Journal of Biotechnology. 135(4):358-65, 2008 Jul 31; Champion KM. Nishihara JC. Joly JC. Arnott D. Similarity of the Escherichia coli proteome upon completion of different biopharmaceutical fermentation processes. [Journal Article] Proteomics. 1(9):1133-48, 2001 Sep; and Horn U. Strittmatter W. Krebber A. Knupfer U. Kujau M. Wenderoth R. Muller K. Matzku S. Pluckthun A. Riesenberg D. High volumetric yields of functional dimeric miniantibodies in Escherichia coli, using an optimized expression vector and high-cell-density fermentation under non-limited growth conditions, Journal Article. Research Support, Non-U.S. Gov't Applied Microbiology & Biotechnology. 46(5-6):524-32, 1996 Dec. The skilled person would also easily be able to test secreted protein to see if the protein is correctly folded using methods well known in the art, such as protein G HPLC, circular dichroism, NMR, X-Ray crystallography and epitope affinity measurement methods.

The present invention will now be described in more detail. All embodiments herein described refer to the first, second and third aspects of the present invention unless specifically stated otherwise.

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

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

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

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

The expression "phenotype of a cell comprising a mutated Tsp gene" in the context of the present invention means the phenotype exhibited by a cell harbouring a mutant Tsp gene. Typically cells comprising a mutant Tsp gene may lyse, especially at high cell densities. The lysis of these cells causes any recombinant protein to leak into the

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

The present invention also provides a recombinant gram-negative bacterial cell comprising a mutated Tsp gene, wherein the mutated Tsp gene encodes a Tsp protein having reduced protease activity or is a knockout mutated Tsp gene, a mutant spr gene encoding a mutatnt spr and a polynucleotide sequence encoding an antibody or an antigen binding fragment thereof specific for TNF

In a preferred embodiment the protein of interest expressed by the cells according to the present invention is an anti-TNF antibody, more preferably an anti-TNF Fab', as described in WO01/094585 ~~(the contents of which are incorporated herein by reference)~~.

In a one embodiment the antibody having specificity for human TNFα, comprises a heavy chain wherein the variable domain comprises a CDR having the sequence shown in SEQ ID NO:26 for CDRH1, the sequence shown in SEQ ID NO:27 or SEQ ID NO:32 for CDRH2 or the sequence shown in SEQ ID NO:28 for CDRH3.

In one embodiment the antibody comprises a light chain wherein the variable domain comprises a CDR having the sequence shown in SEQ ID NO:29 for CDRL1, the sequence shown in SEQ ID NO:30 for CDRL2 or the sequence shown in SEQ ID NO:31 for CDRL3.

The CDRs given in SEQ IDS NOS:26 and 28 to 32 referred to above are derived from a mouse monoclonal antibody hTNF40. However, SEQ ID NO:27 consists of a hybrid CDR. The hybrid CDR comprises part of heavy chain CDR2 from mouse monoclonal antibody hTNF40 (SEQ ID NO:32) and part of heavy chain CDR2 from a human group 3 germline V region sequence.

In one embodiment the antibody comprises a heavy chain wherein the variable domain comprises a CDR having the sequence shown in SEQ ID NO:26 for CDRH1, the sequence shown in SEQ ID NO:27 or SEQ ID NO:32 for CDRH2 or the sequence shown in SEQ ID NO:28 for CDRH3 and a light chain wherein the variable domain comprises a CDR having the sequence shown in SEQ ID NO:29 for CDRL1, the sequence shown in SEQ ID NO:30 for CDRL2 or the sequence shown in SEQ ID NO:31 for CDRL3.

In one embodiment the antibody comprises SEQ ID NO:26 for CDRH1, SEQ ID NO: 27 or SEQ ID NO:32 for CDRH2, SEQ ID NO:28 for CDRH3, SEQ ID NO:29 for

CDRL1, SEQ ID NO:30 for CDRL2 and SEQ ID NO:31 for CDRL3. Preferably the antibody comprises SEQ ID NO:27 for CDRH2.

The anti-TNF antibody is preferably a CDR-grafted antibody molecule. In a preferred embodiment the variable domain comprises human acceptor framework regions and non-human donor CDRs.

Preferably the antibody molecule has specificity for human TNF (formerly known as TNFα), wherein the light chain comprises the light chain variable region of SEQ ID NO: 11 and the heavy chain comprises the heavy chain variable region of SEQ ID NO: 12.

The anti-TNF antibody is preferably a Fab or Fab' fragment.

Preferably the antibody molecule having specificity for human TNF is a Fab' and has a light chain sequence comprising or consisting of SEQ ID NO: 13 and a heavy chain sequence comprising or consisting of SEQ ID NO: 14.

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

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 radioiodide, radioisotopes, chelated metals, nanoparticles and reporter groups such as fluorescent compounds or compounds which may be detected by NMR or ESR spectroscopy. Effector molecular may be attached to the antibody or fragment thereof by any suitable method, for example an antibody fragment may be modified to attach at least one effector molecule as described in WO05/003171 or WO05/003170 ~~(the contents of which are incorporated herein by reference)~~. WO05/003171 or WO05/003170 also describe suitable effector molecules.

In one embodiment the antibody or fragment thereof, such as a Fab, is PEGylated to generate a product with the required properties, for example similar to the whole antibodies, if required. For example, the antibody may be a PEGylated anti-TNF- α Fab', as described in WO01/094585, preferably having attached to one of the cysteine residues at the C-terminal end of the heavy chain a lysyl-maleimide-derived group wherein each of the two amino groups of the lysyl residue has covalently linked to it a methoxypoly(ethyleneglycol) residue having a molecular weight of about 20,000 Da, such

that the total average molecular weight of the methoxypoly(ethyleneglycol) residues is about 40,000Da, more preferably the lysyl-maleimide-derived group is [1-[[[2-[[3-(2,5-dioxo-1-pyrrolidinyl)-1-oxopropyl]amino]ethyl]amino]-carbonyl]-1,5-pentanediyl]bis(iminocarbonyl).

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

In one embodiment one or more *E.coli* host proteins that in the wild type are known to co-purify with the recombinant protein of interest during purification are selected for genetic modification, as described in Humphreys et al. "Engineering of *Escherichia coli* to improve the purification of periplasmic Fab' fragments: changing the pI of the chromosomally encoded PhoS/PstS protein", Protein Expression and Purification 37 (2004) 109-118 and WO04/035792 ~~(the contents of which are incorporated herein by reference)~~. The use of such modified host proteins improves the purification process for proteins of interest, especially antibodies, produced in *E.col* by altering the physical properties of selected *E.coli* proteins so they no longer co-purify with the recombinant antibody. Preferably the *E.coli* protein that is altered is selected from one or more of Phosphate binding protein (PhoS/PstS), Dipeptide binding protein (DppA), Maltose binding protein (MBP) and Thioredoxin.

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

Also preferred is the modification of specific residues of the contaminating *E.coli* protein to alter its physical properties, either alone or in combination with the addition of N or C terminal tags. Such changes can include insertions or deletions to alter the size of the protein or amino acid substitutions to alter pI or hydrophobicity. In one embodiment these residues are located on the surface of the protein. In a preferred embodiment surface residues of the PhoS protein are altered in order to reduce the pI of the protein. Preferably

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.

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

In the embodiments of the present invention wherein a polynucleotide sequence comprises two or more encoding sequences for two or more proteins of interest, for example an antibody light chain and antibody heavy chain, the polynucleotide sequence may comprise one or more internal ribosome entry site (IRES) sequences which allows translation initiation in the middle of an mRNA. An IRES sequence may be positioned between encoding polynucleotide sequences to enhance separate translation of the mRNA to produce the encoded polypeptide sequences.

The expression vector preferably also comprises a dicistronic message for producing the antibody or antigen binding fragment thereof as described in WO 03/048208 or WO2007/039714 ~~(the contents of which are incorporated herein by reference)~~. 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: 34), IGS2 (SEQ ID NO: 35), IGS3 (SEQ ID NO: 36) and IGS4 (SEQ ID NO: 37).

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

Further suitable transcriptional regulators including promoters and terminators and protein targeting methods may be found in "Strategies for Achieving High-Level

curve was made by diluting Bovine Serum Albumin standard over a range of 25-1000µg/mL. Samples were diluted in water so that the optical density fell within the linear range of the method (5 to 10 times), and 33 µL of sample or standard were mixed with 1 mL of coomassie reagent. After incubating for 10 minutes at room temperature, the OD_{595nm} was read on a spectrophotometer with coomassie reagent as a blank. The total proteins concentration was calculated based on the standard curve. The results are shown in Figure 10.

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 appendant claims~~.

CLAIMS

1. A recombinant gram-negative bacterial cell comprising a mutant spr gene, Uniprot/ Swiss-Prot P0AFV4, encoding a spr protein having a mutation at one or more amino acids selected from N31Y, R62C, I70T, Q73R, C94A, S95F, V98E, Q99P, R100G, L108S, Y115F, D133A, V135D, V135G, L136P, G140C, R144C, H145A, G147C and H157A and wherein the cell has reduced Tsp protein activity compared to a wild-type cell.

2. A cell according to clam 1, wherein the mutant spr gene encodes a spr protein having the mutations S95F and Y115F.

3. A cell according to claims 1 or 2, wherein the cell further comprises one or more of the following mutated genes:

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

   b) 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

   c) a mutated OmpT gene, wherein the mutated OmpT gene encodes a OmpT protein having reduced protease activity or is a knockout mutated OmpT gene.

4. A cell according to claim 1, 2 or 3, wherein the cell comprises a mutated Tail-specific protease (Tsp) gene encoding a Tsp protein having reduced protease activity or is a knockout mutated Tsp gene.

5. A cell according to claim 4, wherein the cell's genome is isogenic to a wild-type bacterial cell except for the mutated spr gene and the mutated Tsp gene.

6. A cell according to any one of claims 1 to 5, wherein the cell has a knockout mutated Tsp gene which comprises a mutation to the gene start codon and/or one or more stop codons positioned downstream of the gene start codon and upstream of the gene stop codon.

7. A cell according to claim 6, wherein the knockout mutated Tsp gene comprises a restriction marker site created by a missense mutation to the gene start codon and optionally one or more further point mutations.

8. A cell according to claim 7, wherein the knockout mutated Tsp gene comprises SEQ ID NO:3.

9. A cell according to any one of claims 1 to 8, wherein the cell is *E. coli*.

10. A cell according to any preceding claim, wherein the cell comprises a polynucleotide sequence encoding a protein of interest.