

DORMANCY INDUCED MYCOBACTERIUM PROTEINS

Field of the Invention

The present invention relates to proteins expressed in dormant
5 *Mycobacterium* and to the use of such polypeptides in the prophylaxis, diagnosis and treatment of mycobacterial infections.

Background to the Invention

Upon infection with *Mycobacterium tuberculosis* most individuals mount an
10 effective immune response causing the infection to enter a latent state. The tubercle bacillus may remain in the host for years without causing the symptoms of tuberculosis, with possible reactivation later in life. Little is known about the state in which the bacilli survive in the host during latent infection. Some evidence suggests that cells survive in a state that is similar to the state of bacilli in non-oxygen limiting
15 or hypoxic stationary phase culture.

Mycobacteria are obligate aerobes, i.e. they require oxygen for growth. However, tubercle bacilli encounter hypoxic environments in acute disease as well as in latent infection. Recent genetic evidence suggests that the capability of tubercle bacilli to adapt to hypoxic conditions plays a role *in vivo*. A link has been
20 established between oxygen starvation and drug resistance. Upon depletion of oxygen in culture the bacillus enters a hypoxic growth phase then terminates growth and develops into a dormant form. Importantly, the dormant form of the bacterium is resistant against conventional anti-mycobacterials. Hence, hypoxic dormant bacteria could, at least in part, be responsible for the observed persistence of the pathogen
25 during chemotherapy.

The Wayne dormancy culture system (Wayne and Hayes (1996) Infect. Immun. 64: 2062-2069) is based on growth of the bacilli under oxygen-limited conditions in sealed stirred tubes. Initially the cultures grow exponentially and consume oxygen rapidly. A temporal oxygen gradient is generated and the cultures
30 terminate growth when the oxygen concentration reaches a hypoxic threshold level. Bacilli in the hypoxic stationary phase are in a state of synchronised non-replicating persistence or dormancy.

Our knowledge of the molecules involved in the mycobacterial dormancy response is fragmentary. Elegant biochemical work has shown that dormant bacilli adapt their metabolism to anaerobiosis by switching to nitrate respiration and reductive amination of glyoxylate. Genetic analysis has demonstrated that the
5 stringent response plays a crucial role in the adaptation to hypoxic conditions and stationary phase survival.

Summary of the Invention

The present inventors have identified four proteins that are up-regulated upon
10 termination of growth of the attenuated BCG Pasteur ATCC 35734 strain of *Mycobacterium bovis* in the Wayne dormancy culture system. The inventors have also shown that two of these proteins are upregulated in *Mycobacterium bovis* maintained in a non-oxygen limiting stationary phase. These proteins play a role in the development of the dormant state and in the maintenance of viability during
15 dormancy. The proteins are novel screening targets for the identification and development of novel pharmaceutical agents. These agents may be used in the treatment, prophylaxis and/or diagnosis of mycobacterial infections such as tuberculosis.

Accordingly, the present invention provides:

- 20 - a method for the identification of an anti-mycobacterial agent that modulates the activity and /or expression of a protein, which method comprises:
 - (i) contacting a test agent and a protein selected from RV3133c, Rv2623, Rv2626c, a variant of Rv3133c, Rv2623 or Rv2626c and a fragment of Rv3133c, RV2623, Rv2626c or said variant, or a polynucleotide or
25 expression vector encoding said protein;
 - (ii) monitoring the effect of the test agent on the activity and/or expression of said protein, thereby determining whether the test agent modulates the activity and/or expression of a protein expressed by a *Mycobacterium* in non-oxygen limiting stationary phase, hypoxic
30 stationary phase or hypoxic growth phase.
- an agent which is identifiable by a method of the invention;
- an antibody specific for a protein selected from Rv3133c, Rv2623 or

Rv2626c;

- a pharmaceutical composition comprising a pharmaceutically effective carrier and as an active ingredient an effective amount of an agent or an antibody according to the invention;
- 5 - a vaccine composition comprising as an active ingredient an effective amount of a protein selected from Rv3133c, Rv2623, Rv2626c and a variant of any thereof, or an immunogenic fragment any said protein, and a pharmaceutically effective carrier;
- an agent, antibody, pharmaceutical composition or vaccine composition
10 according to the invention for use in a method of treatment of the human or animal body by therapy or in a diagnostic method practised on the human or animal body;
- use of an agent, antibody, pharmaceutical composition or vaccine composition according to the invention in the manufacture of a medicament
15 for the diagnosis, prophylaxis or treatment of a mycobacterial infection;
- a method of treating a subject suffering from a mycobacterial infection, which method comprises administering to said subject a therapeutically effective amount of an agent, antibody, pharmaceutical composition or vaccine composition according to the invention;
- 20 - a method for detecting a mycobacterial infection in a sample, which method comprises detecting the presence of a protein expressed by a *Mycobacterium* in non-oxygen limiting stationary phase, hypoxic stationary phase or hypoxic growth phase or a nucleic acid encoding said protein in said sample, wherein said protein is selected from Rv3133c, Rv2623, Rv2626c and a variant of
25 any thereof; and
- an *in vitro* or *in vivo* method for diagnosing a mycobacterial infection in a subject which method comprises monitoring expression of a protein expressed by a *Mycobacterium* in non-oxygen limiting stationary phase, hypoxic stationary phase or hypoxic growth phase, wherein said protein is
30 selected from Rv3133c, Rv2623, Rv2626c and a variant of any thereof.
- use of a protein selected from Rv3133c, Rv2623, Rv2626c, a variant of Rv3133c, Rv2623 or Rv2626c and a fragment of Rv3133c, Rv2623, Rv2626c

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or said variant in a method for the identification of an anti-mycobacterial agent.

- use of a protein selected from Rv3133c, Rv2623, Rv2626c, a variant of Rv3133c, Rv2623 or Rv2626c and a fragment of Rv3133c, Rv2623, Rv2626c
- or said variant in a method for the identification of an agent for diagnosing a dormant mycobacterial infection.

Preferably the mycobacterial infection is tuberculosis.

Brief Description of the Figures

10 Fig. 1. shows the growth of BCG under the conditions of the Wayne dormancy culture system. Log A_{600} as a function of time is shown. Viable counts at selected time points are indicated. An aerobic exponential preculture was diluted to $A_{600} = 0.005$ with Dubos Tween-albumin broth (Difco) and incubated in sealed tubes under gentle stirring conditions. f and d indicate fading and complete decolorization

15 of the oxygen indicator methylene blue. Mean values and standard deviations from four independent experiments are shown. Viable counts were determined by plating appropriate dilutions of the cultures on Dubos oleic albumin agar. For all cfu determinations, cultures were checked microscopically for any clumping of cells. Significant clumping was not observed. Arrows A to D indicate time points when

20 samples were taken for the two-dimensional electrophoretic analysis of protein contents shown in Fig. 2.

Fig. 2. shows the temporal profile of protein contents of BCG grown in the Wayne dormancy culture system. Protein extracts were prepared at the time points A to D indicated by the arrows in Fig. 1 and 100 μ g of total protein was subjected to

25 two dimensional electrophoresis. A-D show silver-stained gels corresponding to the 4 time points. Arrows labeled 1 to 4 indicate dormancy-induced protein spots. The arrowhead shows the 22 kD alkyl hydroperoxide reductase (Ahpc, Rv2428) which has been found to be down-regulated in the hypoxic stationary phase (Sherman et al. (1999) Biofactors 10:211-217). Sizes are indicated in kilodaltons. The experiment

30 was carried out four times with the same results. Growth phase-dependent protein contents were also analysed using isoelectric focussing strips pH 3-10. No additional dormancy-induced proteins were detected.

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Fig. 3. shows dormancy-induced proteins. Protein numbers 1 to 4 correspond to protein spots 1 to 4 in Fig. 2. The protein names are according to the *M. tuberculosis H37Rv* genome annotation. To predict the domain architecture of the dormancy-induced proteins their sequence (derived from the *M. tuberculosis H37Rv* genome data base TubercuList (Institut Pasteur, Paris, France, [http://genolist.pasteur.fr/TubercuList/], Data Release R2, March 22, 1999)) was searched against the protein domain families data base Pfam (Sanger Centre, UK [http://www.sanger.ac.uk/Software/Pfam/], version 5.5 September 2000). The result of the search is shown schematically. HSP20, domain found in 20 kD family of heat shock proteins (Accession number PF00011). res_reg, receiver domain found in response regulators (Accession number PF00072). GerE, helix-turn-helix DNA binding domain (luxR subfamily) found in transcriptional regulators (Accession number PF00582). CBS, small modules of unknown function found in diverse proteins. Paire of CBS domains dimerise to form a stable globular domain (Accession number PF00571).

Fig. 4. shows steady state levels of mRNAs encoding dormancy-induced proteins in exponentially growing and dormant cultures. Autoradiograms of Northern blots of total RNA from exponentially growing (lane A, from time point A in Fig.1) and hypoxic stationary phase cultures (lane D, from time point D in Fig. 1) are shown. The blots were hybridised with [α -³²P]dATP labeled probes specific to the transcripts encoding the dormancy-induced proteins: 1, 16kD antigen; 2, 23, kD response regulator; 3, 32 kD conserved hypothetical protein; 4, 16 kD conserved hypothetical protein (Fig. 3). X-ray films were exposed for 1 day. The bottom panel shows the blots after re-hybridisation with a probe specific to 16s rRNA demonstrating equal loading of ribosomal RNA. 5 μ g of total RNA was loaded. The experiment was repeated once with the same results. The up-regulation of the transcripts was confirmed independently by reverse transcriptase – PCR analysis. Sizes are indicated in bases.

Fig. 5 shows the growth of BCG in roller bottles. Exponentially growing pre-cultures were diluted and grown in roller bottles. Optical density as a function of time is shown. Arrows A to D indicate time points when samples were taken for the

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two-dimensional gel electrophoretic analysis of protein contents shown in Fig. 6.

The experiment was carried out four times with the same results and a representative growth curve is shown.

Fig. 6 shows the temporal profile of protein contents of BCG grown in roller
 5 bottles. Protein extracts were prepared at time points A to D indicated by the arrows
 in Fig. 5 and subjected to two dimensional gel electrophoresis. A-D show silver-
 stained gels corresponding to the 4 time points. Arrows labeled 1 and 2 indicate
 stationary phase-induced protein spots. The arrowhead shows the 22 kD alkyl
 hydroperoxide reductase (AhpC, Rv2428) which was found to be down-regulated in
 10 the stationary phase. The experiment was carried out four times and a representative
 set of gels is shown.

Fig. 7 shows a multiple sequence alignment of the 14 kD stationary phase and
 hypoxic stationary phase induced tubercle bacillus protein and the most similar
 orthologs found in other bacteria. The sequence of the 143 amino acid mycobacterial
 15 protein Rv2626c as derived from the *M. tuberculosis* H37Rv genome data base
 TubercuList (Institut Pasteur, Paris, France, [<http://genolist.pasteur.fr/TubercuList/>])
 was searched against the non-redundant GenBank data base (National Center for
 Biotechnology Information, Bethesda, USA, [<http://www.ncbi.nlm.nih.gov/>]) to
 identify orthologs in other bacteria. *M. tub.*: 143 amino acid protein Rv2626c from
 20 *Mycobacterium tuberculosis*, accession number A70573; *S. coe.*: 141 amino acid
 protein from *Streptomyces coelicolor*, accession number CAB62687; *P. aer.*: 138
 amino acid protein from *Pseudomonas aeruginosa*, accession number AAG05547,
B. sub.: 140 amino acid protein from *Bacillus subtilis*, accession number B69824. To
 predict the domain architecture of the proteins their sequence was searched against
 25 the protein domain families data base Pfam (Sanger Centre, UK,
 [<http://www.sanger.ac.uk/Software/Pfam/>]). The positions of two predicted CBS
 domains (Accession number PF00571) are indicated above the alignment.

Brief Description of the Sequences

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SEQ ID NO:1 shows the nucleotide sequence of Rv3133c from the H37Rv strain of
Mycobacterium tuberculosis.

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- SEQ ID NO:2 shows the amino acid sequence of Rv3133c from the H37Rv strain of *Mycobacterium tuberculosis*.
- SEQ ID NO:3 shows the nucleotide sequence of Rv2623 from the H37Rv strain of *Mycobacterium tuberculosis*.
- 5 SEQ ID NO:4 shows the amino acid sequence of Rv2623 from the H37Rv strain of *Mycobacterium tuberculosis*.
- SEQ ID NO:5 shows the nucleotide sequence of Rv2626c from the H37Rv strain of *Mycobacterium tuberculosis*.
- SEQ ID NO:6 shows the amino acid sequence of Rv2626c from the H37Rv strain of
- 10 *Mycobacterium tuberculosis*.
- SEQ ID NO:7 shows the nucleotide sequence of Rv3132c from the H37Rv strain of *Mycobacterium tuberculosis*.
- SEQ ID NO:8 shows the amino acid sequence of Rv3132c from the H37Rv strain of *Mycobacterium tuberculosis*.
- 15 SEQ ID NO:9 shows the amino acid sequence of a variant of Rv2626c from *Streptomyces coelicolor*.
- SEQ ID NO:10 shows the amino acid sequence of a variant of Rv2626c from *Pseudomonas aeruginosa*.
- SEQ ID NO:11 shows the amino acid sequence of a variant of Rv2626c from
- 20 *Bacillus subtilis*.

Detailed Description of the Invention

Proteins

- The present invention relates to dormancy-induced *Mycobacterium* proteins.
- 25 Rv3133c is shown in SEQ ID NO: 1 and SEQ ID NO: 2, Rv2623 is shown in SEQ ID NO: 3 and SEQ ID NO: 4 and Rv2626c is shown in SEQ ID NO: 5 and SEQ ID NO: 6. Sequence information for the dormancy-induced mycobacterial proteins of the invention is taken from the complete genome sequence of the H37Rv strain of *Mycobacterium tuberculosis* (Cole *et al.*, (1998) Nature 393: 537-544) and can be
- 30 found at <http://genolist.pasteur.fr/TubercuList/>.

Proteins of the invention may be in a substantially isolated form. It will be understood that the protein may be mixed with carriers or diluents which will not

interfere with the intended purpose of the polypeptide and still be regarded as substantially isolated. In particular an isolated protein of the invention will be separated from other *Mycobacterium* proteins. For example, an isolated polypeptide may be obtained by separating the polypeptide from other Mycobacterial proteins on a 2D-gel and extracting the polypeptide from the gel. A protein of the invention may also be in a substantially purified form, in which case it will generally comprise the polypeptide in a preparation in which more than 50%, e.g. more than 80%, 90%, 95% or 99%, by weight of the protein in the preparation is a protein of the invention. Routine methods, can be employed to purify and/or synthesise the proteins according to the invention. Such methods are well understood by persons skilled in the art, and include techniques such as those disclosed in Sambrook *et al*, Molecular Cloning: a Laboratory Manual, 2nd Edition, CSH Laboratory Press, 1989, the disclosure of which is included herein in its entirety by way of reference.

The term "variant" refers to a homologues of Rv3133c, Rv2623 or Rv2626c derived from other strains of *Mycobacterium* such as the attenuated BCG Pasteur ATCC 35734 strain, or from other microorganisms such as bacteria including *Streptomyces*, *Pseudomonas* and *Bacillus*.

The term "variant" refers to a protein which is sufficiently similar to Rv3133c, Rv2623 or Rv2626c that the variant is capable of eliciting an immune response to Rv3133c, Rv2623 or Rv2626c. Preferably the variant can be used to raise antibodies to Rv3133c, Rv2623 or Rv2626c. More preferably the variant can be used to generate CD8 T-cells that respond to Rv3133c, Rv2623 or Rv2626c.

Preferably a variant of Rv3133c is capable of binding DNA. More preferably a variant of Rv3133c is capable of binding a sensor histidine protein kinase such as Rv3132c. A variant of Rv3133c preferably has transcriptional regulatory activity.

Typically, polypeptides with more than about 65% identity preferably at least 80% or at least 90% and particularly preferably at least 95% at least 97% or at least 99% identity, with the amino acid sequences of SEQ ID NO: 2, SEQ ID NO: 4 or SEQ ID NO: 6 are considered as variants of the proteins. Such variants include allelic variants and the deletion, modification or addition of single amino acids or groups of amino acids within the protein sequence, as long as the polypeptide maintains the ability to generate an immune response which is effective against Rv3133c, Rv2623

or Rv2626c proteins.

Amino acid substitutions may be made, for example from 1, 2 or 3 to 10, 20 or 30 substitutions. The modified protein generally retains activity as an immunogen. Conservative substitutions may be made, for example according to the following Table. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other.

ALIPHATIC	Non-polar	G A P
		I L V
	Polar-uncharged	C S T M
		N Q
	Polar-charged	D E
		K R
AROMATIC		H F W Y

Shorter polypeptide sequences are within the scope of the invention. For example, a peptide of at least 20 amino acids or up to 50, 60, 70, 80, 100, 150, 200, 300, 400 or 500 amino acids in length is considered to fall within the scope of the invention as long as it is capable of eliciting antibodies or a CD8 T-cell response to Rv3133c, Rv2623 or Rv2626c.

Proteins for use in the invention may be chemically modified, e.g. post-translationally modified. For example, they may be glycosylated or comprise

modified amino acid residues. They may also be modified by the addition of histidine residues to assist their purification.

Polynucleotides

The invention also includes nucleotide sequences for use in methods of the invention that encode for Rv3133c, Rv2323 or Rv2626c or variant or fragment of any thereof as well as nucleotide sequences which are complementary thereto. The nucleotide sequence may be RNA or DNA. Nucleotide sequence information is provided in SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 5. Such nucleotides can be isolated from cells or synthesised according to methods well known in the art, as described by way of example in Sambrook *et al*, 1989.

Typically a polynucleotide for use in the invention comprises a contiguous sequence of nucleotides which is capable of hybridizing under selective conditions to the coding sequence or the complement of the coding sequence of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 5.

A polynucleotide for use in the invention can hybridize to the coding sequence or the complement of the coding sequence of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 5 at a level significantly above background. The signal level generated by the interaction between a polynucleotide of the invention and the coding sequence or complement of the coding sequence of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 5 is typically at least 10 fold, preferably at least 100 fold, as intense as interactions between other polynucleotides and the coding sequence of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 5. The intensity of interaction may be measured, for example, by radiolabelling the probe, e.g. with ³²P. Selective hybridisation may typically be achieved using conditions of medium to high stringency. However, such hybridisation may be carried out under any suitable conditions known in the art (see Sambrook *et al*, 1989. For example, if high stringency is required suitable conditions include from 0.1 to 0.2 x SSC at 60 °C up to 65 °C. If lower stringency is required suitable conditions include 2 x SSC at 60 °C.

The coding sequence of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 5 may be modified by nucleotide substitutions, for example from 1, 2 or 3 to 10, 25, 50 or 100 substitutions. The polynucleotide of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 5 may alternatively or additionally be modified by one or more insertions

and/or deletions and/or by an extension at either or both ends. The modified polynucleotide generally encodes a polypeptide which has immunogenic activity. Alternatively, a polynucleotide encodes a DNA-binding portion of Rv3133c or a fragment of Rv3133c capable of binding a sensor histidine protein kinase such as
5 Rv3132c. Degenerate substitutions may be made and/or substitutions may be made which would result in a conservative amino acid substitution when the modified sequence is translated, for example as shown in the Table above.

A nucleotide sequence which is capable of selectively hybridizing to the complement of the DNA coding sequence of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ
10 ID NO: 5 will generally have at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98% or at least 99% sequence identity to the coding sequence of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 5 over a region of at least 20, preferably at least 30, for instance at least 40, at least 60, more preferably at least 100 contiguous nucleotides or most preferably over the full length of SEQ ID NO: 1,
15 SEQ ID NO: 3 or SEQ ID NO: 5.

For example the UWGCG Package provides the BESTFIT program which can be used to calculate homology (for example used on its default settings) (Devereux *et al* (1984) *Nucleic Acids Research* 12, p387-395). The PILEUP and BLAST algorithms can be used to calculate homology or line up sequences (typically
20 on their default settings), for example as described in Altschul (1993) *J. Mol. Evol.* 36:290-300; Altschul *et al* (1990) *J. Mol. Biol.* 215:403-10.

Software for performing BLAST analyses is publicly available through the National Centre for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). This algorithm involves first identifying high scoring sequence pair (HSPs) by
25 identifying short words of length W in the query sequence that either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighbourhood word score threshold (Altschul *et al*, 1990). These initial neighbourhood word hits act as seeds for initiating searches to find HSPs containing them. The word hits are extended in both
30 directions along each sequence for as far as the cumulative alignment score can be increased. Extensions for the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved

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value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a word length (W) of 11, the
5 BLOSUM62 scoring matrix (see Henikoff and Henikoff (1992) *Proc. Natl. Acad. Sci. USA* 89: 10915-10919) alignments (B) of 50, expectation (E) of 10, M=5, N=4, and a comparison of both strands.

The BLAST algorithm performs a statistical analysis of the similarity between two sequences; see e.g., Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90: 5873-5787. One measure of similarity provided by the BLAST algorithm
10 is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a sequence is considered similar to another sequence if the smallest sum probability in comparison of the first sequence to the second
15 sequence is less than about 1, preferably less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

Any combination of the above mentioned degrees of sequence identity and minimum sizes may be used to define polynucleotides of the invention, with the more stringent combinations (i.e. higher sequence identity over longer lengths) being
20 preferred. Thus, for example a polynucleotide which has at least 90% sequence identity over 25, preferably over 30 nucleotides forms one aspect of the invention, as does a polynucleotide which has at least 95% sequence identity over 40 nucleotides.

The polynucleotides have utility in production of the proteins according to the invention, which may take place *in vitro*, *in vivo* or *ex vivo*. The nucleotides may be
25 involved in recombinant protein synthesis or indeed as therapeutic agents in their own right, utilised in nucleic acid vaccines or gene therapy techniques. Nucleotides complementary to those encoding Rv3133c, Rv2323 or Rv2626c, or antisense sequences, may also be used in gene therapy.

Vectors

30 The present invention also utilizes expression vectors that comprise nucleotide sequences encoding the proteins or variants thereof of the invention. Such expression vectors are routinely constructed in the art of molecular biology and may

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for example involve the use of plasmid DNA and appropriate initiators, promoters, enhancers and other elements, such as for example polyadenylation signals which may be necessary, and which are positioned in the correct orientation, in order to allow for protein expression. Other suitable vectors would be apparent to persons skilled in the art. By way of further example in this regard we refer to Sambrook *et al.* 1989.

Preferably, a polynucleotide for use in the invention in a vector is operably linked to a control sequence which is capable of providing for the expression of the coding sequence by the host cell, i.e. the vector is an expression vector. The term “operably linked” refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A regulatory sequence, such as a promoter, “operably linked” to a coding sequence is positioned in such a way that expression of the coding sequence is achieved under conditions compatible with the regulatory sequence.

15 Cells

The invention also utilises cells that have been modified to express Rv3133c, Rv2323 or Rv2626c or a variant thereof. Such cells include transient, or preferably stable higher eukaryotic cell lines, such as mammalian cells or insect cells, using for example a baculovirus expression system, lower eukaryotic cells, such as yeast or prokaryotic cells such as bacterial or mycobacterial cells. Particular examples of cells which may be modified by insertion of vectors encoding a polypeptide according to the invention include mammalian HEK293T, CHO, HeLa, BHK, 3T3 and COS cells.

Antibodies

25 According to another aspect, the present invention also relates to antibodies specific for a protein that is up-regulated under hypoxic conditions or in stationary phase culture and in particular antibodies that are specific for a protein selected from Rv3133c, Rv2623 and Rv2626c. Such antibodies are for example useful in purification, isolation or screening methods involving immunoprecipitation techniques or, indeed, as therapeutic agents in their own right.

30 Antibodies may be raised against specific epitopes of the proteins according to the invention. Such antibodies may be used to block Rv3133c, or a variant

thereof, binding to DNA or to a sensor histidine protein kinase such as Rv3132c. An antibody, or other compound, "specifically binds" to a protein when it binds with preferential or high affinity to the protein for which it is specific but does substantially bind, or binds with only low affinity, to other proteins. A variety of protocols for competitive binding or immunoradiometric assays to determine the specific binding capability of an antibody are well known in the art (see for example Maddox *et al*, J. Exp. Med. 158, 1211-1226, 1993). Such immunoassays typically involve the formation of complexes between the specific protein and its antibody and the measurement of complex formation.

Antibodies of the invention may be antibodies to human polypeptides or fragments thereof. For the purposes of this invention, the term "antibody", unless specified to the contrary, includes fragments which bind a polypeptide of the invention. Such fragments include Fv, F(ab') and F(ab')₂ fragments, as well as single chain antibodies. Furthermore, the antibodies and fragment thereof may be chimeric antibodies, CDR-grafted antibodies or humanised antibodies.

Antibodies may be used in a method for detecting polypeptides of the invention in a biological sample, which method comprises:

- I providing an antibody of the invention;
- II incubating a biological sample with said antibody under conditions which allow for the formation of an antibody-antigen complex; and
- III determining whether antibody-antigen complex comprising said antibody is formed.

A sample may be for example a tissue extract, blood, serum and saliva. Antibodies of the invention may be bound to a solid support and/or packaged into kits in a suitable container along with suitable reagents, controls, instructions, etc. Antibodies may be linked to a revealing label and thus may be suitable for use in methods of *in vivo* or *in vitro* imaging of Rv3133c, Rv2623 or Rv2626c, for example, in a method of diagnosis.

Antibodies of the invention can be produced by any suitable method. Means for preparing and characterising antibodies are well known in the art, see for example Harlow and Lane (1988) "Antibodies: A Laboratory Manual", Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. For example, an antibody may be

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produced by raising antibody in a host animal against the whole polypeptide or a fragment thereof, for example an antigenic epitope thereof, herein after the "immunogen".

A method for producing a polyclonal antibody comprises immunising a
5 suitable host animal, for example an experimental animal, with the immunogen and isolating immunoglobulins from the animal's serum. The animal may therefore be inoculated with the immunogen, blood subsequently removed from the animal and the IgG fraction purified.

A method for producing a monoclonal antibody comprises immortalising
10 cells which produce the desired antibody. Hybridoma cells may be produced by fusing spleen cells from an inoculated experimental animal with tumour cells (Kohler and Milstein (1975) *Nature* 256, 495-497).

An immortalized cell producing the desired antibody may be selected by a conventional procedure. The hybridomas may be grown in culture or injected
15 intraperitoneally for formation of ascites fluid or into the blood stream of an allogenic host or immunocompromised host. Human antibody may be prepared by *in vitro* immunisation of human lymphocytes, followed by transformation of the lymphocytes with Epstein-Barr virus.

For the production of both monoclonal and polyclonal antibodies, the
20 experimental animal is suitably a goat, rabbit, rat or mouse. If desired, the immunogen may be administered as a conjugate in which the immunogen is coupled, for example via a side chain of one of the amino acid residues, to a suitable carrier. The carrier molecule is typically a physiologically acceptable carrier. The antibody obtained may be isolated and, if desired, purified.

25 Monitoring activity

An important aspect of the present invention is the use of proteins that are upregulated under hypoxic conditions or in stationary phase culture in screening methods. The screening methods may be used to identify substances that bind to hypoxic growth phase, hypoxic stationary phase or non-oxygen limiting stationary
30 phase induced proteins and in particular which bind to Rv3133c, Rv2623 or Rv2626c. Screening methods may also be used to identify agonists or antagonists which may modulate Rv3133c activity, inhibitors or activators of Rv3133c

transcriptional activity, and/or agents which up-regulate or down-regulate Rv3133c, Rv2623 or Rv2626c expression.

Any suitable format may be used for the assay. In general terms such screening methods may involve contacting a dormancy-induced or a stationary-phase
5 induced protein with a test agent and monitoring for binding of the test agent to the protein. The protein may be incubated with a test agent. Modulation of Rv3133c activity may be determined. In a preferred aspect, the assay is a cell-based assay. Preferably the assay may be carried out in a single well of a microtitre plate. Assay formats which allow high throughput screening are preferred.

10 Modulator activity can be determined by contacting cells expressing a dormancy-induced or a stationary-phase induced protein with a substance under investigation and by monitoring an effect mediated by the protein. The protein may be naturally or recombinantly expressed. Preferably, the assay is carried out *in vitro* using cells expressing recombinant protein. Preferably, control experiments are
15 carried out on cells which do not express the protein of interest to establish whether the observed responses are the result of activation of the protein. Typically the cells are transfected with a *Mycobacterium* and grown in oxygen deficient conditions. More preferably, the cells are mycobacterial cells grown in conditions suitable for hypoxic growth, conditions that induce the hypoxic stationary phase or conditions
20 that induce the stationary phase in the presence of oxygen.

The binding of a test agent to a dormancy-induced or a stationary-phase induced protein can be determined directly. For example, a radiolabelled test agent can be incubated with a dormancy-induced or a stationary-phase induced protein and binding of the test agent to the protein can be monitored.

25 Agents that inhibit the interaction of Rv3133c or a variant thereof with DNA or with a sensor histidine protein kinase may also be identified through a yeast 2-hybrid assay or other protein interaction assay such as a co-immunoprecipitation or an ELISA based technique.

Assays may be carried out using cells expressing Rv3133c, and incubating
30 such cells with the test agent optionally in the presence of a sensor histidine protein kinase Rv3132c binding of Rv3133c to the sensor histidine protein kinase may be determined. Alternatively, binding of Rv3133c to DNA or transcriptional activity

may be monitored. The results of the assay are typically compared to the results obtained using the same assay in the absence of the test agent.

Monitoring expression

Assays may also be carried out to identify agents which modify Rv3133c, Rv2623 or Rv2626c expression, for example agents which up- or down- regulate expression. Such assays may be carried out for example by using antibodies for Rv3133c, Rv2623 or Rv2626c to monitor levels of expression following induction of dormant condition. Alternatively, expression may be monitored by determining the effect of the test agent on Rv3133c, Rv 2623 or Rv2626c mRNA levels. Preferably, mRNA levels are monitored in a *Mycobacterium* grown in oxygen-deprived conditions.

Additional control experiments may be carried out.

Test agents

Suitable test agents which can be tested in the above assays include combinatorial libraries, defined chemical entities and compounds, peptide and peptide mimetics, oligonucleotides and natural product libraries, such as display (e.g. phage display libraries) and antibody products.

Typically, organic molecules will be screened, preferably small organic molecules which have a molecular weight of from 50 to 2500 daltons. Candidate products can be biomolecules including, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof. Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. Known pharmacological agents may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification, etc. to produce structural analogs.

Test substances may be used in an initial screen of, for example, 10 substances per reaction, and the substances of these batches which show inhibition or activation tested individually. Test substances may be used at a concentration of from 1nM to 1000µM, preferably from 1µM to 100µM, more preferably from 1µM to 10µM.

Diagnostic agent

Diagnostic methods for the detection of nucleic acid molecules, such as mRNA, encoding a polypeptide of the invention may involve their amplification, e.g. by PCR (the experimental embodiment set forth in U.S. Patent No. 4,683,202), ligase chain reaction (Barany, 1991, Proc. Natl. Acad. Sci. USA 88:189-193), self sustained sequence replication (Guatelli *et al.*, 1990, Proc. Natl. Acad. Sci. USA 87:1874-1878), transcriptional amplification system (Kwoh *et al.*, 1989, Proc. Natl. Acad. Sci. USA 86:1173-1177), Q-Beta Replicase (Lizardi *et al.*, 1988, Bio/Technology 6:1197) or any other nucleic acid amplification method, followed by the detection of the amplified molecules using techniques well known to those of skill in the art. These detection schemes are especially useful for the detection of nucleic acid molecules if such molecules are present in very low numbers.

Medical uses

Another aspect of the present invention is the use of the agents that have been identified by screening techniques referred to above in the treatment of mycobacterial infection. The treatment may be therapeutic or prophylactic. The condition of a patient suffering from a mycobacterial infection can thus be improved. The patient may be a human or animal subject. Generally the animal subject is a mammal, typically one which can be naturally or artificially infected by a *Mycobacterium*. The human or animal subject may be a primate, cow or badger. The subject is typically one which can be naturally or artificially infected by a *Mycobacterium*. The subject may be at risk of a mycobacterial infection, typically because it is resident in a location in which mycobacterial infection is endemic. The subject may be susceptible to mycobacterial infection due to malnutrition or infection by other pathogens, such as HIV. The *Mycobacterium* is typically pathogenic and capable of infecting mammals, such as those mammals discussed above. The *Mycobacterium* is typically *M.tuberculosis*, *M.marinum*, *M.kansasii*, *M. bovis* or *M.avium*.

Substances identified according to the screening methods outlined above may be formulated with standard pharmaceutically acceptable carriers and/or excipients as is routine in the pharmaceutical art. For example, a suitable substance may be dissolved in physiological saline or water for injections. The exact nature of a formulation will depend upon several factors including the particular substance to be

administered and the desired route of administration. Suitable types of formulation are fully described in Remington's Pharmaceutical Sciences, Mack Publishing Company, Eastern Pennsylvania, 17th Ed. 1985, the disclosure of which is included herein of its entirety by way of reference.

5 A vaccine composition typically also comprises an adjuvant. The adjuvant may target the peptide to antigen presenting cells (APCs) or to compartments in the antigen processing pathway, for example acting as a carrier protein. The sequence may stimulate a T helper response, such as a response that favours a CD8 T cell response, and thus may comprise a T helper (e.g. Th1) cell epitope.

10 Preferred substances which enhance immunogenicity include sequence from the hepatitis B core antigen, sequence from a stress protein or sequence from *Clostridium tetani* neurotoxin fragment C. The stress protein is typically a bacterial (e.g. mycobacterial) heat shock protein (HSP) or a protein which has homology with such a protein, such as mycobacterial or *E. coli* proteins of the HSP 60 and HSP 70
15 families (e.g. HSP 65 or HSP 71 of mycobacteria) or mammalian homologue (e.g. gp96 of mice or humans, Anthony *et al.* (1999) Vaccine 17, 373-83).

The substance may cause the polypeptide or vector to adopt a particulate form. The substance may be a virus or virus-like particle (such as a yeast Ty particle, e.g. as in Allsopp *et al.* (1996) Eur. J. Immunol. 26, 1951-9). The substance may be a
20 cytokine, such as a cytokine which stimulates a MHC class I restricted T cell response or favourable MHC class II restricted T cell response (e.g. IL-2, IL-7, IL-12, IFN or GM-CSF). The substance may be, for example, CFA, a muramyl dipeptide (e.g. of a mycobacterial cell wall), monophosphoryl lipid A, lipopolysaccharide (e.g. from *B. abortus*), liposomes, SAF-1, a saponin (e.g. Quil A), keyhole limpet
25 hemocyanin, beta 2-microglobulin, mannan (e.g. oxidised mannan), an acrylic based microbead, or an emulsion (e.g. oil in water or water in oil) such as soybean emulsion (e.g. as in Hioe *et al.* (1996) Vaccine 14, 412-8).

The substances may be administered by enteral or parenteral routes such as via oral, buccal, anal, pulmonary, intravenous, intra-arterial, intramuscular,
30 intraperitoneal, topical or other appropriate administration routes.

The particular route of administration used may aid the stimulating of a CD8 T cell response, and thus the polypeptide vector may be provided in a form suitable

for administering by such a route. Delivery by an intramuscular route or by biolistic means is preferred.

A therapeutically effective amount of a modulator is administered to a patient suffering from a mycobacterial infection. An effective amount of a polypeptide of the invention, or a fragment thereof, capable of generating an immune response is administered to a subject at risk of a mycobacterial infection. Stimulation of an immune response can typically be monitored by detecting antibodies directed against Rv3133c, Rv2623 or Rv2626c. Antibodies specific for such proteins may be detected using an ELISA assay.

The dose of a modulator may be determined according to various parameters, especially according to the substance used; the age, weight and condition of the patient to be treated; the route of administration; and the required regimen. A physician will be able to determine the required route of administration and dosage for any particular patient. A typical daily dose is from about 0.1 to 50 mg per kg of body weight, according to the activity of the specific modulator, the age, weight and conditions of the subject to be treated, the type and severity of the degeneration and the frequency and route of administration. Preferably, daily dosage levels are from 5 mg to 2 g.

A vaccine composition is preferably administered in a single dose. One or more, for example, two, three or four, further doses may be required for long term protection against a *Mycobacterium* infection. Further doses may be against a *Mycobacterium* infection. Further doses may be administered after a period of from 1 month to 15 years after the initial dose, for example, 1, 2, 3, 4, 5, 8, 10, 12 or 15 years. Several further doses may be administered at intervals after the initial dose, for example at 3, 5, 10 or 15 year intervals.

Nucleic acid encoding a polypeptide of the invention may be administered to a mammal as a nucleic acid vaccine. Nucleic acid encoding the polypeptide may be administered by any available technique. For example, the nucleic acid may be introduced by needle injection, preferably intradermally, subcutaneously or intramuscularly. Alternatively, the nucleic acid may be delivered directly across the skin using a nucleic acid delivery device such as particle-mediated gene delivery. The nucleic acid may be administered topically to the skin, or to mucosal surfaces for

example by intranasal, oral, intravaginal or intrarectal administration.

Uptake of nucleic acid constructs may be enhanced by several known transfection techniques, for example those including the use of transfection agents. Examples of these agents includes cationic agents, for example, calcium phosphate
5 and DEAE-Dextran and lipofectants, for example, lipofectam and transfectam. The dosage of the nucleic acid to be administered can be altered. Typically the nucleic acid is administered in the range of 1pg to 1mg, preferably to 1pg to 10µg nucleic acid for particle mediated gene delivery and 10µg to 1mg for other routes.

Generally a low dose of antigen favours the development of a CD8 T cell
10 response. Thus a suitable low dose of the polypeptide or vector may be administered to prevent mycobacterial infection. The polypeptide or vector may thus be in an amount and concentration that is suitable for administering to provide an appropriate low dose. In a preferred embodiment the vector is administered in the form of "naked DNA".

15 The following Examples illustrate the invention.

Example 1: Detection and identification of dormancy-induced proteins.

BCG were grown in the Wayne dormancy culture system. Bacilli were harvested at various time points (Fig. 1, arrows A-D), washed twice in phosphate-buffered saline, resuspended in lysis buffer (9 M urea, 4% CHAPS, 50 mM DTT,
20 pefabloc [1 mg ml⁻¹], pepstatin [1 µg ml⁻¹], leupeptin [1 µg ml⁻¹] and disrupted with 0.5 mm glass beads using a Mini Bead Beater (Biospec). Protein concentrations were determined using the BioRad protein assay reagents and protocols. 100 µg of total protein were subjected to isoelectric focusing using pH 4-7 Immobiline Dry
25 Strips and an IPGphor isoelectric focusing unit as recommended by the manufacturer (Amersham Pharmacia) for 62000 Vhrs. For the separation in the second dimension 12.5% sodium dodecyl sulfate polyacrylamide gels were used (Protean Ixi system, BioRad) and proteins were detected by silver staining. Fig. 2. shows a representative set of two-dimensional gels. Four proteins showed a drastic increase in their steady
30 state level in the hypoxic stationary phase (Fig. 2, arrows 1-4). The proteins were not (arrow 1, 2, 4) or only weakly (arrow 3) detectable in the extracts from exponentially

growing cultures (Fig. 2A) and appeared as major spots immediately upon oxygen-starvation induced termination of growth (Fig. 2B). Elevated levels of the proteins were maintained throughout the hypoxic stationary phase (Fig. 2C, D). To identify the dormancy-induced proteins, the excised gel spots were subjected to in-gel
 5 digestion with trypsin to recover the peptides (Shevchenko *et al.* (1996) Anal. Chem. 68: 850-858). Peptide sequence tags were generated from selected peptides by nanoelectrospray tandem mass spectrometry using a quadrupole/time-of-flight hybrid instrument (QSTAR, PE Sciex). Protein identity was revealed by searching sequence databases with a combination of the peptide sequence tags and the mass information.
 10 Protein 1 was the 16 kD antigen Rv2031c previously reported to be induced in oxygen-starved cultures of tubercle bacilli (Yuan *et al.* (1996) J. Bacteriol. 178: 4484-4492). Protein 2 was the 23 kD response regulator Rv3133cc. Proteins 3 and 4 were the 32 kD conserved hypothetical protein Rv2623 and the 16 kD (14kD on SDS PAGE) conserved hypothetical protein Rv2626c, respectively (Fig. 3). Protein
 15 names and Rv numbers are according to the *M. tuberculosis* H37Rv genome annotation (Cole *et al.* (1998) Nature 393: 537 – 544).

Example 2: Transcript levels of dormancy-induced proteins.

To determine whether the increase of the steady state level of the dormancy-
 20 induced proteins correlates with an increase in the steady state level of their mRNAs, total RNA was isolated from exponentially growing and hypoxic stationary phase cultures and subjected to Northern blot analysis as described (Hutter and Dick (1999) FEMS Microbiol. Lett. 178; 63-69). Probes were isolated by PCR using BCG genomic DNA as template. The primers were derived from the *M. tuberculosis*
 25 H37Rv genome sequence and were as follows: 1, Rv2031c (GCCACCACCCTTCCCGTTCAG, ATGTCGTCCTCGTCAGCACCTACC); 2, Rv3133cc (TCGTAGGTGTAGGCGGGTTC, CGGCGATCTGCTTGTTGGT); 3, Rv2623 (GGCAGCCGTTCCACATTG, GGCTGATCGCGCACCACCAC); 4, Rv2626c (CCACCGCACGCGACATCAT, CGGAACACGGCGGACCTG); 16S
 30 rRNA (GCCTGGGAAACTGGGTCTAA, TCTCCACCTACCGTCAATCC). The identity of the PCR fragments was confirmed by sequencing using a Perkin-Elmer ABI Prism 377 automated sequencer. Fig. 4 shows high levels of the transcripts for

all four proteins in dormant bacilli. In exponential growing culture the transcripts were not detectable or were only weakly detectable. This result indicates that the expression of the proteins is regulated at the transcriptional level.

The up-regulation of the steady state level of the 16 kD antigen mRNA in oxygen-starved dormant culture is in apparent contradiction to the down-regulation of the transcript in nonpagitated cultures of the tubercle bacillus observed by Hu *et al.* (1999) *J. Bacteriol.* 181: 1380-1387. The 16 kD antigen had been previously reported to be induced in oxygen-starved stationary phase cultures of tubercle bacilli. The protein belongs to the 20 kD small heat shock protein family (Fig. 3) and was shown to possess chaperon function ascribed to other members of the family. The three new dormancy-induced proteins were predicted by the *M. tuberculosis* H37Rv genome project (Cole *et al.*). However, biochemical or genetic data for these proteins are not available. Two of the three new dormancy-induced proteins are annotated as 'conserved hypothetical proteins' (Cole *et al.* (1998) *Nature* 393: 537-544). A similarity search against the protein domain families (pfam) database (1) suggested that the 32 kD conserved hypothetical protein shown in SEQ ID NO: 4 contains two 'Universal Stress Protein' (Usp) domains (Fig. 3). This domain is found in the UspA protein in *E. coli*. UspA is up-regulated in stationary phase cultures and plays a role in the survival of growth-arrested cells (Diez *et al.* (2000) *Mol. Microbiol.* 36: 1494-1503). The 16 kD conserved hypothetical protein shown in SEQ ID NO: 6 is predicted to contain two 'Cystathionine-beta synthase' (CBS) domains (Fig. 3). CBS domains are found in functionally diverse proteins. A function for this domain is not known. Most intriguing is the dormancy-dependent up-regulation of the 23 kD response regulator shown in SEQ ID NO: 2. This response regulator contains a 'helix-turn-helix' DNA binding domain and is thus likely to act as a phosphorylation-dependent transcription factor (Fig. 3). Response regulators, together with their respective sensor histidine protein kinase, are part of two-component signal transduction systems and play key roles in a variety of developmental and adaptive processes in bacteria. Thus, it is conceivable that the 23 kD response regulator plays a role in the control of the mycobacterial dormancy response. Inspection of the genomic locus surrounding the gene encoding the 23 kD response regulator in *M. tuberculosis* H37Rv showed that the gene appears to form an operon with the gene

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Rv3132c annotated as putative sensor histidine protein kinase (Cole *et al*). The genomic organisation is identical in BCG and could indicate that this putative kinase represents the sensor involved in the control of the activity of the dormancy-induced 23 kD response regulator.

5

Example 3: Detection and identification of stationary phase - induced proteins

M. bovis BCG were grown at 37°C in Dubos Tween-albumin broth (BD Bioscience). Bacilli were sub-cultured once in liquid medium until they reached an early exponential growth phase ($A_{800} = 0.2 - 0.3$) before inoculation to an experimental culture. To grow experimental cultures medium was dispensed in 100 ml aliquots to roller bottles, 10 x 14 cm, and pre-cultures were diluted to $A_{600} = 0.05$ (5×10^6 cfu/ml). Cultures were aerated by incubation on a roller apparatus at 1 rpm. The roller bottles were opened daily for turbidity measurements and to allow exchange of the air. Growth was monitored by measuring the optical density of the cultures in a Ultrospec 3000 spectrophotometer (Pharmacia).

Protein extracts were prepared from different time points corresponding to the exponential phase, entry into stationary phase, early and late stationary phase (Fig. 5, arrows A-D). For the preparation of protein extracts the cells were washed twice in phosphate-buffered saline, resuspended in lysis buffer (9 M urea, 4% CHAPS, 50 mM DTT, pefabloc [1 mg ml^{-1}], pepstatin [$1 \text{ } \mu\text{g ml}^{-1}$], leupeptin [$1 \text{ } \mu\text{g ml}^{-1}$]) and disrupted with 0.5 mm glass beads using a Mini Bead Beater (Biospec). Protein concentrations were determined using the BioRad protein assay reagents and protocols. 100 μg of total protein was subjected to isoelectric focussing using pH 4-7 Immobiline Dry Strips and a IPGphor isoelectric focusing unit as recommended by the manufacture (Amersham Pharmacia) for 62000 Vhrs. For the separation in the second dimension 12.5% sodium dodecyl sulfate polyacrylamide gels were used (Protean IIxi system, BioRad). Proteins were detected by silver staining and the gels were evaluated by visual inspection. Fig. 6 shows a representative set of two-dimensional gels. Two proteins of 16kD and 14kD showed a drastic increase in their steady state level in the stationary phase (Fig. 6, arrows 1 and 2). The two proteins were not detectable in the extracts from exponentially growing cultures (Fig. 6A) and

25

appeared as major spots immediately upon termination of growth (Fig. 6B). Elevated levels of the proteins were maintained throughout the stationary phase (Fig. 6C, D). The 16 kD protein is the BCG counterpart of the 16 kD antigen (Rv2031c). The 14 kD polypeptide is the counterpart of the protein Rv2626c predicted by the *M. tuberculosis* H37Rv genome project.

The marked up-regulation of the 16 kD antigen and the 14 kD protein immediately upon termination of growth suggests that they play roles in the entry into the stationary phase and/or in maintaining viability of stationary phase bacilli. The 16 kD antigen has been previously reported to be induced in stationary phase cultures of tubercle bacilli (Yuan *et al.* (1996) J. Bacteriol. 178: 4484-4492).

Example 4: Homologues of the stationary phase-induced and dormancy-induced Rv2626c protein

Biochemical or genetic data for the new stationary phase-induced 14 kD protein/dormancy-induced 16 kD protein shown in SEQ ID NO:6 are not available. A similarity search against the protein domain families (pfam) database suggested that the protein consists of a pair of CBS domains (Figs. 3 and 7). The recently discovered CBS domain is named after Cystathionine Beta Synthase where it was originally identified. The domain is usually present as a pair and this CBS domain-dimer associates to form a single compact structure. Pairs of CBS domains are found in a large number of functionally diverse proteins such as inosine-monophosphate dehydrogenases and chloride channels. Although the role of the CBS domain in these proteins is unclear, it may be involved in protein-protein interaction and protein regulation. In contrast to these proteins that contain a pair of CBS domains in the context of other, unrelated domains, the small stationary phase-induced mycobacterial protein appears to consist of only a pair of CBS domains, not fused to other domains (Figs. 3 and 7). It is interesting to note that small molecular weight proteins with the same predicted domain architecture and about 35% sequence identity can be found in other bacteria such as *Streptomyces coelicolor*, *Pseudomonas aeruginosa* and *Bacillus subtilis* (Fig. 7). This suggests that the Rv2626c dormancy-induced and stationary phase-induced mycobacterial protein is a member of a new bacterial protein family.

Example 5: Overexpression, purification and phosphorylation of the 23kD response regulator protein.

The dormancy-induced response regulator Rv3133c is predicted to function
5 as a phosphorylation-dependent transcription regulator. Thus, it is likely that the protein binds to target promoters and recruits RNA polymerase to initiate transcription of essential dormancy genes. Hence, prevention of DNA binding by inhibitors should abolish response regulator function. Under the assumption that a cognate promoter sequence is available, an *in vitro* DNA binding assay can be carried
10 out to screen for inhibitors of the response regulator's DNA binding activity.

Using gene specific primers derived from the *M. tuberculosis* H37Rv genome sequence (Cole *et al.* (1998)) the coding sequence of the Rv3133c gene is isolated by PCR and a glutathione S-transferase fusion protein is constructed, overexpressed and purified as described in Ulijasz *et al.* (2000), Biochemistry 39, 11427-11424 and
15 Sambrook *et al.* (1989) Molecular cloning, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. The purified protein is phosphorylated as described in Ulijasz *et al.* using 50 mM lithium acetyl phosphate.

Example 6: Isolation and radioactive labelling of the promoter DNA

20 The cognate promoter DNA is PCR amplified using primers derived from the *M. tuberculosis* H37Rv genome sequence (Cole *et al.* (1998)) and 5'-end labelled with γ -³²P-ATP and T4 polynucleotide kinase according to standard methods as described in Sambrook *et al.* (1989).

25 **Example 7: Binding of the phosphorylated response regulator to its cognate promoter and gel shift analysis of the binding**

Protein-DNA complexes are formed in a total volume of 15 μ l which contain 4 pmol of phosphorylated response regulator which was preincubated with the appropriate inhibitors for 10 min at room temperature in 20 mM HEPES (pH 7.2), 5
30 mM MgCl₂, 0.1 mM Na₂EDTA, 0.5 mM CaCl₂, 10% glycerol and 0.5 μ g of salmon DNA as described in Ulijasz *et al.* (2000). To start the reaction, ³²P-labeled promoter

DNA (0.65 ng in 1 μ l) is added, and the reaction mixture is incubated on ice for 15 min.

The resultant complexes are analyzed by non-denaturing polyacrylamide gel electrophoresis (PAGE). Gels are dried and exposed to a phosphorimager as
5 described in Ulijasz *et al.* (2000).

An inhibitor of the response regulator will prevent the binding of the protein to its promoter and hence reduce complex formation of the two components. Complex formation, or the absence of complex formation is detectable in the gel shift analysis.

CLAIMS

1. A method for the identification of an anti-mycobacterial agent that
5 modulates the activity and/or expression of a protein expressed by a *Mycobacterium*
in non-oxygen limiting stationary phase, hypoxic stationary phase or hypoxic growth
phase, which method comprises:
 - (i) contacting a test agent and a protein selected from Rv3133c, Rv2623,
Rv2626c, a variant of Rv3133c, Rv2623 or Rv2626c and a fragment
10 of Rv3133c, RV2623, Rv2626c or said variant, or a polynucleotide or
expression vector encoding said protein;
 - (ii) monitoring the effect of the test agent on the activity and/or
expression of said protein, thereby determining whether the test agent
modulates the activity and/or expression of a protein expressed by a
15 *Mycobacterium* in non-oxygen limiting stationary phase, hypoxic
stationary phase or hypoxic growth phase.
2. A method according to claim 1 wherein step (ii) comprises monitoring
binding of said protein to the test agent.
3. A method according to claim 1 wherein step (ii) comprises monitoring
20 binding of Rv3133c, a variant thereof or a fragment of either thereof to DNA.
4. A method according to claim 1 wherein step (ii) comprises monitoring
binding of Rv3133c, a variant thereof or a fragment of either thereof to a sensor
histidine protein kinase.
5. A method according to claim 4 wherein the sensor histidine protein
25 kinase is Rv3132c, a variant thereof or a fragment of either thereof.
6. A method according to claim 1 wherein step (ii) comprises
monitoring the transcriptional activity of a gene regulated by Rv3133c, a variant
thereof or a fragment of either thereof.
7. A method for the identification of a diagnostic agent that binds to a
30 protein expressed by a *Mycobacterium* in non-oxygen limiting stationary phase,
hypoxic stationary phase or hypoxic growth phase, or to a polynucleotide encoding
said protein, which method comprises:
 - (i) contacting a test agent and a protein selected from Rv3133c, Rv2623,

Rv2626c, a variant of Rv3133c, Rv2623, Rv2626c or a fragment of Rv3133c, Rv2623, Rv2626c or said variant, or a polynucleotide encoding said protein;

- 5 (ii) monitoring any interaction between the test agent and said protein or said polynucleotide, thereby determining whether the test agent binds a protein or polynucleotide expressed by a *Mycobacterium* in non-oxygen limiting stationary phase, hypoxic stationary phase or hypoxic growth phase.

8. A method according to any preceding claim wherein said agent is a
10 variant or fragment of a protein expressed by a *Mycobacterium* in non-oxygen limiting stationary phase, hypoxic stationary phase or hypoxic growth phase, a polynucleotide which encodes a variant or fragment of said protein or a polynucleotide which hybridises under stringent conditions to a sequence encoding said protein.

15 9. An agent which is identifiable by a method according to any preceding claim.

10. An agent according to claim 9 which inhibits activity and/or expression of a protein expressed by a *Mycobacterium* in non-oxygen limiting stationary phase hypoxic stationary phase or hypoxic growth phase.

20 11. An antibody specific for a protein selected from Rv3133c, Rv2623 or Rv2626c.

12. A pharmaceutical composition comprising a pharmaceutically effective carrier and as an active ingredient an effective amount of an agent according to claim 9 or 10 or an antibody according to claim 11.

25 13. A vaccine composition comprising as an active ingredient an effective amount of a protein selected from Rv3133c, Rv2623, Rv2626c and a variant of any thereof, or an immunogenic fragment any said protein, and a pharmaceutically effective carrier.

14. An agent according to claim 9 or 10, an antibody according to claim
30 11, a pharmaceutical composition according to claim 12 or a vaccine composition according to claim 13 for use in a method of treatment of the human or animal body by therapy or in a diagnostic method practised on the human or animal body.

15. Use of an agent according to claim 9 or 10, an antibody according to claim 11, a pharmaceutical composition according to claim 12 or a vaccine composition according to claim 13 in the manufacture of a medicament for the diagnosis, prophylaxis or treatment of a mycobacterial infection.

5 16. Use according to claim 15 wherein said mycobacterial infection is tuberculosis.

17. A method of treating a subject suffering from a mycobacterial infection, which method comprises administering to said subject a therapeutically effective amount of an agent according to claim 9 or 10, an antibody according to claim 11 or a pharmaceutical composition according to claim 12.

18. Use of a prophylactically effective amount of a protein selected from Rv3133c, Rv2626c, Rv2623, a variant of any thereof and a fragment of any said protein, an agent according to claim 9 or 10, an antibody according to claim 11 or a vaccine composition according to claim 13 in the manufacture of a medicament for the prevention of a mycobacterial infection.

19. A method for detecting a mycobacterial infection in a sample, which method comprises detecting the presence of a protein expressed by a *Mycobacterium* in non-oxygen limiting stationary phase, hypoxic stationary phase or hypoxic growth phase or a nucleic acid encoding said protein in said sample, wherein said protein is selected from Rv3133c, Rv2623, RV2626c and a variant of any thereof.

20. A method according to claim 19 which comprises:

- (i) contacting a sample and an agent according to claim 9 or an antibody according to claim 11; and
- (ii) monitoring binding of said agent or antibody to said sample, thereby determining whether said sample comprises a protein expressed by a *Mycobacterium* in non-oxygen limiting stationary phase, hypoxic stationary phase or hypoxic growth phase, or a nucleic acid encoding said protein and hence whether said sample is infected with a *Mycobacterium*.

21. A method according to claim 20 wherein said nucleic acid is RNA.

22. An *in vitro* method of diagnosing a mycobacterial infection in a subject, which method comprises a method according to any one of claims 19 to 21

and wherein said sample is a sample from said subject.

23. An *in vitro* or *in vivo* method for diagnosing a mycobacterial infection in a subject which method comprises monitoring expression of a protein expressed by a *Mycobacterium* in non-oxygen limiting stationary phase, hypoxic stationary phase or hypoxic growth phase, wherein said protein is selected from Rv3133c, Rv2623, Rv2626c and a variant of any thereof.

24. A method according to claim 23, which method comprises administering to a subject at risk of mycobacterial infection an agent according to claim 9 or an antibody according to claim 11 and monitoring the response to the said agent or antibody.

25. A method according to any one of claims 17 to 24 wherein said mycobacterial infection is tuberculosis.

26. A method of obtaining a protein selected from Rv3133c, Rv2623, Rv2626c and a variant thereof, which method comprises maintaining a *Mycobacterium* under aerobic or anaerobic conditions suitable for inducing non-oxygen limiting stationary phase, hypoxic stationary phase or hypoxic growth phase expressed proteins and isolating the said protein.

27. Use of a protein selected from Rv3133c, Rv2623, Rv2626c, a variant of Rv3133c, Rv2623 or Rv2626c and a fragment of Rv3133c, Rv2623, Rv2626c or said variant in a method for the identification of an anti-mycobacterial agent.

28. Use of a protein selected from Rv3133c, Rv2623, Rv2626c, a variant of Rv3133c, Rv2623 or Rv2626c and a fragment of Rv3133c, Rv2623, Rv2626c or said variant in a method for the identification of an agent for diagnosing a dormant mycobacterial infection.

WO 02/48391

PCT/EP01/14551

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 gcc gcg gcc gcg ctg atg gcc aag ctg cgc ggt gcc gcc gag aag cag
 432
 Ala Ala Ala Ala Leu Met Ala Lys Leu Arg Gly Ala Ala Glu Lys Gln
 10 130 135 140
 gac ccg cta tca ggc ctt acc gac cag gag cgg acg cta ctg ggc ctg
 480
 Asp Pro Leu Ser Gly Leu Thr Asp Gln Glu Arg Thr Leu Leu Gly Leu
 15 145 150 155 160
 ctt agc gag ggc ctg acc aac aag cag atc gcc gac cga atg ttc cta
 528
 Leu Ser Glu Gly Leu Thr Asn Lys Gln Ile Ala Asp Arg Met Phe Leu
 20 165 170 175
 gcc gaa aag acg gtg aag aac tac gtg tcg cgg ttg ctg gcc aag ctg
 576
 Ala Glu Lys Thr Val Lys Asn Tyr Val Ser Arg Leu Leu Ala Lys Leu
 25 180 185 190
 ggc atg gaa cgt cgg acg caa gcc gcg gta ttc gcg acg gag ttg aag
 624
 Gly Met Glu Arg Arg Thr Gln Ala Ala Val Phe Ala Thr Glu Leu Lys
 30 195 200 205
 cgc tcg cgg cca ccc ggt gat gga cca
 651
 Arg Ser Arg Pro Pro Gly Asp Gly Pro
 35 210 215
 <210> 2
 <211> 217
 40 <212> PRT
 <213> Mycobacterium tuberculosis
 <400> 2
 Val Val Lys Val Phe Leu Val Asp Asp His Glu Val Val Arg Arg Gly
 45 1 5 10 15
 Leu Val Asp Leu Leu Gly Ala Asp Pro Glu Leu Asp Val Val Gly Glu
 50 20 25 30
 Ala Gly Ser Val Ala Glu Ala Met Ala Arg Val Pro Ala Ala Arg Pro
 55 35 40 45
 Asp Val Ala Val Leu Asp Val Arg Leu Pro Asp Gly Asn Gly Ile Glu
 50 55 60
 60
 Leu Cys Arg Asp Leu Leu Ser Arg Met Pro Asp Leu Arg Cys Leu Ile
 65 70 75 80

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3
 Leu Thr Ser Tyr Thr Ser Asp Glu Ala Met Leu Asp Ala Ile Leu Ala
 85 90 95

5 Gly Ala Ser Gly Tyr Val Val Lys Asp Ile Lys Gly Met Glu Leu Ala
 100 105 110

10 Arg Ala Val Lys Asp Val Gly Ala Gly Arg Ser Leu Leu Asp Asn Arg
 115 120 125

15 Ala Ala Ala Ala Leu Met Ala Lys Leu Arg Gly Ala Ala Glu Lys Gln
 130 135 140

Asp Pro Leu Ser Gly Leu Thr Asp Gln Glu Arg Thr Leu Leu Gly Leu
 145 150 155 160

20 Leu Ser Glu Gly Leu Thr Asn Lys Gln Ile Ala Asp Arg Met Phe Leu
 165 170 175

25 Ala Glu Lys Thr Val Lys Asn Tyr Val Ser Arg Leu Leu Ala Lys Leu
 180 185 190

30 Gly Met Glu Arg Arg Thr Gln Ala Ala Val Phe Ala Thr Glu Leu Lys
 195 200 205

35 Arg Ser Arg Pro Pro Gly Asp Gly Pro
 210 215

<210> 3
 <211> 891
 <212> DNA
 40 <213> Mycobacterium tuberculosis

<220>
 <221> CDS
 45 <222> (1)..(891)

<400> 3
 50 atg tca tcg ggc aat tca tct ctg gga att atc gtc ggg atc gac gat
 48
 Met Ser Ser Gly Asn Ser Ser Leu Gly Ile Ile Val Gly Ile Asp Asp
 1 5 10 15

55 tca ccg gcc gca cag gtt gcg gtg cgg tgg gca gct cgg gat gcg gag
 96
 Ser Pro Ala Ala Gln Val Ala Val Arg Trp Ala Ala Arg Asp Ala Glu
 20 25 30

60 ttg cga aaa atc cct ctg acg ctc gtg cac gcg gtg tcg ccg gaa gta
 144
 Leu Arg Lys Ile Pro Leu Thr Leu Val His Ala Val Ser Pro Glu Val
 35 40 45

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4

gcc acc tgg ctg gag gtg cca ctg ccg ccg ggc gtg ctg cga tgg cag
 192
 Ala Thr Trp Leu Glu Val Pro Leu Pro Pro Gly Val Leu Arg Trp Gln
 50 55 60

5 cag gat cac ggg cgc cac ctg atc gac gac gca ctc aag gtg gtt gaa
 240
 Gln Asp His Gly Arg His Leu Ile Asp Asp Ala Leu Lys Val Val Glu
 65 70 75 80

10 cag gct tcg ctg cgc gct ggt ccc ccc acg gtc cac agt gaa atc gtt
 288
 Gln Ala Ser Leu Arg Ala Gly Pro Pro Thr Val His Ser Glu Ile Val
 85 90 95

15 ccg gcg gca gcc gtt ccc aca ttg gtc gac atg tcc aaa gac gca gtg
 336
 Pro Ala Ala Ala Val Pro Thr Leu Val Asp Met Ser Lys Asp Ala Val
 100 105 110

20 ctg atg gtc gtg ggt tgt ctc gga agt ggg cgg tgg ccg ggc cgg ctg
 384
 Leu Met Val Val Gly Cys Leu Gly Ser Gly Arg Trp Pro Gly Arg Leu
 115 120 125

25 ctc ggt tcg gtc agt tcc ggc ctg ctc cgc cac gcg cac tgt ccg gtc
 432
 Leu Gly Ser Val Ser Ser Gly Leu Leu Arg His Ala His Cys Pro Val
 130 135 140

30 gtg atc atc cac gac gaa gat tcg gtg atg ccg cat ccc cag caa gcg
 480
 Val Ile Ile His Asp Glu Asp Ser Val Met Pro His Pro Gln Gln Ala
 145 150 155 160

35 ccg gtg cta gtt ggc gtt gac ggc tcg tcg gcc tcc gag ctg gcg acc
 528
 Pro Val Leu Val Gly Val Asp Gly Ser Ser Ala Ser Glu Leu Ala Thr
 165 170 175

40 gca atc gca ttc gac gaa gcg tcg cgg cga aac gtg gac ctg gtg gcg
 576
 Ala Ile Ala Phe Asp Glu Ala Ser Arg Arg Asn Val Asp Leu Val Ala
 180 185 190

45 ctg cac gca tgg agc gac gtc gat gtg tcg gag tgg ccc gga atc gat
 624
 Leu His Ala Trp Ser Asp Val Asp Val Ser Glu Trp Pro Gly Ile Asp
 195 200 205

50 tgg ccg gca act cag tcg atg gcc gag cag gtg ctg gcc gag cgg ttg
 672
 Trp Pro Ala Thr Gln Ser Met Ala Glu Gln Val Leu Ala Glu Arg Leu
 210 215 220

55 gcg ggt tgg cag gag cgg tat ccc aac gta gcc ata acc cgc gtg gtg
 720
 Ala Gly Trp Gln Glu Arg Tyr Pro Asn Val Ala Ile Thr Arg Val Val
 225 230 235 240

60 gtg cgc gat cag ccg gcc cgc cag ctc gtc caa cgc tcc gag gaa gcc
 768
 Val Arg Asp Gln Pro Ala Arg Gln Leu Val Gln Arg Ser Glu Glu Ala
 245 250 255

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5

5 cag ctg gtc gtg gtc ggc agc cgg ggc cgc ggc ggc tac gcc gga atg
 816
 Gln Leu Val Val Val Gly Ser Arg Gly Arg Gly Gly Tyr Ala Gly Met
 260 265 270

10 ctg gtg ggg tcg gta ggc gaa acc gtt gct cag ctg gcg cgg acg ccg
 864
 Leu Val Gly Ser Val Gly Glu Thr Val Ala Gln Leu Ala Arg Thr Pro
 275 280 285

15 gtc atc gtg gca cgc gag tcg ctg act
 891
 Val Ile Val Ala Arg Glu Ser Leu Thr
 290 295

20 <210> 4
 <211> 297
 <212> PRT
 <213> Mycobacterium tuberculosis

25 <400> 4
 Met Ser Ser Gly Asn Ser Ser Leu Gly Ile Ile Val Gly Ile Asp Asp
 1 5 10 15

30 Ser Pro Ala Ala Gln Val Ala Val Arg Trp Ala Ala Arg Asp Ala Glu
 20 25 30

35 Leu Arg Lys Ile Pro Leu Thr Leu Val His Ala Val Ser Pro Glu Val
 35 40 45

40 Ala Thr Trp Leu Glu Val Pro Leu Pro Pro Gly Val Leu Arg Trp Gln
 50 55 60

45 Gln Asp His Gly Arg His Leu Ile Asp Asp Ala Leu Lys Val Val Glu
 65 70 75 80

50 Gln Ala Ser Leu Arg Ala Gly Pro Pro Thr Val His Ser Glu Ile Val
 85 90 95

55 Pro Ala Ala Ala Val Pro Thr Leu Val Asp Met Ser Lys Asp Ala Val
 100 105 110

60 Leu Met Val Val Gly Cys Leu Gly Ser Gly Arg Trp Pro Gly Arg Leu
 115 120 125

65 Leu Gly Ser Val Ser Ser Gly Leu Leu Arg His Ala His Cys Pro Val
 130 135 140

70 Val Ile Ile His Asp Glu Asp Ser Val Met Pro His Pro Gln Gln Ala
 145 150 155 160

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6

Pro Val Leu Val Gly Val Asp Gly Ser Ser Ala Ser Glu Leu Ala Thr
 165 170 175

5
 Ala Ile Ala Phe Asp Glu Ala Ser Arg Arg Asn Val Asp Leu Val Ala
 180 185 190

10 Leu His Ala Trp Ser Asp Val Asp Val Ser Glu Trp Pro Gly Ile Asp
 195 200 205

15 Trp Pro Ala Thr Gln Ser Met Ala Glu Gln Val Leu Ala Glu Arg Leu
 210 215 220

20 Ala Gly Trp Gln Glu Arg Tyr Pro Asn Val Ala Ile Thr Arg Val Val
 225 230 235 240

Val Arg Asp Gln Pro Ala Arg Gln Leu Val Gln Arg Ser Glu Glu Ala
 245 250 255

25 Gln Leu Val Val Val Gly Ser Arg Gly Arg Gly Gly Tyr Ala Gly Met
 260 265 270

30 Leu Val Gly Ser Val Gly Glu Thr Val Ala Gln Leu Ala Arg Thr Pro
 275 280 285

35 Val Ile Val Ala Arg Glu Ser Leu Thr
 290 295

<210> 5
 <211> 429
 40 <212> DNA
 <213> Mycobacterium tuberculosis

<220>
 45 <221> CDS
 <222> (1)..(429)

<400> 5
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 Met Thr Thr Ala Arg Asp Ile Met Asn Ala Gly Val Thr Cys Val Gly
 1 5 10 15

55 gaa cac gag acg cta acc gct gcc gct caa tac atg cgt gag cac gac
 96
 Glu His Glu Thr Leu Thr Ala Ala Ala Gln Tyr Met Arg Glu His Asp
 20 25 30

60 atc ggc gcg ttg ccg atc tgc ggg gac gac gac cgg ctg cac ggc atg
 144
 Ile Gly Ala Leu Pro Ile Cys Gly Asp Asp Asp Arg Leu His Gly Met
 35 40 45

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```

          7
ctc acc gac cgc gac att gtg atc aaa ggc ctg gct gcg ggc cta gac
192
Leu Thr Asp Arg Asp Ile Val Ile Lys Gly Leu Ala Ala Gly Leu Asp
  50          55          60
5  ccg aat acc gcc acg gct ggc gag ttg gcc cgg gac agc atc tac tac
240
Pro Asn Thr Ala Thr Ala Gly Glu Leu Ala Arg Asp Ser Ile Tyr Tyr
  65          70          75          80
10 gtc gat gcg aac gca agc atc cag gag atg ctc aac gtc atg gaa gaa
288
Val Asp Ala Asn Ala Ser Ile Gln Glu Met Leu Asn Val Met Glu Glu
      85          90          95
15 cat cag gtc cgc cgt gtt ccg gtc atc tca gag cac cgc ttg gtc gga
336
His Gln Val Arg Arg Val Pro Val Ile Ser Glu His Arg Leu Val Gly
      100          105          110
20 atc gtc acc gaa gcc gac atc gcc cga cac ctg ccc gag cac gcc att
384
Ile Val Thr Glu Ala Asp Ile Ala Arg His Leu Pro Glu His Ala Ile
      115          120          125
25 gtg cag ttc gtc aag gca atc tgc tcg ccc atg gcc ctc gcc agc
429
Val Gln Phe Val Lys Ala Ile Cys Ser Pro Met Ala Leu Ala Ser
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30
<210> 6
<211> 143
<212> PRT
35 <213> Mycobacterium tuberculosis

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45 Glu His Glu Thr Leu Thr Ala Ala Ala Gln Tyr Met Arg Glu His Asp
      20          25          30
50 Ile Gly Ala Leu Pro Ile Cys Gly Asp Asp Asp Arg Leu His Gly Met
      35          40          45
55 Leu Thr Asp Arg Asp Ile Val Ile Lys Gly Leu Ala Ala Gly Leu Asp
      50          55          60
60 Pro Asn Thr Ala Thr Ala Gly Glu Leu Ala Arg Asp Ser Ile Tyr Tyr
      65          70          75          80
60 Val Asp Ala Asn Ala Ser Ile Gln Glu Met Leu Asn Val Met Glu Glu
      85          90          95

His Gln Val Arg Arg Val Pro Val Ile Ser Glu His Arg Leu Val Gly

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8
100 105 110

5 Ile Val Thr Glu Ala Asp Ile Ala Arg His Leu Pro Glu His Ala Ile
 115 120 125

10 Val Gln Phe Val Lys Ala Ile Cys Ser Pro Met Ala Leu Ala Ser
 130 135 140

15 <210> 7
 <211> 1734
 <212> DNA
 <213> Mycobacterium tuberculosis

20 <220>
 <221> CDS
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 Met Thr Thr Gly Gly Leu Val Asp Glu Asn Asp Gly Ala Ala Met Arg
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30 cca ctg cgt cac acg ctc tcc caa cta cgc ctg cac gag ctg ctg gtc
 96
 Pro Leu Arg His Thr Leu Ser Gln Leu Arg Leu His Glu Leu Leu Val
 20 25 30

35 gag gtg cag gac cgg gtc gag cag atc gtc gag ggc cgg gac cgc ctc
 144
 Glu Val Gln Asp Arg Val Glu Gln Ile Val Glu Gly Arg Asp Arg Leu
 35 40 45

40 gat ggt ctg gtg gag gcc atg ctc gtg gtc aca gcg ggc ctg gac ctg
 192
 Asp Gly Leu Val Glu Ala Met Leu Val Val Thr Ala Gly Leu Asp Leu
 50 55 60

45 gag gca acc cta cgc gct atc gtg cat tca gcg acc agc ctt gtc gat
 240
 Glu Ala Thr Leu Arg Ala Ile Val His Ser Ala Thr Ser Leu Val Asp
 65 70 75 80

50 gcg cgc tat ggc gct atg gag gtg cac gac cgg cag cat cgg gta ttg
 288
 Ala Arg Tyr Gly Ala Met Glu Val His Asp Arg Gln His Arg Val Leu
 85 90 95

55 cac ttt gtc tat gaa ggc atc gac gag gag acc gtt cgg cgg atc ggc
 336
 His Phe Val Tyr Glu Gly Ile Asp Glu Glu Thr Val Arg Arg Ile Gly
 100 105 110

60 cac cta ccg aaa ggc cta ggc gtc atc ggg ctg ctc atc gaa gat ccc
 384
 His Leu Pro Lys Gly Leu Gly Val Ile Gly Leu Leu Ile Glu Asp Pro
 115 120 125

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9

aaa ccg tta cgg ctg gac gat gtt tct gcg cac ccg gcc tcg att ggt
432
Lys Pro Leu Arg Leu Asp Asp Val Ser Ala His Pro Ala Ser Ile Gly
130 135 140

5 ttt ccg ccg tat cat ccg ccg atg cgt acc ttc ctc ggg gta ccg gtt
480
Phe Pro Pro Tyr His Pro Pro Met Arg Thr Phe Leu Gly Val Pro Val
145 150 155 160

10 cgg gtg cgc gat gaa tcg ttc ggc act ctg tac ctg act gac aag acc
528
Arg Val Arg Asp Glu Ser Phe Gly Thr Leu Tyr Leu Thr Asp Lys Thr
165 170 175

15 aac ggg caa ccg ttc agc gac gac gac gag gtt ctg gtc cag gcg ctg
576
Asn Gly Gln Pro Phe Ser Asp Asp Asp Glu Val Leu Val Gln Ala Leu
180 185 190

20 gcg gcc gcc gcg ggt atc gca gtc gcg aat gcc ccg ctc tac cag cag
624
Ala Ala Ala Ala Gly Ile Ala Val Ala Asn Ala Arg Leu Tyr Gln Gln
195 200 205

25 gct aag gcg cgt cag tcg tgg atc gag gcc acc cgt gac atc gcc acc
672
Ala Lys Ala Arg Gln Ser Trp Ile Glu Ala Thr Arg Asp Ile Ala Thr
210 215 220

30 gag ttg ttg tcc ggc acc gaa ccc gcg acg gtg ttc ccg ctt gtc gcc
720
Glu Leu Leu Ser Gly Thr Glu Pro Ala Thr Val Phe Arg Leu Val Ala
225 230 235 240

35 gcg gag gcg ctc aag ctg acg gcg gct gac gct gcc ctg gta gcc gtt
768
Ala Glu Ala Leu Lys Leu Thr Ala Ala Asp Ala Ala Leu Val Ala Val
245 250 255

40 ccc gtc gac gag gac atg cct gcc gct gac gtg ggg gag ctg ctg gtg
816
Pro Val Asp Glu Asp Met Pro Ala Ala Asp Val Gly Glu Leu Leu Val
260 265 270

45 att gaa aca gtc ggc agc gct gtg gct tcc att gtt ggg cga acg att
864
Ile Glu Thr Val Gly Ser Ala Val Ala Ser Ile Val Gly Arg Thr Ile
275 280 285

50 ccg gtg gcg ggc gcg gtg ctg ccg gag gtc ttc gtc aac ggc att ccg
912
Pro Val Ala Gly Ala Val Leu Arg Glu Val Phe Val Asn Gly Ile Pro
290 295 300

55 cga ccg gtc gac ccg gtc gat ttg gaa ggc ctg gac gaa ctg gcc gac
960
Arg Arg Val Asp Arg Val Asp Leu Glu Gly Leu Asp Glu Leu Ala Asp
305 310 315 320

60 gca ggt ccg gcg ctg ctg ttg ccg ctg ccg gcc aga ggt acc gta gcg
1008
Ala Gly Pro Ala Leu Leu Leu Pro Leu Arg Ala Arg Gly Thr Val Ala
325 330 335

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10

ggt gtc gtt gtt gtg ctg agt caa ggc ggt cca ggg gct ttc acc gac
 1056
 5 Gly Val Val Val Val Leu Ser Gln Gly Gly Pro Gly Ala Phe Thr Asp
 340 345 350

gaa caa ctc gag atg atg gcc gcg ttc gcc gac cag gcc gcg ctg gct
 1104
 10 Glu Gln Leu Glu Met Met Ala Ala Phe Ala Asp Gln Ala Ala Leu Ala
 355 360 365

tgg caa ttg gcc act tcg caa cgt cgg atg cgc gaa ctc gac gta ctg
 1152
 15 Trp Gln Leu Ala Thr Ser Gln Arg Arg Met Arg Glu Leu Asp Val Leu
 370 375 380

acc gac cgg gat cgt atc gcc cgt gac ctc cat gac cat gtc atc cag
 1200
 20 Thr Asp Arg Asp Arg Ile Ala Arg Asp Leu His Asp His Val Ile Gln
 385 390 395 400

cgg ctc ttc gcg att ggc ctg gct ttg cag ggt gct gtc ccg cac gaa
 1248
 25 Arg Leu Phe Ala Ile Gly Leu Ala Leu Gln Gly Ala Val Pro His Glu
 405 410 415

cgt aat cct gaa gtg cag caa cga ctc tcg gac gtg gta gac gat ctg
 1296
 30 Arg Asn Pro Glu Val Gln Gln Arg Leu Ser Asp Val Val Asp Asp Leu
 420 425 430

caa gac gtt ata cag gaa atc cgg acc acc att tat gac ctg cac gga
 1344
 35 Gln Asp Val Ile Gln Glu Ile Arg Thr Thr Ile Tyr Asp Leu His Gly
 435 440 445

gca tcg cag ggt atc act cgg ctc cgg cag cga atc gat gcg gcc gta
 1392
 40 Ala Ser Gln Gly Ile Thr Arg Leu Arg Gln Arg Ile Asp Ala Ala Val
 450 455 460

gcc caa ttt gcc gac tcg ggg ttg cgc acc agc gtt caa ttc gtg ggt
 1440
 45 Ala Gln Phe Ala Asp Ser Gly Leu Arg Thr Ser Val Gln Phe Val Gly
 465 470 475 480

cca ttg tcg gtg gtc gac agc gcg ctc gcc gat cag gcc gag gcg gtg
 1488
 50 Pro Leu Ser Val Val Asp Ser Ala Leu Ala Asp Gln Ala Glu Ala Val
 485 490 495

gtt cgg gaa gcg gtc agc aac gcg gtt cgc cat gcg aag gcc agc acg
 1536
 55 Val Arg Glu Ala Val Ser Asn Ala Val Arg His Ala Lys Ala Ser Thr
 500 505 510

ttg acc gtc cgg gtc aaa gtc gac gac gac ttg tgc atc gag gtg acc
 1584
 60 Leu Thr Val Arg Val Lys Val Asp Asp Asp Leu Cys Ile Glu Val Thr
 515 520 525

gac aac ggc cgc ggg ctg ccc gac gag ttc acc gga agc ggc tta acg
 1632
 Asp Asn Gly Arg Gly Leu Pro Asp Glu Phe Thr Gly Ser Gly Leu Thr

11

	530		535		540
aac ctg cgg cag cgg gca gag cag gcc ggc ggc gaa ttc acc ctc gcg 1680					
5 Asn Leu Arg Gln Arg Ala Glu Gln Ala Gly Gly Glu Phe Thr Leu Ala 545 550 555 560					
agc gta ccg ggc gcg agc gga aca gtg ctg cga tgg tca gca ccg ttg 1728					
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tcg cag 1734					
15 Ser Gln					
<210> 8					
20 <211> 578					
<212> PRT					
<213> Mycobacterium tuberculosis					
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Pro Leu Arg His Thr Leu Ser Gln Leu Arg Leu His Glu Leu Leu Val 20 25 30					
Glu Val Gln Asp Arg Val Glu Gln Ile Val Glu Gly Arg Asp Arg Leu 35 40 45					
Asp Gly Leu Val Glu Ala Met Leu Val Val Thr Ala Gly Leu Asp Leu 40 50 55 60					
Glu Ala Thr Leu Arg Ala Ile Val His Ser Ala Thr Ser Leu Val Asp 65 70 75 80					
Ala Arg Tyr Gly Ala Met Glu Val His Asp Arg Gln His Arg Val Leu 85 90 95					
His Phe Val Tyr Glu Gly Ile Asp Glu Glu Thr Val Arg Arg Ile Gly 100 105 110					
His Leu Pro Lys Gly Leu Gly Val Ile Gly Leu Leu Ile Glu Asp Pro 115 120 125					
Lys Pro Leu Arg Leu Asp Asp Val Ser Ala His Pro Ala Ser Ile Gly 60 130 135 140					
Phe ProPro Tyr His Pro Pro Met Arg Thr Phe Leu Gly Val Pro Val 145 150 155 160					

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12

5 Arg Val Arg Asp Glu Ser Phe Gly Thr Leu Tyr Leu Thr Asp Lys Thr
 165 170 175
 10 Asn Gly Gln Pro Phe Ser Asp Asp Asp Glu Val Leu Val Gln Ala Leu
 180 185 190
 15 Ala Ala Ala Ala Gly Ile Ala Val Ala Asn Ala Arg Leu Tyr Gln Gln
 195 200 205
 20 Glu Leu Leu Ser Gly Thr Glu Pro Ala Thr Val Phe Arg Leu Val Ala
 225 230 235 240
 25 Ala Glu Ala Leu Lys Leu Thr Ala Ala Asp Ala Ala Leu Val Ala Val
 245 250 255
 30 Pro Val Asp Glu Asp Met Pro Ala Ala Asp Val Gly Glu Leu Leu Val
 260 265 270
 35 Ile Glu Thr Val Gly Ser Ala Val Ala Ser Ile Val Gly Arg Thr Ile
 275 280 285
 40 Arg Arg Val Asp Arg Val Asp Leu Glu Gly Leu Asp Glu Leu Ala Asp
 305 310 315 320
 45 Ala Gly Pro Ala Leu Leu Leu Pro Leu Arg Ala Arg Gly Thr Val Ala
 325 330 335
 50 Gly Val Val Val Val Leu Ser Gln Gly Gly Pro Gly Ala Phe Thr Asp
 340 345 350
 55 Glu Gln Leu Glu Met Met Ala Ala Phe Ala Asp Gln Ala Ala Leu Ala
 355 360 365
 60 Trp Gln Leu Ala Thr Ser Gln Arg Arg Met Arg Glu Leu Asp Val Leu
 370 375 380
 Thr Asp Arg Asp Arg Ile Ala Arg Asp Leu His Asp His Val Ile Gln
 385 390 395 400
 Arg Leu Phe Ala Ile Gly Leu Ala Leu Gln Gly Ala Val Pro His Glu
 405 410 415

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13

Arg Asn Pro Glu Val Gln Gln Arg Leu Ser Asp Val Val Asp Asp Leu
 420 425 430
 5

Gln Asp Val Ile Gln Glu Ile Arg Thr Thr Ile Tyr Asp Leu His Gly
 435 440 445
 10

Ala Ser Gln Gly Ile Thr Arg Leu Arg Gln Arg Ile Asp Ala Ala Val
 450 455 460
 15

Ala Gln Phe Ala Asp Ser Gly Leu Arg Thr Ser Val Gln Phe Val Gly
 465 470 475 480
 20

Pro Leu Ser Val Val Asp Ser Ala Leu Ala Asp Gln Ala Glu Ala Val
 485 490 495
 25

Val Arg Glu Ala Val Ser Asn Ala Val Arg His Ala Lys Ala Ser Thr
 500 505 510
 30

Leu Thr Val Arg Val Lys Val Asp Asp Asp Leu Cys Ile Glu Val Thr
 515 520 525
 35

Asp Asn Gly Arg Gly Leu Pro Asp Glu Phe Thr Gly Ser Gly Leu Thr
 530 535 540
 40

Asn Leu Arg Gln Arg Ala Glu Gln Ala Gly Gly Glu Phe Thr Leu Ala
 545 550 555 560
 45

Ser Val Pro Gly Ala Ser Gly Thr Val Leu Arg Trp Ser Ala Pro Leu
 565 570 575
 50

Ser Gln
 45

<210> 8
 <211> 578
 <212> PRT
 <213> Mycobacterium tuberculosis
 50

<400> 8
 55

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 1 5 10 15
 60

Pro Leu Arg His Thr Leu Ser Gln Leu Arg Leu His Glu Leu Leu Val
 20 25 30
 65

Glu Val Gln Asp Arg Val Glu Gln Ile Val Glu Gly Arg Asp Arg Leu
 35 40 45

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14

Asp Gly Leu Val Glu Ala Met Leu Val Val Thr Ala Gly Leu Asp Leu
 50 55 60

5
 Glu Ala Thr Leu Arg Ala Ile Val His Ser Ala Thr Ser Leu Val Asp
 65 70 75 80

10 Ala Arg Tyr Gly Ala Met Glu Val His Asp Arg Gln His Arg Val Leu
 85 90 95

15 His Phe Val Tyr Glu Gly Ile Asp Glu Glu Thr Val Arg Arg Ile Gly
 100 105 110

His Leu Pro Lys Gly Leu Gly Val Ile Gly Leu Leu Ile Glu Asp Pro
 115 120 125

20 Lys Pro Leu Arg Leu Asp Asp Val Ser Ala His Pro Ala Ser Ile Gly
 130 135 140

25 Phe Pro Pro Tyr His Pro Pro Met Arg Thr Phe Leu Gly Val Pro Val
 145 150 155 160

30 Arg Val Arg Asp Glu Ser Phe Gly Thr Leu Tyr Leu Thr Asp Lys Thr
 165 170 175

35 Asn Gly Gln Pro Phe Ser Asp Asp Asp Glu Val Leu Val Gln Ala Leu
 180 185 190

40 Ala Ala Ala Ala Gly Ile Ala Val Ala Asn Ala Arg Leu Tyr Gln Gln
 195 200 205

Ala Lys Ala Arg Gln Ser Trp Ile Glu Ala Thr Arg Asp Ile Ala Thr
 210 215 220

45 Glu Leu Leu Ser Gly Thr Glu Pro Ala Thr Val Phe Arg Leu Val Ala
 225 230 235 240

50 Ala Glu Ala Leu Lys Leu Thr Ala Ala Asp Ala Ala Leu Val Ala Val
 245 250 255

55 Pro Val Asp Glu Asp Met Pro Ala Ala Asp Val Gly Glu Leu Leu Val
 260 265 270

Ile Glu Thr Val Gly Ser Ala Val Ala Ser Ile Val Gly Arg Thr Ile
 275 280 285

60 Pro Val Ala Gly Ala Val Leu Arg Glu Val Phe Val Asn Gly Ile Pro
 290 295 300

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15

Arg Arg Val Asp Arg Val Asp Leu Glu Gly Leu Asp Glu Leu Ala Asp
 305 310 315 320

5 Ala Gly Pro Ala Leu Leu Leu Pro Leu Arg Ala Arg Gly Thr Val Ala
 325 330 335

10 Gly Val Val Val Val Leu Ser Gln Gly Gly Pro Gly Ala Phe Thr Asp
 340 345 350

15 Glu Gln Leu Glu Met Met Ala Ala Phe Ala Asp Gln Ala Ala Leu Ala
 355 360 365

20 Trp Gln Leu Ala Thr Ser Gln Arg Arg Met Arg Glu Leu Asp Val Leu
 370 375 380

Thr Asp Arg Asp Arg Ile Ala Arg Asp Leu His Asp His Val Ile Gln
 385 390 395 400

25 Arg Leu Phe Ala Ile Gly Leu Ala Leu Gln Gly Ala Val Pro His Glu
 405 410 415

30 Arg Asn Pro Glu Val Gln Gln Arg Leu Ser Asp Val Val Asp Asp Leu
 420 425 430

35 Gln Asp Val Ile Gln Glu Ile Arg Thr Thr Ile Tyr Asp Leu His Gly
 435 440 445

40 Ala Ser Gln Gly Ile Thr Arg Leu Arg Gln Arg Ile Asp Ala Ala Val
 450 455 460

Ala Gln Phe Ala Asp Ser Gly Leu Arg Thr Ser Val Gln Phe Val Gly
 465 470 475 480

45 Pro Leu Ser Val Val Asp Ser Ala Leu Ala Asp Gln Ala Glu Ala Val
 485 490 495

50 Val Arg Glu Ala Val Ser Asn Ala Val Arg His Ala Lys Ala Ser Thr
 500 505 510

55 Leu Thr Val Arg Val Lys Val Asp Asp Asp Leu Cys Ile Glu Val Thr
 515 520 525

Asp Asn Gly Arg Gly Leu Pro Asp Glu Phe Thr Gly Ser Gly Leu Thr
 530 535 540

60 Asn Leu Arg Gln Arg Ala Glu Gln Ala Gly Gly Glu Phe Thr Leu Ala
 545 550 555 560

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16

Ser Val Pro Gly Ala Ser Gly Thr Val Leu Arg Trp Ser Ala Pro Leu
565 570 575

5 Ser Gln

10 <210> 9
<211> 141
<212> PRT
<213> Streptomyces coelicolor

15 <400> 9

Met Thr Arg Arg Ile Arg Asp Val Met Ser Pro Ala Ala Val Ala Val
1 5 10 15

20 Glu Pro Met Thr Thr Val Ala Arg Ala Ala Arg Leu Met Arg Glu Glu
20 25 30

25 Asp Val Gly Asp Val Leu Val Thr Tyr Asp Cys Asp Leu Phe Gly Val
35 40 45

Leu Thr Asp Arg Asp Ile Val Leu Arg Gly Val Ala Asp Gly Arg Asp
50 55 60

30 Ser Glu Ala Thr Thr Val Gly Ala Val Cys Thr Pro Pro Pro Val Val
65 70 75 80

Thr Leu Glu Pro Asp Asp Thr Thr Asp Arg Ala Ala Glu Leu Met Ala
85 90 95

35 Arg His Ala Val Arg Arg Leu Pro Val Val Glu His Gly Gly Val Pro
100 105 110

Val Gly Val Val Thr Leu Gly Asp Leu Ala Ala Thr Asp Asp Pro His
40 115 120 125

Ser Ala Leu Ala Asp Ile Ser Arg Ala Ala Pro Gly His
130 135 140

45 <210> 10
<211> 138
<212> PRT
<213> Pseudomonas aeruginosa

50 <400> 10

Met Lys Ile Ser Asp Ile Met Thr Arg Asn Val Gln Val Ala Asp Pro
55 1 5 10 15

Gln Gln Ser Ile Arg Glu Ala Ala Ala Thr Met Ala Arg Ile Asp Ser
20 25 30

60 Gly Ala Leu Leu Val Gly Glu Gly Asp Arg Leu Val Gly Met Ile Thr
35 40 45

Asp Arg Asp Ile Ala Ile Arg Ala Val Ala Gly Gly Leu Ser Gly Asp
50 55 60

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17

Thr Pro Leu Gly Arg Ile Met Ser Gly Asp Ile His Tyr Cys Phe Glu
 65 70 75 80
 5 Asp Glu Asp Val Gln His Val Ala Arg Asn Met Ala Asp Ile Gln Met
 85 90 95
 Arg Arg Leu Pro Val Leu Asn Arg Glu Lys Arg Leu Val Gly Val Val
 100 105 110
 10 Ser Leu Gly Asn Ile Ala Ser Cys Arg Asp Gln Ala Ser Ser Ala Thr
 115 120 125
 Val Leu Gln Gly Val Ala Gln Ala His Tyr
 15 130 135
 <210> 11
 <211> 140
 20 <212> PRT
 <213> Bacillus subtilis
 <400> 11
 25 Met Ser Ser Val Lys Asp Thr Met Thr Thr Gln Val Ala Thr Val Ser
 1 5 10 15
 Pro Asn Gln Thr Ile Gln Glu Ala Ala Ser Leu Met Lys Gln His Asn
 30 20 25 30
 Val Gly Ala Ile Pro Val Val Glu Gln Gly Val Leu Lys Gly Met Leu
 35 35 40 45
 35 Thr Asp Arg Asp Ile Ala Leu Arg Thr Thr Ala Gln Gly Arg Asp Gly
 50 55 60
 Gln Thr Pro Val Ser Glu Val Met Ser Thr Glu Leu Val Ser Gly Asn
 65 70 75 80
 40 Pro Asn Met Ser Leu Glu Asp Ala Ser Gln Leu Met Ala Gln His Gln
 85 90 95
 Ile Arg Arg Leu Pro Ile Val Asp Gln Asn Asn Leu Val Gly Ile Val
 45 100 105 110
 Ala Leu Gly Asp Leu Ala Val Asn Gln Met Ser Asn Glu Ser Ala Gly
 115 120 125
 50 Ser Ala Leu Thr Asn Ile Ser His Gln Asn Ile His
 130 135 140

55