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FROM M. TUBERCULOSIS**(60) Provisional application No. 60/116,673, filed on Jan.
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hagen S (DK)(21) Appl. No.: **11/196,018**(22) Filed: **Aug. 2, 2005**(57) **ABSTRACT****Related U.S. Application Data**(60) Division of application No. 10/138,473, filed on May
2, 2002, now Pat. No. 6,982,085, which is a continu-
ation-in-part of application No. 10/060,428, filed on
Jan. 29, 2002, now abandoned, which is a continua-
tion-in-part of application No. 09/415,884, filed on
Oct. 8, 1999, now abandoned.

The present invention is based on the identification and
characterization of a number of novel *M. tuberculosis*
derived proteins and protein fragments. The invention is
directed to the polypeptides and immunologically active
fragments thereof, the genes encoding them, immunological
compositions such as diagnostic reagents containing the
polypeptides.

TB DIAGNOSTIC BASED ON ANTIGENS FROM M. TUBERCULOSIS

[0001] This application is a divisional of co-pending

[0002] U.S. application Ser. No. 10/138,473, filed on May 2, 2002, which is a continuation-in-part of U.S. patent application Ser. No. 10/060,428, filed Jan. 29, 2002, which is a continuation-in-part of U.S. application Ser. No. 09/415,884, filed Oct. 8, 1999, which is a non-provisional of U.S. application Ser. No. 60/116,673, filed Jan. 21, 1999, which claims priority to

[0003] U.S. patent application Ser. No. 09/050,739, filed 30 Mar. 1998, which claims priority from U.S. Provisional Application No. 60/044,624, filed 18 Apr. 1997, U.S. Provisional Application No. 60/070,488, filed 5 Jan. 1998, and Danish Patent Applications Nos. DK 1997 00376, filed 2 Apr. 1997, and DK 1997 01277, filed 10 Nov. 1997;

[0004] U.S. patent application Ser. No. 09/791,171, filed 20 Feb. 2001, which is a divisional of the above mentioned U.S. patent application Ser. No. 09/050,739, claiming the same priorities; and

[0005] U.S. patent application Ser. No. 09/415,884, filed 8 Oct. 1999, which claims priority from U.S. Provisional Application No. 60/116,673, filed 21 Jan. 1999 and Danish Patent Application No. DK 1998 01281, filed 8 Oct. 1998.

[0006] Each of these patent applications as well as all documents cited in the text of this application, and references cited in the documents referred to in this application (including references cited in the aforementioned patent applications or during their prosecution) are hereby incorporated herein by reference and for which priority is claimed under 35 U.S.C. § 120.

FIELD OF INVENTION

[0007] The present invention relates to a number of immunologically active, novel polypeptide fragments derived from *Mycobacterium tuberculosis*, diagnostics and other immunologic compositions containing the fragments as immunogenic components, and methods of production and use of the polypeptides. The invention also relates to novel nucleic acid fragments derived from *M. tuberculosis* which are useful in the preparation of the polypeptide fragments of the invention or in the diagnosis of infection with *M. tuberculosis*. The invention further relates to certain fusion polypeptides.

GENERAL BACKGROUND

[0008] Human tuberculosis caused by *Mycobacterium tuberculosis* (*M. tuberculosis*) is a severe global health problem, responsible for approx. 3 million deaths annually, according to the WHO. The worldwide incidence of new tuberculosis (TB) cases had been falling during the 1960s and 1970s but during recent years this trend has markedly changed in part due to the advent of AIDS and the appearance of multidrug resistant strains of *M. tuberculosis*.

[0009] In 1998 Cole et al published the complete genome sequence of *M. tuberculosis* and predicted the presence of approximately 4000 open reading frames (Cole et al 1998). Nucleotide sequences are described, and putative protein

sequences. However importantly, this sequence information cannot be used to predict if the DNA is translated and expressed as proteins in vivo. More importantly, it is not possible on the basis of the sequences, to predict whether a given sequence will encode an immunogenic or an inactive protein. The only way to determine if a protein is recognized by the immune system during or after an infection with *M. tuberculosis* is to produce the given protein and test it in an appropriate assay as described herein.

[0010] Short term-culture filtrate (ST-CF) is a complex mixture of proteins released from *M. tuberculosis* during the first few days of growth in a liquid medium (Andersen et al., 1991). Culture filtrates has been suggested to hold protective antigens recognized by the host in the first phase of TB infection (Andersen et al. 1991, Orme et al. 1993). Recent data from several laboratories have demonstrated that experimental subunit vaccines based on culture filtrate antigens can provide high levels of acquired resistance to TB (Pal and Horwitz, 1992; Roberts et al., 1995; Andersen, 1994; Lindblad et al., 1997). Culture filtrates are, however, complex protein mixtures and until now very limited information has been available on the molecules responsible for this protective immune response.

[0011] It is thus an object of the present invention to provide a composition for the determination of an immune response against a virulent *Mycobacterium* such as a diagnostic reagent for the diagnosis of an infection with a virulent *Mycobacterium*.

SUMMARY OF THE INVENTION

[0012] It is surprisingly demonstrated herein that several polypeptides isolated from the cell wall, cell membrane or cytosol and short term culture filtrate (STCF) are recognized by human tuberculosis antisera.

[0013] Therefore it is considered likely that these polypeptides, either alone or in combination, can be useful as diagnostic reagents in the diagnosis of tuberculosis.

[0014] The present inventors contemplate that in order to achieve a very high sensitivity for a serodiagnostic TB reagent it is important to combine two or more TB antigens, or alternatively, to use recombinant fusions proteins comprising at least two proteins or B cell epitopes. The antibody response of tuberculosis is heterogeneous with considerable person-to-person variance to which antigens that are recognized by the antibodies and therefore it can be an advantage to use combinations of proteins (e.g. in protein cocktails) which may increase the sensitivity and be recognized by sera from a high proportion of infected individuals. Especially, it is advantageous to combine from two to four antigens which will give a higher sensitivity than the single antigen and still a high specificity (more than 90%).

[0015] Thus, the invention is related to detection of infections caused by species of the tuberculosis complex (*M. tuberculosis*, *M. bovis*, *M. africanum*) by the use of a combination of two or more polypeptides comprising a *M. tuberculosis* antigen or an immunogenic portion or other variant thereof, or by the use of two or more DNA sequences encoding a *M. tuberculosis* antigen or an immunogenic portion or other variant thereof.

DETAILED DISCLOSURE OF THE INVENTION

[0016] The invention relates to polypeptides which induce specific antibody responses in a TB patient as determined by

an ELISA technique or a western blot when the whole blood is diluted 1:20 in PBS and stimulated with the polypeptide in a concentration of at the most 20 µg/ml and induces an OD of at least 0.1 in ELISA, or a visual response in western blot.

[0017] Any polypeptide fulfilling the above property and which is obtainable from either the cell wall, cell membrane, the cytosol or STCF of the tuberculosis complex is within the scope of the present invention.

[0018] In an important embodiment, the invention relates to a composition comprising a combination of two or more (e.g. 2, 3, 4, 5, 6, 7 or more) substantially pure polypeptides, which comprises one or more amino acid sequences selected from

[0019] (a) Rv0652, Rv2462c, Rv1984c, Rv2185c, Rv1636, Rv3451, Rv3872, Rv3354 and Rv2623

[0020] (b) an immunogenic portion of any one of the sequences in (a); and/or

[0021] (c) an amino acid sequence analogue having at least 70% sequence identity to any one of the sequences in (a) or (b) and at the same time being immunogenic;

for use as a pharmaceutical or diagnostic reagent.

[0022] Also, the invention relates to a composition comprising one or more fusion polypeptides, which comprises one or more amino acid sequences selected from

[0023] (a) Rv0652, Rv2462c, Rv1984c, Rv2185c, Rv1636, Rv3451, Rv3872, Rv3354 and Rv2623

[0024] (b) an immunogenic portion of any one of the sequences in (a); and/or

[0025] (c) an amino acid sequence analogue having at least 70% sequence identity to any one of the sequences in (a) or (b) and at the same time being immunogenic;

and at least one fusion partner.

[0026] The fusion partner comprises preferably a polypeptide fragment selected from

[0027] (a) a polypeptide fragment derived from a virulent mycobacterium, such as ESAT-6, MPB64, MPT64, TB10.4, CFP10, RD1-ORF5, RD1-ORF2, Rv1036, Ag85A, Ag85B, Ag85C, 19 kDa lipoprotein, MPT32, MPB59 and alpha-crystallin;

[0028] (b) a polypeptide as defined above; and/or

[0029] (c) at least one immunogenic portion of any of such polypeptides in (a) or (b).

[0030] In another embodiment, the invention relates to an immunogenic composition comprising a composition according to the invention.

[0031] In a further embodiment, the invention relates to the use of a composition as defined above for the preparation of a pharmaceutical composition, e.g. for diagnosis of tuberculosis caused by virulent mycobacteria, e.g. by *Mycobacterium tuberculosis*, *Mycobacterium africanum* or *Mycobacterium bovis*.

[0032] In a still further embodiment, the invention relates to a diagnostic tool comprising a combination of two or more substantially pure polypeptides, which comprises one or more amino acid sequences selected from

[0033] (a) Rv0652, Rv2462c, Rv1984c, Rv2185c, Rv1636, Rv3451, Rv3872, Rv3354 and Rv2623

[0034] (b) an immunogenic portion of any one of the sequences in (a); and/or

[0035] (c) an amino acid sequence analogue having at least 70% sequence identity to any one of the sequences in (a) or (b) and at the same time being immunogenic

[0036] Also, the invention relates to a substantially pure polypeptide, which comprises an amino acid sequence selected from

[0037] (a) Rv0652, Rv2462c, Rv1984c, Rv2185c, Rv1636, Rv3451, Rv3872, Rv3354 and Rv2623

[0038] (b) an immunogenic portion of any one of the sequences in (a); and/or

[0039] (c) an amino acid sequence analogue having at least 70% sequence identity to any one of the sequences in (a) or (b) and at the same time being immunogenic

for use in preparing a composition according to the invention or a diagnostic tool according to the invention.

[0040] In a further embodiment, the invention relates to a nucleic acid fragments in isolated form which

[0041] (a) comprises one or more nucleic acid sequences which encodes a polypeptide as defined above, or comprises a nucleic acid sequence complementary thereto; or

[0042] (b) has a length of at least 10 nucleotides and hybridizes readily under stringent hybridization conditions with a nucleotide sequence selected from Rv0652, Rv2462c, Rv1984c, Rv2185c, Rv1636, Rv3451, Rv3872, Rv3354 and Rv2623 nucleotide sequences or a sequence complementary thereto, or with a nucleotide sequence selected from a sequence in (a).

[0043] The nucleic acid fragment is preferably a DNA fragment.

[0044] In another embodiment, the invention relates to the use of a nucleic acid fragment according to the invention for the preparation of a composition for the diagnosis of tuberculosis caused by virulent mycobacteria, e.g. by *Mycobacterium tuberculosis*, *Mycobacterium africanum* or *Mycobacterium bovis*.

[0045] The invention also relates to a replicable expression vector, which comprises a nucleic acid, fragment according to the invention, and to a transformed cell harbouring at least one such vector.

[0046] In another embodiment, the invention relates to a method for producing a polypeptide according to the invention, comprising

[0047] (a) inserting a nucleic acid fragment according to the invention into a vector which is able to replicate in a host cell, introducing the resulting recombinant vector into the host cell, culturing the host cell in a culture medium under conditions sufficient to effect expression of the polypeptide, and recovering the polypeptide from the host cell or culture medium;

[0048] (b) isolating the polypeptide from a whole mycobacterium, e.g. *Mycobacterium tuberculosis*, *Mycobacte-*

rium africanum or *Mycobacterium bovis*, from culture filtrate or from lysates or fractions thereof; or

[0049] (c) synthesizing the polypeptide e.g. by solid or liquid phase peptide synthesis.

[0050] The invention also relates to a method of diagnosing tuberculosis caused by virulent mycobacteria, e.g. by *Mycobacterium tuberculosis*, *Mycobacterium africanum* or *Mycobacterium bovis*, in an animal, including a human being, comprising intradermally injecting, in the animal, a composition according to the invention, a positive skin response at the location of injection being indicative of the animal having tuberculosis, and a negative skin response at the location of injection being indicative of the animal not having tuberculosis.

[0051] A monoclonal or polyclonal antibody, which is specifically reacting with a polypeptide according to the invention in an immuno assay, or a specific binding fragment of said antibody for use as a diagnostic reagent, is also a part of the invention.

[0052] In a further embodiment, the invention relates to a method for diagnosing previous or ongoing infection with a virulent mycobacterium, said method comprising

[0053] (a) contacting a subject sample, e.g. a blood sample, with a composition according to the invention or a diagnostic tool according to the invention,

[0054] (b) detecting binding of an antibody, said binding being an indication that said subject is infected by *Mycobacterium tuberculosis* or is susceptible to *Mycobacterium tuberculosis* infection.

[0055] In an important embodiment, the invention relates to a serodiagnostic composition comprising a combination of two or more substantially pure polypeptides, which comprises one or more amino acid sequences selected from

[0056] (a) Rv0652, Rv2462c, Rv1983c, Rv2185c, Rv1636, Rv3451, Rv3872, Rv3354 and Rv2623;

[0057] (b) an immunogenic portion of any one of the sequences in (a); and/or

[0058] (c) an amino acid sequence analogue having at least 70% sequence identity to any one of the sequences in (a) or (b) and at the same time being immunogenic.

[0059] Throughout this specification, unless the context requires otherwise, the word "comprise", or variations thereof such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

[0060] By the term "a polypeptide" in the present application is generally understood a polypeptide of the invention, as will be described later. It is also within the meaning of "a polypeptide" that several polypeptides can be used, i.e. in the present context "a" means "at least one" unless explicitly indicated otherwise. The "polypeptide" is used to refer to short peptides with a length of at least two amino acid residues and at most 10 amino acid residues, oligopeptides (11-100 amino acid residues), and longer peptides (the usual interpretation of "polypeptide", i.e. more than 100 amino acid residues in length) as well as proteins (the functional entity comprising at least one peptide, oligopep-

tide, or polypeptide which may be chemically modified by being phosphorylated, glycosylated, by being lipidated, or by comprising prosthetic groups). The definition of polypeptides comprises native forms of peptides/proteins in *Mycobacteria* as well as recombinant proteins or peptides in any type of expression vectors transforming any kind of host, and also chemically synthesized polypeptides. Within the scope of the invention is a polypeptide which is at least 6 amino acids long, preferably 7, such as 8, 9, 10, 11, 12, 13, 14 amino acids long, preferably at least 15 amino acids, such as 15, 16, 17, 18, 19, 20 amino acids long. However, also longer polypeptides having a length of e.g. 25, 50, 75, 100, 125, 150, 175 or 200 amino acids are within the scope of the present invention.

[0061] In the present context the term "purified polypeptide" and substantially pure polypeptide fragment" means a polypeptide preparation which contains at most 5% by weight of other polypeptide material with which it is natively associated (lower percentages of other polypeptide material are preferred, e.g. at most 4%, at most 3%, at most 2%, at most 1%, and at most 1/2%). It is preferred that the substantially pure polypeptide is at least 96% pure, i.e. that the polypeptide constitutes at least 96% by weight of total polypeptide material present in the preparation, and higher percentages are preferred, such as at least 97%, at least 98%, at least 99%, at least 99.25%, at least 99.5%, and at least 99.75%. It is especially preferred that the polypeptide is in "essentially pure form", i.e. that the polypeptide is essentially free of any other antigen with which it is natively associated, i.e. free of any other antigen from bacteria belonging to the tuberculosis complex. This can be accomplished by preparing the polypeptide by means of recombinant methods in a non-mycobacterial host cell as will be described in detail below, or by synthesizing the polypeptide by the well-known methods of solid or liquid phase peptide synthesis, e.g. by the method described by Merrifield or variations thereof.

[0062] By the terms "somatic protein" or "protein derived from the cell wall, the cell membrane or the cytosol", or by the abbreviation "SPE" is understood a polypeptide or a protein extract obtainable from a cell or a part. By the terms "culture filtrate protein", or by the abbreviation "STCF" is understood a complex mixture of proteins released from *M. tuberculosis* during the first few days of growth in a liquid medium.

[0063] By the term "non-naturally occurring polypeptide" is understood a polypeptide that does not occur naturally. This means that the polypeptide is substantially pure, and/or that the polypeptide has been synthesized in the laboratory, and/or that the polypeptide has been produced by means of recombinant technology.

[0064] The "tuberculosis-complex" has its usual meaning, i.e. the complex of mycobacteria causing TB which are *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium bovis* BCG, and *Mycobacterium africanum*.

[0065] By the term "virulent *Mycobacterium*" is understood a bacterium capable of causing the tuberculosis disease in a mammal including a human being. Examples of virulent *Mycobacteria* are *M. tuberculosis*, *M. africanum*, and *M. bovis*.

[0066] By "a TB patient" is understood an individual with culture or microscopically proven infection with virulent

Mycobacteria, and/or an individual clinically diagnosed with TB and who is responsive to anti-TB chemotherapy. Culture, microscopy and clinical diagnosis of TB is well known by the person skilled in the art.

[0067] By the term “PPD positive individual” is understood an individual with a positive Mantoux test or an individual where PPD induces an increase in in vitro recall response determined by release of IFN- γ of at least 1,000 pg/ml from Peripheral Blood Mononuclear Cells (PBMC) or whole blood, the induction being performed by the addition of 2.5 to 5 μ g PPD/ml to a suspension comprising about 1.0 to 2.5 $\times 10^5$ PBMC, the release of IFN- γ being assessable by determination of IFN- γ in supernatant harvested 5 days after the addition of PPD to the suspension compared to the release of IFN- γ without the addition of PPD.

[0068] By the term “delayed type hypersensitivity reaction” is understood a T-cell mediated inflammatory response elicited after the injection of a polypeptide into or application to the skin, said inflammatory response appearing 72-96 hours after the polypeptide injection or application.

[0069] By the term “IFN- γ ” is understood interferon-gamma.

[0070] By the terms “analogue” and “subsequence” when used in connection with polypeptides is meant any polypeptide having the same immunological characteristics as the polypeptides of the invention shown in any of SEQ ID NOs: 8, 30, 34, 38, 149, 64, 10 or 88. Thus, included is also a polypeptide from a different source, such as from another bacterium or even from a eukaryotic cell.

[0071] When referring to an “immunologically equivalent” polypeptide is herein meant that the polypeptide, when formulated in a diagnostic agent (i.e. together with a pharmaceutically acceptable carrier or vehicle and optionally an adjuvant), will elicit a diagnostically significant immune response in a mammal indicating previous or ongoing sensitization with antigens derived from mycobacteria belonging to the tuberculosis complex; this diagnostically significant immune response can be in the form of a delayed type hypersensitivity reaction which can e.g. be determined by a skin test, or a serological test. A diagnostically significant response in a skin test setup will be a reaction which gives rise to a skin reaction which is at least 5 mm in diameter and which is at least 65% (preferably at least 75% such as at the least 85%) of the skin reaction (assessed as the skin reaction diameter) elicited by the parent polypeptide comprising SEQ ID NO: 8, 30, 34, 38, 149, 64, 10 and 88.

[0072] When the term “nucleotide” is used in the following, it should be understood in the broadest sense. That is, most often the nucleotide should be considered as DNA. However, when DNA can be substituted with RNA, the term nucleotide should be read to include RNA embodiments which will be apparent for the person skilled in the art. For the purposes of hybridization, PNA or LNA may be used instead of DNA. PNA has been shown to exhibit a very dynamic hybridization profile and is described in Nielsen P E et al., 1991, *Science* 254: 1497-1500). LNA (Locked Nucleic Acids) is a recently introduced oligonucleotide analogue containing bicyclo nucleoside monomers (Koshkin et al., 1998, 54, 3607-3630; Nielsen, N. K. et al. *J. Am. Chem. Soc.* 1998, 120, 5458-5463).

[0073] The term “stringent” when used in conjunction with hybridization conditions is as defined in the art, i.e. the

hybridization is performed at a temperature not more than 15-20° C. under the melting point T_m , cf. Sambrook et al, 1989, pages 11.45-11.49. Preferably, the conditions are “highly stringent”, i.e. 5-10° C. under the melting point T_m .

[0074] The terms “analogue” or “subsequence” when used in connection with the nucleotide fragments of the invention are thus intended to indicate a nucleotide sequence which encodes a polypeptide exhibiting identical or substantially identical immunological properties to a polypeptide encoded by the nucleotide fragment of the invention shown in any of SEQ ID NOs: 7, 29, 33, 37, 148, 63, 9 or 87 allowing for minor variations which do not have an adverse effect on the ligand binding properties and/or biological function and/or immunogenicity as compared to any of the polypeptides of the invention or which give interesting and useful novel binding properties or biological functions and immunogenicities etc. of the analogue and/or subsequence. The analogous nucleotide fragment or nucleotide sequence may be derived from a bacterium, a mammal, or a human or may be partially or completely of synthetic origin. The analogue and/or subsequence may also be derived through the use of recombinant nucleotide techniques.

[0075] The term “subsequence” when used in connection with the nucleic acid fragments of the invention is intended to indicate a continuous stretch of at least 10 nucleotides which exhibits the above hybridization pattern. Normally this will require a minimum sequence identity of at least 70% with a subsequence of the hybridization partner having SEQ ID NO: 7, 29, 33, 37, 148, 63, 9 or 87. It is preferred that the nucleic acid fragment is longer than 10 nucleotides, such as at least 15, at least 20, at least 25, at least 30, at least 35, at least 40, at least 45, at least 50, at least 55, at least 60, at least 65, at least 70, and at least 80 nucleotides long, and the sequence identity should preferably also be higher than 70%, such as at least 75%, at least 80%, at least 85%, at least 90%, at least 92%, at least 94%, at least 96%, and at least 98%. It is most preferred that the sequence identity is 100%. Such fragments may be readily prepared by, for example, directly synthesizing the fragment by chemical means, by application of nucleic acid reproduction technology, such as the PCR technology of U.S. Pat. No. 4,603,102, or by introducing selected sequences into recombinant vectors for recombinant production.

[0076] Furthermore, the terms “analogue” and “subsequence” are intended to allow for variations in the sequence such as substitution, insertion (including introns), addition, deletion and rearrangement of one or more nucleotides, which variations do not have any substantial effect on the polypeptide encoded by a nucleotide fragment or a subsequence thereof. The term “substitution” is intended to mean the replacement of one or more nucleotides in the full nucleotide sequence with one or more different nucleotides, “addition” is understood to mean the addition of one or more nucleotides at either end of the full nucleotide sequence, “insertion” is intended to mean the introduction of one or more nucleotides within the full nucleotide sequence, “deletion” is intended to indicate that one or more nucleotides have been deleted from the full nucleotide sequence whether at either end of the sequence or at any suitable point within it, and “rearrangement” is intended to mean that two or more nucleotide residues have been exchanged with each other.

[0077] It is well known that the same amino acid may be encoded by various codons, the codon usage being related,

inter alia, to the preference of the organisms in question expressing the nucleotide sequence. Thus, at least one nucleotide or codon of a nucleotide fragment of the invention may be exchanged by others which, when expressed, results in a polypeptide identical or substantially identical to the polypeptide encoded by the nucleotide fragment in question.

[0078] The term "sequence identity" indicates a quantitative measure of the degree of homology between two amino acid sequences of equal length or between two nucleotide sequences of equal length. If the two sequences to be compared are not of equal length, they must be aligned to best possible fit. The sequence identity can be calculated as

$$\frac{(N_{ref} - N_{dif})100}{N_{ref}},$$

wherein N_{dif} is the total number of nonidentical residues in the two sequences when aligned and wherein N_{ref} is the number of residues in one of the sequences. Hence, the DNA sequence AGTCAGTC will have a sequence identity of 75% with the sequence AATCAATC ($N_{dif}=2$ and $N_{ref}=8$). A gap is counted as non-identity of the specific residue(s), i.e. the DNA sequence AGTGTC will have a sequence identity of 75% with the DNA sequence AGTCAGTC ($N_{dif}=2$ and $N_{ref}=8$). Sequence identity can alternatively be calculated by the BLAST program e.g. the BLASTP program or the BLASTN program (Pearson W. R and D. J. Lipman (1988) PNAS USA 85:2444-2448)(www.ncbi.nlm.nih.gov/BLAST). In one aspect of the invention, alignment is performed with the global align algorithm with default parameters as described by X. Huang and W. Miller. Adv. Appl. Math. (1991) 12:337-357, available at http://www.ch.embnet.org/software/LALIGN_form.html.

[0079] The sequence identity is used here to illustrate the degree of identity between the amino acid sequence of a given polypeptide and the amino acid sequence shown in SEQ ID NO: 8, 30, 34, 38, 149, 64, 10 and 88. The amino acid sequence to be compared with the amino acid sequence shown in SEQ ID NO: 8, 30, 34, 38, 149, 64, 10 and 88 may be deduced from a DNA sequence, e.g. obtained by hybridization as defined below, or may be obtained by conventional amino acid sequencing methods. The sequence identity is preferably determined on the amino acid sequence of a mature polypeptide, i.e. without taking any leader sequence into consideration.

[0080] As appears from the above disclosure, polypeptides which are not identical to the polypeptides having SEQ ID NO: 8, 30, 34, 38, 149, 64, 10 and 88 are embraced by the present invention. The invention allows for minor variations which do not have an adverse effect on immunogenicity compared to the parent sequences and which may give interesting and useful novel binding properties or biological functions and immunogenicities etc.

[0081] Each polypeptide fragment may thus be characterized by specific amino acid and nucleic acid sequences. It will be understood that such sequences include analogues and variants produced by recombinant methods wherein such nucleic acid and polypeptide sequences have been modified by substitution, insertion, addition and/or deletion of one or more nucleotides in said nucleic acid sequences to

cause the substitution, insertion, addition or deletion of one or more amino acid residues in the recombinant polypeptide. When the term DNA is used in the following, it should be understood that for the number of purposes where DNA can be substituted with RNA, the term DNA should be read to include RNA embodiments which will be apparent for the man skilled in the art. For the purposes of hybridization, PNA may be used instead of DNA, as PNA has been shown to exhibit a very dynamic hybridization profile (PNA is described in Nielsen P E et al., 1991, Science 254: 1497-1500).

[0082] The nucleotide sequence to be modified may be of cDNA or genomic origin as discussed above, but may also be of synthetic origin. Furthermore, the sequence may be of mixed cDNA and genomic, mixed cDNA and synthetic or genomic and synthetic origin as discussed above. The sequence may have been modified, e.g. by site-directed mutagenesis, to result in the desired nucleic acid fragment encoding the desired polypeptide.

[0083] The nucleotide sequence may be modified using any suitable technique which results in the production of a nucleic acid fragment encoding a polypeptide of the invention.

[0084] The modification of the nucleotide sequence encoding the amino acid sequence of the polypeptide of the invention should be one which does not impair the immunological function of the resulting polypeptide.

[0085] In particular, the invention relates to a polypeptide obtained from *M. tuberculosis*, which polypeptide has at least one of the following properties:

[0086] i) it induces a specific antibody response in a TB patient as determined by an ELISA technique or a western blot when the whole blood is diluted 1:20 in PBS and stimulated with the polypeptide in a concentration of at the most 20 µg/ml and induces an OD of at least 0.1 in ELISA, or a visual response in western blot.

[0087] ii) it induces a positive DTH response determined by intradermal injection or local application patch of at most 100 µg of the polypeptide to an individual who is clinically or subclinically infected with a virulent *Mycobacterium*, a positive response having a diameter of at least 10 mm 72-96 hours after the injection or application,

[0088] iii) it induces a positive DTH response determined by intradermal injection or local application patch of at most 100 µg of the polypeptide to an individual who is clinically or subclinically infected with a virulent *Mycobacterium*, a positive response having a diameter of at least 10 mm 72-96 hours after the injection, and preferably does not induce a such response in an individual who has a cleared infection with a virulent *Mycobacterium*.

[0089] Any polypeptide fulfilling one or more of the above properties and which is obtainable from either the cell wall, cell membrane, the cytosol or STCF is within the scope of the present invention.

[0090] The property described in i) will in particular be satisfied, if the ELISA is performed as follows: the polypeptide of interest in the concentration of 1 to 10 µg/ml is coated on a 96 wells polystyrene plate (NUNC, Denmark) and after a washing step with phosphate buffer pH 7.3, containing 0.37 M NaCl and 0.5% Tween-20 the serum or plasma from

a TB patient is applied in dilution's from 1:10 to 1:1000 in PBS with 1% Tween-20. Binding of an antibody to the polypeptide is determined by addition of a labeled (e.g. peroxidase labeled) secondary antibody and reaction is thereafter visualized by the use of OPD and H₂O₂ as described by the manufacturer (DAKO, Denmark). The OD value in each well is determined using an appropriate ELISA reader.

[0091] In a preferred embodiment the western blot is performed as follows: The polypeptide is applied in concentrations from 1-40 µg to a SDS-PAGE and after electrophoresis the polypeptide is transferred to a membrane e.g. nitrocellulose or PVDF. The membrane is thereafter washed in phosphate buffer, pH 7.3, containing 0.37 M NaCl and 0.5% Tween-20 for 30 min. The sera obtained from one or more TB patients were diluted 1:10 to 1:1000 in phosphate buffer pH 7.3 containing 0.37 M NaCl. The membrane is hereafter washed four times five minutes in binding buffer and incubated with peroxidase- or phosphates-labeled secondary antibody. Reaction is then visualized using the staining method recommended by the manufacture (DAKO, Denmark).

[0092] The property described in ii) will in particular be satisfied if the polypeptide does not induce such a response in an individual not infected with a virulent *Mycobacterium*, i.e. an individual who has been BCG vaccinated or infected with *Mycobacterium avium* or sensitized by non-tuberculosis *Mycobacterium*. In a preferred embodiment the amount of polypeptide intradermally injected or applied is 90 µg, such as 80 µg, 70 µg, 60 µg, 50 µg, 40 µg, or 30 µg. In another embodiment of the invention, the diameter of the positive response is at least 11 mm, such as 12 mm, 13 mm, 14 mm, or 15 mm. In a preferred embodiment the induration of erythema or both could be determined after administration of the polypeptide by intradermal injection, patch test or multipuncture. The reaction diameter could be positive after more than 48, such as 72 or 96 hours.

[0093] The property described in ii) will in particular be satisfied if the polypeptide does not induce such a response in an individual cleared of an infection with a virulent *Mycobacterium*, i.e. which does not have any positive culture or microscopically proven ongoing infection with virulent *Mycobacterium*. The comments on property ii) regarding the amount of polypeptide intradermally injected or applied and the diameter of the positive response are equally relevant to property iii).

[0094] In immunodiagnostics, it is often possible and practical to prepare antigens from segments of a known immunogenic protein or polypeptide. Certain epitopic regions may be used to produce responses similar to those produced by the entire antigenic polypeptide. Potential antigenic or immunogenic regions may be identified by any of a number of approaches, e.g., Jameson-Wolf or Kyte-Doolittle antigenicity analyses or Hopp and Woods (Hopp et Woods, (1981), Proc Natl Acad Sci USA 78/6:3824-8) hydrophobicity analysis (see, e.g., Jameson and Wolf, (1988) Comput Appl Biosci, 4(1):181-6; Kyte and Doolittle, (1982) J Mol Biol, 157(1):105-32; or U.S. Pat. No. 4,554, 101). Hydrophobicity analysis assigns average hydrophilicity values to each amino acid residue; from these values average hydrophilicities can be calculated and regions of greatest hydrophilicity determined. Using one or more of

these methods, regions of predicted antigenicity may be derived from the amino acid sequence assigned to the polypeptides of the invention. Alternatively, in order to identify relevant T-cell epitopes which are recognized during an immune response, it is also possible to use a "brute force" method: Since T-cell epitopes are linear, deletion mutants of polypeptides will, if constructed systematically, reveal what regions of the polypeptide are essential in immune recognition. A presently preferred method utilises overlapping oligomers (preferably synthetic ones having a length of e.g. 20 amino acid residues) derived from the polypeptide. A preferred T-cell epitope is a T-helper cell epitope or a cytotoxic T-cell epitope.

[0095] Although the minimum length of a T-cell epitope has been shown to be at least 6 amino acids, it is normal that such epitopes are constituted of longer stretches of amino acids. Hence it is preferred that the polypeptide fragment of the invention has a length of at least 7 amino acid residues, such as at least 8, at least 9, at least 10, at least 12, at least 14, at least 16, at least 18, at least 20, at least 22, at least 24, or at least 30 amino acid residues.

[0096] B-cell epitopes may be linear or spatial. The three-dimensional structure of a protein is often such that amino acids, which are located distant from each other in the one-dimensional structure, are located near to each other in the folded protein. Within the meaning of the present context, the expression epitope is intended to comprise the one- and three-dimensional structure as well as mimics thereof. The term is further intended to include discontinuous B-cell epitopes. The linear B-cell epitopes can be identified in a similar manner as described for the T-cell epitopes above. However, when identifying B-cell epitopes the assay should be an ELISA using overlapping oligomers derived from the polypeptide as the coating layer on a microtiter plate as described elsewhere.

[0097] A non-naturally occurring polypeptide, an analogue, a subsequence, a T-cell epitope and/or a B-cell epitope of any of the described polypeptides are defined as any non-naturally occurring polypeptide, analogue, subsequence, T-cell epitope and/or B-cell epitope of any of the polypeptides inducing a specific antibody response in a TB patient.

[0098] Preferred embodiments of the invention are the specific polypeptides which have been identified and analogues and subsequences thereof. It has been noted that none of the identified polypeptides in the examples include a signal sequence. Table 1 lists the antigens of the invention.

TABLE 1

The antigens of the invention by the names used herein as well as by reference to relevant SEQ ID NOs of N-terminal sequences, full amino acid sequences and sequences of nucleotides encoding the antigens			
Antigen	Sanger ID	Nucleotide sequence SEQ ID NO:	Amino acid sequence SEQ ID NO:
TB15A	Rv1636	7	8
TB16	Rv2185c	29	30
TB32	Rv2623	33	34
TB51	Rv2462c	37	38
CFP8A	Rv3354	148	149
CFP16	Rv0652	63	64

TABLE 1-continued

The antigens of the invention by the names used herein as well as by reference to relevant SEQ ID NOs of N-terminal sequences, full amino acid sequences and sequences of nucleotides encoding the antigens			
Antigen	Sanger ID	Nucleotide sequence SEQ ID NO:	Amino acid sequence SEQ ID NO:
CFP21	Rv1984c	9	10
CFP23	Rv3451	55	56
RD1-ORF3	Rv3872	87	88

Until the present invention was made, it was unknown that the polypeptides Rv1636, Rv2185c, Rv2623, Rv2462c, Rv3354, Rv0652, Rv1984c, Rv3451 or Rv3872 with the amino acid sequences disclosed in SEQ ID NOs: 8, 30, 34, 38, 149, 64, 10, 56 and 88 are expressed in live virulent *Mycobacterium*. These polypeptides in purified form, or non-naturally occurring, i.e. recombinantly or synthetically produced, are considered part of the invention. It is understood that a polypeptide which has the above mentioned property and has a sequence identity of at least 80% with any of the amino acid sequences shown in SEQ ID NOs: 8, 30, 34, 38, 149, 64, 10, 56 and 88 or has a sequence identity of at least 80% to any subsequence thereof is considered part of the invention. In a preferred embodiment the sequence identity is at least 80%, such as 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 99% or 99.5%. Furthermore, any T cell epitope of the polypeptides disclosed in SEQ ID NOs: 8, 30, 34, 38, 149, 64, 10, 56, and 88 is considered part of the invention. Also, any B-cell epitope of the polypeptides disclosed in SEQ ID NOs: 8, 30, 34, 38, 149, 64, 10, 56 and 88 is considered part of the invention.

[0099] The invention also relates to a replicable expression vector which comprises a nucleic acid fragment defined above, especially a vector which comprises a nucleic acid fragment encoding a polypeptide fragment of the invention. The vector may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication; examples of such a vector are a plasmid, phage, cosmid, mini-chromosome and virus. Alternatively, the vector may be one which, when introduced in a host cell, is integrated in the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

[0100] Expression vectors may be constructed to include any of the DNA segments disclosed herein. Such DNA might encode an antigenic protein specific for virulent strains of mycobacteria or even hybridization probes for detecting mycobacteria nucleic acids in samples. Longer or shorter DNA segments could be used, depending on the antigenic protein desired. Epitopic regions of the proteins expressed or encoded by the disclosed DNA could be included as relatively short segments of DNA. A wide variety of expression vectors is possible including, for example, DNA segments encoding reporter gene products useful for identification of heterologous gene products and/

or resistance genes such as antibiotic resistance genes which may be useful in identifying transformed cells.

[0101] The vector of the invention may be used to transform cells so as to allow propagation of the nucleic acid fragments of the invention or so as to allow expression of the polypeptide fragments of the invention. Hence, the invention also pertains to a transformed cell harbouring at least one such vector according to the invention, said cell being one which does not natively harbour the vector and/or the nucleic acid fragment of the invention contained therein. Such a transformed cell (which is also a part of the invention) may be any suitable bacterial host cell or any other type of cell such as a unicellular eukaryotic organism, a fungus or yeast, or a cell derived from a multicellular organism, e.g. an animal or a plant. It is especially in cases where glycosylation is desired that a mammalian cell is used, although glycosylation of proteins is a rare event in prokaryotes. Normally, however, a prokaryotic cell is preferred such as a bacterium belonging to the genera *Mycobacterium*, *Salmonella*, *Pseudomonas*, *Bacillus* and *Escherichia*. It is preferred that the transformed cell is an *E. coli*, *B. subtilis*, or *M. bovis* BCG cell, and it is especially preferred that the transformed cell expresses a polypeptide according to the invention. The latter opens for the possibility to produce the polypeptide of the invention by simply recovering it from the culture containing the transformed cell. In the most preferred embodiment of this part of the invention the transformed cell is *Mycobacterium bovis* BCG strain: Danish 1331, which is the *Mycobacterium bovis* strain Copenhagen from the Copenhagen BCG Laboratory, Statens Seruminstitut, Denmark.

[0102] The nucleic acid fragments of the invention allow for the recombinant production of the polypeptides fragments of the invention. However, also isolation from the natural source is a way of providing the polypeptide fragments as is peptide synthesis.

[0103] Therefore, the invention also pertains to a method for the preparation of a polypeptide fragment of the invention, said method comprising inserting a nucleic acid fragment as described in the present application into a vector which is able to replicate in a host cell, introducing the resulting recombinant vector into the host cell (transformed cells may be selected using various techniques, including screening by differential hybridization, identification of fused reporter gene products, resistance markers, anti-antigen antibodies and the like), culturing the host cell in a culture medium under conditions sufficient to effect expression of the polypeptide (of course the cell may be cultivated under conditions appropriate to the circumstances, and if DNA is desired, replication conditions are used), and recovering the polypeptide from the host cell or culture medium; or

[0104] isolating the polypeptide from a short-term culture filtrate; or

[0105] isolating the polypeptide from whole mycobacteria of the tuberculosis complex or from lysates or fractions thereof, e.g. cell wall containing fractions, or

[0106] synthesizing the polypeptide by solid or liquid phase peptide synthesis.

[0107] The medium used to grow the transformed cells may be any conventional medium suitable for the purpose.

A suitable vector may be any of the vectors described above, and an appropriate host cell may be any of the cell types listed above. The methods employed to construct the vector and effect introduction thereof into the host cell may be any methods known for such purposes within the field of recombinant DNA. In the following a more detailed description of the possibilities will be given:

[0108] In general, of course, prokaryotes are preferred for the initial cloning of nucleic sequences of the invention and constructing the vectors useful in the invention. For example, in addition to the particular strains mentioned in the more specific disclosure below, one may mention by way of example, strains such as *E. coli* K12 strain 294 (ATCC No. 31446), *E. coli* B, and *E. coli* X 1776 (ATCC No. 31537). These examples are, of course, intended to be illustrative and not limiting.

[0109] Prokaryotes are also preferred for expression. The aforementioned strains, as well as *E. coli* W3110 (F-, lambda-, prototrophic, ATCC No. 273325), bacilli such as *Bacillus subtilis*, or other enterobacteriaceae such as *Salmonella typhimurium* or *Serratia marcescens*, and various *Pseudomonas* species may be used. Especially interesting are rapid-growing mycobacteria, e.g. *M. smegmatis*, as these bacteria have a high degree of resemblance with mycobacteria of the tuberculosis complex and therefore stand a good chance of reducing the need of performing posttranslational modifications of the expression product.

[0110] In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. For example, *E. coli* is typically transformed using pBR322, a plasmid derived from an *E. coli* species (see, e.g., Bolivar et al., 1977, Gene 2: 95). The pBR322 plasmid contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR plasmid, or other microbial plasmids or phages must also contain, or be modified to contain, promoters which can be used by the microorganism for expression.

[0111] Those promoters most commonly used in recombinant DNA construction include the B-lactamase (penicillinase) and lactose promoter systems (Chang et al., (1978), Nature, 35:515; Itakura et al., (1977), Science 198:1056; Goeddel et al., (1979), Nature 281:544) and a tryptophan (trp) promoter system (Goeddel et al., (1979) Nature 281:544; EPO Appl. Publ. No. 0036776). While these are the most commonly used, other microbial promoters have been discovered and utilized, and details concerning their nucleotide sequences have been published, enabling a skilled worker to ligate them functionally with plasmid vectors (Siebwenlist et al., (1980), Cell, 20:269). Certain genes from prokaryotes may be expressed efficiently in *E. coli* from their own promoter sequences, precluding the need for addition of another promoter by artificial means.

[0112] After the recombinant preparation of the polypeptide according to the invention, the isolation of the polypeptide may for instance be carried out by affinity chromatography (or other conventional biochemical procedures based on chromatography), using a monoclonal antibody which substantially specifically binds the polypeptide according to

the invention. Another possibility is to employ the simultaneous electroelution technique described by Andersen et al. in J. Immunol. Methods 161: 29-39.

[0113] According to the invention the post-translational modifications involves lipidation, glycosylation, cleavage, or elongation of the polypeptide.

[0114] Individuals infected with virulent *Mycobacteria* can generally be divided into two groups. The first group has an infection with a virulent *Mycobacterium* e.g. contacts of TB patients. The virulent *Mycobacterium* may have established colonies in the lungs, but the individual has, as yet, no symptoms of TB. The second group has clinical symptoms of TB, as a TB patient.

[0115] In one embodiment of the invention, any of the above mentioned polypeptides are used for the manufacture of a diagnostic reagent that preferably distinguishes a subclinically or clinically infected individual (group I and group II) from an individual who has been BCG vaccinated or infected with *Mycobacterium avium* or sensitized by non-tuberculosis *Mycobacterium* (NTM), and may distinguish a subclinically or clinically infected individual from an individual who has cleared a previous infection with a virulent *Mycobacterium*. It is most likely that specific polypeptides derived from SPE or ST-CF will identify group I and/or group II from individuals not infected with virulent *Mycobacteria* in the same way as ESAT-6 and CFP10 (P. Ravn et al., (1998), J. Infectious Disease 179:63745).

[0116] In another embodiment of the invention, any of the above discussed polypeptides are used for the manufacture of a diagnostic reagent for the diagnosis of an infection with a virulent *Mycobacterium*. One embodiment of the invention provides a diagnostic reagent for differentiating an individual who is clinically or subclinically infected with a virulent *Mycobacterium* from an individual not infected with virulent *Mycobacterium*, i.e. an individual who has been BCG vaccinated or infected with *Mycobacterium avium* or sensitized by non-tuberculosis *Mycobacterium* (NTM). Such a diagnostic reagent will distinguish between an individual in group I and/or II of the infection stages above, from an individual who has been vaccinated against TB.

[0117] Another embodiment of the invention provides a diagnostic reagent for differentiating an individual who is clinically or subclinically infected with a virulent *Mycobacterium* from an individual who has a cleared infection with a virulent *Mycobacterium*. Such a diagnostic reagent will distinguish between an individual in group I and/or II of the infection stages above, from an individual who has cleared the infection.

[0118] Determination of an infection with virulent *Mycobacterium* will be instrumental in the, still very laborious, diagnostic process of tuberculosis. A number of possible diagnostic assays and methods can be envisaged (some more specifically described in the examples and the list of properties): a sample comprising whole blood or mononuclear cells (i.a. T-lymphocytes) from a patient could be contacted with a sample of one or more polypeptides of the invention. This contacting can be performed in vitro and a positive reaction could e.g. be proliferation of the T-cells or release of cytokines such as IFN- γ into the extracellular phase (e.g. into a culture supernatant).

[0119] Alternatively, a sample of a possibly infected organ may be contacted with an antibody raised against a polypep-

tide of the invention. The demonstration of the reaction by means of methods well-known in the art between the sample and the antibody will be indicative of ongoing infection and could be used to monitor treatment effect by reduction in responses.

[0120] It is of course also a possibility to demonstrate the presence of anti-Mycobacterial antibodies in serum by contacting a serum sample from a subject with at least one of the polypeptide fragments of the invention and using well-known methods for visualising the reaction between the antibody and antigen such as ELISA, Western blot, precipitation assays.

[0121] The invention also relates to a method of diagnosing infection caused by a virulent *Mycobacterium* in a mammal, including a human being, comprising locally applying (patch test) or intradermally injecting (Mantoux test) a polypeptide of the invention. These tests are both called a delayed hypersensitivity reaction (DTH). A positive skin response at the location of injection or application is indicative of the mammal including a human being, being infected with a virulent *Mycobacterium*, and a negative skin response at the location of injection or application is indicative of the mammal including a human being not having TB. A positive response is a skin reaction having a diameter of at least 5 mm larger than background, but larger reactions are preferred, such as at least 1 cm, 1.5 cm, and at least 2 cm in diameter. A skin reaction is here to mean erythema or induration of the skin, as directly measured. The composition used as the skin test reagent can be prepared in the same manner as described for the vaccines above.

[0122] In human volunteers, the generation of a significant immune response can alternatively be defined as the ability of the reagent being tested to stimulate an in vitro recall response by peripheral blood cells from at least 30% of PPD positive individuals previously vaccinated with that reagent or infected with a virulent *Mycobacterium*, said recall response being defined as proliferation of T cells or the production of cytokine(s) which is higher than the responses generated by cells from unimmunized or uninfected control individuals, with a 95% confidence interval as defined by an appropriate statistical analysis such as a Student's two-tailed T test.

[0123] The polypeptides according to the invention may be potential drug targets. The fact that certain of the disclosed antigens are not present in *M. bovis* BCG but are present in virulent mycobacteria point them out as interesting drug targets; the antigens may constitute receptor molecules or toxins which facilitate the infection by the mycobacterium, and if such functionalities are blocked the infectivity of the mycobacterium will be diminished.

[0124] To determine particularly suitable drug targets among the antigens of the invention, the gene encoding at least one of the polypeptides of the invention and the necessary control sequences can be introduced into avirulent strains of mycobacteria (e.g. BCG) so as to determine which of the polypeptides are critical for virulence. Once particular proteins are identified as critical for/contributory to virulence, anti-mycobacterial agents can be designed rationally to inhibit expression of the critical genes or to attack the critical gene products. For instance, antibodies or fragments thereof (such as Fab and (Fab')₂ fragments can be prepared against such critical polypeptides by methods known in the

art and thereafter used as prophylactic or therapeutic agents. Alternatively, small molecules can be screened for their ability to selectively inhibit expression of the critical gene products, e.g. using recombinant expression systems which include the gene's endogenous promoter, or for their ability to directly interfere with the action of the target. These small molecules are then used as therapeutics or as prophylactic agents to inhibit mycobacterial virulence.

[0125] Alternatively, anti-mycobacterial agents which render a virulent *mycobacterium* avirulent can be operably linked to expression control sequences and used to transform a virulent mycobacterium. Such anti-mycobacterial agents inhibit the replication of a specified mycobacterium upon transcription or translation of the agent in the mycobacterium. Such a "newly avirulent" mycobacterium would constitute a superb alternative to the above described modified BCG for vaccine purposes since it would be immunologically very similar to a virulent *mycobacterium* compared to e.g. BCG.

[0126] Once a particular interesting polypeptide has been identified, the biological function of that polypeptide may be tested. The polypeptides may constitute receptor molecules or toxins which facilitates the infection by the *Mycobacterium* and if such functionality is blocked, the infectivity of the virulent *Mycobacterium* will be diminished.

[0127] The biological function of particular interesting polypeptides may be tested by studying the effect of inhibiting the expression of the polypeptides on the virulence of the virulent *Mycobacterium*. This inhibition may be performed at the gene level such as by blocking the expression using antisense nucleic acid, PNA or LNA or by interfering with regulatory sequences or the inhibition may be at the level of translation or post-translational processing of the polypeptide.

[0128] Once a particular polypeptide according to the invention is identified as critical for virulence, an anti-mycobacterial agent might be designed to inhibit the expression of that polypeptide. Such anti-mycobacterial agent might be used as a prophylactic or therapeutic agent. For instance, antibodies or fragments thereof, such as Fab and (Fab')₂ fragments, can be prepared against such critical polypeptides by methods known in the art and thereafter used as prophylactic or therapeutic agents

[0129] A monoclonal or polyclonal antibody, which is specifically reacting with a polypeptide of the invention in an immuno assay, or a specific binding fragment of said antibody, is also a part of the invention. The production of such polyclonal antibodies requires that a suitable animal be immunized with the polypeptide and that these antibodies are subsequently isolated, suitably by immune affinity chromatography. The production of monoclonals can be effected by methods wellknown in the art, since the present invention provides for adequate amounts of antigen for both immunization and screening of positive hybridomas.

[0130] As will appear from the examples, a number of the polypeptides of the invention are natively translation products which include a leader sequence (or other short peptide sequences), whereas the product which can be isolated from short-term culture filtrates from bacteria belonging to the tuberculosis complex are free of these sequences. Although it may in some applications be advantageous to produce

these polypeptides recombinantly and in this connection facilitate export of the polypeptides from the host cell by including information encoding the leader sequence in the gene for the polypeptide, it is more often preferred to either substitute the leader sequence with one which has been shown to be superior in the host system for effecting export, or to totally omit the leader sequence (e.g. when producing the polypeptide by peptide synthesis. Hence, a preferred embodiment of the invention is a polypeptide which is free from amino acid residues -32 to -1 in SEQ ID NO: 10 and/or -33 to -1 in SEQ ID NO: 56.

[0131] In another preferred embodiment, the polypeptide fragment of the invention is free from any signal sequence; this is especially interesting when the polypeptide fragment is produced synthetically but even when the polypeptide fragments are produced recombinantly it is normally acceptable that they are not exported by the host cell to the periplasm or the extracellular space; the polypeptide fragments can be recovered by traditional methods (cf. the discussion below) from the cytoplasm after disruption of the host cells, and if there is need for refolding of the polypeptide fragments, general refolding schemes can be employed, cf. e.g. the disclosure in WO 94/18227 where such a general applicable refolding method is described.

[0132] As mentioned above, it will normally be interesting to omit the leader sequences from the polypeptide fragments of the invention. However, by producing fusion polypeptides, superior characteristics of the polypeptide fragments of the invention can be achieved. For instance, fusion partners which facilitate export of the polypeptide when produced recombinantly, fusion partners which facilitate purification of the polypeptide, and fusion partners which enhance the immunogenicity of the polypeptide fragment of the invention are all interesting possibilities. Therefore, the invention also pertains to a fusion polypeptide comprising at least one polypeptide fragment defined above and at least one fusion partner. The fusion partner can, in order to enhance immunogenicity, e.g. be selected from the group consisting of another polypeptide fragment as defined above (so as to allow for multiple expression of relevant epitopes), and an other polypeptide derived from a bacterium belonging to the tuberculosis complex, such as ESAT-6, MPB64, MPT64, and MPB59 or at least one T-cell epitope of any of these antigens. Other immunogenicity enhancing polypeptides which could serve as fusion partners are T-cell epitopes (e.g. derived from the polypeptides ESAT-6, MPB64, MPT64, or MPB59) or other immunogenic epitopes enhancing the immunogenicity of the target gene product, e.g. lymphokines such as IFN- γ , IL-2 and IL-12. In order to facilitate expression and/or purification the fusion partner can e.g. be a bacterial fimbrial protein, e.g. the pilus components pilin and papA; protein A; the ZZ-peptide (ZZ-fusions are marketed by Pharmacia in Sweden); the maltose binding protein; glutathione S-transferase; β -galactosidase; or polyhistidine.

[0133] Also a method of determining the presence of virulent *Mycobacterium* nucleic acids in a mammal, including a human being, or in a sample, comprising incubating the sample with a nucleic acid sequence of the invention or a nucleic acid sequence complementary thereto, and detecting the presence of hybridized nucleic acids resulting from the incubation (by using the hybridization assays which are well-known in the art), is included in the invention. Such a

method of diagnosing TB might involve the use of a composition comprising at least a part of a nucleotide sequence as defined above and detecting the presence of nucleotide sequences in a sample from the animal or human being to be tested which hybridizes with the nucleic acid sequence (or a complementary sequence) by the use of PCR techniques.

[0134] In certain aspects, the DNA sequence information provided by this invention allows for the preparation of relatively short DNA (or RNA or PNA) sequences having the ability to specifically hybridize to mycobacterial gene sequences. In these aspects, nucleic acid probes of an appropriate length are prepared based on a consideration of the relevant sequence. The ability of such nucleic acid probes to specifically hybridize to the mycobacterial gene sequences lend them particular utility in a variety of embodiments. Most importantly, the probes can be used in a variety of diagnostic assays for detecting the presence of pathogenic organisms in a given sample. However, either uses are envisioned, including the use of the sequence information for the preparation of mutant species primers, or primers for use in preparing other genetic constructs.

[0135] In one embodiment of the invention a composition is produced comprising as the effective component a micro-organism, the micro-organism is a bacterium such as *Mycobacterium*, *Salmonella*, *Pseudomonas* and *Escherichia*, preferably *Mycobacterium bovis* BCG wherein at least one, such as at least 2 copies, such as at least 5 copies of a nucleotide fragment comprising a nucleotide sequence encoding a polypeptide of the invention has been incorporated into the genome of the micro-organism or introduced as a part of an expression vector in a manner allowing the micro-organism to express and optionally secrete the polypeptide. In a preferred embodiment, the composition comprises at least 2 different nucleotide sequences encoding at least 2 different polypeptides of the invention.

[0136] Another part of the invention pertains to a nucleic acid fragment in isolated form which

[0137] 1) comprises a nucleic acid sequence which encodes a polypeptide or fusion polypeptide as defined above, or comprises a nucleic acid sequence complementary thereto, and/or

[0138] 2) has a length of at least 10 nucleotides and hybridizes readily under stringent hybridization conditions (as defined in the art, i.e. 5-10° C. under the melting point T_m , cf. Sambrook et al, 1989, pages 11.45-11.49) with a nucleic acid fragment which has a nucleotide sequence selected from

[0139] SEQ ID NO: 7 or a sequence complementary thereto,

[0140] SEQ ID NO: 29 or a sequence complementary thereto,

[0141] SEQ ID NO: 33 or a sequence complementary thereto,

[0142] SEQ ID NO: 37 or a sequence complementary thereto,

[0143] SEQ ID NO: 148 or a sequence complementary thereto,

[0144] SEQ ID NO: 63 or a sequence complementary thereto,

[0145] SEQ ID NO: 9 or a sequence complementary thereto,

[0146] SEQ ID NO: 55 or a sequence complementary thereto,

[0147] SEQ ID NO: 87 or a sequence complementary thereto

[0148] It is preferred that the nucleic acid fragment is a DNA fragment.

[0149] To provide certainty of the advantages in accordance with the invention, the preferred nucleic acid sequence when employed for hybridization studies or assays includes sequences that are complementary to at least a 10 to 40, or so, nucleotide stretch of the selected sequence. A size of at least 10 nucleotides in length helps to ensure that the fragment will be of sufficient length to form a duplex molecule that is both stable and selective. Molecules having complementary sequences over stretches greater than 10 bases in length are generally preferred, though, in order to increase stability and selectivity of the hybrid, and thereby improve the quality and degree of specific hybrid molecules obtained.

[0150] A preferred immunologic composition according to the present invention comprising at least two different polypeptide fragments, each different polypeptide fragment being a polypeptide or a fusion polypeptide defined above. It is preferred that the immunologic composition comprises between 3-20 different polypeptide fragments or fusion polypeptides.

EXAMPLES

Example 1A

Isolation of CFP21

[0151] ST-CF was precipitated with ammonium sulphate at 80% saturation. The precipitated proteins were removed by centrifugation and after resuspension washed with 8 M urea. CHAPS and glycerol were added to a final concentration of 0.5% (w/v) and 5% (v/v) respectively and the protein solution was applied to a Rotofor isoelectrical Cell (BioRad). The Rotofor Cell had been equilibrated with an 8 M urea buffer containing 0.5% (w/v) CHAPS, 5% (v/v) glycerol, 3% (v/v) Biolyt 3/5 and 1% (v/v) Biolyt 4/6 (BioRad). Isoelectric focusing was performed in a pH gradient from 3-6. The fractions were analyzed on silver-stained 10-20% SDS-PAGE. Fractions with similar band patterns were pooled and washed three times with PBS on a Centriprep concentrator (Amicon) with a 3 kDa cut off membrane to a final volume of 1-3 ml. An equal volume of SDS containing sample buffer was added and the protein solution boiled for 5 min before further separation on a Prep Cell (BioRad) in

a matrix of 16% polyacrylamide under an electrical gradient. Fractions containing pure proteins with an molecular mass from 17-30 kDa were collected.

N-terminal Sequencing and Amino Acid Analysis

[0152] CFP21 was washed with water on a Centricon concentrator (Amicon) with cutoff at 10 kDa and then applied to a ProSpin concentrator (Applied Biosystems) where the proteins were collected on a PVDF membrane. The membrane was washed 5 times with 20% methanol before sequencing on a Procise sequencer (Applied Biosystems).

[0153] The following N-terminal sequence was obtained:

For CFP21: DPXSDIAVVFARGTH (SEQ ID NO:150)

"X" denotes an amino acid which could not be determined by the sequencing method used, whereas a "/" between two amino acids denotes that the sequencing method could not determine which of the two amino acids is the one actually present.

Homology Searches in the Sanger Database

[0154] For CFP21 the N-terminal amino acid sequence was used for a homology search using the blast program of the Sanger *Mycobacterium tuberculosis* database:

<http://www.sanger.ac.uk/pathogens/TB-blast-server.html>.

[0155] Thereby, the following information was obtained:

CFP21

[0156] A sequence 100% identical to the 14 determined amino acids of CFP21 was found at MTCY39. From the N-terminal sequencing it was not possible to determine amino acid number 3; this amino acid is a C in MTCY39. The amino acid C can not be detected on a Sequencer which is probably the explanation of this difference.

[0157] Within the open reading frame the translated protein is 217 amino acids long. The N-terminally determined sequence from the protein purified from culture filtrate starts at amino acid 33 in agreement with the presence of a signal sequence that has been cleaved off. This gives a length of the mature protein of 185 amino acids, which corresponds to a theoretical molecular weight at 18657 Da, and a theoretical pI at 4.6. The observed weight in a SDS-PAGE is 21 kDa.

[0158] In a 193 amino acids overlap the protein has 32.6% identity to a cutinase precursor with a length of 209 amino acids (CUTI_ALTBR P41744).

[0159] A comparison of the 14 N-terminal determined amino acids with the translated region (RD2) deleted in *M. bovis* BCG revealed a 100% identical sequence (mb3484) (Mahairas et al. (1996)).

CFP21:

(SEQ ID NO:10)

1 MTPRSLVRIV GVVVATTLLAL VSAPAGGRAA HADPCSDIAV

41 VFARGTHQAS GLGDVGEAFV DSLTSQVGGR SIGVYAVNYP ASDDYRASAS

-continued

91 NGSDASAH1 QRTVASCNT RIVLGGYSQG ATVIDLSTSA MPPAVADHVA
 141 AVALFGPESS GFSSMLWGGG SLPTIGPLYS SKTINLCAPD DPICITGGGNI
 191 MAHVSIVQSG MTSQAATFAA NRLDHAG

Cloning of the Gene Encoding CFP21

[0160] The gene encoding CFP21 was cloned into the expression vector pMCT6, by PCR amplification with gene specific primers, for recombinant expression in *E. coli* of the proteins.

[0161] PCR reactions contained 10 ng of *M. tuberculosis* chromosomal DNA in 1× ow salt Taq+buffer from Stratagene supplemented with 250 mM of each of the four nucleotides (Boehringer Mannheim), 0.5 mg/ml BSA (IgG technology), 1% DMSO (Merck), 5 pmoles of each primer and 0.5 unit Tag+ DNA polymerase (Stratagene) in 10 µl reaction volume. Reactions were initially heated to 94° C. for 25 sec. and run for 30 cycles according to the following program; 94° C. for 10 sec., 55° C. for 10 sec. and 72° C. for 90 sec, using thermocycler equipment from Idaho Technology.

[0162] The DNA fragments were subsequently run on 1% agarose gels, the bands were excised and purified by Spin-X spin columns (Costar) and cloned into pBluescript SK II+–T vector (Stratagene). Plasmid DNA was thereafter prepared from clones harbouring the desired fragments, digested with suitable restriction enzymes and subcloned into the expression vector pMCT6 in frame with 8 histidine residues which are added to the N-terminal of the expressed proteins. The resulting clones were hereafter sequenced by use of the dideoxy chain termination method adapted for supercoiled DNA using the Sequenase DNA sequencing kit version 1.0 (United States Biochemical Corp., USA) and by cycle sequencing using the Dye Terminator system in combination with an automated gel reader (model 373A; Applied Biosystems) according to the instructions provided. Both strands of the DNA were sequenced.

[0163] For cloning the following gene specific primers were used:

[0164] CFP21: Primers used for cloning of cfp21:

(SEQ ID NO:151)
 OPBR-55: ACAGATCTGCGCATGCGGATCCGTGT

(SEQ ID NO:152)
 OPBR-56: TTTTCCATGGTCATCCGGCGTGATCGAG

[0165] OPBR-55 and OPBR-56 create BglIII and NcoI sites, respectively, used for the cloning in pMCT6.

Expression/Purification of Recombinant CFP21 Protein.

[0166] Expression and metal affinity purification of recombinant proteins was undertaken essentially as described by the manufacturers. For each protein, 1 l LB-media containing 100 µg/ml ampicillin, was inoculated with 10 ml of an overnight culture of XL1-Blue cells harbouring recombinant pMCT6 plasmids. Cultures were shaken at 37° C. until they reached a density of OD₆₀₀=0.4-0.6. IPTG was hereafter added to a final concentration of 1 mM and the

cultures were further incubated 4-16 hours. Cells were harvested, resuspended in 1× sonication buffer +8 M urea and sonicated 5×30 sec. with 30 sec. pausing between the pulses. After centrifugation, the lysate was applied to a column containing 25 ml of resuspended Talon resin (Clontech, Palo Alto, USA). The column was washed and eluted as described by the manufacturers.

[0167] After elution, all fractions (1.5 ml each) were subjected to analysis by SDS-PAGE using the Mighty Small (Hoefer Scientific Instruments, USA) system and the protein concentrations were estimated at 280 nm. Fractions containing recombinant protein were pooled and dialysed against 3 M urea in 10 mM Tris-HCl, pH 8.5. The dialysed protein was further purified by FPLC (Pharmacia, Sweden) using a 6 ml Resource-Q column, eluted with a linear 0-1 M gradient of NaCl. Fractions were analyzed by SDS-PAGE and protein concentrations were estimated at OD₂₈₀. Fractions containing protein were pooled and dialysed against 25 mM Hepes buffer, pH 8.5.

[0168] Finally the protein concentration and the LPS content were determined by the BCA (Pierce, Holland) and LAL (Endosafe, Charleston, USA) tests, respectively.

Example 1B

Identification of RD1-ORF3.

[0169] In an effort to control the threat of TB, attenuated bacillus Calmette-Guérin (BCG) has been used as a live attenuated vaccine. BCG is an attenuated derivative of a virulent *Mycobacterium bovis*. The original BCG from the Pasteur Institute in Paris, France was developed from 1908 to 1921 by 231 passages in liquid culture and has never been shown to revert to virulence in animals, indicating that the attenuating mutation(s) in BCG are stable deletions and/or multiple mutations which do not readily revert. While physiological differences between BCG and *M. tuberculosis* and *M. bovis* has been noted, the attenuating mutations which arose during serial passage of the original BCG strain has been unknown until recently. The first mutations described are the loss of the gene encoding MPB64 in some BCG strains (Li et al., 1993, Oettinger and Andersen, 1994) and the gene encoding ESAT-6 in all BCG strain tested (Harboe et al., 1996), later 3 large deletions in BCG have been identified (Mahairas et al., 1996). The region named RD1 includes the gene encoding ESAT-6 and an other (RD2) the gene encoding MPT64. Both antigens have been shown to have diagnostic potential and ESAT-6 has been shown to have properties as a vaccine candidate (cf. PCT/DK94/00273 and PCT/DK/00270). In order to find new *M. tuberculosis* specific diagnostic antigens as well as antigens for a new vaccine against TB, the RD1 region (17.499 bp) of *M. tuberculosis* H37Rv has been analyzed for Open Reading Frames (ORF). ORFs with a minimum length of 96 bp have been predicted using the algorithm described by Borodovsky and McIninch (1993), in total 27 ORFs have been predicted, 20 of these have possible diagnostic and/or vaccine poten-

tial, as they are deleted from all known BCG strains. The predicted ORFs include ESAT-6 (RD1-ORF7) and CFP10 (RD1-ORF6) described previously (Sørensen et al., 1995), as a positive control for the ability of the algorithm. In the present is described the potential of 1 of the predicted antigens for diagnosis of TB.

Identification of rd1-orf3.

[0170] The nucleotide sequence of rd1-orf3 from *M. tuberculosis* H37Rv is set forth in SEQ ID NO: 87. The deduced amino acid sequence of RD1-ORF2 is set forth in SEQ ID NO: 88.

[0171] The DNA sequence rd1-orf3 (SEQ ID NO: 87) contained an open reading frame starting with an ATG codon at position 2807-2809 and ending with a termination codon (TAA) at position 3101-3103 (position numbers referring to the location in RD1). The deduced amino acid sequence (SEQ ID NO: 88) contains 98 residues corresponding to a molecular weight of 9,799.

Cloning of rd1-orf3.

[0172] Rd1-orf3 was PCR cloned in the pMST24 (Theisen et al., 1995) expression vector. Chromosomal DNA from *M. tuberculosis* H37Rv was used as template in the PCR reactions. Oligonucleotides were synthesized on the basis of the nucleotide sequence from the RD1 region (Accession no. U34848). The oligonucleotide primers were engineered to include an restriction enzyme site at the 5' end and at the 3' end by which a later subcloning was possible. Primers are listed in table 2.

[0173] rd1-orf3. A SmaI site was engineered immediately 5' of the first codon of rd1-orf3, and a NcoI site was incorporated right after the stop codon at the 3' end. The gene rd1-orf3 was subcloned in pMST24, giving pTO87.

[0174] The PCR fragments were digested with the suitable restriction enzymes, purified from an agarose gel and cloned into pMST24. The construct was used to transform the *E. coli* XL1-Blue. Endpoints of the gene fusions were determined by the dideoxy chain termination method. Both strands of the DNA were sequenced.

Purification of recombinant RD1-ORF3.

[0175] The rRD1-ORF3 was fused N-terminally to the (His)₆-tag. Recombinant antigen was prepared as described in example 1a. Purification of recombinant antigen by Ni²⁺ affinity chromatography was also carried out as described in example 1b. Fractions containing purified His-rRD1-ORF3 were pooled. The His-rRD1-ORF3 were extensively dialysed against 10 mM Tris/HCl, pH 8.5, 3 M urea followed by an additional purification step performed on an anion exchange column (Mono Q) using fast protein liquid chromatography (FPLC) (Pharmacia, Uppsala, Sweden). The purification was carried out in 10 mM Tris/HCl, pH 8.5, 3 M urea and protein was eluted by a linear gradient of NaCl from 0 to 1 M. Fractions containing the His-rRD1-ORF3 were pooled and subsequently dialysed extensively against 25 mM Hepes, pH 8.0 before use.

TABLE 2

Sequence of the rd1-orf3 oligonucleotides ^a .		
Orientation and oligonucleotide	Sequences (5'→3')	Position (nt)
Sense RD1-ORF3f	CTTCCCGGGATGGAATAATGTCA C (SEQ ID NO:153)	2807-2822
Antisense RD1-ORF3r	GATGCCATGGTTAGGCGAAGACGC CGGC (SEQ ID NO:154)	3103-3086

^aThe oligonucleotides were constructed from the Accession number U34484 nucleotide sequence (Mahairas et al., 1996). Nucleotides (nt) underlined are not contained in the nucleotide sequence of RD1-ORF's. The positions correspond to the nucleotide sequence of Accession number U34484.

[0176] The nucleotide sequences of rd1-orf3 from *M. tuberculosis* H37Rv are set forth in SEQ ID NO: 87. The deduced amino acid sequences of rd1-orf3 are set forth in SEQ ID NO: 88.

Example 1C

Identification of CFP8A, CFPI6 and CFP23

Identification of CFP16.

[0177] ST-CF was precipitated with ammonium sulphate at 80% saturation. The precipitated proteins were removed by centrifugation and after resuspension washed with 8 M urea. CHAPS and glycerol were added to a final concentration of 0.5% (w/v) and 5% (v/v) respectively and the protein solution was applied to a Rotofor isoelectrical Cell (BioRad). The Rotofor Cell had been equilibrated with a 8M urea buffer containing 0.5% (w/v) CHAPS, 5% (v/v) glycerol, 3% (v/v) Biolyt 3/5 and 1% (v/v) Biolyt 4/6 (BioRad). Isoelectric focusing was performed in a pH gradient from 3-6. The fractions were analyzed on silver-stained 10-20% SDS-PAGE. Fractions with similar band patterns were pooled and washed three times with PBS on a Centriprep concentrator (Amicon) with a 3 kDa cut off membrane to a final volume of 1-3 ml. An equal volume of SDS containing sample buffer was added and the protein solution boiled for 5 min before further separation on a Prep Cell (BioRad) in a matrix of 16% polyacrylamide under an electrical gradient. Fractions containing well separated bands in SDS-PAGE were selected for N-terminal sequencing after transfer to PVDF membrane.

Isolation of CFP8A

[0178] ST-CF was precipitated with ammonium sulphate at 80% saturation and redissolved in PBS, pH 7.4, and dialysed 3 times against 25 mM Piperazin-HCl, pH 5.5, and subjected to chromatofocusing on a matrix of PBE 94 (Pharmacia) in a column connected to an FPLC system (Pharmacia). The column was equilibrated with 25 mM Piperazin-HCl, pH 5.5, and the elution was performed with 10% PB74-HCl, pH 4.0 (Pharmacia). Fractions with similar band patterns were pooled and washed three times with PBS on a Centriprep concentrator (Amicon) with a 3 kDa cut off membrane to a final volume of 1-3 ml and separated on a Prepcell as described above.

N-terminal Sequencing

[0179] Fractions containing CFP8A and CFP16 were blotted to PVDF membrane after Tricine SDS-PAGE (Ploug et al, 1989). The relevant bands were excised and subjected to N-terminal amino acid sequence analysis on a Procise 494 sequencer (Applied Biosystems). The fraction containing CFP25A was blotted to PVDF membrane after 2-DE PAGE (isoelectric focusing in the first dimension and Tricin SDS-PAGE in the second dimension). The relevant spot was excised and sequenced as described above.

[0180] The following N-terminal sequences were obtained:

(SEQ ID NO:155)

CFP8A: DPVDDAFIAKLNTAG

(SEQ ID NO:156)

CFP16: AKLSTDELLDAFKEM

N-terminal Homology Searching in the Sanger Database and Identification of the Corresponding Genes.

[0181] The N-terminal amino acid sequence from each of the proteins was used for a homology search using the blast program of the Sanger *Mycobacterium tuberculosis* database:

<http://www.sanger.ac.uk/projects/m-tuberculosis/TB-blast-server>.

[0182] For CFP8A and CFP16 the following information was obtained:

[0183] CFP8A: A sequence 80% identical to the 15 N-terminal amino acids was found on contig TB_1884. The N-terminally determined sequence from the protein purified from culture filtrate starts at amino acid 32. This gives a length of the mature protein of 98 amino acids corresponding to a theoretical MW of 9700 Da and a pi of 3.72. This is in good agreement with the observed MW on SDS-PAGE at approximately 8 kDa. The full length protein has a theoretical MW of 12989 Da and a pi of 4.38.

[0184] CFP16: The 15 aa N-terminal sequence was found to be 100% identical to a sequence found on cosmid MTCY20H1.

[0185] The identity is found within an open reading frame of 130 amino acids length corresponding to a theoretical MW of CFP16 of 13440.4 Da and a pi of 4.59. The observed molecular weight in an SDS-PAGE gel is 16 kDa.

Use of Homology Searching in the EMBL Database for Identification of CFP23.

[0186] Homology searching in the EMBL database (using the GCG package of the Biobase, Århus-DK) with the amino acid sequences of two earlier identified highly immunoreactive ST-CF proteins, using the TFASTA algorithm, revealed that these proteins (CFP21 and CFP25) belong to a family of fungal cutinase homologs. Among the most homologous sequences were also two *Mycobacterium tuberculosis* sequences found on cosmid MTCY13E12. The first, MTCY13E12.04 has 46% and 50% identity to CFP25 and CFP21 respectively. The second, MTCY13E12.05, has also 46% and 50% identity to CFP25 and CFP21. The two proteins share 62.5% aa identity in a 184 residues overlap.

On the basis of the high homology to the strong T-cell antigens CFP21 and CFP25, respectively, it is believed that CFP19A and CFP23 are possible new T-cell antigens.

[0187] The first reading frame encodes a 254 amino acid protein of which the first 26 aa constitute a putative leader peptide that strongly indicates an extracellular location of the protein. The mature protein is thus 228 aa in length corresponding to a theoretical MW of 23149.0 Da and a Pi of 5.80. The protein is named CFP23.

[0188] The second reading frame encodes an 231 aa protein of which the first 44 aa constitute a putative leader peptide that strongly indicates an extracellular location of the protein. The mature protein is thus 187 aa in length corresponding to a theoretical MW of 19020.3 Da and a Pi of 7.03. The protein is named CFP19A.

[0189] The presence of putative leader peptides in both proteins (and thereby their presence in the ST-CF) is confirmed by theoretical sequence analysis using the signalP program at the Expasy molecular Biology server (<http://expasy.hcuge.ch/www/tools.html>).

Searching for Homologies to CFP16 and CFP23 in the EMBL Database.

[0190] The amino acid sequences derived from the translated genes of the individual antigens were used for homology searching in the EMBL and Genbank databases using the TFASTA algorithm, in order to find homologous proteins and to address eventual functional roles of the antigens.

[0191] CFP16: RpIL gene, 130 aa. Identical to the *M. bovis* 50s ribosomal protein L7/L12 (acc. No P37381).

[0192] CFP23: CFP23 has between 38% and 46% identity to several cutinases from different fungal sp.

[0193] In addition CFP23 has 46% identity and 61% similarity to CFP25 as well as 50% identity and 63% similarity to CFP21 (both proteins are earlier isolated from the ST-CF).

Cloning of the Genes Encoding CFP8A, CFP16 and CFP23

[0194] The genes encoding CFP8A, CFP16 and CFP23 were all cloned into the expression vector pMCT6, by PCR amplification with gene specific primers, for recombinant expression in *E. coli* of the proteins.

[0195] PCR reactions contained 10 ng of *M. tuberculosis* chromosomal DNA in 1× low salt Taq+ buffer from Stratagene supplemented with 250 mM of each of the four nucleotides (Boehringer Mannheim), 0.5 mg/ml BSA (IgG technology), 1% DMSO (Merck), 5 pmols of each primer and 0.5 unit Tag+ DNA polymerase (Stratagene) in 10 ml reaction volume. Reactions were initially heated to 94° C. for 25 sec. and run for 30 cycles of the program; 94° C. for 10 sec., 55° C. for 10 sec. and 72° C. for 90 sec, using thermocycler equipment from Idaho Technology.

[0196] The DNA fragments were subsequently run on 1% agarose gels, the bands were excised and purified by Spin-X spin columns (Costar) and cloned into pBluescript SK II+-T vector (Stratagene). Plasmid DNA was hereafter prepared from clones harbouring the desired fragments, digested with suitable restriction enzymes and subcloned into the expression vector pMCT6 in frame with 8 histidines which are added to the N-terminal of the expressed proteins. The

resulting clones were hereafter sequenced by use of the dideoxy chain termination method adapted for supercoiled DNA using the Sequenase DNA sequencing kit version 1.0 (United States Biochemical Corp., USA) and by cycle sequencing using the Dye Terminator system in combination with an automated gel reader (model 373A; Applied Biosystems) according to the instructions provided. Both strands of the DNA were sequenced.

[0197] For cloning of the individual antigens, the following gene specific primers were used:

[0198] CFP8A: Primers used for cloning of cfp8A:

(SEQ ID NO:157)
CFP8A-F: CTGAGATCTATGAACCTACGGCGCC

(SEQ ID NO:158)
CFP8A-R: CTCCCATGGTACCCTAGGACCCGGGCGCCCGGC

[0199] CFP8A-F and CFP8A-R create Bg/II and NcoI sites, respectively, used for the cloning in pMCT6.

[0200] CFP16: Primers used for cloning of cfp16:

(SEQ ID NO:159)
OPBR-104: CCGGGAGATCTATGGCAAAGCTCTCCACCGACG

(SEQ ID NO:160)
OPBR-105: CGCTGGGCGAGCTACTTGACGGTGACGGTGG

[0201] OPBR-104 and OPBR-105 create Bg/II and NcoI sites, respectively, used for the cloning in pMCT6.

[0202] CFP23: Primers used for cloning of cfp23:

(SEQ ID NO:161)
OPBR-86: CCTTGGGAGATCTTTGGACCCCGGTTGC

(SEQ ID NO:162)
OPBR-87: GACGAGATCTTATGGGCTTACTGAC

[0203] OPBR-86 and OPBR-87 both create a Bg/II site used for the cloning in pMCT6.

Expression/purification of recombinant CFP8A, CFP16 and CFP23 proteins.

[0204] Expression and metal affinity purification of recombinant proteins was undertaken essentially as described by the manufacturers. For each protein, 1 l LB-media containing 100 µg/ml ampicillin, was inoculated with 10 ml of an overnight culture of XL1-Blue cells harbouring recombinant pMCT6 plasmids. Cultures were shaken at 37° C. until they reached a density of OD₆₀₀ = 0.4-0.6. IPTG was hereafter added to a final concentration of 1 mM and the cultures were further incubated 4-16 hours. Cells were harvested, resuspended in 1× sonication buffer+8 M urea and sonicated 5×30 sec. with 30 sec. pausing between the pulses.

[0205] After centrifugation, the lysate was applied to a column containing 25 ml of resuspended Talon resin (Clontech, Palo Alto, USA). The column was washed and eluted as described by the manufacturers.

[0206] After elution, all fractions (1.5 ml each) were subjected to analysis by SDS-PAGE using the Mighty Small

(Hoefer Scientific Instruments, USA) system and the protein concentrations were estimated at 280 nm. Fractions containing recombinant protein were pooled and dialysed against 3 M urea in 10 mM Tris-HCl, pH 8.5. The dialysed protein was further purified by FPLC (Pharmacia, Sweden) using a 6 ml Resource-Q column, eluted with a linear 0-1 M gradient of NaCl. Fractions were analyzed by SDS-PAGE and protein concentrations were estimated at OD₂₈₀. Fractions containing protein were pooled and dialysed against 25 mM Hepes buffer, pH 8.5.

[0207] Finally the protein concentration and the LPS content were determined by the BCA (Pierce, Holland) and LAL (Endosafe, Charleston, USA) tests, respectively.

Example 2

Species Distribution of cfp21, cfp23 and rd1-orf3.

Presence of cfp21, cfp23 and rd1-orf3 in Different *Mycobacterial* species.

[0208] The Southern blotting was carried out as described previously (Oettinger and Andersen, 1994) with the following modifications: 2 µg of genomic DNA was digested with PvuII, electrophoresed in an 0.8% agarose gel, and transferred onto a nylon membrane (Hybond N-plus; Amersham International plc, Little Chalfont, United Kingdom) with a vacuum transfer device (Milliblot, TM-v; Millipore Corp., Bedford, Mass.).

[0209] The cfp21, cfp23 and rd1-orf3 gene fragments were amplified by PCR from the recombinant pMCT6 plasmids encoding the individual genes. The primers used (same as the primers used for cloning) are described in example 1a and 1b. The results are summarized in Table 3.

TABLE 3

Interspecies analysis of the cfp21 and rd1-orf3 genes by Southern blotting.				
Species and strain	cfp21	cfp23	rd1-orf3	
1. <i>M. tub.</i> H37Rv	+	+	+	
2. <i>M. bovis</i>	+	+	+	
3. <i>M. bovis</i>	N.D.	+	-	
BCG Danish 1331				
4. <i>M. bovis</i>	+	+	-	
BCG Japan				
5. <i>M. avium</i>	+	+	-	
6. <i>M. kansasii</i>	-	+	-	
7. <i>M. marinum</i>	+	+	-	
8. <i>M. scrofulaceum</i>	+	+	-	
9. <i>M. intercellulare</i>	+	+	-	
10. <i>M. fortuitum</i>	-	+	-	
11. <i>M. xenopi</i>	+	+	-	
12. <i>M. szulgai</i>	+	+	-	

+, positive reaction;
-, no reaction,
N.D. not determined.

Example 3

Total Extraction of Proteins from Dead *M. tuberculosis* Bacteria.

[0210] 1.5×10⁹ bacteria/ml *M. tuberculosis* was heat treated at 55° C. for 1.5 hours and checked for sterility. 10 ml of these heat killed bacteria was centrifuged at 2000 g for 40 min; the supernatant was discharged and the pellet

resuspended in PBS containing 0.5% Tween 20 and used as the antigen source. The pellet was sonicated with 20 rounds of 90 seconds and centrifuged 30 min at 5000 g to remove unbroken cells. The supernatant containing soluble proteins as well as cell wall and cell membrane components was extracted twice with 10% SDS to release proteins inserted in the cell wall and membrane compartments. After a centrifugation at 20,000 g for 30 min the supernatant was precipitated with 8 volume of cold acetone and resuspended in PBS at a protein concentration of 5 mg/ml and named: Somatic Proteins Extract (SPE).

Example 3A

Subcellular Fractionation of *Mycobacterium Tuberculosis*

[0211] 1.5×10^9 colony forming units (CFU/ml) of *M. tuberculosis* H37Rv were inactivated by heat-killing at 60° C. for 1.5 hour. The heat-killed *Mycobacteria* was centrifuged at 3,000×g for 20 min; the supernatant was discarded and the pellet was resuspended in cold PBS. This step was repeated twice. After the final wash, the pellet was resuspended in a homogenizing buffer consisting of PBS supplemented with 10 mM EDTA and 1 mM of phenylmethylsulfonyl fluoride in a ratio of 1 ml buffer per 0.5 g of heat-killed *Mycobacteria*. The sample was sonicated on ice for 15 min (1-min-pulser-on/10-sec-pulser off) and subsequently lysed three times with a French Pressure Cell at 12,000 lb/in². The lysate was centrifuged at 27,000×g for 20 min; the pellet was washed in homogenizing buffer and recentrifuged. The pooled supernatants contained a mixture of cytosol and membrane components, while the pellet represented the crude cell wall.

Preparation of Cell Wall

[0212] The cell wall pellet, resuspended in homogenizing buffer, was added RNase and DNase to a final concentration of 1 mg/ml and incubated overnight at 4° C. The cell wall was washed twice in homogenizing buffer, twice in homogenizing buffer saturated with KCl, and twice with PBS. Soluble proteins were extracted from the cell wall by a 2 hour incubation with 2% SDS at 6° C. The insoluble cell wall core was removed by a centrifugation at 27,000×g for 20 min and the SDS-extraction was repeated. Finally, the pooled supernatants were precipitated with 6 volumes of chilled acetone and resuspended in PBS.

Preparation of Cytosol and Membrane:

[0213] To separate the cytosol and the membrane fraction, the pooled supernatants were ultracentrifuged at 100,000×g for 2 hours at 5° C. The cytosol proteins in the supernatant were precipitated with acetone and resuspended in PBS. The pellet, representing the membrane fraction, was washed in PBS, ultracentrifuged, and finally resuspended in PBS.

Triton X-114 Extraction of Cell Wall and Membrane:

[0214] To prepare protein fractions largely devoid of lipoarabinomannan, the cell wall and the membrane fraction were subjected to extraction with precondensed Triton X-114. Triton X-114 was added to the protein sample at a final concentration of 4%. The solution was mixed on ice for 60 min and centrifuged at 20,000×g for 15 min at 4° C. The pellet containing residual insoluble material was extracted

once more (membrane) or twice (cell wall), while the supernatant was warmed to 37° C. to condense the Triton X-114. After centrifugation of the supernatant at 12,000 x g for 15 min, the aqueous phase and detergent phase were separated. The aqueous phase and detergent phase were washed twice with Triton X-114 and PBS, respectively. The combined aqueous phases and residual insoluble material containing the majority of proteins were pooled, precipitated with acetone, and resuspended in PBS.

Example 4A

Identification of Proteins from the Cytosolic Fraction

Use of Patient Sera to Identify *M. Tuberculosis* Antigens

[0215] This example illustrates the identification of antigens from the cytosol fraction by screening with serum from *M. tuberculosis* infected individuals in western blot. The reaction with serum was used as an indication that the proteins are recognized immunologically.

Identification of Abundant Proteins

[0216] As immunity to tuberculosis is not B-cell but T-cell mediated, reactivity with serum from TB patients was not the only selection criterion used to identify proteins from the cytosol. Further proteins were selected by virtue of their abundance in the cytosol.

[0217] The cytosol was precipitated with ammonium sulphate at 80% saturation. The non-precipitated proteins were removed by centrifugation and precipitated proteins were resuspended in 20 mM imidazole, pH 7.0. The protein solution was applied to a DEAE Sepharose 6B column, equilibrated with 20 mM imidazole. Bound protein was eluted from the column using a salt gradient from 0 to 1 M NaCl, in 20 mM imidazole. Fractions collected during elution was analyzed on a silver stained 10-20% SDS-PAGE and on 2 dimensional electrophoresis. Fractions containing well separated bands were selected for 2D electrophoresis and blotted to PVDF, after which spots, visualised by staining with Coomassie Blue, were selected for N-terminal sequencing.

[0218] The following N-terminal sequence was obtained:

ForTB15A: SAYKTVVVGTDXSX (SEQ ID NO:163)

[0219] No sequence identity was found, when searching the Sanger database using the blast program. However, when the blast program at Swiss-blast was used, a sequence was obtained.

TB15A

[0220] For the determined N-terminal sequence of TB15 a 78% identical sequence was found in CY01 B2.28. The X at position 13 of the determined N-terminal sequence corresponds to a G in MTCY01 B2.28 and the X at position 15 to a D. Within the open reading frame the translated protein is 146 amino acids long. The N-terminal sequence of the protein identified in the cytosol starts at amino acid no 2, with the N-terminal Met cleaved off.

[0221] This gives a protein of 146 amino acids, which corresponds to a theoretical molecular mass of 15 313 Da and a theoretical pI of 5.60. The observed mass in SDS-PAGE is 16 kDa.

[0222] The highest sequence identity, 32% in a 34 amino acid overlap, was found to a conserved protein of *Methanobacterium thermoautotrophicum*.

Example 4B

Identification of Proteins from the Cell Wall

Identification of TB16, TB32 and TB51.

[0223] Proteins contained in the cell wall fraction were separated by 2-D electrophoresis. A sample containing 120 mg protein was subjected to isoelectric focusing in a pH gradient from 4 to 7. The second dimension separation (SDS-PAGE) was carried out in a 10-20% acrylamide gradient. After blotting onto a PVDF membrane, proteins could be visualised by Coomassie blue staining.

N-terminal Ssequencing.

[0224] The relevant spots were excised from the PVDF membrane and subjected to N-terminal sequencing using a Procise sequencer (Applied Biosystems). The following N-terminal sequences were obtained:

TB16: ADKTTQTIIYIDADPG	(SEQ ID NO:164)
TB32: SGNSSLGIIVGIDD	(SEQ ID NO:165)
TB51: MKSTVEQLSPTRVRI	(SEQ ID NO:166)

N-terminal Sequence Identity Searching and Identification of the Corresponding Genes.

[0225] The N-terminal amino acid sequence from each of the proteins identified was used for a sequence identity search using the tblastn program at NCBI: <http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-blast?form=0>

[0226] The following information was obtained:

TB16:

[0227] The 15 aa N-terminal sequence was found to be 100% identical to a sequence found within the *Mycobacterium tuberculosis* sequence MTV021. The identity is found within an open reading frame of 144 amino acids length corresponding to a theoretical molecular mass of 16294 Da and a pi of 4.64. The apparent molecular mass in an SDS-PAGE gel is 17 kDa. The amino acid sequence shows some similarity to other hypothetical Mycobacterial proteins.

TB32:

[0228] The 14 aa N-terminal sequence was found to be 100% identical to a sequence found within the *Mycobacterium tuberculosis* sequence MTCY1A10. The identity is found within an open reading frame of 297 amino acids length corresponding to a theoretical molecular mass of 31654 Da and a pi of 5.55. The apparent molecular mass in an SDS-PAGE gel is 33 kDa. The amino acid sequence shows some similarity to other hypothetical Mycobacterial proteins.

TB51:

[0229] The 15 aa N-terminal sequence was found to be 100% identical to a sequence found within the *Mycobacterium tuberculosis* sequence MTV008. The identity is found

within an open reading frame of 466 amino acids length corresponding to a theoretical molecular mass of 50587 Da and a pi of 4.3. The apparent molecular mass in an SDS-PAGE gel is 56 kDa. The amino acid sequence shows similarities to trigger factor from several organisms. Possible chaperone protein.

Example 4C

Cloning of the Genes Encoding TB15A, TB16, TB32 and TB51

[0230] The genes encoding TB15A, TB16, TB32 and TB51 were all cloned into the *E. coli* expression vector pMCT3, by PCR amplification with gene specific primers. Each PCR reaction contained 10 ng of *M. tuberculosis* chromosomal DNA in 1× low salt Taq+ buffer (Stratagene) supplemented with 250 μ M of each of the four nucleotides (Boehringer Mannheim), 0.5 mg/ml BSA (IgG technology), 1% DMSO (Merck), 5 pmoles of each primer, and 0.5 unit Taq+DNA polymerase (Stratagene) in 10 μ l reaction volume. Reactions were initially heated to 94° C. for 25 sec. and run for 30 cycles according to the following program; 94° C. for 10 sec., 55° C. for 10 sec., and 72° C. for 90 sec., using thermocycler equipment from Idaho Technology.

[0231] The PCR fragment was ligated with TA cloning vector pCR® 2.1 (Invitrogen) and transformed into *E. coli*. Plasmid DNA was thereafter prepared from clones harbouring the desired fragment, digested with suitable restriction enzymes and subcloned into the expression vector pMCT3 in frame with 6 histidine residues which are added to the N-terminal of the expressed proteins. The resulting clones were hereafter sequenced by cycle sequencing using the Dye Terminator system in combination with an automated gel reader (model 373A; Applied Biosystems) according to the instructions provided. Both strands of the DNA were sequenced.

[0232] Expression and metal affinity purification of recombinant proteins was undertaken essentially as described by the manufacturers. For each protein, 1 l LB-media containing 100 μ g/ml ampicillin, was inoculated with 10 ml of an overnight culture of XL1-Blue cells harbouring recombinant pMCT3 plasmids. Cultures were shaken at 37° C. until they reached a density of OD₆₀₀=0.4-0.6. IPTG was hereafter added to a final concentration of 1 mM and the cultures were further incubated 4-16 hours. Cells were harvested, resuspended in 1× sonication buffer +8 M urea and sonicated 5×30 sec. with 30 sec. pausing between the pulses. After centrifugation, the lysate was applied to a column containing 10 ml of resuspended Talon resin (Clontec, Palo Alto, USA). The column was washed and eluted as described by the manufacturers.

[0233] After elution, all fractions (1.5 ml each) were subjected to analysis by SDS-PAGE using the Mighty Small (Hofer Scientific Instruments, USA) system and the protein concentrations were estimated at OD₂₈₀ nm. Fractions containing recombinant protein were pooled and dialysed against 3 M urea in 10 mM Tris-HCl, pH 8.5. The dialysed protein was further purified by FPLC (Pharmacia, Sweden) using 1 ml HiTrap columns (Pharmacia, Sweden) eluted with a linear salt gradient from 0-1 M NaCl. Fractions were analysed by SDS-PAGE and protein concentrations were estimated at OD₂₈₀ nm. Fractions containing protein were pooled and dialysed against 25 mM Hepes buffer, pH 8.5.

[0234] Finally, the protein concentration and the LPS content were determined by the BCA (Pierce, Holland) and LAL (Endosafe, Charleston, USA) tests, respectively.

[0235] For cloning of the individual proteins, the following gene specific primers were used:

[0236] TB15A: Primers used for cloning of TB15A:

(SEQ ID NO:167)
TB15A-F: CTG CCA TGG CTA GGT GGT GTG CAC GAT C

(SEQ ID NO: 168)
TB15A-R: CTG AAG CTT ATG AGC GCC TAT AAG ACC

[0237] TB15-F and TB15-R create NcoI and HindIII sites, respectively, used for the cloning in pMCT3.

[0238] TB16: Primers used for cloning OF TB16:

(SEQ ID NO:169)
TB16-F: CTG AGA TCT GCG GAG AAG ACG ACA GAG

(SEQ ID NO:170)
TB16-R: CTC CCA TGG TAC CGG AAT CAC TCA GCC

[0239] TB16-F and TB16-R create BG/II and NcoI sites, respectively, used for the cloning in pMCT3.

[0240] TB32: Primers used for cloning of TB32:

(SEQ ID NO:171)
TB32-F: CTG AGA TCT ATG TCA TCG GGC AAT TCA

(SEQ ID NO:172)
TB32-R: CTC CCA TGG CTAC CTA AGT CAG CGA CTC GCG

[0241] TB32-F and TB32-R create BG/II and NcoI sites, respectively, used for the cloning in pMCT3.

[0242] TB51: Primers used for cloning of TB51:

(SEQ ID NO:173)
TB51-F: CTG AGA TCT GTG AAG AGC ACC GTC GAG

(SEQ ID NO:174)
TB51-R: CTC CCA TGG GTC ATA CGG TCA CGT TGT

[0243] TB51-F and TB51-R create BG/II and NcoI sites, respectively, used for the cloning in pMCT3.

Example 5

Evaluation of Immunological Aactivity of Identified Somatic Proteins

The use of Polypeptides as Diagnostic Reagents:

[0244] A polypeptide has diagnostic potential in humans when it is inducing significantly higher responses in patients with microscopy or culture positive tuberculosis compared to PPD positive or PPD negative individuals with no known history of TB infection or exposure to *M. tuberculosis* but who may or may not have received a prior BCG vaccination, have been exposed to non-tuberculous mycobacteria (NTM), or be actively infected with *M. avium*. To identify polypeptides capable of discriminating between the above men-

tioned groups, the level of response and the frequency of positive responders to the polypeptide is compared. By positive responders are meant i) reactivity by human serum or plasma from TB patients with the polypeptide using conventional antibody ELISA/Western blot or ii) in vivo delayed type hypersensitivity response to the polypeptide which is at least 5 mm higher than the response induced by a control material.

[0245] The diagnostic potential of polypeptides will initially be evaluated in 10 individuals with TB infection and 10 individuals with no known exposure to virulent Mycobacteria. High specificity, >80%, will be the most important selection criteria for these polypeptides and a sensitivity >80% is desirable but sensitivity >30% is acceptable as combinations of several specific antigens may be preferred in a cocktail of diagnostic reagent recognized by different individuals.

Skin Test Reaction in TB Infected Guinea Pigs

[0246] To identify polypeptides as antigens with the potential as TB diagnostic reagents the ability of the proteins to induce a skin test response will be evaluated in the guinea pig model where groups of guinea pigs have been infected with either *M. tuberculosis* or *M. avium* or vaccinated with BCG.

[0247] To evaluate the response in *M. tuberculosis* infected guinea pigs, female outbred guinea pigs will be infected via an ear vein with 1×10^4 CFU of *M. tuberculosis* H37Rv in 0.2 ml of PBS or aerosol infected (in an exposure chamber of a Middlebrook Aerosol Generation device) with 1×10^5 CFU/ml of *M. tuberculosis* Erdman given rise to 10-15 granulomas per animal in the lung. After 4 weeks skin test will be performed with the polypeptides diluted in 0.1 ml of PBS and 24 hours after the injection reaction diameter is measured.

[0248] To evaluate the response in *M. avium* infected guinea pigs, female outbred guinea pigs will be infected intradermally with 2×10^6 CFU of a clinical isolate of *M. avium* (Atyp.1443; Statens Serum Institut, Denmark). Skin test are performed 4 weeks after with the polypeptides diluted in 0.1 ml of PBS and 24 hours after the injection reaction diameter is measured.

[0249] To evaluate the response in BCG vaccinated guinea pigs, female outbred guinea pigs will be sensitized intradermally with 2×10^6 CFU of BCG (BCG Danish 1331; Statens Serum Institut). Skin test are performed 4 weeks after with the polypeptides diluted in 0.1 ml of PBS and 24 hours after the injection reaction diameter is measured.

[0250] If a polypeptide induces a significant reaction in animal infected with *M. tuberculosis* but not in BCG vaccinated guinea pigs this polypeptide may have a potential as a diagnostic reagent to differentiate between BCG vaccinated and *M. tuberculosis* infected individuals, which will hereafter be evaluated in the human population.

[0251] If a polypeptide induces a reaction in *M. tuberculosis* infected guinea pigs but not in guinea pigs infected with *M. avium*, this polypeptide may have a potential as a diagnostic reagent with respect to differentiate between an individual infected with *M. tuberculosis* and an individual infected with Mycobacteria not belonging to the tuberculosis complex. The polypeptide may also have a potential as a

diagnostic reagent to differentiate between a *M. avium* and a *M. tuberculosis* infected individual.

Example 6A

Serological Recognition of the Recombinant Produced Proteins

[0252] To test the potential of the proteins as serological antigens sera was collected from 8 TB patients and 4 healthy BCG non-vaccinated controls and were assayed for antibodies recognizing the recombinantly produced proteins in an ELISA assay as follows: Each of the sera was absorbed with Promega *E. coli* extract (S37761) for 4 hours at room temperature and the supernatants collected after centrifugation. 0.5 ug of the proteins in Carbonatbuffer at pH 9.6 were absorbed over night at 5° C. to a polystyrene plate (Maxisorp, Nunc). The plates were washed in PBS-0.05% Tween-20 and the sera applied in a dilution of 1:100. After 1 hour of incubation the plates were washed 3 times with PBS-0.05% Tween-20 and 100 ul per well of peroxidase-conjugated Rabbit Anti-Human IgA, IgG, IgM was applied in a dilution of 1:8000. After 1 hour of incubation the plates were washed 3 times with PBS-0.05% Tween-20. 100 ul of substrate (TMB PLUS, Kem-En-Tec) was added per well and the reaction stopped after 30 min with 0.2 M Sulphuric acid and the absorbance was read at 405 nm. The results are shown in table 4.

TABLE 4

Serological recognition of the proteins by TB patients (n = 8) and healthy controls (n = 4). The percentage of responders as well as the number of persons responding in each group is indicated. The cut-off values for positive responses are OD 0.2 for CFP8A, CFP16, CFP23 and RD1-ORF3, OD 0.25 for CFP21, TB15A and TB16 and OD 0.3 for TB51.		
Protein	Percent (n) responders	Percent (n) positive healthy controls
CFP8A	63 (5)	0 (0)
CFP16	50 (4)	0 (0)
CFP21	80 (6)	0 (0)
CFP23	50 (4)	0 (0)
RD1-ORF3	25 (2)	0 (0)
TB15A	25 (2)	0 (0)
TB16	100 (8)	0 (0)
TB51	13 (1)	0 (0)

[0253] As shown in table 4 all the proteins are recognized by at least 13% of the tested TB patients. CFP8A, CFP16 and CFP21 are recognized by 50% or more of the TB patients tested and most extraordinary all the tested TB patients recognized TB16. In addition, CFP8A, CFP16, CFP21, CFP23, RD1-ORF3, TB15A, TB16 and TB51 were recognized with a very high OD (>0.5) by some of the TB patients indicating a particular high amount of specific antibodies to these proteins. None of the proteins are recognized by healthy non-BCG vaccinated controls, which demonstrates the potential of these proteins to differentiate between *M. tuberculosis* infected individuals and healthy individuals. All these proteins are therefore excellent diagnostic candidates.

Example 6B

Serological Recognition of Single Recombinant Produced Proteins and Mixtures of the Recombinant Produced Proteins

[0254] To evaluate the potential of 39 recombinantly produced proteins as serological antigens were sera collected from 42 TB patients and 32 healthy controls and assayed for antibodies recognizing the recombinantly produced proteins in an ELISA assay as follows: Each of the sera was absorbed with Promega *E. coli* extract (S3761) for 4 hours at room temperature and the supernatants collected after centrifugation. 0.5 ug of the proteins in Carbonatbuffer at pH 9.6 were absorbed over night at 5° C. to a polystyrene plate (Maxisorp, Nunc). The plates were washed in PBS-0.05% Tween-20 and the sera applied in a dilution of 1:100. After 1 hour of incubation the plates were washed 3 times with PBS-0.05% Tween-20 and 100 ul per well of peroxidase-conjugated Rabbit Anti-Human IgA, IgG, IgM was applied in a dilution of 1:8000. After 1 hour of incubation the plates were washed 3 times with PBS-0.05% Tween-20. 100 ul of substrate (TMB PLUS, Kem-En-Tec) was added per well and the reaction stopped after 30 min with 0.2 M Sulphuric acid and the absorbance was read at 405 nm.

[0255] The results were evaluated for all the 39 tested proteins and on the basis of these results 7 antigens were selected for their superior abilities as serological antigens as shown in Table 5. For comparison has the result for the well known serological antigen 38 kDa also been shown in table 5.

TABLE 5

Serological recognition of the proteins by TB patients (n = 42) and healthy controls (n = 32). The number of responders as well as the calculated sensitivity and specificity is indicated for each antigen. Cut-off is defined as MeanControl + 3 SD for the individual antigen.						
Protein	TB patients		Healthy controls		Sensitivity	Specificity
	Positive	High responders*	Positive	High responders*		
CFP8a	11	2	1	0	26%	96.9%
TB15A	7	2	1	0	17%	96.9%
CFP16	10	6	1	0	24%	96.9%
TB16	23	9	0	0	55%	100%
CFP21	13	3	1	0	31%	96.9%
CFP23 ^a	12	3	1	0	23%	97%
TB32	9	2	1	0	21%	96.9%
TB51	14	5	0	0	33%	100%
RD1-ORF3	6	5	0	0	14%	100%
38 kDa	6	2	0	0	14%	100%

*High responders defined as OD values > MeanControl + 6 SDControl for each individual antigen.

^a53 TB patients and 33 healthy controls were assayed for antibodies recognizing CFP23

[0256] For a diagnostic reagent for TB it is crucial to have a high specificity in order not to obtain false positive results which may lead to anti-TB treatment of healthy people. We therefore selected the serological antigens on the criteria of either the ability to induce a high specificity (more than 90%) combined with high sensitivity or the ability to enhance the sensitivity of a protein cocktail when combined with other antigens without compromising the high specificity. Also included in table 5 is the 38 kDa antigen which

is well documented antigen and is believed to be one of the most promising serological proteins (Cole, R. A., et al 1996). As shown in table 5 the 38 kDa antigen has a sensitivity of 14% in the tested patient group and all the selected antigens shown in table 5 performs similar or with a higher sensitivity that the 38 kDa antigen without compromising the specificity (all selected antigen have a specificity more than 96%). In particular are TB16 and TB51 outstanding with a sensitivity of respectively 55% and 33% and a specificity of 100%. Also important is the fact that all these selected antigens induces a very high response in two or more donors which demonstrates their potency as diagnostic reagents. For a diagnostic TB reagent it is important to achieve a very high sensitivity and as demonstrated in table 6 this be achieved by combining the antigens identified above. In practice this can be accomplished either by mixing the antigens in the same well in the ELISA plate or by combining the results from multiple wells incubated with the same blood sample. Alternatively the proteins of interests can be produced as recombinant fusions proteins comprising of at least two proteins or B cell epitopes and the resulting fusion molecule can hereafter can used in the serological assays.

[0257] The response of tuberculosis is heterogeneous with considerable person-to-person variance to which antigens that are recognized by the antibodies (Lyashchenko, K. et al 1998) and therefore, can it be an advantage to use combinations proteins (e.g. in protein cocktails) which may increase the sensitivity and be recognized by sera from a high proportion of infected individuals.

TABLE 6

Calculated sensitivity (sens.) and specificity (spec.) of selected antigen combinations			
#	Antigens	Sens.	Spec.
2	TB16 + TB51	62%	100%
2	TB15A + TB16	64%	97%
2	TB16 + CFP21	67%	97%
3	TB15A + TB16 + TB51	71%	97%
3	CFP16 + TB16 + CFP21	71%	94%
3	TB16 + CFP21 + TB51	74%	97%
3	TB15A + TB16 + CFP21	74%	94%
4	CFP16 + CFP17 + CFP21 + TB51	64%	94%
4	CFP8A + CFP16 + TB16 + CFP21	76%	94%
4	CFP16 + TB16 + CFP21 + TB51	79%	94%

[0258] For the combinations shown in table 6 it is advantageous to combine from two to four antigens which will give a higher sensitivity than the single antigen and still a high specificity (more than 90%). In particular is the combination of CFP16+TB16+CFP21+TB51 and TB16+CFP21+TB51 and TB15A+TB16+CFP21 very efficient in this study population. The combinations shown in table 6 are only examples and other useful combinations can be envisaged as up to eight antigens may be combined and lead to increased sensitivity. In addition, can other antigens be combined with the above defined proteins for example the 38 kDa antigen which may be combined with any of the above described antigens and may increase the sensitivity. In this respect it is of importance that it has been observed that different populations react to different antigens (Julian, E. et al 2000, Lyashchenko, K. et al 1998) and it may therefore be necessary to define individual combinations for different

populations. Therefore, combinations which does not give high sensitivity in the tested study population may be very efficient as diagnostic reagents when tested in another population.

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atg ttg tgg ggc ggc ggg tcg ttg ccg aca atc ggt ccg ctg tat agc			710
Met Leu Trp Gly Gly Gly Ser Leu Pro Thr Ile Gly Pro Leu Tyr Ser			
	125 130 135		
tct aag acc ata aac ttg tgt gct ccc gac gat cca ata tgc acc gga			758
Ser Lys Thr Ile Asn Leu Cys Ala Pro Asp Asp Pro Ile Cys Thr Gly			
	140 145 150		
ggc gcc aat att atg gcg cat gtt tcg tat gtt cag tcg ggg atg aca			806
Gly Gly Asn Ile Met Ala His Val Ser Tyr Val Gln Ser Gly Met Thr			
	155 160 165 170		

-continued

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agc cag gcg gcg aca ttc gcg gcg aac agg ctc gat cac gcc gga tga      854
Ser Gln Ala Ala Thr Phe Ala Ala Asn Arg Leu Asp His Ala Gly
          175                      180                      185

```

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tcaaagactg ttgtccctat accgctgggg ctgtagtcga tgtacaccgg ctggaatctg      914

```

```

aagggaaga acccggtatt catcaggccg gatgaaatga cggtcggggc gtaatcgttt      974

```

```

gtgttgaacg cgtagagccg atcaccgccg gggctggtgt agacctcaat gtttgtgttc     1034

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```

gccggcaggg ttccggatcc                                           1054

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```

<210> SEQ ID NO 10

```

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<211> LENGTH: 217

```

```

<212> TYPE: PRT

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<213> ORGANISM: Mycobacterium tuberculosis

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<400> SEQUENCE: 10

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Met Thr Pro Arg Ser Leu Val Arg Ile Val Gly Val Val Val Ala Thr
-32   -30           -25           -20

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Thr Leu Ala Leu Val Ser Ala Pro Ala Gly Gly Arg Ala Ala His Ala
  -15           -10           -5

```

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Asp Pro Cys Ser Asp Ile Ala Val Val Phe Ala Arg Gly Thr His Gln
  1         5         10         15

```

```

Ala Ser Gly Leu Gly Asp Val Gly Glu Ala Phe Val Asp Ser Leu Thr
        20        25        30

```

```

Ser Gln Val Gly Gly Arg Ser Ile Gly Val Tyr Ala Val Asn Tyr Pro
        35        40        45

```

```

Ala Ser Asp Asp Tyr Arg Ala Ser Ala Ser Asn Gly Ser Asp Asp Ala
        50        55        60

```

```

Ser Ala His Ile Gln Arg Thr Val Ala Ser Cys Pro Asn Thr Arg Ile
        65        70        75        80

```

```

Val Leu Gly Gly Tyr Ser Gln Gly Ala Thr Val Ile Asp Leu Ser Thr
        85        90        95

```

```

Ser Ala Met Pro Pro Ala Val Ala Asp His Val Ala Ala Val Ala Leu
       100       105       110

```

```

Phe Gly Glu Pro Ser Ser Gly Phe Ser Ser Met Leu Trp Gly Gly Gly
       115       120       125

```

```

Ser Leu Pro Thr Ile Gly Pro Leu Tyr Ser Ser Lys Thr Ile Asn Leu
       130       135       140

```

```

Cys Ala Pro Asp Asp Pro Ile Cys Thr Gly Gly Gly Asn Ile Met Ala
       145       150       155       160

```

```

His Val Ser Tyr Val Gln Ser Gly Met Thr Ser Gln Ala Ala Thr Phe
       165       170       175

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Ala Ala Asn Arg Leu Asp His Ala Gly
       180       185

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<210> SEQ ID NO 11

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<400> SEQUENCE: 11

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<210> SEQ ID NO 12

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<400> SEQUENCE: 12

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<210> SEQ ID NO 13

<400> SEQUENCE: 13

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<210> SEQ ID NO 14

<400> SEQUENCE: 14

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<210> SEQ ID NO 15

<400> SEQUENCE: 15

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<210> SEQ ID NO 16

<400> SEQUENCE: 16

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<210> SEQ ID NO 17

<400> SEQUENCE: 17

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<210> SEQ ID NO 18

<400> SEQUENCE: 18

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<210> SEQ ID NO 19

<400> SEQUENCE: 19

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<210> SEQ ID NO 20

<400> SEQUENCE: 20

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<210> SEQ ID NO 21

<400> SEQUENCE: 21

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<210> SEQ ID NO 22

<400> SEQUENCE: 22

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<210> SEQ ID NO 23

<400> SEQUENCE: 23

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<210> SEQ ID NO 24

<400> SEQUENCE: 24

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<210> SEQ ID NO 25

<400> SEQUENCE: 25

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<210> SEQ ID NO 26

<400> SEQUENCE: 26

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<210> SEQ ID NO 27

<400> SEQUENCE: 27

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<210> SEQ ID NO 28

<400> SEQUENCE: 28

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<210> SEQ ID NO 29

<211> LENGTH: 435

<212> TYPE: DNA

<213> ORGANISM: Mycobacterium tuberculosis

<220> FEATURE:

<221> NAME/KEY: CDS

<222> LOCATION: (1)...(435)

<400> SEQUENCE: 29

gtg gcg gac aag acg aca cag acg att tac atc gac gcg gat cca ggc	48
Val Ala Asp Lys Thr Thr Gln Thr Ile Tyr Ile Asp Ala Asp Pro Gly	
1 5 10 15	

gag gtg atg aag gcg atc gcc gac atc gaa gcc tac ccg caa tgg att	96
Glu Val Met Lys Ala Ile Ala Asp Ile Glu Ala Tyr Pro Gln Trp Ile	
20 25 30	

tcg gag tat aag gaa gtc gag atc cta gag gcc gac gac gag ggc tac	144
Ser Glu Tyr Lys Glu Val Glu Ile Leu Glu Ala Asp Asp Glu Gly Tyr	
35 40 45	

ccg aaa cga gcg cga atg ttg atg gac gca gcc atc ttc aaa gac acc	192
Pro Lys Arg Ala Arg Met Leu Met Asp Ala Ala Ile Phe Lys Asp Thr	
50 55 60	

ttg atc atg tcc tac gag tgg ccg gaa gac cgc caa tcg ctt agc tgg	240
Leu Ile Met Ser Tyr Glu Trp Pro Glu Asp Arg Gln Ser Leu Ser Trp	
65 70 75 80	

act ctc gaa tcc agc tcg ctg cta aag tcc ctc gaa ggc acg tat cgc	288
Thr Leu Glu Ser Ser Ser Leu Leu Lys Ser Leu Glu Gly Thr Tyr Arg	
85 90 95	

ttg gcg ccc aag ggt tct ggc act gag gtc acc tac gag ctt gcc gtc	336
Leu Ala Pro Lys Gly Ser Gly Thr Glu Val Thr Tyr Glu Leu Ala Val	
100 105 110	

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gac ctt gct gtc ccc atg atc ggg atg ctc aag cgt aag gcg gaa cgc      384
Asp Leu Ala Val Pro Met Ile Gly Met Leu Lys Arg Lys Ala Glu Arg
      115              120              125

agg ttg ata gac ggc gcg ttg aag gat ctg aag aaa cga gtc gag ggc      432
Arg Leu Ile Asp Gly Ala Leu Lys Asp Leu Lys Lys Arg Val Glu Gly
      130              135              140

tga                                                                    435

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<210> SEQ ID NO 30
<211> LENGTH: 144
<212> TYPE: PRT
<213> ORGANISM: Mycobacterium tuberculosis

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<400> SEQUENCE: 30

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Met Ala Asp Lys Thr Thr Gln Thr Ile Tyr Ile Asp Ala Asp Pro Gly
  1             5             10             15

Glu Val Met Lys Ala Ile Ala Asp Ile Glu Ala Tyr Pro Gln Trp Ile
      20             25             30

Ser Glu Tyr Lys Glu Val Glu Ile Leu Glu Ala Asp Asp Glu Gly Tyr
      35             40             45

Pro Lys Arg Ala Arg Met Leu Met Asp Ala Ala Ile Phe Lys Asp Thr
      50             55             60

Leu Ile Met Ser Tyr Glu Trp Pro Glu Asp Arg Gln Ser Leu Ser Trp
      65             70             75             80

Thr Leu Glu Ser Ser Ser Leu Leu Lys Ser Leu Glu Gly Thr Tyr Arg
      85             90             95

Leu Ala Pro Lys Gly Ser Gly Thr Glu Val Thr Tyr Glu Leu Ala Val
      100            105            110

Asp Leu Ala Val Pro Met Ile Gly Met Leu Lys Arg Lys Ala Glu Arg
      115            120            125

Arg Leu Ile Asp Gly Ala Leu Lys Asp Leu Lys Lys Arg Val Glu Gly
      130            135            140

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<210> SEQ ID NO 31

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<400> SEQUENCE: 31

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<210> SEQ ID NO 32

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<400> SEQUENCE: 32

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<210> SEQ ID NO 33
<211> LENGTH: 894
<212> TYPE: DNA
<213> ORGANISM: Mycobacterium tuberculosis
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (1)...(891)

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<400> SEQUENCE: 33

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```

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

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

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20	25	30	
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	
gcc acc tgg ctg gag gtg cca ctg ccg ccg gcc 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	
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
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	
ccg gcg gca gcc gtt ccc aca ttg gtc gac atg tcc aaa gac gca gtg			336
Pro Ala Ala Val Pro Thr Leu Val Asp Met Ser Lys Asp Ala Val			
100	105	110	
ctg atg gtc gtg ggt tgt ctc gga agt ggg ccg tgg ccg gcc ccg ctg			384
Leu Met Val Val Gly Cys Leu Gly Ser Gly Arg Trp Pro Gly Arg Leu			
115	120	125	
ctc ggt tcg gtc agt tcc gcc 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	
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
ccg gtg cta gtt gcc gtt gac gcc tcg tcg gcc tcc gag ctg gcg acc			528
Pro Val Leu Val Val Asp Gly Ser Ser Ala Ser Glu Leu Ala Thr			
165	170	175	
gca atc gca ttc gac gaa gcg tcg ccg 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	
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	
tgg ccg gca act cag tcg atg gcc gag cag gtg ctg gcc gag ccg ttg			672
Trp Pro Ala Thr Gln Ser Met Ala Glu Gln Val Leu Ala Glu Arg Leu			
210	215	220	
gcg ggt tgg cag gag ccg 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
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	
cag ctg gtc gtg gtc gcc agc ccg gcc cgc gcc gcc tac gcc gga atg			816
Gln Leu Val Val Val Gly Ser Arg Gly Arg Gly Gly Tyr Ala Gly Met			
260	265	270	
ctg gtg ggg tcg gta gcc gaa acc gtt gct cag ctg gcg ccg acg ccg			864
Leu Val Gly Ser Val Gly Glu Thr Val Ala Gln Leu Ala Arg Thr Pro			
275	280	285	
gtc atc gtg gca cgc gag tcg ctg act tag			894
Val Ile Val Ala Arg Glu Ser Leu Thr			
290	295		

<210> SEQ ID NO 34

<211> LENGTH: 297

<212> TYPE: PRT

<213> ORGANISM: Mycobacterium tuberculosis

-continued

<400> SEQUENCE: 34

Met Ser Ser Gly Asn Ser Ser Leu Gly Ile Ile Val Gly Ile Asp Asp
1 5 10 15
Ser Pro Ala Ala Gln Val Ala Val Arg Trp Ala Ala Arg Asp Ala Glu
20 25 30
Leu Arg Lys Ile Pro Leu Thr Leu Val His Ala Val Ser Pro Glu Val
35 40 45
Ala Thr Trp Leu Glu Val Pro Leu Pro Pro Gly Val Leu Arg Trp Gln
50 55 60
Gln Asp His Gly Arg His Leu Ile Asp Asp Ala Leu Lys Val Val Glu
65 70 75 80
Gln Ala Ser Leu Arg Ala Gly Pro Pro Thr Val His Ser Glu Ile Val
85 90 95
Pro Ala Ala Ala Val Pro Thr Leu Val Asp Met Ser Lys Asp Ala Val
100 105 110
Leu Met Val Val Gly Cys Leu Gly Ser Gly Arg Trp Pro Gly Arg Leu
115 120 125
Leu Gly Ser Val Ser Ser Gly Leu Leu Arg His Ala His Cys Pro Val
130 135 140
Val Ile Ile His Asp Glu Asp Ser Val Met Pro His Pro Gln Gln Ala
145 150 155 160
Pro Val Leu Val Gly Val Asp Gly Ser Ser Ala Ser Glu Leu Ala Thr
165 170 175
Ala Ile Ala Phe Asp Glu Ala Ser Arg Arg Asn Val Asp Leu Val Ala
180 185 190
Leu His Ala Trp Ser Asp Val Asp Val Ser Glu Trp Pro Gly Ile Asp
195 200 205
Trp Pro Ala Thr Gln Ser Met Ala Glu Gln Val Leu Ala Glu Arg Leu
210 215 220
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
Gln Leu Val Val Val Gly Ser Arg Gly Arg Gly Gly Tyr Ala Gly Met
260 265 270
Leu Val Gly Ser Val Gly Glu Thr Val Ala Gln Leu Ala Arg Thr Pro
275 280 285
Val Ile Val Ala Arg Glu Ser Leu Thr
290 295

<210> SEQ ID NO 35

<400> SEQUENCE: 35

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<210> SEQ ID NO 36

<400> SEQUENCE: 36

000

<210> SEQ ID NO 37

<211> LENGTH: 1401

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<212> TYPE: DNA
<213> ORGANISM: Mycobacterium tuberculosis
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (1)...(1398)

<400> SEQUENCE: 37

gtg aag agc acc gtc gag cag ttg agc ccc acc cgg gtt cgt atc aac      48
Val Lys Ser Thr Val Glu Gln Leu Ser Pro Thr Arg Val Arg Ile Asn
  1             5             10             15

gtg gag gtg cca ttc gcc gag ctt gag ccg gat ttc cag cgg gcc tac      96
Val Glu Val Pro Phe Ala Glu Leu Glu Pro Asp Phe Gln Arg Ala Tyr
      20             25             30

aaa gag ctg gcc aaa cag gtg cgg ctg ccc ggc ttc cgg ccc ggg aag     144
Lys Glu Leu Ala Lys Gln Val Arg Leu Pro Gly Phe Arg Pro Gly Lys
      35             40             45

gcg ccg gcc aaa cta ctc gaa gcc cgc atc ggc cgg gag gcc atg ctg     192
Ala Pro Ala Lys Leu Leu Glu Ala Arg Ile Gly Arg Glu Ala Met Leu
      50             55             60

gat caa atc gtc aac gat gcg ctg ccc agc cgg tac gga cag gcg gtg     240
Asp Gln Ile Val Asn Asp Ala Leu Pro Ser Arg Tyr Gly Gln Ala Val
      65             70             75             80

gcc gag tcg gat gtc caa ccg ctc ggc cgg ccc aac atc gag gtg acc     288
Ala Glu Ser Asp Val Gln Pro Leu Gly Arg Pro Asn Ile Glu Val Thr
      85             90             95

aag aag gag tac ggc cag gac ctg caa ttc acc gcc gag gtc gac atc     336
Lys Lys Glu Tyr Gly Gln Asp Leu Gln Phe Thr Ala Glu Val Asp Ile
      100            105            110

cgc ccg aag atc agt ccc ccg gac ctg agc gcg ctg acg gtc tcg gtg     384
Arg Pro Lys Ile Ser Pro Pro Asp Leu Ser Ala Leu Thr Val Ser Val
      115            120            125

gat ccg atc gaa atc ggt gag gac gac gtc gac gcc gaa ctg cag tcg     432
Asp Pro Ile Glu Ile Gly Glu Asp Asp Val Asp Ala Glu Leu Gln Ser
      130            135            140

tta cgt acc cgg ttc ggc acc ctg acc gcg gtg gac cgg ccg gtg gcc     480
Leu Arg Thr Arg Phe Gly Thr Leu Thr Ala Val Asp Arg Pro Val Ala
      145            150            155            160

gtc ggc gac gtc gtc tcg atc gac ttg tct gcc acg gtc gac gga gag     528
Val Gly Asp Val Val Ser Ile Asp Leu Ser Ala Thr Val Asp Gly Glu
      165            170            175

gac ata ccg aac gca gcc gct gag gga ctc tcc cac gag gtc ggc tcc     576
Asp Ile Pro Asn Ala Ala Ala Glu Gly Leu Ser His Glu Val Gly Ser
      180            185            190

ggc cgg ctc atc gca ggt ctc gac gac gcg gtt gtt ggt ctg tcc gcc     624
Gly Arg Leu Ile Ala Gly Leu Asp Asp Ala Val Val Gly Leu Ser Ala
      195            200            205

gac gag tcc cgg gtc ttc acc gcc aag ctg gca gcc ggc gag cac gcc     672
Asp Glu Ser Arg Val Phe Thr Ala Lys Leu Ala Ala Gly Glu His Ala
      210            215            220

ggg cag gaa gct cag gtt acc gtc acg gtc agg tcg gtt aag gag cgc     720
Gly Gln Glu Ala Gln Val Thr Val Thr Val Arg Ser Val Lys Glu Arg
      225            230            235            240

gaa cta cca gag ccc gac gac gaa ttc gcg cag tta gcc agc gag ttc     768
Glu Leu Pro Glu Pro Asp Asp Glu Phe Ala Gln Leu Ala Ser Glu Phe
      245            250            255

gac agc atc gac gaa ttg cgg gcc agc ctc agc gac cag gtg cgc cag     816
Asp Ser Ile Asp Glu Leu Arg Ala Ser Leu Ser Asp Gln Val Arg Gln
      260            265            270

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gcc aag cgc gcc cag cag gcc gag cag att cga aac gcc acc atc gat Ala Lys Arg Ala Gln Gln Ala Glu Gln Ile Arg Asn Ala Thr Ile Asp 275 280 285	864
gcg cta ctc gaa cag gtc gac gtg ccg ttg ccg gag tcg tat gtg cag Ala Leu Leu Glu Gln Val Asp Val Pro Leu Pro Glu Ser Tyr Val Gln 290 295 300	912
gcc caa ttc gac agc gtg ctg cac agc gcg ctc agc ggt ctt aat cac Ala Gln Phe Asp Ser Val Leu His Ser Ala Leu Ser Gly Leu Asn His 305 310 315 320	960
gac gaa gcc ccg ttc aat gag ttg ctc gtc gag caa ggc tcg tca cgc Asp Glu Ala Arg Phe Asn Glu Leu Leu Val Glu Gln Gly Ser Ser Arg 325 330 335	1008
gcg gcg ttc gat gcc gag gcg cgc acc gcc tca gaa aag gac gtc aag Ala Ala Phe Asp Ala Glu Ala Arg Thr Ala Ser Glu Lys Asp Val Lys 340 345 350	1056
agg cag ctg ttg cta gac gcc ctg gcc gat gag ctg cag gtc caa gtt Arg Gln Leu Leu Leu Asp Ala Leu Ala Asp Glu Leu Gln Val Gln Val 355 360 365	1104
ggc cag gat gat ctg acc gaa cga ctg gtg acg acg tct ccg caa tac Gly Gln Asp Asp Leu Thr Glu Arg Leu Val Thr Thr Ser Arg Gln Tyr 370 375 380	1152
ggc atc gag ccg cag cag ctg ttc ggc tac ctc caa gag cgc aac cag Gly Ile Glu Pro Gln Gln Leu Phe Gly Tyr Leu Gln Glu Arg Asn Gln 385 390 395 400	1200
ctg ccg acc atg ttc gct gac gtg ccg cgc gag ctg gcg atc agg gcc Leu Pro Thr Met Phe Ala Asp Val Arg Arg Glu Leu Ala Ile Arg Ala 405 410 415	1248
gca gtg gag gcg gcg acg gtc acc gac agt gac gga aac acg atc gat Ala Val Glu Ala Ala Thr Val Thr Asp Ser Asp Gly Asn Thr Ile Asp 420 425 430	1296
acc agt gag ttc ttc ggc aag cgt gtg tcg gcc ggt gag gct gag gag Thr Ser Glu Phe Phe Gly Lys Arg Val Ser Ala Gly Glu Ala Glu Glu 435 440 445	1344
gcc gaa ccg gca gac gag ggt gcc gcg ccg gcg gcg tcc gac gaa gcg Ala Glu Pro Ala Asp Glu Gly Ala Ala Arg Ala Ala Ser Asp Glu Ala 450 455 460	1392
aca acg tga Thr Thr 465	1401

<210> SEQ ID NO 38

<211> LENGTH: 466

<212> TYPE: PRT

<213> ORGANISM: Mycobacterium tuberculosis

<400> SEQUENCE: 38

Met Lys Ser Thr Val Glu Gln Leu Ser Pro Thr Arg Val Arg Ile Asn 1 5 10 15
Val Glu Val Pro Phe Ala Glu Leu Glu Pro Asp Phe Gln Arg Ala Tyr 20 25 30
Lys Glu Leu Ala Lys Gln Val Arg Leu Pro Gly Phe Arg Pro Gly Lys 35 40 45
Ala Pro Ala Lys Leu Leu Glu Ala Arg Ile Gly Arg Glu Ala Met Leu 50 55 60
Asp Gln Ile Val Asn Asp Ala Leu Pro Ser Arg Tyr Gly Gln Ala Val 65 70 75 80
Ala Glu Ser Asp Val Gln Pro Leu Gly Arg Pro Asn Ile Glu Val Thr

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85					90					95					
Lys	Lys	Glu	Tyr	Gly	Gln	Asp	Leu	Gln	Phe	Thr	Ala	Glu	Val	Asp	Ile
			100					105					110		
Arg	Pro	Lys	Ile	Ser	Pro	Pro	Asp	Leu	Ser	Ala	Leu	Thr	Val	Ser	Val
		115					120						125		
Asp	Pro	Ile	Glu	Ile	Gly	Glu	Asp	Asp	Val	Asp	Ala	Glu	Leu	Gln	Ser
		130					135						140		
Leu	Arg	Thr	Arg	Phe	Gly	Thr	Leu	Thr	Ala	Val	Asp	Arg	Pro	Val	Ala
				150									155		160
Val	Gly	Asp	Val	Val	Ser	Ile	Asp	Leu	Ser	Ala	Thr	Val	Asp	Gly	Glu
				165					170					175	
Asp	Ile	Pro	Asn	Ala	Ala	Ala	Glu	Gly	Leu	Ser	His	Glu	Val	Gly	Ser
			180					185					190		
Gly	Arg	Leu	Ile	Ala	Gly	Leu	Asp	Asp	Ala	Val	Val	Gly	Leu	Ser	Ala
		195					200						205		
Asp	Glu	Ser	Arg	Val	Phe	Thr	Ala	Lys	Leu	Ala	Ala	Gly	Glu	His	Ala
							215						220		
Gly	Gln	Glu	Ala	Gln	Val	Thr	Val	Thr	Val	Arg	Ser	Val	Lys	Glu	Arg
				230									235		240
Glu	Leu	Pro	Glu	Pro	Asp	Asp	Glu	Phe	Ala	Gln	Leu	Ala	Ser	Glu	Phe
				245					250					255	
Asp	Ser	Ile	Asp	Glu	Leu	Arg	Ala	Ser	Leu	Ser	Asp	Gln	Val	Arg	Gln
			260					265					270		
Ala	Lys	Arg	Ala	Gln	Gln	Ala	Glu	Gln	Ile	Arg	Asn	Ala	Thr	Ile	Asp
		275						280					285		
Ala	Leu	Leu	Glu	Gln	Val	Asp	Val	Pro	Leu	Pro	Glu	Ser	Tyr	Val	Gln
							295						300		
Ala	Gln	Phe	Asp	Ser	Val	Leu	His	Ser	Ala	Leu	Ser	Gly	Leu	Asn	His
				310									315		320
Asp	Glu	Ala	Arg	Phe	Asn	Glu	Leu	Leu	Val	Glu	Gln	Gly	Ser	Ser	Arg
				325					330					335	
Ala	Ala	Phe	Asp	Ala	Glu	Ala	Arg	Thr	Ala	Ser	Glu	Lys	Asp	Val	Lys
				340					345					350	
Arg	Gln	Leu	Leu	Leu	Asp	Ala	Leu	Ala	Asp	Glu	Leu	Gln	Val	Gln	Val
			355					360					365		
Gly	Gln	Asp	Asp	Leu	Thr	Glu	Arg	Leu	Val	Thr	Thr	Ser	Arg	Gln	Tyr
							375						380		
Gly	Ile	Glu	Pro	Gln	Gln	Leu	Phe	Gly	Tyr	Leu	Gln	Glu	Arg	Asn	Gln
				390									395		400
Leu	Pro	Thr	Met	Phe	Ala	Asp	Val	Arg	Arg	Glu	Leu	Ala	Ile	Arg	Ala
				405					410					415	
Ala	Val	Glu	Ala	Ala	Thr	Val	Thr	Asp	Ser	Asp	Gly	Asn	Thr	Ile	Asp
				420					425					430	
Thr	Ser	Glu	Phe	Phe	Gly	Lys	Arg	Val	Ser	Ala	Gly	Glu	Ala	Glu	Glu
				435				440					445		
Ala	Glu	Pro	Ala	Asp	Glu	Gly	Ala	Ala	Arg	Ala	Ala	Ser	Asp	Glu	Ala
				450				455						460	
Thr	Thr														

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<400> SEQUENCE: 39

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<210> SEQ ID NO 40

<400> SEQUENCE: 40

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<210> SEQ ID NO 41

<400> SEQUENCE: 41

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<210> SEQ ID NO 42

<400> SEQUENCE: 42

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<210> SEQ ID NO 43

<400> SEQUENCE: 43

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<210> SEQ ID NO 44

<400> SEQUENCE: 44

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<210> SEQ ID NO 45

<400> SEQUENCE: 45

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<210> SEQ ID NO 46

<400> SEQUENCE: 46

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<210> SEQ ID NO 47

<400> SEQUENCE: 47

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<210> SEQ ID NO 48

<400> SEQUENCE: 48

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<210> SEQ ID NO 49

<400> SEQUENCE: 49

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<210> SEQ ID NO 50

<400> SEQUENCE: 50

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<210> SEQ ID NO 51

<400> SEQUENCE: 51

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<210> SEQ ID NO 52

<400> SEQUENCE: 52

000

<210> SEQ ID NO 53

<400> SEQUENCE: 53

000

<210> SEQ ID NO 54

<400> SEQUENCE: 54

000

<210> SEQ ID NO 55

<211> LENGTH: 950

<212> TYPE: DNA

<213> ORGANISM: Mycobacterium tuberculosis

<220> FEATURE:

<221> NAME/KEY: CDS

<222> LOCATION: (133)...(918)

<400> SEQUENCE: 55

tgggctcggc actggtcttc ccacggtggc gcgctgattt ctccccacgg taggcgttgc 60

gacgcatgtt cttcaccgtc tatccacagc taccgacatt tgctccggct ggatcgcggg 120

taaaattccg tc gtg aac aat cga ccc atc cgc ctg ctg aca tcc ggc agg 171
Met Asn Asn Arg Pro Ile Arg Leu Leu Thr Ser Gly Arg
-30 -25gct ggt ttg ggt gcg ggc gca ttg atc acc gcc gtc gtc ctg ctc atc 219
Ala Gly Leu Gly Ala Gly Ala Leu Ile Thr Ala Val Val Leu Leu Ile
-20 -15 -10 -5gcc ttg ggc gct gtt tgg acc ccg gtt gcc ttc gcc gat gga tgc ccg 267
Ala Leu Gly Ala Val Trp Thr Pro Val Ala Phe Ala Asp Gly Cys Pro
1 5 10gac gcc gaa gtc acg ttc gcc cgc gcc acc gcc gag ccg ccc gga atc 315
Asp Ala Glu Val Thr Phe Ala Arg Gly Thr Gly Glu Pro Pro Gly Ile
15 20 25ggg cgc gtt ggc cag gcg ttc gtc gac tcg ctg cgc cag cag act ggc 363
Gly Arg Val Gly Gln Ala Phe Val Asp Ser Leu Arg Gln Gln Thr Gly
30 35 40atg gag atc gga gta tac ccg gtg aat tac gcc gcc agc cgc cta cag 411
Met Glu Ile Gly Val Tyr Pro Val Asn Tyr Ala Ala Ser Arg Leu Gln
45 50 55 60ctg cac ggg gga gac ggc gcc aac gac gcc ata tcg cac att aag tcc 459
Leu His Gly Gly Asp Gly Ala Asn Asp Ala Ile Ser His Ile Lys Ser
65 70 75

-continued

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atg gcc tcg tca tgc ccg aac acc aag ctg gtc ttg ggc ggc tat tcg      507
Met Ala Ser Ser Cys Pro Asn Thr Lys Leu Val Leu Gly Gly Tyr Ser
      80                      85                      90

cag ggc gca acc gtg atc gat atc gtg gcc ggg gtt ccg ttg ggc agc      555
Gln Gly Ala Thr Val Ile Asp Ile Val Ala Gly Val Pro Leu Gly Ser
      95                      100                     105

atc agc ttt ggc agt ccg cta cct gcg gca tac gca gac aac gtc gca      603
Ile Ser Phe Gly Ser Pro Leu Pro Ala Ala Tyr Ala Asp Asn Val Ala
      110                     115                     120

gcg gtc gcg gtc ttc ggc aat ccg tcc aac cgc gcc ggc gga tcg ctg      651
Ala Val Ala Val Phe Gly Asn Pro Ser Asn Arg Ala Gly Gly Ser Leu
      125                     130                     135                     140

tcg agc ctg agc ccg cta ttc ggt tcc aag gcg att gac ctg tgc aat      699
Ser Ser Leu Ser Pro Leu Phe Gly Ser Lys Ala Ile Asp Leu Cys Asn
      145                     150                     155

ccc acc gat ccg atc tgc cat gtg ggc ccc ggc aac gaa ttc agc gga      747
Pro Thr Asp Pro Ile Cys His Val Gly Pro Gly Asn Glu Phe Ser Gly
      160                     165                     170

cac atc gac ggc tac ata ccc acc tac acc acc cag gcg gct agt ttc      795
His Ile Asp Gly Tyr Ile Pro Thr Tyr Thr Thr Gln Ala Ala Ser Phe
      175                     180                     185

gtc gtg cag agg ctc cgc gcc ggg tcg gtg cca cat ctg cct gga tcc      843
Val Val Gln Arg Leu Arg Ala Gly Ser Val Pro His Leu Pro Gly Ser
      190                     195                     200

gtc ccg cag ctg ccc ggg tct gtc ctt cag atg ccc ggc act gcc gca      891
Val Pro Gln Leu Pro Gly Ser Val Leu Gln Met Pro Gly Thr Ala Ala
      205                     210                     215                     220

ccg gct ccc gaa tcg ctg cac ggt cgc tgacgctttg tcagtaagcc cataaaa      945
Pro Ala Pro Glu Ser Leu His Gly Arg
      225

tcgcbg      950

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<210> SEQ ID NO 56

<211> LENGTH: 262

<212> TYPE: PRT

<213> ORGANISM: Mycobacterium tuberculosis

<400> SEQUENCE: 56

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Met Asn Asn Arg Pro Ile Arg Leu Leu Thr Ser Gly Arg Ala Gly Leu
      -30                      -25                      -20

Gly Ala Gly Ala Leu Ile Thr Ala Val Val Leu Leu Ile Ala Leu Gly
      -15                      -10                      -5

Ala Val Trp Thr Pro Val Ala Phe Ala Asp Gly Cys Pro Asp Ala Glu
      1                      5                      10                      15

Val Thr Phe Ala Arg Gly Thr Gly Glu Pro Pro Gly Ile Gly Arg Val
      20                      25                      30

Gly Gln Ala Phe Val Asp Ser Leu Arg Gln Gln Thr Gly Met Glu Ile
      35                      40                      45

Gly Val Tyr Pro Val Asn Tyr Ala Ala Ser Arg Leu Gln Leu His Gly
      50                      55                      60

Gly Asp Gly Ala Asn Asp Ala Ile Ser His Ile Lys Ser Met Ala Ser
      65                      70                      75

Ser Cys Pro Asn Thr Lys Leu Val Leu Gly Gly Tyr Ser Gln Gly Ala
      80                      85                      90                      95

Thr Val Ile Asp Ile Val Ala Gly Val Pro Leu Gly Ser Ile Ser Phe
      100                     105                     110

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Gly Ser Pro Leu Pro Ala Ala Tyr Ala Asp Asn Val Ala Ala Val Ala
115 120 125
Val Phe Gly Asn Pro Ser Asn Arg Ala Gly Gly Ser Leu Ser Ser Leu
130 135 140
Ser Pro Leu Phe Gly Ser Lys Ala Ile Asp Leu Cys Asn Pro Thr Asp
145 150 155
Pro Ile Cys His Val Gly Pro Gly Asn Glu Phe Ser Gly His Ile Asp
160 165 170 175
Gly Tyr Ile Pro Thr Tyr Thr Thr Gln Ala Ala Ser Phe Val Val Gln
180 185 190
Arg Leu Arg Ala Gly Ser Val Pro His Leu Pro Gly Ser Val Pro Gln
195 200 205
Leu Pro Gly Ser Val Leu Gln Met Pro Gly Thr Ala Ala Pro Ala Pro
210 215 220
Glu Ser Leu His Gly Arg
225

<210> SEQ ID NO 57

<400> SEQUENCE: 57

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<210> SEQ ID NO 58

<400> SEQUENCE: 58

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<210> SEQ ID NO 59

<400> SEQUENCE: 59

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<210> SEQ ID NO 60

<400> SEQUENCE: 60

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<210> SEQ ID NO 61

<400> SEQUENCE: 61

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<210> SEQ ID NO 62

<400> SEQUENCE: 62

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<210> SEQ ID NO 63

<211> LENGTH: 550

<212> TYPE: DNA

<213> ORGANISM: Mycobacterium tuberculosis

<220> FEATURE:

<221> NAME/KEY: CDS

<222> LOCATION: (101)...(490)

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<400> SEQUENCE: 63

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ggcccggtc gggccgccc tgcaggaaaa gaaggcctgc ccaggcccag actcagccga    60
gtagtacccc agtaccaccac accaggaagg accgcccac atg gca aag ctc tcc    115
                               Met Ala Lys Leu Ser
                               1           5

acc gac gaa ctg ctg gac gcg ttc aag gaa atg acc ctg ttg gag ctc    163
Thr Asp Glu Leu Leu Asp Ala Phe Lys Glu Met Thr Leu Leu Glu Leu
                10                15                20

tcc gac ttc gtc aag aag ttc gag gag acc ttc gag gtc acc gcc gcc    211
Ser Asp Phe Val Lys Lys Phe Glu Glu Thr Phe Glu Val Thr Ala Ala
                25                30                35

gct cca gtc gcc gtc gcc gcc gcc ggt gcc gcc ccg gcc ggt gcc gcc    259
Ala Pro Val Ala Val Ala Ala Ala Gly Ala Ala Pro Ala Gly Ala Ala
                40                45                50

gtc gag gct gcc gag gag cag tcc gag ttc gac gtg atc ctt gag gcc    307
Val Glu Ala Ala Glu Glu Gln Ser Glu Phe Asp Val Ile Leu Glu Ala
                55                60                65

gcc gcc gac aag aag atc gcc gtc atc aag gtg gtc cgg gag atc gtt    355
Ala Gly Asp Lys Lys Ile Gly Val Ile Lys Val Val Arg Glu Ile Val
                70                75                80                85

tcc gcc ctg gcc ctc aag gag gcc aag gac ctg gtc gac gcc gcg ccc    403
Ser Gly Leu Gly Leu Lys Glu Ala Lys Asp Leu Val Asp Gly Ala Pro
                90                95                100

aag ccg ctg ctg gag aag gtc gcc aag gag gcc gcc gac gag gcc aag    451
Lys Pro Leu Leu Glu Lys Val Ala Lys Glu Ala Ala Asp Glu Ala Lys
                105                110                115

gcc aag ctg gag gcc gcc gcc gcc acc gtc acc gtc aag tagctctgcc ca    502
Ala Lys Leu Glu Ala Ala Gly Ala Thr Val Thr Val Lys
                120                125                130

gcgtgttctt ttgcgtctgc tcggcccgta gcgaacactg cgcccgt                550

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<210> SEQ ID NO 64

<211> LENGTH: 130

<212> TYPE: PRT

<213> ORGANISM: Mycobacterium tuberculosis

<400> SEQUENCE: 64

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Met Ala Lys Leu Ser Thr Asp Glu Leu Leu Asp Ala Phe Lys Glu Met
 1           5           10           15

Thr Leu Leu Glu Leu Ser Asp Phe Val Lys Lys Phe Glu Glu Thr Phe
 20           25           30

Glu Val Thr Ala Ala Ala Pro Val Ala Val Ala Ala Gly Ala Ala
 35           40           45

Pro Ala Gly Ala Ala Val Glu Ala Ala Glu Glu Gln Ser Glu Phe Asp
 50           55           60

Val Ile Leu Glu Ala Ala Gly Asp Lys Lys Ile Gly Val Ile Lys Val
 65           70           75           80

Val Arg Glu Ile Val Ser Gly Leu Gly Leu Lys Glu Ala Lys Asp Leu
 85           90           95

Val Asp Gly Ala Pro Lys Pro Leu Leu Glu Lys Val Ala Lys Glu Ala
100          105          110

Ala Asp Glu Ala Lys Ala Lys Leu Glu Ala Ala Gly Ala Thr Val Thr
115          120          125

Val Lys
130

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<210> SEQ ID NO 65

<400> SEQUENCE: 65

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<210> SEQ ID NO 66

<400> SEQUENCE: 66

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<210> SEQ ID NO 67

<400> SEQUENCE: 67

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<210> SEQ ID NO 68

<400> SEQUENCE: 68

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<210> SEQ ID NO 69

<400> SEQUENCE: 69

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<210> SEQ ID NO 70

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<210> SEQ ID NO 71

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<210> SEQ ID NO 72

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<210> SEQ ID NO 73

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<210> SEQ ID NO 74

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<210> SEQ ID NO 75

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<210> SEQ ID NO 77

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<210> SEQ ID NO 78

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<210> SEQ ID NO 79

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<210> SEQ ID NO 80

<400> SEQUENCE: 80

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<210> SEQ ID NO 81

<400> SEQUENCE: 81

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<210> SEQ ID NO 82

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<210> SEQ ID NO 83

<400> SEQUENCE: 83

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<210> SEQ ID NO 84

<400> SEQUENCE: 84

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<210> SEQ ID NO 85

<400> SEQUENCE: 85

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<210> SEQ ID NO 86

<400> SEQUENCE: 86

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<210> SEQ ID NO 87
<211> LENGTH: 450
<212> TYPE: DNA
<213> ORGANISM: Mycobacterium tuberculosis
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (107)...(400)

<400> SEQUENCE: 87

agcccggttaa tcgagttcgg gcaatgctga ccatcggtt tgtttccggc tataaccgaa      60
cggtttgtgt acgggataca aatacaggga gggaagaagt aggcaa atg gaa aaa      115
                                   Met Glu Lys
                                   1

atg tca cat gat ccg atc gct gcc gac att ggc acg caa gtg agc gac      163
Met Ser His Asp Pro Ile Ala Ala Asp Ile Gly Thr Gln Val Ser Asp
   5              10              15

aac gct ctg cac gcc gtg acg gcc gcc tcg acg gcg ctg acg tcg gtg      211
Asn Ala Leu His Gly Val Thr Ala Gly Ser Thr Ala Leu Thr Ser Val
  20              25              30              35

acc ggg ctg gtt ccc gcg ggg gcc gat gag gtc tcc gcc caa gcg gcg      259
Thr Gly Leu Val Pro Ala Gly Ala Asp Glu Val Ser Ala Gln Ala Ala
   40              45              50

acg gcg ttc aca tcg gag gcc atc caa ttg ctg gct tcc aat gca tcg      307
Thr Ala Phe Thr Ser Glu Gly Ile Gln Leu Leu Ala Ser Asn Ala Ser
   55              60              65

gcc caa gac cag ctc cac cgt gcg gcc gaa gcg gtc cag gac gtc gcc      355
Ala Gln Asp Gln Leu His Arg Ala Gly Glu Ala Val Gln Asp Val Ala
   70              75              80

cgc acc tat tcg caa atc gac gac gcc gcc gcc gcc gtc ttc gcc taata      405
Arg Thr Tyr Ser Gln Ile Asp Asp Gly Ala Ala Gly Val Phe Ala
   85              90              95

ggccccaac acatcgagg gagtgatcac catgctgtgg cagcgc      450

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<210> SEQ ID NO 88
<211> LENGTH: 98
<212> TYPE: PRT
<213> ORGANISM: Mycobacterium tuberculosis

<400> SEQUENCE: 88

Met Glu Lys Met Ser His Asp Pro Ile Ala Ala Asp Ile Gly Thr Gln
  1              5              10              15

Val Ser Asp Asn Ala Leu His Gly Val Thr Ala Gly Ser Thr Ala Leu
  20              25              30

Thr Ser Val Thr Gly Leu Val Pro Ala Gly Ala Asp Glu Val Ser Ala
  35              40              45

Gln Ala Ala Thr Ala Phe Thr Ser Glu Gly Ile Gln Leu Leu Ala Ser
  50              55              60

Asn Ala Ser Ala Gln Asp Gln Leu His Arg Ala Gly Glu Ala Val Gln
  65              70              75              80

Asp Val Ala Arg Thr Tyr Ser Gln Ile Asp Asp Gly Ala Ala Gly Val
  85              90              95

Phe Ala

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<210> SEQ ID NO 89

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<400> SEQUENCE: 89

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<210> SEQ ID NO 90

<400> SEQUENCE: 90

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<210> SEQ ID NO 91

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<210> SEQ ID NO 92

<400> SEQUENCE: 92

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<210> SEQ ID NO 93

<400> SEQUENCE: 93

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<210> SEQ ID NO 94

<400> SEQUENCE: 94

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<210> SEQ ID NO 95

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<210> SEQ ID NO 96

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<210> SEQ ID NO 99

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<210> SEQ ID NO 100

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<210> SEQ ID NO 103

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<210> SEQ ID NO 104

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<210> SEQ ID NO 105

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<210> SEQ ID NO 106

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<210> SEQ ID NO 113

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<210> SEQ ID NO 115

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<210> SEQ ID NO 116

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<210> SEQ ID NO 117

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<210> SEQ ID NO 118

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<210> SEQ ID NO 119

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<210> SEQ ID NO 120

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<210> SEQ ID NO 121

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<210> SEQ ID NO 122

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<210> SEQ ID NO 123

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<210> SEQ ID NO 124

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<210> SEQ ID NO 125

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<210> SEQ ID NO 126

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<210> SEQ ID NO 127

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<210> SEQ ID NO 130

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<210> SEQ ID NO 131

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<210> SEQ ID NO 132

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<210> SEQ ID NO 140

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<210> SEQ ID NO 141

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<210> SEQ ID NO 142

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<210> SEQ ID NO 143

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<210> SEQ ID NO 144

<400> SEQUENCE: 144

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<210> SEQ ID NO 145

<400> SEQUENCE: 145

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<210> SEQ ID NO 146

<400> SEQUENCE: 146

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<210> SEQ ID NO 147

<400> SEQUENCE: 147

000

<210> SEQ ID NO 148

<211> LENGTH: 540

<212> TYPE: DNA

<213> ORGANISM: Mycobacterium tuberculosis

<220> FEATURE:

<221> NAME/KEY: CDS

<222> LOCATION: (105)...(491)

<400> SEQUENCE: 148

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atcgtgtcgt atcgagaacc ccggccggta tcagaacgcg ccagagcgca aacotttata      60
acttcgtgtc ccaaattgta cgaccatgga ccaaggttcc tgag atg aac cta cgg      116
                                     Met Asn Leu Arg
                                     1

cgc cat cag acc ctg acg ctg cga ctg ctg gcg gca tcc gcg ggc att      164
Arg His Gln Thr Leu Thr Leu Arg Leu Leu Ala Ala Ser Ala Gly Ile
   5              10              15              20

ctc agc gcc gcg gcc ttc gcc gcg cca gca cag gca aac ccc gtc gac      212
Leu Ser Ala Ala Phe Ala Ala Pro Ala Gln Ala Asn Pro Val Asp
   25              30              35

gac gcg ttc atc gcc gcg ctg aac aat gcc ggc gtc aac tac ggc gat      260
Asp Ala Phe Ile Ala Ala Leu Asn Asn Ala Gly Val Asn Tyr Gly Asp
   40              45              50

ccg gtc gac gcc aaa gcg ctg ggt cag tcc gtc tgc ccg atc ctg gcc      308
Pro Val Asp Ala Lys Ala Leu Gly Gln Ser Val Cys Pro Ile Leu Ala
   55              60              65

gag ccc gcc ggg tcg ttt aac acc gcg gta gcc agc gtt gtg gcg cgc      356
Glu Pro Gly Gly Ser Phe Asn Thr Ala Val Ala Ser Val Val Ala Arg
   70              75              80

gcc caa gcc atg tcc cag gac atg gcg caa acc ttc acc agt atc gcg      404
Ala Gln Gly Met Ser Gln Asp Met Ala Gln Thr Phe Thr Ser Ile Ala
   85              90              95              100

att tcg atg tac tgc ccc tcg gtg atg gca gac gtc gcc agc ggc aac      452
Ile Ser Met Tyr Cys Pro Ser Val Met Ala Asp Val Ala Ser Gly Asn
   105              110              115

ctg ccg gcc ctg cca gac atg ccg ggg ctg ccc ggg tcc taggcgtgcg cg      503

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Leu Pro Ala Leu Pro Asp Met Pro Gly Leu Pro Gly Ser
120 125

gctcctagcc ggtccctaac ggatcgatcg tggatgc

540

<210> SEQ ID NO 149
<211> LENGTH: 129
<212> TYPE: PRT
<213> ORGANISM: Mycobacterium tuberculosis

<400> SEQUENCE: 149

Met Asn Leu Arg Arg His Gln Thr Leu Thr Leu Arg Leu Leu Ala Ala
1 5 10 15

Ser Ala Gly Ile Leu Ser Ala Ala Ala Phe Ala Ala Pro Ala Gln Ala
20 25 30

Asn Pro Val Asp Asp Ala Phe Ile Ala Ala Leu Asn Asn Ala Gly Val
35 40 45

Asn Tyr Gly Asp Pro Val Asp Ala Lys Ala Leu Gly Gln Ser Val Cys
50 55 60

Pro Ile Leu Ala Glu Pro Gly Gly Ser Phe Asn Thr Ala Val Ala Ser
65 70 75 80

Val Val Ala Arg Ala Gln Gly Met Ser Gln Asp Met Ala Gln Thr Phe
85 90 95

Thr Ser Ile Ala Ile Ser Met Tyr Cys Pro Ser Val Met Ala Asp Val
100 105 110

Ala Ser Gly Asn Leu Pro Ala Leu Pro Asp Met Pro Gly Leu Pro Gly
115 120 125

Ser

<210> SEQ ID NO 150
<211> LENGTH: 15
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: N-terminal sequence of CFP21
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (3)..(3)
<223> OTHER INFORMATION: Xaa = any amino acid, unknown, or other

<400> SEQUENCE: 150

Asp Pro Xaa Ser Asp Ile Ala Val Val Phe Ala Arg Gly Thr His
1 5 10 15

<210> SEQ ID NO 151
<211> LENGTH: 26
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OPBR-55 primer used for cloning of CFP21

<400> SEQUENCE: 151

acagatctgc gcatgcggat ccgtgt

26

<210> SEQ ID NO 152
<211> LENGTH: 28
<212> TYPE: DNA

-continued

<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: OPBR-56 primer used for cloning of CFP21

<400> SEQUENCE: 152

ttttccatgg tcatccggcg tgatcgag 28

<210> SEQ ID NO 153
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Sense oligonucleotide of RD1-ORF3f

<400> SEQUENCE: 153

cttcccggga tggaaaaaat gtcac 25

<210> SEQ ID NO 154
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Antisense oligonucleotide of RD1-ORF3r

<400> SEQUENCE: 154

gatgccatgg ttaggcgaag acgccgc 28

<210> SEQ ID NO 155
<211> LENGTH: 15
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: N-terminal sequence of CFP8A

<400> SEQUENCE: 155

Asp Pro Val Asp Asp Ala Phe Ile Ala Lys Leu Asn Thr Ala Gly
1 5 10 15

<210> SEQ ID NO 156
<211> LENGTH: 15
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: N-terminal sequence of CFP16

<400> SEQUENCE: 156

Ala Lys Leu Ser Thr Asp Glu Leu Leu Asp Ala Phe Lys Glu Met
1 5 10 15

<210> SEQ ID NO 157
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Gene specific primer CFP8A-F used for cloning
of the individual antigens

<400> SEQUENCE: 157

ctgagatcta tgaacctacg gcgcc 25

<210> SEQ ID NO 158
<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence

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<220> FEATURE:
<223> OTHER INFORMATION: Gene specific primer CFP8A-R used for cloning
      of the individual antigens

<400> SEQUENCE: 158

ctcccatggt accctaggac ccgggcagcc ccggc                               35

<210> SEQ ID NO 159
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Gene specific primer OPBR-104 used for cloning
      of the individualantigens

<400> SEQUENCE: 159

ccgggagatc tatggcaaag ctctccaccg acg                               33

<210> SEQ ID NO 160
<211> LENGTH: 32
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Gene specific primer OPBR-105 used for cloning
      of the individualantigens

<400> SEQUENCE: 160

cgctgggcag agctacttga cggtgacggt gg                               32

<210> SEQ ID NO 161
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Gene specific primer OPBR-86 used for cloning
      of the individual antigens

<400> SEQUENCE: 161

ccttgggaga tctttggacc ccggttgc                               28

<210> SEQ ID NO 162
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Gene specific primer OPBR-87 used for cloning
      of the individual antigens

<400> SEQUENCE: 162

gacgagatct tatgggctta ctgac                               25

<210> SEQ ID NO 163
<211> LENGTH: 15
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: N-terminal sequence of TB15A
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (13)..(15)
<223> OTHER INFORMATION: any Xaa = any amino acid, unknown, or other

<400> SEQUENCE: 163

Ser Ala Tyr Lys Thr Val Val Val Gly Thr Asp Asp Xaa Ser Xaa
1           5           10           15

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<210> SEQ ID NO 164
<211> LENGTH: 15
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: N-terminal sequence of TB16

<400> SEQUENCE: 164

Ala Asp Lys Thr Thr Gln Thr Ile Tyr Ile Asp Ala Asp Pro Gly
1 5 10 15

<210> SEQ ID NO 165
<211> LENGTH: 14
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: N-terminal sequence of TB32

<400> SEQUENCE: 165

Ser Gly Asn Ser Ser Leu Gly Ile Ile Val Gly Ile Asp Asp
1 5 10

<210> SEQ ID NO 166
<211> LENGTH: 15
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: N-terminal sequence of TB51

<400> SEQUENCE: 166

Met Lys Ser Thr Val Glu Gln Leu Ser Pro Thr Arg Val Arg Ile
1 5 10 15

<210> SEQ ID NO 167
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer TB15A-F used for cloning of TB15A

<400> SEQUENCE: 167

ctgccatggc taggtggtgt gcacgac 28

<210> SEQ ID NO 168
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer TB15A-R used for cloning of TB15A

<400> SEQUENCE: 168

ctgaagctta tgagcgccta taagacc 27

<210> SEQ ID NO 169
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer TB16A-F used for cloning of TB16

<400> SEQUENCE: 169

ctgagatctg cggacaagac gacacag 27

-continued

<210> SEQ ID NO 170
 <211> LENGTH: 27
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Primer TB16A-R used for cloning of TB16

<400> SEQUENCE: 170

ctcccatggt accggaatca ctcagcc

27

<210> SEQ ID NO 171
 <211> LENGTH: 27
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Primer TB32-F used for cloning TB32

<400> SEQUENCE: 171

ctgagatcta tgtcatcggt caattca

27

<210> SEQ ID NO 172
 <211> LENGTH: 31
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Primer TB32-R used for cloning TB32

<400> SEQUENCE: 172

ctcccatggc tacctaagtc agcgactcgc g

31

<210> SEQ ID NO 173
 <211> LENGTH: 27
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Primer TB51-F used for cloning TB51

<400> SEQUENCE: 173

ctgagatctg tgaagagcac cgctcgag

27

<210> SEQ ID NO 174
 <211> LENGTH: 27
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Primer TB32-R used for cloning TB32

<400> SEQUENCE: 174

ctcccatggg tcatacggtc acgttgt

27

1. A pharmaceutical composition comprising a combination of two or more substantially pure polypeptides, of which one or more comprises at least one amino acid sequence selected from the group consisting of:

- a) Rv3354 (SEQ ID NO. 148);
- b) an immunogenic portion of the sequence in (a); and
- c) an amino acid sequence analogue having at least 70% sequence identity to any one of the sequences in (a) or (b) and at the same time being immunogenic.

2. A composition according to claim 1 comprising at least one fusion polypeptides, which comprises one or more amino acid sequences selected from the group consisting of

- a) Rv3354 (SEQ ID NO. 148);
 - b) an immunogenic portion of any one of the sequences in (a); and/or
 - c) an amino acid sequence analogue having at least 70% sequence identity to any one of the sequences in (a) or (b) and at the same time being immunogenic;
- and at least one fusion partner.

3. A composition according to claim 2, wherein each fusion partner comprises a polypeptide fragment selected from the group consisting of:

(a) a polypeptide fragment derived from a virulent mycobacterium, such as ESAT-6, MPB64, MPT64, TB10.4, CFP10, RD1-ORF5, RD1-ORF2, Rv1036, Ag85A, Ag85B, Ag85C, 19 kDa lipoprotein, MPT32, MPB59 and alpha-crystallin;

(b) a polypeptide selected from the group consisting of Rv0652, Rv2462c, Rv1984c, Rv2185c, Rv1636, Rv3451, Rv3872, Rv3354 and Rv2623 and an amino acid sequence analogue having at least 70% sequence identity to any one said sequences and at the same time being immunogenic;

(c) at least one immunogenic portion of any of such polypeptides in (a) or (b).

4. An immunogenic composition comprising a composition according to claim 1.

5. Use of a composition according to claim 1 for the preparation of a pharmaceutical composition, e.g. for diagnosis of tuberculosis caused by virulent mycobacteria, e.g. by *Mycobacterium tuberculosis*, *Mycobacterium africanum* or *Mycobacterium bovis*.

6. A diagnostic tool comprising a combination of two or more substantially pure polypeptides, of which at least one comprises at least one amino acid sequence selected from the group consisting of:

(a) Rv3354 (SEQ ID NO. 148);

(b) an immunogenic portion of the sequence in (a); and

(c) an amino acid sequence analogue having at least 70% sequence identity to any one of the sequences in (a) or (b) and at the same time being immunogenic

7. A substantially pure polypeptide, which comprises an amino acid sequence selected from the group consisting of:

(a) Rv3354 (SEQ ID NO. 148);

(b) an immunogenic portion of the sequence in (a); and

(c) an amino acid sequence analogue having at least 70% sequence identity to any one of the sequences in (a) or (b) and at the same time being immunogenic

8. Nucleic acid fragments in isolated form which

(a) comprises one or more nucleic acid sequences which encodes a polypeptide as defined in claim 7, or comprises a nucleic acid sequence complementary thereto; or

(b) has a length of at least 10 nucleotides and hybridizes readily under stringent hybridization conditions with a nucleotide sequence selected from Rv0652, Rv2462c, Rv1983c, Rv2185c, Rv1636, Rv3451, Rv3872, Rv3354 and Rv2623 nucleotide sequences or a sequence complementary thereto, or with a nucleotide sequence selected from a sequence in (a).

9. A nucleic acid fragment according to claim 8, which is a DNA fragment.

10. Use of a nucleic acid fragment according to claim 8 for the preparation of a composition for the diagnosis of tuberculosis caused by virulent mycobacteria, e.g. by *Mycobacterium tuberculosis*, *Mycobacterium africanum* or *Mycobacterium bovis*.

11. A replicable expression vector, which comprises a nucleic acid fragment according to claim 8.

12. A transformed cell harbouring at least one vector according to claim 11.

13. A method for producing a polypeptide according to claim 7, comprising

(a) inserting a nucleic acid fragment according to claim 8 into a vector which is able to replicate in a host cell, introducing the resulting recombinant vector into the host cell, culturing the host cell in a culture medium under conditions sufficient to effect expression of the polypeptide, and recovering the polypeptide from the host cell or culture medium;

(b) isolating the polypeptide from a whole *mycobacterium*, e.g. *Mycobacterium tuberculosis*, *Mycobacterium africanum* or *Mycobacterium bovis*, from culture filtrate or from lysates or fractions thereof; or

(c) synthesizing the polypeptide e.g. by solid or liquid phase peptide synthesis.

14. A method of diagnosing tuberculosis caused by virulent mycobacteria, e.g. by *Mycobacterium tuberculosis*, *Mycobacterium africanum* or *Mycobacterium bovis*, in an animal, including a human being, comprising intradermally injecting, in the animal, a composition according to claim 1, a positive skin response at the location of injection being indicative of the animal having tuberculosis, and a negative skin response at the location of injection being indicative of the animal not having tuberculosis.

15. A monoclonal or polyclonal antibody, which is specifically reacting with a polypeptide according to claim 7 in an immuno assay, or a specific binding fragment of said antibody for use as a diagnostic reagent.

16. A method for diagnosing previous or ongoing infection with a virulent mycobacterium, said method comprising

(a) contacting a subject sample, e.g. a blood sample, with a composition according to claim 1 or a diagnostic tool according to claim 6,

(b) detecting binding of an antibody, said binding being an indication that said subject is infected by *Mycobacterium tuberculosis* or is susceptible to *Mycobacterium tuberculosis* infection.

17. A serodiagnostic composition comprising a combination of two or more substantially pure polypeptides, of which at least one or more comprises at least one amino acid sequence selected from

(a) Rv3354 (SEQ ID NO. 148);

(b) an immunogenic portion of the sequence in (a); and

(c) an amino acid sequence analogue having at least 70% sequence identity to any one of the sequences in (a) or (b) and at the same time being immunogenic.

18. A substantially pure polypeptide according to claim 7, which comprises at least one amino acid sequence selected from the group consisting of:

(a) Rv3354 (SEQ ID NO. 148);

(b) an immunogenic portion of the sequence in (a); and

(c) an amino acid sequence analogue having at least 70% sequence identity to any one of the sequences in (a) or (b) and at the same time being immunogenic;

and at least one fusion partner.

19. A substantially pure polypeptide according to claim 18, wherein each fusion partner comprises a polypeptide fragment selected from the group consisting of:

(a) a polypeptide fragment derived from a virulent *mycobacterium*, such as ESAT-6, MPB64, MPT64, TB10.4, CFP10, RD1-ORF5, RD1-ORF2,

Rv1036, Ag85A, Ag85B, Ag85C, 19 kDa lipoprotein, MPT32, MPB59 and alpha-crystallin;

(b) a polypeptide selected from the group consisting of Rv0652, Rv2462c, Rv1984c, Rv2185c, Rv1636, Rv3451, Rv3872, Rv3354 and Rv2623 and an amino acid sequence analogue having at least 70% sequence identity to any one said sequences and at the same time being immunogenic;

(c) at least one immunogenic portion of any of such polypeptides in (a) or (b).

20. A composition comprising a combination of two or more substantially pure polypeptides, of which one or more comprises one or more amino acid sequence selected from the group consisting of:

a) Rv3354 (SEQ ID NO. 148);

b) an immunogenic portion of the sequence in (a); and

c) an amino acid sequence analogue having at least 70% sequence identity to any one of the sequences in (a) or (b) and at the same time being immunogenic;

and of which one or more comprises one or more amino acid sequences selected from the group consisting of:

d) Rv0652, Rv2462c, Rv1984c, Rv2185c, Rv1636, Rv3451, Rv3872, Rv3354 and Rv2623;

e) an immunogenic portion of any one of the sequences in (d); and

f) an amino acid analogue having at least 70% sequence identity to any one of the sequences in (d) or (e) and at the same time being immunogenic.

* * * * *