop codons positioned downstream of the gene start codon and upstream of the gene stop codon thereby preventing expression of the OmpT protein. The mutation to the start codon may be a missense mutation of one, two or all three of the nucleotides of the start codon. A suitable mutated knockout OmpT sequence is shown in SEQ ID NO: 24. Alternatively or additionally the start codon may be mutated by an insertion or deletion frameshift mutation.

In one embodiment the gram-negative bacterial cell according to the present invention does not carry a knockout mutated ompT gene, such as being deficient in chromosomal ompT.

In one embodiment the gram-negative bacterial cell according to the present invention does not carry a knockout mutated degP gene, such as being deficient in chromosomal degP. In one embodiment the gram-negative bacterial cell according to the present invention does not carry a mutated degP gene.

5 In one embodiment the gram-negative bacterial cell according to the present invention does not carry a knockout mutated ptr gene, such as being deficient in chromosomal ptr.

Many genetically engineered mutations including knockout mutations involve the use of antibiotic resistance markers which allow the selection and identification of successfully mutated cells. However, as discussed above, there are a number of disadvantages to using  
10 antibiotic resistance markers.

A further embodiment of the present invention the cell does not comprise an antibiotic resistance marker and overcomes the above disadvantages of using antibiotic resistance markers wherein the mutated Tsp gene, the mutated spr gene and optionally the mutated DegP gene and/or a mutated ptr gene and/or a mutated OmpT gene, are mutated to comprise one or more  
15 restriction marker sites. The restriction sites are genetically engineered into the gene and are non-naturally occurring. The restriction marker sites are advantageous because they allow screening and identification of correctly modified cells which comprise the required chromosomal mutations. Cells which have been modified to carry one or more of the mutated protease genes may be analyzed by PCR of genomic DNA from cell lysates using  
20 oligonucleotide pairs designed to amplify a region of the genomic DNA comprising a non-naturally occurring restriction marker site. The amplified DNA may then be analyzed by agarose gel electrophoresis before and after incubation with a suitable restriction enzyme capable of digesting the DNA at the non-naturally occurring restriction marker site. The presence of DNA fragments after incubation with the restriction enzyme confirms that the cells  
25 have been successfully modified to carry the one or more mutated genes.

In the embodiment wherein the cell carries a knockout mutated ptr gene having the DNA sequence of SEQ ID NO: 6, the oligonucleotide primer sequences shown in SEQ ID NO: 17 and SEQ ID NO: 18 may be used to amplify the region of the DNA comprising the non-naturally occurring Asc I restriction site from the genomic DNA of transformed cells. The amplified  
30 genomic DNA may then be incubated with Ase I restriction enzyme and analyzed by gel electrophoresis to confirm the presence of the mutated ptr gene in the genomic DNA.

In the embodiment wherein the cell comprises a knockout mutated Tsp gene having the DNA sequence of SEQ ID NO: 3 or nucleotides 7 to 2048 of SEQ ID NO:3, the oligonucleotide primer sequences shown in SEQ ID NO: 15 and SEQ ID NO:16 may be used to amplify the  
35 region of the DNA comprising the non-naturally occurring Ase I restriction site from the genomic DNA of transformed cells. The amplified genomic DNA may then be incubated with Ase I restriction enzyme and analyzed by gel electrophoresis to confirm the presence of the mutated Tsp gene in the genomic DNA.

In the embodiment wherein the cell comprises a mutated DegP gene having the DNA  
40 sequence of SEQ ID NO: 9, the oligonucleotide primer sequences shown in SEQ ID NO: 19 and

SEQ ID NO:20 may be used to amplify the region of the DNA comprising the non-naturally occurring Ase I restriction site from the genomic DNA of transformed cells. The amplified genomic DNA may then be incubated with Ase I restriction enzyme and analyzed by gel electrophoresis to confirm the presence of the mutated DegP gene in the genomic DNA.

5       The one or more restriction sites may be introduced by any suitable mutation including by one or more deletion, insertion, point, missense, nonsense and frameshift mutations. A restriction site may be introduced by the mutation of the start codon and/or mutation to introduce the one or more stop codons, as described above. This embodiment is advantageous because the restriction marker site is a direct and unique marker of the knockout mutations  
10       introduced.

      A restriction maker site may be inserted which comprises an in-frame stop codon, such as an Ase I restriction site. This is particularly advantageous because the inserted restriction site serves as both a restriction marker site and a stop codon to prevent full transcription of the gene coding sequence. For example, in the embodiment wherein a stop codon is introduced to the ptr  
15       gene by introduction of an Ase I site, this also creates a restriction site, as shown in Figure 7A. For example, in the embodiment wherein a stop codon is introduced to the Tsp gene at codon 21 by introduction of an Ase I site, this also creates a restriction site, as shown in Figure 7B.

      A restriction marker site may be inserted by the mutation to the start codon and optionally one or more further point mutations. In this embodiment the restriction marker site is  
20       preferably an EcoR I restriction site. This is particularly advantageous because the mutation to the start codon also creates a restriction marker site. For example, in the embodiment wherein the start codon of the ptr gene is changed to ATT, this creates an EcoR I marker site, as shown in Figure 7A. For example, in the embodiment wherein the start codon (codon 3) of the Tsp gene is changed from ATG to TCG, as shown in Figure 7B, a further point mutation of codon 2  
25       from AAC to AAT and mutation of codon 3 ATG to TCG creates an EcoR I restriction marker site, as shown in Figure 7B.

      In the embodiment of the present invention wherein the cell carries a mutated OmpT gene, the one or more restriction sites may be introduced by any suitable mutation including by one or more deletion, insertion, point, missense, nonsense and frameshift mutations. For  
30       example, in the embodiment wherein the OmpT gene comprises the mutations D210A and H212A, these mutations introduce silent HindIII restriction site which may be used as a selection marker.

      In the DegP gene or the spr gene, a marker restriction site may be introduced using silent codon changes. For example, an Ase I site may be used as a silent restriction marker site,  
35       wherein the TAA stop codon is out-of-frame, as shown in Figure 7C for the mutated DegP gene.

      In the embodiments of the present invention, wherein the ptr gene and/or the Tsp gene are mutated to encode a Protease III or Tsp having reduced protease activity, one or more marker restriction sites may be introduced using silent codon changes.

      The recombinant gram-negative bacterial cell according to the present invention may be  
40       produced by any suitable means. The skilled person knows of suitable techniques which may be

used to replace a chromosomal gene sequence with a mutated gene sequence. Suitable vectors may be employed which allow integration into the host chromosome by homologous recombination.

Suitable gene replacement methods are described, for example, in Hamilton et al (New  
5 Method for Generating Deletions and Gene Replacements in *Escherichia coli*, Hamilton C. M.  
et al., *Journal of Bacteriology* Sept. 1989, Vol. 171, No. 9 p 4617-4622), Skorupski et al  
(Positive selection vectors for allelic exchange, Skorupski K and Taylor R. K., *Gene*, 1996, 169,  
47-52), Kiel et al (A general method for the construction of *Escherichia coli* mutants by  
homologous recombination and plasmid segregation, Kiel J.A.K.W. et al, *Mol Gen Genet* 1987,  
10 207:294-301), Blomfield et al (Allelic exchange in *Escherichia coli* using the *Bacillus subtilis*  
*sacB* gene and a temperature sensitive pSC101 replicon, Blomfield I. C. et al., *Molecular*  
*Microbiology* 1991, 5(6), 1447-1457) and Ried et al. (An *nptI-sacB-sacR* cartridge for  
constructing directed, unmarked mutations in Gram-negative bacteria by marker exchange-  
eviction mutagenesis, Ried J. L. and Collmer A., *Gene* 57 (1987) 239-246). A suitable plasmid  
15 which enables homologous recombination/replacement is the pKO3 plasmid (Link et al., 1997,  
*Journal of Bacteriology*, 179, 6228-6237).

Successfully mutated strains may be identified using methods well known in the art including colony PCR DNA sequencing and colony PCR restriction enzyme mapping.

In the embodiment wherein the cell comprises two or more mutated chromosomal genes,  
20 the mutated genes may be introduced into the gram-negative bacterium on the same or different  
vectors.

In one embodiment the gram-negative bacterial cell according to the present invention does not carry a knockout mutated *ompT* gene, such as being deficient in chromosomal *ompT*.

In one embodiment the cells according to the present disclosure only contain the three  
25 characterizing mutations of mutated *spr*, reduced *Tsp* and express *FkpA*, *Skp* or a combination  
of *FkpA* and *Skp*.

In one embodiment the cell line according to the present disclosure consists of a mutated  
*Tsp* gene and a mutated *spr* gene, and an *FkpA* gene.

In one embodiment the cell line according to the present disclosure consists of a mutated  
30 *Tsp* gene and a mutated *spr* gene, and a *Skp* gene.

In one embodiment the cell line according to the present disclosure consists of a mutated  
*Tsp* gene and a mutated *spr* gene, an *FkpA* gene and a *Skp* gene.

In one embodiment the gene or genes capable of expressing or overexpressing one or  
more proteins capable of facilitating protein folding, such as *FkpA*, *Skp*, *SurA*, *PPiA* and *PPiD*  
35 are transiently transformed into the cell, for example in an expression vector optionally  
comprising a polynucleotide sequence encoding an antibody or binding fragment thereof.

In one embodiment the polynucleotide sequence encoding the antibody and the  
polynucleotide encoding *FkpA* and/or *Skp* are inserted into separate expression vectors.

For production of products comprising both heavy and light chains, the cell line may be  
40 transformed with two vectors, a first vector encoding a light chain polypeptide and a second

vector encoding a heavy chain polypeptide. Alternatively, a single vector may be used, the vector including sequences encoding light chain and heavy chain polypeptides.

Alternatively, the polynucleotide sequence encoding the antibody and the polynucleotide encoding FkpA and/or Skp are inserted into one vector. Preferably the vector comprises the sequences encoding the light and heavy chain polypeptides of the antibody.

The present invention also provides an expression vector comprising a recombinant polynucleotide encoding FkpA and/or Skp and an antibody or an antigen binding fragment thereof specific for human FcRn. The expression vector is a multi-cistronic vector comprising the polynucleotide sequence encoding FkpA and/or Skp and the polynucleotide sequence encoding the antibody.

The multicistronic vector may be produced by an advantageous cloning method which allows repeated sequential cloning of polynucleotide sequences into a vector. The method uses compatible cohesive ends of a pair of restrictions sites, such as the "AT" ends of Ase I and Nde I restriction sites. A polynucleotide sequence comprising a coding sequence and having compatible cohesive ends, such as a AseI-NdeI fragment, may be cloned into a restrictions site in the vector, such as Nde I. The insertion of the polynucleotide sequence destroys the 5' restriction site but creates a new 3' restriction site, such as NdeI, which may then be used to insert a further polynucleotide sequence comprising compatible cohesive ends. The process may then be repeated to insert further sequences. Each polynucleotide sequence inserted into the vector comprises non-coding sequence 3' to the stop codon which may comprise an Ssp I site for screening, a Shine Dalgarno ribosome binding sequence, an A rich spacer and an NdeI site encoding a start codon.

A diagrammatic representation of the creation of a vector comprising a polynucleotide sequence encoding a light chain of an antibody (LC), a heavy chain of an antibody (HC), an FkpA polynucleotide sequences and a further polynucleotide sequence is shown in Figure 7d.

The cell according to the present invention preferably comprises an expression vector as defined above.

In the embodiment wherein the cell also expresses one or more further proteins as follows: one or more proteins capable of facilitating protein folding, such as, skp, SurA, PPiA and PPiD; and optionally one or more protein capable of facilitating protein secretion or translocation, such as SecY, SecE, SecG, SecYEG, SecA, SecB, FtsY and Lep; the one or more further protein may be expressed from one or more polynucleotides inserted into the same vector as the polynucleotide encoding FkpA and/or the polynucleotide sequence encoding the antibody. Alternatively the one or more polynucleotides may be inserted into separate vectors.

The expression vector may be produced by inserting one or more expression cassettes as defined above into a suitable vector. Alternatively, the regulatory expression sequences for directing expression of the polynucleotide sequence may be contained in the expression vector and thus only the encoding region of the polynucleotide may be required to complete the expression vector.

The polynucleotide encoding FkpA and/or Skp and/or the polynucleotide encoding the antibody is suitably inserted into a replicable vector, typically an autonomously replicating expression vector, for expression in the cell under the control of a suitable promoter for the cell. Many vectors are known in the art for this purpose and the selection of the appropriate vector may depend on the size of the nucleic acid and the particularly cell type.

Examples of expression vectors which may be employed to transform the host cell with a polynucleotide according to the invention include:

- a plasmid, such as pBR322 or pACYC184, and/or
- a viral vector such as bacterial phage
- a transposable genetic element such as a transposon

Such expression vectors usually comprise a plasmid origin of DNA replication, an antibiotic selectable marker, a promoter and transcriptional terminator separated by a multi-cloning site (expression cassette) and a DNA sequence encoding a ribosome binding site.

The promoters employed in the present invention can be linked to the relevant polynucleotide directly or alternatively be located in an appropriate position, for example in a vector such that when the relevant polypeptide is inserted the relevant promoter can act on the same. In one embodiment the promoter is located before the encoding portion of the polynucleotide on which it acts, for example a relevant promoter before each encoding portion of polynucleotide. "Before" as used herein is intended to imply that the promoter is located at the 5 prime end in relation to the encoding polynucleotide portion.

The promoters may be endogenous or exogenous to the host cells. Suitable promoters include lac, tac, trp, phoA, Ipp, Arab, tet and T7.

One or more promoters employed may be inducible promoters. In the embodiment wherein the polynucleotide encoding FkpA and/or Skp and the polynucleotide encoding the antibody are inserted into one vector, the nucleotide sequences encoding FkpA and the antibody may be under the control of a single promoter or separate promoters. In the embodiment wherein the nucleotide sequences encoding FkpA and/or Skp and the antibody are under the control of separate promoters, the promoter may be independently inducible promoters.

Promoters for use in bacterial systems also generally contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding the polypeptide of interest. The promoter can be removed from the bacterial source DNA by restriction enzyme digestion and inserted into the vector containing the desired DNA.

The expression vector preferably also comprises a dicistronic message for producing the antibody or antigen binding fragment thereof as described in WO03/048208 or WO2007/039714. Preferably the upstream cistron contains DNA coding for the light chain of the antibody and the downstream cistron contains DNA coding for the corresponding heavy chain, and the dicistronic intergenic sequence (IGS) preferably comprises a sequence selected from IGS1 (SEQ ID NO: 23), IGS2 (SEQ ID NO: 24), IGS3 (SEQ ID NO: 25) and IGS4 (SEQ ID NO: 26).

The terminators may be endogenous or exogenous to the host cells. A suitable terminator is *rrnB*.

Further suitable transcriptional regulators including promoters and terminators and protein targeting methods may be found in “Strategies for Achieving High-Level Expression of Genes in *Escherichia coli*” Savvas C. Makrides, *Microbiological Reviews*, Sept 1996, p 512-538.

The FkpA polynucleotide inserted into the expression vector preferably comprises the nucleic acid encoding the FkpA signal sequences and the FkpA coding sequence. The vector preferably contains a nucleic acid sequence that enables the vector to replicate in one or more selected host cells, preferably to replicate independently of the host chromosome. Such sequences are well known for a variety of bacteria.

In one embodiment the FkpA and/or Skp and/or the protein of interest comprises a histidine-tag at the N-terminus and/or C-terminus.

The antibody molecule may be secreted from the cell or targeted to the periplasm by suitable signal sequences. Alternatively, the antibody molecules may accumulate within the cell's cytoplasm. Preferably the antibody molecule is targeted to the periplasm.

The polynucleotide encoding the antibody may be expressed as a fusion with another polypeptide, preferably a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature polypeptide. The heterologous signal sequence selected should be one that is recognized and processed by the host cell. For prokaryotic host cells that do not recognize and process the native or a eukaryotic polypeptide signal sequence, the signal sequence is substituted by a prokaryotic signal sequence. Suitable signal sequences include OmpA, PhoA, LamB, PelB, DsbA and DsbC. In an embodiment where the cell comprises a polynucleotide sequence encoding a heavy chain of the antibody and a polynucleotide sequence encoding a light chain of the antibody, each polynucleotide may comprise a signal sequence, such as OmpA.

Construction of suitable vectors containing one or more of the above-listed components employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and re-ligated in the form desired to generate the plasmids required. General methods by which the vectors may be constructed, transfection methods and culture methods are well known to those skilled in the art. In this respect, reference is made to “Current Protocols in Molecular Biology”, 1999, F. M. Ausubel (ed), Wiley Interscience, New York and the Maniatis Manual produced by Cold Spring Harbor Publishing.

Standard techniques of molecular biology may be used to prepare DNA sequences coding for the antibody. Desired DNA sequences may be synthesised completely or in part using oligonucleotide synthesis techniques. Site-directed mutagenesis and polymerase chain reaction (PCR) techniques may be used as appropriate.

Embodiments of the invention described herein with reference to the polynucleotide apply equally to alternative embodiments of the invention, for example vectors, expression

cassettes and/or host cells comprising the components employed therein, as far as the relevant aspect can be applied to same.

The cell according to the present invention may further comprise a polynucleotide sequence encoding a protein of interest. The polynucleotide sequence encoding the protein of interest may be exogenous or endogenous. The polynucleotide sequence encoding the protein of interest may be integrated into the host's chromosome or may be non-integrated in a vector, typically a plasmid.

In one embodiment the cell according to the present invention expresses a protein of interest. "Protein of interest" in the context of the present specification is intended to refer to polypeptide for expression, usually a recombinant polypeptide. However, the protein of interest may be an endogenous protein expressed from an endogenous gene in the host cell.

As used herein, a "recombinant polypeptide" refers to a protein that is constructed or produced using recombinant DNA technology. The protein of interest may be an exogenous sequence identical to an endogenous protein or a mutated version thereof, for example with attenuated biological activity, or fragment thereof, expressed from an exogenous vector. Alternatively, the protein of interest may be a heterologous protein, not normally expressed by the host cell.

The protein of interest may be any suitable protein including a therapeutic, prophylactic or diagnostic protein.

The skilled person would easily be able to test secreted protein to see if the protein is correctly folded using methods well known in the art, such as protein G HPLC, circular dichroism, NMR, X-Ray crystallography and epitope affinity measurement methods.

In one embodiment the protein of interest is useful in the treatment of diseases or disorders including immunological disorders and/or autoimmune diseases.

In one embodiment autoimmune disease is selected from acute disseminated encephalomyelitis (ADEM), acute necrotizing hemorrhagic leukoencephalitis, addison's disease, agammaglobulinemia, alopecia areata, amyloidosis, ANCA-associated vasculitis, ankylosing spondylitis, anti-GBM/Anti-TBM nephritis, antiphospholipid syndrome (APS), autoimmune angioedema, autoimmune aplastic anemia, autoimmune dysautonomia, autoimmune hepatitis, autoimmune hyperlipidemia, autoimmune immunodeficiency, autoimmune inner ear disease (AIED), autoimmune myocarditis, autoimmune pancreatitis, autoimmune retinopathy, autoimmune thrombocytopenic purpura (ATP), autoimmune thyroid disease, autoimmune urticarial, axonal & neuronal neuropathies, balo disease, Behcet's disease, bullous pemphigoid, cardiomyopathy, Castleman disease, celiac disease, chagas disease, chronic fatigue syndrome, chronic inflammatory demyelinating polyneuropathy (CIDP), chronic recurrent multifocal osteomyelitis (CRMO), Churg-Strauss syndrome, cicatricial pemphigoid/benign mucosal pemphigoid, Crohn's disease, Cogans syndrome, cold agglutinin disease, congenital heart block, coxsackie myocarditis, CREST disease, essential mixed cryoglobulinemia, demyelinating neuropathies, dermatitis herpetiformis, dermatomyositis, devic's disease (neuromyelitis optica), dilated cardiomyopathy, discoid lupus, dressler's syndrome, endometriosis, eosinophilic

angiocentric fibrosis, eosinophilic fasciitis, erythema nodosum, experimental allergic  
 encephalomyelitis, Evans syndrome, fibromyalgia, fibrosing alveolitis, giant cell arteritis  
 (temporal arteritis), glomerulonephritis, Goodpasture's syndrome, granulomatosis with  
 polyangiitis (GPA) see Wegener's, Graves' disease, Guillain-Barre syndrome, Hashimoto's  
 5 encephalitis, Hashimoto's thyroiditis, hemolytic anemia, Henoch-Schonlein purpura, herpes  
 gestationis, hypogammaglobulinemia, idiopathic hypocomplementemic tubulointestinal  
 nephritis, idiopathic thrombocytopenic purpura (ITP), IgA nephropathy, IgG4-related disease,  
 IgG4-related sclerosing disease, immunoregulatory lipoproteins, inflammatory aortic aneurysm,  
 inflammatory pseudotumour, inclusion body myositis, insulin-dependent diabetes (type1),  
 10 interstitial cystitis, juvenile arthritis, juvenile diabetes, Kawasaki syndrome, Kuttner's tumour,  
 Lambert-Eaton syndrome, leukocytoclastic vasculitis, lichen planus, lichen sclerosus, ligneous  
 conjunctivitis, linear IgA disease (LAD), Lupus (SLE), Lyme disease, chronic, mediastinal  
 fibrosis, meniere's disease, microscopic polyangiitis, Mikulicz's syndrome, mixed connective  
 tissue disease (MCTD), Mooren's ulcer, Mucha-Habermann disease, multifocal fibrosclerosis,  
 15 multiple sclerosis, myasthenia gravis, myositis, narcolepsy, neuromyelitis optica (Devic's),  
 neutropenia, ocular cicatricial pemphigoid, optic neuritis, ormond's disease (retroperitoneal  
 fibrosis), palindromic rheumatism, PANDAS (Pediatric Autoimmune neuropsychiatric disorders  
 associated with Streptococcus), paraneoplastic cerebellar degeneration, paraproteinemic  
 polyneuropathies, paroxysmal nocturnal hemoglobinuria (PNH), Parry Romberg syndrome,  
 20 Parsonnage-Turner syndrome, pars planitis (peripheral uveitis), pemphigus vulgaris, periaortitis,  
 periarthritis, peripheral neuropathy, perivenous encephalomyelitis, pernicious anemia, POEMS  
 syndrome, polyarteritis nodosa, Type I, II, & III autoimmune polyglandular syndromes,  
 polymyalgia rheumatic, polymyositis, postmyocardial infarction syndrome, postpericardiotomy  
 syndrome, progesterone dermatitis, primary biliary cirrhosis, primary sclerosing cholangitis,  
 25 psoriasis, psoriatic arthritis, idiopathic pulmonary fibrosis, pyoderma gangrenosum, pure red  
 cell aplasia, Raynauds phenomenon, reflex sympathetic dystrophy, Reiter's syndrome, relapsing  
 polychondritis, restless legs syndrome, retroperitoneal fibrosis (Ormond's disease), rheumatic  
 fever, rheumatoid arthritis, Riedel's thyroiditis, sarcoidosis, Schmidt syndrome, scleritis,  
 scleroderma, Sjogren's syndrome, Sperm & testicular autoimmunity, stiff person syndrome,  
 30 subacute bacterial endocarditis (SBE), Susac's syndrome, sympathetic ophthalmia, Takayasu's  
 arteritis, temporal arteritis/giant cell arteritis, thrombotic, thrombocytopenic purpura (TTP),  
 Tolosa-Hunt syndrome, transverse myelitis, ulcerative colitis, undifferentiated connective tissue  
 disease (UCTD), uveitis, vasculitis, vesiculobullous dermatosis, vitiligo, Waldenstrom  
 macroglobulinaemia, warm idiopathic haemolytic anaemia and Wegener's granulomatosis (now  
 35 termed Granulomatosis with Polyangiitis (GPA)).

In one embodiment the neurological disorder is selected from chronic inflammatory  
 demyelinating polyneuropathy (CIDP), Guillain-Barre syndrome, paraproteinemic  
 polyneuropathies, neuromyelitis optica (NMO, NMO spectrum disorders or NMO spectrum  
 diseases), and myasthenia gravis.

In one embodiment the immunology haematology disorder is selected from idiopathic thrombocytopenic purpura (ITP), thrombotic thrombocytopenic purpura (TTP), warm idiopathic haemolytic anaemia, Goodpasture's syndrome, and transplantation donor mismatch due to anti-HLA antibodies.

5 In one embodiment the disease is selected from myasthenia gravis, neuro- myelitis optica, CIDP, Guillaume-Barre Syndrome, Para-proteinemic Poly neuropathy, Refractory Epilepsy, ITP/TTP, hemolytic anemia, Goodpasture's Syndrome, ABO mismatch, Lupus nephritis, renal vasculitis, sclero-derma, fibrosing alveolitis, dilated cardio-myopathy, Grave's Disease, Type 1 diabetes, auto-immune diabetes, pemphigus, sclero-derma, lupus, ANCA  
10 vasculitis, dermatomyositis, Sjogren's disease and rheumatoid arthritis.

In one embodiment the dermatology disorder is selected from bullous pemphigoid, pemphigus vulgaris, ANCA-associated vasculitis and dilated cardiomyopathy.

In one embodiment the antibodies or fragments according to the present disclosure may be employed in prophylaxis or treatment of alloimmune diseases associated with allogenic organ  
15 or tissue transplantation or certain neonatal conditions.

The protein may be a proteolytically-sensitive polypeptide, i.e. proteins that are prone to be cleaved, susceptible to cleavage, or cleaved by one or more gram-negative bacterial, such as *E. coli*, proteases, either in the native state or during secretion. In one embodiment the protein of interest is proteolytically-sensitive to a protease selected from DegP, Protease III and Tsp. In  
20 one embodiment the protein of interest is proteolytically-sensitive to the protease Tsp. In one embodiment the protein of interest is proteolytically-sensitive to the proteases DegP and Protease III. In one embodiment the protein of interest is proteolytically sensitive to the proteases DegP and Tsp. In one embodiment the protein of interest is proteolytically-sensitive to the proteases Tsp and Protease III. In one embodiment the protein of interest is proteolytically  
25 sensitive to the proteases DegP, Protease III and Tsp.

Preferably the protein is a eukaryotic polypeptide.

The protein of interest expressed by the cells according to the invention may, for example be an immunogen, a fusion protein comprising two heterologous proteins or an antibody. Antibodies for use as the protein of interest include monoclonal, multi-valent, multi-  
30 specific, humanized, fully human or chimeric antibodies. The antibody can be from any species but is preferably derived from a monoclonal antibody, a human antibody, or a humanized fragment. The antibody can be derived from any class (e.g. IgG, IgE, IgM, IgD or IgA) or subclass of immunoglobulin molecule and may be obtained from any species including for example mouse, rat, shark, rabbit, pig, hamster, camel, llama, goat or human. Parts of the  
35 antibody fragment may be obtained from more than one species for example the antibody fragments may be chimeric. In one example the constant regions are from one species and the variable regions from another.

The antibody may be a complete antibody molecule having full length heavy and light chains or a fragment thereof, e.g. VH, VL, VHH, Fab, modified Fab, Fab', F(ab')<sub>2</sub>, Fv, scFv

fragment, or a dual specificity antibody, such as a Fab-dAb or Fab-Fv, as described in WO2009/040562 and WO2010/035012.

In one embodiment the protein is a Fab'.

The antibody may be specific for any target antigen. The antigen may be a cell-associated protein, for example a cell surface protein on cells such as bacterial cells, yeast cells, T-cells, endothelial cells or tumour cells, or it may be a soluble protein. Antigens of interest may also be any medically relevant protein such as those proteins upregulated during disease or infection, for example receptors and/or their corresponding ligands. Particular examples of cell surface proteins include adhesion molecules, for example integrins such as  $\beta 1$  integrins e.g. VLA-4, E-selectin, P selectin or L-selectin, CD2, CD3, CD4, CD5, CD7, CD8, CD11a, CD11b, CD18, CD19, CD20, CD23, CD25, CD33, CD38, CD40, CD40L, CD45, CDW52, CD69, CD134 (OX40), ICOS, BCMP7, CD137, CD27L, CDCP1, CSF1 or CSF1-Receptor, DPCR1, DPCR1, dudulin2, FLJ20584, FLJ40787, HEK2, KIAA0634, KIAA0659, KIAA1246, KIAA1455, LTBP2, LTK, MAL2, MRP2, nectin-like2, NKCC1, PTK7, RAIG1, TCAM1, SC6, BCMP101, BCMP84, BCMP11, DTD, carcinoembryonic antigen (CEA), human milk fat globulin (HMFG1 and 2), MHC Class I and MHC Class II antigens, KDR and VEGF, and where appropriate, receptors thereof.

Soluble antigens include interleukins such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-12, IL-13, IL-14, IL-16 or IL-17, such as IL17A and/or IL17F, viral antigens for example respiratory syncytial virus or cytomegalovirus antigens, immunoglobulins, such as IgE, interferons such as interferon  $\alpha$ , interferon  $\beta$  or interferon  $\gamma$ , tumour necrosis factor TNF (formerly known as tumour necrosis factor- $\alpha$ ), tumor necrosis factor- $\beta$ , colony stimulating factors such as G-CSF or GM-CSF, and platelet derived growth factors such as PDGF- $\alpha$ , and PDGF- $\beta$  and where appropriate receptors thereof. Other antigens include bacterial cell surface antigens, bacterial toxins, viruses such as influenza, EBV, HepA, B and C, bioterrorism agents, radionuclides and heavy metals, and snake and spider venoms and toxins.

In one embodiment the antigen is FcRn.

Antibodies for use in the present disclosure may be obtained using any suitable method known in the art. The FcRn polypeptide/protein including fusion proteins and mutants thereof, including cells (recombinantly or naturally) expressing the polypeptide (such as activated T cells) can be used to produce antibodies which specifically recognise FcRn. The polypeptide may be the 'mature' polypeptide or a biologically active fragment or derivative thereof. The human protein is registered in Swiss-Prot under the number P55899. In one embodiment the immunogen is the FcRn alpha chain or a fragment thereof.

Polypeptides, for use to immunize a host, may be prepared by processes well known in the art from genetically engineered host cells comprising expression systems or they may be recovered from natural biological sources. In the present application, the term "polypeptides" includes peptides, polypeptides and proteins. These are used interchangeably unless otherwise specified. The FcRn polypeptide may in some instances be part of a larger protein such as a fusion protein for example fused to an affinity tag.

Antibodies generated against the FcRn polypeptide may be obtained, where immunisation of an animal is necessary, by administering the polypeptides to an animal, preferably a non-human animal, using well-known and routine protocols, see for example Handbook of Experimental Immunology, D. M. Weir (ed.), Vol 4, Blackwell Scientific Publishers, Oxford, England, 1986). Many warm-blooded animals, such as rabbits, mice, rats, sheep, cows, camels or pigs may be immunized. However, mice, rabbits, pigs and rats are generally most suitable.

In one embodiment, the antibody may be used to functionally alter the activity of the antigen of interest. For example, the antibody may neutralize, antagonize or agonise the activity of said antigen, directly or indirectly or simply blocks binding of the normal ligand thereto.

The present invention also provides a recombinant gram-negative bacterial cell comprising a mutated Tsp gene, wherein the mutated Tsp gene encodes a Tsp protein having reduced protease activity or is a knockout mutated Tsp gene, a mutant spr gene encoding a mutant spr, a gene capable of expressing or overexpressing one or proteins capable of facilitating protein folding and a polynucleotide sequence encoding an antibody or an antigen binding fragment thereof specific for FcRn.

Various anti-FcRn antibodies and fragments are shown in sequences 36 to 74.

In one embodiment the heavy chain comprises 1, 2 or 3 CDRs independently selected from SEQ ID NO: 36, 37 and 38.

In one embodiment the light chain comprises 1, 2 or 3 CDRs independently selected from SEQ ID NO: 39, 40 and 41.

In one embodiment the antibody heavy chain comprises the sequence given in SEQ ID NO:36 for CDR-H1, the sequence given in SEQ ID NO:37 for CDR-H2 and the sequence given in SEQ ID NO:38 for CDRH3.

In one embodiment the antibody light chain comprises the sequence given in SEQ ID NO:39 for CDR-L1, the sequence given in SEQ ID NO:40 for CDR-L2 and the sequence given in SEQ ID NO:41 for CDRL3.

In one embodiment the antibody heavy chain comprises the sequence given in SEQ ID NO:36 for CDR-H1, the sequence given in SEQ ID NO:37 for CDR-H2, the sequence given in SEQ ID NO:38 for CDRH3, the sequence given in SEQ ID NO:39 for CDR-L1, the sequence given in SEQ ID NO:40 for CDR-L2 and the sequence given in SEQ ID NO:41 for CDRL3.

In one embodiment the cell expresses an antibody molecule with a sequence disclosed herein.

Antibody molecules include antibodies and binding fragments thereof.

Suitably, the humanised antibody according to the present invention has a variable domain comprising human acceptor framework regions as well as one or more of the CDRs provided specifically herein. Thus, provided in one embodiment is a humanised antibody which binds human FcRn wherein the variable domains comprise human acceptor framework regions and non-human donor CDRs. In one example the light chain variable domain comprises the sequence given in SEQ ID NO:50 and the heavy chain variable domain comprises the sequence

given in SEQ ID NO:58. In one example the light chain comprises the sequence given in SEQ ID NO:54 and the heavy chain comprises the sequence given in SEQ ID NO:62.

After expression, antibody fragments may be further processed, for example by conjugation to another entity such as an effector molecule.

5       The term effector molecule as used herein includes, for example, antineoplastic agents, drugs, toxins (such as enzymatically active toxins of bacterial or plant origin and fragments thereof e.g. ricin and fragments thereof) biologically active proteins, for example enzymes, other antibody or antibody fragments, synthetic or naturally occurring polymers, nucleic acids and fragments thereof e.g. DNA, RNA and fragments thereof, radionuclides, particularly  
10       radioiodide, radioisotopes, chelated metals, nanoparticles and reporter groups such as fluorescent compounds or compounds which may be detected by NMR or ESR spectroscopy. Effector molecular may be attached to the antibody or fragment thereof by any suitable method, for example an antibody fragment may be modified to attach at least one effector molecule as described in WO05/003171 or WO05/003170. WO05/003171 or WO05/003170 also describe  
15       suitable effector molecules.

      In one embodiment the antibody or fragment thereof, such as a Fab, is PEGylated to generate a product with the required properties, for example similar to the whole antibodies, if required. For example, the antibody may be a PEGylated anti-TNF-  $\alpha$  Fab', as described in WO01/094585, preferably having attached to one of the cysteine residues at the C-terminal end  
20       of the heavy chain a lysyl-maleimide-derived group wherein each of the two amino groups of the lysyl residue has covalently linked to it a methoxypoly(ethyleneglycol) residue having a molecular weight of about 20,000 Da, such that the total average molecular weight of the methoxypoly(ethyleneglycol) residues is about 40,000Da, more preferably the lysyl-maleimide-derived group is [1-[[[2-[[3-(2,5-dioxo-1-pyrrolidinyl)-1-oxopropyl]amino]ethyl]amino]-  
25       carbonyl]-1,5-pentanediy]bis(iminocarbonyl).

      In one embodiment a Fab or Fab' according to the present disclosure is conjugated to a human serum albumin molecule or a starch molecule.

      The cell may also comprise further polynucleotide sequences encoding one or more further proteins of interest.

30       In one embodiment one or more E. coli host proteins that in the wild type are known to co-purify with the recombinant protein of interest during purification are selected for genetic modification, as described in Humphreys et al. "Engineering of Escherichia coli to improve the purification of periplasmic Fab' fragments: changing the pI of the chromosomally encoded PhoS/PstS protein", Protein Expression and Purification 37 (2004) 109-118 and WO04/035792.

35       The use of such modified host

      proteins improves the purification process for proteins of interest, especially antibodies, produced in E.coli by altering the physical properties of selected E.coli proteins so they no longer co-purify with the recombinant antibody. Preferably the E.coli protein that is altered is selected from one or more of Phosphate binding protein (PhoS/PstS), Dipeptide binding protein  
40       (DppA), Maltose binding protein (MBP) and Thioredoxin.

In one embodiment a physical property of a contaminating host protein is altered by the addition of an amino acid tag to the C-terminus or N-terminus. In a preferred embodiment the physical property that is altered is the isoelectric point and the amino acid tag is a poly-aspartic acid tag attached to the C-terminus. In one embodiment the E.coli proteins altered by the addition of said tag are Dipeptide binding protein (DppA), Maltose binding protein (MBP), Thioredoxin and Phosphate binding protein (PhoS/PstS). In one specific embodiment the pI of the E. coli Phosphate binding protein (PhoS/PstS) is reduced from 7.2 to 5.1 by the addition of a poly-aspartic acid tag (polyD), containing 6 aspartic acid residues to the C-terminus.

Also preferred is the modification of specific residues of the contaminating E. coli protein to alter its physical properties, either alone or in combination with the addition of N or C terminal tags. Such changes can include insertions or deletions to alter the size of the protein or amino acid substitutions to alter pI or hydrophobicity. In one embodiment these residues are located on the surface of the protein. In a preferred embodiment surface residues of the PhoS protein are altered in order to reduce the pI of the protein. Preferably residues that have been implicated to be important in phosphate binding (Bass, US5,304,472) are avoided in order to maintain a functional PhoS protein. Preferably lysine residues that project far out of the surface of the protein or are in or near large groups of basic residues are targeted. In one embodiment, the PhoS protein has a hexa poly-aspartic acid tag attached to the C-terminus whilst surface residues at the opposite end of the molecule are targeted for substitution. Preferably selected lysine residues are substituted for glutamic acid or aspartic acid to confer a greater potential pI change than when changing neutral residues to acidic ones. The designation for a substitution mutant herein consists of a letter followed by a number followed by a letter. The first letter designates the amino acid in the wild-type protein. The number refers to the amino acid position where the amino acid substitution is being made, and the second letter designates the amino acid that is used to replace the wild-type amino acid. In preferred mutations of PhoS in the present invention lysine residues (K) 275, 107, 109, 110, 262, 265, 266, 309, 313 are substituted for glutamic acid (E) or glutamine (Q), as single or combined mutations, in addition lysine(K)318 may be substituted for aspartic acid (D) as a single or combined mutation. Preferably the single mutations are K262E, K265E and K266E. Preferably the combined mutations are K265/266E and K110/265/266E. More preferably, all mutations are combined with the polyaspartic acid (polyD) tag attached at the C-terminus and optionally also with the K318D substitution. In a preferred embodiment the mutations result in a reduction in pI of at least 2 units. Preferably the mutations of the present invention reduce the pI of PhoS from 7.2 to between about 4 and about 5.5. In one embodiment of the present invention the pI of the PhoS protein of E. coli is reduced from 7.2 to about 4.9, about 4.8 and about 4.5 using the mutations polyD K318D, polyD K265/266E and polyD K110/265/266E respectively.

The polynucleotide encoding the protein of interest may be expressed as a fusion with another polypeptide, preferably a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature polypeptide. The heterologous signal sequence selected should be one that is recognized and processed by the host cell. For prokaryotic host

cells that do not recognize and process the native or a eukaryotic polypeptide signal sequence, the signal sequence is substituted by a prokaryotic signal sequence. Suitable signal sequences include OmpA, PhoA, LamB, PelB, DsbA and DsbC.

Construction of suitable vectors containing one or more of the above-listed components  
5 employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and re-ligated in the form desired to generate the plasmids required.

In one embodiment an expression cassette is employed in the present invention to carry the polynucleotide encoding the protein of interest which typically comprises one or more protein coding sequences encoding one or more proteins of interest and one or more regulatory  
10 expression sequences. The one or more regulatory expression sequences may include a promoter. The one or more regulatory expression sequences may also include a 3' untranslated region such as a termination sequence. Suitable promoters are discussed in more detail below.

In one embodiment, the cell according to the present invention comprises a vector, such as plasmid. The vector preferably comprises one or more of the expression cassettes as defined  
15 above.

In the embodiment where the protein of interest is an antibody comprising both heavy and light chains, the cell line may be transfected with two vectors, a first vector encoding a light chain polypeptide and a second vector encoding a heavy chain polypeptide. Alternatively, a single vector may be used, the vector including sequences encoding light chain and heavy chain  
20 polypeptides.

The vector for use in the present invention may be produced by inserting an expression cassette as defined above into a suitable vector. Alternatively, the regulatory expression sequences for directing expression of the polynucleotide sequence encoding a protein of interest may be contained in the vector and thus only the encoding region of the polynucleotide may be  
25 required to complete the vector.

Examples of vectors which may be employed to transform the host cell with a polynucleotide according to the invention include: a plasmid, such as pBR322 or pACYC184, and/or a viral vector such as bacterial phage, a transposable genetic element such as a transposon.

Many forms of expression vector are available. Such vectors usually comprise a plasmid  
30 origin of DNA replication, an antibiotic selectable marker a promoter and transcriptional terminator separated by a multi-cloning site (expression cassette) and a DNA sequence encoding a ribosome binding site.

The promoters employed in the present invention can be linked to the relevant polynucleotide directly or alternatively be located in an appropriate position, for example in a  
35 vector such that when the relevant polypeptide is inserted the relevant promoter can act on the same. In one embodiment the promoter is located before the encoding portion of the polynucleotide on which it acts, for example a relevant promoter before each encoding portion of polynucleotide. "Before" as used herein is intended to imply that the promoter is located at the 5 prime end in relation to the encoding polynucleotide portion.

The promoters may be endogenous or exogenous to the host cells. Suitable promoters include lac, tac, trp, phoA, Ipp, Arab, tet and T7.

One or more promoters employed may be inducible promoters.

Expression units for use in bacterial systems also generally contain a Shine-Dalgarno (S. D.) ribosome sequence operably linked to the DNA encoding the polypeptide of interest.

The expression vector preferably also comprises a dicistronic message for producing the antibody or antigen binding fragment thereof as described in WO 03/048208 or WO2007/039714. Preferably the upstream cistron contains DNA coding for the light chain of the antibody and the downstream cistron contains DNA coding for the corresponding heavy chain, and the dicistronic intergenic sequence (IGS) preferably comprises a sequence selected from IGS1 (SEQ ID NO: 38), IGS2 (SEQ ID NO: 39), IGS3 (SEQ ID NO: 40) and IGS4 (SEQ ID NO: 41).

The terminators may be endogenous or exogenous to the host cells. A suitable terminator is rmb.

Further suitable transcriptional regulators including promoters and terminators and protein targeting methods may be found in "Strategies for Achieving High-Level Expression of Genes in Escherichia coli" Savvas C. Makrides, Microbiological Reviews, Sept 1996, p 512-538.

The antibody molecule may be secreted from the cell or targeted to the periplasm by suitable signal sequences. Alternatively, the antibody molecules may accumulate within the cell's cytoplasm. Preferably the antibody molecule is targeted to the periplasm.

Embodiments of the invention described herein with reference to the polynucleotide apply equally to alternative embodiments of the invention, for example vectors, expression cassettes and/or host cells comprising the components employed therein, as far as the relevant aspect can be applied to same.

The present invention also provides a method for producing a recombinant protein of interest comprising expressing the recombinant protein of interest in a recombinant gram-negative bacterial cell as described above in the first or second aspect of the present invention.

The gram negative bacterial cell and protein of interest preferably employed in the method of the present invention are described in detail above.

When the polynucleotide encoding the protein of interest is exogenous the polynucleotide may be incorporated into the host cell using any suitable means known in the art. Typically, the polynucleotide is incorporated as part of an expression vector which is transformed into the cell. Accordingly, in one aspect the cell according to the present invention comprises an expression cassette comprising the polynucleotide encoding the protein of interest.

The polynucleotide sequence can be transformed into a cell using standard techniques, for example employing rubidium chloride, PEG or electroporation.

The method according to the present invention may also employ a selection system to facilitate selection of stable cells which have been successfully transformed with the polynucleotide encoding the protein of interest. The selection system typically employs co-

transformation of a polynucleotide sequence encoding a selection marker. In one embodiment, each polynucleotide transformed into the cell further comprises a polynucleotide sequence encoding one or more selection markers. Accordingly, the transformation of the polynucleotide encoding the protein of interest and the one or more polynucleotides encoding the marker occurs together and the selection system can be employed to select those cells which produce the desired proteins.

Cells able to express the one or more markers are able to survive/grow/multiply under certain artificially imposed conditions, for example the addition of a toxin or antibiotic, because of the properties endowed by the polypeptide/gene or polypeptide component of the selection system incorporated therein (e.g. antibiotic resistance). Those cells that cannot express the one or more markers are not able to survive/grow/multiply in the artificially imposed conditions. The artificially imposed conditions can be chosen to be more or less vigorous, as required.

Any suitable selection system may be employed in the present invention. Typically the selection system may be based on including in the vector one or more genes that provides resistance to a known antibiotic, for example a tetracycline, chloramphenicol, kanamycin or ampicillin resistance gene. Cells that grow in the presence of a relevant antibiotic can be selected as they express both the gene that gives resistance to the antibiotic and the desired protein.

In one embodiment, the method according to the present invention further comprises the step of culturing the transformed cell in a medium to thereby express the protein of interest.

An inducible expression system or a constitutive promoter may be used in the present invention to express the protein of interest. Suitable inducible expression systems and constitutive promoters are well known in the art.

Any suitable medium may be used to culture the transformed cell. The medium may be adapted for a specific selection system, for example the medium may comprise an antibiotic, to allow only those cells which have been successfully transformed to grow in the medium.

The cells obtained from the medium may be subjected to further screening and/or purification as required. The method may further comprise one or more steps to extract and purify the protein of interest as required.

The polypeptide may be recovered from the strain, including from the cytoplasm, periplasm, or supernatant.

In one embodiment the antibody is isolated from the periplasm.

In one embodiment the post induction feed rate is in the range 5 to 7.5g/h, such as about 7g/h.

The specific method (s) used to purify a protein depends on the type of protein. Suitable methods include fractionation on immuno-affinity or ion-exchange columns; ethanol precipitation; reversed-phase HPLC; hydrophobic-interaction chromatography; chromatography on silica; chromatography on an ion-exchange resin such as S-SEPHAROSE and DEAE; chromatofocusing; ammonium-sulfate precipitation; and gel filtration.

Antibodies may be suitably separated from the culture medium and/or cytoplasm extract and/or periplasm extract by conventional antibody purification procedures such as, for example, protein A-Sepharose, protein G chromatography, protein L chromatography, thiophilic, mixed mode resins, His-tag, FLAGTag, hydroxylapatite chromatography, gel electrophoresis, dialysis, affinity chromatography, Ammonium sulphate, ethanol or PEG fractionation/precipitation, ion exchange membranes, expanded bed adsorption chromatography (EBA) or simulated moving bed chromatography..

The method may also include a further step of measuring the quantity of expression of the protein of interest and selecting cells having high expression levels of the protein of interest. The method may also including one or more further downstream processing steps such as PEGylation of the protein of interest, such as an antibody or antibody fragment.

One or more method steps described herein may be performed in combination in a suitable container such as a bioreactor. The antibodies and fragments according to the present disclosure may be employed in treatment or prophylaxis.

Where technically appropriate embodiments of the invention may be combined. Embodiments are described herein as comprising certain features/elements. The disclosure also extends to separate embodiments consisting or consisting essentially of said features/elements.

The present invention is further described by way of illustration only in the following examples, which refer to the accompanying Figures.

## **EXAMPLES**

### **Example 1: Generation of Cell Lines**

The generation of MXE016 and MXE017 is given in WO2011/086136.

**Generation of plasmids for anti-FcRn Fab' expression and anti-FcRn Fab' with FkpA, anti-FcRn Fab' with skp and anti-FcRn Fab with both FkpA and skp expression**

A plasmid was constructed containing both the heavy and light chain sequences of an anti-FcRn Fab (SEQ ID NOs: 63 and 55, respectively. Plasmid pTTOD 1519.g57 Fab', was constructed using conventional restriction cloning methodologies which can be found in Sambrook *et al* 1989, Molecular cloning: a laboratory manual. CSHL press, N.Y. The plasmid pTTOD 1519.g57 Fab' contained the following features; a strong *tac* promoter and *lac* operator sequence. The plasmid contained a unique EcoRI restriction site after the coding region of the Fab' heavy chain, followed by a non-coding sequence containing a strong ribosome binding site and then a unique NdeI restriction site.

The Fab light chain, heavy chain genes were transcribed as a single polycistronic message. DNA encoding the signal peptide from the *E. coli* OmpA protein was fused to the 5' end of both light and heavy chain gene sequences, which directed the translocation of the polypeptides to the *E. coli* periplasm. Transcription was terminated using a dual transcription terminator *rrnB t1t2*. The *lacIq* gene encoded the constitutively expressed Lac I repressor protein. This repressed transcription from the *tac* promoter until de-repression was induced by the presence of

allolactose or IPTG. The origin of replication used was p15A, which maintained a low copy number. The plasmid contained a tetracycline resistance gene for antibiotic selection.

Plasmid pTTOD 1519.g57 Fab' FkpA, an expression vector for the anti-FcRn Fab and FkpA (a periplasmic polypeptide), was constructed by ligating FkpA to the 3' end of the Fab' sequence of pTTOD 1519.g57 Fab' using the EcoRI and NdeI sites (See Figure 7d). The FkpA (SEQ ID NO:30) was synthetically constructed to remove 2 Puv II sites, an SfuI site, a BamHI site and an EcoRI, such that the new construct encoded for a 5' EcoRI site followed by a strong ribosome binding site, followed by the native start codon, signal sequence and mature sequence of FkpA, terminating in a C-terminal His tag and finally a strong ribosome binding site followed by a non-coding NdeI site. The EcoRI-NdeI restriction fragment was restricted and ligated into the expression vector such that all three polypeptides: Fab' light chain, Fab' heavy chain and FkpA were encoded on a single polycistronic mRNA.

Plasmid pTTOD 1519.g57 Fab' Skp, an expression vector for the anti-FcRn Fab and Skp (a periplasmic polypeptide), was constructed by ligating skp to the 3' end of the Fab' sequence of plasmid pTTOD 1519.g57 Fab' using the EcoRI and NdeI sites. The skp (SEQ ID NO:34) was synthetically constructed to remove 4 Pst I sites and an EcoRV site, such that the new construct encoded for a 5' EcoRI site followed by a strong ribosome binding site, followed by the native start codon, signal sequence and mature sequence of Skp, terminating in a C-terminal His tag and finally a strong ribosome binding site followed by a non-coding NdeI site. The EcoRI-NdeI restriction fragment was restricted and ligated into the expression vector such that all three polypeptides: Fab' light chain, Fab' heavy chain and skp were encoded on a single polycistronic mRNA.

Plasmid pTTOD 1519.g57 Fab' FkpA Skp an expression vector for the anti-FcRn Fab, FkpA and Skp (both periplasmic polypeptides), was constructed by ligating Skp into plasmid pTTOD 1519.g57 Fab' FkpA, at the 3' end of the FkpA sequence using the NdeI site. The Skp (SEQ ID NO:34) was synthetically constructed to remove 4 Pst I sites and an EcoRV site, such that the construct encoded for a 5' Ase I site including the native start codon, signal sequence and mature sequence of skp, terminating in a C-terminal His tag and finally a non-coding NdeI site. The AseI-NdeI restriction fragment was restricted and ligated into the expression vector such that all four polypeptides: Fab' light chain, Fab' heavy chain, FkpA and Skp were encoded on a single polycistronic mRNA.

**Fermentation** of pTTOD 1519.g57 Fab', pTTOD 1519.g57 Fab' FkpA, pTTOD 1519.g57 Fab' Skp and pTTOD 1519.g57 Fab' FkpA skp in *E.coli* W3110, MXE012 (*E. coli* W3110 spr H145A), MXE016 (*E. coli* W3110  $\Delta$ Tsp, spr C94A) and MXE017 (*E. coli* W3110  $\Delta$ Tsp, spr H145A)

The *E. coli* strains W3110, MXE012, MXE016 and MXE017 were transformed with the plasmids pTTOD 1519.g57 Fab', pTTOD 1519.g57 Fab' FkpA, pTTOD 1519.g57 Fab' Skp and pTTOD 1519.g57 Fab' FkpA Skp generated in Example 1. The transformation of the strains was carried out using the method found in Chung C.T *et al* Transformation and storage of bacterial

cells in the same solution. PNAS 86:2172-2175 (1989). These transformed strains were tested for expression by fermentation.

**Example 2: Effect of chaperones on Fab' expression:** The strains shown in **Figure 1** were tested in 1L and 2.5L fermentation experiments comparing expression of Fab':

5 **Growth medium, inoculum and fermentation steps.** The fermentation process was initiated by preparing an inoculum from a vial of the cell bank and amplifying through several pre-culture stages (flask and reactors) before seeding of the production fermenter. In the production fermenter, the cells were grown in defined media to high density in batch and fed-batch mode. When the desired cell density was reached expression of the Fab' was induced by the addition of  
10 IPTG. The Fab' expression is targeted to the *E. coli* periplasmic space, where Fab' accumulates throughout the course of the induction phase. A carbon source feed was applied during the induction phase to control expression and cell growth. Temperature, dissolved oxygen (pO<sub>2</sub>) and pH were controlled to maintain the culture within optimal culture conditions.

**Measurement of biomass concentration and growth rate.** Biomass concentration was  
15 determined by measuring the optical density of cultures at 600 nm.

**Periplasmic Extraction.** Cells were collected from culture samples by centrifugation. The supernatant fraction was retained (at -20°C) for further analysis. The cell pellet fraction was resuspended to the original culture volume in extraction buffer (100 mM Tris-HCl, 10 mM EDTA; pH 7.4). Following incubation at 60°C for approximately 10 to 12 hours the extract was  
20 clarified by centrifugation and the supernatant fraction used fresh or retained (at -20°C) for analysis.

**Fab' quantification.** Fab' concentrations in periplasmic extracts and culture supernatants were determined by using Protein G HPLC. A HiTrap Protein-G HP 1ml column (GE-Healthcare or equivalent) was loaded with analyte (approximately neutral pH, 30°C, 0.2µm filtered) at  
25 2ml/min, the column was washed with 20mM phosphate, 50mM NaCl pH 7.4 and then Fab' was eluted using an injection of 50mM Glycine/HCl pH 2.7. Eluted Fab' was measured by A280 on an Agilent 1100 or 1200 HPLC system and quantified by reference to a standard curve of a purified Fab' protein of known concentration.

**Figure 1** shows the results of 46 fermentations on the 5L scale performed with various  
30 combinations of host cells and "chaperone". W3110 is a wild-type *E. coli* strain. The various combinations were: wild type with no chaperone, wild-type with FkpA and Skp, MXE016 mutant spr and Δ Tsp published in WO2011/086136, MXE016 and FkpA, MXE016 and Skp, MXE016 and FkpA and Skp, MXE017 disclosed in WO2011/086136, MXE017 and FkpA and Skp.

35 The results show that MXE016, MXE016 and FkpA, MXE016 and FkpA and Skp, and MXE017 and FkpA and Skp showed the best levels of expression.

**Example 3: Effect of post induction feed rate**

The effect of three different post induction feed rates 5.4, 6.0 and 7.0g/h was tested on MXE016 and MXE016 + FkpA.

**Growth medium, inoculum and fermentation steps.** The fermentation process was initiated by preparing an inoculum from a vial of the cell bank and amplifying through several pre-culture stages (flask and reactors) before seeding of the production fermenter. In the production fermenter, the cells were grown in defined media to high density in batch and fed-batch mode.

5 When the desired cell density was reached expression of the Fab' was induced by the addition of IPTG. The Fab' expression is targeted to the *E. coli* periplasmic space, where Fab' accumulates throughout the course of the induction phase. A carbon source feed was applied during the induction phase to control expression and cell growth, in this experiment the feed rate set at three separate setpoints (shown in the figure). Temperature, dissolved oxygen (pO<sub>2</sub>) and pH  
10 were controlled to maintain the culture within optimal culture conditions.

**Figure 2** shows strain MXE016 transformed with plasmids expressing either no chaperone or FkpA. The figure shows the effect of increasing the post-induction feed rate on Fab' production and retention in the periplasm both with and without FkpA. The data shows that when the feed rate is increased without FkpA expression the additional Fab' produced is lost to the  
15 supernatant. This is not the case with expression of FkpA where the additional Fab' produced at higher feed rates is retained within the periplasm resulting in a higher titre.

#### **Example 4: Analysis of yield and cell viability**

Figures 3A and B show effect on cell viability and Fab' titre in MXE016 transformed with plasmids expressing either no chaperone or FkpA at 20L scale. Figure 3A shows that cell  
20 viability is lower where no FkpA is present. 3b shows that Fab'titres were higher and less variable for MXE016 + FkpA.

Figure 4 shows the higher titre with MXE016+FkpA results in 30% more Fab' following primary Fab product recovery.

Figure 5 and 6 show SDS-PAGE and anti-his-tag western blots of samples from the primary  
25 recovery of MXE016 with no chaperone and MXE016 with FkpA expression.

Samples were loaded according to Fab' concentration with 1 µg of Fab' loaded per lane. Gels were 4-20% Tris-Glycine Novex and were run under non-reducing conditions at 125V for 2 hours. Gels were stained with Sypro Ruby stain and imaged. The western blots were transferred to a PVDF membrane using an Invitrogen iBlot system and then blocked with 1% casein  
30 solution. The membranes were then probed with an anti-his HRP conjugate antibody (Novagen). The blots were then developed with ECL solution and imaged using an Amersham Life Sciences HyperProcessor.

While this invention has been particularly shown and described with reference to preferred embodiments, it will be understood to those skilled in the art that various changes in form and  
35 detail may be made without departing from the scope of the invention as defined by the appendant claims.

41a

SEQUENCE LISTING IN ELECTRONIC FORM

In accordance with Section 111(1) of the Patent Rules, this description contains a sequence listing in electronic form in ASCII text format (file: 28779-9 Seq 07-OCT-14 v1.txt).

A copy of the sequence listing in electronic form is available from the Canadian Intellectual Property Office.

CLAIMS:

## 1. A recombinant gram-negative bacterial cell comprising:

- a. a mutant spr gene encoding a spr protein having a mutation at one or more amino acids selected from D133, H145, H157, N31, R62, I70, Q73, C94, S95, V98, Q99, R100, L108, Y115, V135, L136, G140, R144 and G147 and
- b. an expression vector comprising a gene(s) expressing or overexpressing one or more proteins capable of facilitating protein folding selected from FkpA, Skp or a combination of FkpA and Skp,

wherein the cell:

- has reduced Tsp protein activity compared to a wild-type cell,
- does not comprise a recombinant polynucleotide encoding DsbC, and
- is isogenic to a wild-type bacterial cell except for the mutant spr gene and except for the reduced Tsp protein activity as compared to the wild-type cell.

2. The cell according to claim 1, wherein the mutant spr gene encodes an spr protein having one or more mutations selected from D133A, H145A, H157A, N31Y, R62C, I70T, Q73R, C94A, S95F, V98E, Q99P, R100G, L108S, Y115F, V135D, V135G, L136P, G140C, R144C and G147C.

3. The cell according to claim 1, wherein the mutant spr gene encodes a spr protein having a mutation in amino acid C94 and the mutation H145A.

4. The cell according to claim 1, wherein the mutant spr gene encodes a spr protein having one or more mutations selected from D133A, H145A and H157A.

5. The cell according to claim 1, wherein the mutant spr gene encodes a spr protein having the mutation C94A.

6. The cell according to any one of claims 1 to 5, wherein the mutant spr gene is integrated into the genome of the cell.

7. The cell according to any one of claims 1 to 6, wherein the gene(s) encoding one or more proteins capable of facilitating protein folding are transiently transfected into the cell.

8. The cell according to any one of claims 1 to 7, wherein the protein capable of facilitating protein folding is FkpA.

9. The cell according to any one of claims 1 to 8, wherein the cell further comprises one or more of the following mutated genes:

a. a mutated DegP gene which encodes a DegP protein having chaperone activity and reduced protease activity;

b. a mutated ptr gene which encodes a Protease III protein having reduced protease activity or which is a knockout mutated ptr gene; and

c. a mutated OmpT gene which encodes a OmpT protein having reduced protease activity or which is a knockout mutated OmpT gene.

10. The cell according to any one of claims 1 to 9, wherein the cell comprises a mutated Tsp gene encoding a Tsp protein having reduced protease activity or is a knockout mutated Tsp gene.

11. The cell according to any one of claims 1 to 10, wherein the cell has a knockout mutated Tsp gene which comprises a mutation to the gene start codon and/or one or more stop codons positioned downstream of the gene start codon and upstream of the gene stop codon.

12. The cell according to claim 11, wherein the knockout mutated Tsp gene comprises a restriction marker site created by a missense mutation to the gene start codon and optionally one or more further point mutations.

13. The cell according to claim 12, wherein the knockout mutated Tsp gene comprises SEQ ID NO:3.

14. The cell according to any one of claims 1 to 13, wherein the cell is E. coli.

15. The cell according to claim 14 wherein the E coli is strain K12 or W3110.

16. The cell according to any one of claims 1 to 15, wherein the cell further comprises a polynucleotide sequence encoding a protein of interest.

17. The cell according to claim 16, wherein the protein of interest is an antibody or an antigen binding fragment thereof.

18. The cell according to claim 17, wherein the antibody or antigen binding fragment thereof binds specifically to FcRn.

19. A method for producing a protein of interest comprising culturing a recombinant gram negative bacterial cell as defined in any one of claims 16 to 18 in a culture medium under conditions effective to express the recombinant protein of interest and recovering the recombinant protein of interest from the periplasm of the recombinant gram-negative bacterial cell and/or the culture medium.

20. The method according to claim 19, wherein the method further comprises recovering the protein of interest from the cell.

21. The method according to claim 20, wherein the protein of interest is recovered from the periplasm and/or the supernatant.

Figure 1

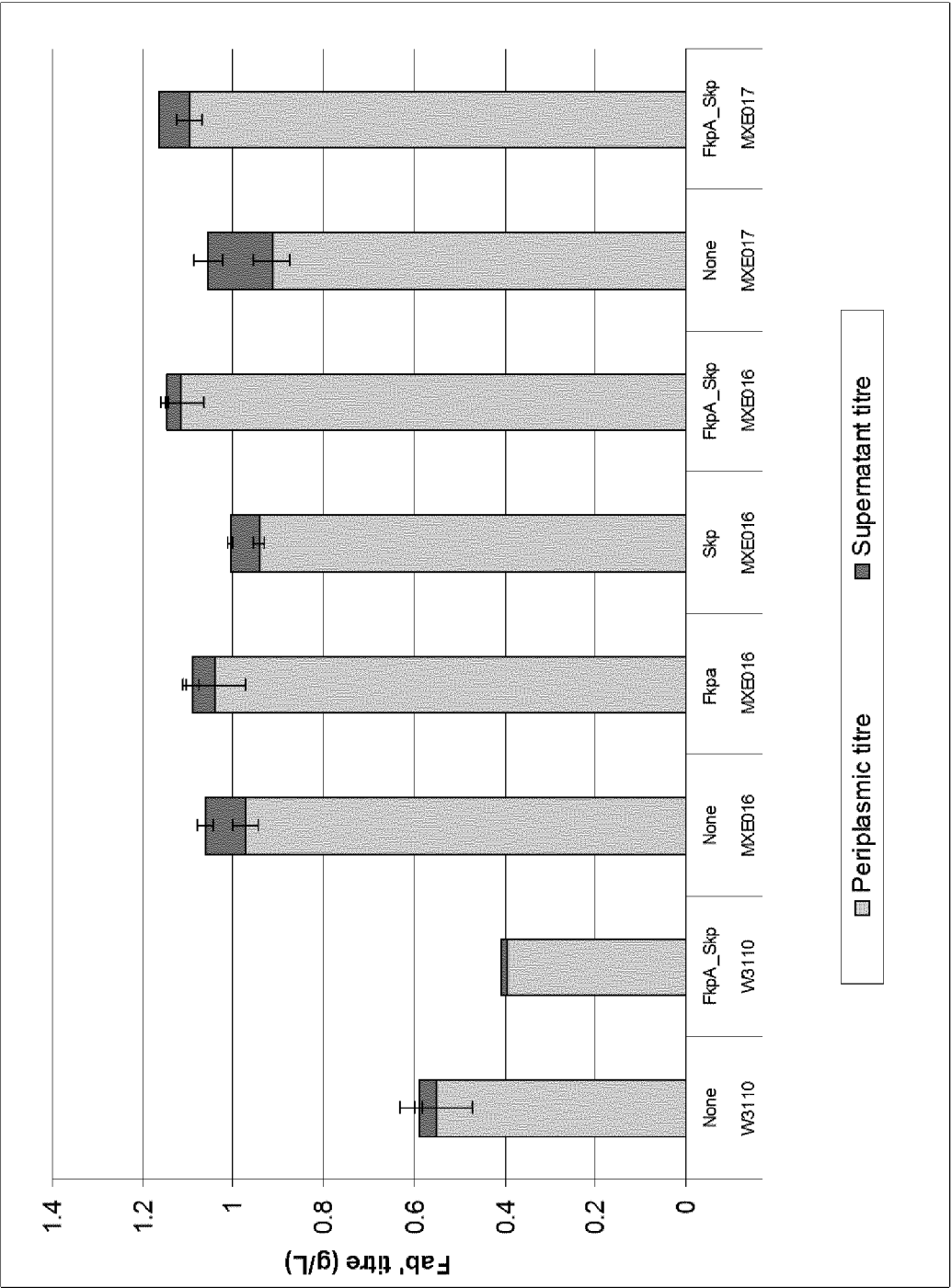


Figure 2 Feed Rate Variation Experiments

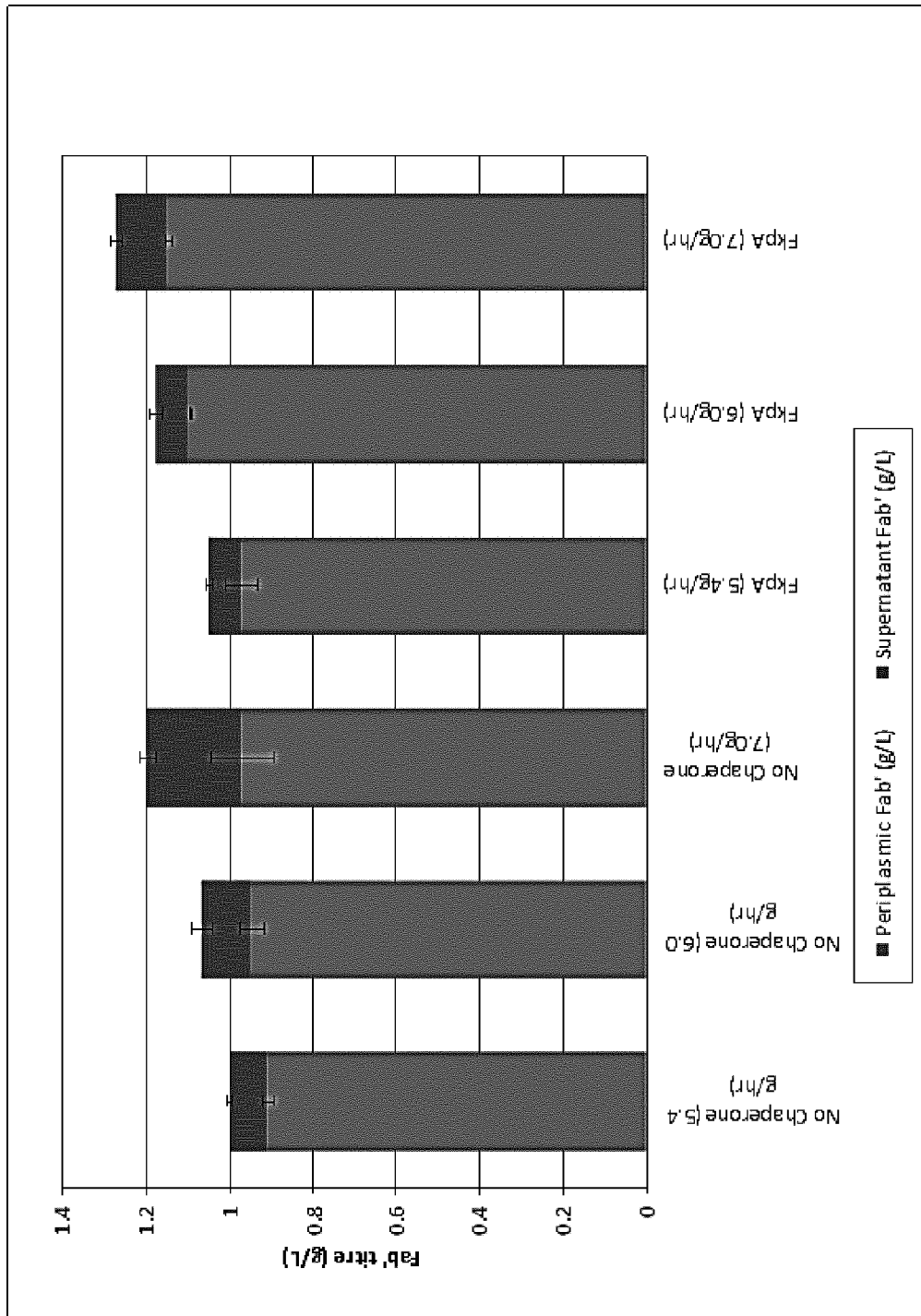


Figure 3A Cell Viability

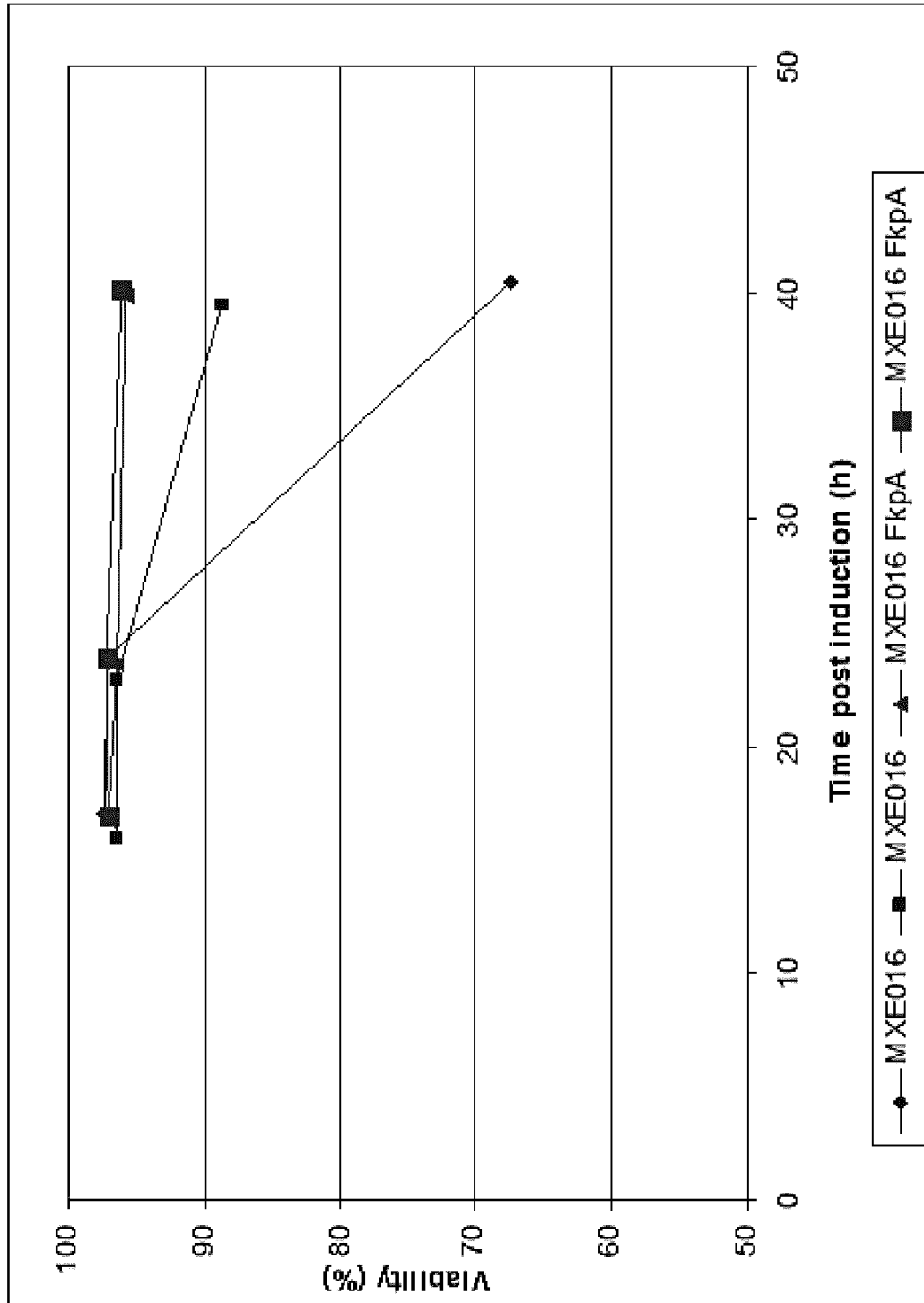
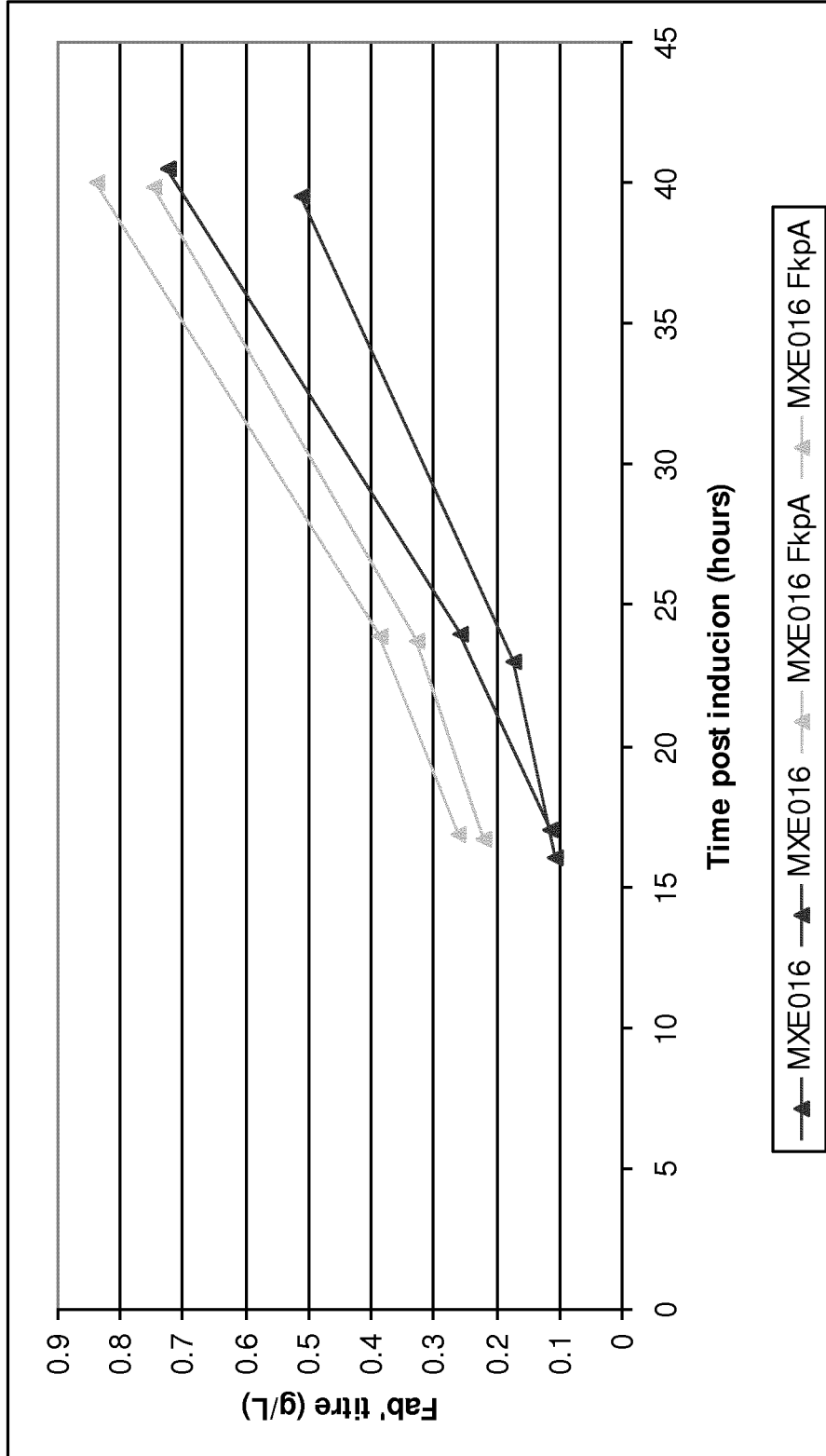


Figure 3B



**Figure 4** Harvest from 20 Litre Pilot Scale Productions and Primary Recovery for “MXE016” Cell Lines

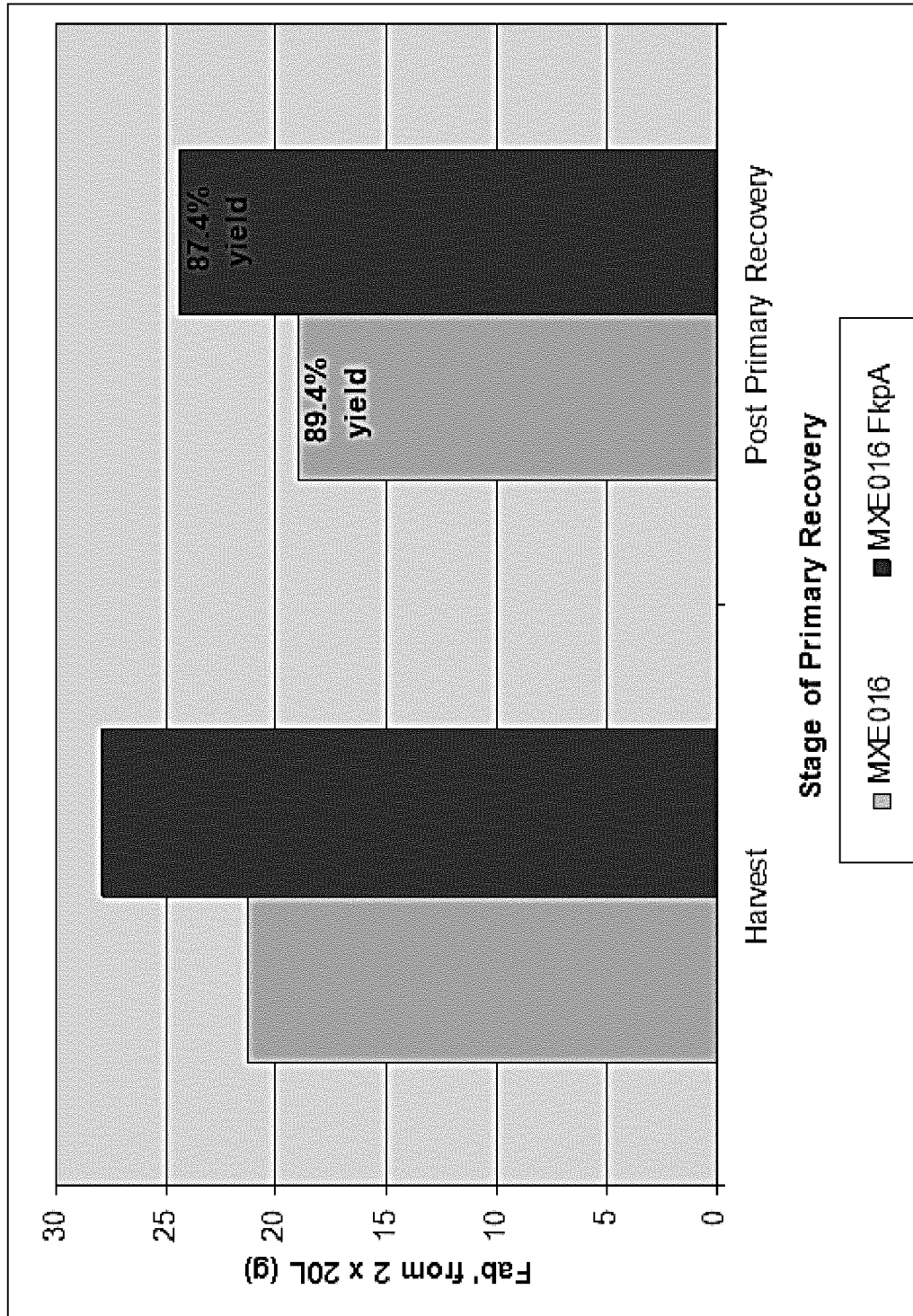
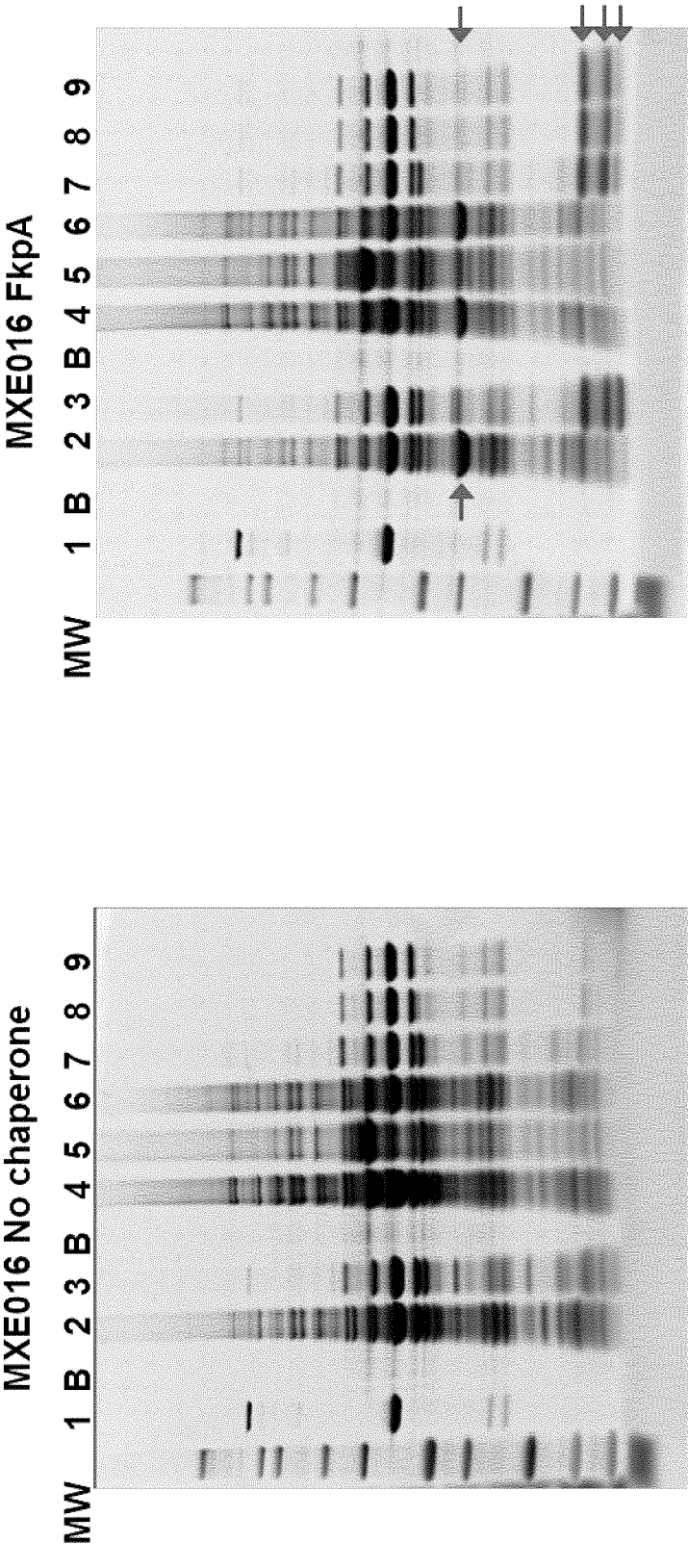
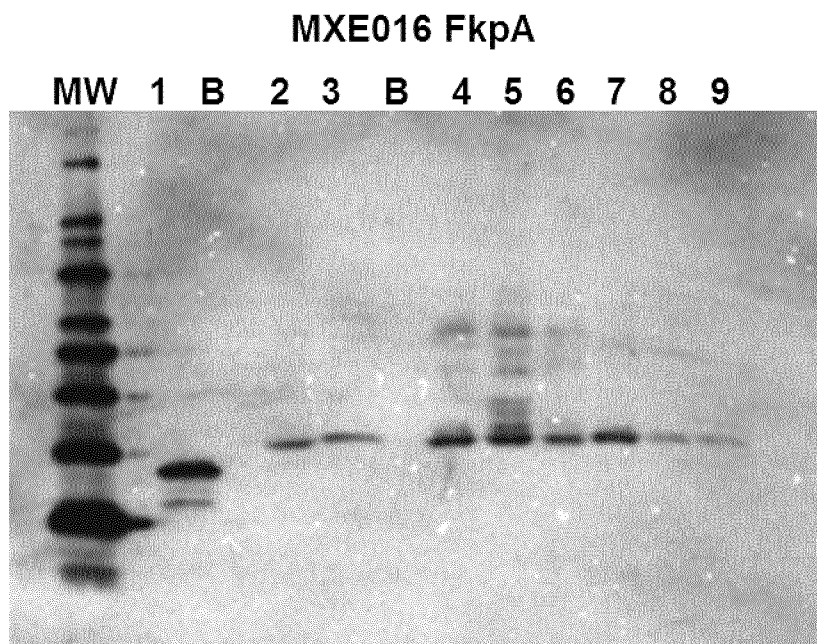


Figure 5 Primary Recovery From 20 Litre Batch Run on SDS-PAGE Stained Gel Under Non-Reducing Conditions



- 1 – X Fab’ Standard;
- 2 - 30°C small scale extract;
- 3 - 60°C small scale extract;
- 4 – Supernatant of heavy phase;
- 5 – Light phase
- 6 – Post buffer addition;
- 7 – Post extraction;
- 8 – Post extract centrifugation
- 9 – Post filtration

**Figure 6 Western Blot Under Non-Reducing Conditions for a 20 L Pilot Scale Batch**

1 – His-tag positive control (DsbC)

2 - 30°C small scale extract

3 - 60°C small scale extract

4 – Supernatant of heavy phase

5 – Light phase

6 – Post buffer addition

7 – Post extraction

8 – Post extract centrifugation

9 – Post filtration

**Figure 7A Mutation in Various Genes****Wild type ptr (protease III) 5' (SEQ ID NOs 81 and 82)**

```

      *   M   P   R   S   T   W   F   K   A   L   L   L   L   V
TGA ATG CCC CGC AGC ACC TGG TTC AAA GCA TTA TTG TTG TTA GTT

      A   L   W   A   P   L   S
GCC CTT TGG GCA CCC TTA AGT

```

**Mutated Δ ptr (protease III) 5' (SEQ ID NOs 79 and 80)**

```

      EcoR I
      ~~~~~~
      *   I   P   R   S   T   W   F   K   A   L   L   L   L   V
TGA ATT CCC CGC AGC ACC TGG TTC AAA GCA TTA TTG TTG TTA GTT

                        Ase I
                        ~~~~~~
      A   L   W   A   H   *   C
GCC CTT TGG GCA CAT TAA TGT

```

**Figure 7B****Wild type Tsp 5' (SEQ ID NOs 75 and 76)**

```

      M   N   M   F   F   R   L   T   A   L   A   G   L   L   A
ATG AAC ATG TTT TTT AGG CTT ACC GCG TTA GCT GGC CTG CTT GCA

      I   A   G   Q   T   F   A
ATA GCA GGC CAG ACC TTC GCT

```

**Mutated Δ Tsp 5' (SEQ ID NOs 77 and 78)**

```

      EcoR I
      ~~~~~~
      M   N   S   F   L   G   L   P   R   *   L   A   C   L   Q
ATG AAT TCG TTT TTA GGC TTA CCG CGT TAG CTG GCC TGC TTG CAA

                        Ase I
                        ~~~~~~
      *   Q   A   R   H   *   L
TAG CAG GCC AGA CAT TAA TGT

```

Figure 7C

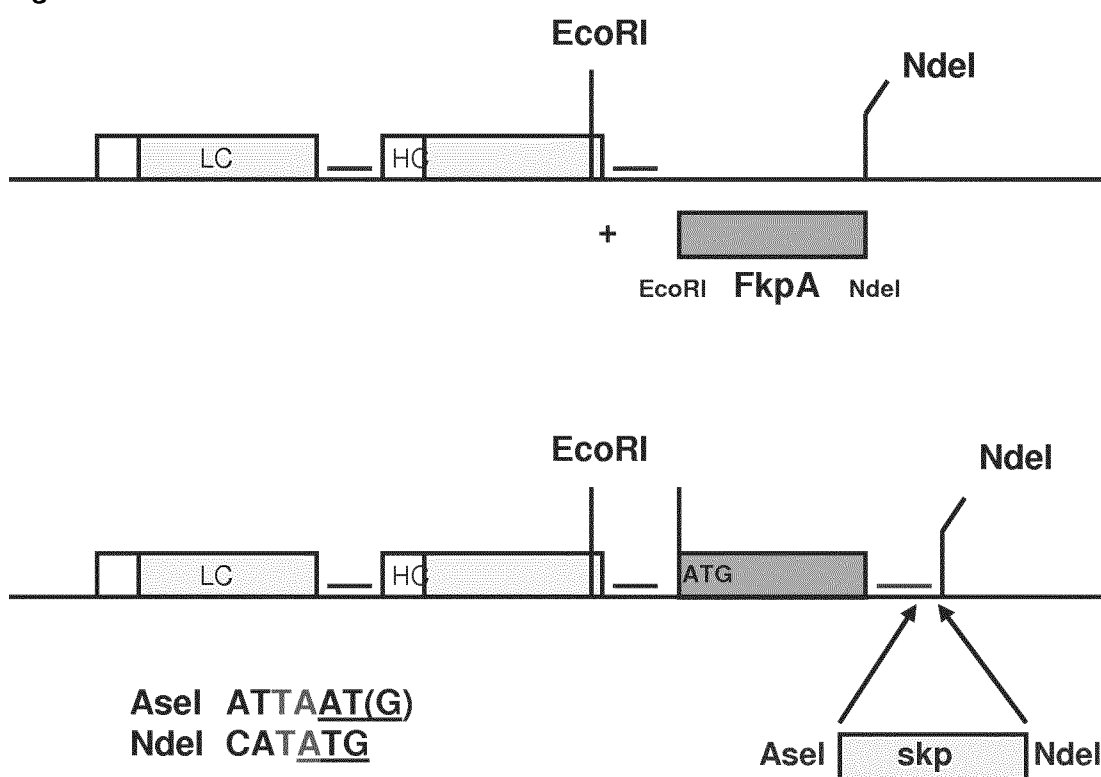
## Wild type DegP

|     |     |     |     |     |     |     |     |     |            |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|------------|-----|-----|
| 202 | D   | A   | A   | I   | N   | R   | G   | N   | <b>S</b>   | G   | G   |
| 949 | GAT | GCA | GCG | ATC | AAC | CGT | GGT | AAC | <b>TCC</b> | GGT | GGT |

## Mutated DegP S210A

|     |     |     |     |                |     |     |     |            |          |     |   |
|-----|-----|-----|-----|----------------|-----|-----|-----|------------|----------|-----|---|
|     |     |     |     | <i>Ase I</i>   |     |     |     |            |          |     |   |
|     |     |     |     | ~~~~~          |     |     |     |            |          |     |   |
| 202 | D   | A   | A   | I              | N   | R   | G   | N          | <b>A</b> | G   | G |
| 949 | GAT | GCA | GCG | <b>ATT AAT</b> | CGT | GGT | AAC | <b>GCC</b> | GGT      | GGT |   |

Figure 7D Gene Insertion



**FIGURE 8A** SEQ ID NO:1 is the DNA sequence of the wild-type Tsp gene including the 6 nucleotides ATGAAC upstream of the start codon.

ATGAACATGTTTTT TAGGCTTACCGCGTTAGCTGGCCTGCTTGCAATAGCAGGCCAGACCTTCGCTGTAGAA  
 GATATCACGCGTGCTGATCAAATTCCGGTATTAAAGGAAGAGACGCAGCATGCGACGGTAAGTGAGCGCGTA  
 ACGTCGCGCTTACCCGTTCTCATTATCGCCAGTTTCGACCTCGATCAGGCATTTTCGGCCAAAATCTTTGAC  
 CGCTACCTGAATCTGCTCGATTACAGCCACAACGTGCTGCTGGCAAGCGATGTTGAACAGTTTCGCGAAAAAG  
 AAAACCGAGTTAGGCGATGAAGTGCCTTCAGGCAAACTCGACGTTTCTACGATCTCTACAATCTGGCGCAA  
 AAGCGCCGTTTTGAGCGTTACCAGTACGCTTTGTCTGGTACTGGAAAAGCCGATGGATTTACCGGCAACGAC  
 ACTTATAACCTTGACCGCAGCAAAGCGCCCTGGCCGAAAAACGAGGCTGAGTTGAACGCGCTGTGGGACAGT  
 AAAGTCAAATTCGACGAGTTAAGCCTGAAGCTGACAGGAAAAACGGATAAAGAAATTCGTGAAACCCTGACT  
 CGCCGCTACAAATTTGCCATTCTGTCGTCTGGCGCAAACCAACAGCGAAGATGTTTTCTCGCTGGCAATGACG  
 GCGTTTGC GCGTGAAATCGACCCGCATACCAACTATCTTTCCCCGCGTAATACCGAACAGTTCAACACTGAA  
 ATGAGTTTGTCTGCTGGAAGGTATTGGCGCAGTGCTGCAAATGGATGATGACTACACCGTTATCAATTTCGATG  
 GTGGCAGGTGGTCCGGCAGCGAAGAGTAAAGCTATCAGCGTTGGTGACAAAATTGTCTGGTGTGGTCAAACA  
 GGCAAGCCGATGGTTGACGTGATTGGCTGGCGTCTTGATGATGTGGTTGCCTTAATTAAAGGGCCGAAGGGC  
 AGTAAAGTTCGTCTGGAAATTTTACCTGCTGGTAAAGGGACCAAGACCCGTAAGTGAACGTTGACCCGTGAA  
 CGTATTCTGCTCTCGAAGACCGCGCGGTTAAAATGTCTGGTGAAGACCGTCGGTAAAGAGAAAGTCGGCGTGCTG  
 GATATTCCGGGCTTCTATGTGGGTTTGACAGACGATGTCAAAGTGCAACTGCAGAACTGGAAAAACAGAAT  
 GTCAGCAGCGTCATCATCGACCTGCGTAGCAATGGCGGTGGGGCGTTAACTGAAGCCGTATCGCTCTCCGGT  
 CTGTTTATTCTGCGGGTCCCATTGTTTACGGTCCGCGATAACAACGGCAAGGTTCTGTAAGATAGCGATACC  
 GACGGACAGGTTTTCTATAAAGGCCCGCTGGTGGTGCTGGTTGACCGCTTCAGTGCTTCGGCTTCAGAAATC  
 TTTGCCGCGCAATGCAGGATTACGGTCGTGCGCTGGTTGTGGGTGAACCGACGTTTGGTAAAGGCACCGTT  
 CAGCAATACCGTTCATTGAACCGTATTTACGATCAGATGTTACGTCCTGAATGGCCAGCGCTGGGTTCTGTG  
 CAGTACACGATCCAGAAATTTCTATCGCGTTAACGGCGGCAGTACGCAACGTAAAGGCGTAACGCCAGACATC  
 ATCATGCCGACGGGTAATGAAGAAACGGAAACGGGTGAGAAATTCGAAGATAACGCGCTGCCGTGGGATAGC  
 ATTGATGCCGCGACTTATGTGAAATCAGGAGATTTAACGGCCTTTGAACCGGAGCTGCTGAAGGAACATAAT  
 GCGCGTATCGCGAAAGATCCTGAGTTCCAGAACATCATGAAGGATATCGCGCGCTTCAACGCTATGAAGGAC  
 AAGCGCAATATCGTTTTCTCTGAATTACGCTGTGCGTGAGAAAGAGAATAATGAAGATGATGCGACGCGTCTG  
 GCGCGTTTGAACGAACGCTTTAAACGCGAAGGTAAACCGGAGTTGAAGAACTGGATGATCTACCGAAAGAT  
 TACCAGGAGCCGATCCTTATCTGGATGAGACGGTGAATATCGCACTCGATCTGGCGAAGCTTGAAAAAGCC  
 AGACCCGCGGAACAACCCGCTCCCGTCAAGTAA

**FIGURE 8B** SEQ ID NO:2 is the amino acid sequence of the wild-type Tsp protein.

MFFRLTALAGLLAIAGQTFAVEDITRADQIPVLKEETQHATVSESVTSRFRTRSHYRQFDLDQAFSAKIFDRY  
 LNLLDYSHNVLLASDVEQFAKKKTELGDELRSGKLDVFDLYNLAQKRRFERYQYALSVLEKPMDFGTGNDTY  
 NLDRSKAPWPKNELNALWDSKVKFDELSLKLTKGTDKEIRETLTRYKFAIRRLAQTNSEDVFSLAMTAF  
 AREIDPHTNYLSPRNTEQFNTEMSLSLEGIGAVLQMDDDYTVINSMVAGGPAAKSKAISVGDKIVGVGQTGK  
 PMVDVIGWRLDDVVALIKGPKGSKVRLEILPAGKGTKTRTVTLTRERIRLEDRAVKMSVKTVGKEKVGVLDI  
 PGFYVGLTDDVKVQLQKLEKQNVSSVIIDLRNNGGALTEAVSLSGLFIPAGPIVQVRDNNGVKREDSDTDG  
 QVFKYKGPLVLVDRFSASASEIFAAMQDYGRALVVGEPTFGKGTQQYRSLNRIYDQMLRPEWPALGSVQY  
 TIQKFYRVNGGSTQRKGVTPDIIMPTGNEETETGEKFEDNALPWDSIDAATYVKSGDLTAFEPELLKEHNAR  
 IAKDPEFQNMKDIARFNAMKDKRNIVSLNYAVREKENNEDDATRLARLNERFKREGKPELKKLDDLDPKDYQ  
 EPDPYLDETVNIALDLAKLEKARPAEQPAPVK\*

**FIGURE 8C**

SEQ ID NO:3 is the DNA sequence of a mutated knockout Tsp gene including the 6 nucleotides ATGAAT upstream of the start codon.

```

ATGAATTCGTTTTTAGGCTTACCGCGTTAGCTGGCCTGCTTGCAATAGCAGGCCAGACATTAATTGTAGAAG
ATATCACGCGTGCTGATCAAATTCGGTATTAAAGGAAGAGACGCAGCATGCGACGGTAAGTGAGCGCGTAA
CGTCGCGCTTCACCCGTTCTCATTATCGCCAGTTCGACCTCGATCAGGCATTTTCGGCCAAAATCTTTGACC
GCTACCTGAATCTGCTCGATTACAGCCACAACGTGCTGCTGGCAAGCGATGTTGAACAGTTCGCGAAAAAGA
AAACCGAGTTAGGCGATGAACTGCGTTCAGGCAAACTCGACGTTTTCTACGATCTCTACAATCTGGCGCAAA
AGCGCCGTTTTGAGCGTTACCAGTACGCTTTGTGCGTACTGGAAAAGCCGATGGATTTACCCGGCAACGACA
CTTATAACCTTGACCGCAGCAAAGCGCCCTGGCCGAAAAACGAGGCTGAGTTGAACGCGCTGTGGGACAGTA
AAGTCAAATTCGACGAGTTAAGCCTGAAGCTGACAGGAAAAACGGATAAAGAAATTCGTGAAACCCTGACTC
GCCGCTACAAATTTGCCATTTCGTCGTCTGGCGCAAACCAACAGCGAAGATGTTTTCTCGCTGGCAATGACGG
CGTTTTCGCGGTGAAATCGACCCGCATACCAACTATCTTTCCCGCGTAATACCGAACAGTTCAACACTGAAA
TGAGTTTGTGCGTGAAGGTATTGGCGCAGTGCTGCAAATGGATGATGACTACACCGTTATCAATTCGATGG
TGGCAGGTGGTCCGGCAGCGAAGAGTAAAGCTATCAGCGTTGGTGACAAAATTGTGCGGTGTTGGTCAAACAG
GCAAGCCGATGGTTGACGTGATTGGCTGGCGTCTTGATGATGTGGTTGCCTTAATTAAAGGGCCGAAGGGCA
GTAAAGTTTCGTCTGGAATTTTACCTGCTGGTAAAGGGACCAAGACCCGTAAGTGTACGTTGACCCGTGAAC
GTATTCGTCTCGAAGACCGCGCGGTTAAATGTGCGTGAAGACCGTCGGTAAAGAGAAAGTCGGCGTGCTGG
ATATTCGGGCTTCTATGTGGGTTTGACAGACGATGTCAAAGTGCAACTGCAGAACTGGAAAAACAGAATG
TCAGCAGCGTCATCATCGACCTGCGTAGCAATGGCGGTGGGGCGTTAACTGAAGCCGATCGCTCTCCGGTC
TGTTTTATTCTGCGGGTCCCATTTGTTTCAGGTCCGCGATAACAACGGCAAGGTTTCGTGAAGATAGCGATACCG
ACGGACAGGTTTTCTATAAAGGCCCGCTGGTGGTGCTGGTTGACCGCTTCAGTGCTTCGGCTTCAGAAATCT
TTGCCGCGGCAATGCAGGATTACGGTCGTGCGCTGGTTGTGGGTGAACCGACGTTTGGTAAAGGCACCGTTC
AGCAATACCGTTCATTGAACCGTATTTACGATCAGATGTTACGTCCTGAATGGCCAGCGCTGGGTTCTGTGC
AGTACACGATCCAGAAATTCATCGCGTTAACGGCGCAGTACGCAACGTAAAGGCGTAACGCCAGACATCA
TCATGCCGACGGGTAATGAAGAAACGGAAACGGGTGAGAAATTCGAAGATAACGCGCTGCCGTGGGATAGCA
TTGATGCCGCGACTTATGTGAAATCAGGAGATTTAACGGCCTTTGAACCGGAGCTGCTGAAGGAACATAATG
CGCGTATCGCGAAAGATCCTGAGTTCCAGAACATCATGAAGGATATCGCGCGCTTCAACGCTATGAAGGACA
AGCGCAATATCGTTTCTCTGAATTACGCTGTGCGTGAGAAAGAGAATAATGAAGATGATGCGACGCGTCTGG
CGCGTTTGAACGAACGCTTTAAACGCGAAGGTAAACCGGAGTTGAAGAACTGGATGATCTACCGAAAGATT
ACCAGGAGCCGGATCCTTATCTGGATGAGACGGTGAATATCGCACTCGATCTGGCGAAGCTTGAAAAAGCCA
GACCCGCGGAACAACCCGCTCCCGTCAAGTAA

```

SEQ ID NO:4 is the DNA sequence of the wild-type Protease III gene.

```

ATGCCCCGAGCACCTGGTTCAAAGCATTATTGTTGTTAGTTGCCCTTTGGGCACCCTTAAGTCAGGCAGAA
ACGGGATGGCAGCCGATTACAGGAAACCATCCGTAAAAGTGATAAAGATAACCGCCAGTATCAGGCTATACGT
CTGGATAACGGTATGGTGGTCTTGCTGGTTTTCTGATCCGCAGGCAGTTAAATCGCTCTCGGCGCTGGTGGTG
CCCGTTGGGTCGCTGGAAGATCCCGAGGCGTACCAGGGGCTGGCACATTACCTTGAACATATGAGTCTGATG
GGGTGCAAAAAGTACCCGCGAGGCTGACAGTCTGGCCGAATATCTCAAAATGCACGGCGGTAGTCACAATGCC
AGCACTGCGCCGATATCGCACGGCTTTCTATCTGGAAGTTGAGAACGACGCCTTGCTGGTGCGGTAGACCGC
CTGGCCGATGCTATTGCTGAACCTTTGCTCGACAAGAAATATGCCGAACGTGAGCGTAATGCGGTGAACGCT
GAATTAACCATGGCGCGTACGCGTGACGGGATGCGCATGGCACAGGTCAGCGCAGAAACCATTAACCCGGCA
CACCCCGGTTCAAAGTTTTCTGGTGGTAACCTCGAACTTTAAGCGACAAACCTGGTAATCCGGTGCAGCAG
GCGCTGAAAGATTTCCACGAGAAGTACTATTCCGCCAATTTGATGAAGGCGGTTATTTACAGTAATAAACCG
CTGCCGGAGTTGGCAAAAATGGCGGCGGACACCTTTGGTCGCGTGCCGAACAAAGAGAGCAAAAAACCGGAA
ATCACCGTGCCGGTAGTCACCGACGCGCAAAAGGGCATTATCATTACGTCCCTGCGCTGCCGCGTAAA
GTGTTGCGCGTTGAGTTTCGCATCGATAACAACCTCAGCGAAGTTCCGTAGTAAAACCGATGAATTGATTACC
TATCTGATTGGCAATCGCAGCCCAGGTACACTTTCTGACTGGCTGCAAAAGCAGGGATTAGTTGAGGGCATT
AGCGCCAACCTCCGATCCTATCGTCAACGGCAACAGCGGCGTATTAGCGATCTCTGCGTCTTTAACCGATAAA
GGCCTGGCTAATCGCGATCAGGTTGTGGCGGCAATTTTAGCTATCTCAATCTGTTACGTGAAAAAGGCATT

```

**FIGURE 8C continued**

GATAAACAACTACTTCGATGAACTGGCGAATGTGCTGGATATCGACTTCCGTTATCCGTCGATCACCCGTGAT  
 ATGGATTACGTCGAATGGCTGGCAGATACCATGATTCGCGTTCTCTGTTGAGCATACGCTGGATGCAGTCAAT  
 ATTGCCGATCGGTACGATGCTAAAGCAGTAAAGGAACGCTCTGGCGATGATGACGCCGCAGAATGCGCGTATC  
 TGGTATATCAGCCCCGAAAAGAGCCGCACAAACAAACGGCTTACTTTGTTCGATGCGCCGTATCAGGTCGATAAA  
 ATCAGCGCACAACTTTTCGCCGACTGGCAGAAAAAGCCGCCGACATTGCGCTCTCTTTGCCAGAGCTTAAC  
 CCTTATATTCTGATGATTTCTCGCTGATTAAGTCAGAGAAGAAATACGACCATCCAGAGCTGATTGTTGAT  
 GAGTCGAATCTGCGCGTGGTGTATGCGCCAAGCCGTTATTTTGGCAGCGAGCCCCAAAGCTGATGTCAGCCTG  
 ATTTTTCGTAATCCGAAAGCCATGGACAGCGCCCCGCAATCAGGTGATGTTTTCGCTCAATGATTATCTCGCA  
 GGGCTGGCGCTTGATCAGTTAAGCAACCAGGCGTCGGTTGGTGGCATAAGTTTTTCCACCAACGCTAACAAC  
 GGCCTTATGGTTAATGCTAATGGTTACACCCAGCGCTCTGCCGCAGCTGTTCCAGGCATTGCTCGAGGGGTAC  
 TTTAGCTATACCGCTACGGAAGATCAGCTTGAGCAGGCGAAGTCCTGGTATAACCAGATGATGGATTCCGCA  
 GAAAAGGGTAAAGCGTTTGAGCAGGCGATTATGCCCAGCGCAGATGCTCTCGCAAGTGCCGTACTTCTCGCGA  
 GATGAACGGCGTAAATTTTGGCCTCCATTACGTTGAAAGAGGTGCTGGCCTATCGCGACGCCTTAAATCA  
 GGGGCTCGACCAGAGTTTATGGTTATCGGCAACATGACCGAGGCCAGGCAACAACGCTGGCACGCGATGTG  
 CAAAAACAGTTGGGCGCTGATGGTTCAGAGTGGTGTGAAACAAAGATGTAGTGGTCGATAAAAAACAATCC  
 GTCATCTTTGAAAAAGCCGGTAACAGCACCGACTCCGCACTGGCAGCGGTATTTGTACCGACTGGCTACGAT  
 GAATACACCAGCTCAGCCTATAGCTCTCTGTTGGGGCAGATCGTACAGCCGTGGTCTACAATCAGTTGCGT  
 ACCGAAGAACAATTGGGCTATGCCGTGTTTGCCTTCCAATGAGCGTGGGGCGTCAGTGGGGCATGGGCTTC  
 CTTTTGCAAAGCAATGATAAACAGCCTTCATTCTTGTGGGAGCGTTACAAGGCGTTTTTCCCAACCGCAGAG  
 GCAAAATTGCGAGCGATGAAGCCAGATGAGTTTTCGCAAAATCCAGCAGGCGGTAATTACCCAGATGCTGCAG  
 GCACCGCAAACGCTCGGCCGAAGAAGCATCGAAGTTAAGTAAAGATTTTCGATCGCGGCAATATGCGCTTCGAT  
 TCGCGTGATAAAATCGTGGCCAGATAAACTGCTGACGCCGCAAAAACCTTGCTGATTTCTTCCATCAGGCG  
 GTGGTCGAGCCGCAAGGCATGGCTATTCTGTTCGAGATTTCCGGCAGCCAGAACGGGAAAGCCGAATATGTA  
 CACCCTGAAGGCTGGAAAGTGTGGGAGAACGTCAGCGCGTTGCAGCAAACAATGCCCTGATGAGTGAAAAG  
 AATGAGTGA

SEQ ID NO:5 is the amino acid sequence of the wild-type Protease III protein.

MPRSTWFKALLLLVALWAPLSQAETGWQPIQETIRKSDKDNRQYQAIRLDNGMVLLVSDPQAVKSLSALVV  
 PVGSLEDPEAYQGLAHYLEHMSLMGSKKYPQADSLAEYLKMHGGSHNASTAPYRTAFYLEVENDALPGAVDR  
 LADAI AEPLLDK KYAERERNAVNAELTMARTRDGMRAQVSAETINPAHPGSKFSGGNLETLSDKPGNPVQQ  
 ALKDFHEKYYSANLMKAVIYSNKPLPELAKMAADTFGRVPNKESKKPEITVPVVTDAQKGII IHYPALPRK  
 VLRVEFRIDNNSAKFRSKTDELITYLIGNRSPGTLSDWLQKQGLVEGISANS DPIVNGNSGVLAI SASLTDK  
 GLANRDQVVAIFSYLNLLEK GIDKQYFDELANVLDIDFRYPSITRDM DYVEWLADTMIRVPVEHTLDAVN  
 IADRYDAKAVKERLAMMTPQNARIWYISPKEPHNKTAYFVDAPYQVDKISAQTFADWQKKAADIALSLPELN  
 PYIPDDFSLIKSEKKYDHPELIVDESNLRVVYAPSR YFASEPKADVSLILRNPKAMDSARNQVMFALNDYLA  
 GLALDQLSNQASVGGISFSTNANNGLMVNANGYTQRLPQLFQALLEGYFSYTATEDQLEQAKSWYNQMMDSA  
 EKGKA FEQAIMPAQMLSQVPYFSRDERRKILPSITLKEVLAYRDALKSGARPEFMVIGNMTEAQATTLARDV  
 QKQLGADGSEWCRNKDVVVDKKQSVIFEKAGNSTDSALAAVFVPTGYDEYTSSAYS SLLGQIVQPWFYNQLR  
 TEEQLGYAVFAFPMSVGRQWGMGFLQSN DKQPSFLWERYKAFFPTAEAKLRAMKPDEFAQIQQAVITQMLQ  
 APQTLGEEASKLSKDFDRGNMRFDSRDKIVAQIKLLTPQKLADFFHQAVVEPQGMAILSQISGSQNGKAEYV  
 HPEGWKVWENV SALQQTMPLMSEKNE\*

**FIGURE 8D**

SEQ ID NO:6 is the DNA sequence of a mutated knockout Protease III gene.

```
ATTCCTCCCGCAGCACCTGGTTCAAAGCATTATTGTTGTTAGTTGCCCTTTGGGCACATTAATGTCAGGCAGAA
ACGGGATGGCAGCCGATTACAGGAAACCATCCGTAAAAGTGATAAAGATAACCGCCAGTATCAGGCTATACGT
CTGGATAACGGTATGGTGGTCTTGCTGGTTTCTGATCCGCAGGCAGTTAAATCGCTCTCGGCGCTGGTGGTG
CCCCTTGGGTCGCTGGAAGATCCCGAGGCGTACCAGGGGCTGGCACATTACCTTGAACATATGAGTCTGATG
GGGTCGAAAAAGTACCCGCAGGCTGACAGTCTGGCCGAATATCTCAAATGCACGGCGGTAGTCACAATGCC
AGCACTGCGCCGTATCGCACGGCTTTCTATCTGGAAGTTGAGAACGACGCCTTGCTGGTGGGTAGACCGC
CTGGCCGATGCTATTGCTGAACCTTTGCTCGACAAGAAATATGCCGAACGTGAGCGTAATGCGGTGAACGCT
GAATTAACCATGGCGCGTACGCGTGACGGGATGCGCATGGCACAGGTCAGCGCAGAAACCATTAACCCGGCA
CACCCCGGTTCAAAGTTTTCTGGTGGTAACCTCGAAACTTTAAGCGACAAACCTGGTAATCCGGTGCAGCAG
GCGCTGAAAAGATTTCCACGAGAAGTACTATTCCGCCAATTTGATGAAGGCGGTTATTTACAGTAATAAACCG
CTGCCGGAGTTGGCAAAAAATGGCGGCGGACACCTTTGGTCTGCGTGCCGAACAAAGAGAGCAAAAAACCGGAA
ATCACCGTGCCGGTAGTCACCGACGCGCAAAAGGGCATTATCATTACGTCCCTGCGCTGCCGCGTAAA
GTGTTGCGCGTTGAGTTTCGCATCGATAACAACCTCAGCGAAGTTCCGTAGTAAAACCGATGAATTGATTACC
TATCTGATTGGCAATCGCAGCCCAGGTACACTTTCTGACTGGCTGCAAAAGCAGGGATTAGTTGAGGGCATT
AGCGCCAACCTCCGATCCTATCGTCAACGGCAACAGCGGCGTATTAGCGATCTCTGCGTCTTTAACCGATAAA
GGCCTGGCTAATCGCGATCAGGTTGTGGCGGCAATTTTTAGCTATCTCAATCTGTTACGTGAAAAAGGCATT
GATAACAATACTTCGATGAACTGGCGAATGTGCTGGATATCGACTTCCGTTATCCGTCGATCACCCGTGAT
ATGGATTACGTCAATGGCTGGCAGATACCATGATTCGCGTTCTGTTGAGCATAACGCTGGATGCAGTCAAT
ATTGCCGATCGGTACGATGCTAAAGCAGTAAAGGAACGTCTGGCGATGATGACGCCGCAGAATGCGCGTATC
TGGTATATCAGCCCGAAAGAGCCGCACAAAACCGGCTTACTTTGTTCGATGCGCCGTATCAGGTCGATAAA
ATCAGCGCACAACTTTTCGCCGACTGGCAGAAAAAGCCGCCGACATTGCGCTCTCTTTGCCAGAGCTTAAC
CCTTATATTCCTGATGATTTCTCGCTGATTAAAGTCAGAGAAGAAATACGACCATCCAGAGCTGATTGTTGAT
GAGTCGAATCTGCGCGTGGTGTATGCGCCAAGCCGTTATTTTGCCAGCGAGCCCAAAGCTGATGTCAGCCTG
ATTTTGCGTAATCCGAAAGCCATGGACAGCGCCCGCAATCAGGTGATGTTTGCGCTCAATGATTATCTCGCA
GGGCTGGCGCTTGATCAGTTAAGCAACCAGGCGTCGTTGGTGGCATAAGTTTTTCCACCAACGCTAACAAAC
GGCCTTATGGTTAATGCTAATGGTTACACCCAGCGTCTGCCGCAGCTGTTCCAGGCATTGCTCGAGGGGTAC
TTTAGCTATACCGCTACGGAAGATCAGCTTGAGCAGGCGAAGTCTGGTATAACCAGATGATGGATTCCGCA
GAAAAGGGTAAAGCGTTTGAGCAGGCGATTATGCCCAGCGCAGATGCTCTCGCAAGTGCCGTACTTCTCGCGA
GATGAACGGCGTAAATTTTGCCCTCCATTACGTTGAAAGAGGTGCTGGCCTATCGCGACGCCTTAAATCA
GGGGCTCGACCAGAGTTTATGGTTATCGGCAACATGACCGAGGCCAGGCAACAACGCTGGCACGCGATGTG
CAAAAACAGTTGGGCGCTGATGGTTCAGAGTGGTGTGAAACAAAGATGTAGTGGTCGATAAAAAACAATCC
GTCATCTTTGAAAAAGCCGTTAACAGCACCGACTCCGCACTGGCAGCGGTATTTGTACCGACTGGCTACGAT
GAATACACCAGCTCAGCCTATAGCTCTCTGTTGGGGCAGATCGTACAGCCGTGGTTCTACAATCAGTTGCGT
ACCGAAGAACAATTGGGCTATGCCGTGTTTGCGTTTCCAATGAGCGTGGGGCGTCAGTGGGGCATGGGCTTC
CTTTTGCAAAGCAATGATAAACAGCCTTCATTCTTGTTGGGAGCGTTACAAGGCGTTTTTCCCAACCGCAGAG
GCAAAATTGCGAGCGATGAAGCCAGATGAGTTTGCGCAAATCCAGCAGGCGGTAATTACCCAGATGCTGCAG
GCACCGCAAACGCTCGGCGAAGAAGCATCGAAGTTAAGTAAAGATTTTCGATCGCGGCAATATGCGCTTCGAT
TCGCGTGATAAAATCGTGGCCAGATAAACTGCTGACGCCGCAAAAACCTTGCTGATTTCTTCCATCAGGCG
GTGGTCGAGCCGCAAGGCATGGCTATTCTGTGCGCAGATTTCCGGCAGCCAGAACGGGAAAGCCGAATATGTA
CACCTGAAGGCTGGAAAGTGTGGGAGAACGTCAGCGCGTTGCAGCAAACAATGCCCTGATGAGTGAAAAG
AATGAGTGA
```

**FIGURE 8E**

SEQ ID NO:7 is the DNA sequence of the wild-type DegP gene.

ATGAAAAAACCACATTAGCACTGAGTGCCTGGCTCTGAGTTTGGCGTTATCTCCGCTCTCTGCA  
ACGGCGGCTGAGACTTCTTCAGCAACGACAGCCCAGCAGATGCCAAGCCTTGACCGATGCTCGAAAAGGTG  
ATGCCTTCAGTGGTCAGCATTAACTAGAAAGGTAGCACAACCGTTAATACGCCGCGTATGCCGCGTAATTTT  
CAGCAGTTCTTCGGTGATGATTCTCCGTTCTGCCAGGAAGGTTCTCCGTTCCAGAGCTCTCCGTTCTGCCAG  
GGTGGCCAGGGCGGTAATGGTGGCGGCCAGCAACAGAAATTCATGGCGCTGGGTTCCGGCGTCATCATTGAT  
GCCGATAAAGGCTATGTCGTCACCAACAACACGTTGTTGATAACGCGACGGTCATTAAAGTTCAACTGAGC  
GATGGCCGTAAGTTTCGACGCGAAGATGGTTGGCAAAGATCCGCGCTCTGATATCGCGCTGATCCAAATCCAG  
AACCCGAAAAACCTGACCGCAATTAAGATGGCGGATTCTGATGCACTGCGCGTGGGTGATTACACCGTAGCG  
ATTGGTAACCCGTTTGGTCTGGGCGAGACGGTAACCTCCGGGATTGTCTCTGCGCTGGGGCGTAGCGGCCTG  
AATGCCGAAAACTACGAAAACTTCATCCAGACCGATGCAGCGATCAACCGTGGTAACTCCGGTGGTGCCTG  
GTTAACCTGAACGGCGAACTGATCGGTATCAACACCGCGATCCTCGACCGGACGGCGGCAACATCGGTATC  
GGTTTTGCTATCCCGAGTAACATGGTGAAAAACCTGACCTCGCAGATGGTGGAATACGGCCAGGTGAAACGC  
GGTGAGCTGGGTATTATGGGGACTGAGCTGAACTCCGAACTGGCGAAAGCGATGAAAGTTGACGCCCAGCGC  
GGTGCTTTCGTAAGCCAGGTTCTGCCTAATTCCTCCGCTGCAAAAGCGGGCATTAAAGCGGGTGATGTGATC  
ACCTCACTGAACGGTAAGCCGATCAGCAGCTTTGCCGCACTGCGTGCTCAGGTGGGTACTATGCCGGTAGGC  
AGCAAACTGACCCTGGGCTTACTGCGCGACGGTAAGCAGGTTAACGTGAACCTGGAACCTGCAGCAGAGCAGC  
CAGAATCAGGTTGATTCCAGCTCCATCTTCAACGGCATTGAAGGCGCTGAGATGAGCAACAAAGGCAAAGAT  
CAGGGCGTGGTAGTGAACAACGTGAAAACGGGCACTCCGGCTGCGCAGATCGGCCTGAAGAAAGGTGATGTG  
ATTATTGGCGCGAACCAGCAGGCAGTGAAAAACATCGCTGAACTGCGTAAAGTTCTCGACAGCAAACCGTCT  
GTGCTGGCACTCAACATTCAGCGCGGCGACAGCACCATCTACCTGTTAATGCAGTAA

SEQ ID NO:8 is the amino acid sequence of the wild-type DegP protein.

MKKTTLALSALSLGLALSPLSATAAETSSATTAQQMPSLAPMLEKVMPSVVSINVEGSTTVNTPRMPRNF  
QQFFGDDSPFCQEGSPFQSSPFCQGGQGGNGGQQQKFMALGSGVIIDADKGYVVTNNHVVDNATVIKVLQS  
DGRKFDKMGKDPKSDIALIQIQNPKNLTAIKMADSDALRVGDYTVAGNPFGLGETVTSIGIVSALGRSGL  
NAENYENFIQTDAAINRGNSSGALVNLNGELIGINTAILAPDGGNIGIGFAIPSNMVKNLTSQMVEYGVQKR  
GELGIMGTELNSELAKAMKVDAQRGAFFVSQVLPNSSAAKAGIKAGDVITSLNGKPISSFAALRAQVGTMPVG  
SKLTLGLLRDQKQVNVNLELQQSSQNQVDSSSIENGIEGAEMSNGKDKQGVVVNNVKTGTPAAQIGLKKGDV  
IIGANQQAVKNIAELRKVLDSKPSVLALNIQRGDSTIYLLMQ

SEQ ID NO:9 is the DNA sequence of a mutated DegP gene.

ATGAAAAAACCACATTAGCACTGAGTGCCTGGCTCTGAGTTTGGCGTTATCTCCGCTCTCTGCA  
ACGGCGGCTGAGACTTCTTCAGCAACGACAGCCCAGCAGATGCCAAGCCTTGACCGATGCTCGAAAAGGTG  
ATGCCTTCAGTGGTCAGCATTAACTAGAAAGGTAGCACAACCGTTAATACGCCGCGTATGCCGCGTAATTTT  
CAGCAGTTCTTCGGTGATGATTCTCCGTTCTGCCAGGAAGGTTCTCCGTTCCAGAGCTCTCCGTTCTGCCAG  
GGTGGCCAGGGCGGTAATGGTGGCGGCCAGCAACAGAAATTCATGGCGCTGGGTTCCGGCGTCATCATTGAT  
GCCGATAAAGGCTATGTCGTCACCAACAACACGTTGTTGATAACGCGACGGTCATTAAAGTTCAACTGAGC  
GATGGCCGTAAGTTTCGACGCGAAGATGGTTGGCAAAGATCCGCGCTCTGATATCGCGCTGATCCAAATCCAG  
AACCCGAAAAACCTGACCGCAATTAAGATGGCGGATTCTGATGCACTGCGCGTGGGTGATTACACCGTAGCG  
ATTGGTAACCCGTTTGGTCTGGGCGAGACGGTAACCTCCGGGATTGTCTCTGCGCTGGGGCGTAGCGGCCTG  
AATGCCGAAAACTACGAAAACTTCATCCAGACCGATGCAGCGATTAATCGTGGTAAACGCCGGTGGTGCCTG  
GTTAACCTGAACGGCGAACTGATCGGTATCAACACCGCGATCCTCGACCGGACGGCGGCAACATCGGTATC  
GGTTTTGCTATCCCGAGTAACATGGTGAAAAACCTGACCTCGCAGATGGTGGAATACGGCCAGGTGAAACGC  
GGTGAGCTGGGTATTATGGGGACTGAGCTGAACTCCGAACTGGCGAAAGCGATGAAAGTTGACGCCCAGCGC  
GGTGCTTTCGTAAGCCAGGTTCTGCCTAATTCCTCCGCTGCAAAAGCGGGCATTAAAGCGGGTGATGTGATC  
ACCTCACTGAACGGTAAGCCGATCAGCAGCTTTGCCGCACTGCGTGCTCAGGTGGGTACTATGCCGGTAGGC  
AGCAAACTGACCCTGGGCTTACTGCGCGACGGTAAGCAGGTTAACGTGAACCTGGAACCTGCAGCAGAGCAGC  
CAGAATCAGGTTGATTCCAGCTCCATCTTCAACGGCATTGAAGGCGCTGAGATGAGCAACAAAGGCAAAGAT  
CAGGGCGTGGTAGTGAACAACGTGAAAACGGGCACTCCGGCTGCGCAGATCGGCCTGAAGAAAGGTGATGTG  
ATTATTGGCGCGAACCAGCAGGCAGTGAAAAACATCGCTGAACTGCGTAAAGTTCTCGACAGCAAACCGTCT  
GTGCTGGCACTCAACATTCAGCGCGGCGACAGCACCATCTACCTGTTAATGCAGTAA

**FIGURE 8F**

SEQ ID NO:10 is the amino acid sequence of a mutated DegP protein.

MKKTTLALSALALSLGLALSPLSATAAETSSATTAQQMPSLAPMLEKVMPSVVSINVEGSTTVNTPRMPRNF  
 QQFFGDDSPFCQEGSPFQSSPFCQGGQGGNGGGQQQKFMALGSGVIIADADKGYVVTNNHVVDNATVIKVQLS  
 DGRKFDAKMVGKDPRSDIALIQIQNPKNLTAIKMADSDALRVGDYTVAGNPFGLGETVTSGIVSALGRSGL  
 NAENYENFIQTDAAINRGNAGGALVNLNGELIGINTAILAPDGGNIGIGFAIPSNMVKNLTSQMVEYGQVVKR  
 GELGIMGTELNSELAKAMKVDAQRGAFVSQVLPNSSAAKAGIKAGDVITSLNGKPISSFAALRAQVGTMFVG  
 SKLTLGLLRDQKQVNVNLELQQSSQNQVDSSSIFNGIEGAEMSNGKGDQGVVVNNVKTGTPAAQIGLKKGDV  
 IIGANQQAVKNIAELRKVLDSKPSVLALNIQRGDSTIYLLMQ

SEQ ID NO: 11 is the sequence of the 5' oligonucleotide primer for the region of the mutated DegP gene comprising the Ase I restriction site.

CTGCCTGCGATTTTCGCCGGAACG

SEQ ID NO: 12 is the sequence of the 3' oligonucleotide primer for the region of the mutated DegP gene comprising the Ase I restriction site.

CGCATGGTACGTGCCACGATATCC

SEQ ID NO: 13 is the sequence of the 5' oligonucleotide primer for the region of the mutated Tsp gene comprising the Ase I restriction site.

GGGAAATGAACCTGAGCAAAACGC

SEQ ID NO: 14 is the sequence of the 3' oligonucleotide primer for the region of the mutated Protease III gene comprising the Ase I restriction site.

GGGAAAGGCGGCGGAACCGCCTAG

SEQ ID NO: 15 is the sequence of the 5' oligonucleotide primer for the region of the mutated Protease III gene comprising the Ase I restriction site.

CTACTGTGCCAGCGGTGGTAATGG

SEQ ID NO: 16 is the sequence of the 3' oligonucleotide primer for the region of the mutated Tsp gene comprising the Ase I restriction site.

GCATCATAATTTTCTTTTACCTC

SEQ ID NO: 17 is the DNA sequence of the wild-type spr gene.

ATGGTCAAATCTCAACCGATTTTGAGATATATCTTGCGCGGGATTCCCGCGATTGCAGTAGCGGTTCTGCTT  
 TCTGCATGTAGTGCAAATAACACCGCAAAGAATATGCATCCTGAGACACGTGCAGTGGGTAGTGAAACATCA  
 TCACTGCAAGCTTCTCAGGATGAATTTGAAAACCTGGTTTCGTAATGTCGACGTAAAATCGCGAATTATGGAT  
 CAGTATGCTGACTGGAAAGGCGTACGTTATCGTCTGGGCGGCAGCACTAAAAAGGTATCGATTGTTCTGGT  
 TTCGTACAGCGTACATTCCGTGAGCAATTTGGCTTAGAACTTCCGCGTTCGACTTACGAACAGCAGGAAATG  
 GGTAATCTGTTTCCCGCAGTAATTTGCGTACGGGTGATTTAGTTCTGTTCCGTGCCGGTTCAACGGGACGC  
 CATGTCGGTATTTATATCGGCAACAATCAGTTTGTCCATGCTTCCACCAGCAGTGGTGTTATTATTTCCAGC  
 ATGAATGAACCGTACTGGAAGAAGCGTTACAACGAAGCACGCCGGTTCTCAGCCGCAGC

**FIGURE 8G**

SEQ ID NO: 18 is the amino acid sequence of the wild-type spr gene including the signal sequence which is the first 26 amino acid residues.

MVKSQPIILRYILRGIPAIHAVVLLSACSANNTAKNMHPETRAVGSETSSLQASQDEFENLVRNVDVKSIRIMD  
QYADWKGVRYRLGGSTKKGIDCSGFVQRTFREQFGLLELPSTYEQQEMGKSVSRNLRTGDLVLFragSTGR  
HVGIIYIGNNQFVHASTSSGVIISSMNEPYWKKRYNEARRVLSRS

SEQ ID NO: 19 the non-mutated spr gene without the signal sequence.

CSANNTAKNMHPETRAVGSETSSLQASQDEFENLVRNVDVKSIRIMDQYADWKGVRYRLGGSTKKGIDCSGFV  
QRTFREQFGLLELPSTYEQQEMGKSVSRNLRTGDLVLFragSTGRHVGIIYIGNNQFVHASTSSGVIISSMN  
EPYWKKRYNEARRVLSRS

SEQ ID NO: 20 a mutated OmpT sequence comprising D210A and H212A mutations.

ATGCGGGCGAACTTCTGGGAATAGTCCTGACAACCCCTATTGCGATCAGCTCTTTTGCTTCTACCGAGACT  
TTATCGTTTACTCCTGACAACATAAATGCGGACATTAGTCTTGGAACCTCTGAGCGGAAAAACAAAAGAGCGT  
GTTTATCTAGCCGAAGAAGGAGGCCGAAAAGTCAGTCAACTCGACTGGAAATTCAATAACGCTGCAATTATT  
AAAGGTGCAATTAATTGGGATTTGATGCCCCAGATATCTATCGGGGCTGCTGGCTGGACAACCTCTCGGCAGC  
CGAGGTGGCAATATGGTCGATCAGGACTGGATGGATTCCAGTAACCCCGGAACCTGGACGGATGAAAGTAGA  
CACCCTGATACACAACCTCAATTATGCCAACGAATTTGATCTGAATATCAAAGGCTGGCTCCTCAACGAACCC  
AATTACCGCTGGGACTCATGGCCGGATATCAGGAAAGCCGTTATAGCTTTACAGCCAGAGGTGGTTCCTAT  
ATCTACAGTTCTGAGGAGGGATTTCAGAGATGATATCGGCTCCTTCCCGAATGGAGAAAGAGCAATCGGCTAC  
AAACAACGTTTTTAAATGCCCTACATTGGCTTGACTGGAAGTTATCGTTATGAAGATTTTGAACCTCGGTGGC  
ACATTTAAATACAGCGGCTGGGTGGAATCATCTGATAACGCTGAAGCTTATGACCCGGGAAAAAGAATCACT  
TATCGCAGTAAGGTCAAAGACCAAAATTACTATTCTGTTGCAGTCAATGCAGGTATTACGTACACCTAAC  
GCAAAAGTTTATGTTGAAGGCGCATGGAATCGGGTTACGAATAAAAAAGGTAATACTTCACTTTATGATCAC  
AATAATAACACTTCAGACTACAGCAAAAATGGAGCAGGTATAGAAACTATAACTTCATCACTACTGCTGGT  
CTTAAGTACACATTTTAA

SEQ ID NO: 21 a mutated OmpT sequence comprising D210A and H212A mutations.

MRAKLLGIVLTTPIAISSFASTETLSFTPDNINADISLGLSGKTKERVYLAEEGGRKVSQLDWKFNNAAII  
KGAINWDLMPQISIGAAGWTTLGSRGGMVDQDWMDSNPGTWTDSESRHPTQLNYANEFDLNKGWLLNEP  
NYRLGLMAGYQESRYSFTARGGSYIYSSEEGFRDDIGSFNGERAIGYKQRFKMPYIGLTGSYRYEDFELGG  
TFKYSGWVSSDNAEAYDPGKRITYRSKVKDQNYYSVAVNAGYYVTPNAKVYVEGAWNVRVTNKKGNTSLYDH  
NNNTSDYSKNGAGIENYNFITTAGLKYTF

SEQ ID NO: 22 is the nucleotide sequence of a mutated knockout OmpT sequence.

ATTCGGGGCGAACTTCTGGGAATAGTCCTGACAACCCCTATTGCGATCAGCTCTTTTGCTTCTACCGAGACT  
TTATCGTTTACTCCTGACAACATAAATGCGGACATTAGTCTTGGAACCTCTGAGCGGAAAAACAAAAGAGCGT  
GTTTATCTAGCCGAAGAAGGAGGCCGAAAAGTCAGTCAACTCGACTGGAAATTCAATAACGCTGCAATTATT  
AAAGGTGCAATTAATTGGGATTTGATGCCCCAGATATCTATCGGGGCTGCTGGCTGGACAACCTCTCGGCAGC  
CGAGGTGGCAATATGGTCGATCAGGACTGGATGGATTCCAGTAACCCCGGAACCTGGACGGATGAAAGTAGA  
CACCCTGATACACAACCTCAATTATGCCAACGAATTTGATCTGAATATCAAAGGCTGGCTCCTCAACGAACCC  
AATTACCGCTGGGACTCATGGCCGGATATCAGGAAAGCCGTTATAGCTTTACAGCCAGAGGTGGTTCCTAT  
ATCTACAGTTCTGAGGAGGGATTTCAGAGATGATATCGGCTCCTTCCCGAATGGAGAAAGAGCAATCGGCTAC  
AAACAACGTTTTTAAATGCCCTACATTGGCTTGACTGGAAGTTATCGTTATGAAGATTTTGAACCTCGGTGGC  
ACATTTAAATACAGCGGCTGGGTGGAATCATCTGATAACGATGAACACTATGACCCGGGAAAAAGAATCACT  
TATCGCAGTAAGGTCAAAGACCAAAATTACTATTCTGTTGCAGTCAATGCAGGTATTACGTACACCTAAC  
GCAAAAGTTTATGTTGAAGGCGCATGGAATCGGGTTACGAATAAAAAAGGTAATACTTCACTTTATGATCAC  
AATAATAACACTTCAGACTACAGCAAAAATGGAGCAGGTATAGAAACTATAACTTCATCACTACTGCTGGT  
CTTAAGTACACATTTTAA

**FIGURE 8H**

SEQ ID NO: 23 shows the sequence of the OmpA oligonucleotide adapter.

CGATTGAATGGAGAACTTGAATTCGGGCGAACTTCTGGGAATAG

SEQ ID NO: 24 shows a cassette encoding intergenic sequence 1 (IGS1) for E. coli Fab expression.

AAGTTTTAATAGAGGAGAGTGTTAATGAAGAAG

SEQ ID NO: 25 shows the oligonucleotide cassette encoding intergenic sequence 2 (IGS2) for E. coli Fab expression.

AAGTTTTAATAGAGGGGAGTGTTAAATGAAGAAG

SEQ ID NO: 26 shows a cassette encoding intergenic sequence 3 (IGS3) for E. coli Fab expression.

AAGCTTTAATAGAGGAGAGTGTTGAGGAGGAAAAAATGAAGAAA

SEQ ID NO: 27 shows a cassette encoding intergenic sequence 4 (IGS4) for E. coli Fab expression.

AAGCTTTAATAGAGGAGAGTGTTGACGAGGATTATATAATGAAGAAA

SEQ ID NO: 28 is the DNA sequence of the wild-type FkpA gene.

ATGAAATCACTGTTTAAAGTAACGCTGCTGGCGACCACAATGGCCGTTGCCCTGCATGCACCAATCACTTTT  
GCTGCTGAAGCTGCAAAACCTGCTACAGCTGCTGACAGCAAAGCAGCGTTCAAAAATGACGATCAGAAATCA  
GCTTATGCACTGGGTGCCTCGCTGGGTGCTTACATGGAAAACCTCTCTAAAAGAACAAGAAAACTGGGCATC  
AAACTGGATAAAGATCAGCTGATCGCTGGTGTTCAGGATGCATTTGCTGATAAGAGCAAACCTCTCCGACCAA  
GAGATCGAACAGACTCTACAAGCATTGCAAGCTCGCGTGAAGTCTTCTGCTCAGGCGAAGATGGAAAAAGAC  
GCGGCTGATAACGAAGCAAAAGGTAAAGAGTACCGCGAGAAATTTGCCAAAGAGAAAGGTGTGAAAACCTCT  
TCAACTGGTCTGGTTTATCAGGTAGTAGAAGCCGGTAAAGGCGAAGCACCAGAAAGACAGCGATACTGTTGTA  
GTGAACTACAAAGGTACGCTGATCGACGGTAAAGAGTTCGACAACTCTTACACCCGTGGTGAACCGCTTTCT  
TTCCGTCTGGACGGTGTATCCCGGGTTGGACAGAAGGTCTGAAGAACATCAAGAAAGGCGGTAAGATCAAA  
CTGGTTATTCCACCAGAACTGGCTTACGGCAAAGCGGGTGTTCGGGGATCCACCGAATTCTACCCTGGTG  
TTTGACGTAGAGCTGCTGGATGTGAAACCAGCGCCGAAGGCTGATGCAAAGCCGGAAGCTGATGCGAAAGCC  
GCAGATTCTGCTAAAAAA

SEQ ID NO: 29 is the protein sequence of the wild-type FkpA gene.

MKSLFKVTL LATTMAVALHAPITFAAEAAKPATAADSKAAFKNDDQKSAYALGASLGRYMENSLKEQEKLGI  
KLDKDQLIAGVQDAFADKSKLSQEI EQTLQAFEARKVSSAQAKMEKDAADNEAKGKEYREKFAKEKGVKTS  
STGLVYQVVEAGKEAPKDSDTV VVNYKGT LIDGKEFDNSYTRGEPLSFRLDGVI PGWTEGLKNIKKGGKIK  
LVIPPELAYGKAGVPGIPPNSTLVFDVELLDVKPAPKADAKPEADAKAADS AKK

SEQ ID NO: 30 is the DNA sequence of the FkpA his tagged gene.

ATGAAATCACTGTTTAAAGTAACGCTGCTGGCGACCACAATGGCCGTTGCCCTGCACGCACCAATCACTTTT  
GCTGCTGAAGCTGCAAAACCTGCTACTGCTGCTGACAGCAAAGCAGCGTTCAAAAATGACGATCAGAAATCA  
GCTTATGCACTGGGTGCCTCGCTGGGTGCTTACATGGAAAACCTCTCTAAAAGAACAAGAAAACTGGGCATC  
AAACTGGATAAAGATCAACTGATCGCTGGTGTTCAGGATGCATTTGCTGATAAGAGCAAACCTCTCCGACCAA  
GAGATCGAACAGACTCTACAAGCATTGCAAGCTCGCGTGAAGTCTTCTGCTCAGGCGAAGATGGAAAAAGAC  
GCGGCTGATAACGAAGCAAAAGGTAAAGAGTACCGCGAGAAATTTGCCAAAGAGAAAGGTGTGAAAACCTCT  
TCAACTGGTCTGGTTTATCAGGTAGTAGAAGCCGGTAAAGGCGAAGCACCAGAAAGACAGCGATACTGTTGTA  
GTGAACTACAAAGGTACGCTGATCGACGGTAAAGAGTTCGACAACTCTTACACCCGTGGTGAACCGCTTTCT  
TTCCGTCTGGACGGTGTATCCCGGGTTGGACAGAAGGTCTGAAGAACATCAAGAAAGGCGGTAAGATAAAA  
CTGGTTATTCCACCAGAACTGGCTTACGGCAAAGCGGGTGTTCGGGGATCCACCAAATTCTACCCTGGTG  
TTTGACGTAGAGCTGCTGGATGTGAAACCAGCGCCGAAGGCTGATGCAAAGCCGGAAGCTGATGCGAAAGCC  
GCAGATTCTGCTAAAAAACCATCACCATCACCAC

**FIGURE 8I**

SEQ ID NO: 31 is the protein sequence of the FkpA his tagged gene.

MKSLFKVTLTLLATTMAVALHAPITFAAEAAKPATAADSKAAFKNDQKSAYALGASLGRYMENSLKEQEKLGI  
KLDKDKQLIAGVQDAFADKSKLSDQEIEQTLQAFEARVKSSAQAKMEKDAADNEAKGKEYREKFAKEKGVKTS  
STGLVYQVVEAGKGEAPKDSDTVVVNYKGTLDGKEFDNSYTRGEPLSFRLDGVIPGWTEGLKNIKKGGKIK  
LVIPPELAYGKAGVPGIPPNSTLVFDELVDVKPAPKADAKPEADAKAADSAAKHHHHHH

SEQ ID NO: 32 is the DNA sequence of the wild-type skp gene.

GTGAAAAAGTGGTTATTAGCTGCAGGTCTCGGTTTAGCACTGGCAACTTCTGCTCAGGCGGCTGACAAAATT  
GCAATCGTCAACATGGGCAGCCTGTTCCAGCAGGTAGCGCAGAAAACCGGTGTTTCTAACACGCTGGAAAAT  
GAGTTCAAAGGCCGTGCCAGCGAACTGCAGCGTATGGAACCGATCTGCAGGCTAAAATGAAAAAGCTGCAG  
TCCATGAAAAGCGGGCAGCGATCGCACTAAGCTGGAAAAAGACGTGATGGCTCAGCGCCAGACTTTTGCTCAG  
AAAGCGCAGGCTTTTGAGCAGGATCGCGCACGTCGTTCCAACGAAGAACGCGGCAAACCTGGTTACTCGTATC  
CAGACTGCTGTGAAATCCGTTGCCAACAGCCAGGATATCGATCTGGTTGTTGATGCAAACGCCGTTGCTTAC  
AACAGCAGCGATGTAAAAGACATCACTGCCGACGTACTGAAACAGGTAAATAA

SEQ ID NO: 33 is the protein sequence of the wild-type skp gene.

MKKWLLAAGLGLALATSAQAADKIAIVNMGSLFQQVAQKTGVSNTLENEFKGRASELQRMETDLQAKMKKLQ  
SMKAGSDRTKLEKDVMAQRQTFAQKAQAFEQDRARRSNEERGKLVTRIQTAVKSVANSQDIDLVDANAVAY  
NSSDVKDITADVLKQVK

SEQ ID NO: 34 is the DNA sequence of the skp his tagged gene.

ATGAAAAAGTGGTTATTAGCCGCAGGTCTCGGTTTAGCACTGGCAACTTCTGCTCAGGCGGCTGACAAAATT  
GCAATCGTCAACATGGGCAGCCTGTTCCAGCAGGTAGCGCAGAAAACCGGTGTTTCTAACACGCTGGAAAAT  
GAGTTCAAAGGCCGTGCCAGCGAACTCCAGCGTATGGAACCGATCTCCAGGCTAAAATGAAAAAGCTGCAA  
TCCATGAAAAGCGGGCAGCGATCGCACTAAGCTGGAAAAAGACGTGATGGCTCAGCGCCAGACTTTTGCTCAG  
AAAGCGCAGGCTTTTGAGCAGGATCGCGCACGTCGTTCCAACGAAGAACGCGGCAAACCTGGTTACTCGTATC  
CAGACTGCTGTGAAATCCGTTGCCAACAGCCAGGAAATCGATCTGGTTGTTGATGCAAACGCCGTTGCTTAC  
AACAGCAGCGATGTAAAAGACATCACTGCCGACGTACTGAAACAGGTAAACACCATCACCATCACCAC

SEQ ID NO: 35 is the protein sequence of the skp his tagged gene.

MKKWLLAAGLGLALATSAQAADKIAIVNMGSLFQQVAQKTGVSNTLENEFKGRASELQRMETDLQAKMKKLQ  
SMKAGSDRTKLEKDVMAQRQTFAQKAQAFEQDRARRSNEERGKLVTRIQTAVKSVANSQEIDLVDANAVAY  
NSSDVKDITADVLKQVKHHHHHH

**CA170\_1519 Ab sequences****CDRH1**

GFTFSNYGMV

SEQ ID NO: 36

**CDRH2**

YIDSDGDNTYYRDSVKG

SEQ ID NO: 37

**CDRH3**

GIVRPFLY

SEQ ID NO: 38

**CDRL1**

KSSQSLVGASGKTYLY

SEQ ID NO: 39

**CDRL2**

LVSTLDS

SEQ ID NO: 40

**CDRL3**

LQGTHFPHT

SEQ ID NO: 41

**FIGURE 8J****Rat Ab 1519 VL region** SEQ ID NO: 42

DVVMQTPLS LSVALGQPAS ISCKSSQSLV GASGKTYLYW LFQRSGQSPK RLIYLVSTLD  
SGIPDRFSGS GAETDFTLKI RRVEADDLGV YYCLQGTHFP HTFGAGTKLE LK

**Rat Ab 1519 VL region** SEQ ID NO: 43

gatgttgtga tgaccagac tccactgtct ttgtcgggtg cccttggaca accagcctcc  
atctcttgca agtcaagtca gagcctcgta ggtgctagt gaaagacata tttgtattgg  
ttatttcaga ggtccggcca gtctccaaag cgactaatct atctggtgtc cacactggac  
tctggaattc ctgataggtt cagtggcagt ggagcagaga cagattttac tcttaaaatc  
cgcagagtgg aagccgatga tttgggagtt tattactgct tgcaaggtag acattttcct  
cacacgtttg gagctgggac caagctggaa ttgaaa

**Rat Ab 1519 VL region with signal sequence underlined and italicised** SEQ ID NO: 44

*MMSPAQFLFLMLW**IQGTSG*DVVMQTPLSLSVALGQPASISCKSSQSLVGASGKTYLYWLFQRSGQSPK  
RLIYLVSTLD SGIPDRFSGSGAETDFTLKIRRVEADDLGVYYCLQGTHFPHTFGAGTKLELK

**Rat Ab 1519 VL region with signal sequence underlined and italicised** SEQ ID NO: 45

*atgatgagtc ctgcccagtt cctgtttctg ctgatgctct ggattcaggg aaccagtgg*  
gatgttgtga tgaccagac tccactgtct ttgtcgggtg cccttggaca accagcctcc  
atctcttgca agtcaagtca gagcctcgta ggtgctagt gaaagacata tttgtattgg  
ttatttcaga ggtccggcca gtctccaaag cgactaatct atctggtgtc cacactggac  
tctggaattc ctgataggtt cagtggcagt ggagcagaga cagattttac tcttaaaatc  
cgcagagtgg aagccgatga tttgggagtt tattactgct tgcaaggtag acattttcct  
cacacgtttg gagctgggac caagctggaa ttgaaa

**Rat Ab 1519 VH region** SEQ ID NO: 46

EVPLVESGGG SVQPGRSMKL SCVVS GF TFS NYGMVWVRQA PKKGLEWVAY IDSDGDNTYY  
RDSVKGRFTI SRNNAKSTLY LQMDSLRSED TATYYCTTGI VRPFLYWGQG TTVTVS

**Rat Ab 1519 VH region** SEQ ID NO: 47

gaggtgccgc tgggtggagtc tggggggcgc tcagtgcagc ctgggaggtc catgaaactc  
tctgtgtag tctcaggatt cactttcagt aattatggca tgggtctgggt ccgccaggct  
ccaaagaagg gtctggagtg ggtcgcatat attgattctg atggtgataa tacttactac  
cgagattccg tgaagggccg attcactatc tccagaaata atgcaaaaag caccctatat  
ttgcaaatgg acagtctgag gtctgaggac acggccactt attactgtac aacaggggatt  
gtccggccct ttctctattg gggccaagga accacgggtc cgtctcgc

**Rat Ab 1519 VH region with signal sequence underlined and italicised** SEQ ID NO: 48

*MDISLSLAFL VLF**IKGVRCE* VPLVESGGGS VQPGRSMKLS CVVSGFTFSN YGMVWVRQAP  
KKGLEWVAYI DSDGDNTYYR DSVKGRFTIS RNNAKSTLYL QMDSLRSED TATYYCTTGIV  
RPFLYWGQGT TTVTVS

**Rat Ab 1519 VH region with signal sequence underlined and italicised** SEQ ID NO: 49

*atggacatca gtctcagctt ggcttttcctt gtccttttca taaaagggtgt ccggtgtgag*  
gtgccgctgg tggagtctgg gggcggtcga gtgcagcctg ggaggtccat gaaactctcc  
tgtgtagtct caggattcac ttctcagtaat tatggcatgg tctgggtccg ccaggctcca  
aagaagggtc tggagtgggt cgcataatatt gattctgatg gtgataatac ttactaccga  
gattccgtga agggccgatt cactatctcc agaaataatg caaaaagcac cctatatattg  
caaatggaca gtctgaggtc tgaggacacg gccacttatt actgtacaac agggattgtc  
cggccctttc tctattgggg ccaaggaacc acggtcaccg tctcgc

**FIGURE 8K****1519 gL20 V-region SEQ ID NO: 50**

DIQMTQSPSS LSASVGDRV ITCKSSQSLV GASGKTYLYW LFQKPGKAPK RLIYLVSTLD  
SGIPSRFSGS GSGTEFTLTI SSLQPEDFAT YYCLQGTHFP HTFGQGTKLE IK

**1519 gL20 V-region SEQ ID NO: 51**

gatatccaga tgaccagag tccaagcagt ctctccgcca gcgtaggcga tcgtgtgact  
attacctgta aaagctccca gtccctggtg ggtgcaagcg gcaaaaccta cctgtactgg  
ctcttccaga aaccgggcaa agctccgaaa cgcctgatct atctggtgtc taccctggat  
agcggatttc cgtctcgttt ctccggtagc ggtagcggta ccgaattcac gctgaccatt  
agctccctcc agccggagga ctttgctacc tattactgcc tccagggcac tcattttccg  
cacactttcg gccagggtac caaactggaa atcaaaa

**1519 gL20 V-region with signal sequence underlined and italicized SEQ ID NO: 52**

*MKKTAAIAIAVALAGFATVAQ*ADIQMTQSPSSLSASVGDRVITITCKSSQSLVGASGKTYLYWLFQKPGKAPKR  
LIYLVSTLD SGIPSRFSGSGSGTEFTLTISLQPEDFATYYCLQGTHFPHTFGQGTKLEIK

**1519 gL20 V-region with signal sequence underlined and italicized SEQ ID NO: 53**

*atgaaaaaga cagctatcgc aattgcagtg gccttggtcg gtttcgctac cgtagcgcaa*  
*gct*gatatcc agatgaccca gagtccaagc agtctctccg ccagcgtagg cgatcgtgtg  
actattacct gtaaaagctc ccagtccttg gtgggtgcaa gcggcaaaac ctacctgtac  
tggtctcttc agaaaccggg caaagctccg aaacgcctga tctatctggt gtctaccctg  
gatagcggta ttccgtctcg tttctccggt agcggtagcg gtaccgaatt cacgctgacc  
attagctccc tccagccgga ggactttgct acctattact gcctccaggg cactcatttt  
ccgcacactt tcggccaggg taccaaaactg gaaatcaaa

**1519 gL20 light chain (V + constant) SEQ ID NO: 54**

DIQMTQSPSS LSASVGDRV ITCKSSQSLV GASGKTYLYW LFQKPGKAPK RLIYLVSTLD  
SGIPSRFSGS GSGTEFTLTI SSLQPEDFAT YYCLQGTHFP HTFGQGTKLE IKRTVAAPSV  
FIFPPSDEQL KSGTASVVCL LNNFYPREAK VQWKVDNALQ SGNSQESVTE QDSKDSTYSL  
SSTLTLSKAD YEKHKVYACE VTHQGLSSPV TKSFNREGC

**1519 gL20 light chain (V + constant) SEQ ID NO: 55**

gatatccaga tgaccagag tccaagcagt ctctccgcca gcgtaggcga tcgtgtgact  
attacctgta aaagctccca gtccctggtg ggtgcaagcg gcaaaaccta cctgtactgg  
ctcttccaga aaccgggcaa agctccgaaa cgcctgatct atctggtgtc taccctggat  
agcggatttc cgtctcgttt ctccggtagc ggtagcggta ccgaattcac gctgaccatt  
agctccctcc agccggagga ctttgctacc tattactgcc tccagggcac tcattttccg  
cacactttcg gccagggtac caaactggaa atcaaactgta cggtagcggc cccatctgtc  
ttcatcttcc cgccatctga tgagcagttg aaatctggaa ctgcctctgt tgtgtgcctg  
ctgaataact tctatcccag agaggccaaa gtacagtga aggtggataa cgccctccaa  
tcgggtaact cccaggagag tgtcacagag caggacagca aggacagcac ctacagcctc  
agcagcacc tgacgctgag caaagcagac tacgagaaac acaaagtcta cgcctgcgaa  
gtcaccatc agggcctgag ctcaccagta acaaaaagt ttaatagagg ggagtgt

**1519 gL20 light chain with signal sequence underlined and italicized SEQ ID NO: 56**

*MKKTAAIAIAV ALAGFATVAQ*ADIQMTQSPSLSASVGDRVITITCKSSQSLVGASGKTYLY  
WLFQKPGKAP KRLIYLVSTL DSGIPSRFSGSGSGTEFTLTISLQPEDFA TYYCLQGTHF  
PHTFGQGTKL EIKRTVAAPS VFIFPPSDEQ LKSGTASVVC LLNNFYPREA KVQWKVDNAL  
QSGNSQESVT EQDSKDSTYS LSSTLTLSKA DYEKHKVYAC EVTHQGLSSP VTKSFNRGEC

**FIGURE 8L**

1519 gL20 light chain with signal sequence underlined and italicized SEQ ID NO: 57

atgaaaaaga cagctatcgc aattgcagtg gccttggctg gtttcgctac cgtagcgcaa  
gctgatatcc agatgaccca gagtccaagc agtctctccg ccagcgtagg cgatcgtgtg  
 actattacct gtaaaagctc ccagtccctg gtgggtgcaa gcggcaaaac ctacctgtac  
 tggctcttcc agaaaccggg caaagctccg aaacgcctga tctatctggg gtctaccctg  
 gatagcggta ttccgtctcg tttctccggg agcggtagcg gtaccgaatt cacgctgacc  
 attagctccc tccagccgga ggactttgct acctattact gcctccaggg cactcatttt  
 ccgcacactt tcggccaggg taccaaactg gaaatcaaac gtacggtagc ggccccatct  
 gtcttcatct tcccgccatc tgatgagcag ttgaaatctg gaactgcctc tgttgtgtgc  
 ctgctgaata acttctatcc cagagaggcc aaagtacagt ggaagggtgga taacgccctc  
 caatcgggta actcccagga gagtgtcaca gagcaggaca gcaaggacag cacctacagc  
 ctacgcagca ccctgacgct gagcaaagca gactacgaga aacacaaagt ctacgcctgc  
 gaagtcaccc atcagggcct gagctcacca gtaacaaaaa gttttaatag aggggagtg

1519 gH20 V-region SEQ ID NO: 58

EVPLVESGGG LVQPGGSLRL SCAVSGFTFS NYGMVWVRQA PGKGLEWVAY IDSDGDNTYY  
 RDSVKGRFTI SRDPAKSSLY LQMNSLRAED TAVYYCTTGI VRPFLYWGGQ TLVTVS

1519 gH20 V-region SEQ ID NO: 59

gaggttccgc tggtcgagtc tggaggcggg cttgtccagc ctggaggag cctgcgtctc  
 tcttgtgcag tatctggctt caggttctcc aactacggtg tgggtgtggg tcgtcaggct  
 ccaggtaaag gtctggaatg ggtggcgtat attgactccg acggcgacaa cacctactat  
 cgcgactctg tgaaaggctc cttcaccatt tcccgcgata acgccaatc cagcctgtac  
 ctgcagatga acagcctgcg tgctgaagat actgcggtgt actattgcac cactggcatc  
 gtgcgtccgt ttctgtattg gggtcagggt accctcgta ctgtctcg

1519 gH20 V-region with signal sequence underlined and italicized SEQ ID NO: 60

MKKTATATAV ALAGFATVAQ AEVPLVESGG GLVQPGGSLR LSCAVSGFTF SNYGMVWVRQ  
APGKGLEWVA YIDSDGDNTY YRDSVKGRFT ISRDNKSSL YLQMNSLRAE DTAVYYCTTG  
 IVRPFLYWGG GTLVTVS

1519 gH20 V-region with signal sequence underlined and italicized SEQ ID NO: 61

atgaagaaga ctgctatagc aattgcagtg gcgctagctg gtttcgccac cgtggcgcaa  
gctgagggtc cgctggtcga gtctggaggc gggcttgctc agcctggagg gagcctgcgt  
 ctctcttctg cagtatctgg cttcacgttc tccaactacg gtatggtgtg ggttcgtcag  
 gctccaggta aaggctctgga atgggtggcg tatattgact ccgacggcga caacacctac  
 tatcgcgact ctgtgaaagg tcgcttcacc atttcccgcg ataacgcaa atccagcctg  
 tacctgcaga tgaacagcct gcgtgctgaa gatactgcgg tgtactattg caccactggc  
 atcgtgcgtc cgtttctgta ttgggggtcag ggtaccctcg ttactgtctc g

1519gH20 Fab' heavy chain (V + human gamma-1 CH1 + hinge) SEQ ID NO: 62

EVPLVESGGG LVQPGGSLRL SCAVSGFTFS NYGMVWVRQA PGKGLEWVAY IDSDGDNTYY  
 RDSVKGRFTI SRDPAKSSLY LQMNSLRAED TAVYYCTTGI VRPFLYWGGQ TLVTVSSAST  
 KGPSVFPLAP SSKSTSGGTA ALGCLVKDYF PEPVTVSWNS GALTSGVHTF PAVLQSSGLY  
 SLSSVVTVPV SSLGTQTYIC NVNHKPSNTK VDKKVEPKSC DKHTTCAA

**FIGURE 8M****1519gH20 Fab' heavy chain (V + human gamma-1 CH1 + hinge) SEQ ID NO: 63**

gaggttccgc tggtcgagtc tggaggcggg cttgtccagc ctggagggag cctgcgtctc  
 tcttgtgcag tatctggctt cacgttctcc aactacggta tgggtgtggg tctgcaggct  
 ccaggtaaag gtctggaatg ggtggcgtat attgactccg acggcgacaa cacctactat  
 cgcgactctg tgaaaggctg cttcaccatt tcccgcgata acgccaaatc cagcctgtac  
 ctgcagatga acagcctgcg tgcgaagat actgcggtgt actattgcac cactggcatc  
 gtgcgtccgt ttctgtattg gggtcagggg accctcgtaa ctgtctcgag cgcttctaca  
 aagggcccat cggctcttccc cctggcaccc tcctccaaga gcacctctgg gggcacagcg  
 gccctgggct gcctgggtcaa ggactacttc cccgaaccgg tgacgggtgtc gtggaactca  
 ggcgccctga ccagcgggcg gcacaccttc ccggctgtcc tacagtcctc aggactctac  
 tccctcagca gcgtgggtgac cgtgccctcc agcagcttgg gcaccagac ctacatctgc  
 aacgtgaatc acaagcccag caacaccaag gtcgacaaga aagttgagcc caaatcttgt  
 gacaaaactc acacatgcgc cgcg

**1519gH20 Fab' heavy chain with signal sequence underlined and italicized SEQ ID NO: 64**

*MKKTALIAIAV* *ALAGFATVAQ* *AEVPLVESGG* GLVQPGGSLR LSCAVSGFTF SNYGMVWVRQ APGKGLEWVA  
 YIDSDGNTY YRDSVKGRFT ISRDNAKSSL YLQMNSLRAE DTAVYYCTTG IVRPFLYWQG GTLVTVSSAS  
 TKGPSVFPLA PSSKSTSGGT AALGCLVKDY FPEPVTVSWN SGALTSGVHT FPAVLQSSGL YSLSSVVTVP  
 SSSLGTQTYI CNVNHKPSNT KVDKKVEPKS CDKTHTC

**1519gH20 Fab' heavy chain with signal sequence underlined and italicized SEQ ID NO: 65**

*atgaagaaga* *ctgctatagc* *aattgcagtg* *gcgctagctg* *gtttcgccac* *cgtggcgcaa*  
*gctgaggttc* cgctgggtcga gtctggaggc gggcttgtcc agcctggagg gagcctgcgt  
 ctctcttgtg cagtatctgg cttcacgttc tccaactacg gtatgggtgtg ggttcgtcag  
 gctccaggta aaggctctgga atgggtggcg tatattgact ccgacggcga caacacctac  
 tatcgcgact ctgtgaaagg tcgcttcacc atttcccgcg ataacgcaa atccagcctg  
 tacctgcaga tgaacagcct gcgtgctgaa gatactgcg tgtactattg caccactggc  
 atcgtgcgtc cgtttctgta ttgggggtcag ggtaccctcg ttactgtctc gagcgcttct  
 acaaagggcc catcggtctt ccccttgcca cctcctcca agagcacctc tgggggcaca  
 gcggccctgg gctgcctgggt caaggactac ttccccgaac cggtgacggg gtcgtggaac  
 tcaggcgccc tgaccagcgg cgtgcacacc ttccccggctg tcctacagtc ctcaggactc  
 tactccctca gcagcgtggg gaccgtgcc tccagcagct tgggcacca gacctacatc  
 tgcaacgtga atcacaagcc cagcaacacc aaggctcgaca agaaagttga gccc aaatct  
 tgtgacaaaa ctcacacatg cgccgcg

**1519gH20 IgG4 heavy chain (V + human gamma-4P constant) SEQ ID NO: 66**

EVPLVESGGG LVQPGGSLRL SCAVSGFTFS NYGMVWVRQA PGKGLEWVAY IDSDGDNTRY  
 RDSVKGRFTI SRDNAKSSLY LQMNSLRAED TAVYYCTTGI VRPFLYWQG TLVTVSSAST  
 KGPSVFPLAP CSRSTSESTA ALGCLVKDYF PEPVTVSWNS GALTSGVHTF PAVLQSSGLY  
 SLSSVVTVPSS SSLGTKTYTC NVDHKPSNTK VDKRVESKYG PPCPPCPAPE FLGGPSVFLF  
 PPKPKDTLMI SRTPEVTCVV VDVSQEDPEV QFNWYVDGVE VHNATKPRE EQFNSTYRVV  
 SVLTVLHQDW LNGKEYKCKV SNKGLPSSIE KTISKAKGQP REPQVYTLPP SQEEMTKNQV  
 SLTCLVKGFY PSDIAVEWES NGQPENNYKT TPPVLDSGDS FFLYSRLTVD KSRWQEGNVF  
 SCSVMHEALH NHYTQKSLSL SLGK

**FIGURE 8N**

1519gH20 IgG4 heavy chain (V + human gamma-4P constant, exons underlined) SEQ ID NO: 67

gaggtaccacttgtggaaagcggaggaggtcttgtgcagcctggaggaagtttacgtctctcttgtgctgtgtctggctt  
caccttctccaattacggaatggtctgggtcagacaagcacctggaaaggtcttgaatgggtggcctatatattgactctg  
acggggacaacacctactatcgggattccgtgaaaggacgcttcacaatctcccgagataacgccaaagagctcactgtac  
ctgcagatgaatagcctgagagccgaggatactgccgtgtactattgcacaacgggaatcgtaggccttttctgtactg  
gggacagggcaccttgggttactgtctcgagcgcttctacaaagggcccatccgtcttccccctggcgccctgctccagga  
gcacctccgagagcacagccgcccctgggctgcctgggtcaaggactacttccccgaaccgggtgacgggtgtcgtggaactca  
ggcgccctgaccagcggcgtgcacaccttccccggctgtcctacagtcctcaggactctactccctcagcagcgtggtgac  
cgcgccctccagcagcttggggcacgaagacctacacctgcacaagcccagcaaacaccaaggtggacaagagagttggtga  
gagggcagcacagggaggggagggtgtctgctggaagccaggctcagccctcctgcctggacgcaccccggtgtgcagcc  
ccagcccagggcagcaaggcatgccccatctgtctcctcaccggaggcctctgaccacccactcatgccaggggagag  
ggtcttctggatttttccaccaggctccgggcagccacaggctggatgcccctaccccaggccctgcgcatacaggggca  
ggtgctgcgctcagacctgccaaagagccatatccgggaggacctgcccctgacctaaagcccaccccaaggccaaactc  
tccactccctcagctcagacaccttctctcctcccagatctgagtaactcccaatcttctctctgcagagtcctcaaatatg  
gtcccccatgcccaccatgcccaggtaagccaacccaggcctcgccctccagctcaaggcgggacagggtgccctagagta  
gcctgcatccagggacaggcccagccgggtgtgacgcattccacctccatctcttccctcagcacctgagttcctggggg  
gaccatcagctcttctgttccccccaaaacccaaggacactctcatgatctcccgaccctgaggtcacgtgcgtggtg  
gtggacgtgagccaggaagaccccagggtccagttcaactggtagctggatggcgtggaggtgcataatgccaagacaaa  
gccgcccaggagcagttcaacagcacgtaccgtgtgggtcagcgtcctcaccgtcctgcaccaggactggctgaacggca  
aggagtacaagtgaaggtctccaacaaaggcctcccgtcctccatcgagaaaaccatctccaaagccaaaggtgggacc  
cacggggtgcgagggccacatggacagaggtcagctcgcccacccctctgcctgggagtgaccgtgtgccaacctctg  
tccctacagggcagccccgagagccacagggtgtacacctgcccccatcccaggaggagatgaccaagaaccagggtcagc  
ctgacctgcctgggtcaaaggcttctaccccagcgacatcgccgtggagtgaggagcaatgggcagccgggagaacaacta  
caagaccagcctcccgtgtggtgactccgacggctccttcttctctacagcagggttaaccgtggacaagagcagggtggc  
aggaggggaatgtcttctcatgctccgtgatgcatgaggctctgcacaaccactacacacagaagagcctctccctgtct  
ctgggtaaa

## 1519gL20 FabFv light chain SEQ ID NO: 68

DIQMTQSPSS LSASVGDRVIT ITCKSSQSLV GASGKTYLYW LFQKPGKAPK RLIYLVSTLD  
 SGIPSRFSGS GSGTEFTLTI SSLQPEDFAT YYCLQGTHFP HTFGQGTKLE IKRTVAAPSV  
 FIFPPSDEQL KSGTASVVCL LNNFYPREAK VQWKVDNALQ SGNSQESVTE QDSKDSTYSL  
 SSTLTLSKAD YEKHKVYACE VTHQGLSSPV TKSFNREGES GGGSGGGGS GGGGSDIQMT  
 QSPSSVSASV GDRVITITCQS SPSVWSNFLS WYQQKPGKAP KLLIYEASKL TSGVPSRFSG  
 SSGSGTDFTLT ISSLQPEDFA TYYCGGYS ISDTTFGCGT KVEIKRT

## 1519gL20 FabFv light chain SEQ ID NO: 69

gataaccaga tgaccagag cccatctagc ttatccgctt ccgttggtga tcgctgaca  
 attacgtgta agagctccca atctctcgtg ggtgcaagt gcaagacct tctgtactgg  
 ctctttcaga agcctggcaa ggcacaaaaa cggctgatct atctggtgtc tacccttgac  
 tctgggatac cgtcacgatt ttccggatct gggagcggaa ctgagttcac actcacgatt  
 tcatcgctgc aaccggagga ctttgctacc tactactgcc tgcaaggcac tcatttccct  
 cacactttcg gccaggggac aaaactcgaa atcaaactgta cggtagcggc cccatctgtc  
 ttcatcttcc cgccatctga tgagcagttg aaatctggaa ctgcctctgt tgtgtgcctg  
 ctgaataact tctatcccag agaggccaaa gtacagtggaa aggtggataa cgccctccaa  
 tcgggtaact cccaggagag tgtcacagag caggacagca aggacagcac ctacagcctg  
 agcagcacc tgacgctgtc taaagcagac tacgagaaac acaaagtgtg cgctgcgaa  
 gtcacccatc agggcctgag ctcaccagta acaaaaagtt ttaatagagg ggagtgtagc  
 ggtggcggtg gcagtgggtg gggaggctcc ggaggtggcg gttcagacat acaaatgacc  
 cagagtcctt catcggtatc cgcgtccgtt ggcgataggg tgactattac atgtcaaagc  
 tctcctagcg tctggagcaa ttttctatcc tggtatcaac agaaaccggg gaaggctcca  
 aaacttctga tttatgaagc ctcgaaactc accagtggag ttccgtcaag attcagtggc  
 tctggatcag ggacagactt cacgttgaca atcagttcgc tgcaaccaga ggactttgag  
 acctactatt gtgggtggagg ttacagtagc ataagtata cgacatttgg gtgcggtact  
 aaggtggaaa tcaaacgtac c

**FIGURE 80****1519gL20 FabFv light chain with signal sequence underlined and italicised SEQ ID NO: 70**

MSVPTQVLGLLLLWLTDARCDIQMTQSPSSLSASVGDRTITCKSSQSLVGASGKTYLYWLFQKPGKAPKRLIYLV  
STLDSGIPSRFSGSGSGTEFTLTISLQPEDFATYYCLOGTHFPHTFGQGTKLEIKRTVAAPSVFIFPPSDEQLKS  
GTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKSTYLSSTLTLSKADYEKHKVYACEVTHQGLSS  
PVTKSFNRGECSSGGGSGGGGSGGGGSDIQMTQSPSSVSASVGDRTITCQSSPSVWSNFLSWYQQKPGKAPKLLI  
YEASKLTSGVPSRFSGSGSGTDFTLTISLQPEDFATYYCGGYSISDITFGCGTKVEIKRT

**1519gL20 FabFv light chain with signal sequence underlined and italicised SEQ ID NO: 71**

atgtctgtccccacccaagtcctcggactcctgctactctggcttacagatgccagatgcgatatccagatg  
accagagcccatctagcttatccgcttccgttgggtgatcgcgtagacaattacgtgtaagagctcccaatct  
ctcgtgggtgcaagtggcaagacctatctgtactggctctttcagaagcctggcaaggcaccaaaacggctg  
atctatctgggtgtctacccttgactctgggataccgtcacgattttccggatctgggagcggaaactgagttc  
acactcacgattttcatcgctgcaacccgaggactttgctacctactactgctgcaaggcactcatttccct  
cacactttcggccaggggacaaaactcgaaatcaaacgtacggttagcgccccatctgtcttcatcttcccg  
ccatctgatgagcagttgaaatctggaactgcctctgttgtgtgctgctgaataacttctatcccagagag  
gccaaagtacagtgggaaggtggataacgccctccaatcgggttaactcccaggagagtgtcacagagcaggac  
agcaaggacagcacctacagcctgagcagcacctgacgtgtctaaagcagactacgagaaacacaaagtg  
tacgctgcaagtcacccatcagggcctgagctcaccagtaacaaaaagttttaatagaggggagtgtgac  
gggtggcggtggcagtggtgggggaggtccggaggtggcggttcagacatacaaatgaccagagtccttca  
tcggtatccgcgtccgttggcgatagggtgactattacatgtcaaagctctcctagcgtctggagcaatttt  
ctatcctggtatcaacagaaaccggggaaggtccaaaacttctgatttatgaagcctcgaaaactcaccagt  
ggagttccgtcaagattcagtggtctctggatcagggacagacttcacgttgacaatcagttcgctgcaacca  
gaggactttgcgacctactattgtggtggaggttacagtagcataagtgatacgacatttgggtgcggtact  
aaggtggaaatcaaacgtacc

**1519gH20 FabFv heavy chain SEQ ID NO: 72**

EVPLVESGGGLVQPGGSLRLSCAVSGFTFSNYGMVWVRQAPGKGLEWVAYIDSDGDNTYYRDSVKGRFTISR  
DNAKSSLYLQMNSLRAEDTAVYYCTTGIVRPFLYWGGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGC  
LVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVE  
PKSCSGGGGSGGGGTGGGGSEVQLLES GGGLVQPGGSLRLSCAVSGIDLSNYAINWVRQAPGKCLEWIGIIW  
ASGTTFYATWAKGRFTISRDN SKNTVYLQMNSLRAEDTAVYYCARTVPGYSTAPYFDLWGQGT LTVTVSS

**1519gH20 FabFv heavy chain SEQ ID NO: 73**

gaggtaccacttgtggaaagcggaggaggtcctgtgcagcctggaggaagtttacgtctctcttctgtgctgtg  
tctggcttcaccttctccaattacggaatgggtctgggtcagacaagcacctggaaagggctctgaatgggtg  
gcctatattgactctgacggggacaacacctactatcgggattccgtgaaaggacgcttcacaatctcccga  
gataacgccaaagagctcactgtacctgcagatgaatagcctgagagccgaggatactgccgtgtactattgc  
acaacgggaatcggttaggccttttctgtactggggacagggcaccttggttactgtctcgagcgcgctccaca  
aagggcccatcggtcttccccctggcaccttctccaagagcacctctgggggcacagcggccctgggctgc  
ctggtcaaggactacttccccgaaccagtgcggtgtcgtggaaactcaggtgccctgaccagcggcggttcac  
accttccccggtgtctctacagtcttcaggactctactccctgagcagcgtggtgacctgacctccagcagc  
ttgggcacccagacctacatctgcaacgtgaatcacaagcccagcaacaccaaggtcgataagaaagttgag  
cccaaactcttgtagtggaggtgggggctcaggtggaggcgggaccggtggaggtggcagcgaggttcaactg  
cttgagctctggaggaggcctagtcacagcctggaggagcctgcgtctctcttctgtgcagtaagcggcatcgac  
ctgagcaattacgccatcaactgggtgagacaagctccggggaagtgttagaatggatcgggtataatatgg  
gccagtgggacgaccttttatgctacatgggcgaaaggaaggtttacaattagccgggacaatagcaaaaac  
accgtgtatctccaaatgaactccttgcgagcagaggacacggcggtgtactattgtgctcgactgtcca  
ggttatagcactgcacctacttcgatctgtggggacaagggaccctgggtgactgtttcaagt

**1519gH20 FabFv heavy chain with signal sequence underlined and italicised SEQ ID NO: 74**

MEWSWVFLFSLVTTGVHSEVPLVESGGGLVQPGGSLRLSCAVSGFTFSNYGMVWVRQAPGKGLEWVAYIDS  
DGDNTYYRDSVKGRFTISRDN AKSSLYLQMNSLRAEDTAVYYCTTGIVRPFLYWGGTLVTVSSASTKGPSV  
FPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQT  
YICNVNHKPSNTKVDKKVEPKSCSGGGGSGGGGTGGGGSEVQLLES GGGLVQPGGSLRLSCAVSGIDLSNYA  
INWVRQAPGKCLEWIGIIWASGTTFYATWAKGRFTISRDN SKNTVYLQMNSLRAEDTAVYYCARTVPGYSTA  
PYFDLWGQGT LTVTVSS

# Harvest from 20 Litre Pilot Scale Productions and Primary Recovery for “MXE016” Cell Lines

