promoter or separate promoters. In the embodiment wherein the nucleotide sequences encoding DsbC and the protein of interest are under the control of separate promoters, the promoter may be independently inducible promoters.

Expression units 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.

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: 36), IGS2 (SEQ ID NO: 37), IGS3 (SEQ ID NO: 38) and IGS4 (SEQ ID NO: 39).

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

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

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

In one embodiment, the 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.

In one aspect of the present invention there is provided a recombinant gram-negative bacterial cell comprising a mutant spr gene encoding a mutant spr protein, a wild-type Tsp gene and a polynucleotide sequence encoding an antibody or an antigen binding fragment thereof specific for TNF. The wild-type chromosomal Tsp gene is preferably a non-recombinant chromosomal Tsp gene. Preferably, the cell further comprises a recombinant polynucleotide encoding DsbC.

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:28 for CDRH1, the sequence shown in SEQ ID NO:29 or SEQ ID NO:34 for CDRH2 or the sequence shown in SEQ ID NO:30 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:31 for CDRL1, the sequence shown in SEQ ID NO:32 for CDRL2 or the sequence shown in SEQ ID NO:33 for CDRL3.

The CDRs given in SEQ IDS NOS:28 and 30 to 34 referred to above are derived from a mouse monoclonal antibody hTNF40. However, SEQ ID NO:29 consists of a hybrid CDR. The hybrid CDR comprises part of heavy chain CDR2 from mouse monoclonal antibody hTNF40 (SEQ ID NO:34) 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:28 for CDRH1, the sequence shown in SEQ ID NO:29 or SEQ ID NO:34 for CDRH2 or the sequence shown in SEQ ID NO:30 for CDRH3 and a light chain wherein the variable domain comprises a CDR having the sequence shown in SEQ ID NO:31 for CDRL1, the sequence shown in SEQ ID NO:32 for CDRL2 or the sequence shown in SEQ ID NO:33 for CDRL3.

In one embodiment the antibody comprises SEQ ID NO:28 for CDRH1, SEQ ID NO: 29 or SEQ ID NO:34 for CDRH2, SEQ ID NO:30 for CDRH3, SEQ ID NO:31 for

CDRL1, SEQ ID NO:32 for CDRL2 and SEQ ID NO:33 for CDRL3. Preferably the antibody comprises SEQ ID NO:29 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

CLAIMS

1. A method for producing a recombinant protein of interest comprising culturing a recombinant gram-negative bacterial cell in a culture medium under conditions effective to express the recombinant protein of interest and recovering the recombinant protein of interest from the periplasm of the recombinant gram-negative bacterial cell and/or the culture medium, characterized in that the recombinant gram-negative bacterial cell comprises

(a) a mutant *spr* gene encoding a mutant spr protein capable of suppressing the phenotype of a cell comprising a mutated *Tsp* gene having one or more mutations selected from N31Y, R62C, I70T, Q73R, C94A, S95F, V98E, Q99P, R100G, L108S, Y115F, D133A, V135D, V135G, L136P, G140C, R144C, H145A, G147C, H157A and W174R according to the amino acid sequence of SEQ ID NO:21, and

~~1.~~ (b) a non-recombinant wild-type chromosomal *Tsp* gene.

~~2. The method according to claim 1, wherein the mutant *spr* gene encodes a spr protein having a mutation at one or more amino acids selected from N31, R62, I70, Q73, C94, S95, V98, Q99, R100, L108, Y115, D133, V135, L136, G140, R144, H145, G147, H157 and W174 according to the amino acid sequence of SEQ ID NO:21.~~

~~3. The method according to claim 2, wherein the mutant *spr* gene encodes an spr protein having one or more mutations selected from N31Y, R62C, I70T, Q73R, C94A, S95F, V98E, Q99P, R100G, L108S, Y115F, D133A, V135D, V135G, L136P, G140C, R144C, H145A, G147C, H157A and W174R according to the amino acid sequence of SEQ ID NO:21.~~

~~4.~~2. The method according to clam ~~3~~1, wherein the mutant *spr* gene encodes a spr protein having the mutations S95F and Y115F according to the amino acid sequence of SEQ ID NO:21.

~~5.~~3. The method according to claim ~~3~~1, wherein the spr protein mutation is H145A according to the amino acid sequence of SEQ ID NO:21.

~~6.~~4. The method according to any one of claims 1 to ~~5~~3, wherein the cell is isogenic to a wild-type bacterial cell except for the mutated *spr* gene.

~~7.~~5. The method according to any one of claims 1 to ~~6~~4, wherein the cell further comprises a recombinant polynucleotide encoding DsbC.

~~8.~~6. The method according to any one of claims 1 to ~~7~~5, wherein the cell further comprises one of 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.

~~9.~~7. The method according to any one of claims 1 to ~~8~~6, wherein the cell is *E. coli*.

~~10.~~8. The method according to any one of claims 1 to ~~9~~7, wherein the cell comprises a vector comprising the recombinant polynucleotide encoding DsbC and the polynucleotide sequence encoding a protein of interest.

~~11.~~9. The method according to claim ~~10~~8, wherein the vector comprises a promoter which controls the expression of the recombinant polynucleotide encoding DsbC and the polynucleotide sequence encoding a protein of interest.

~~12.~~10. The method according to claim ~~10~~ 8 or ~~11~~9, wherein the protein of interest is an antibody or an antigen binding fragment thereof.

~~13.~~11. The method according to claim ~~12~~10, wherein the antibody or antigen binding fragment thereof is specific for TNF.

~~14.~~12. A method according to any one of claims 1 to ~~13~~11, wherein the recombinant protein of interest is recovered from the periplasm and/or the supernatant.

~~15.~~13. The method according to claim ~~14~~12, which comprises the further downstream processing step of PEGylation of the protein of interest.