BACTERIAL HOST STRAIN EXPRESSING RECOMBINANT DSBC

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.

BACKGROUND OF THE INVENTION

Bacterial cells, such as *E. coli*, are commonly used for producing recombinant proteins that do not require glycosylation. 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 human insulin.

Despite the many advantages to using bacterial cells to produce recombinant proteins, there are still significant limitations including poor cell health phenotype.

Accordingly, there is still a need to provide new bacterial strains which provide advantageous means for producing recombinant proteins.

The generation of humoral and cell-mediated immunity is orchestrated by the interaction of activated helper T cells with antigen-presenting cells ("APCs") and effector T cells. Activation of the helper T cells is not only dependent on the interaction of the antigen-specific T-cell receptor ("TCR") with its cognate peptide-MHC ligand, but also requires the coordinate binding and activation by a number of cell adhesion and costimulatory molecules.

The natural receptor binding to CD40 is CD40 ligand (CD40-L = CD154) a critical costimulatory molecule that is expressed on the surface of CD4+ T cells in an activation-dependent, temporally-restricted manner. CD154 is also expressed, following activation, on a subset of CD8+ T cells, basophils, mast cells, eosinophils, natural killer cells, B cells, macrophages, dendritic cells and platelets. CD40 is constitutively and widely expressed on the surface of many cell types, including B cells and other antigen presenting cells.

Signaling through CD40 after engagement with CD154 initiates a cascade of cellular events that results in the activation of the CD40 receptor-bearing cells and optimal CD4+ T cell priming. More specifically, the binding of CD154 to CD40 promotes the differentiation of B cells into antibody secreting cells and memory B cells.

The pivotal role of CD154 in regulating the function of both the humoral and cell-mediated immune response has provoked great interest in the use of inhibitors of this pathway for therapeutic immunomodulation. As such, anti-CD154 antibodies have been shown to be

beneficial in a wide variety of models of immune response to other therapeutic proteins or gene therapy, allergens, autoimmunity and transplantation (see, e.g., US 5,474,771 and WO 2008/118356 which are incorporated herein by reference in their entirety).

There is a need in the art to efficiently and cost effectively produce high amounts of antibodies or antibody fragments interfering with the interaction of CD40 and CD154 suitable for therapeutic applications.

SUMMARY OF THE INVENTION

The present invention provides a recombinant gram-negative bacterial cell comprising:

a) a recombinant polynucleotide encoding DsbC; and

b) one or more polynucleotides encoding an antibody or an antigen binding fragment thereof specifically binding to CD154.

More specifically the present invention provides a recombinant gram-negative bacterial cell, characterized in that the cell:

a) comprises a recombinant polynucleotide encoding DsbC;

b) has reduced Tsp protein activity compared to a wild-type cell, and

c) one or more polynucleotides encoding an antibody or an antigen binding fragment thereof specifically binding to CD154.

In one embodiment the cell comprises a wild-type *spr* gene or a mutated *spr* gene, for example capable of suppressing reduced activity Tsp phenotype.

The gram-negative bacterial cell according to the present invention shows advantageous growth and protein production phenotypes.

More specifically the present invention provides a recombinant gram-negative bacterial cell comprising a recombinant polynucleotide encoding a DsbC polypeptide comprising a histidine (His)-tag, preferably wherein the DsbC polypeptide comprises the sequence his-his-his-his-his-his (6x histidine).

More specifically the present invention provides a recombinant gram-negative bacterial cell comprising a recombinant polynucleotide comprising a polynucleotide with the sequence according to SEQ ID NO:45 or SEQ ID NO:51.

The present invention also provides an expression vector comprising a recombinant polynucleotide encoding DsbC and an antibody or an antigen binding fragment thereof specifically binding to CD154.

More specifically the present invention provides an expression vector comprising a recombinant polynucleotide encoding a DsbC polypeptide comprising a histidine (His)-tag, preferably wherein the DsbC polypeptide comprises the sequence his-his-his-his-his-his (6x histidine).

More specifically the present invention an expression vector comprising a recombinant polynucleotide comprising a polynucleotide with the sequence according to SEQ ID NO:45 or SEQ ID NO:51.

The present invention also provides a method for producing an antibody or an antigen binding fragment thereof specifically binding to CD154 comprising:

a) culturing a recombinant gram-negative bacterial cell as defined above in a culture medium under conditions effective to express the antibody or the antigen binding fragment thereof specifically binding to CD154 and the recombinant polynucleotide encoding DsbC; and

b) recovering the antibody or an antigen binding fragment thereof specifically binding to CD154 from the periplasm of the recombinant gram-negative bacterial cell and/or the culture medium.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the construction of a vector for use in producing a cell according to an embodiment of the present invention.

Figure 2 shows the structure of a compound comprising a modified Fab' fragment covalently linked via a cysteine residue to a lysyl-maleimide linker wherein each amino group on the lysyl residue has covalently attached to it a methoxy PEG residue wherein n is between about 420 to 450.

Figure 3 shows the growth profile of anti-CD154 Fab' expressing strain W3110 and the growth profile of anti-CD154 Fab' and recombinant DsbC expressing strain MXE016 (W3110 ΔTsp, spr C94A). It can be seen that the MXE016 strain expressing recombinant DsbC exhibits improved growth profile and growth rate in the initial batch phase compared to the W3110 strain.

Figure 4 shows total Fab' yield (g/L) from the periplasm (closed symbols) and supernatant (open symbols) from the MXE016 strain expressing recombinant DsbC compared to control strain W3110. The DsbC expressing strain shows higher periplasmic Fab' expression compared to W3110. Further, the MXE016 strain expressing DsbC shows equivalent extracellular Fab' levels compared to strain W3110.

Figure 5 shows the results of a reverse phase HPLC analysis of fermentation extractions. The wild-type strain W3110 expressing anti-CD154 Fab' exhibits a high level of degraded Kappa light chains (light chain [LC] fragments). In contrast, strain MXE016 (W3110 ΔTsp, spr C94A) expressing recombinant DsbC and anti-CD154 Fab' exhibits hardly any light chain fragments due to the absence of Tsp protease activity.

Figure 6 shows the harvest of anti-CD154 Fab' (g/L) from fermentations in strain W3110 and in strain MXE016 (W3110 ΔTsp, spr C94A) expressing recombinant DsbC. The harvest from strain MXE016 (W3110 ΔTsp, spr C94A) expressing recombinant DsbC is substantially higher and exhibits substantially less extracellular Fab' which is beneficial as extracellular Fab' is a marker of cell lysis risk and the extracellular Fab is not easily recovered using the same process as used for periplasmic Fab'.

Figure 7 shows the viability of (a) strain W3110 cells and (b) strain MXE016 (W3110 ΔTsp, spr C94A) cells expressing recombinant DsbC in both cases (a) and (b) expressing anti-CD154 Fab' (g/L). The strain MXE016 cells (W3110 ΔTsp, spr C94A) expressing recombinant DsbC exhibit a higher viability.

Figure 8 shows total Fab' yield (g/L) from fermentations of MXE016 strains expressing anti-CD154 Fab'. The right bar represents an MXE016 strain expressing additionally recombinant DsbC. The MXE016 strain expressing recombinant DsbC strain exhibits a higher yield.

Figure 9 shows polynucleotide and amino acid sequences of a region within the ptr gene that was mutated.

Figure 10 shows polynucleotide and amino acid sequences of a region within the Tsp gene that was mutated.

Figure 11 shows polynucleotide and amino acid sequences of a region within the DegP gene that was mutated

BRIEF DESCRIPTION OF THE SEQUENCES

SEQ ID NO: 1 shows the amino acid sequence of CDRH1 of an anti-CD154 antibody.

SEQ ID NO: 2 shows the amino acid sequence of CDRH2 of an anti-CD154 antibody.

SEQ ID NO: 3 shows the amino acid sequence of CDRH3 of an anti-CD154 antibody.

SEQ ID NO: 4 shows the amino acid sequence of CDRL1 of an anti-CD154 antibody.

SEQ ID NO: 5 shows the amino acid sequence of CDRL2 of an anti-CD154 antibody.

SEQ ID NO: 6 shows the amino acid sequence of CDRL3 of an anti-CD154 antibody.

SEQ ID NO: 7 shows the polynucleotide and amino acid sequence of the variable light chain (gL4) of an anti-CD154 antibody (342).

SEQ ID NO: 8 shows the amino acid sequence of the variable light chain (gL4) of an anti-CD154 antibody (342).

SEQ ID NO: 9 shows the polynucleotide and amino acid sequence of the variable heavy chain (gH1) of an anti-CD154 antibody (342).

SEQ ID NO: 10 shows the amino acid sequence of the variable heavy chain (gH1) of an anti-CD154 antibody (342).

SEQ ID NO: 11 shows the polynucleotide and amino acid sequence comprising the variable and constant region of the light chain (gL4) of an anti-CD154 antibody fragment.

SEQ ID NO: 12 shows the amino acid sequence comprising the variable and constant region of the light chain (gL4) of an anti-CD154 antibody fragment.

SEQ ID NO: 13 shows the polynucleotide and amino acid sequence of a heavy chain fragment of an anti-CD154 antibody comprising the variable and the CH1 region with deletions in the hinge region.

SEQ ID NO: 14 shows the amino acid sequence of a heavy chain fragment of an anti-CD154 antibody comprising the variable and the CH1 region with deletions in the hinge region.

SEQ ID NO: 15 shows the polynucleotide and amino acid sequence of a heavy chain fragment of an anti-CD154 antibody comprising the variable, the CH1 and the hinge region.

SEQ ID NO: 16 shows the amino acid sequence of a heavy chain fragment of an anti-CD154 antibody comprising the variable, the CH1 and the hinge region.

SEQ ID NO: 17 shows the polynucleotide and amino acid sequence of a kappa light chain of an anti-CD154 antibody (342) including the signal peptide (amino acids 1-22).

SEQ ID NO: 18 shows the amino acid sequence of a kappa light chain of an anti-CD154 antibody (342) including the signal peptide (amino acids 1-22).

SEQ ID NO: 19 shows the polynucleotide and amino acid sequence of the entire heavy chain of an aglycosylated IgG_4 anti-CD154 antibody.

SEQ ID NO: 20 shows the amino acid sequence of the entire heavy chain of an aglycosylated IgG_4 anti-CD154 antibody.

SEQ ID NO: 21 shows the polynucleotide sequence coding for gL4 and gH1 (no hinge).

SEQ ID NO: 22 shows the polynucleotide sequence coding for gL4 and gH1.

SEQ ID NO: 23 shows the polynucleotide and amino acid sequence for wild-type *E. coli* spr (GenBank accession no. D86610).

SEQ ID NO: 24 shows the amino acid sequence for wild-type *E. coli* spr (GenBank accession no. D86610).

SEQ ID NO: 25 shows the polynucleotide and amino acid sequence for wild-type *E. coli* Tsp with the signal sequence (GenBank accession no. M75634).

SEQ ID NO: 26 shows the amino acid sequence for wild-type *E. coli* Tsp with the signal peptide (GenBank accession no. M75634).

SEQ ID NO: 27 shows the amino acid sequence for wild-type *E. coli* DsbC (NCBI Reference Sequence AP_003452).

SEQ ID NO: 28 shows the polynucleotide sequence for knockout mutated Tsp gene.

SEQ ID NO: 29 shows the polynucleotide and amino acid sequence for wild-type *E. coli* DegP.

SEQ ID NO: 30 shows the amino acid sequence for wild-type *E. coli* DegP.

SEQ ID NO: 31 shows the polynucleotide and amino acid sequence for DegP comprising the point mutation S210A and an Ase I restriction marker.

SEQ ID NO: 32 shows the amino acid sequence for DegP comprising the point mutation S210A and an Ase I restriction marker.

SEQ ID NO: 33 to 36 show dicistronic intergenic sequences (IGS) IGS1, IGS2, IGS3 and IGS4, respectively.

SEQ ID NO: 37 shows the polynucleotide and amino acid sequence for wild-type *E. coli* OmpT.

SEQ ID NO: 38 shows the amino acid sequence for wild-type *E. coli* OmpT.

SEQ ID NO: 39 shows the polynucleotide and amino acid sequence for knockout mutated *E. coli* OmpT.

SEQ ID NO: 40 shows the amino acid sequence for knockout mutated *E. coli* OmpT.

SEQ ID NO: 41 shows the polynucleotide and amino acid sequence of the mutated *E. coli* OmpT comprising the point mutations D210A and H212A.

SEQ ID NO: 42 shows the amino acid sequence of the *E. coli* OmpT comprising the point mutations D210A and H212A.

SEQ ID NO: 43 shows the polynucleotide and amino acid sequence of wild-type *E. coli* DsbC.

SEQ ID NO: 44 shows the amino acid sequence of wild-type *E. coli* DsbC.

SEQ ID NO: 45 shows the polynucleotide and amino acid sequence of *E. coli* DsbC lacking an EcoRI restriction site with a His-tag.

SEQ ID NO: 46 shows the amino acid sequence of *E. coli* DsbC lacking an EcoRI restriction site with a His-tag.

SEQ ID NO: 47 shows the a polynucleotide sequence of primer 6283 Tsp 3'.

SEQ ID NO: 48 shows the a polynucleotide sequence of primer 6283 Tsp 5'.

SEQ ID NO: 49 shows the polynucleotide and amino acid sequence of wild-type *E. coli* ptr (protease III according to GenBank accession number X06227).

SEQ ID NO: 50 shows the amino acid sequence of wild-type *E. coli* ptr (protease III according to GenBank accession number X06227).

SEQ ID NO: 51 shows the polynucleotide and amino acid sequence of wild-type *E. coli* DsbC with a His-tag.

SEQ ID NO: 52 shows the amino acid sequence of wild-type *E. coli* DsbC with a His-tag.

DETAILED DESCRIPTION OF THE INVENTION

Tsp (also known as Prc) is a 60kDa periplasmic protease. The first known substrate of 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* (Hara et al. 4799-813;Nagasawa et al. 5890-93) 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, Keiler, and Sauer 295-99;Silber and Sauer 237-40) 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^r marker.

The reduction of Tsp (prc) activity is desirable to reduce the proteolysis of proteins of interest. Fab proteolysis may manifest itself as the presence of impurities such as a fragment which can be referred to as the light chain impurity.

However, it was found that cells lacking protease prc show thermosensitive growth at low osmolarity. Hara *et al* isolated thermoresistant revertants containing extragenic suppressor (spr) mutations (Hara et al. 63-72). 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).

Protein disulphide isomerase is an enzyme that catalyzes the formation and breakage of disulphide bonds between cysteine residues within proteins as they fold. It is known to co-express proteins which catalyze the formation of disulphide bonds to improve protein expression in a host cell. WO 98/56930 discloses a method for producing heterologous disulfide bond-containing polypeptides in bacterial cells wherein a prokaryotic disulfide isomerase, such as DsbC or DsbG is co-expressed with a eukaryotic polypeptide. US 6,673,569 discloses an artificial operon comprising polynucleotides encoding each of DsbA, DsbB, DsbC and DsbD for use in producing a foreign protein. EP0786009 discloses a process for producing a heterologous polypeptide in bacteria wherein the expression of nucleic acid encoding DsbA or DsbC is induced prior to the induction of expression of nucleic acid encoding the heterologous polypeptide.

DsbC is a prokaryotic protein found in the periplasm of *E. coli* which catalyzes the formation of disulphide bonds in *E. coli*. DsbC has an amino acid sequence length of 236 (including signal peptide) and a molecular weight of 25.6 KDa (UniProt No. P0AEG6). DsbC was first identified in 1994 (Missiakas, Georgopoulos, and Raina 2013-20;Shevchik, Condemine, and Robert-Baudouy 2007-12).

It has been surprisingly found that the over-expression of DsbC in a gram-negative bacterial cell reduces lysis during cultivation of cells lacking protease Tsp. Accordingly, the present inventors have provided a new strain having advantageous properties for producing a protein of interest.

The gram-negative bacterial cell having the above specific combination of genetic modifications shows advantageous growth and protein production phenotypes.

In one embodiment the cell's genome is preferably isogenic to a wild-type bacterial cell except for the modification required to reduce Tsp protein activity compared to a wild-type cell.

In a further embodiment the cell according to the present invention has reduced Tsp protein activity compared to a wild-type cell and comprises a recombinant polynucleotide encoding DsbC and an altered spr protein. In this embodiment the cell's genome is preferably isogenic to a wild-type bacterial cell except for the mutated spr gene and a modification leading to reduced or absent expression of the Tsp protein compared to a wild-type cell.

The terms "protein" and "polypeptide" are used interchangeably herein, unless the context indicates otherwise. "Peptide" is intended to refer to 20 or less amino acids.

The term "polynucleotide" includes a gene, DNA, cDNA, RNA, mRNA, and analogues thereof including, but not limited to, locked nucleic acid (LNA), peptide nucleic acid (PNA), morpholino nucleic acid, glycol nucleic acid (GNA) and threose nucleic acid (TNA), etc. unless the context indicates otherwise.

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

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 recombinant polynucleotide encoding DsbC and one or more polynucleotides encoding an antibody or an antigen binding fragment thereof specifically binding to CD154. 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 recombinant polynucleotide(s).

The expressions "cell", "cell line", "cell culture" and "strain" are used interchangeably.

The term "isogenic" in the context of the present invention means that the cell comprises the same or substantially the same nucleic acid sequence(s) compared to wild-type cell except for the elements incorporated therein that characterize the invention, for example the recombinant polynucleotide encoding DsbC and the one or more polynucleotides encoding an antibody or an antigen binding fragment thereof specifically binding to CD154 and optionally a modification leading to reduced or absent expression of the Tsp protein, and optionally a mutated spr gene. In this embodiment the cell according to the present invention comprises no further non-naturally occurring or genetically engineered changes to its genome.

In one embodiment wherein the polynucleotide encoding DsbC and/or the one or more polynucleotides encoding the antibody or antigen binding fragment thereof are inserted into the cell's genome, the cell according to the present invention may have substantially the same genomic sequence compared to the wild-type cell except for the polynucleotide encoding DsbC and/or the one or more polynucleotides encoding the antibody or antigen binding fragment thereof and optionally a modification resulting in a reduced or absent expression of the Tsp protein or the expression of a Tsp protein with reduced protease activity, and optionally a mutated spr gene coding for a protein with reduced activity as compared to the wild-type, taking into account any naturally occurring mutations which may occur. In one embodiment, wherein the polynucleotide encoding DsbC and/or the one or more polynucleotides encoding the antibody or antigen binding fragment thereof are inserted into the cell's genome, the cell according to the present invention may have exactly the same genomic sequence compared to the wild-type cell except for the polynucleotide encoding DsbC and/or the one or more polynucleotides encoding the antibody or antigen binding fragment thereof.

The polynucleotide encoding DsbC may be present on a suitable expression vector transformed into the cell and/or integrated into the host cell's genome. In the embodiment where the polynucleotide encoding DsbC is inserted into the host cell's genome, the cell of the present invention differs from a wild-type cell due to the inserted polynucleotide encoding the DsbC. In this embodiment, the host cell's genome may be isogenic compared to a wild-type cell genome except for the recombinant polynucleotide encoding DsbC.

Preferably the polynucleotide encoding DsbC is in an expression vector in the cell thereby causing minimal disruption to the host cell's genome.

The one or more polynucleotides encoding the antibody or an antigen binding fragment thereof specifically binding to CD154 may be contained within a suitable expression vector transformed into the cell and/or integrated into the host cell's genome. In the embodiment where the polynucleotide encoding the antibody or antigen binding fragment thereof specifically binding to CD154 is inserted into the host's genome, the cell of the present invention differs from a wild-type cell due to the inserted polynucleotide(s) encoding the antibody or antigen binding fragment thereof. In this embodiment, the host cell's genome may be isogenic compared to a wild-type cell genome except for the polynucleotide(s) encoding the antibody or antigen binding fragment thereof. Preferably the polynucleotide encoding the protein of interest is in an expression vector in the cell thereby causing minimal disruption to the host cell's genome.

In one embodiment the recombinant polynucleotide encoding DsbC and the polynucleotide encoding the antibody or antigen binding fragment thereof specifically binding to CD154 are inserted into the host's genome. In this embodiment, the cell of the present invention differs from a wild-type cell due to the inserted recombinant polynucleotide encoding DsbC and the one or more polynucleotide encoding the antibody or antigen binding fragment thereof and optionally a modification resulting in a reduced or absent expression of the Tsp protein or the expression of a Tsp protein with reduced protease activity, and optionally a mutated spr gene coding for a protein with reduced activity as compared to the wild-type. In this embodiment, the host cell's genome may be isogenic compared to a wild-type cell genome except for the recombinant polynucleotide encoding DsbC and the one or emore polynucleotides encoding the antibody or antigen binding fragment thereof.

In a preferred embodiment the recombinant polynucleotide encoding DsbC and the polynucleotide encoding the antibody or antigen binding fragment thereof specifically binding to CD154 are present in the same or different expression vectors in the cell thereby causing minimal disruption to the host cell's genome. In this embodiment the cell's genome may be substantially the same or exactly the same compared to the genome of a wild-type cell.

In one embodiment there is provided a recombinant *E. coli* cell that has reduced Tsp activity and optionally an spr gene or a mutant thereof, wherein modifications to the Tsp activity and any mutation in the spr gene are effected through changes in the cells genome. The cell according to this embodiment may be transformed with a vector such as a plasmid encoding vector DsbC and the antibody or a binding fragment thereof specifically binding to CD154. In one embodiment the vector or plasmid is not integrated into the genome of the cell.

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 recombinant polynucleotide or genetically engineered mutations. An example of a wild-type strain of *E. coli* is the K-12 strain and its pedigree strain W3110. *E. coli* strain K-12 has been in cultivation for 90 years now (Bachmann 525-57). *E. coli* strain K-12 and its pedigree strains such as W3110 are well known in the art. Strain W3110 is available for example from the American Tissue Culture Collection (ATCC) under catalog no. 27325. W3110 has the genotype: F^-, λ^-, IN(rrnD-rrnE)1, rph-1.

The present inventors have provided a recombinant gram-negative bacterial cell suitable for expressing an antibody or an antigen binding fragment thereof specifically binding to CD154 which comprises a recombinant polynucleotide encoding DsbC.

The cells according to the present invention comprise a recombinant polynucleotide encoding DsbC. As used herein, a "recombinant polypeptide" refers to a protein that is constructed or produced using recombinant DNA technology. The polynucleotide encoding DsbC may be identical to the endogenous polynucleotide encoding DsbC found in wild-type bacterial cells. Alternatively, the recombinant polynucleotide encoding DsbC is a mutated version of the wild-type DsbC polynucleotide, for example being altered such that the restriction site removed from the DsbC protein, such as an EcoRI site, and/or the his-tag. An example modified DsbC polynucleotide for use in the present invention is shown in SEQ ID NO: 45, which encodes the his-tagged DsbC sequence shown in SEQ ID NO: 46.

DsbC is characterized by the presence of an active site comprising amino acids –CXXC- wherein XX represents the amino acids GY. Variants of DsbC include wherein each X represents an amino acid independently selected (with the proviso that XX does not represent GY). Examples of XX include NY, SF, TF, MF, GF, HH, VH, SH, RF, FA, GA, MA, GI or AV.

In one embodiment the host cell of the invention comprises a variant of DsbC, for example where the active site is altered, in particular as described above.

In one embodiment the variant of DsbC has at least the biological activity of the wild type protein, for example as measured in an *in vitro* assay.

In one embodiment the variant of DsbC has at greater biological activity than the wild type protein, for example as measured in an *in vitro* assay.

In one embodiment the variant of DsbC has an alteration in the active site –CXXC- wherein XX represents NY, SF, TF, MF, GF, HH, VH, SH.

In one embodiment the DsbC is wild-type.

The present inventors have identified that the selection of the expression of recombinant polynucleotide encoding DsbC in a bacterial cell, provides an improved host cell for expressing an antibody or an antigen binding fragment thereof specifically binding to CD154. The cells provided by the present invention have improved cell health and growth phenotype compared to wild-type bacterial cells.

Improved cell health as employed herein is intended to refer to one or more improved properties in comparison to cells which do not carry the features according to the present invention, for example a lower propensity for cell lysis at the growth phase or after induction of

expression of a heterologous protein in the cell or other beneficial property as known to the skilled artisan.

The cells according to the present invention exhibit improved protein, such as antibody or antibody fragment, production yield compared to wild-type bacterial cells. The improved protein yield may be the rate of protein production and/or the duration of protein production from the cell. The improved protein yield may be the periplasmic protein yield and/or the supernatant protein yield. The recombinant bacterial cells may be capable of faster rate of production of the protein, such as an antibody or fragment thereof and, therefore, the same quantity of a protein of interest may be produced in a shorter time compared to a non-mutated bacterial cell. 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 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 the antibody.

Advantageously the reduced Tsp protein activity and/or the co-expression of DsbC leads to reduced generation of the undesirable impurity referred to herein as the light chain fragment (LC), see for example Figure 5.

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 et al. 193-202;Backlund et al. 358-65) which are incorporated herein by reference in their entirety. 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.

In one embodiment the cell according to the present invention also expresses or overexpresses as compared to the corresponding wild-type cell, such as e.g. the *E. coli* W3110 K-12 strain, one or more further proteins as follows:

- one or more proteins capable of facilitating protein folding, such as FkpA, Skp, SurA, PPiA and PPiD; and/or
- one or more protein capable of facilitating protein secretion or translocation, such as SecY, SecE, SecG, SecYEG, SecA, SecB, FtsY and Lep; and/or

- one or more proteins capable of facilitating disulphide bond formation, such as DsbA, DsbB, DsbD, DsbG.

One of more of the above proteins may be integrated into the cell's genome and/or inserted in an expression vector.

In one embodiment the cell according to the present invention does not express or expresses at a level which is at least 50%, 75% or 90% lower than the level of the corresponding wild-type cell, such as e.g. the *E. coli* W3110 K-12 strain, one or more of the following further proteins:

- one or more proteins capable of facilitating protein folding, such as FkpA, Skp, SurA, PPiA and PPiD;
- one or more protein capable of facilitating protein secretion or translocation, such as SecY, SecE, SecG, SecYEG, SecA, SecB, FtsY and Lep; and
- one or more proteins capable of facilitating disulphide bond formation, such as DsbA, DsbB, DsbD, DsbG.

In one embodiment the cell according to the present invention also expresses one or more further proteins selected from FkpA, Skp and a combination thereof.

In one embodiment the cell further comprises one or more of the following mutated genes:

a) a mutated spr gene;

b) a mutated Tsp gene, wherein the mutated Tsp gene encodes a Tsp protein having reduced protease activity or is a knockout mutated Tsp gene;

c) a mutated DegP gene encoding a DegP protein having chaperone activity and reduced protease activity;

d) 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

e) a mutated OmpT gene, wherein the mutated OmpT gene encodes a OmpT protein having reduced protease activity for example as shown in SEQ ID NO: 42 or is a knockout mutated OmpT gene for example as shown in SEQ ID NO: 40.

In a basic embodiment of the invention the gram-negative bacterial cell does not carry a knockout mutated Tsp gene, such as being deficient in chromosomal Tsp.

The latter mutation is particular important in production of antibodies and fragments thereof specifically binding to CD154 because Tsp protease activity may result in cleavage of the antibody product in the elbow regions, thereby generating a by-product in significant quantities and reducing yield of the desired product.

Thus in one embodiment the cell according to the present invention comprises a mutated Tsp gene, wherein the mutated Tsp gene encodes a Tsp protein having reduced protease activity or is a knockout mutated Tsp gene and also encodes a DsbC protein in addition to an antibody or fragment specific to CD154.

In embodiments of the present invention the cell further comprises a mutated spr gene. 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: 24 with the signal sequence at the N-terminus (amino acids 1-26 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 amino acid 1 of the Spr protein is the first amino acid (Met) shown in SEQ ID NO: 24.

In the embodiments wherein the cell according to the present invention comprises a mutated spr gene, the mutated spr gene is preferably the cell's chromosomal spr gene. The mutated spr gene encodes a Spr protein capable of suppressing a disadvantageous phenotype associated with a cell 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 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 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 this phenotype of thermosensitive growth at low osmolarity and the cell exhibits reduced lysis, particularly at a high cell density. This "thermosensitive 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 is apparent 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 preferably comprise a mutant spr gene encoding a spr protein having a change of 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, H157 and W174, more preferably at one or more amino acids selected from C94, S95, V98, Y115, D133, V135, H145, G147, H157 and W174. Preferably the mutant spr gene encodes a spr protein having a change of 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, more preferably 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 amino acid changes. Preferably, the mutant spr gene encodes a spr protein having a change of one or more amino acids selected from N31, R62, I70, Q73, S95, V98, Q99, R100, L108, Y115, D133, V135, L136, G140, R144 and G147, more preferably 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 amino acid changes.

The present inventors have identified spr changes which are capable of suppressing the growth phenotype of a cell comprising a mutated Tsp gene.

The inventors have also surprisingly found that cells carrying a recombinant DsbC gene, a mutated spr gene and having reduced Tsp protein activity compared to a wild-type cell exhibit increased cell growth rate and increased cell survival duration compared to a cell comprising a mutated Tsp gene. Specifically, cells carrying a recombinant DsbC gene, a change in the spr protein and having reduced Tsp protein activity exhibit reduced cell lysis during cultivation compared to cells carrying a mutated Tsp gene.

The change of one or more of the above spr amino acids may be the result of 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 change is with respect to one, two or three of the catalytic triad of amino acid residues of C94, H145, and H157 (Aramini et al. 9715-17).

Accordingly, the mutated spr gene may comprise:

- a mutation affecting the amino acid C94; or
- a mutation affecting the amino acid H145; or
- a mutation affecting the amino acid H157; or

- a mutation affecting the amino acids C94 and H145; or
- a mutation affecting the amino acids C94 and H157; or
- a mutation affecting the amino acids H145 and H157; or
- a mutation affecting the amino acids C94, H145 and H157.

In this embodiment, the spr protein preferably does not have any further amino acid changes.

One, two or three of C94, H145 and H157 may be changed 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 changed to a small amino acid such as Gly or Ala. Accordingly, the spr protein may have one, two or three of the mutations resulting in C94A (i.e. cysteine at position 94 changed to alanine), H145A (i.e. histidine at position 145 changed to alanine) and H157A (i.e. histidine at position 157 changed to alanine). Preferably, the spr gene comprises the missense mutation leading to 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. 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 a preferred embodiment the mutant spr protein comprises a change of 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 change of 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 affecting the amino acid N31; or
- a mutation affecting the amino acid R62; or
- a mutation affecting the amino acid I70; or
- a mutation affecting the amino acid Q73; or
- a mutation affecting the amino acid S95; or
- a mutation affecting the amino acid V98; or

- a mutation affecting the amino acid Q99; or
- a mutation affecting the amino acid R100; or
- a mutation affecting the amino acid L108; or
- a mutation affecting the amino acid Y115; or
- a mutation affecting the amino acid D133; or
- a mutation affecting the amino acid V135; or
- a mutation affecting the amino acid L136; or
- a mutation affecting the amino acid G140; or
- a mutation affecting the amino acid R144; or
- a mutation affecting the amino acid G147.

In one embodiment the mutant spr gene comprises multiple mutations affecting the 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.

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 changed 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 changed to a small amino acid such as Gly or Ala.

In a preferred embodiment the spr protein comprises one or more of the following changes: N31Y, R62C, I70T, Q73R, S95F, V98E, Q99P, R100G, L108S, Y115F, D133A, V135D or V135G, L136P, G140C, R144C and G147C. Preferably the spr protein comprises

one or more of the following changes: S95F, V98E, Y115F, D133A, V135D or V135G and G147C. In this embodiment, the spr protein preferably does not have any further amino acid changes.

In one embodiment the spr protein has only one amino acid change selected from N31Y, R62C, I70T, Q73R, C94A, S95F, V98E, Q99P, R100G, L108S, Y115F, D133A, V135D or V135G, L136P, G140C, R144C and G147C, in particular C94A. In this embodiment, the spr protein preferably does not have any further amino acid changes.

In a further embodiment the spr protein has multiple changes 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.

Preferably, the mutant spr gene encodes an spr protein having a amino acid changes selected from C94A, D133A, H145A and H157A, in particular C94A.

In a further embodiment the mutated spr gene encodes a spr protein having the amino acid change W174R. In an alternative embodiment the spr protein does not have the amino acid change W174R.

The cell according to the present invention has 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.

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.

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 cell according to the present invention carries 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.

As used herein, "Tsp gene" means a gene encoding protease Tsp (also known as Prc) 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: 25 and the sequence of the wild-type Tsp protein is shown in SEQ ID NO: 26.

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.

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 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 the art, such as the method described in Keiler *et al.* (Keiler and Sauer 28864-68) which is incorporated by reference herein in its entirety wherein the protease activity of Tsp was tested.

Tsp has been reported in Keiler *et al* (*supra*) as having an active site comprising residues 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 leading to the amino acid changes S430A, D441A, K455A, K455H, K455R, G375A, G376A, E433A and T452A had no detectable protease activity. It is further reported that the mutated Tsp gene

leading to S430C displayed about 5-10% wild-type activity. Accordingly, the Tsp mutation to produce a protein having reduced protease activity may comprise a mutation, such as a missense mutation leading to a change of 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 affecting one, two or all three of the active site residues S430, D441 and K455.

Accordingly the mutated Tsp gene may comprise:

- a mutation affecting the amino acid S430; or
- a mutation affecting the amino acid D441; or
- a mutation affecting the amino acid K455; or
- a mutation affecting the amino acids S430 and D441; or
- a mutation affecting the amino acids S430 and K455; or
- a mutation affecting the amino acids D441 and K455; or
- a mutation affecting the amino acids S430, D441 and K455.

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

The expression "knockout mutated Tsp gene" in the context of the present invention means that the Tsp 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 one embodiment the Tsp 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 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, as shown in Figure 10. 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, as shown in Figure 10. 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, as shown in Figure 10.

In a preferred embodiment the knockout mutated Tsp gene has the DNA sequence of SEQ ID NO: 25, which includes the 6 nucleotides ATGAAT upstream of the start codon. The mutations which have been made in the knockout mutated Tsp sequence of SEQ ID NO: 25 are shown in Figure 10. In one embodiment the mutated Tsp gene has the DNA sequence of nucleotides 7 to 2048 of SEQ ID NO: 25.

In embodiments of the present invention the cell comprises a mutated DegP gene. As used herein, "DegP" means a gene encoding DegP protein (also known as HtrA), which has dual function as a chaperone and a protease. The sequence of the wild-type DegP gene is shown in SEQ ID NO: 29 and the sequence of the non-mutated DegP protein is shown in SEQ ID NO: 30.

At low temperatures DegP functions as a chaperone and at high temperatures DegP has a preference to function as a protease .

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, e.g. wherein the protease and chaperone activities of DegP were tested on MalS, a natural substrate of DegP.

DegP is a serine protease and has an active center consisting of a catalytic triad of amino acid residues of His105, Asp135 and Ser210. The DegP mutation to produce a protein having chaperone activity and reduced protease activity may comprise a mutation, such as a missense mutation affecting one, two or three of His105, Asp135 and Ser210.

Accordingly, the mutated DegP gene may comprise:

- a mutation affecting the amino acid His105; or
- a mutation to affecting the amino acid; or
- a mutation to affecting the amino acid; or
- a mutation affecting the amino acids His105 and Asp135; or

- a mutation affecting the amino acids His105 and Ser210; or
- a mutation affecting the amino acids Asp135 and Ser210; or
- a mutation affecting the amino acids His105, Asp135 and Ser210.

One, two or three of His105, Asp135 and Ser210 may be changed 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 changed 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 changed to Lys or Arg, polar His105 being changed to a non-polar amino acid such as Gly, Ala, Val or Leu and small hydrophilic Ser210 being changed to a large or hydrophobic residue such as Val, Leu, Phe or Tyr. Preferably, the DegP gene comprises the alteration S210A, as shown in Figure 11, which has been found to produce a protein having chaperone activity but not protease activity.

DegP has two PDZ domains, PDZ1 (residues 260-358) and PDZ2 (residues 359-448), which mediate protein-protein interaction. 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.

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 11.

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: 31 and the encoded protein sequence is shown in SEQ ID NO: 27. The mutations which have been made in the mutated DegP sequence of SEQ ID NO: 32 are shown in Figure 11.

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 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 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 SEQ ID NO: 39.

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.

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.

In one embodiment the gram-negative bacterial cell according to the present invention does not carry a mutated spr gene.

Any suitable gram-negative bacterium may be used as the parental cell for producing the 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.

A drawback associated with strains previously created and used to express recombinant proteins involves the use of mutations of genes involved in cell metabolism and DNA replication such as, for example *phoA, fhuA, 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, periplasmic leakage, recombinant protein expression yield and 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 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 a preferred embodiment, the cells carry only the minimal mutations to introduce the recombinant polynucleotide encoding DsbC and the one or more polynucleotides encoding the antibody or antigen-binding fragment thereof and optionally a mutation resulting in reduced Tsp protease activity and optionally an spr gene or a mutant thereof.

In one embodiment wherein the polynucleotide encoding DsbC and/or the one or more polynucleotide encoding the antibody or antigen binding fragment thereof are inserted into the cell's genome only minimal mutations are made to the cell's genome to introduce recombinant polynucleotide encoding DsbC and/or the antibody. In a further embodiment wherein the recombinant polynucleotide encoding DsbC and the polynucleotide encoding the antibody are present in the same or different expression vectors, the genome is preferably isogenic to a wild-type cell genome.

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. 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 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 disruptions to the cell genome.

In a preferred embodiment, the cell is isogenic to a wild-type *E. coli* cell except for the recombinant polynucleotide encoding DsbC and the one or more polynucleotides encoding an antibody or antigen binding fragment thereof specifically binding to CD154 and optionally a mutation resulting in reduced Tsp protease activity and optionally an spr gene or a mutant thereof.

In one embodiment there is provided a cell isogenic to an *E. coli* strain W3110 except with reduced Tsp activity and an spr gene or a mutant thereof, for use with a plasmid suitable for expressing DsbC and an antibody or an antigen binding fragment thereof specifically binding to CD154.

More preferably the cell according to the present invention is isogenic to an *E. coli* strain W3110 except for the recombinant polynucleotide encoding DsbC and the one or more polynucleotides encoding an antibody or an antigen binding fragment thereof specifically binding to CD154.

The cell provided by the present invention comprises one or more polynucleotides encoding an antigen binding antibody fragment with specificity for CD154.

A cell comprising as employed herein is intended to refer to where the entity concerned is integrated into the cell's genome or where the cell contains a vector such as a plasmid containing and generally for expressing the entity.

The antibody or antibody fragment may be a multi-valent, multi-specific, humanized, fully human or chimeric. The antibody or antibody fragment can be from any species but is preferably derived from a monoclonal antibody, a human antibody, or a humanized fragment. The antibody fragment 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 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 fragment may be a VH, VL, VHH, Fab, modified Fab, Fab', $F(ab')_2$ or Fv fragment; a light chain or heavy chain monomer or dimer; as well as a diabody; triabody; tetrabody; minibody; domain antibody or a single chain antibody, e.g. a single chain Fv in which the heavy and light chain variable domains are joined by a peptide linker, Fab-Fv, or a dual specificity antibody, such as a Fab-dAb, as described in WO 2009/040562. Similarly, the heavy and light chain variable regions may be combined with other antibody domains as appropriate. Antibody fragments are known in the art (Holliger and Hudson 1126-36).

The antibody specifically binding to CD154, is preferably the antibody 342 described in WO 2008/118356 (the contents of which are incorporated herein by reference) or comprises the CDRs or variable heavy and light chain regions of the antibody 342 described in WO 2008/118356. The antibody fragment specifically binding to CD154, is preferably derived from the antibody 342 described in WO 2008/118356 and/or comprises the CDRs or variable heavy and light chain regions of said antibody.

In a one embodiment the antibody or antibody fragment specifically binding to CD154, comprises a heavy chain wherein the variable domain comprises three CDRs wherein the CDRs are selected from SEQ ID NO:1 for CDRH1, SEQ ID NO: 2 for CDRH2 and SEQ ID NO: 3 for CDRH3.

In one embodiment the antibody or antibody fragment specifically binding to CD154 comprises a light chain wherein the variable domain comprises three CDRs wherein the CDRs

are selected from SEQ ID NO: 4 for CDRL1, SEQ ID NO: 5 for CDRL2 and SEQ ID NO: 6 for CDRL3.

In one embodiment the antibody or antibody fragment specifically binding to CD154 comprises a heavy chain comprising the sequence of SEQ ID NO:1 for CDRH1, the sequence of SEQ ID NO: 2 for CDRH2 and the sequence of SEQ ID NO: 3 for CDRH3.

In one embodiment the antibody or antibody fragment specifically binding to CD154 comprises a light chain comprising the sequence of SEQ ID NO: 4 for CDRL1, the sequence of SEQ ID NO: 5 for CDRL2 and the sequence of SEQ ID NO: 6 for CDRL3.

In one embodiment the antibody or antibody fragment specifically binding to CD154 comprises a heavy chain comprising the sequence of SEQ ID NO: 1 for CDRH1, the sequence of SEQ ID NO: 2 for CDRH2 and the sequence of SEQ ID NO: 3 for CDRH3 and a light chain comprising the sequence of SEQ ID NO: 4 for CDRL1, the sequence of SEQ ID NO: 5 for CDRL2 and the sequence of SEQ ID NO: 6 for CDRL3.

The antibody is preferably a CDR-grafted antibody molecule and typically the variable domain comprises human acceptor framework regions and non-human donor CDRs.

Preferably, the antibody comprises the light chain variable domain (SEQ ID NO: 7) and the heavy chain variable domain (SEQ ID NO: 9).

Preferably the antibody is a Fab fragment. Preferably the Fab fragment has a heavy chain comprising or consisting of the sequence given as SEQ ID NO: 14 and a light chain comprising or consisting of the sequence given as SEQ ID NO: 12. The amino acid sequences given in SEQ ID NO:14 and SEQ ID NO:12 are preferably encoded by the nucleotide sequences given in SEQ ID NO:13 and SEQ ID NO:11, respectively.

Alternatively, it is preferred that the antibody fragment is a modified Fab fragment wherein the modification is the addition to the C-terminal end of its heavy chain one or more amino acids to allow the attachment of an effector or reporter molecule. Preferably, the additional amino acids form a modified hinge region containing one or two cysteine residue to which the effector or reporter molecule may be attached as known in the art (see e.g. WO 98/25971 which is incorporated herein in its entirety).

The cell according to the present invention comprises DNA sequence encoding the antibody. Preferably, the DNA sequence encodes the heavy and the light chain of the antibody.

In one preferred embodiment, the DNA sequence encodes a light chain and comprises the sequence shown herein.

The DNA sequence of the may comprise synthetic DNA, for instance produced by chemical processing, cDNA, genomic DNA or any combination thereof.

The constant region domains of the antibody, if present, may be selected having regard to the proposed function of the antibody molecule, and in particular the effector functions which may be required. For example, the constant region domains may be human IgA, IgD, IgE, IgG or IgM domains. In particular, human IgG constant region domains may be used, especially of the IgG_1 and IgG_3 isotypes when the antibody molecule is intended for therapeutic uses and antibody effector functions are required. Alternatively, IgG_2 and IgG_4 isotypes may be used when the antibody molecule is intended for therapeutic purposes and antibody effector functions are not required, e.g. for simply blocking CD154 activity.

The antibody may be useful in the treatment of diseases or disorders including inflammatory diseases and disorders, immune disease and disorders, fibrotic disorders and cancers.

The term "inflammatory disease" or "autoimmune disorder" and "immune disease or disorder" includes rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, juvenile arthritis, Still's disease, Hashimoto's thyroiditis, Graves' disease, Sjogren's syndrome, Goodpasture's syndrome, Addison's disease, vasculitis, including ANCA-associated vasculitis and Wegener's granulomatosis, primary biliary cirrhosis, sclerosing cholangitis, autoimmune hepatitis polymyalgia rheumatica, Guillain-Barre syndrome, antiphospholipid syndrome, idiopathic thrombocytopaenia, autoimmune haemolytic anaemia, pernicious anaemia, pemphigus vulgars, dermatomyositis, bullous pemphigoid, Henoch-Schönlein purpura, Muckle Wells disease, psoriasis, Crohn's disease, ulcerative colitis, SLE (Systemic Lupus Erythematosus), Celiac disease, asthma, allergic rhinitis, atopic dermatitis, multiple sclerosis, vasculitis, Type I diabetes mellitus, transplantation and graft-versus-host disease.

The term "fibrotic disorder", as used herein refers to a disorder characterized by the formation or development of excess fibrous connective tissue in an organ or tissue, frequently as a reparative or reactive process. A fibrotic disorder can affect single organs, such as the lungs (for example without limitation idiopathic pulmonary fibrosis, interstitial lung disease), the liver, the intestine, the kidney, the heart or the skin, or affect multiple organs, for example without limitation in systemic sclerosis. The term fibrotic disorder also relates to scarring of the skin. Scars of the skin include, but are not limited to, keloid scars, contracture scars that occur, for example without limitation after skin burn, hypertrophic scars and acne scars.

The host cell of the invention may also comprise further polynucleotide encoding one or more further proteins of interest.

The recombinant gram-negative bacterial cell according to the present invention may be produced by any suitable means.

The skilled person knows suitable techniques which may be used to insert the recombinant polynucleotide encoding DsbC and the polynucleotide encoding the antibody. The recombinant polynucleotide encoding DsbC and/or the polynucleotide encoding the antibody may be integrated into the cell's genome using a suitable vector such as pKO3 described in Link *et al.* which is incorporated herein by reference in its entirety (Link, Phillips, and Church 6228-37).

Alternatively or additionally, the recombinant polynucleotide encoding DsbC and/or the polynucleotide encoding the antibody may be non-integrated in a recombinant expression cassette. In one embodiment an expression cassette is employed in the present invention to carry the polynucleotide encoding DsbC and/or the polynucleotide encoding the antibody which typically comprises a protein coding sequence encoding DsbC, one or more protein coding sequences encoding the antibody and one or more regulatory 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 gene encoding DsbC and/or the antibody or fragment thereof is/are integrated into the genome of the host cell to create stable cell line.

In one embodiment, the cell according to the present invention comprises one or more expression vectors, such as plasmid. The vector preferably comprises one or more of the expression cassettes as defined above. The host cell preferably comprises an expression vector comprising DNA encoding an antibody or an antigen binding fragment thereof specifically binding to CD154 as described above. Preferably the expression vector comprises a polynucleotide sequence encoding a light chain and a polynucleotide sequence encoding a heavy chain of the antibody or an antigen binding fragment thereof specifically binding to CD154.

In a preferred embodiment, the expression vector is an *E. coli* expression vector.

In one embodiment the polynucleotide sequence encoding the antibody and the polynucleotide encoding DsbC are inserted into separate expression vectors.

For production of products comprising both heavy and light chains, the cell line may be 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 DsbC 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 DsbC and an antibody or an antigen binding fragment thereof specifically binding to CD154. The expression vector is a multi-cistronic vector comprising the polynucleotide sequence encoding DsbC 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 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), a DsbC polynucleotide sequence and a further polynucleotide sequence is shown in Figure 1.

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 FkpA, Skp, SurA, PPiA and PPiD; and/or

- one or more protein capable of facilitating protein secretion or translocation, such as SecY, SecE, SecG, SecYEG, SecA, SecB, FtsY and Lep; and/or
- one or more proteins capable of facilitating disulphide bond formation, such as DsbA, DsbB, DsbD, DsbG;

the one or more further protein may be expressed from one or more polynucleotides inserted into the same vector as the polynucleotide encoding DsbC and/or the one or more polynucleotides encoding the antibody or antigen binding fragment thereof specifically binding to CD154. 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 DsbC and/or the polynucleotide encoding the antibody or antigen binding fragment thereof specifically binding to CD154 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 DsbC and the polynucleotide encoding the antibody are inserted into one vector, the nucleotide sequences encoding DsbC and the antibody may be under the control of a single promoter or separate promoters. In the embodiment wherein the nucleotide sequences encoding DsbC 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-Dalgamo (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 WO 03/048208 or WO 2007/039714 (the contents of which are incorporated herein by reference in their entirety). 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: 33), IGS2 (SEQ ID NO: 34), IGS3 (SEQ ID NO: 35) and IGS4 (SEQ ID NO: 36).

A preferably expression vector comprises a tricistronic message for producing the light chain and the heavy chain of the antibody or antigen binding fragment thereof as described above and a message for producing the recombinant DsbC, preferably comprising a his-tag.

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 Makrides *et al.* which is incorporated herein by reference in its entirety(Makrides 512-38).

The DsbC polynucleotide inserted into the expression vector preferably comprises the nucleic acid encoding the DsbC signal sequence and the DsbC coding sequence. The DsbC protein may also be directed to the periplasm under by genetic fusion to other signal peptides, for example such as those from the proteins: OmpA, MalB, PelB, PhoA, PhoS, LppA, DsbA. 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 DsbC 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 encoding a heavy chain of the antibody and a polynucleotide 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. .

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 present invention also provides a method for producing an antibody or an antigen binding fragment thereof specifically binding to CD154 comprising:

culturing a recombinant gram-negative bacterial cell as defined above in a culture medium under conditions effective to express the antibody or the antigen binding fragment thereof specifically binding to CD154 and the recombinant polynucleotide encoding DsbC; and

recovering the antibody or an antigen binding fragment thereof specifically binding to CD154 from the periplasm of the recombinant gram-negative bacterial cell and/or the culture medium.

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

The recombinant polynucleotide encoding DsbC and the polynucleotide encoding the antibody or antigen binding fragment thereof specifically binding to CD154 may be incorporated into the host cell using any suitable means known in the art. As discussed above, typically the polynucleotide encoding DsbC and the polynucleotide encoding the antibody are incorporated as part of the same or separate expression vectors which are transformed into the cell.

The polynucleotide encoding DsbC and the polynucleotide encoding the antibody or antigen binding fragment thereof specifically binding to CD154 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 encoding a selection marker. In one embodiment, each polynucleotide transformed into the cell further comprises a polynucleotide encoding one or more selection markers. Accordingly, the transformation of the polynucleotides encoding DsbC and the antibody or antigen binding fragment thereof specifically binding to CD154 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.

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

In one embodiment wherein the polynucleotide encoding DsbC and the polynucleotide encoding the antibody are under the control of the same or separate inducible promoters, the expression of the polynucleotide(s) encoding the antibody and the recombinant polynucleotide encoding DsbC is induced by adding an inducer to the culture medium.

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 antibody or antigen binding fragment thereof may be recovered and purified from the strain, including from the cytoplasm, periplasm, or supernatant. Suitable methods include fractionation on immunoaffnity 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.

In one embodiment the method further comprises separating the antibody or antigen binding fragment thereof from DsbC.

Antibodies or antigen binding fragments thereof 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.

One or more method steps described herein may be performed in combination in a suitable container such as a bioreactor.

After expression, the antibody may be further processed, for example by conjugation to another entity such as an effector molecule. Accordingly, the method according to the present invention may comprise a further step of attaching an effector molecule to the antibody.

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 radio-iodide, 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 WO 2005/003171 or WO 2005/003170 (the contents of which are incorporated herein by reference in their entirety). WO 2005/003171 or WO 2005/003170 also describe suitable effector molecules.

The antibody may have a macrocycle, for chelating a heavy metal atom, or a toxin, such as ricin, attached to it by a covalent bridging structure. Alternatively, procedures of recombinant DNA technology may be used to produce an antibody molecule in which the Fc fragment (CH2, CH3 and hinge domains), the CH2 and CH3 domains or the CH3 domain of a complete immunoglobulin molecule has (have) been replaced by, or has attached thereto by peptide linkage, a functional non-immunoglobulin protein, such as an enzyme or toxin molecule. In the embodiment wherein the antibody is a modified Fab fragment having at the

C-terminal end of its heavy chain one or more amino acids to allow attachment of an effector or reporter molecule, the additional amino acids preferably form a modified hinge region containing one or two cysteine residues to which the effector or reporter molecule may be attached.

A preferred effector group is a polymer molecule, which may be attached to the modified Fab fragment to increase its half-life *in vivo*.

The polymer molecule may, in general, be a synthetic or a naturally occurring polymer, for example an optionally substituted straight or branched chain polyalkylene, polyalkenylene or polyoxyalkylene polymer or a branched or unbranched polysaccharide, e.g. a homo- or hetero- polysaccharide.

Particular optional substituents which may be present on the above-mentioned synthetic polymers include one or more hydroxy, methyl or methoxy groups. Particular examples of synthetic polymers include optionally substituted straight or branched chain poly(ethyleneglycol), poly(propyleneglycol) poly(vinylalcohol) or derivatives thereof, especially optionally substituted poly(ethyleneglycol) such as methoxypoly(ethyleneglycol) or derivatives thereof. Particular naturally occurring polymers include lactose, amylose, dextran, glycogen or derivatives thereof. "Derivatives" as used herein is intended to include reactive derivatives, for example thiol-selective reactive groups such as maleimides and the like. The reactive group may be linked directly or through a linker segment to the polymer. It will be appreciated that the residue of such a group will in some instances form part of the product as the linking group between the antibody fragment and the polymer.

The size of the polymer may be varied as desired, but will generally be in an average molecular weight range from 500 Da to 50000 Da, preferably from 5000 Da to 40000 Da and more preferably from 25000 Da to 40000 Da. The polymer size may in particular be selected on the basis of the intended use of the product. Thus, for example, where the product is intended to leave the circulation and penetrate tissue, for example for use in the treatment of an inflammation, it may be advantageous to use a small molecular weight polymer, for example with a molecular weight of around 5000 Da. For applications where the product remains in the circulation, it may be advantageous to use a higher molecular weight polymer, for example having a molecular weight in the range from 25000 Da to 40000 Da.

Particularly preferred polymers include a polyalkylene polymer, such as a poly(ethyleneglycol) or, especially, a methoxypoly(ethyleneglycol) or a derivative thereof, and especially with a molecular weight in the range from about 25000 Da to about 40000 Da.

Each polymer molecule attached to the modified antibody fragment may be covalently linked to the sulphur atom of a cysteine residue located in the fragment. The covalent linkage will generally be a disulphide bond or, in particular, a sulphur-carbon bond.

Where desired, the antibody fragment may have one or more effector or reporter molecules attached to it. The effector or reporter molecules may be attached to the antibody fragment through any available amino acid side-chain or terminal amino acid functional group located in the fragment, for example any free amino, imino, hydroxyl or carboxyl group. One or more effector or reporter molecules may be attached to an amino acid at or towards the C-terminal end of the heavy chain and/or the light chain of the antibody.

An activated polymer may be used as the starting material in the preparation of polymer-modified antibody fragments as described above. The activated polymer may be any polymer containing a thiol reactive group such as an α-halocarboxylic acid or ester, e.g. iodoacetamide, an imide, e.g. maleimide, a vinyl sulphone or a disulphide. Such starting materials may be obtained commercially (for example from Shearwater Polymers Inc., Huntsville, AL, USA) or may be prepared from commercially available starting materials using conventional chemical procedures.

Where it is desired to obtain an antibody fragment linked to an effector or reporter molecule, this may be prepared by standard chemical or recombinant DNA procedures in which the antibody fragment is linked either directly or via a coupling agent to the effector or reporter molecule either before or after reaction with the activated polymer as appropriate. Particular chemical procedures include, for example, those described in WO 93/62331 and WO 92/22583. Alternatively, where the effector or reporter molecule is a protein or polypeptide the linkage may be achieved using recombinant DNA procedures, for example as described in WO 86/01533 and EP-A-0392745.

Preferably, the modified Fab fragment provided by the method of the present invention is PEGylated (i.e. has PEG (poly(ethyleneglycol)) covalently attached thereto) according to the method disclosed in EP-A-0948544. Preferably the antibody is a PEGylated modified Fab fragment as shown in Figure 2. As shown in Figure 2, the modified Fab fragment has attached to one of the cysteine residues at the C-terminal end of the modified hinge region 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 20000 Da, such that the total average molecular weight of the methoxypoly(ethyleneglycol) residues is about 40000 Da, more preferably the lysyl-maleimide-derived group is [1-[[[2-[[3-(2,5-dioxo-1-pyrrolidinyl)-1-oxopropyl]amino]ethyl]amino]-

carbonyl]-1,5-pentanediyl]bis(iminocarbonyl). A lysine residue is covalently linked to the maleimide group. To each of the amine groups on the lysine residue is attached a methoxypoly(ethyleneglycol) polymer having a molecular weight of approximately 20000 Da. The total molecular weight of the entire effector molecule is therefore approximately 40000 Da.

Accordingly, the method according to the present invention preferably comprises attaching to one of the cysteine residues at the C-terminal end of the heavy chain a lysyl-maleimide group wherein each amino group of the lysyl residue has covalently linked to it a methoxypoly(ethyleneglycol) residue having a molecular weight of about 20000 Da.

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 (US 5,304,472) are avoided in order to maintain a functional PhoS protein.

EXAMPLES

Cell Lines

For all experiments the *E. coli* cell line W3110 was used as the parental wild-type cell line.

Cell lines were created carrying the following mutations:

a. a mutated Tsp gene;

b. a mutated Tsp gene and carrying recombinant DsbC;

c. a mutated Tsp gene and a mutated spr gene;

d. a mutated Tsp gene and a mutated spr gene and carrying recombinant DsbC.

Example 1 Generation of cell line carrying mutated Tsp gene MXE001 (ΔTsp)

The MXE001 strain was generated as follows:

The Tsp cassette was moved as Sal I, Not I restriction fragments into similarly restricted pKO3 plasmids. The pKO3 plasmid uses the temperature sensitive mutant of the pSC101 origin of replication (*RepA*) along with a chloramphenicol marker to force and select for chromosomal integration events. The *sacB* gene which encodes for levansucrase is lethal to *E. coli* grown on sucrose and hence (along with the chloramphenicol marker and pSC101 origin) is used to force and select for de-integration and plasmid curing events. This methodology had been described previously (Hamilton et al. 4617-22;Blomfield et al. 1447-57). The pKO3 system removes all selective markers from the host genome except for the inserted gene.

The following plasmids were constructed:

pMXE191 comprising the knockout mutated Tsp gene as shown in the SEQ ID NO: 28 comprising *EcoR I* and *Ase I* restriction markers.

The plasmid was then transformed into electro-competent competent *E. coli* W3110 cells prepared using the method found in Miller and Nickoloff which is incorporated herein by reference in its entirety (Miller and Nickoloff 105-13).

Day 1: 40 µl of *E.coli* cells were mixed with (10 pg) 1 µl of pKO3 DNA in a chilled BioRad 0.2 cm electroporation cuvette before electroporation at 2500V, 25 µF and 200Ω. 1000 µl of 2xPY was added immediately, the cells recovered by shaking at 250 rpm in an incubator at 30°C for 1 hour. Cells were serially 1/10 diluted in 2xPY before 100 µl aliquots were plated out onto 2xPY agar plates containing chloramphenicol at 20 µg/ml prewarmed at 30°C and 43°C. Plates were incubated overnight at 30°C and 43°C.

Day 2: The number of colonies grown at 30°C gave an estimate of the efficiency of electroporation whilst colonies that survive growth at 43°C represent potential integration events. Single colonies from the 43°C plate were picked and resuspended in 10 ml of 2xPY. 100 µl of this was plated out onto 2xPY agar plates containing 5% (w/v) sucrose pre-warmed to 30°C to generate single colonies. Plates were incubated overnight at 30°C.

Day 3: Colonies here represent potential simultaneous de-integration and plasmid curing events. If the de-integration and curing events happened early on in the growth, then the bulk of the colony mass will be clonal. Single colonies were picked and replica plated onto 2xPY agar

that contained either chloramphenicol at 20 µg/ml or 5% (w/v) sucrose. Plates were incubated overnight at 30°C.

Day 4: Colonies that both grow on sucrose and die on chloramphenicol represent potential chromosomal replacement and plasmid curing events. These were picked and screened by PCR with a mutation specific oligonucleotide. Colonies that generated a positive PCR band of the correct size were struck out to produce single colonies on 2xPY agar containing 5% (w/v) sucrose and the plates were incubated overnight at 30°C.

Day 5: Single colonies of PCR positive, chloramphenicol sensitive and sucrose resistant *E. coli* were used to make glycerol stocks, chemically competent cells and act as PCR templates for a PCR reaction with 5' and 3' flanking oligonucleotide primers to generate PCR product for direct DNA sequencing using Taq polymerase.

Cell strain MXE001 was tested to confirm successful modification of genomic DNA carrying the mutated Tsp gene by PCR amplification of the region of the Tsp gene comprising a non-naturally occurring Ase I restriction site, (SEQ ID NO: 28), using oligonucleotides primers. The amplified regions of the DNA were then analyzed by gel electrophoresis before and after incubation with Ase I restriction enzyme to confirm the presence of the non-naturally occurring Ase I restriction site in the mutated genes. This method was carried out as follows:

The following oligos were used to amplify, using PCR, genomic DNA from prepared *E. coli* cell lysates from MXE001 and W3110:

6284 Tsp 3' 5'-GCATCATAATTTTCTTTTTACCTC-3' (SEQ ID NO: 47)

6283 Tsp 5' 5'-GGGAAATGAACCTGAGCAAAACGC-3' (SEQ ID NO: 48)

The lysates were prepared by heating a single colony of cells for 10 minutes at 95°C in 20 µl of 1 x PCR buffer. The mixture was allowed to cool to room temperature then centrifugation at 13,200 rpm for 10 minutes. The supernatant was removed and labeled as 'cell lysate'.

Each strain was amplified using the Tsp oligonucleotide pair.

The DNA was amplified using a standard PCR procedure.

5 µl Buffer x10 (Roche)

1 µl	dNTP mix (Roche, 10mM mix)
1.5 µl	5' oligo (5 pmol)
1.5 µl	3' oligo (5 pmol)
2 µl	Cell lysate
0.5 µl	Taq DNA polymerase (Roche 5 U/µl)
38.5 µl	H_2O

PCR cycle.

94°C	1 minute
94°C	1 minute
55°C	1 minute (repeated for 30 cycles)
72°C	1 minute
72°C	10 minutes

Once the reactions were complete 25 µl was removed to a new microfuge tube for digestion with Ase I. To the 25 µl of PCR reaction 19 µl of H_2O, 5 µl of buffer 3 (New England Biolabs®), 1 µl of Ase I (New England Biolabs®) was added, mixed and incubated at 37°C for 2 hours.

To the remaining PCR reaction 5 µl of loading buffer (x6) was added and 20 µl was loaded onto a 0.8% TAE 200 ml agarose gel (Invitrogen®) plus ethidium bromide (5 µl of 10 mg/ml stock) and run at 100 V for 1 hour. 10 µl of size marker (Perfect DNA marker 0.1-12Kb, Novagen®) was loaded in the final lane.

Once the Ase I digestions were complete 10 µl of of loading buffer (x6) was added and 20 µl was loaded onto a 0.8% TAE agarose gel (Invitrogen®) plus ethidium bromide (5 µl of 10mg/ml stock) and run at 100 V for 1 hour. 10 µl of size marker (Perfect DNA marker 0.1-12 Kb, Novagen®) was loaded in the final lane. Both gels were visualized using UV transluminator.

The genomic fragment amplified showed the correct sized band of 2.8 Kb for Tsp. Following digestion with Ase I this confirmed the presence of the introduced Ase I sites in the Tsp deficient strain MXE001 but not in the W3110 control.

MXE001: genomic DNA amplified using the Tsp primer set and the resulting DNA was digested with Ase I to produce 2.2 and 0.6 Kbps bands.

W3110 PCR amplified DNA was not digested by Ase I restriction enzyme.

Example 2 Generation of cell lines carrying mutated spr gene and cell lines carrying a mutated Tsp gene and a mutated spr gene

The spr mutations were generated and selected for using a complementation assay.

The spr gene (SEQ ID NO: 23) was mutated using the Clontech® random mutagenisis diversity PCR kit which introduced 1 to 2 mutations per 1000 bp. The mutated spr PCR DNA was cloned into an inducible expression vector [pTTO CDP870] which expresses CDP870 Fab' (as described in WO 01/94585) along with the spr mutant. This ligation was then electro-transformed into an *E.coli* strain comprising a deletion variant of Tsp (ΔTsp) (designed MXE001) prepared using the method found in Miller *et al.* (Miller and Nickoloff 105-13). The following protocol was used, 40 µl of electro competent MXE001, 2.5µl of the ligation (100 pg of DNA) was added to a 0.2 cm electroporation cuvette, electro-transformation was performed using as BioRad® Genepulser Xcell® with the following conditions, 2500 V, 25 µF and 200Ω. After the electro-transformation 1 ml of S.OC. medium (Invitrogen® catalog: 18045-088) (pre-warmed to 37°C) was added and the cells left to recover at 37°C for 1 hour with gentle agitation.

The cells where plated onto hypotonic agar [5 g/L yeast extract, 2.5 g/L Tryptone, 15 g/L Agar (all Difco®)] and incubated at 40°C. Cells which formed colonies were re-plated onto HLB at 43°C to confirm restoration of the ability to grow under low osmotic conditions at high temperature to the MXE001 strain. Plasmid DNA was prepared from the selected clones and sequenced to identify spr mutations.

Using this method eight single, one double mutation and two multiple mutations in the spr protein were isolated which complemented the ΔTsp phenotype as follows:

1. V98E
2. D133A
3. V135D
4. V135G
5. G147C
6. S95F and Y115F
7. I70T
8. N31T, Q73R, R100G, G140C
9. R62C, Q99P, R144C
10. L108S

11. L136P

The individual mutations 1 to 5 identified above and three catalytic triad mutations of spr (C94A, H145A, H157A) and W174R were used to generate new strains using either the wild-type W3110 *E. coli* strain (genotype: F- LAM- IN (rrnD-rrnE)1 rph1 (ATCC catalog no. 27325) to create spr mutated strains or MXE001 strain from Example 1 to make combined ΔTsp/mutant spr strains.

The following mutant *E. coli* cell strains were generated using a gene replacement vector system using the pKO3 homologous recombination/replacement plasmid (Link *et al.*, *supra*), as described in Example 1 for the generation of MXE001.

Table 1

Mutant *E. coli* Strain	**Genotype**	**Spr Vectors**
MXE001	ΔTsp	-
MXE008	ΔTsp, spr D133A	pMXE339, pK03 spr D133A (-SalI)
MXE009	ΔTsp, spr H157A	pMXE345, pK03 spr H157A (-SalI)
MXE010	spr G147C	pMXE338, pK03 spr G147C (-SalI)
MXE011	spr C94A	pMXE343, pK03 spr C94A (-SalI)
MXE012	spr H145A	pMXE344, pK03 spr H145A (-SalI)
MXE013	spr W174R	pMXE346, pK03 spr W174R (-SalI)
MXE014	ΔTsp, spr V135D	pMXE340, pK03 spr V135D (-SalI)
MXE015	ΔTsp, spr V98E	pMXE342, pK03 spr V98E (-SalI)
MXE016	ΔTsp, spr C94A	pMXE343, pK03 spr C94A (-SalI)
MXE017	ΔTsp, spr H145A	pMXE344, pK03 spr H145A (-SalI)
MXE018	ΔTsp, spr V135G	pMXE341, pK03 spr V135G (-SalI)

The mutant spr integration cassettes were moved as Sal I, Not I restriction fragments into similarly restricted pKO3 plasmids.

For all experiments the *E.coli* cell line W3110 was used as the wild-type cell line and the *E.coli* cell line W3110 ΔTsp, spr C94A (MX016).

Example 3 - Generation of plasmid for anti-CD154 Fab' and DsbC expression

A plasmid was constructed containing both the heavy and light chain sequences of an anti-CD154 Fab (SEQ ID NOs: 13 and 11, respectively) and the sequence encoding DsbC (SEQ ID NO: 27).

Plasmid pMXE351 (pTTOD_DsbC), an expression vector for the anti-CD154 Fab and DsbC (a periplasmic polypeptide), was constructed using conventional restriction cloning methodologies. The plasmid pMXE351 contained the following features; a strong *tac* promoter and *lac* operator sequence. As shown in Figure 1, the plasmid contained a unique EcoRI restriction site after the coding region of the Fab' heavy chain, followed by a non-coding sequence and then a unique NdeI restriction site. The DsbC gene was PCR cloned using W3110 crude chromosomal DNA as a template. An EcoRI site was removed from the wild type DsbC sequence by PCR overlap extension such that the PCR product 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 DsbC, terminating in a C-terminal His tag and finally a non-coding NdeI site. The EcoRI-NdeI PCR fragment was restricted and ligated into the expression vector such that all three polypeptides: Fab' light chain, Fab' heavy chain and DsbC were encoded on a single polycistronic mRNA.

The Fab light chain, heavy chain genes and DsbC gene 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.

Example 4 – Expression of anti-CD154 Fab' and DsbC in *E.coli* W3110 and MXE016 (*E. coli* W3110 ΔTsp, spr C94A)

Expression of anti-CD154 Fab' and DsbC in E. coli W3110 ΔTsp, spr C94A

The *E.coli* W3110 ΔTsp, spr C94A cell strain (MXE016) was transformed with the plasmid pMXE351 generated in Example 3. The transformation of the strains was carried out using the method found in Chung C.T *et al.* (Chung, Niemela, and Miller 2172-75).

Expression of anti-CD154 Fab'in E. coli W3110

The *E. coli* W3110 cell strain was transformed with plasmid pTTOD, an expression vector for the anti-CD154 Fab', which was constructed using conventional restriction cloning methodologies. The plasmid pTTOD contained the following features; a strong *tac* promoter and *lac* operator sequence. The Fab light and heavy chain genes were transcribed as a single dicistronic 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. The transformation of the strains was carried out using the method found in Chung C.T *et al.* (Chung, Niemela, and Miller 2172-75).

Example 5 – Growth of mutated *E. coli* strains and expression of anti-CD154 Fab' in mutated *E. coli* strains using high density fermentations

The strains produced in Example 4 were tested in fermentation experiments comparing growth and expression of an anti-CD154 Fab':

Growth medium, inoculum and fermentation steps. The fermentation process is 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 are grown in defined media to high density in batch and fed-batch mode. When the desired cell density is reached expression of the Fab' is 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 is applied during the induction phase to control expression and cell growth. Temperature, dissolved oxygen (pO_2) and pH are controlled to maintain the culture within optimal culture conditions.

Measurement of biomass concentration and growth rate. Biomass concentration was 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 16 hours the extract was

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 1 ml column (GE-Healthcare® or equivalent) was loaded with analyte (approximately neutral pH, 30°C, 0.2 µm filtered) at 2 ml/min, the column was washed with 20 mM phosphate, 50 mM NaCl pH 7.4 and then Fab' eluted using an injection of 50 mM 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.

Example 6 – Level of light chain fragments in fermentation extractions of mutated *E. coli* strains

The fermentations presented in Example 2 were tested for light chain fragment level of anti-CD154 Fab' in periplasmic extractions.

Light chain fragment quantification. Quantitative estimation of the level of Fab' and Fab' proteolytic fragments was achieved by high-temperature reversed phase HPLC. Separation is performed on a Poroshell® 300SB-C8 reversed phase column (Agilent Technologies®, Product No. 660750-906) at a temperature of 80°C. The equilibration solvent is HPLC water, 0.1 % (v/v) TFA, and the elution solvent is 80:20 (v/v) 1-propanol:acetonitrile, 0.03 % (v/v) TFA. Separation is performed at a flow rate of 2.0 mL/min, by means of a linear gradient of 16-38 % solvent B in 4.4 min. Detection was by UV absorbance at 214 nm. Data were processed by manual integration, and the quantity of Fab proteolytic fragments expressed as % peak area relative to the intact Fab peak.

The present invention also provides a therapeutic or diagnostic composition comprising the antibody produced by the method of the present invention in combination with a pharmaceutically acceptable excipient, diluent or carrier.

The present invention also provides a process for preparation of a therapeutic or diagnostic composition comprising admixing the antibody produced by the method of the present invention together with a pharmaceutically acceptable excipient, diluent or carrier.

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 detail may be made without departing from the scope of the invention as defined by the appending claims.

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CLAIMS

1) A recombinant gram-negative bacterial cell comprising:

a) an expression vector comprising a recombinant polynucleotide encoding DsbC; and

b) one or more polynucleotides encoding an antibody or an antigen binding fragment thereof specifically binding to CD154;

c) a mutated Tsp gene encoding a Tsp protein having 50% or less of the protease activity of a wild-type non-mutated Tsp protein as shown in SEQ ID NO: 26 and;

d) a mutated spr gene encoding an spr protein whose wild-type sequence is shown in SEQ ID NO: 24 comprising a mutation affecting the amino acid C94 ; and

wherein said gram-negative bacterial cell is selected from E. coli strains K12 and W3110, and wherein the bacterial cell is isogenic to said strains except for the recombinant polynucleotide encoding DsbC, the one or more polynucleotides encoding an antibody or an antigen binding fragment thereof specifically binding to CD154 and the mutated Tsp gene and the mutated spr gene.

2) The gram-negative bacterial cell according to claim 1, wherein DsbC comprises a histidine-tag at the N-terminus or the C-terminus.

3) The gram-negative bacterial cell according to claim 1 or 2, wherein the expression vector comprises a polynucleotide having the sequence given in SEQ ID NO:45 or SEQ ID NO:51.

4) The gram-negative bacterial cell according to any one of claims 1 to 3, wherein the antibody or antigen binding fragment thereof comprises a heavy chain variable domain comprising three CDRs having the sequence given in SEQ ID NO: 1 for CDRH1, SEQ ID NO: 2, for CDRH2 and SEQ ID NO: 3 for CDRH3 and a variable domain light chain comprising three CDRs having the sequence given in SEQ ID NO: 4 for CDRL1, SEQ ID NO: 5 for CDRL2 and SEQ ID NO: 6 for CDRL3.

5) The gram-negative bacterial cell according to claim 4, wherein the one or more polynucleotides encode an antibody comprising the light chain variable region sequence given in SEQ ID NO:8 and the heavy chain variable region given in SEQ ID NO:10.

6) The gram-negative bacterial cell according to any of claims 1 to 5, wherein the antibody is a Fab or Fab' fragment.

7) The gram-negative bacterial cell according to claim 6, wherein the Fab or Fab' fragment comprises a light chain having the sequence given in SEQ ID NO:12 and a heavy chain having the sequence given in SEQ ID NO:14 or 16.

8) The gram-negative bacterial cell according to any one of claims 1 to 7, wherein the gram-negative bacterial cell comprises a first expression vector comprising a recombinant polynucleotide encoding DsbC and a second expression vector comprising one or more polynucleotides encoding an antibody or an antigen binding fragment thereof specifically binding to CD154.

9) The gram-negative bacterial cell according to any one of claims 1 to 7, wherein the expression vector comprising a recombinant polynucleotide encoding DsbC additionally comprises one or more polynucleotides encoding an antibody or an antigen binding fragment thereof specifically binding to CD154.

10) The gram-negative bacterial cell according to any preceding claim, wherein the mutation in the spr gene encoding an spr protein results in a change of the amino acid cysteine to alanine at position 94 (C94A).

11) The gram-negative bacterial cell according to any of claims 1 to 10, which comprises an expression vector comprising a recombinant polynucleotide encoding DsbC and a dicistronic message for producing an antibody or antigen binding fragment thereof specifically binding to CD154, in which 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, characterised in that the dicistronic message comprises a sequence selected from IGS1 (SEQ ID NO: 33), IGS2 (SEQ ID NO: 34), IGS3 (SEQ ID NO: 35) and IGS4 (SEQ ID NO: 36).

12) A method for producing an antibody or an antigen binding fragment thereof specifically binding to CD154 comprising:

a) culturing a recombinant gram-negative bacterial cell as defined in any of claims 1 to 11 in a culture medium under conditions effective to express the antibody or the antigen binding fragment thereof specifically binding to CD154 and the recombinant polynucleotide encoding DsbC; and

b) recovering the antibody or an antigen binding fragment thereof specifically binding to CD154 from the periplasm of the recombinant gram-negative bacterial cell and/or the culture medium.

13) A method according to claims 12, wherein the method further comprises a step of attaching an effector molecule to an amino acid at or towards the C-terminal end of the heavy chain and/or the light chain of the antibody.

14) A method according to claim 13, wherein the effector molecule comprises poly(ethyleneglycol) or methoxypoly(ethyleneglycol).

15) A method according to claim 14, wherein the method comprises attaching to one of the cysteine residues at the C-terminal end of the heavy chain a lysyl-maleimide group wherein each amino group of the lysyl residue has covalently linked to it a methoxypoly(ethyleneglycol) residue having a molecular weight of about 20,000 Da.