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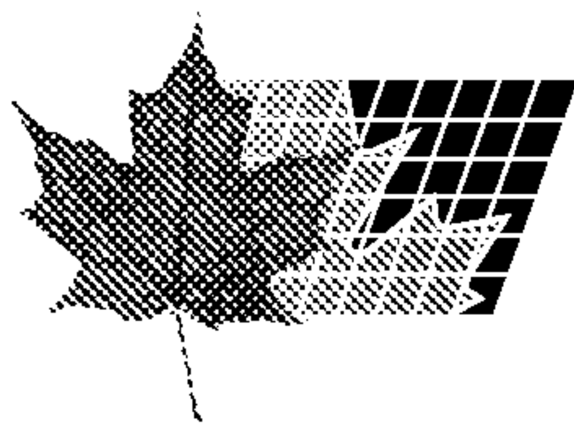

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(54) **VACCINS RECOMBINES A BASE DE MYCOBACTERIES**
(54) **MYCOBACTERIUM RECOMBINANT VACCINES**

(57) Cette invention se rapporte à des vaccins recombinés à base de mycobactéries destinés au traitement de maladies intracellulaires, que l'on a développé au moyen d'un système d'administration d'antigènes se présentant sous la forme de souches de mycobactéries, d'un système de transfert génétique se présentant sous la forme de vecteurs d'expression et de clonage non pathogènes et de techniques associées. On a ainsi développé des produits combinant des adjuvants mycobactériens immuno-régulateurs, non toxiques, des antigènes exogènes immuno-stimulateurs, non toxiques, spécifiques d'une variété de maladies et des quantités non toxiques de cytokines qui renforcent la voie T_H-1. Les vecteurs mycobactériens d'expression et de clonage incluent à la fois des vecteurs extra-cellulaires et des vecteurs d'intégration.

(57) Mycobacterium recombinant vaccines for treatment of intracellular diseases have been developed utilizing an antigen delivery system in the form of Mycobacterium strains, a genetic transfer system in the form of cloning nonpathogenic and expression vectors, and related technologies to provide products combining nontoxic immuno-regulating Mycobacterium adjuvants, nontoxic immuno-stimulating exogenous antigens specific for a variety of diseases, and nontoxic amounts of cytokines that boost the T_H-1 pathway. Cloning and expression Mycobacterium vectors include both extra-nuclear and integrative vectors.

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(57) Abstract

Mycobacterium recombinant vaccines for treatment of intracellular diseases have been developed utilizing an antigen delivery system in the form of *Mycobacterium* strains, a genetic transfer system in the form of cloning nonpathogenic and expression vectors, and related technologies to provide products combining nontoxic immuno-regulating *Mycobacterium* adjuvants, nontoxic immuno-stimulating exogenous antigens specific for a variety of diseases, and nontoxic amounts of cytokines that boost the T_H-1 pathway. Cloning and expression *Mycobacterium* vectors include both extra-nuclear and integrative vectors.

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MYCOBACTERIUM RECOMBINANT VACCINES

TECHNICAL FIELD OF THE INVENTION

The present invention relates to DNA constructs for cloning and methods of cloning *mycobacterium* genes.

BACKGROUND OF THE INVENTION

The mammalian immune system comprises both humoral and cellular components which are interrelated but have different roles. Although both arms of the immune system involve helper T cells, the outcome of the immune response depends on which subclass of T cells is involved. Helper T lymphocytes are produced by two maturation pathways (T_H-1 and T_H-2), are grouped according to cluster differentiation (CD4 and CD8), and secrete different cytokines. Both components of the immune system constantly scan and survey what is displayed in association with the molecules of the major histocompatibility complex (MHC), at the cell surface.

The humoral immune response involves helper T lymphocytes produced by the T cell maturation pathway T_H-2. Cells of this pathway secrete cytokines such as Interleukin 4 (IL-4), IL-5, IL-6, IL-9, IL-10 and tumor necrosis factor (TNF). These cytokines inactivate macrophage proliferation, contributing to a down-regulation of the T_H-1 response. TNF causes tissue inflammation and necrosis when released at high levels, which are the indications of failure of the overall immune system in many diseases. CD4+ T lymphocytes become activated through contact with antigens displayed in association with MHC class II molecules (MHC II), at the surface of macrophages and antigen presenting cells. Antibodies are produced by B cells when they interact with these activated CD4+ T lymphocytes. The MHC II molecules reside in the vesicles that engulf and destroy extracellular materials. Thus, their location within the cell gives them their specific function in monitoring the content of these vesicles. They specifically bind to antigens that have been enzymatically processed in the lysosomes of the immune cells after phagocytosis. The humoral immune response is required to protect the extracellular environment against extracellular antigens and parasites through antibodies which can be effective in neutralizing infectious agents. However, the humoral immune response cannot eliminate whole cells that become diseased, it causes tissue destruction and necrosis, and it is not effective in fighting intracellular diseases.

Consequently, the body relies on the cellular immune response for protection from pathologies that start in the intracellular environment. Cellular immune response is carried out through cytotoxic immune cells which are capable of killing diseased cells. The cellular immune response involves helper T lymphocytes produced by the T cell maturation pathway TH-1. Cells of this pathway secrete cytokines such as IL-2, IL-12, IL-15, gamma Interferon (IFN), lymphotoxin, and Granylocyte Macrophage Colony Stimulating Factor (GMCSF). These cytokines activate macrophages. The cytotoxic T lymphocytes are CD8+ T cells that become activated through contact with antigens associated with MHC class I molecules (MHC I). MHC I molecules reside around the protein factories such as the endoplasmic reticulum. Thus, their location within the cell gives them their specific function of monitoring the output and transport of materials produced inside the cell. They specifically bind to antigens that have been synthesized in the intracellular environment like in the case of cancer or intracellular diseases. The cellular immune response protects against chronic intracellular diseases such as intracellular infection, parasitism and cancer, by activating the macrophages and facilitating the detection and lysis of diseased cells. The result is the formation of a granuloma which is the paradigm of protective immunity in intracellular diseases.

Although the immune system has evolved to be efficient in selecting the target antigens against which an immune response is delivered, it does not always succeed in selecting the appropriate combination of the humoral and cellular immune components necessary to contain or eliminate the disease. For example, intracellular diseases resulting from genetic disorders, cancer, infections, allergies and autoimmune reactions are particularly difficult to treat and continue to be life threatening illnesses despite the advances in detection, diagnosis and treatment. Many of these diseases are able to circumvent the immune system and progress without challenge. For others with a long latency period, diagnosis is often made too late. Some display multi-resistance profiles against drug treatment or have their disease processes originating in environments accessible only to high doses of existing drugs. Many of these

drug treatments have high toxic side effects. Treating with chemotherapy is expensive and may be implemented only after significant expansion of the pathological process, or if there is transmission of infection and damage to the host. Although these diseases may elicit an immune response, they usually compromise its effectiveness by suppressing or mimicking the MHC molecules. In this type of illness, a TH-1 immune response favors protection, while down-regulation of this pathway, conversion to TH-2 during the chronic course of the disease, or up-regulation of the pathway TH-2 is detrimental to the host. Accordingly, a shift to TH-1 response or up-regulation of the TH-1 pathway should be beneficial on its own, and when associated with appropriate chemotherapy, would mount an effective response to resistance, chronicity, and disease. Therefore, treatment methods for intracellular diseases are needed which favor a TH-1 immune response rather than a TH-2 response.

Cancers are caused by genetic alterations that disrupt the metabolic activities of the cell. These genetic changes can result from hereditary and/or environmental factors including infections by pathogenic viruses. Like in other intracellular diseases, cellular immunity plays a major role in the host defense against cancer. Traditionally, cancer immunotherapies were designed to boost the cellular immune response by using specific and non-specific stimuli, including: 1) passive cancer immunotherapies where antibodies have been administered to patients, showing success only in rare cases; 2) active cancer immunotherapies where materials expressing cancer antigens have been administered to patients (e.g., the injection of whole or fractions of cancer cells that have been irradiated, modified chemically, or genetically) showing little impact in experimental tumor models; and 3) the combination of adoptive lymphocytes and IL-2, which caused regression of tumors in mice and metastic melanoma in humans. Tumor infiltrating lymphocytes (TIL) capable of mediating tumor regression are lymphoid cells that can be grown from single cell suspensions of the tumor incubated with IL-2. Thus, antigens recognized by TIL are more likely to be involved *in vivo* in anticancer immune response, and the cDNA and the amino-acid sequences of several of these antigens have

been identified. While these findings have opened new opportunities for the development of cancer specific immunotherapies, treatment methods based on mixing cancer antigens or the cloning and expression of the genes encoding these antigens into a delivery system that favors a T_H-1 response rather than a T_H-2 response to these antigens are needed.

Intracellular infections are caused by bacteria, viruses, parasites, and fungi. These infectious agents are either present free in the environment or carried by untreated hosts. Humans, animals and plants can serve as hosts, and if not treated, they can act as reservoirs facilitating the further spreading of such agents to others. Intracellular pathogens such as *M. tuberculosis*, *M. leprae*, and tumor viruses cause disease worldwide in millions of people each year. It is estimated that *M. tuberculosis* infects at least thirty million people/year and will cause an average of three million deaths/year during this decade, making tuberculosis (TB) the number one cause of death from a single infectious agent (World Health Organization, 1996). TB occurs most commonly in developing countries, but the prevalence of TB has increased recently in the U.S., as well as in developing countries, due to an increase in the number of immune compromised individuals with HIV infection. The risk of TB infection has also increased in individuals with diabetes, hemophilia, lymphomas, leukemias, and other malignant neoplasms, because these individuals have compromised immune systems. Leprosy and viruses which cause neoplasia are also important intracellular pathogens worldwide. Leprosy presently causes disease in more than twelve million people, and at least 15% of human cancers are thought to be caused by neoplastic transformation of cells by viruses.

Intracellular infections with highly virulent strains are quickly resolved resulting in death or cure of the patient. However, organisms of lower virulence can persist in the host and develop chronic diseases. *Mycobacterium* infections develop through a spectrum that ranges from a state of high resistance associated with cellular immunity to an opposite extreme of low resistance associated with humoral immunity. For example, leprosy is caused

by *Mycobacterium leprae* which remains uncultivable. The disease manifests an immuno-histological spectrum with six groups. At one end of the spectrum, there is the polar tuberculous leprosy (TT), a paucibacillary form of the disease which is characterized by a strong TH-1 immune response and a bacteriolytic effect that lead to granuloma formation and restrict the growth of *M. leprae*, respectively. At the opposite end of the spectrum there is the polar lepromatous leprosy (LL), a multibacillary form of the disease which is characterized by a strong but inefficient TH-2 immune response and a down-regulation of the TH-1 pathway. During the chronic course of the disease the levels of IL-2 and cells with IL-2 receptors diminish, the T cells become defective in their functions, and *M. leprae* proliferates unrestricted within the macrophages and the schwann cells. With this immune failure the clearance of the bacteria is markedly retarded, and the patient continues to harbor bacilli in the tissues even after prolonged drug therapy. The antibodies react with circulating antigens forming immune complexes that lead to tissue damage, necrosis and organ failure. Between these two extremes there are four borderline forms of leprosy reflecting the different balances achieved by the body between TH-1 and TH-2 immune responses. Likewise, tuberculosis caused by *Mycobacterium tuberculosis* also manifests an immuno-clinical spectrum with multiple (four) groups. The reactive polar group (RR) is associated with a TH-1 immune response while the opposite pole (UU) is unreactive and is associated with a TH-2 immune response. Therefore, there are clear indications that the TH-1 immune response is the main defense mechanism in leprosy and tuberculosis. Thus, treatment and immuno-prophylaxis against these diseases should be aimed at enhancing the TH-1 pathway.

Allergic diseases are characterized by the sustained production of Ig E molecules against common environmental antigens. This production is dependent of IL-4 and is inhibited by gamma interferon. Thus, the allergic reactions involve a TH-2 immune response which requires a low level of stimulation by allergens. Therefore, preferable treatment for allergies would

include the following: switching to a TH-1 immune response, which requires a high level of stimulation; activating CD8+ T cells and the production of gamma interferon; reducing the production of Ig E and recruitment of eosinophils and mast cells; and increasing the threshold concentration of the allergen to trigger a reaction.

Mycobacterium gene products, especially heat shock proteins, show homologies with bacterial, viral, parasitic, mycotic, and tumor antigens suggesting that these similarities may reflect regions in *Mycobacterium* antigens which can serve as potential inducers of cross immunity to different diseases. Heat shock proteins are overexpressed by stressed cells in many pathologies including infections, cancer, and autoimmune diseases. Thus, vaccinated individuals would have circulating cytotoxic T lymphocytes (CTL) that can interact and lyse the stressed cells, while the expression of putative autoimmunity antigenic domains in a susceptible host may lead to the suppression of the immune response and the chronicity of the disease. (Labidi, et al. 1992. "Cloning and DNA sequencing of the *Mycobacterium fortuitum* var. *fortuitum* plasmid, pAL 5000," *Plasmid* 27:130-140).

The available methods for prophylaxis and treatment of intracellular diseases include antibiotics, chemotherapy, and vaccines. Antibiotics have not been effective in treating diseases caused by *M. tuberculosis* or *M. leprae* because the lipid-rich cell wall of a mycobacteria is impermeable to antibiotics. Likewise, antibiotics have no effect on viral pathogenesis. Chemotherapy as a means of prophylaxis for high-risk individuals can be effective against *M. tuberculosis* or *M. leprae*, but it has disadvantages. Chemotherapeutic agents have undesirable side-effects in the patient, are costly, and lead to the potential existence of multi-drug resistant *Mycobacterium* strains. In addition to these disadvantages, chemotherapy as a means of treating active TB, leprosy, and virus-induced neoplasms has minimal effect since it is used only after significant disease progression. Consequently, vaccination is the therapy of choice because it provides the best protection at the lowest cost with the least number of undesirable side-effects.

Early vaccines administered as protection against acute infections were developed using antigens to initiate an immune response regardless of its nature or its mechanism. The aim was to protect against acute infections where a TH-2 immune response may be efficient. These vaccines were made of a variety of crude antigens including killed or attentuated whole cells, toxins, and other structural components derived from the pathogen. Bacterial products such as peptidoglycan, lipoproteins, lipopolysaccharides, and mycolic acids were used as therapeutic and prophylactic agents in several diseases. The administration of non-specific stimulants derived from *Corynebacterium parvum, Streptococci, Serratia marcescens,* and *Mycobacterium,* to cancer patients showed some efficacy and concomitantly enhanced the immune response against the disease. Adjuvants were developed to stimulate the immune response to antigenic material. One such adjuvant was complete Freund's adjuvant, which consisted of killed *Mycobacterium tuberculosis* suspended in oil and emulsified with aqueous antigen solution. This preparation was found to be too toxic for human use. (Riott, et al., *Immunology*, 5th ed., Mosby, Philadelphia, pp. 332, 370 (1998).

Following these first steps, efforts have been made to isolate and to develop single antigens and even single epitopes into vaccines. Molecular techniques have been used for the last two decades to clone the genes and map the domains of the corresponding proteins. However, individual antigens or cytokines did not reproduce the same physiological effects like a whole bacterial adjuvant. For example, antigen development for *M. tuberculosis, M. leprae*, and other intracellular parasites were fruitless because the dogma of the specific protective antigens or epitopes could not accurately define a protective antigen for these diseases. The dogma, furthermore, has ignored the fact that the immune response to a pathogen is a coherent response to a mosaic complex of epitopes displayed by the pathogen with some epitopes conferring protection and other epitopes mediating virulence and immunopathology. These vaccines have been unsuccessful in establishing the favored TH-1 response over the TH-2 response.

Early vaccines were also not potent against intracellular diseases. The vaccines were inefficient, short-lived, or triggered inappropriate immune responses similar to hypersensitivity reactions in allergic diseases that result in necrosis, which worsens the outcome of the pathological process in many chronic infections such as tuberculosis and leprosy. For example, BCG (Bacille-Calmette Guérin) is a vaccine that has been used for TB and leprosy prophylaxis, but has questionable efficacy. BCG is an attenuated live vaccine derived from *M. bovis*, a *Mycobacterium* strain that is closely related to *M. tuberculosis.* BCG has been only marginally effective against leprosy and is not currently recommended for leprosy prophylaxis. Results from controlled studies to determine the efficacy of BCG vaccines for TB prophylaxis have been conflicting. Estimates of BCG efficacy from placebo-controlled studies range from no efficacy to 80% efficacy. A large scale BCG trial in India (n=360,000 people) showed that BCG failed to provide a protective effect against the onset of pulmonary TB. Other studies have shown that BCG produces an inconsistent, fluctuating immunity. Because no effective vaccine has been developed to protect against leprosy or virus-induced cancers, and because BCG is unreliable for TB prophylaxis, a more effective vaccine is needed. An example of such new vaccines would combine selective antigens with potent adjuvants and stimulate the cellular immune response to deliver a lasting protective immunogen.

In U.S. Patent No. 3,956,481, Jolles et al. discloses a hydrosoluble extract of mycobacteria suitable as an adjuvant, wherein delipidated bacterial residues are subjected either to a mild extraction process or treatment with pyridine followed by treatment with ethanol or water. These extracts were found to be toxic in humans, discouraging their use as a vaccine.

In U.S. Patent No. 4,036,953, Adam et al. discloses an adjuvant for enhancing the effects of a vaccine, wherein the adjuvant is prepared by disrupting mycobacteria or Nocardia cells; separating and removing waxes, free lipids, proteins, and nucleic acids; digesting delipidated material from the cell

wall with a murolytic enzyme; and collecting the soluble portion. Adjuvants of this type were also noted to be toxic in humans.

In U.S. Patent No. 4,724,144, Rook, et al. discloses an immuntherapeutic agent comprising antigenic material from killed *Mycobacterium vaccae* cells useful for the treatment of diseases such as tuberculosis and leprosy. The vaccine has been shown to be effective against persistent microorganisms which survived long exposure to chemotherapeutic agents. Although the vaccine shows improved immune response, it is limited only to antigens endogenous to *Mycobacterium vaccae*.

In U.S. Patent No. 5,599,545, Stanford, et al. discloses an immunotherapeutic agent comprising killed *Mycobacterium vaccae* cells in combination with an antigen exogenous to mycobacteria which promotes a TH-1 response. The exogenous antigen may be combined with the killed *Mycobacterium vaccae* by admixture, chemical conjugation or absorption, or alternatively produced by expression of an exogenous gene in *Mycobacterium vaccae* via plasmid, cosmid, viral or other expression vector, or inserted into the genome. While these compositions promote the TH-1 immune response, they were limited only to killed *Mycobacterium vaccae* cells. Further, the patent provides no guidance as to how to make *Mycobacterium* expression vectors, or how to incorporate the expression vectors into either a plasmid, cosmid, or viral expression vector, or how to integrate the expression vector into the genome.

In U.S. Patent No. 5,583,038, Stover disclosed an expression vector for expressing a protein or polypeptide in a bacterium which comprises a first DNA sequence encoding at least a secretion signal of a lipoprotein and a second DNA sequence encoding a desired protein, protein fragment, polypeptide, or peptide heterologous to the bacterium which expresses the desired protein, etc. Stover demonstrated use of an origin of replication recognized in *Mycobacterium* and the desirability of eliminating sequences not necessary for plasmid replication, e.g., reducing a pAL5000 plasmid fragment containing such an origin of replication to 1910 base pairs. Stover also discloses use of an attP-integrase gene fragment from mycobacteriophage L5 to transform *M. smegmatis* and BCG.

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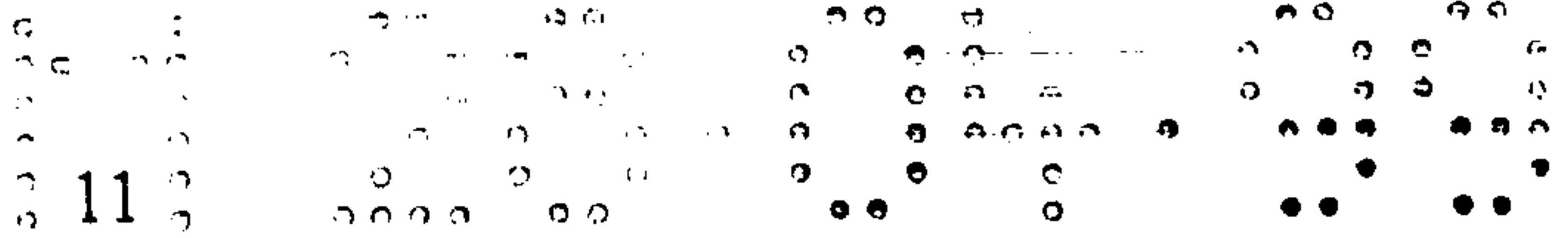

WO 92/01783 disclosed a DNA which includes a first DNA sequence containing a phage integration gene and a second DNA sequence encoding a protein or polypeptide heterologous to the mycobacterium in which the DNA is to be integrated for integrating DNA into a mycobacterium chromosome and
5 then administering the mycobacteria as a vaccine and/or therapeutic agent. WO 92/01783 also disclosed use of an origin of replication recognized in *Mycobacterium* and the desirability of eliminating sequences not necessary for plasmid replication, e.g., reducing a pAL5000 plasmid fragment containing such an origin of replication to 1910 base pairs, and the use of an attP-
10 integrase gene fragment from mycobacteriophage L5 to transform *M. smegmatis* and BCG.

David, et al. (David, et al. 1992. *Plasmid* 28:267-271) discloses a plasmid shuttle vector for *E. coli* and mycobacteria constructed from an *E. coli* plasmid containing the ColE1 origin, a 2.6 kb *Pst*I fragment from
15 bacteriophage D29, and kanamycin resistance gene, which successfully transformed *Mycobacterium smegmatis*. Mistakenly reporting that transformation was achieved due to an origin of replication from the D29 fragment, David, et al. did not teach the use of a minimal functional component of D29 comprising an attachment site and and integrase gene.
20 With respect to *Mycobacterium* diseases, advances made in the area of genetic tools and vaccine strategy included: the isolation, characterization and sequencing of the *Mycobacterium* plasmid pAL 5000; the identification of the kanamycin resistance gene as a selection marker for *Mycobacterium*; the development of the first *Escherichia coli (E. coli)/Mycobacterium* shuttle
25 vectors; the construction of *M. tuberculosis* and *M. leprae* genomic libraries; and the expression of *Mycobacterium* DNA in *E. coli*. (Labidi, et al. 1984. "Plasmid profiles of *Mycobacterium fortuitum* complex isolates," *Curr. Microbiol.* 11, 235-240; Labidi, et al. 1985. "Cloning and expression of mycobacterial plasmid DNA in *Escherichia coli*," *FEMS Microbiol Lett.* 30,
30 221-225; Labidi, et al. 1985. "Restriction endonuclease mapping and cloning of *Mycobacterium fortuitum* var. *fortuitum* plasmid pAL 5000," *Ann. Insti. Pasteur/Microbiol.* 136B, 209-215; Labidi, et al. May 8-13, 1988. "Nucleotide sequence analysis of a 5.0 kilobase plasmid from

AMENDED SHEET

Mycobacterium fortuitum," *Abstract U6* of the 88th Annual Meeting of the American Society for Microbiology, Miami, Florida, USA; Labidi, et al. 1992. "Cloning and DNA sequencing of the *Mycobacterium fortuitum* var. *fortuitum* plasmid, pAL 5000," *Plasmid* 27, 130-140; Labidi, A. January,
5 1986. "Contribution to a plan of action for research in molecular biology and immunology of mycobacteria," *Ph.D. Thesis*. University of Paris and Pasteur Institute, Paris, France). Such advancements have opened the way for the application of recombinant DNA technology to *Mycobacterium*. (Lazraq, et al. 1990. Conjugative transfer of a shuttle plasmid from *Escherichia*
10 *coli* to *Mycobacterium smegmatis*. *FEMS Microbiol. Lett.* 69, 135-138; Konicek, et al. 1991. Gene manipulation in mycobacteria. *Folia Microbiol.* 36(5), 411-422; and Falkinham, III, J.O. and J.T. Crawford. 1994. Plasmids, p. 185-198. *In* Barry Bloom (*ed.*), Tuberculosis: Pathogenesis, protection and control. American Society for Microbiology, Washington, D.C.).

15 The *Mycobacterium* expression vectors resulting from such advancements are not suitable for vaccine development because: 1) the expression vectors are large so the vectors have limited cloning capacity and low transformation efficiency (calculated as the number of transformants obtained per microgram of vector DNA), 2) the vectors lack multiple-cloning
20 sites, 3) the protocols for transformation of mycobacteria with these expression plasmids result in inefficient transformation, 4) the spectrum of mycobacteria transformed by the vectors is restricted because transformation is host-dependent, and 5) the current expression plasmids do not stably transform mycobacteria. Therefore, suitable *Mycobacterium* expression vectors are
25 needed which can provide efficient transformation and stable expression of multiple protective immunogens in mycobacteria.

Suitable antigen delivery systems using nonpathogenic *Mycobacterium* strains, cloning vectors, and *Mycobacterium* expression vectors have now been found which contain protective immunogens that specifically stimulate a cell-
30 mediated immune response by the induction of T_H-1 cells, or cytotoxic T lymphocytes, and provide a consistent, prolonged immunity to intracellular pathogens.

BRIEF DESCRIPTION OF THE DRAWINGS:

Fig. 1 depicts a sequence of the origin of replication in *E. coli* (695 bp). The underlined base indicates the replication point.

Fig. 2 depicts a sequence for the kanamycin gene (932 bp). The underlined sequences are in the 5' to 3' order: the (-35) region for the gene, the (-10) region for the gene, the ribosomal binding site region for the gene, the starting codon (ATG), and the stop codon (TAA).

Fig. 3A depicts a sequence of the pAL 5000 origin of replication (1463 bp) obtained by restriction enzymes analysis. The numbers in superscript indicate the position of the nucleotides in the published sequence of pAL 5000 (Labidi, et al. 1992. "Cloning and DNA sequencing of the *Mycobacteriumfortuitum* var. *fortuitum* plasmid, pAL 5000," *Plasmid* 27:130-140). The underlined sequences indicate in the 5' to 3' order: the position of the forward (F_c, F_1, F_2, and F_3), and the reverse (R_4, R_3, R_2, R_1, and R_C) primers used in PCR analysis, respectively.

Fig. 3B depicts a sequence of the pAL 5000 origin of replication (1382 bp) obtained after PCR analysis. The numbers in superscript indicate the position of the nucleotides in the published sequence of pAL 5000 (Labidi, et al. 1992. *Plasmid* 27:130-140). The underlined sequences indicate in the 5' to 3' order: the position of the forward (F_1, F_2 and F_3), and the reverse (R_4, R_3, R_2 and R_1) primers used in PCR analysis, respectively.

Fig. 4A depicts a sequence of the attachment site (*attP*) and the integrase gene (*int*) of the Mycobacteriophage D_{29}, obtained by restriction enzymes analysis (1631 bp). The numbers in superscript indicate the position of the nucleotides in the sequence. The underlined sequences delimited by numbered nucleotides indicate in the 5' to 3' order: the position of the forward (F_c, F_1, F_2, F_3, and F_4) and the reverse (R_4, R_3, R_2, R_1, and R_c) primers used in PCR analysis, respectively. The underlined sequences not delimited by numbered nucleotides indicate in the 5' to 3' order: the attachment site (*attP*), the (-35) region for the gene (*int*), the (-10) region for the integrase gene (*int*), the ribosomal binding site region for the integrase gene (*int*), and the starting

codon (ATG) for the integrase gene (*int*). The stop codon for the integrase gene (*int*) is the TGA^{1531}.

Fig. 4B depicts a sequence of the attachment site (*attP*) and the integrase gene (*int*) of the Mycobacteriophage D_{29}, obtained after PCR analysis (1413 bp). The numbers in superscript indicate the position of the nucleotides in the sequence. The underlined sequences delimited by numbered nucleotides indicate in the 5' to 3' order: the position of the forward (F_3, and F_4) and the reverse (R_4, R_3, and R_2) primers used in PCR analysis, respectively. The underlined sequences not delimited by numbered nucleotides indicate in the 5' to 3' order: the attachment site (*attP*), the (-35) region for the gene (*int*), the (-10) region for the integrase gene (*int*), the ribosomal binding site region for the integrase gene (*int*), and the starting codon (ATG) for the integrase gene (*int*). The stop codon for the integrase gene (*int*) is the TGA^{1531}.

Fig. 4C depicts a sequence of the attachment site (*attP*) and the integrase gene (*int*) of the Mycobacteriophage D_{29}, obtained after PCR analysis (1374 bp). The numbers in superscript indicate the position of the nucleotides in the sequence. The underlined sequences delimited by numbered nucleotides indicate in the 5' to 3' order: the position of the forward (F_4) and the reverse (R_4, R_3, and R_2) primers used in PCR analysis, respectively. The underlined sequences not delimited by numbered nucleotides indicate in the 5' to 3' order: the attachment site (*attP*), the (-35) region for the gene (*int*), the (-10) region for the integrase gene (*int*), the ribosomal binding site region for the integrase gene (*int*), and the starting codon (ATG) for the integrase gene (*int*). The stop codon for the integrase gene (*int*) is the TGA^{1531}.

Fig. 5 depicts a sequence for the kanamycin gene promoter(102 bp) and the first ATG codon. The underlined sequences are in the 5' to 3' order: the (-35) region for the gene, the (-10) region for the gene, the ribosomal binding site region for the gene, and the starting codon (ATG).

Fig. 6 depicts a sequence of the pAL 5000 fragment containing the open reading frame ORF 2 (2096 bp). The numbers in superscript indicate the position of the nucleotides in the published sequence of pAL 5000 (Labidi,

et al. 1992. *Plasmid* 27:130-140). The underlined sequence (**GGATCC**) is the unique *Bam* HI site which is spanned by the ORF 2 promoter.

Fig. 7 is a gene map of a representative genetic transfer system, wherein "C-ter/anch/seq." = C terminal anchoring sequence; "MCS/express." = multiple
5 cloning site for expression; "N-ter/lead/seq." = N terminal leading sequence; "*Myco*/Prom." = *Mycobacterium* promoter; "Rep/Integ/*Myco*" = *Mycobacterium* origin of replication or phage attachment site and integrase gene (either one or the other but not both is present in a given vector); "MCS/gen/clon." = multiple cloning site for general cloning;
10 "univ/select/mark." = universal selection marker; and "ori/*E.coli*" = *E. coli* origin of replication.

DETAILED DESCRIPTION OF THE INVENTION

The therapeutic or prophylactic vaccines of the present invention combine a protective immunogen with one or more *Mycobacterium* strains acting as a delivery system and an adjuvant, preferably in addition to cytokines and appropriate chemotherapy. The rationale is that the *Mycobacterium* cells will be ingested by macrophages and remain within the macrophage, blocking the killing mechanism of the macrophage while synthesizing the protective immunogen. The immunogen will be processed and presented on the macrophage cell surface to T cells, resulting in TH-1 cell activation and a cell-mediated immune response that is protective against the intracellular disease.

One aspect of the present invention uses an antigen delivery system in the form of a nonpathogenic *Mycobacterium* strain to provide products combining nontoxic immuno-regulating *Mycobacterium* adjuvants, nontoxic immuno-stimulating protective immunogens specific for a variety of diseases, and nontoxic amounts of cytokines that boost the TH-1 pathway. Preferably, the present invention uses a protective immunogen delivery system in the form of a nonpathogenic *Mycobacterium* strain, a genetic transfer system in the form of cloning vectors, and expression vectors to carry and express selected genes in the delivery system.

Protective immunogen delivery system

The protective immunogens of the present invention form pure non-necrotizing complete granuloma. Such immunogens can be protein antigens or other immunogenic products produced by culturing and killing the diseased cell or infectious microorganism, by separating and purifying the immunogens from natural or recombinant sources, or by the cloning and expression into a *Mycobacterium* delivery system of the genes encoding these protein antigens or the enzymes necessary to modify an endogenous lipid to a stage where it is immunogenic and specific. The protective immunogens of the present invention include antigens associated with: 1) cancer including but not limited to lung, colorectum, breast, stomach, prostate, pancreas, bladder, liver, ovary, esophagus, oral and pharynx, kidney, non-Hodgkin's, brain, cervix, larynx,

myeloma, corpus uteri, melanoma, thyroid, Hodgkin's, and testis; 2) bacterial infections including but not limited to mycobacteriosis (e.g., tuberculosis and leprosy), Neisseria infections (e.g., gonorrhea and meningitis), brucellosis, plague, spirochetosis (e.g., trypanosomiasis, Lyme disease and tularemia),
5 rickettsiosis (e.g., typhus, rickettsialpox, and anaplasmosis), chlamydiosis (e.g., trachoma, pneumonia, atherosclerosis, and urethritis), and Whipple's disease; 3) parasitic diseases including but not limited to malaria, leishmania, trypanosomiasis, and toxoplasmosis; 4) viral diseases including but not limited to measles, hepatitis, T-cell leukemia, dengue, AIDS, lymphomas, herpes, and
10 warts; 5) autoimmune diseases including but not limited to rheumatoid arthritis, ankylosing spondylitis, and Reiter's syndrome; 6) allergy diseases including but not limited to asthma, hay fever, atopic eczema, and food allergies; 7) veterinary diseases including but not limited to feline immunodeficiency, equine infectious anemia, avian flue, heartworm, and canine flea allergy; and 8) other
15 diseases including but not limited to leukemia, multiple sclerosis, bovine spongiform (BSE), and myoencephalitis (ME). These antigens can be used singly or in combination in one vaccine. When a combination of antigens is used, they can be administered together at one time or they can be administered separately at different times.

20 Preferred endogenous lipid protective immunogens for the treatment of tuberculosis, leprosy, and other mycobacterioses include but are not limited to complex lipid heteropolymers such as the phenolic glycolipids PGL I and PGL Tb1, the sulfolipid SL I, the diacyl-trehalose DAT and the lipo-oligosaccharide LOS. These lipid immunogens are not synthesized, or modified to their final
25 forms by all *Mycobacterium* species. Therefore, the host strain must provide the necessary precursors to synthesize the desired final immunogenic products. When using an expression vector, the expression system must provide the necessary genes that encode the necessary enzymes to modify the lipid to a stage where it is immunogenic.

30 The mycobacterial adjuvant of the present invention is one that boosts the TH-1 immune response, and preferably down-regulates the TH-2 response.

The *Mycobacterium* strains are characterized by their lack of pathogenicity to mammals and their capacity to be ingested mammalian macrophages. The *Mycobacterium* strains of the present invention may be live or dead upon administration. When the vaccines of the present invention are administered to immunocompromised patients, only dead *Mycobacterium* strains are used. Preferable *Mycobacterium* strains can be obtained from the American Type Culture Collection (Rockville, MD). One or more types of *Mycobacterium* species may be utilized in the preparation of a vaccine. Examples include but are not limited to nonpathogenic *Mycobacterium vaccae*, *Mycobacterium gastri*, *Mycobacterium triviale*, *Mycobacterium aurum*, *Mycobacterium thermoresistible*, *Mycobacterium chitae*, *Mycobacterium duvalii*, *Mycobacterium flavescens*, *Mycobacterium nonchromogenicum*, *Mycobacterium neoaurum*, and *Mycobacterium bovis* BCG. *M. bovis* BCG and *M. gastri* are the only known *Mycobacterium* species that have precursors for producing *M. tuberculosis* and *M. leprae* lipids; therefore, *M. gastri* must be used if the precursors of exogenous lipids are to be expressed in a vaccine for TB or leprosy. *M. gastri* and *M. triviale* can be found in the gastrointestinal tract, and are, thus, important for use in oral vaccines. The *Mycobacterium* adjuvants of the present invention can utilize either one *Mycobacterium* strain or multiple strains; however, when killed *Mycobacterium vaccae* is used, it is preferably administered in combination with other *Mycobacterium* species.

Preferably, the vaccine of the present invention also comprises cytokines that associate with the TH-1 pathway. Examples of such cytokines include but are not limited to gamma interferon (IFN), interleukin(IL)-2, IL-12, IL-15 and granulocyte macrophage colony stimulating factor (GMCSF).

Additionally, the vaccine of the present invention may also be administered in combination with appropriate chemotherapy for treatment of patients with active diseases. If a live *Mycobacterium* strain is used as an adjuvant, appropriate chemotherapy must be selected that does not interfere with the adjuvant function of the live *Mycobacterium*. Examples of

appropriate concommitant chemotherapy is Taxol-R for the treatment of cancer or protein inhibitors for AIDS treatment.

The protective immunogens, cytokines, and concommitant chemotherapy may be produced separately in a synthetic or in a recombinant form, purified by any conventional technique. They may be used in parallel with, mixed with, or conjugated to live or dead *Mycobacterium* cells of interest.

Genetic transfer system

The genetic transfer system of the present invention comprises cloning vectors where the genes of interest are cloned and the transformation technique is used to introduce and express the recombinant molecules into the delivery system. Previous cloning vectors which have been used in *Mycobacterium* species include the extrachromosomal *M. fortuitum* plasmid pAL 5000 (Labidi, et al. 1992. "Cloning and DNA sequencing of the *Mycobacterium fortuitum* var. *fortuitum* plasmid, pAL 5000," *Plasmid* 27:130-140) which replicate extrachromosomally and the mycobacteriophage D_{29}. (Forman, et al. 1954. "Bacteriophage active against virulent *Mycobacterium tuberculosis*: isolation and activity," *Am J Public Health* 44:1326-1333) Mycobacteriophage D_{29} is a large spectrum virulent phage able to infect and efficiently reproduce itself in cultivated *Mycobacterium* species and attach itself to uncultivated *M. leprae*.

New cloning vectors have now been developed which are generally made of either origin(s) of replication or integration system(s), selection marker(s), and multiple cloning site(s) (MCS). The cloning vectors are comprised of the minimum functional sizes of various components including the following components: the *E. coli* replicon, the kanamycin selection marker, the pAL 5000 origin of replication, and the D_{29} attachment site (*attP*) and integrase gene (*int*). Using conventional deletion techniques, the coding region for each component have been reduced to the point that further loss of base pairs resulted in loss of function, hence the designation of minimum functional size. The sequences for each minimum functional component are given as

follows: origin of replication in *E. coli* (695 bp) as SEQ ID NO:1 and Fig. 1; kanamycin gene (932 bp) as SEQ ID NO:2 and Fig. 2; origin of replication in pAL 5000 (1463 bp) obtained by restriction enzyme analysis as SEQ ID NO:3 and Fig. 3A; origin of replication in pAL 5000 (1382 bp) obtained after PCR analysis as SEQ ID NO:4 and Fig. 3B; Mycobacteriophage D_{29} attachment site and integrase gene (1631 bp) obtained by restriction enzyme analysis as SEQ ID NO:5 and Fig. 4A; Mycobacteriophage D_{29} attachment site and integrase gene (1413 bp) obtained after PCR analysis as SEQ ID NO:6 and Fig. 4B; and Mycobacteriophage D_{29} attachment site and integrase gene (1374 bp) obtained after PCR analysis as SEQ ID NO:7 and Fig. 4C. It is well understood in the art of deletion techniques that while the above-identified sequences provide the coding regions for each minimum functional component, an additional loss of a few base pairs from the minimum functional component could still result in a functional component of the present invention.

Numerous *E. coli* origins of replication are commercially available and can be utilized in the present invention. For example, the *E. coli* origin of replication ColE1 is found in most commercially available plasmid vectors designed for *E. coli*. Although the replication point is usually indicated for these vectors, the smallest fragment that can support an efficient replication in *E. coli* has not heretofore been specified. Using the commercially available plasmid vector pNEB 193 (Guan C., New England Biolabs Inc., USA, 1993) as starting material, it has now been determined through restriction endonuclease deletions, cloning, and transformation analysis that the smallest DNA fragment that can support an efficient ColE1 replication in *E. coli* is limited to a 695 bp sequence given in SEQ ID NO:1 and Fig. 1. This *E. coli* origin of replication of minimum functional size has been successfully utilized in the construction of *E.coli* cloning vectors and *E. coli-Mycobacterium* shuttle vectors of the present invention.

While a variety of selection markers are available for the selection of transformed cells and can be used in the present invention, the *Streptococcus faecalis* 1489 bp gene coding for resistance to kanamycin has been selected as

a representative selection marker for *Mycobacterium* (Labidi, et al. 1992. "Cloning and DNA sequencing of the *Mycobacterium fortuitum* var. *fortuitum* plasmid, pAL 5000," *Plasmid* 27:130-140; Labidi, et al. 1985. "Restriction endonuclease mapping and cloning of *Mycobacterium fortuitum* var. *fortuitum* plasmid pAL 5000," *Ann. Insti. Pasteur/Microbiol.* 136B, 209-215). While this gene is well established as the selection marker for *Mycobacterium* (Konicek, et al. 1991. *Folia Microbiol.* 36(5), 411-422), the smallest fragment capable of supporting kanamycin selection in *Mycobacterium* has not heretofore been established. It has now been found that the minimal functional sequence for this gene is about 932 bp as shown in SEQ ID:NO2 and Fig. 2. The kanamycin gene of minimum functional size described herein has been successfully utilized in the construction of *E. coli* cloning vectors and *E. coli-Mycobacterium* shuttle vectors of the present invention.

Vectors containing a plasmid origin of replication do not usually integrate in the chromosome of the host strain. Thus, they are extra-chromosomal vectors. The replication and maintenance in *Mycobacterium* strains of the extra-chromosomal vectors developed in this study, are supported by the origin of replication of the *Mycobacterium fortuitum* plasmid pAL 5000. Labidi, et al. 1984. "Plasmid profiles of *Mycobacterium fortuitum* complex isolates," *Curr. Microbiol.* 11, 235-240. The pAL 5000 plasmid is the most thoroughly studied *Mycobacterium* plasmid and has been used worldwide to develop vectors for genetic transfer in *Mycobacterium* (Falkinham, III, J.O. and J.T. Crawford. 1994. Plasmids, p. 185-198. *In* Barry Bloom (*ed.*), Tuberculosis: Pathogenesis, protection and control. American Society for Microbiology, Washington, D.C.). Functional analysis of the pAL 5000 plasmid has indicated the location of two open reading frames coding for a 20 KDa and a 65 KDa protein, respectively, and a 2579 bp fragment containing its origin of replication (Labidi, et al. 1992. *Plasmid* 27:130-140). In the present invention, the 2579 bp fragment was reduced through deletions with restriction enzymes to a 1463 bp fragment extending from nucleotide 4439 to nucleotide 1079 without loosing its function (SEQ ID NO:3 and Fig. 3A). It has been

found that the 1247 bp fragments extending from nucleotide 4439 to nucleotide 863, and the 1315 bp fragment extending from nucleotide 4587 to nucleotide 1079 do not support replication in *Mycobacterium* (SEQ ID NO:3 and Fig. 3A). Thus, the role of the sequences extending from nucleotide 4439 to nucleotide 4587, and from nucleotide 863 to nucleotide 1079 have now been investigated. In the absence of usable restrictions sites in these two areas of the pAL 5000 sequence, sets of forward and reverse primers that span the two areas have been designed. PCR is then used to amplify the different fragments which are subsequently cloned into an *E. coli* replicon containing the kanamycin gene. Using PCR analysis technique, the minimal functional pAL 5000 origin of replication has been reduced to a 1382 bp fragment extending from nucleotide 4468 to nucleotide 1027 as given in SEQ ID NO:4 and Fig. 3B. Although it has been determined that the 1383 bp fragment extending from nucleotide 4519 to nucleotide 1079, and the 1356 bp fragment extending from nucleotide 4439 to nucleotide 972 did not support replication in *Mycobacterium*, it is further believed that some of the 51 bp sequence extending from nucleotide 4468 to nucleotide 4518 and the 55 bp sequence extending from nucleotide 973 to nucleotide 1027 also might not be needed for replication. This pAL 5000 origin of replication of minimum functional size described herein has been successfully utilized in the *Mycobacterium* cloning vectors and construction of *E. coli-Mycobacterium* shuttle vectors of the present invention.

Vectors can also include a phage attachment site (*attP*) and its accompanying integrase gene. A preferred embodiment of the present invention comprises the attachment site (*attP*) and the integrase gene (int) of the Mycobacteriophage D_{29} (Forman, et al. 1954. *Am J Public Health* 44:1326-1333). The phage D_{29} is a large spectrum virulent phage able to infect cultivated *Mycobacterium* species and efficiently reproduce itself. To develop integrative vectors, a map of its attachment site (*att P*) and integrase gene (*int*) has been determined by constructing a set of hybrid plasmids containing overlapping fragments of D_{29} genome. The recombinant plasmids were then

electroporated into the *Mycobacterium* strains and plated on LB medium containing 50 *ug*/ml kanamycin. A plasmid containing a 2589 bp fragment generated *Mycobacterium* transformants. The 2589 bp fragment was isolated and further analyzed. After establishing its restriction map, another set of hybrid plasmids were constructed containing overlapping segments of the 2589 bp fragment. These recombinant plasmids were electroporated into the *Mycobacterium* strains then plated on selective media. The smallest fragment still able to generate kanamycin resistant *Mycobacterium* transformants were isolated and sequenced using a double strand plasmid template and sequenase version 2.0 (USB, Cleveland, Ohio, USA). The sequence analysis indicated that the fragment size was 1631 bp, which comprised from 5' to 3' the phage attachment site (attP), the integrase gene promoter and the integrase gene (*int*) (SEQ ID NO:5 and Fig. 4A). Subsequent deletions studies regarding the 1631 bp were performed. A 1413 bp originating from base pair 119 to 1531 , illustrated in Fig. 4B afforded a high transformation efficiency. Additional deletion studies resulted in a 1374 bp fragment originating from base pair 158 to 1531, illustrated in Fig. 4C. The 1374 bp fragment generated *Mycobacterium* transformants, but the transformation efficiency was 100 times lower and the incubation time becomes much longer, probably due to low efficiency of integration and stability. It is believed that some of the 39 bp sequence extending from nucleotide 119 to nucleotide 157 might not be needed for integration. These D_{29} (*AttP*), (*int*) and the preceding sequence as described above are the smallest phage DNA fragment so far used in the construction of *Mycobacterium* integrative expression vector and *E. coli/ Mycobacterium* integrative shuttle vectors.

The MCS is a synthetic fragment of DNA containing the recognition sites for certain restriction enzymes that do not cut in the vector sequence. The choice of enzymes to be included in the MCS is based on their frequent use in cloning and their availability. Representative enzymes include *Bam*H I, *Eco*R V, and *Pst* I.

From these minimal functional components, cloning vectors have been developed which maximize the capacity for multiple cloning sites. Preferably, the cloning vectors comprise each component at its minimal functional size. For example, extra-chromosomal cloning vectors have been constructed by assembling the minimum functional fragments for the *E. coli* origin of replication, the pAL 5000 origin of replication, the kanamycin gene, and the MCS. Exemplary integrative cloning vectors have the same structure except the origin of pAL 5000 is replaced by the *attP* and the integrase gene of D_{29}. When each component of the cloning vector is reduced to its smallest functional size, the vectors have a size of about 3 Kb and a transformation efficiency about 10^8. Each vector has a theoretically unlimited cloning capacity and is capable of transforming *Mycobacterium* species. Each cloning vector is presented in Table I.

Fig. 7 presents a genetic map of an exemplary cloning and expression vector. The present invention does not require any particular ordering of the functional components within the cloning vector.

Further, the cloning vectors of the present invention, do not require that each component contained in the vector be reduced to its minimum functional size. The degree to which the minimal functional components are utilized in each cloning vector is dictated ultimately by the application of the vaccine and the maximum transformation size. For example, an integrative cloning vector may contain the minimal functional component for the attachment site and integrase gene while the selection marker is larger than its minimal functional size. Such an arrangement can arise because the cloning vector contains only one site for cloning a protective immunogen, thereby allowing other components of the vector to range in size as long as the vector is of a small enough size to allow for efficient transformation into *Mycobacterium* cells.

Preferably, the present invention uses an *E. coli-Mycobacterium* shuttle vector constructed by applying various recombinant DNA techniques. The constructed vector can be efficiently transformed into either an *E. coli* or *Mycobacterium* host, allowing selected mycobacterial genes to be exponentially

cloned and expressed. Preferably, the *E. coli-Mycobacterium* shuttle vector uses a selection marker that can be expressed in both genera. One shuttle vector is comprised of a kanamycin selection marker, an origin of replication for *E. coli*, and an origin of replication for the *Mycobacterium* plasmid pAL 5000. Another shuttle vector is comprised of a kanamycin selection marker, an origin of replication for *E. coli*, and an attachment site and integrase gene of the Bacteriophage D29. Each component of the constructed shuttle vector has been reduced to its smallest functional size thereby enhancing its cloning and transformation efficiency.

By reducing the vector components to their minimum functional size, the cloning vectors have the capacity for a multiple cloning site with a large number of restriction sites. Therefore, the genetic transfer system of the present invention preferably comprises cloning vectors for more than one protective immunogen. When more than one *Mycobacterium* strain is used in a vaccine, the genetic transfer system of each *Mycobacterium* strain comprises cloning vectors for one or more protective immunogens.

Transformation

Mycobacterium strains have been successfully transformed through electroporation. (Labidi, et al. 1992. "Cloning and DNA sequencing of the *Mycobacteriumfortuitum* var. *fortuitum* plasmid, pAL 5000," *Plasmid* 27:130-140) It is understood that other transformation techniques developed for *Mycobacterium* would be useful in the present invention. The electroporation techniques of the present invention are described in Example 3, and the results are given in Table 1. The vector designs, culture medium, and the transformation technique described have improved significantly the transformation efficiency for *Mycobacterium* species and brought it for the first time to a level comparable to that obtained with *E. coli*.

The integrative vectors containing the attachment site (*attP*) and the integrase gene (*int*) of the phage D_{29} have been found to integrate into the chromosomes of their hosts at a region complementary of the region (*attP*). This region is the bacterial attachment site (*attB*) and is located between the genes encoding the Proline transfer RNA ($tRNA^{Pro}$) and the Glycine transfer RNA ($tRNA^{Gly}$).

Table I: Vectors

vectors	Definition	M.W (bp)	Marker[1]	Replication Origin[2]	Efficiency[3]	Expression[4]	Reference
pBR 322	An *E. coli* cloning vector.	4361	ApTC	ColE1	(10^8;0)		Bolivar F., *et al.* 1977a&b
pUC 19	An *E. coli* cloning vector.	2686	Ap	ColE1	(10^9;0)		Yanish-Perron C., *et al.* 1985
pBS II KS (+)	An *E. coli* cloning vector.	2961	Ap	ColE1	(10^9;0)		Short et al. 1988
pNEB 193	A pUC 19 derivative vector.	2713	Ap	ColE1	(10^9;0)		Guan C. 1993., New England Biolabs, USA.
D_{29}	A large spectrum mycobacteriophage	52000	Lysis	D_{29}	(0;100%)		Forman S., *et al.* 1954

vectors	Definition	M.W (bp)	Marker[1]	Replication Origin[2]	Efficiency[3]	Expression[4]	Reference
pAL 5000	A plasmid isolated from *Mycobacterium fortuitum* var *fortuitum*. pAL 5000 was the first plasmid to be used for vector construction and gene manipulation in *Mycobacterium*. *Mycobacterium* cloning and expression vectors are generally based on pAL 5000.	4821	Cryptic	pAL 5000	(0;100%)	ORF2	Labidi A., 1986., Labidi A., *et al.* (1984,1985, 1988,1992)
pAL 04	Cloning of pAL 5000 into pBR 322 at the *Eco*R V.	9182	Ap	ColE1/pAL 5000	(10^6;10^5)	ORF2	Labidi A., 1986., Labidi A., *et al.* 1985a
pAL 15	Cloning of pAL 5000 into pBR 322 at the *Eco*R I site.	9182	ApTc	ColE1	(10^6;0)	ORF2	Labidi A., 1986., Labidi A., *et al.* 1985a
pAL 64	Cloning of pAL 5000 into pBR 322 at the *Bam*H I site.	9182	Ap	ColE1/pAL 5000	(10^6;10^5)	None	Labidi A., 1986., Labidi A., *et al.* 1985a
pAL 12	Cloning of a 1489 bp *Cla* I/*Cla* I DNA fragment encoding a 3'-5"-aminoglycoside phosphotransferase type III, into pAL 15	10671	ApTcKm	ColE1	(10^6;0)	ORF2	Labidi A., 1986., Labidi A., *et al.* 1985b

vectors	Definition	M.W (bp)	Marker[1]	Replication Origin[2]	Efficiency[3]	Expression[4]	Reference
pAL 21	The Kanamycin resistance gene (type III) is cloned into pAL 15 in the opposite orientation per comparison to pAL 12.	10671	ApTcKm	ColE1	(10^6;0)	ORF2	Labidi A., 1986., Labidi A., et *al*. 1985b
pAL 23	Cloning of a 1489 bp *Cla* I/*Cla* I DNA fragment encoding a 3'-5"-aminoglycoside phosphotransferase type III, into pAL 64	10671	ApKm	ColE1/pAL 5000	(10^6;10^5)	None	Labidi A., 1986., Labidi A., *et al*. 1985b
pAL 32	The Kanamycin resistance gene (type III) is cloned into pAL 64 in the opposite orientation per comparison to pAL 23. Not only were pAL 23 and pAL 32 the first *E. coli* - *Mycobacterium* shuttle vectors to be described in the literature, but also for the first time the Kanamycin resistance gene was introduced as a selection marker for *Mycobacterium*.	10671	ApKm	ColE1/pAL 5000	(10^6;10^5)	None	Labidi A., 1986., Labidi A., *et al*. 1985b
pAL 401	pNEB 193/*Tfi* I digested and self ligated.	2573	AP	ColE1	(10^9;0)		This study

vectors	Definition	M.W (bp)	Marker[1]	Replication Origin[2]	Efficiency[3]	Expres-sion[4]	Reference
pAL 402A	Cloning of a 1240 bp *Pst* I/*Pst* I DNA fragment encoding a 3'-5"-aminoglycoside phosphotransferase type I, into pAL 401.	3813	ApKm	ColE1	(10^8;0)		This study
pAL 402B	The Kanamycin resistance gene (type I)is cloned into pAL 401 in the opposite orientation per comparison to pAL 402A.	3813	ApKm	ColE1	(10^8;0)		This study
pAL 403	pAL 402B/(*Ecl*136 II + *Eam* 1105I) digested, Klenow filled and self ligated.	2421	Km	ColE1	(10^9;0)		This study
pAL 404	pAL 402A/*Ava* II digested and self ligated.	2372	Km	ColE1	(10^9;0)		This study
pAL 405	pAL 404/*Dra* I digested and self ligated.	2353	Km	ColE1	(10^9;0)		This study
pAL 406	pAL 402A/(*Eam*1105 I + *Ecl* 136II) digested, Klenow filled and self ligated.	4842	Km	ColE1	(10^8;0)		This study
pAL 407	pAL 403/*Dra* I digested and self ligated.	2235	Km	ColE1	(10^9;0)		This study
pAL 408	pAL 404/(*Bsm*F I + *Dra* I) digested, Klenow filled and self ligated.	1970	Km	ColE1	(10^{10};0)		This study

vectors	Definition	M.W (bp)	Marker[1]	Replication Origin[2]	Efficiency[3]	Expres-sion[4]	Reference
pAL 409	pAL 403/(*Afl* III + *Bsm*F I) digested, Klenow filled and self ligated.	1934	Km	ColE1	(10^{10};0)		This study
pAL 410	pAL 404/(*Afl* III + *Sph* I) digested, Klenow filled and self ligated.	2147	Km	ColE1	(10^{9};0)		This study
pAL 411	pAL 407/(*Afl* III + *Bsm*F I) digested, klenow filled and self ligated.	1748	Km	ColE1	(10^{10};0)		This study
pAL 412	pAL 408/(*Afl* III + *Sph* I) digested, Klenow filled and self ligated.	1745	Km	ColE1	(10^{10};0)		This study
pAL 413A	Cloning of a 2590 bp *Pst* I/*Pst* I DNA fragment isolated from the phage D_{29} into pAL 411 at the *Pst* I site.	4333	Km	ColE1/D_{29}	10^{8};10^{7}		This study
pAL 413B	The 2590 bp *Pst* I/*Pst* I DNA fragment is cloned into pAL 411 in the opposite orientation per comparison to pAL 413A.	4333	Km	ColE1/D_{29}	(10^{8};10^{7})		This study
pAL 414A	The 2590 bp *Pst* I/*Pst* I DNA fragment is cloned into pAL 412 at the Pst I site.	4330	Km	ColE1/D_{29}	(10^{8};10^{7})		This study

vectors	Definition	M.W (bp)	Marker[1]	Replication Origin[2]	Efficiency[3]	Expres-sion[4]	Reference
pAL 414B	The 2590 bp *Pst* I/*Pst* I DNA fragment is cloned into pAL 412 in the opposite orientation per comparison to pAL 414A.	4330	Km	ColE1/D_{29}	(10^8;10^7)		This study
pAL 415	pAL 402 A/(*Acc* I + *Bsm*F I) digested, Klenow filled and self ligated.	3541	ApKm	ColE1	(10^8;0)		This study
pAL 416	pAL 415/(*Afl* III + *Pst* I) digested, klenow filled and self ligated.	3310	ApKm	ColE1	(10^9;0)		This study
pAL 417A	Cloning of pAL 5000 into pAL 416 at the *Bam*H I site.	8131	ApKm	ColE1/pAL 5000	(10^6;10^5)	None	This study
pAL 417B	The pAL 5000 sequence is cloned into pAL 416 in the opposite orientation per comparison to pAL 417A.	8131	Apkm	ColE1/pAL 5000	(10^6;10^5)	None	This study
pAL 418	pAL 411/(*Dra* III + *Pst* I) digested, klenow filled and self ligated.	1721	Km	ColE1	(Low;0)		This study
pAL 418a	pAL 411/(*Bsp*M I + *Pst* I) digested, Klenow filled and self ligated.	1741	Km	ColE1	(Low;0)		This study

vectors	Definition	M.W (bp)	Marker[1]	Replication Origin[2]	Efficiency[3]	Expres-sion[4]	Reference
pAL 418b	pAL 411/(*BsrD* I + *Pst* I) digested, Klenow filled and self ligated.	1698	Km	ColE1	(0;0)		This study
pAL 418c	pAL 411/(*Eco57* I + *Pst* I) digested, Klenow filled and self ligated.	1526	Km	ColE1	(Low;0)		This study
pAL 418d	pAL 411/(*AlwN* I + *Pst* I) digested, Klenow filled and self ligated.	1393	Km	ColE1	(0;0)		This study
pAL 418e	pAL 411/(*AspH* I + *Pst* I) digested, Klenow filled and self ligated.	1294	Km	ColE1	(0;0)		This study
pAL 419	pAL 412/*Bfa* I digested and self ligated.	1492	Km	ColE1	(Low;0)		This study
pAL 419a	pAL 412/(*Nsp* I + *Pst* I) digested, Klenow filled and self ligated.	1735	Km	ColE1	(10^{10};0)		This study
pAL 419b	pAL 412/(*Drd* I + *Pst* I) digested, Klenow filled and self ligated.	1631	Km	ColE1	(0;0)		This study
pAL 419c	pAL 412/(*Hae* II + *Pst* I) digested, Klenow filled and self ligated	1491	Km	ColE1	(0;0)		This study

vectors	Definition	M.W (bp)	Marker[1]	Replication Origin[2]	Efficiency[3]	Expres-sion[4]	Reference
pAL 419d	pAL 412/(*Apa*L I + *Pst* I) digested, Klenow filled and self ligated.	1425	Km	ColE1	(0;0)		This study
pAL 419e	pAL 412/(*Asp*H I + *Pst* I) digested, Klenow filled and self ligated.	1421	Km	ColE1	(0;0)		This study
pAL 419f	pAL 412/(*Alw*N I + *Pst* I) digested, Klenow filled and self ligated.	1323	Km	ColE1	(0;0)		This study
pAL 420	pAL 417A/(*Dra* I + *Eco*R V) digested and self ligated.	5606	Km	ColE1/pAL 5000	(10^8;10^7)		This study
pAL 421	pAL 417A/(*Hpa* I + *Xba* I) digested, Klenow filled and self ligated.	6852	ApKm	ColE1/pAL 5000	(10^7;10^6)		This study
pAL 422	pAL 417B/(*Dra* I + *Hpa* I) digested and self ligated.	5316	Km	ColE1/pAL 5000	(10^8;10^7)		This study
pAL 423	pAL 417B/(*Eco*R V + *Xba* I) digested, Klenow filled and self ligated.	7142	ApKm	ColE1/pAL 5000	(10^7;10^6)	None	This study
pAL 424	pAL 417A/(*Dra* I partial + *Eco*R V) digested and self ligated.	6317	Km	ColE1/pAL 5000	(10^7;10^6)		This study
pAL 425A	pAL 421/(*Dra* I + *Eco*R V) digested and self ligated.	4327	Km	ColE1/pAL 5000	(10^8;10^7)		This study

vectors	Definition	M.W (bp)	Marker[1]	Replication Origin[2]	Efficiency[3]	Expression[4]	Reference
pAL 425B	pAL 423/(*Dra* I + *Hpa* I) digested and self ligated.	4327	Km	ColE1/pAL 5000	(10^8;10^7)		This study
pAL 426	pAL 424/(*Hpa* I + *Xba* I) digested, Klenow filled and self ligated.	5038	Km	ColE1/pAL 5000	(10^8;10^7)		This study
pAL 427	pAL 417A/(*Dra* I + *Hpa* I) digested and self ligated.	3027	Km	ColE1	(10^9;0)		This study
pAL 428	pAL 417A/(*Eco*R V + *Xba* I) digested, Klenow filled and self ligated.	4272	ApKm	ColE1	(10^8;0)		This study
pAL 429	pAL 417A/(*Eco*R V + *Hpa* I) digested and self ligated.	5552	ApKm	ColE1	(10^8;0)		This study
pAL 430	pAL 426/(*Dra* I + *Sgr*A I) digested, klenow filled and self ligated.	3601	Km	ColE1	(10^8;0)		This study
pAL 431	pAL 426/(*Asc* I + *Dra* I) digested, Klenow filled and self ligated.	3214	Km	ColE1	(10^9;0)		This study
pAL 432	pAL 426/(*Bgl* II + *Dra* I) digested, klenow filled and self ligated.	3128	Km	ColE1	(10^9;0)		This study

vectors	Definition	M.W (bp)	Marker[1]	Replication Origin[2]	Efficiency[3]	Expres-sion[4]	Reference
pAL 433	pAL 426/(*Dra* I + *Eco*R I) digested, Klenow filled and self ligated.	2890	Km	ColE1	(10^9;0)		This study
pAL 434	pAL 426/(*Mun* I + *Xba* I) digested, Klenow filled and self ligated.	4498	Km	ColE1/pAL 5000	(10^8;10^7)		This study
pAL 435	pAL 426/(*Nco* I + *Xba* I) digested, Klenow filled and self ligated.	4126	Km	ColE1	(10^8;0)		This study
pAL 436	pAL 426/(*Eco*R I + *Xba* I) digested, Klenow filled and self ligated.	3900	Km	ColE1	(10^8;0)		This study
pAL 437	pAL 426/(*Bgl* II + *Xba* I) digested, Klenow filled and self ligated.	3662	Km	ColE1	(10^8;0)		This study
pAL 438	pAL 434/(*Dra* I + *Psp*1405 I) digested, Klenow filled and self ligated.	3774	Km	ColE1/pAL 5000	(10^8;10^7)		This study
pAL 439	pAL 434/(*Bsi*W I + *Dra* I) digested, Klenow filled and self ligated.	3372	Km	ColE1/pAL 5000	(10^9;10^8)		This study
pAL 440	pAL 439/(*Bcg* I + *Xba* I) digested, Klenow filled and self ligated.	3156	Km	ColE1	(10^9;0)		This study

vectors	Definition	M.W (bp)	Marker[1]	Replication Origin[2]	Efficiency[3]	Expression[4]	Reference
pAL 441	pAL 439/(*Msl* I + *Xba* I) digested, Klenow filled and self ligated.	3009	Km	ColE1	(10^9;0)		This study
pAL 442	pAL 414B/(*Bbs* I + *Nsp* I) digested, Klenow filled and self ligated.	4189	Km	ColE1/D_{29}	(10^8;10^7)		This study
pAL 443	pAL 414B/(*Bpm* I + *Nsp* I) digested, Klenow filled and self ligated.	3978	Km	ColE1/D_{29}	(10^8;10^7)		This study
pAL 444	pAL 414B/(*Afl* II + *Nsp* I) digested, Klenow filled and self ligated.	3389	Km	ColE1/D_{29}	(10^9;10^8)		This study
pAL 445	pAL 414B/(*BsaA* I + *Nsp* I) digested, Klenow filled and self ligated.	3370	Km	ColE1/D_{29}	(10^9;10^8)		This study
pAL 446	pAL 414B/(*BamH* I + *Nsp* I) digested, Klenow filled and self ligated.	3195	Km	ColE1	(10^9;0)		This study
pAL 447	pAL 445/(*Pst* I + *Sal* I) digested, Klenow filled and self ligated.	3071	Km	ColE1	(10^9;0)		This study
pAL 448	pAL 434/(*Dra* I + *Nsp* I partial) digested, Klenow filled and self ligated.	3210	Km	ColE1/pAL 5000	(10^9;10^8)		This study

vectors	Definition	M.W (bp)	Marker[1]	Replication Origin[2]	Efficiency[3]	Expres-sion[4]	Reference
pAL 449	pAL 434/(*Age* I + *Dra* I) digested, Klenow filled and self ligated.	3061	Km	ColE1	(10^9;0)		This study
pAL 450	pAL 445/(*Msl* I + *Pst* I) digested, Klenow filled and self ligated.	3183	Km	ColE1	(10^9;0)		This study
pAL 451	pAL 445/(*Bgl* I + *Pst* I) digested, Klenow filled and self ligated.	3111	Km	ColE1	(10^9;0)		This study
pAL 452	pAL 448/(*Msl* I + *Hinc* II) digested and self ligated. pAL 5000's ORF 1 is deleted.	2655	Km	ColE1	(10^9;0)		This study
pAL 453	pAL 448/(*Bsa*A I + *Hinc* II) digested and self ligated. pAL 5000's ORF 1 is reduced and is out of frame.	3055	Km	ColE1	(10^9;0)		This study
pAL 454	pAL 448/(*Aos* I + *Bsa*A I) digested and self ligated. pAL 5000's ORF 1 is reduced and is out of frame.	3173	Km	ColE1	(10^9;0)		This study
pAL 455	pAL 448/(*Aos* I + *Psh*A I) digested and self ligated. pAL 5000's ORF 1 is reduced and is out of frame.	3067	Km	ColE1	(10^9;0)		This study

vectors	Definition	M.W (bp)	Marker[1]	Replication Origin[2]	Efficiency[3]	Expres-sion[4]	Reference
pAL 456	pAL 448/(*Msl* I + *Psh*A I) digested and self ligated. pAL 5000's ORF 1 is reduced and is out of frame.	2990	Km	ColE1	(10^9;0)		This study
pAL 457	pAL 448/(*Aos* I + *Hinc* II) digested and self ligated. pAL 5000's ORF 1 is reduced and is in frame.	3018	Km	ColE1	(10^9;0)		This study
pAL 458	pAL 448/(*Hinc* II + *Psh*A I) digested and self ligated. pAL 5000's ORF 1 is reduced and is out of frame.	2875	Km	ColE1	(10^9;0)		This study
pAL 459	pAL 448/(*Aos* I + *Msl* I) digested and self ligated. pAL 5000's ORF 1 is reduced and is in frame.	2847	Km	ColE1	(10^9;0)		This study
pAL 460	pAL 448/(*Bsa*A I + *Msl* I) digested and self ligated. pAL 5000's ORF 1 is reduced and is out of frame.	2810	Km	ColE1	(10^9;0)		This study
pAL 461	pAL 448/(*Bsa*A I + *Psh*A I) digested and self ligated. pAL 5000's ORF 1 is reduced and is in frame.	3030	Km	ColE1	(10^9;0)		This study

vectors	Definition	M.W (bp)	Marker[1]	Replication Origin[2]	Efficiency[3]	Expres-sion[4]	Reference
pAL 462	pAL 423/(*Bam*H I + *Bst*X I) digested, Klenow filled and self ligated. pAL 5000's ORF 2 is fused in frame to Lac Z.	6798	Km	ColE1/pAL 5000	(10^7;10^6)	None	This study
pAL 463	pAL 423/(*Bst*X I + *Eco*R I partial) digested, Klenow filled and self ligated. pAL 5000's ORF 2 is fused in frame to Lac Z.	6768	Km	ColE1/pAL 5000	(10^7;10^6)	None	This study
pAL 464	pAL 462/(*Dra* I + *Kas* I) digested, Klenow filled and self ligated. Lac Z is reduced at the C-terminal.	5442	Km	ColE1/pAL 5000	(10^8;100^7)	None	This study
pAL 465	pAL 463/(*Dra* I + *Kas* I) digested, Klenow filled and self ligated. Lac Z is reduced at the C-terminal.	5412	Km	ColE1/pAL 5000	(10^8;10^7)	None	This study
pAL 466A	Cloning of the pAL 5000 into pAL 416 at the *Eco*R I site.	8131	APKm	ColE1	(10^6;0)	ORF2	This study
pAL 466B	The pAL 5000 sequence is cloned into pAL 416 in the opposite orientation per comparison to pAL 466A.	8131	APKm	ColE1	(10^6;0)	ORF2	This study

vectors	Definition	M.W (bp)	Marker[1]	Replication Origin[2]	Efficiency[3]	Expres-sion[4]	Reference
pAL 467	pAL 448/(*Apa*L I + *Eco*57 I) digested, Klenow filled and self ligated. The origin of replication in *E. coli* is destroyed.	2980	Km	pAL 5000	$(0;10^8)$		This study
pAL 468	pAL 414B/(*Bsi*W I + *Sal* I) digested, Klenow filled and self ligated.	3473	Km	ColE1/D_{29}?	$(10^9;10^3\text{-}10^5?)$		This study
pAL 469	pAL 414B/(*Bam*H I + *Bsi*W I) digested, Klenow filled and self ligated.	4038	Km	ColE1/D_{29}?	$(10^8;0\text{-}10^3?)$		This study
pAL 470	pAL 466A/(*Dra* I + *Afl* III) digested, Klenow filled and self ligated.	6598	Km	ColE1	$(10^7;0)$	ORF2	This study
pAL 471	pAL 466A/(*Dra* I + *Mun* I) digested, Klenow filled and self ligated.	6016	Km	ColE1	$(10^7;0)$	ORF2	This study
pAL 472	pAL 466A/(*Dra* I + *Bal* I) digested and self ligated.	5734	Km	ColE1	$(10^7;0)$	ORF2	This study
pAL 473	pAL 466A/(*Dra* I + *Hpa* I) digested and self ligated.	5472	Km	ColE1	$(10^8;0)$	ORF2	This study
pAL 474	pAL 466A/(*Dra* I + *Apa* I) digested, Klenow filled and self ligated.	5064	Km	ColE1	$(10^8;0)$	ORF2	This study

vectors	Definition	M.W (bp)	Marker[1]	Replication Origin[2]	Efficiency[3]	Expres-sion[4]	Reference
pAL 475	pAL 466A/(*Dra* I + *Bst*E II) digested, Klenow filled and self ligated.	5013	Km	ColE1	(10^8;0)	ORF2	This study
pAL 476	pAL 466B/(*Pac* I + *Afl* III) digested, Klenow filled and self ligated.	8079	ApKm	ColE1	(10^6;0)	ORF2	This study
pAL 477	pAL 466B/(*Pac* I + *Mun* I) digested, Klenow filled and self ligated.	7497	ApKm	ColE1	(10^7;0)	ORF2	This study
pAL 478	pAL 466B/(*Pac* I + *Bal* I) digested, Klenow filled and self ligated.	7215	ApKm	ColE1	(10^7;0)	ORF2	This study
pAL 479	pAL 466B/(*Pac* I + *Hpa* I) digested, Klenow filled and self ligated.	6953	ApKm	ColE1	(10^7;0)	ORF2	This study
pAL 480	pAL 466B/(*Pac* I + *Apa* I) digested, Klenow filled and self ligated.	6545	ApKm	ColE1	(10^7;0)	ORF2	This study
pAL 481	pAL 466B/(*Pac* I + *Bst*E II) digested, Klenow filled and self ligated.	6494	ApKm	ColE1	(10^7;0)	ORF2	This study
pAL 482	pAL 445/(*Dsa* I + *Sal* I) digested, Klenow filled and self ligated.	3341	Km	ColE1	(10^9;0)		This study

vectors	Definition	M.W (bp)	Marker[1]	Replication Origin[2]	Efficiency[3]	Expres-sion[4]	Reference
pAL 483	pAL 445/(*Bsi*W I + *Sal* I) digested, Klenow filled and self ligated.	2513	Km	ColE1	(10^9;0)		This study
pAL 484	pAL 445/(*Aat* II + *Bsi*W I) digested, Klenow filled and self ligated.	3259	Km	ColE1	(10^9;0)		This study
pAL 485	pAL 445/(*Bsr*B I + *Aat* II) digested, Klenow filled and self ligated.	3222	Km	ColE1/D_{29}?	(10^9;0-10^3?)		This study
pAL 486	pAL 445/(*Bsr*B I + *Bam*H I) digested, Klenow filled and self ligated.	3333	Km	ColE1/D_{29}?	(10^9;0-10^3?)		This study
pAL 487	pAL 445/(*Dsa* I + *Bgl* I) digested, Klenow filled and self ligated.	3360	Km	ColE1	(10^9;0)		This study
pAL 488	pAL 445/(*Bgl* I + *Msl* I) digested, Klenow filled and self ligated.	3298	Km	ColE1	(10^9;0)		This study
pAL 490	pAL 414A/(*Nsp* I + *Sal* I) digested, Klenow filled and self ligated.	4025	Km	ColE1	(10^8;0)		This study
pAL 491	pAL 414A/(*Nsp* I + *Bsa* I) digested, Klenow filled and self ligated.	3356	Km	ColE1	(10^9;0)		This study

vectors	Definition	M.W (bp)	Marker[1]	Replication Origin[2]	Efficiency[3]	Expres-sion[4]	Reference
pAL 492	pAL 414A/(*Nsp* I + *BsiW* I) digested, Klenow filled and self ligated.	3164	Km	ColE1	(10^9;0)		This study
pAL 493	pAL 414A/(*Nsp* I + *Aat* II) digested, Klenow filled and self ligated.	3049	Km	ColE1	(10^9;0)		This study
pAL 494	pAL 414A/(*Nsp* I + *Bam*H I) digested, Klenow filled and self ligated.	2868	Km	ColE1	(10^9;0)		This study
pAL 495	pAL 414A/(*Nsp* I + *BbrP* I) digested, Klenow filled and self ligated.	2689	Km	ColE1	(10^9;0)		This study
pAL 496	pAL 468/(*Nsp* I + *Bam*H I) digested, Klenow filled and self ligated.	2338	Km	ColE1	(10^9;0)		This study
pAL 497	pAL 468/*Bsa* I digested and self ligated.	2513	Km	ColE1/?	(10^9;0?)		This study
pAL 498	pAL 468/(*Bam*H I + *Aat* II) digested, Klenow filled and self ligated.	3288	Km	ColE1	(10^9;0)		This study
pAL 499	pAL 468/(*Bam*H I + *Acy* I) digested, Klenow filled and self ligated.	3291	Km	ColE1	(10^9;0)		This study

vectors	Definition	M.W (bp)	Marker[1]	Replication Origin[2]	Efficiency[3]	Expression[4]	Reference
pAL 500							This study
pAL 501	pAL 420/(*Bst*X I + *Mun* I) digested, Klenow filled and self ligated.	4142	Km	ColE1/pAL 5000	(10^8;10^7)		This study
pAL 502	pAL 501/(*Bsi*W I + *Psp*1406 I) digested, Klenow filled and self ligated.	3742	Km	ColE1/pAL 5000	(10^8;10^7)		This study
pAL 503	pAL 466A unmethylated/ *Bsa*B I digested and self ligated.	7490	ApKm	ColE1	(10^7;0)	ORF2	This study
pAL 504	pAL 466B unmethylated/ *Bsa*B I digested and self ligated.	7490	ApKm	ColE1	(10^7;0)	ORF2	This study
pAL 505	pAL 466A/ *Bsa* I digested, Klenow filled and self ligated.	4913	Km	ColE1	(10^7;0)	ORF2	This study
pAL 506	pAL 466B/ *Nco* I digested and self ligated.	6484	ApKm	ColE1	(10^7;0)	ORF2	This study
pAL 507	pAL 466A/(*Bsa* I + *Bst*X I) digested, Klenow filled and self ligated.	4747	Km	ColE1	(10^8;0)	ORF2	This study
pAL 508	pAL 466B/(*Nco* I + *Bst*X I) digested, Klenow filled and self ligated.	6295	ApKm	ColE1	(10^7;0)	ORF2	This study

vectors	Definition	M.W (bp)	Marker[1]	Replication Origin[2]	Efficiency[3]	Expres-sion[4]	Reference
pAL 509	A 577 bp DNA fragment was PCR amplified from pAL 5000 (bp 1969 to bp 2546) using an *Eco*R I forward primer and a *Bam*H I reverse primer. 10 bp were added to each primer to create the restriction sites. Thus the DNA fragment amplified was 597 bp. The 597 bp DNA fragment was digested with *Eco*R I and *Bam*H I then cloned into pBS II KS(+)digested with the same enzymes	3528	Ap	ColE1	$(10^8;0)$		This study
pAL 510	pAL 507/(*Pac* I + *Bsi*W I) digested, Klenow filled and self ligated.	3689	Km	ColE1	$(10^8;0)$	ORF2	This study
pAL 511	pAL 507/(*Pac* I + *Psp*1406 I) digested, Klenow filled and self ligated.	3285	Km	ColE1	$(10^9;0)$	ORF2	This study
pAL 512	pAL 507/(*Pac* I + *Eco*R V) digested, Klenow filled and self ligated.	3270	Km	ColE1	$(10^9;0)$	ORF2	This study
pAL 513	pAL 507/(*Dra* I + *Csp* I) digested, Klenow filled and self ligated.	4373	Km	ColE1	$(10^8;0)$	ORF2	This study

vectors	Definition	M.W (bp)	Marker[1]	Replication Origin[2]	Efficiency[3]	Expres-sion[4]	Reference
pAL 514	pAL 508/(*Dra* I + *Bsi*W I) digested, Klenow filled and self ligated.	3756	Km	ColE1	(10^8;0)	ORF2	This study
pAL 515	pAL 508/(*Dra* I + *Eco*R V) digested, Klenow filled and self ligated.	3337	Km	ColE1	(10^9;0)	ORF2	This study
pAL 516	pAL 508/(*Dra* I + *Acc* III) digested, Klenow filled and self ligated.	3219	Km	ColE1	(10^9;0)	ORF2	This study
pAL 517	pAL 475/(*Pac* I + *Bsi*W I) digested, Klenow filled and self ligated.	3955	Km	ColE1	(10^8;0)	ORF2	This study
pAL 518	pAL 475/(*Pac* I + *Eco*R V) digested, Klenow filled and self ligated.	3536	Km	ColE1	(10^8;0)	ORF2	This study
pAL 519	pAL 475/(*Pac* I + *Acc* III) digested, Klenow filled and self ligated.	3418	Km	ColE1	(10^9;0)	ORF2	This study
pAL 520	pAL 481/(*Dra* I + *Bsi*W I) digested, Klenow filled and self ligated.	3955	Km	ColE1	(10^8;0)	ORF2	This study
pAL 521	pAL 481/(*Dra* I + *Eco*R V) digested, Klenow filled and self ligated.	3536	Km	ColE1	(10^8;0)	ORF2	This study

vectors	Definition	M.W (bp)	Marker[1]	Replication Origin[2]	Efficiency[3]	Expres-sion[4]	Reference
pAL 522	pAL 481/(*Dra* I + *Acc* III) digested, Klenow filled and self ligated.	3418	Km	ColE1	$(10^9;0)$	ORF2	This study
pAL 523	A 1462 bp fragment (fig. 3A) was PCR amplified using the control *Xba* I - forward primer F_cX (gc-TCTAGA-tgttcctcctggttggt), the control *Ban* I - reverse primer R_cB (ccg-GGCACC-attgcgttagaaca), and as a template the 2196 bp *Eco*N I ---> *Bal* I fragment of pAL 5000, which contain a functional origin of replication, as determined by restriction analysis in pAL 448. The amplified fragment was digested with (*Ban* I + *Xba* I) and ligated to an 1836 bp (*Ban* I/*Xba* I) fragment originating from pAL 416 and containing the origin of replication for *E. coli* and the kanamycin gene. This plasmid was generated to confirm the results obtained with pAL 448.	3298	km	ColE1/pAL 5000	$(10^9;10^8)$		This study

vectors	Definition	M.W (bp)	Marker[1]	Replication Origin[2]	Efficiency[3]	Expres-sion[4]	Reference
pAL 524	Same as pAL 523 except that the fragment amplified is 1411 bp, and the primers are F_cX and R_1B (ctt-GGCACC-cgcttcctgttctg).	3247	Km	ColE1/pAL 5000	(10^9;10^8)		This study
pAL 525	Same as pAL 523 except that the fragment amplified is 1356 bp, and the primers are F_cX and R_2B (cgc-GGCACC-gtgtccagatgcag).	3192	Km	ColE1	(10^9;0)		This study
pAL 526	Same as pAL 523 except that the fragment amplified is 1356 bp, and the primers are F_cX and R_3B (cgc-GGCACC-gtgtccagatgcag).	3142	Km	ColE1	(10^9;0)		This study
pAL 527	Same as pAL 523 except that the fragment amplified is 1356 bp, and the primers are F_cX and R_4B (agt-GGCACC-g).	3097	Km	ColE1	(10^9;0)		This study
pAL 528	Same as pAL 523 except that the fragment amplified is 1433 bp, and the primers are F_1X (gt-TCTAGA-gggtgctcggctgtcgc) and R_cB.	3269	Km	ColE1/pAL 5000	(10^9;10^8)		This study

vectors	Definition	M.W (bp)	Marker[1]	Replication Origin[2]	Efficiency[3]	Expression[4]	Reference
pAL 529	Same as pAL 523 except that the fragment amplified is 1382 bp, and the primers are F_2X (gg-TCTAGA-ggggagtgtgcagtt) and R_cB.	3218	Km	ColE1	(10^9;0)		This study
pAL 530	Same as pAL 523 except that the fragment amplified is 1331 bp, and the primers are F_3X (gc-TCTAGA-ctcgtgtcggaccat) and R_cB.	3167	Km	ColE1	(10^9;0)		This study

vectors	Definition	M.W (bp)	Marker[1]	Replication Origin[2]	Efficiency[3]	Expres-sion[4]	Reference
pAL 531	A 1630 bp fragment (fig. 4A) was PCR amplified using the control *Nsp* I - forward primer F_cN (acct-GCATGT-gtgagagaatcttca), the control *Pst* I - reverse primer R_cP (cc-CTGCAG-atcgtctacacgcccaa), and as a template the 1677 bp *Afl* II ---> *Dra* III fragment of pAL 413, which contain a functional *attP* and *int* gene of D_{29}, as determined by restriction analysis in pAL 445. The amplified fragment was digested with (*Nsp* I + *Pst* I) and ligated to an 1745 bp (*Nsp* I/*Pst* I) fragment originating from pAL 408, and containing the origin of replication for *E. coli* and the kanamycin gene. This plasmid was generated to confirm the results obtained with pAL 445.	3375	Km	ColE1/D_{29}	(10^9;10^8)		This study
pAL 532	Same as pAL 531 except that the fragment amplified is 1583 bp, and the primers are F_cN and R_1P (ga-CTGCAG-agaaaccccctcttga).	3328	Km	ColE1/D_{29}	(10^9;10^8)		This study

vectors	Definition	M.W (bp)	Marker[1]	Replication Origin[2]	Efficiency[3]	Expres-sion[4]	Reference
pAL 533	Same as pAL 531 except that the fragment amplified is 1531 bp, and the primers are F_cN and R_2P (tt-CTGCAG-tcaggggtaatc).	3276	Km	ColE1/D_{29}	(10^9;10^8)		This study
pAL 534	Same as pAL 531 except that the fragment amplified is 1501 bp, and the primers are F_cN and R_3P (tc-CTGCAG-tcgcctcgctatctcct).	3246	Km	ColE1	(10^9;0)		This study
pAL 535	Same as pAL 531 except that the fragment amplified is 1465 bp, and the primers are F_cN and R_4P (gc-CTGCAG-catctggtacttcatcg).	3210	Km	ColE1	(10^9;0)		This study
pAL 536	Same as pAL 531 except that the fragment amplified is 1592 bp, and the primers are F_1N (gatc-GCATGT-accgcccctcgtctg) and R_cP.	3337	Km	ColE1/D_{29}	(10^9;10^8)		This study
pAL 537	Same as pAL 531 except that the fragment amplified is 1543 bp, and the primers are F_2N (cgcc-GCATGT-aggtcgaaggtagca) and R_cP.	3288	Km	ColE1/D_{29}	(10^9;10^8)		This study

vectors	Definition	M.W (bp)	Marker[1]	Replication Origin[2]	Efficiency[3]	Expres-sion[4]	Reference
pAL 538	Same as pAL 531 except that the fragment amplified is 1512 bp, and the primers are F_3N (ttga-GCATGT-cgggcagcataaatg) and R_cP.	3257	Km	ColE1/D_{29}	(10^9;10^8)		This study
pAL 539	Same as pAL 531 except that the fragment amplified is 1473 bp, and the primers are F_4N (ctct-GCATGT-tcgcggccccctctc) and R_cP.	3218	Km	ColE1/D_{29}?	(10^9;low)		This study
pAL 540	pNEB 193/(*Sap* I + *Hind* III) digested, klenow filled and self ligated. The promoter of LacZ alpha is deleted. The LacZ frame did not change.	2474	Ap	ColE1	(10^9;0)		This study
pAL 541	The pAL 5000 fragment extending from nucleotide 1898 to nucleotide 2898 was PCR amplified using the *Pst* I - forward primer (tca-CTGCAG-cgtcggtgtaga)and the *Xba* I - reverse primer (ctc-TCTAGA-gcctccgagcggat), digested with (*Pst* I + *Xba* I) and cloned into (*Pst* I + *Xba* I) digested pAL 540.	3457	Ap	ColE1	(10^9;0)		This study

vectors	Definition	M.W (bp)	Marker[1]	Replication Origin[2]	Efficiency[3]	Expres-sion[4]	Reference
pAL 542	pAL 519/(*BsaB* I + *Ecl*136 II) digested and self ligated.	2904	Km	ColE1	(10^9;0)		This study
pAL 543	pAL 519/(*BsaB* I + *SgrA* I) digested, klenow filled and self ligated.	2820	Km	ColE1	(10^9;0)		This study
pAL 544	pAL 519/(*Xba* I + *Not* I) digested, klenow filled and self ligated.	3297	Km	ColE1	(10^9;0)		This study
pAL 545	pAL 519/(*Xba* I + *Aat* I) digested, klenow filled and self ligated.	3202	Km	ColE1	(10^9;0)		This study
pAL 546	pAL 519/(*Xba* I + *Age* I) digested, Klenow filled and self ligated.	2768	Km	ColE1	(10^9;0)		This study
pAL 547	pAL 519/(*Xba* I + *Pvu* II) digested, klenow filled and self ligated.	2678	Km	ColE1	(10^9;0)		This study
pAL 548	pAL 519/(*Xba* I + *Kas* I) digested, klenow filled and self ligated.	2618	Km	ColE1	(10^9;0)		This study
pAL 549	pAL 522/(*Xba* I + *Ecl*136 II) digested, klenow filled and self ligated.	2700	Km	ColE1	(10^9;0)		This study

vectors	Definition	M.W (bp)	Marker[1]	Replication Origin[2]	Efficiency[3]	Expres-sion[4]	Reference
pAL 550	pAL 522/(*Xba* I + *Sgr*A I) digested, klenow filled and self ligated.	2616	Km	ColE1	(10^9;0)		This study
pAL 551	pAL 522/(*Acc* III + *Not* I) digested, klenow filled and self ligated.	3306	Km	ColE1	(109^9;0)		This study
pAL 552	pAL 522/(*Acc* III + *Bss*H II) digested, klenow filled and self ligated.	3237	Km	ColE1	(10^9;0)		This study
pAL 553	pAL 522/(*Acc* III + *Age* I) digested, klenow filled and self ligated.	2777	Km	ColE1	(10^9;0)		This study
pAL 554	pAL 522/(*Acc* III + *Pvu* II) digested, klenow filled and self ligated.	2687	Km	ColE1	(10^9;0)		This study
pAL 555	pAl 522/(*Acc* III + *Kas* I) digested, klenow filled and self ligated.	2627	Km	ColE1	(10^9;0)		This study
pAL 556	Same as pAL 541 except that the amplified pAL 5000 fragment extends from nucleotide 1898 to nucleotide 2190, and the *Xba* I - reverse primer is (tcgc-TCTAGA-cgattggacggcatt).	2749	Ap	ColE1	(10^9;0)		This study

vectors	Definition	M.W (bp)	Marker[1]	Replication Origin[2]	Efficiency[3]	Expres-sion[4]	Reference
pAL 557	Same as pAL 541 except that the amplified pAL 5000 fragment extends from nucleotide 1898 to nucleotide 2343, and the *Xba* I - reverse primer is (attgg-TCTAGA-ttgcagacgcgcatt).	2902	Ap	ColE1	$(10^9;0)$		This study
pAL 558	Same as pAL 541 except that the amplified pAL 5000 fragment extends from nucleotide 1898 to nucleotide 2535, and the *Xba* I - reverse primer is (aggg-TCTAGA-gatgtggcgggcatt).	3094	Ap	ColE1	$(10^9;0)$		This study
pAL 559	Same as pAL 541 except that the amplified pAL 5000 fragment extends from nucleotide 1898 to nucleotide 3159, and the *Xba* I - reverse primer is (gcg-TCTAGA-gggcgcggcgttgt).		AP	ColE1	$(10^9;0)$		This study

vectors	Definition	M.W (bp)	Marker[1]	Replication Origin[2]	Efficiency[3]	Expres-sion[4]	Reference
pAL 560	The kanamycin gene promoter (fig. 6) was PCR amplified using the *Pst* I - forward primer (caa-CTGCAG-gttgtgtctcaaaatc) and the *Xba* I - reverse primer (cgtt-TCTAGA-tgaatatggctcata), digested with (*Pst* I + *Xba* I) and cloned into (*Pst* I + *Xba* I) digested pAL 540. The Kanamycin gene promoter replaces the LacZ alpha promoter.	2575	Ap	ColE1	(10^9;0)	LacZ	This study

1 Indicates the marker used to select transformants. Ap, Tc and Km were used at 100 ug/ml, 15 ug/ml and 50 ug/ml respectively.

2 Indicates the remaining functional origin of replication and/or integration in the vector.

3 Indicates the transformation efficiencies obtained by electroporation for *E. coli* and *Mycobacterium* respectively. The efficiencies for D_{29} and pAL 5000 were set arbitrarily at 100% when these vectors are used with their host strains respectively.

4 Indicates ORFs other than the one(s) involved in replication, expressed from pAL 5000 in *E. coli* mini-cells.

5 Abbreviations: MW = Molecular Weight, bp = base pair, Ap = Ampicillin, Tc = Tetracycline, Km = Kanamycin, ORF = Open Reading Frame.

Expression vectors

The expression vectors of the present invention are made by inserting functional promoters from plasmid or chromosomal origin into the cloning vectors which serve as backbones. The expression vectors are tailored to carry and express selected genes in the delivery system. They contain in their structures the genetic information necessary for their auto-replication in the cytoplasm, or their integration into the chromosome of the host. They provide the promoter and the regulatory sequences necessary for 1) gene expression, and if necessary, 2) the secretion of the gene product out of the cytoplasm to the cell membrane structure or to the extracellular environment.

While the kanamycin gene is a preferred selection marker for the present invention, it is also well expressed in a wide range of hosts including *Mycobacterium* and *E. coli* species, and therefore, vectors containing the promoter of this gene can express foreign genes in *E. coli* and *Mycobacterium* strains, respectively. Using conventional PCR techniques, the minimum functional component of this promoter was determined and is given in SEQ ID NO:8 and Fig. 5. The use of a kanamycin promoter to construct *E. coli-Mycobacterium* expression shuttle vectors is reported for the first time.

Another preferred expression vector in the present invention used the promoter of pAL 5000 open reading frame (ORF) 2. An open reading frame (ORF 2) encoding a 60 - 65 KDa protein in *E. coli* minicells was identified in the plasmid pAL 5000. To map the promoter region of this ORF, the 2096 bp fragment containing this open reading frame (SEQ ID NO:9 and Fig. 6) has been isolated. Through restriction endonuclease deletions, cloning, and transformation analysis, a set of hybrid plasmids containing overlapping segments of the 2096 bp fragment were constructed. These recombinant plasmids were electroporated into *E. coli* DS410. Minicells were prepared from transformants and plasmid encoded proteins were analyzed as indicated in

Example 4. The promoter of the ORF was found in the sequence spanning the unique *Bam* HI site in the fragment indicated in Fig. 6.

The products of the invention are administered by injection given intradermal or via other routes (e.g., oral, nasal, subcutaneous, intraperitoneal, 5 intramuscular) in a volume of about 100 microliters containing 10^7 to 10^{11} live or killed cells of recombinant *Mycobacterium*, or the same amount of nonrecombinant *Mycobacterium* cells mixed with, or conjugated to predetermined amounts of the exogenous antigens, the cytokines, and/or the drugs. If the products are being used with patients with active diseases, they 10 should be associated with drug treatments that do not interfere with the live form of the vaccine if it is being used. If the products of the invention are being used separately, they can be administered in any order, at the same or at different sites, and using the same or different routes. The invention takes in consideration that the products are designed to be used in humans or in animals 15 and therefore they must be effective and safe with or without any further pharmaceutical formulation that may add other ingredients.

In summary, the preferred cloning and expression vectors of the present invention comprise an *E. coli-Mycobacterium* shuttle vector which contains the following: an origin of replication for both *E. coli* (*E. coli* replicon) and 20 *Mycobacterium* (pAL 5000 origin of replication), a kanamycin resistance marker, multiple cloning sites, promoters and regulatory sequences for secretion of gene products out of the bacteria and for insertion into the cell membrane, and the attachment site (*att P*) and integrase gene (*int*) of phage D_{29}. Another type of preferred cloning and expression vectors contain all of 25 these elements listed above except the phage D_{29} attachment site and integrase gene. The multiple cloning sites allow cloning of a variety of DNA fragments. The *E. coli* replicon, the pAL 5000 origin of replication, the kanamycin resistance marker, and the D_{29} *attP* site and *int* genes have been mapped and reduced to their minimum functional sizes to maximize the cloning capacity of 30 the vector and to increase the transformation efficiency. A new transformation protocol was developed so that the efficiency with which these vectors

transform *Mycobacterium* strains (10^8 *Mycobacterium* transformants/μg DNA) approaches the transformation efficiency for *E. coli*.

The vaccine system of the present invention has a number of advantages over current vaccines. The major advantage of such a system over current vaccines is the ability to specifically express immunogens that elicit a consistent, protective immune response, i.e., a prolonged activation of T_H-1 cells with concomitant activation of macrophages. Additional advantages include: 1) protective immunogens for more than one intracellular disease can be incorporated into one vaccine, 2) such a genetically engineered vaccine is flexible in that new technology can be easily incorporated to improve the vaccine, and 3) large amounts of immunogen can be synthesized by using a genetically engineered expression vector to induce protective immunity, 4) the *Mycobacterium* itself acts as an adjuvant injected along with the immunogen to induce immunity, 5) the vaccine is naturally targeted to macrophages because the *Mycobacterium* infect these cells, 6) and prolonged immunity will result since a *Mycobacterium* strain remains live within by the macrophages for a long time.

Methodologies for performing various aspects of the present invention are presented below.

DNA, RNA and oligonucleotide primers.

DNA and RNA were extracted and purified at Cytoclonal Pharmaceutics, Inc., Dallas, Texas. The oligonucleotide primers were purchased from National Biosciences Inc., Plymouth, MN., or from Integrated DNA Technologies Inc., Coralville, IA.

Enzymes.

Restriction endonucleases were purchased from United States Biochemical Inc., Cleveland, OH.; New England Biolabs Inc., Beverly, MA.; Promega Inc., Madison, WI.; Stratagene Inc., La Jolla, CA.; MBI Fermantas Inc., Lithuania.; and TaKaRa Biomedicals Inc., Kyoto, Japan. DNA ligase was purchased from Boehringer Mannheim Biochemica Inc., Indianapolis, IN.; Gibco-BRL Inc., Gaithersburg, MD., and New England Biolabs. RNase was

purchased from 5 Prime --------->3 Prime Inc., Boulder, CO. Deoxyribonucleotides and DNA polymerase I (Klenow fragment) were purchased from New England Biolabs. Alkaline phosphatase was purchased from Boehringer Mannheim Biochemica and New England Biolabs. Taq polymerase was purchased from Qiagen Inc., Chatsworth, CA. AMV reverse transcriptase was purchased from Promega Inc. DNase-free RNase and RNase-free DNase were purchased from Ambion Inc., Austin, TX.

Computer software

The computer software Oligo (National Biosciences Inc, Plymouth, MN) and MacVector (Oxford Molecular Group Inc., Campbell, CA) were used to design primers and to analyze nucleic acid and protein sequences.

Preparation of Microorganisms

Bacterial strains and bacteriophages were used from the collection of the Vaccine Program at Cytoclonal Pharmaceutics Inc., Dallas, TX.

Antibiotics ampicillin, kanamycin and tetracycline were purchased from Sigma Chemical Co., Inc. (Saint Louis, MO).

The requirements for *Mycobacterium* species to grow are usually more complex and more diversified than those for *E. coli* strains. Consequently, a general culture medium, hereinafter designated Labidi's medium, has been developed which can support the growth of all *Mycobacterium* species and which contributes to the increased transformation rate of the present invention. The composition of the Labidi's medium per liter contains: about 0.25% proteose peptone No 3; about 0.2% nutrient broth, about 0.075% pyruvic acid, about 0.05% sodium glutamate, about 0.5% albumin fraction V, about 0.7% dextrose, about 0.0004% catalase, about 0.005% oleic acid, $L_{(-)}$ amino-acid complex (about 0.126% alanine, about 0.097% leucine, about 0.089% glycine, about 0.086% valine, about 0.074% arginine, about 0.06% threonine, about 0.059% aspartic acid, about 0.057% serine, about 0.056% proline, about 0.05% glutamic acid, about 0.044% isoleucine, about 0.033% glutamine, about 0.029% phenylalanine, about 0.025% asparagine, about 0.024% lysine, about 0.023% histidine, about 0.021% tyrosine, about 0.02% methionine, about

0.014% tryptophan, and about 0.01% cysteine), about 0.306% Na_2HPO_4, about 0.055% KH_2PO_4, about 0.05% NH_4Cl, about 0.335% NaCl, about 0.0001% $ZnSO_4$, about 0.0001% $CuSO_4$, about 0.0001% $FeCl_3$, about 0.012% $MgSO_4$, about 0.05% Tween 80, and about 0.8% Glycerol (except for *M. bovis*), pH 7.0. A solid form of this medium can be obtained by adding 2.0% agar. Whenever it is necessary, this medium can be supplemented with preferred selection markers and/or with special factors required for the growth of certain species such as mycobactin for *M. paratuberculosis* and hemin X factor for *M. haemophilium*.

For transformation, cultures were grown on Labidi's medium. The cultures were incubated at the appropriate temperature for each strain. Cultures in liquid media were shaken at 150 rpm in a rotatory shaker Gyromax 703 (Amerex Instruments Inc., Hercules, CA).

In growing *Mycobacterium* cells for the vaccine, cultures were grown on protein-free media: [per liter: 6.0% glycerol, 0.75%glucose, 0.4% asparagine, 0.25% Na_2HPO_4, 0.2% citric acid, 0.1% KH_2PO_4, 0.05% ferric ammonium citrate, 0.05% $MgSO_4$, 0.02% Tween 80, 0.0005% $CaCl_2$, 0.0001% $ZnSO_4$, and 0.0001% $CuSO_4$, at a final pH of 7]. Whenever it is necessary, this medium can be supplemented with the required selection markers and/or the growth factors.

For routine culture of *E. coli* strains, the bacteria were cultivated on Luria Broth (LB) medium [per liter of medium: 1% tryptone, 1% NaCl, and 0.5% yeast extract in distilled or deioninzed water]. The solid form of the LB medium was obtained by adding 2.0% agar to the previous formula. When necessary, the medium was supplemented with selection markers. The cultures were incubated at 37°C except if the culture required otherwise. Cultures in liquid media were shaken at 280 rpm in a rotatory shaker Gyromax 703 (Amerex Instruments Inc., Hercules, CA).

Spheroplasts were prepared from *Mycobacterium* cultures as previously described (Labidi, et al. 1984. *Curr. Microbiol.* 11, 235-240). Briefly, the spheroplast solution [for every ml of *Mycobacterium* culture (14

mg of glycine, 60 μg of D-cycloserine, 1 mg of lithium chloride, 200 μg of lysizyme, and 2 mg of EDTA)] was added to the *Mycobacterium* cultures in exponential growth phase, and the incubation was continued for three generations to induce spheroplast formation. The spheroplasts were
5 subsequently collected by centrifugation for 20 min, at 3000 rpm, at 4°C, washed and resuspended in the spheroplast storage solution [per liter, (6.05 gm of tris, 18.5 gm of EDTA, 250 gm of sucrose, and pH adjusted to 7)].

Culturing *Mycobacterium* for Adjuvants

The adjuvants are made of *Mycobacterium* cells harvested after
10 preferably growing the corresponding *Mycobacterium* strains in a liquid protein free medium. The medium is inoculated and incubated at the appropriate temperature. The culture is shaken at 150 rpm for appropriate aeration. The OD_{600} of the culture is monitored daily to determine when the culture reaches stationary phase. At the stationary phase, the number of cells per milliliter is
15 determined through serial dilutions and plating each dilution in triplicate. The culture is sterilely centrifuged for 30 minutes, at 5000 rpm, at 4°C. The pelleted cells are washed twice with ice cold sterile distilled water and pelleted as indicated above. The pellet is re-suspended into pyrogen-free saline (for injection only), to form a suspension of cells ranging from 10^8 - 10^{12} cells per
20 ml. The *Mycobacterium* cell suspension is dispensed into suitable multi-dose vials and used alive, or dead. Preferred methods for killing the *mycobacterium* cells include the use of chemicals, radiation, or intense heat (autoclaving for 30 min, at 15 - 18 psig (104 - 124 kPa) at 120 - 122°C).

DNA and RNA Preparations

25 Plasmid DNA was prepared from *E. coli* strains, as described in prior text (Labidi, et al. 1984. "Plasmid profiles of *Mycobacterium fortuitum* complex isolates," *Curr. Microbiol.* 11, 235-240). 300 μl of spheroplasts were microcentrifuged in another preferred method of the invention. The pellet was resuspended in 360 μl of freshly prepared SI solution [250 mM tris (pH7),
30 50mM EDTA (pH8), 50 mM glucose, and 2.5 μg/ml losozyme]. 240 μl of S II [10% SDS (pH7)] was added and the pellet incubated at 65° C for 15 minutes.

Subsequently, 300 μl of S III [7.5 ammonium acetate (pH 7.5), or 5 M NaCl, or 3 M potassium acetate (pH 5.2), or 3 M sodium acetate (pH 5.2)] was added and the pellet was incubated on ice for 15 minutes and microcentrifuged for 15 minutes at 0° C at 14 Krpm. 2.5 μl of proteinase K (20 mg/ml) was
5 added and incubated at 37° C for 15 minutes. The aqueous phase is extracted three times by adding 250 μl of buffered phenol and 250 μl of chloroform/iso-amyl-alcohol (24:1, v/v) each time. The pellet is vortexed, microcentrifuged for 15 minutes at 14 Krpm at room temperature and the aqueous phase recovered. To the last aqueous phase is added 1 ml of isopropanol, vortex
10 briefly and microcentrifuge for 10 minutes at 14 Krpm at room temperature. The pellet is dried at 37 ° C for 5 minutes and the DNA is dissolved in 50 μl of sterile distilled water.

Total DNA was prepared from *Mycobacterium* strains as described before (Labidi, A., 1986). Another preferred method is to add sterile glass
15 beads to the pellet obtained from 20 ml of spheroplasts. The pellet is vortexed vigorously to have a homogeneous suspension. The suspension is treated with 20 ml of SI, 8 ml of SII, and 14 ml of SIII. The aqueous phase is extracted several times, each time with 10.5 ml of a buffered phenol/chloroform/iso-amyl-alcohol solution. The total DNA is precipitated with 0.6 volume of
20 isopropanol, then dissolved in a cesium chloride gradient and ethidium bromide. The gradient is centrifuged and treated according to techniques that are well established in the art. The plasmid DNA then be separated from the chromosomal DNA.

25 Total RNA was prepared from *E. coli* strains containing the appropriate plasmids and application of a preferred two step protocol. A crude preparation of total RNA was made using the protocol provided with the kit "Ultraspec RNA Isolation System" (Biotex Laboratories Inc., Houston, TX). Since the latter was always contaminated with plasmid DNA, the total RNA was further
30 purified using the protocol provided with the kit "Qiagen Total RNA Isolation"

(Qiagen Inc., Chatsworth, CA). The combination of the two systems efficiently separated total RNA from other contaminating nucleic acids.

Preparation of Electro-competent Cells

Mycobacterium strains can be transformed only through electroporation (Labidi, A., 1986). Therefore, the bacterial cells must be made electro-competent before being subject to this procedure. *E. coli* strains were made electro-competent following the protocol provided with the BRL Cell Porator apparatus (BRL Life Technologies, Gaithersburg, MD).

For *Mycobacterium* strains, a single colony of *Mycobacterium* culture was inoculated into 25 ml of Labidi's medium in a 250 ml screw capped flask. The culture was shaken at 150 rpm at appropriate temperature until the OD_{600} reached 0.7. The culture was checked for contamination by staining. If there was no contamination, a second culture was started by inoculating 50 μl of the first culture into 200 ml of Labidi's medium in a 2000 ml screw capped flask. The culture was shaken at 150 rpm at appropriate temperature until the OD_{600} reached 0.7. The culture was cooled on ice/water for 2 hours, and then the bacterial cells were harvested by centrifugation (7.5 Krpm) for 10 minutes at 4°C. The first pellet was suspended into 31 ml of 3.5% sterile cold glycerol and centrifuged (5 Krpm) for 10 minutes at 4°C. The second pellet was suspended into 12 ml of 7% sterile cold glycerol and centrifuged (3 Krpm) for 10 minutes at 4°C. The third pellet was suspended into 6 ml of 10% sterile cold glycerol and centrifuged (3 Krpm) for 10 minutes at 4°C. The fourth pellet was suspended in a minimum volume of about 2.0 ml of 10.0% sterile cold glycerol, aliquoted into 25.0 μl fractions then used immediately or stored at minus 80°C.

Transformation

The technique of electroporation was applied to *E. coli* and *Mycobacterium* strains. *E. coli* or *Mycobacterium* electro-competent cells (25 μl) were mixed with vector DNA (10 ng in 1 μl), incubated on ice/water for 1 minute then transferred to an electroporation cuvette (0.15 cm gap). The electroporation was conducted with a BRL Cell Porator apparatus Cat. series

1600 equipped with a Voltage Booster Unit Cat. series 1612 (BRL Life Technologies, Gaithersburg, MD). The Voltage Booster Unit was set at a resistance of 4 kiloohms and the Power Supply Unit was set at a capacitance of 330 microfarad, a fast charging speed rate and a low Ohm mode to eliminate extra-resistance. Once the cuvettes were in the safety chamber, the "charge/arm button" was set to "charge", the "up button" was held down until the capacitors voltage displayed in the Power Supply Unit reached 410 volts for *E. coli* and 330 volts for *Mycobacterium* strains. The "charge/arm button" was set to "arm" and the capacitors voltage was allowed to fall down to 400 volts for *E. coli* and to 316 volts for *Mycobacterium* strains. The "trigger button" was pushed to deliver about 2.5 kilovolts for *E. coli* and *Mycobacterium* strains, respectively. These voltage values were displayed on the Voltage Booster Unit. Each voltage value corresponds to 2.5 kilovolts divided by 0.15 cm equals 16.66 kilovolts/cm across the cuvette gap for *E. coli* strains and 1.9 kilovolts divided by 0.15 cm equals 12.66 kilovolts/cm across the cuvette gap for *Mycobacterium* strains. The electroporated cells of each sample were immediately collected with 1 ml of Labidi's medium, transferred to a 15 ml falcon tube with a round bottom (Becton Dickenson Inc., Lincoln Park, NJ) and incubated for one generation time under appropriate temperature and shaking conditions. The cultures were diluted $1:10^2$ to $1:10^5$ into sterile distilled water. The diluted cultures were plated (100 μl) in triplicates on Kanamycin-containing LB and Labidi's media, respectively. The plates were incubated at appropriate temperatures until colonies were visible and easy to count. The numbers counted were averaged and used to calculate transformation efficiencies. A negative and a positive control were included for each species and each experiment.

DNA Sequencing

The DNA was sequenced using a double strand plasmid template and the protocol provided with the kit "Sequenase Version 2.0" (USB, Cleveland, Ohio, USA). The sequence was computer analyzed using MacVector program (Oxford Molecular Group Inc., Campbell, CA).

In Vitro Analysis of Vector's Stability.

Single *Mycobacterium* transformant colonies were grown to saturation on Labidi's medium containing kanamycin (50 μg/ml). The number of generations required to reach saturation is significantly different between slow
5 and rapidly growing mycobacteria. The saturated cultures were diluted to $1{:}10^2$ and to $1{:}10^6$ into antibiotic-free Labidi's medium. The dilution $1{:}10^6$ was immediately plated (0.1 ml per plate) on antibiotic containing Labidi's medium to determine the number of Kanamycin-resistant colonies per ml of culture at the start of the experiment. For calculation purposes, the number of
10 Kanamycin-resistant colonies per ml of this culture was considered to be 100%. Fractions of 0.1 ml of the dilution $1{:}10^2$ were used to inoculate fresh antibiotic-free Labidi's medium and allowed to grow to saturation. This procedure was repeated for six months. Each time the number of Kanamycin-resistant colonies was determined. The proportion of antibiotic-resistant
15 colonies in the culture after the six month period was found to be 96%.

DNA and RNA transactions.

DNA and RNA were treated with the appropriate enzymes respectively, as recommended by the manufacturers.

20 Integration analysis

The integration of vectors containing the attachment site (*attP*) and the integrase gene (*int*) of the Mycobacteriophage D_{29} into the chromosomes of the *Mycobacterium* host strains was analyzed by plasmid DNA preparation and by hybridization using the cloned fragment from the D_{29} genome as a probe.

25 Minicells analysis

Minicells analysis was performed using the *E. coli* DS410, which is a mutant strain of *E. coli* (MinA and MinB). This mutant divides asymmetrically and produces normal cells and small anucleated cells called minicells. The minicells are easily separated from normal cells by their differential
30 sedimentation on a sucrose gradient. If the minicells producing strain contains a multi-copy plasmid, each of its minicells will not have a chromosome but will

carry at least one copy of the plasmid. Since minicells are capable of supporting DNA, RNA and protein synthesis for several hours, they are used as an *in vivo* gene expression system for prokaryotes. The expression product is labeled with S^{35}-methionine and analyzed by protein gel electrophoresis.
5 Nutrient Broth is the medium used in this technique.

Preparation of minicells originated with the preparation of electrocompetent cells of *E. coli* DS410 with the appropriate recombinant plasmids. Each plasmid containing clone is grown overnight in 400 ml NB having the appropriate selection markers. One clone of the non transformed
10 DS410 was grown on 400 ml NB alone to serve as a control.

Three 35 ml sucrose gradients (10-30% w/v) were prepared per clone using M9-mm-S[per liter of medium: 200 gm of sucrose, 100 ml of sterile 10X I- M9-mm, 10 ml of sterile 10 mM $CaCl_2$, and 10 ml of sterile 100 mM Mg SO_4]. The gradients are then placed at minus 70 °C for at least one hour
15 or until the gradients are completely frozen. The gradients are then placed at 4° C overnight to allow the gradient to thaw and to be established. The bacterial cultures are centrifuged for 5 minutes at 2 Krpm at 4° C. The supernatants are then centrifuged for 15 minutes at 8 Krpm at 4° C. Each pellet is subsequently resuspended in 6 ml of M9-mm [per 10X liter of medium:
20 400 mM NaH_2PO_4, 200 mM KH_2PO_4, 80 mM NaCl, and 200 mM NH_4Cl)]. Each 3 ml of cell suspension is layered on top of a sucrose gradient. The gradients are then centrifuged for 18 minutes at 5 Krpm at 4° C. The top one-third of the white transparent minicells band are recovered from each gradient. An equal volume of M9-mm is added to each tube and centrifuged for 10
25 minutes at 10 Krpm at 4° C. Each pellet is subsequently resuspended in 3 ml of M9-mm and the suspension is layered on top of the last gradient and centrifuged for 18 minutes at 5 Krpm at 4° C. The top one-third of the white transparent minicells band is recovered and the optical density is read at 600 nm. The number of cells in the minicells preparation is calculated using the
30 equation of 2 OD_{600}, which equals 10^{10} minicells per ml. Preferably, the level of whole cell contamination is determined in the minicells' preparation. The

minicell suspension is centrifuged for 10 minutes at 10 Krpm at 4° C and resuspended in M9-mm-G [per 100 ml of medium: 30 ml of sterile (100%) glycerol, 1 ml of sterile 10 mM $CaCl_2$, 1 ml of sterile 100 mM $MgSO_4$, and 10 ml of sterile 10X I-M9-mm].

5 The labeling of the plasmid encoded proteins with 5^{35} methionine is achieved by placing 100 μl of minicells in the microcentriuge for 3 minutes at 4° C. The pellet is resuspended in 200 μl of M9-mm and 3 μl of MAM [10.5 gm of methionine assay medium per 100 ml of medium]. The pellet is incubated at 37° C for 90 minutes and 25 μCi of S^{35}-methionine is added. The

10 pellet is incubated at 37° C for 60 minutes. 10 μl of unlabeled MS (0.8 gm of L(-) methionine in 100 ml of distilled water] is added and incubated at 37° C for 5 minutes and microcentrifuged for 3 minutes at room temperature. The pellet is resuspended in 50 μl of BB [per 100 ml of solution, (0.71gm of Na_2HPO_4, 0.27 gm of KH_2PO_4, 0.41 gm of NaC1, and 400 μl of sterile 100

15 mM $MgSO_4$)] and 50 μl of SDS-SB [per 10 ml of solution, (12.5 ml of sterile 1 M tris (pH 6.8), 20 ml of sterile (100%) glycerol, 10 ml of 20% SDS (pH 7.2), 5ml of mercaptoethanol, and 250 μl of 0.4% bromophenol blue)]. The pellet is boiled for 3 minutes, centrifuged, and the top 25 μl of the sample is applied to a 12% SDS-polyacrylamide slab gel.

20 Primer extension analysis

Primer extension analysis was conducted according to the protocol provided with the kit "Primer Extension System" (Promega Inc., Madison, WI).

Ribonuclease protection assay analysis

Ribonuclease protection assay (RPA) was conducted according the

25 protocol provided with the "Ambion HypSpeed RPA Kit" (Ambion Inc. Austin, TX).

DNA amplification by polymerase chain reaction

DNA fragments from the Mycobacteriophage D_{29} genome and *Mycobacterium* plasmid and chromosomal DNA were amplified by polymerase

30 chain reaction using a Progene Programmable Dri-Block Cycler (Techne Inc., Princeton, NJ). The reaction mixture was subject to denaturation (94°C for 3

minutes), followed by 10 cycles of amplification (94°C for 2 minutes, 55°C for 2 minutes, 72°C for 2 minutes), followed by 30 cycles of amplification (94°C for 2 minutes, 63°C for 2 minutes, 72°C for 2 minutes). The programming described above is disclosed for the first time in this report.

5 Examples 1-3 demonstrate the present invention in terms of use of specific antigens in the treatment of various diseases. These examples are illustrative and are not meant to be limiting with regard to the selected antigen and *Mycobacterium* strain nor the application of the *E.coli-Mycobacterium* shuttle.

10 Example 1: Exemplary AIDS Vaccine

If the product is being used to vaccinate against AIDS, *E. coli-Mycobacterium* expression vectors containing genes encoding HIV env, rev, and gag/pol proteins (National Institutes of Health, Bethtesda MD), and genes encoding IL-2, gamma INF and GMCSF (Cytoclonal Pharmaceutics, Inc.,
15 Dallas, Texas) are electroporated into a recipient strain *M. aurum*. The transformants are checked for their plasmid content. A clone containing the expected hybrid plasmid is grown in the protein-free liquid medium. The inoculated medium is incubated at a temperature of 35 to 37°C. The culture is shaken at 150 rpm for appropriate aeration. The OD_{600} of the culture is
20 measured daily, and a growth curve featuring optical densities versus time is established. At the stationary phase, the number of cells per milliliter is determined through serial dilutions (1:10 to 1:10^{10}), and plating in triplicates of each dilution on Labidi's medium. The culture is sterilely centrifuged for 30 minutes, at 5000 rpm, at 4°C. The pelleted cells are washed twice with ice
25 cold sterile distilled water and pelleted as indicated above. The pellet is re-suspended into pyrogen-free saline for injection only, to have a suspension of 10^8 to 10^{12} cells per ml. The *Mycobacterium* cell suspension is dispensed into suitable multi-dose vials. The product is administered by injection given intradermal in a volume of about 100 ul containing 10^7 to 10^{11} cells of
30 recombinant *Mycobacterium*. If a killed form of the vaccine is preferred, the cells can be killed either chemically, by radiation, or by autoclaving for 30 min,

at 15 - 18 psig (104 - 124 kPa) at 120 - 122°C. If a killed form of the vaccine is used, those antigens or cytokines that may be inactivated during the process are added to the product separately, or the recombinant cells are killed by radiation.

5 Example 2: Exemplary Cancer Vaccine

If the product is being used to vaccinate against cancer such as prostate cancer, the gene encoding the cancer antigen such as the prostate cancer antigen PSA (National Institutes of Health, Bethesda, MD), is cloned according to the procedure given in Example 1. The product is prepared and adminstered
10 according to the procedure given in Example 1.

Example 3: Exemplary Allergy Vaccine

If the product is being used for vaccination against allergies such as reactions to the major allergen of birch pollen, the gene encoding the allergen such as the birch pollen allergen BetV1a (Univeristy of Vienna, Austria) is
15 cloned according to the procedure given in Example 1. The product is prepared and adminstered according to the procedure given in Example 1.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Cytoclonal Pharmaceutics, Inc.
(B) STREET: 9000 Harry Hines Blvd, Suite 330
(C) CITY: Dallas
(D) STATE: Texas
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(F) POSTAL CODE (ZIP): 75235
(G) TELEPHONE: (214) 353-2923
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(I) TELEX:

(ii) TITLE OF INVENTION: Mycobacterium Recombinant Vaccines

(iii) NUMBER OF SEQUENCES: 9

(iv) CORRESPONDENCE ADDRESS:
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(B) STREET: 717 N. Harwood, Suite 3400
(C) CITY: Dallas
(D) STATE: Texas
(E) COUNTRY: United States
(F) ZIP: 75201

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 60/042849
(B) FILING DATE: 28-MAR-1997

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Hansen, Eugenia S.
(B) REGISTRATION NUMBER: 31,966
(C) REFERENCE/DOCKET NUMBER: 10365/05602

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 214-981-3300
(B) TELEFAX: 214-981-3400

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 695 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GTTTTTCCAT AGGCTCCGCC CCCCTGACGA GCATCACAAA AATCGACGCT CAAGTCAGAG 60

GTGGCGAAAC CCGACAGGAC TATAAAGATA CCAGGCGTTT CCCCCTGGAA GCTCCCTCGT 120

GCGCTCTCCT GTTCCGACCC TGCCGCTTAC CGGATACCTG TCCGCCTTTC TCCCTTCGGG 180

AAGCGTGGCG CTTTCTCAAT GCTCACGCTG TAGGTATCTC AGTTCGGTGT AGGTCGTTCG 240

CTCCAAGCTG GGCTGTGTGC ACGAACCCCC CGTTCAGCCC GACCGCTGCG CCTTATCCGG 300

TAACTATCGT CTTGAGTCCA ACCCGGTAAG ACACGACTTA TCGCCACTGG CAGCAGCCAC 360

TGGTAACAGG ATTAGCAGAG CGAGGTATGT AGGCGGTGCT ACAGAGTTCT TGAAGTGGTG 420

GCCTAACTAC GGCTACACTA GAAGGACAGT ATTTGGTATC TGCGCTCTGC TGAAGCCAGT 480

TACCTTCGGA AAAAGAGTTG GTAGCTCTTG ATCCGGCAAA CAAACCACCG CTGGTAGCGG 540

TGGTTTTTTT GTTTGCAAGC AGCAGATTAC GCGCAGAAAA AAAGGATCTC AAGAAGATCC 600

TTTGATCTTT TCTACGGGGT CTGACGCTCA GTGGAACGAA AACTCACGTT AAGGGATTTT 660

GGTCATGAGA TTATCAAAAA GGATCTTCAC CTAGA 695

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 932 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GTTGTGTCTC AAAATCTCTG ATGTTACATT GCACAAGATA AAAATATATC ATCATGAACA 60

ATAAAACTGT CTGCTTACAT AAACAGTAAT ACAAGGGGTG TTATGAGCCA TATTCAACGG 120

GAAACGTCTT GCTCGAGGCC GCGATTAAAT TCCAACATGG ATGCTGATTT ATATGGGTAT 180

AAATGGGCTC GCGATAATGT CGGGCAATCA GGTGCGACAA TCTATCGATT GTATGGGAAG 240

CCCGATGCGC CAGAGTTGTT TCTGAAACAT GGCAAAGGTA GCGTTGCCAA TGATGTTACA 300

GATGAGATGG TCAGACTAAA CTGGCTGACG GAATTTATGC CTCTTCCGAC CATCAAGCAT 360

TTTATCCGTA CTCCTGATGA TGCATGGTTA CTCACCACTG CGATCCCCGG GAAAACAGCA 420

TTCCAGGTAT TAGAAGAATA TCCTGATTCA GGTGAAAATA TTGTTGATGC GCTGGCAGTG 480

TTCCTGCGCC GGTTGCATTC GATTCCTGTT TGTAATTGTC CTTTTAACAG CGATCGCGTA 540

TTTCGTCTCG CTCAGGCGCA ATCACGAATG AATAACGGTT TGGTTGATGC GAGTGATTTT 600

GATGACGAGC GTAATGGCTG GCCTGTTGAA CAAGTCTGGA AAGAAATGCA TAAGCTTTTG 660

CCATTCTCAC CGGATTCAGT CGTCACTCAT GGTGATTTCT CACTTGATAA CCTTATTTTT 720

GACGAGGGGA AATTAATAGG TTGTATTGAT GTTGGACGAG TCGGAATCGC AGACCGATAC 780

CAGGATCTTG CCATCCTATG GAACTGCCTC GGTGAGTTTT CTCCTTCATT ACAGAAACGG 840

CTTTTTCAAA AATATGGTAT TGATAATCCT GATATGAATA AATTGCAGTT TCATTTGATG 900

CTCGATGAGT TTTTCTAATC AGAATTGGTT AA 932

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1463 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TGTTCCTCCT GGTTGGTACA GGTGGTTGGG GGTGCTCGGC TGTCGCGGTT GTTCCACCAC 60

CAGGGCTCGA CGGGAGAGCG GGGGAGTGTG CAGTTGTGGG GTGGCCCCTC AGCGAAATAT 120

CTGACTTGGA GCTCGTGTCG GACCATACAC CGGTGATTAA TCGTGGTCTA CTACCAAGCG 180

TGAGCCACGT CGCCGACGAA TTTGAGCAGC TCTGGCTGCC GTACTGGCCG CTGGCAAGCG 240

ACGATCTGCT CGAGGGGATC TACCGCCAAA GCCGCGCGTC GGCCCTAGGC CGCCGGTACA 300

TCGAGGCGAA CCCAACAGCG CTGGCAAACC TGCTGGTCGT GGACGTAGAC CATCCAGACG 360

CAGCGCTCCG AGCGCTCAGC GCCCGGGGGT CCCATCCGCT GCCCAACGCG ATCGTGGGCA 420

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AATACGCGCG GCGTAAGCCG CTCGCATACA TGGCGGCGTG CGCCGAAGGC CTTCGGCGCG 540

CCGTCGACGG CGACCGCAGT TACTCAGGCC TCATGACCAA AAACCCCGGC CACATCGCCT 600

GGGAAACGGA ATGGCTCCAC TCAGATCTCT ACACACTCAG CCACATCGAG GCCGAGCTCG 660

GCGCGAACAT GCCACCGCCG CGCTGGCGTC AGCAGACCAC GTACAAAGCG GCTCCGACGC 720

CGCTAGGGCG GAATTGCGCA CTGTTCGATT CCGTCAGGTT GTGGGCCTAT CGTCCCGCCC 780

TCATGCGGAT CTACCTGCCG ACCCGGAACG TGGACGGACT CGGCCGCGCG ATCTATGCCG 840

AGTGCCACGC GCGAAACGCC GAATTCCCGT GCAACGACGT GTGTCCCGGA CCGCTACCGG 900

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CGTCAGCCAT GGAGGCATTG CTATGAGCGA CGGCTACAGC GACGGCTACA GCGACGGCTA 1140

CAACCGGCAG CCGACTGTCC GCAAAAAGCC GTGACGCGCC GAAGGCGCTC GAATCACCGG 1200

ACTATCCGAA CGCCACGTCG TCCGGCTCGT GGCGCAGGAA CGCAGCGAGT GGCTCGCCGA 1260

GCAGGCTGCA CGCGCGCGAA GCATCCGCGC CTATCACGAC GACGAGGGCC ACTCTTGGCC 1320

GCAAACGGCC AAACATTTCG GGCTGCATCT GGACACCGTT AAGCGACTCG GCTATCGGGC 1380

GAGGAAAGAG CGTGCGGCAG AACAGGAAGC GGCTCAAAAG GCCCACAACG AAGCCGACAA 1440

TCCACCGCTG TTCTAACGCA ATT 1463

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1382 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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GGGTGCTCGG CTGTCGCGGT TGTTCCACCA CCAGGGCTCG ACGGGAGAGC GGGGGAGTGT     60
GCAGTTGTGG GGTGGCCCCT CAGCGAAATA TCTGACTTGG AGCTCGTGTC GGACCATACA    120
CCGGTGATTA ATCGTGGTCT ACTACCAAGC GTGAGCCACG TCGCCGACGA ATTTGAGCAG    180
CTCTGGCTGC CGTACTGGCC GCTGGCAAGC GACGATCTGC TCGAGGGGAT CTACCGCCAA    240
AGCCGCGCGT CGGCCCTAGG CCGCCGGTAC ATCGAGGCGA ACCCAACAGC GCTGGCAAAC    300
CTGCTGGTCG TGGACGTAGA CCATCCAGAC GCAGCGCTCC GAGCGCTCAG CGCCCGGGGG    360
TCCCATCCGC TGCCCAACGC GATCGTGGGC AATCGCGCCA ACGGCCACGC ACACGCAGTG    420
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ATGGCGGCGT GCGCCGAAGG CCTTCGGCGC GCCGTCGACG GCGACCGCAG TTACTCAGGC    540
CTCATGACCA AAAACCCCGG CCACATCGCC TGGGAAACGG AATGGCTCCA CTCAGATCTC    600
TACACACTCA GCCACATCGA GGCCGAGCTC GGCGCGAACA TGCCACCGCC GCGCTGGCGT    660
CAGCAGACCA CGTACAAAGC GGCTCCGACG CCGCTAGGGC GGAATTGCGC ACTGTTCGAT    720
TCCGTCAGGT TGTGGGCCTA TCGTCCCGCC CTCATGCGGA TCTACCTGCC GACCCGGAAC    780
GTGGACGGAC TCGGCCGCGC GATCTATGCC GAGTGCCACG CGCGAAACGC CGAATTCCCG    840
TGCAACGACG TGTGTCCCGG ACCGCTACCG GACAGCGAGG TCCGCGCCAT CGCCAACAGC    900
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GCCACACTCA GTGCGCGCCA GTCGGCCATC TCGCGGAAGG GCGCAGCAGC GCGCACGGCG   1020
GCGAGCACAG TTGCGCGGCG CGCAAAGTCC GCGTCAGCCA TGGAGGCATT GCTATGAGCG   1080
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CGTGACGCGC CGAAGGCGCT CGAATCACCG GACTATCCGA ACGCCACGTC GTCCGGCTCG   1200
TGGCGCAGGA ACGCAGCGAG TGGCTCGCCG AGCAGGCTGC ACGCGCGCGA AGCATCCGCG   1260
CCTATCACGA CGACGAGGGC CACTCTTGGC CGCAAACGGC CAAACATTTC GGGCTGCATC   1320
TGGACACCGT TAAGCGACTC GGCTATCGGG CGAGGAAAGA GCGTGCGGCA GAACAGGAAG   1380
CG                                                                  1382
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(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1631 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GTGAGAGAAT CTTCACTGCA CCAGCTCCGA TCTGGTGTAC CGCCCCTCGT CTGTTGCAGC 60

AGGCGGGGGG CTTTCTTCGT CTGTCGGAGG TCGAAGGTAG CAGATGTGTC GCTGTATCCG 120

GGCAGCATAA ATGCAGGTCA TTAGTGTCGC TCTAAGGTCG CGGCCCCCTC TCGGGGATCC 180

GGTCCTCGGG CTAAAAACCA CCTCTGACCT GTGGAGCGGG CGACGGGAAT CGAACCCGCG 240

TAGCTAGTTT GGAAGTAAGG GGGTCGGCGT GTCACATTCT CCCAGCTCAG ACCCTGTTTT 300

TAGCTCTGAC CCTGTGCGAC CTTGAAGTGG ACAAAAATGC CTGTTCACGG ACACGCAAAG 360

ACGTCTGAAG GTCGCAATAA GGTCGCATTC CGGTAGCCTG TTTCGCATGG CAGCAAGACG 420

GAGAGGATGG GGATCGCTGC GGACCCAGCG CAGCGGTCGA GTGCAAGCGT CGTACGTCAG 480

CCCGATCGAC GGGCAGCGGT ACTTCGGGCC GAGGAACTAC GACAACCGGA TGGACGCCGA 540

AGCGTGGCTC GCGTCTGAGA AGCGGCTGAT CGACAACGAG GAGTGGACCC CGCCGGCCGA 600

GCGCGAGAAG AAGGCTGCGG CGAGTGCCAT CACGGTCGAG GAGTACACCA AGAAGTGGAT 660

CGCCGAGCGA GACCTCGCTG GCGGCACCAA GGATCTCTAC AGCACGCACG CTCGCAAGCG 720

GATCTACCCG GTGTTGGGCG ACACCCCGGT CGCCGAGATG ACCCCCGCCC TTGTCCGGGC 780

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AGTGGCCGGG GAGGTGTTCG AGCACTACCG CGTGGCCGTC TACATCCTGG CGTGGACCAG 1020

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GATGAAGCTC CGCGTGCGCC GGGGCGCGGC CCGCGTCGGC GAGAAGATCG TCGTCGGCAA 1140

CACCAAGACC GTCAGGTCCA AGCGGCCGGT GACCGTGCCG CCTCACGTCG CGGCGATGAT 1200

CCGCGAGCAC ATGGCTGACC GGACGAAGAT GAACAAGGGG CCGGAAGCTC TCCTGGTGAC 1260

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GGCGGCTCAG GCCGGTGCGA CGACCAAGGA GCTGATGGTG CGCCTCGGGC ACACGACTCC 1440

GCGCATGGCG ATGAAGTACC AGATGGCCTC AGCAGCCCGT GACGAGGAGA TAGCGAGGCG 1500

AATGTCGGAG CTGGCAGGGA TTACCCCCTG AAACGCAAAA AGCCCCCCTC CCAAGGCCAT 1560

ACAGCCTCAA GAGGGGGGTT TCTTGTCACT CAGTCCACAC GGTCCATTGG ATCTTGGGCG 1620

TGTAGACGAT C 1631

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1413 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CGGGCAGCAT AAATGCAGGT CATTAGTGTC GCTCTAAGGT CGCGGCCCCC TCTCGGGGAT 60

CCGGTCCTCG GGCTAAAAAC CACCTCTGAC CTGTGGAGCG GGCGACGGGA ATCGAACCCG 120

CGTAGCTAGT TTGGAAGTAA GGGGGTCGGC GTGTCACATT CTCCCAGCTC AGACCCTGTT 180

TTTAGCTCTG ACCCTGTGCG ACCTTGAAGT GGACAAAAAT GCCTGTTCAC GGACACGCAA 240

AGACGTCTGA AGGTCGCAAT AAGGTCGCAT TCCGGTAGCC TGTTTCGCAT GGCAGCAAGA 300

CGGAGAGGAT GGGGATCGCT GCGGACCCAG CGCAGCGGTC GAGTGCAAGC GTCGTACGTC 360

AGCCCGATCG ACGGGCAGCG GTACTTCGGG CCGAGGAACT ACGACAACCG GATGGACGCC 420

GAAGCGTGGC TCGCGTCTGA GAAGCGGCTG ATCGACAACG AGGAGTGGAC CCCGCCGGCC 480

GAGCGCGAGA AGAAGGCTGC GGCGAGTGCC ATCACGGTCG AGGAGTACAC CAAGAAGTGG 540

ATCGCCGAGC GAGACCTCGC TGGCGGCACC AAGGATCTCT ACAGCACGCA CGCTCGCAAG 600

CGGATCTACC CGGTGTTGGG CGACACCCCG GTCGCCGAGA TGACCCCCGC CCTTGTCCGG 660

GCGTGGTGGG CCGGGATGGG TAAGCAGTAC CCGACGGCAC GGCGGCACGC CTACAACGTA 720

CTCCGGGCGG TCATGAATAC CGCTGTAGAG GACAAGCTGG TGTCGGAGAA CCCGTGCCGG 780

ATCGAGCAGA AGGCACCCGC TGAGCGCGAC GTGGAAGCCC TCACACCGGA GGAGCTGGAC 840

GTAGTGGCCG GGGAGGTGTT CGAGCACTAC CGCGTGGCCG TCTACATCCT GGCGTGGACC 900

AGCCTGCGGT TCGGTGAGCT GATCGAGATC CGCCGCAAGG ACATCGTGGA TGACGGCGAG 960

ACGATGAAGC TCCGCGTGCG CCGGGGCGCG GCCCGCGTCG GCGAGAAGAT CGTCGTCGGC 1020

AACACCAAGA CCGTCAGGTC CAAGCGGCCG GTGACCGTGC CGCCTCACGT CGCGGCGATG 1080

ATCCGCGAGC ACATGGCTGA CCGGACGAAG ATGAACAAGG GGCCGGAAGC TCTCCTGGTG 1140

ACCACCACGC GGGGGCAGCG GCTGTCGAAG TCTGCGTTCA CTCGCTCGCT GAAGAAGGGC 1200

TACGCCAAGA TCGGTCGACC GGACCTCCGC ATCCACGACC TCCGGGCCGT GGGAGCCACG 1260

CTGGCGGCTC AGGCCGGTGC GACGACCAAG GAGCTGATGG TGCGCCTCGG GCACACGACT 1320

CCGCGCATGG CGATGAAGTA CCAGATGGCC TCAGCAGCCC GTGACGAGGA GATAGCGAGG 1380

CGAATGTCGG AGCTGGCAGG GATTACCCCC TGA 1413

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1374 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TCGCGGCCCC CTCTCGGGGA TCCGGTCCTC GGGCTAAAAA CCACCTCTGA CCTGTGGAGC 60

GGGCGACGGG AATCGAACCC GCGTAGCTAG TTTGGAAGTA AGGGGGTCGG CGTGTCACAT 120

TCTCCCAGCT CAGACCCTGT TTTTAGCTCT GACCCTGTGC GACCTTGAAG TGGACAAAAA 180

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CTGTTTCGCA TGGCAGCAAG ACGGAGAGGA TGGGGATCGC TGCGGACCCA GCGCAGCGGT 300

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GAGGAGTACA CCAAGAAGTG GATCGCCGAG CGAGACCTCG CTGGCGGCAC CAAGGATCTC 540

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ATGACCCCCG CCCTTGTCCG GGCGTGGTGG GCCGGGATGG GTAAGCAGTA CCCGACGGCA 660

CGGCGGCACG CCTACAACGT ACTCCGGGCG GTCATGAATA CCGCTGTAGA GGACAAGCTG 720

GTGTCGGAGA ACCCGTGCCG GATCGAGCAG AAGGCACCCG CTGAGCGCGA CGTGGAAGCC 780

CTCACACCGG AGGAGCTGGA CGTAGTGGCC GGGGAGGTGT TCGAGCACTA CCGCGTGGCC 840

GTCTACATCC TGGCGTGGAC CAGCCTGCGG TTCGGTGAGC TGATCGAGAT CCGCCGCAAG 900

GACATCGTGG ATGACGGCGA GACGATGAAG CTCCGCGTGC GCCGGGGCGC GGCCCGCGTC 960

GGCGAGAAGA TCGTCGTCGG CAACACCAAG ACCGTCAGGT CCAAGCGGCC GGTGACCGTG 1020

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GGGCCGGAAG CTCTCCTGGT GACCACCACG CGGGGGCAGC GGCTGTCGAA GTCTGCGTTC 1140

ACTCGCTCGC TGAAGAAGGG CTACGCCAAG ATCGGTCGAC CGGACCTCCG CATCCACGAC 1200

CTCCGGGCCG TGGGAGCCAC GCTGGCGGCT CAGGCCGGTG CGACGACCAA GGAGCTGATG 1260

GTGCGCCTCG GGCACACGAC TCCGCGCATG GCGATGAAGT ACCAGATGGC CTCAGCAGCC 1320

CGTGACGAGG AGATAGCGAG GCGAATGTCG GAGCTGGCAG GGATTACCCC CTGA 1374

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 105 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GTTGTGTCTC AAAATCTCTG ATGTTACATT GCACAAGATA AAAATATATC ATCATGAACA 60

ATAAAACTGT CTGCTTACAT AAACAGTAAT ACAAGGGGTG TTATG 105

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2096 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GGTCACCTGC GATCACACCG AGCGTGCAGG TAGCGAAGTC CTCATCACCA CCAGGACGGG 60

CCTGGGCGAT ACCAGCGCCG GGGGCGATCC CGCCAGGAAA TGCCGTCCAA TCGGTGTCCG 120

CGACTGCGGC GGAGCGGACA CTCCGACCAA CACAACAACC AACGTCGTCA TAGCGACGAC 180

GAACCACGAT CGGATGATCC GAATCACTGC GCTGTCCATA CAGGCGGCCA CCCCTCGAAC 240

TCACCAGCTT CAATGCGCGT CTGCAAAGAC TGCCATGGAG CGCTACTCGG GCCGGTCTCA 300

ACGCACTGCT CGAAGAAATC GACAGCGGCC AGTGCACCGA ACTCCTTGTG CTGCTCGGCT 360

TGCAGCTCGG CGCTCCACGT CTTCACCTCG GGCGCGGACA ATTCGACGAC CTTGTTAGCG 420

ATCGACGCAT TGGTCGCCGC AGCAATGCCC GCCACATCCC AGTCCCCTGG ATCGAGGTCG 480

GCGCGGCACA ACAGCTCCGC GATCCGACCC CGATCCAGCG CCTGCCTCAC CACTTTTCGT 540

CGTCGCGGGG CTCACCCGGG TACTGAACCG GATCGCCACT ATCGAAACGG CTACGCGCGG 600

CGGCAGCGGC GGCGCTGGCG GCGGCACGTT CATCACCACC GGACCGGGAA CCAGCGTCGA 660

TTCATCGATG GCCGGCTGAA TCGGCCGGCG TTCGTCGGGC AGCAGGTCCG CGAGCTCGTC 720

GGCATCGATG TACTGCCGGC CGGCGGATCG TCGTCACGCA GAATGTGGGA CACCAGCGCC 780

TTGTCGCGGG CCTCTTCGCC GGTGAGGATC CGCTCGGAGG CGCGGTCGCG GCGCGGCTGT 840

GGCATGTCGG GGCGTGCCGC TCCCCCGGCG CCGCCCATCG GCCCGCCCAT TGGCATTCCG 900

CCCATGCCGC CCATCATTCC TGTGGAGCCA GCTGGCCCGG TCTTCAATGG AGGCAGGCCC 960

GCTGACGGCG ACGTGGAGGC GGTGCGCCCC GAAATCTGGG CCGGATCAAC TCGGCCACCG 1020

GTCACGGTCG GATTGGCGGC CGGTGTTGTC GGTGCGACAA CACCGCCGAC AACGCCGCGC 1080

CCCGCCATCG CCGAACCACG GGGTGGTGGG TGCGTCCGAC CTGCCAGAAT CGTCCCGGCG 1140

TCGCGGCTGC TGCTGAACAC CGCCGAGCCC GCCGCCAGTC GGGAAAGCGC TGGGCATCAT 1200

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GGTCGGGCCG GGGGCCATCG GAGCGGGTGC ACCTGTCGGG GCTGGTGGCG GCGTCAGCGC   1260
CGTCGCCTGC ACCATCGGCC GTGGGCCGCC GACACCTCCG TGGTCGCACC GCCGCCGCCG   1320
ACGATCGTGT CGTCAGCGCC GCCGCCGACG ATGGTGTCGT CCCAACCGTC GCGCGGCTGG   1380
AGGTCGCGGG GCGACCGGAA AATGCCTTTA TCGTGGCCGG ACACCTTGGA ATCGGTGTCC   1440
GGCTCGTCGG GCAGGCCTTC CGTCGCTGAC GTGCACGCGC GCTCCAATCG CTCCAGCGCC   1500
GCCTGGACCT CGGGATCGGC AGCCGTCCCG CCCCGAATGA CCGGGCGGCC GCGGCCGGCC   1560
TCTCCCACCG CACGCAGGGC CGTCGGCGAT TTTCAGCAGG TCGCCGCCCA TTTCCGACAT   1620
CTTTTCCTCG GCGGCCGATC GCCGCACCGG ACCCAATGTC GTCCGGAAAC GGCTCGGCCG   1680
CGATCGACTC CAGCAACGCG GCCATGTCGA TGCGCTCCTG AAACTCGGCC TCGTTGGTCA   1740
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CTTGGACAAG CCAGTTGGAT TCGGAGACAA GCAAATTCAG CCTTAAAAAG GGCGAGGCCC   1920
TGCGGTGGTG GAACACCGCA GGGCCTCTAA CCGCTCGACG CGCTGCACCA ACCAG
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WE CLAIM:

1. A pharmaceutical composition for administration to a human or animal providing a continuous source of a protein of interest into said human or animal upon administration thereto and stimulating the cellular immunity of said human or animal upon administration thereto made according the steps of:

5 (a) cultivating an inoculum of at least one live *Mycobacterium* strain, which is nonpathogenic to said human or animal and capable of sustaining a commensal symbiotic relationship with macrophages in said human or animal, in a liquid culture medium capable of providing sufficient nutrients for growth of said *Mycobacterium* strain at an appropriate temperature to obtain a liquid cell culture;

10 (b) cooling said culture to about 4°C;

(c) centrifuging said cooled culture at about 4°C to obtain a pellet of live *Mycobacterium* cells and a supernatant;

(d) separating said pellet from said supernatant;

(e) washing said pellet by suspending in sterile cold glycerol and

15 centrifuging at about 4°C to obtain electro-competent *Mycobacterium* cells;

(f) mixing said electro-competent *Mycobacterium* cells with integrating vector DNA, said integrating vector DNA comprising a first coding region for a protein of interest cloned under the control of a promoter sequence recognized in *Mycobacterium*, a second coding region for an attachment site and an integrase

20 gene from mycobacteriophage D29, and a third coding region for a selection marker suitable for said *Mycobacterium* strain to form a transformation mixture;

(g) performing electroporation on said transformation mixture to form an electroporated culture comprising transformed *Mycobacterium* cells, said transformed *Mycobacterium* cells having said integrating vector DNA incorporated

25 into the genome of said *Mycobacterium* strain at a transformation efficiency rate of greater than or equal to 10^7 transformants per microgram of vector DNA and being capable of expressing said protein of interest after administration into said human or animal; and

(h) isolating said transformed *Mycobacterium* cells from non-transformed

30 *Mycobacterium* cells by growing said electroporated culture in the presence of a

substance which said selection marker permits transformed *Mycobacterium* cells to be distinguished from non-transformed *Mycobacterium* cells.

2. The composition of claim 1, made by a method further comprising the step of transferring the transformed *Mycobacterium* cell culture of step (h) from said culture medium to a liquid protein-free culture medium and cultivating said transformed *Mycobacterium* cells in said liquid protein-free culture medium under
5 appropriate conditions for vaccine production.

3. The composition of claim 1, wherein said second coding region for said attachment site and integrase gene from said mycobacteriophage D29 essentially consisting of the sequence provided in SEQ ID No:5.

4. The composition of claim 1, wherein said second coding region for said attachment site and integrase gene from said mycobacteriophage D29 essentially consisting of the sequence provided in SEQ ID No:6.

5. The composition of claim 1, wherein said second coding region for said attachment site and integrase gene from said mycobacteriophage D29 essentially consisting of the sequence provided in SEQ ID No:7.

6. The composition of claim 1, wherein said third coding region for said selection marker suitable for said *Mycobacterium* strain comprising a kanamycin selection marker essentially consisting of the sequence provided in SEQ ID No:2.

7. The composition of claim 1, wherein said second coding region for an attachment site and an integrase gene from a mycobacteriophage D29 variant.

8. The composition of claim 1, wherein said *Mycobacterium* strain is selected from the group consisting of *Mycobacterium gastri, Mycobacterium*

5

triviale, Mycobacterium aurum, Mycobacterium thermoresistible, Mycobacterium chitae, Mycobacterium duvalii, Mycobacterium flavescens, Mycobacterium nonchromogenicum, Mycobacterium bovis BCG, Mycobacterium neoaurum, and *Mycobacterium vaccae*.

9. The composition of claim 1, further comprising a cytokine associated with cellular immunity.

10. The composition of claim 1, further comprising a chemotherapeutic agent.

11. A pharmaceutical composition for administration to a human or animal providing a continuous source of a protein of interest into said human or animal upon administration thereto and stimulating the cellular immunity of said human or animal upon administration thereto made according the steps of:

5 (a) cultivating an inoculum of at least one live *Mycobacterium* strain, which is nonpathogenic to said human or animal and capable of sustaining a commensal symbiotic relationship with macrophages in said human or animal, in a liquid culture medium capable of providing sufficient nutrients for growth of said *Mycobacterium* strain at an appropriate temperature to obtain a liquid cell culture;

10 (b) cooling said culture to about 4°C;

(c) centrifuging said cooled culture at about 4°C to obtain a pellet of live *Mycobacterium* cells and a supernatant;

(d) separating said pellet from said supernatant;

(e) washing said pellet by suspending in sterile cold glycerol and

15 centrifuging at about 4°C to obtain electro-competent *Mycobacterium* cells;

(f) mixing said electro-competent *Mycobacterium* cells with integrating vector DNA, said integrating vector DNA comprising a first coding region for a protein of interest cloned under the control of a promoter sequence recognized in

Mycobacterium, a second coding region for the minimal functional component of
20 an attachment site and an integrase gene from mycobacteriophage D29, and a third coding region for a selection marker suitable for said *Mycobacterium* strain to form a transformation mixture;

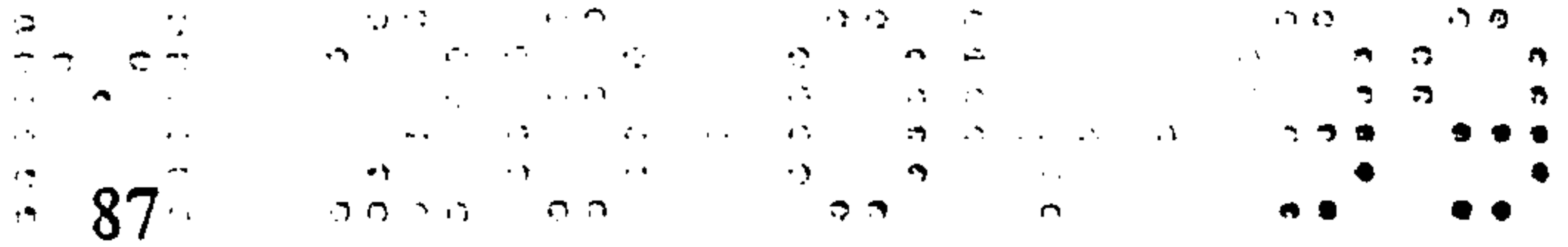
(g) performing electroporation on said transformation mixture to form an electroporated culture comprising transformed *Mycobacterium* cells, said
25 transformed *Mycobacterium* cells having said integrating vector DNA incorporated into the genome of said *Mycobacterium* strain at a transformation efficiency rate of greater than or equal to 10^7 transformants per microgram of vector DNA and being capable of expressing said protein of interest after administration into said human or animal; and

30 (h) isolating said transformed *Mycobacterium* cells from nontransformed *Mycobacterium* cells by growing said electroporated culture in the presence of a substance which said selection marker permits transformed *Mycobacterium* cells to be distinguished from non-transformed *Mycobacterium* cells.

12. The composition of claim 11, made by a method further comprising the step of transferring the transformed *Mycobacterium* cell culture of step (h) from said culture medium to a liquid protein-free culture medium and cultivating said transformed *Mycobacterium* cells in said liquid protein-free culture medium under
5 appropriate conditions for vaccine production.

13. The composition of claim 11, wherein said second coding region for said minimal functional component of said attachment site and integrase gene from said mycobacteriophage D29 essentially consisting of the sequence provided in SEQ ID No:5.

14. The composition of claim 11, wherein said second coding region for said minimal functional component of said attachment site and integrase gene from



said mycobacteriophage D29 essentially consisting of the sequence provided in SEQ ID No:6.

15. The composition of claim 11, wherein said second coding region for said minimal functional component of said attachment site and integrase gene from said mycobacteriophage D29 essentially consisting of the sequence provided in SEQ ID No:7.

16. The composition of claim 11, wherein said second coding region for an attachment site and an integrase gene from a mycobacteriophage D29 variant.

17. The composition of claim 11, wherein said *Mycobacterium* strain is selected from the group consisting of *Mycobacterium gastri, Mycobacterium triviale, Mycobacterium aurum, Mycobacterium thermoresistible, Mycobacterium chitae, Mycobacterium duvalii, Mycobacterium flavescens, Mycobacterium*
5 *nonchromogenicum, Mycobacterium bovis BCG, Mycobacterium neoaurum*, and *Mycobacterium vaccae*.

18. The composition of claim 11, further comprising a cytokine associated with cellular immunity.

19. The composition of claim 11, further comprising a chemotherapeutic agent.

20. A pharmaceutical composition for administration to a human or animal providing a continuous source of a protein of interest into said human or animal upon administration thereto and stimulating the cellular immunity of said human or animal upon administration thereto made according the steps of:
5 (a) cultivating an inoculum of at least one live *Mycobacterium* strain, which is nonpathogenic to said human or animal and capable of sustaining a

commensal symbiotic relationship with macrophages in said human or animal, in a liquid culture medium capable of providing sufficient nutrients for growth of said *Mycobacterium* strain at an appropriate temperature to obtain a liquid cell culture;

10 (b) cooling said culture to about 4°C;

(c) centrifuging said cooled culture at about 4°C to obtain a pellet of live *Mycobacterium* cells and a supernatant;

(d) separating said pellet from said supernatant;

(e) washing said pellet by suspending in sterile cold glycerol and

15 centrifuging at about 4°C to obtain electro-competent *Mycobacterium* cells;

(f) mixing said electro-competent *Mycobacterium* cells with integrating vector DNA, said integrating vector DNA comprising a first coding region for a protein of interest cloned under the control of a promoter recognized in *Mycobacterium*, a second coding region for an attachment site and integrase gene

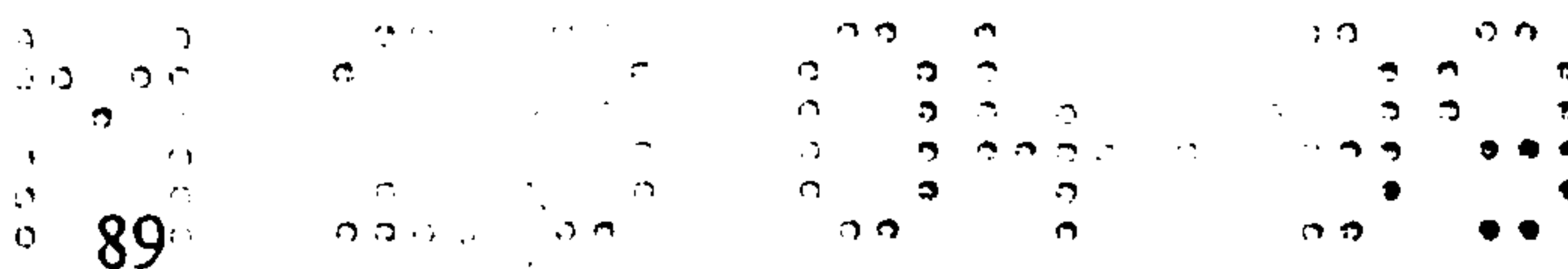
20 from mycobacteriophage D29, and the minimal functional component of a third coding region for a selection marker suitable for said *Mycobacterium* strain to form a transformation mixture;

(g) performing electroporation on said transformation mixture to form an electroporated culture comprising transformed *Mycobacterium* cells, said

25 transformed *Mycobacterium* cells having said integrating vector DNA incorporated into the genome of said *Mycobacterium* strain at a transformation efficiency rate of greater than or equal to 10^7 transformants per microgram of vector DNA and being capable of expressing said protein of interest after administration into said human or animal; and

30 (h) isolating said transformed *Mycobacterium* cells from nontransformed *Mycobacterium* cells by growing said electroporated culture in the presence of a substance which said selection marker permits transformed *Mycobacterium* cells to be distinguished from non-transformed *Mycobacterium* cells.

21. The composition of claim 20, made by a method further comprising the step of transferring the transformed *Mycobacterium* cell culture of step (h) from



said culture medium to a liquid protein-free culture medium and cultivating said transformed *Mycobacterium* cells in said liquid protein-free culture medium under
5 appropriate conditions for vaccine production.

22. The composition of claim 20, wherein said third coding region for said minimal functional component of said selection marker suitable for said *Mycobacterium* strain comprising a kanamycin selection marker essentially consisting of the sequence provided in SEQ ID No:2.

23. The composition of claim 20, wherein said second coding region for an attachment site and an integrase gene from mycobacteriophage D29 variant.

24. The composition of claim 20, wherein said *Mycobacterium* strain is selected from the group consisting of *Mycobacterium gastri, Mycobacterium triviale, Mycobacterium aurum, Mycobacterium thermoresistible, Mycobacterium chitae, Mycobacterium duvalii, Mycobacterium flavescens, Mycobacterium*
5 *nonchromogenicum, Mycobacterium bovis BCG*, *Mycobacterium neoaurum*, and *Mycobacterium vaccae*.

25. The composition of claim 20, further comprising a cytokine associated with cellular immunity.

26. The composition of claim 20, further comprising a chemotherapeutic agent.

27. A pharmaceutical composition for administration to a human or animal providing a continuous source of a protein of interest into said human or animal upon administration thereto and stimulating the cellular immunity of said human or animal upon administration thereto made according the steps of:

5 (a) cultivating an inoculum of at least one live *Mycobacterium* strain, which is nonpathogenic to said human or animal and capable of sustaining a commensal symbiotic relationship with macrophages in said human or animal, in a liquid culture medium capable of providing sufficient nutrients for growth of said *Mycobacterium* strain at an appropriate temperature to obtain a liquid cell culture;

10 (b) cooling said culture to about 4°C;

(c) centrifuging said cooled culture at about 4°C to obtain a pellet of live *Mycobacterium* cells and a supernatant;

(d) separating said pellet from said supernatant;

(e) washing said pellet by suspending in sterile cold glycerol and

15 centrifuging at about 4°C to obtain electro-competent *Mycobacterium* cells;

(f) mixing said electro-competent *Mycobacterium* cells with an extra-chromosomal DNA vector, said extra-chromosomal DNA vector comprising a first coding region for a protein of interest cloned under the control of a promoter sequence recognized in *Mycobacterium*, a second region supporting replication in

20 *Mycobacterium*, and a third coding region for a selection marker suitable for said *Mycobacterium* strain to form a transformation mixture;

(g) performing electroporation on said transformation mixture to form an electroporated culture comprising transformed *Mycobacterium* cells, said *Mycobacterium* cells comprising said extra-chromosomal DNA vector transformed

25 at a transformation efficiency rate of greater than or equal to 10^7 transformants per microgram of vector DNA and being capable of expressing said protein of interest after administration into said human or animal; and

(h) isolating said transformed *Mycobacterium* cells from nontransformed *Mycobacterium* cells by growing said electroporated culture in the presence of a

30 substance which said selection marker permits transformed *Mycobacterium* cells to be distinguished from non-transformed *Mycobacterium* cells.

28. The composition of claim 27, made by a method further comprising the step of transferring the transformed *Mycobacterium* cell culture of step (h) from

said culture medium to a liquid protein-free culture medium and cultivating said transformed *Mycobacterium* cells in said liquid protein-free culture medium under
5 appropriate conditions for vaccine production.

29. The composition of claim 27, wherein said second region supporting replication in *Mycobacterium* essentially consisting of the sequence of an origin of replication of a *Mycobacterium* plasmid provided in SEQ ID No:3.

30. The composition of claim 27, wherein said second region supporting replication in *Mycobacterium* essentially consisting of the sequence of an origin of replication of a *Mycobacterium* plasmid provided in SEQ ID No:4.

31. The composition of claim 27, wherein said coding region for a selection marker suitable for said *Mycobacterium* strain comprising a kanamycin selection marker essentially consisting of the sequence provided in SEQ ID No:2.

32. The composition of claim 27, wherein said *Mycobacterium* plasmid is pAL 5000.

33. The composition of claim 27, wherein said *Mycobacterium* strain is selected from the group consisting of *Mycobacterium gastri, Mycobacterium triviale, Mycobacterium aurum, Mycobacterium thermoresistible, Mycobacterium chitae, Mycobacterium duvalii, Mycobacterium flavescens, Mycobacterium*
5 *nonchromogenicum, Mycobacterium bovis BCG, Mycobacterium neoaurum*, and *Mycobacterium vaccae*.

34. The composition of claim 27, further comprising a cytokine associated with cellular immunity.

35. The composition of claim 27, further comprising a chemotherapeutic agent.

36. A pharmaceutical composition for administration to a human or animal providing a continuous source of a protein of interest into said human or animal upon administration thereto and stimulating the cellular immunity of said human or animal upon administration thereto made according the steps of:

5 (a) cultivating an inoculum of at least one live *Mycobacterium* strain, which is nonpathogenic to said human or animal and capable of sustaining a commensal symbiotic relationship with macrophages in said human or animal, in a liquid culture medium capable of providing sufficient nutrients for growth of said *Mycobacterium* strain at an appropriate temperature to obtain a liquid cell culture;

10 (b) cooling said culture to about 4°C;

(c) centrifuging said cooled culture at about 4°C to obtain a pellet of live *Mycobacterium* cells and a supernatant;

(d) separating said pellet from said supernatant;

(e) washing said pellet by suspending in sterile cold glycerol and

15 centrifuging at about 4°C to obtain electro-competent *Mycobacterium* cells;

(f) mixing said electro-competent *Mycobacterium* cells with an extra-chromosomal DNA vector, said extra-chromosomal DNA vector comprising a first coding region for a protein of interest cloned under the control of a promoter sequence recognized in *Mycobacterium*, a second region for the minimal functional

20 component supporting replication in *Mycobacterium*, and a third coding region for a selection marker suitable for said *Mycobacterium* strain to form a transformation mixture;

(g) performing electroporation on said transformation mixture to form an electroporated culture comprising transformed *Mycobacterium* cells, said

25 *Mycobacterium* cells comprising said extra-chromosomal DNA vector transformed at a transformation efficiency rate of greater than or equal to 10^7 transformants per

microgram of vector DNA and being capable of expressing said protein of interest after administration into said human or animal; and

(h) isolating said transformed *Mycobacterium* cells from nontransformed

30 *Mycobacterium* cells by growing said electroporated culture in the presence of a substance which said selection marker permits transformed *Mycobacterium* cells to be distinguished from non-transformed *Mycobacterium* cells.

37. The composition of claim 36, made by a method further comprising the step of transferring the transformed *Mycobacterium* cell culture of step (h) from said culture medium to a liquid protein-free culture medium and cultivating said transformed *Mycobacterium* cells in said liquid protein-free culture medium under

5 appropriate conditions for vaccine production.

38. The composition of claim 36, wherein said second region for the minimal functional component supporting replication in *Mycobacterium* essentially consisting of the sequence of an origin of replication of a *Mycobacterium* plasmid provided in SEQ ID No:3.

39. The composition of claim 36, wherein said second region for the minimal functional component supporting replication in *Mycobacterium* essentially consisting of the sequence of an origin of replication of a *Mycobacterium* plasmid provided in SEQ ID No:4.

40. The composition of claim 36, wherein said *Mycobacterium* plasmid is pAL 5000.

41. The composition of claim 36, wherein said *Mycobacterium* strain is selected from the group consisting of *Mycobacterium gastri, Mycobacterium triviale, Mycobacterium aurum, Mycobacterium thermoresistible, Mycobacterium chitae, Mycobacterium duvalii, Mycobacterium flavescens, Mycobacterium*

5 *nonchromogenicum, Mycobacterium bovis BCG, Mycobacterium neoaurum*, and *Mycobacterium vaccae*.

42. The composition of claim 36, further comprising a cytokine associated with cellular immunity.

43. The composition of claim 36, further comprising a chemotherapeutic agent.

44. A pharmaceutical composition for administration to a human or animal providing a continuous source of a protein of interest into said human or animal upon administration thereto and stimulating the cellular immunity of said human or animal upon administration thereto made according the steps of:

5 (a) cultivating an inoculum of at least one live *Mycobacterium* strain, which is nonpathogenic to said human or animal and capable of sustaining a commensal symbiotic relationship with macrophages in said human or animal, in a liquid culture medium capable of providing sufficient nutrients for growth of said *Mycobacterium* strain at an appropriate temperature to obtain a liquid cell culture;

10 (b) cooling said culture to about 4°C;

(c) centrifuging said cooled culture at about 4°C to obtain a pellet of live *Mycobacterium* cells and a supernatant;

(d) separating said pellet from said supernatant;

(e) washing said pellet by suspending in sterile cold glycerol and

15 centrifuging at about 4°C to obtain electro-competent *Mycobacterium* cells;

(f) mixing said electro-competent *Mycobacterium* cells with an extra-chromosomal DNA vector, said extra-chromosomal DNA vector comprising a first coding region for a protein of interest cloned under the control of a promoter sequence recognized in *Mycobacterium*, a second region supporting replication in

20 *Mycobacterium*, and a third coding region for the minimal functional component of

a selection marker suitable for said *Mycobacterium* strain to form a transformation mixture;

(g) performing electroporation on said transformation mixture to form an electroporated culture comprising transformed *Mycobacterium* cells, said

25 *Mycobacterium* cells comprising said extra-chromosomal DNA vector transformed at a transformation efficiency rate of greater than or equal to 10^7 transformants per microgram of vector DNA and being capable of expressing said protein of interest after administration into said human or animal; and

(h) isolating said transformed *Mycobacterium* cells from nontransformed

30 *Mycobacterium* cells by growing said electroporated culture in the presence of a substance which said selection marker permits transformed *Mycobacterium* cells to be distinguished from non-transformed *Mycobacterium* cells.

45. The composition of claim 44, made by a method further comprising the step of transferring the transformed *Mycobacterium* cell culture of step (h) from said culture medium to a liquid protein-free culture medium and cultivating said transformed *Mycobacterium* cells in said liquid protein-free culture medium under appropriate conditions for vaccine production.

46. The composition of claim 44, wherein said coding region for the minimal functional component of said selection marker suitable for said *Mycobacterium* strain comprising a kanamycin selection marker essentially consisting of the sequence provided in SEQ ID No:2.

47. The composition of claim 44, wherein said *Mycobacterium* plasmid is pAL 5000.

48. The composition of claim 44, wherein said *Mycobacterium* strain is selected from the group consisting of *Mycobacterium gastri, Mycobacterium triviale, Mycobacterium aurum, Mycobacterium thermoresistible, Mycobacterium*

chitae, Mycobacterium duvalii, Mycobacterium flavescens, Mycobacterium nonchromogenicum, Mycobacterium bovis BCG, Mycobacterium neoaurum, and *Mycobacterium vaccae*.

49. The composition of claim 44, further comprising a cytokine associated with cellular immunity.

50. The composition of claim 44, further comprising a chemotherapeutic agent.

51. A pharmaceutical composition for administration to a human or animal, said composition capable of stimulating the cellular immunity of said human or animal upon administration into said human or animal comprising the steps of:

5 (a) cultivating an inoculum of at least one live *Mycobacterium* strain, which is nonpathogenic to said human or animal and capable of sustaining a commensal symbiotic relationship with macrophages in said human or animal, in a liquid culture medium capable of providing sufficient nutrients for growth of said *Mycobacterium* strain at an appropriate temperature to obtain a liquid cell culture;

10 (b) cooling said culture to about 4°C;

(c) centrifuging said cooled culture to obtain a pellet of live *Mycobacterium* cells and a supernatant;

(d) separating said pellet from said supernatant;

(e) washing said pellet by suspending in sterile cold saline and centrifuging

15 at about 4°C to obtain washed live *Mycobacterium* cells; and

(f) mixing said *live Mycobacterium* cells with an antigen of interest.

52. The composition of claim 51, wherein said *Mycobacterium* strain is selected from the group consisting of *Mycobacterium gastri, Mycobacterium triviale, Mycobacterium aurum, Mycobacterium thermoresistible, Mycobacterium*

chitae, Mycobacterium duvalii, Mycobacterium flavescens, Mycobacterium
5 *nonchromogenicum, Mycobacterium bovis BCG, Mycobacterium neoaurum*, and
Mycobacterium vaccae.

53. The composition of claim 51, further comprising a cytokine associated with cellular immunity.

54. The composition of claim 51, further comprising a chemotherapeutic agent.

55. A pharmaceutical composition for administration to a human or animal, said composition capable of stimulating the cellular immunity of said human or animal upon administration into said human or animal comprising the steps of:

5 (a) cultivating an inoculum of two or more live *Mycobacterium* strains, which are nonpathogenic to said human or animal and capable of sustaining a commensal symbiotic relationship with macrophages in said human or animal, in a liquid culture medium capable of providing sufficient nutrients for growth of said *Mycobacterium* strains at an appropriate temperature to obtain a liquid cell culture;

10 (b) centrifuging said culture to obtain a pellet of live *Mycobacterium* cells and a supernatant;

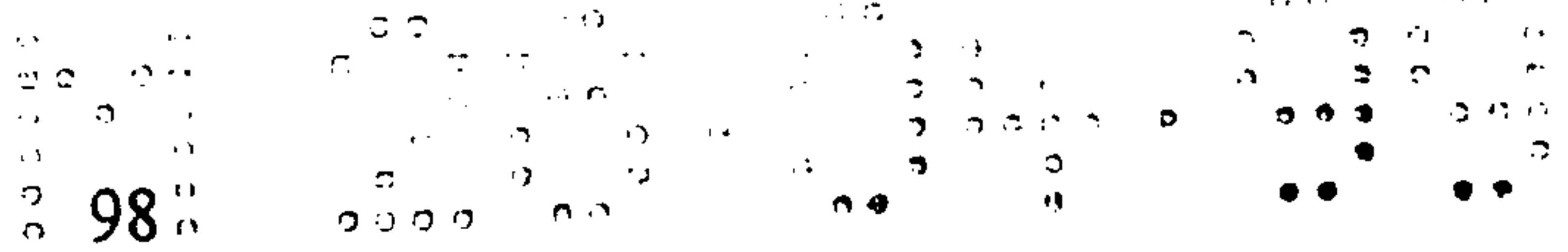
(c) separating said pellet from said supernatant;

(d) washing said pellet and centrifuging to obtain washed *Mycobacterium* cells;

15 (e) killing said washed *Mycobacterium* cells to obtain *Mycobacterium* adjuvant; and

(f) mixing said *Mycobacterium* adjuvant with an antigen of interest.

56. The composition of claim 55, wherein said *Mycobacterium* strains are selected from the group consisting of *Mycobacterium gastri, Mycobacterium*



triviale, Mycobacterium aurum, Mycobacterium thermoresistible, Mycobacterium chitae, Mycobacterium duvalii, Mycobacterium flavescens, Mycobacterium 5 *nonchromogenicum, Mycobacterium bovis BCG, Mycobacterium vaccae*, and *Mycobacterium neoaurum.*

57. The composition of claim 55, further comprising a cytokine associated with cellular immunity.

58. The composition of claim 55, further comprising a chemotherapeutic agent.

59. A shuttle vector comprising a first region supporting replication in *Escherichia coli*, a second coding region for an attachment site and an integrase gene from mycobacteriophage D29, and a third coding region for a selection marker suitable for both *Escherichia coli* and *Mycobacterium*, said first region 5 supporting replication in *Escherichia coli* comprising the minimal functional component of said origin of replication for *Escherichia coli*, wherein said shuttle vector is capable of transforming bacteria at a transformation efficiency rate of greater than or equal to 10^7 transformants per microgram of vector DNA.

60. The shuttle vector according to claim 59, wherein said minimal functional component of said origin of replication for *Escherichia coli* essentially consisting of the sequence provided in SEQ ID NO:1.

61. The shuttle vector according to claim 59, wherein said second coding region for an attachment site and an integrase gene from mycobacteriophage D29 variant.

62. A shuttle vector comprising a first region supporting replication for *Escherichia coli*, a second coding region for an attachment site and an integrase

gene from mycobacteriophage D29, and a third coding region for a selection marker suitable for both *Escherichia coli* and *Mycobacterium*, said second coding region for said attachment site and integrase gene from said mycobacteriophage D29 comprising the minimal functional component of said attachment site and
5 integrase gene from said mycobacteriophage D29, wherein said shuttle vector is capable of transforming bacteria at a transformation efficiency rate of greater than or equal to 10^7 transformants per microgram of vector DNA.

63. The shuttle vector according to claim 62, wherein minimal functional component of said attachment site and integrase gene from said mycobacteriophage D29 essentially consisting of the sequence provided in SEQ ID NO:5.

64. The shuttle vector according to claim 62, wherein minimal functional component of said attachment site and integrase gene from said mycobacteriophage D29 essentially consisting of the sequence provided in SEQ ID NO:6.

65. The shuttle vector according to claim 62, wherein minimal functional component of said attachment site and integrase gene from said mycobacteriophage D29 essentially consisting of the sequence provided in SEQ ID NO:7.

66. The shuttle vector according to claim 62, wherein said second coding region for an attachment site and an integrase gene from a mycobacteriophage D29 variant.

67. A shuttle vector comprising a first region supporting replication in *Escherichia coli*, a second coding region for an attachment site and an integrase gene from mycobacteriophage D29, and a third coding region for a selection marker suitable for both *Escherichia coli* and *Mycobacterium*, said third coding
5 region for said selection marker suitable for both *Escherichia coli* and *Mycobacterium* comprising the minimal functional component of said selection

marker suitable for both *Escherichia coli* and *Mycobacterium*, wherein said shuttle vector is capable of transforming bacteria at a transformation efficiency rate of greater than or equal to 10^7 transformants per microgram of vector DNA.

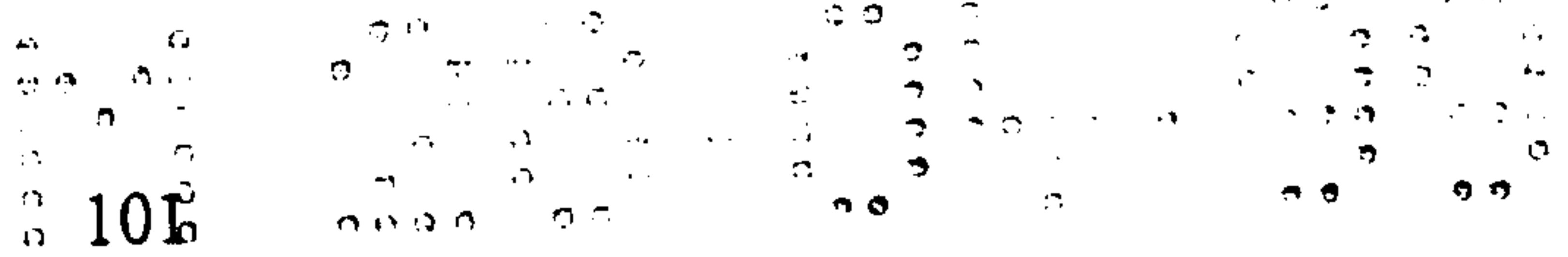
68. The shuttle vector according to claim 67, wherein said minimal functional component of said selection marker suitable for both *Escherichia coli* and *Mycobacterium* comprising a kanamycin selection marker essentially consisting of the sequence provided in SEQ ID NO:2.

69. The shuttle vector according to claim 67, wherein said second coding region for an attachment site and an integrase gene from mycobacteriophage D29 variant.

70. A shuttle vector comprising a first region supporting replication in *Escherichia coli*, a second region supporting replication in *Mycobacterium*, and a third coding region for a selection marker suitable for both *Escherichia coli* and *Mycobacterium*, said first region supporting replication in *Escherichia coli*
5 comprising the minimal functional component of said origin of replication for *Escherichia coli*, wherein said shuttle vector is capable of transforming bacteria at a transformation efficiency rate of greater than or equal to 10^7 transformants per microgram of vector DNA.

71. The shuttle vector according to claim 70, wherein said minimal functional component of said origin of replication for *Escherichia coli* essentially consisting of the sequence provided in SEQ ID No:1.

72. A shuttle vector comprising a first region supporting replication in *Escherichia coli*, a second region supporting replication in *Mycobacterium*, and a third coding region for a selection marker suitable for both *Escherichia coli* and *Mycobacterium*, said second region supporting replication in *Mycobacterium*



5 essentially comprising the minimal functional component of said origin of replication of said *Mycobacterium* plasmid, wherein said shuttle vector is capable of transforming bacteria at a transformation efficiency rate of greater than or equal to 10^7 transformants per microgram of vector DNA.

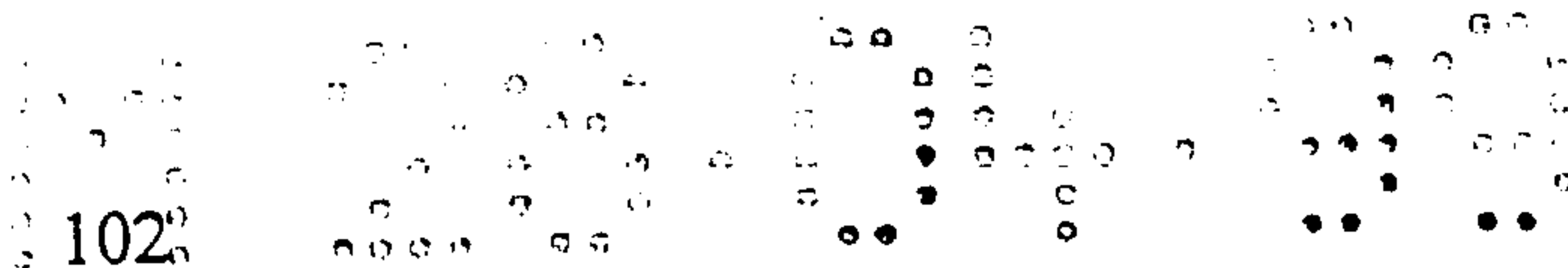
73. The shuttle vector according to claim 72, wherein said minimal functional component of said origin of replication of said *Mycobacterium* plasmid essentially consisting of the sequence provided in SEQ ID No:3.

74. The shuttle vector according to claim 72, wherein said minimal functional component of said origin of replication of said *Mycobacterium* plasmid essentially consisting of the sequence provided in SEQ ID No:4.

75. A shuttle vector comprising a first region supporting replication in *Escherichia coli*, a second region supporting replication in *Mycobacterium*, and a third coding region for a selection marker suitable for both *Escherichia coli* and *Mycobacterium*, said third coding region for a selection marker suitable for both
5 *Escherichia coli* and *Mycobacterium* comprising the minimal functional component of said selection marker suitable for both *Escherichia coli* and *Mycobacterium*, wherein said shuttle vector is capable of transforming bacteria at a transformation efficiency rate of greater than or equal to 10^7 transformants per microgram of vector DNA.

76. The shuttle vector according to claim 75, wherein said minimal functional component of said selection marker suitable for both *Escherichia coli* and *Mycobacterium* comprising a kanamycin selection marker essentially consisting of the sequence provided in SEQ ID NO:2.

77. A vector comprising a first coding region for an attachment site and integrase gene from mycobacteriophage D29 and a second coding region for a



selection marker suitable for *Mycobacterium*, said first coding region for said attachment site and integrase gene from said mycobacteriophage D29 comprising
5 the minimal functional component of said attachment site and integrase gene from said mycobacteriophage D29, wherein said vector is capable of transforming bacteria at a transformation efficiency rate of greater than or equal to 10^7 transformants per microgram of vector DNA.

78. The vector according to claim 77, wherein said minimal functional component of said attachment site and integrase gene from said mycobacteriophage D29 essentially consisting of the sequence provided in SEQ ID No:5.

79. The vector according to claim 77, wherein said minimal functional component of said attachment site and integrase gene from said mycobacteriophage D29 essentially consisting of the sequence provided in SEQ ID No:6.

80. The vector according to claim 77, wherein said minimal functional component of said attachment site and integrase gene from said mycobacteriophage D29 essentially consisting of the sequence provided in SEQ ID No:7.

81. The shuttle vector according to claim 77, wherein said second coding region for an attachment site and an integrase gene from a mycobacteriophage D29 variant.

82. A vector comprising a first coding region for an attachment site and integrase gene from mycobacteriophage D29 and a second coding region for a selection marker suitable for *Mycobacterium*, said second coding region for said selection marker suitable for said *Mycobacterium* strain comprising the minimal
5 functional component of said selection marker suitable for said *Mycobacterium* strain, wherein said shuttle vector is capable of transforming bacteria at a

transformation efficiency rate of greater than or equal to 10^7 transformants per microgram of vector DNA.

83. The vector according to claim 82, wherein said minimal functional component of said selection marker suitable for said *Mycobacterium* strain comprising a kanamycin selection marker essentially consisting of the sequence provided in SEQ ID No:2.

84. The shuttle vector according to claim 82, wherein said second coding region for an attachment site and an integrase gene from mycobacteriophage D29 variant.

85. A vector for carrying and expressing selected genes of a *Mycobacterium* strain comprising a first coding region for an attachment site and integrase gene from mycobacteriophage D29 and a second coding region for a selection marker suitable for *Mycobacterium*, said first coding region for said
5 attachment site and integrase gene from said mycobacteriophage D29 comprising the minimal functional component of said attachment site and integrase gene from said mycobacteriophage D29, wherein said vector is capable of transforming bacteria at a transformation efficiency rate of greater than or equal to 10^7 transformants per microgram of vector DNA.

86. The vector according to claim 85, wherein said minimal functional component of said attachment site and integrase gene from said mycobacteriophage D29 essentially consisting of the sequence provided in SEQ ID No:5.

87. The vector according to claim 85, wherein said minimal functional component of said attachment site and integrase gene from said mycobacteriophage D29 essentially consisting of the sequence provided in SEQ ID No:6.

88. The vector according to claim 85, wherein said minimal functional component of said attachment site and integrase gene from said mycobacteriophage D29 essentially consisting of the sequence provided in SEQ ID No:7.

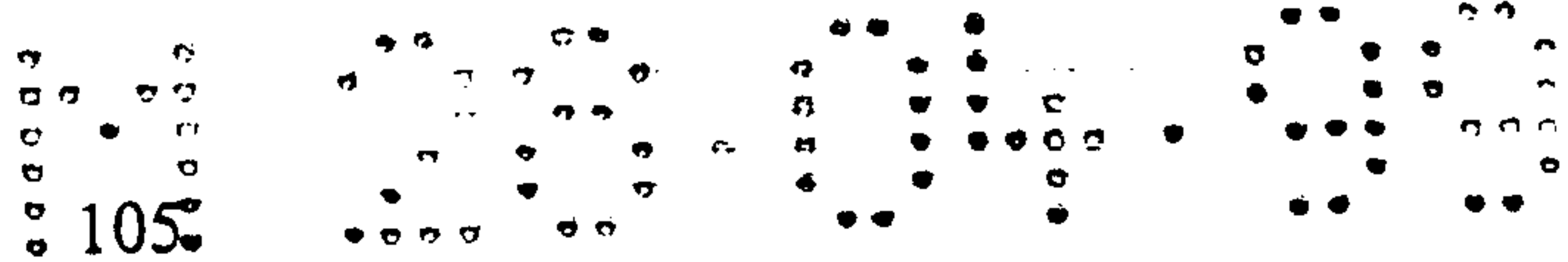
89. The shuttle vector according to claim 85, wherein said second coding region for an attachment site and an integrase gene from a mycobacteriophage D29 variant.

90. A vector for carrying and expressing selected genes of a *Mycobacterium* strain comprising a first coding region for an attachment site and integrase gene from mycobacteriophage D29 and a second coding region for a selection marker suitable for *Mycobacterium*, said second coding region for said
5 selection marker suitable for said *Mycobacterium* strain comprising the minimal functional component of said selection marker suitable for said *Mycobacterium* strain, wherein said vector is capable of transforming bacteria at a transformation efficiency rate of greater than or equal to 10^7 transformants per microgram of vector DNA.

91. The vector according to claim 90, wherein said minimal functional component of said selection marker suitable for said *Mycobacterium* strain comprising a kanamycin selection marker essentially consisting of the sequence provided in SEQ ID No:2.

92. The shuttle vector according to claim 90, wherein said second coding region for an attachment site and an integrase gene from mycobacteriophage D29 variant.

93. A vector comprising a first region supporting replication in *Mycobacterium* and a second coding region for a selection marker suitable for *Mycobacterium*, said first region supporting replication in *Mycobacterium*



comprising the minimal functional component of said origin of replication of said

5 *Mycobacterium* plasmid, wherein said vector is capable of transforming bacteria at a transformation efficiency rate of greater than or equal to 10^7 transformants per microgram of vector DNA.

94. The vector according to claim 93, wherein said minimal functional component of origin of replication of said *Mycobacterium* plasmid essentially consisting of the sequence provided in SEQ ID No:3.

95. The vector according to claim 93, wherein said minimal functional component of origin of replication of said *Mycobacterium* plasmid essentially consisting of the sequence provided in SEQ ID No:4.

96. A vector comprising a first region supporting replication in *Mycobacterium* and a second coding region for a selection marker suitable for *Mycobacterium*, said second coding region for said selection marker suitable for said *Mycobacterium* comprising the minimal functional component of said

5 selection marker suitable for said *Mycobacterium*, wherein said vector is capable of transforming bacteria at a transformation efficiency rate of greater than or equal to 10^7 transformants per microgram of vector DNA.

97. The vector according to claim 96, wherein said minimal functional component of said selection marker suitable for said *Mycobacterium* comprising a kanamycin selection marker essentially consisting of the sequence provided in SEQ ID No:2.

98. A vector comprising a first region supporting replication in *Escherichia coli* and a second coding region for a selection marker suitable for said *Escherichia coli*, said first coding region for an origin of replication for *Escherichia coli*

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comprising the minimal functional component of said origin of replication for said

5 *Escherichia coli.*

99. The vector according to claim 98, wherein said minimal functional component of said origin of replication of said *Escherichia coli* essentially consisting of the sequence provided in SEQ ID No:1.

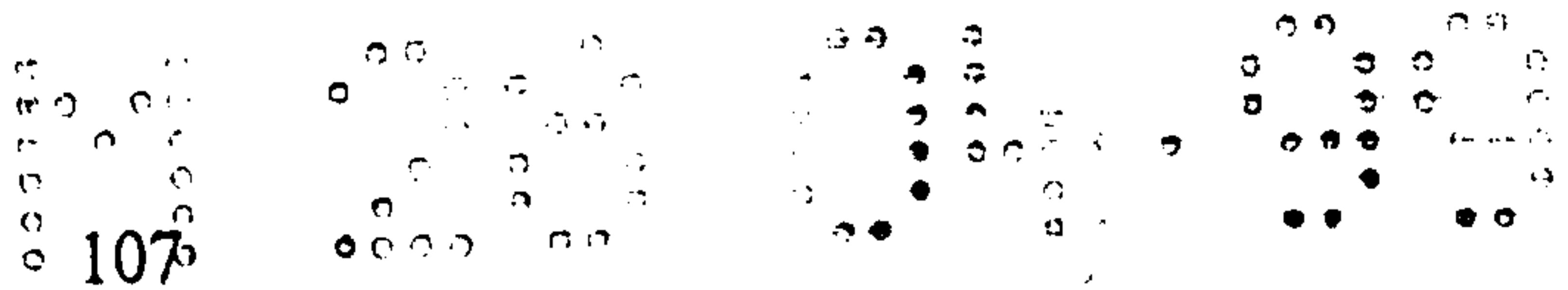
100. A vector comprising a first region supporting replication in *Escherichia coli* and a second coding region for a selection marker suitable for said *Escherichia coli*, said second coding region for a selection marker suitable for said *Escherichia coli* comprising the minimal functional component of said selection

5 marker suitable for said *Escherichia coli*, wherein said vector is capable of transforming bacteria at a transformation efficiency rate of greater than or equal to 10^7 transformants per microgram of vector DNA.

101. The vector according to claim 100, wherein said minimal functional component of said selection marker suitable for said *Escherichia coli* comprising a kanamycin selection marker for said *Escherichia coli* essentially consisting of the sequence provided in SEQ ID No:2.

102. A vector for carrying and expressing selected genes in *Escherichia coli* comprising a first region supporting replication in *Escherichia coli* and a second coding region for a selection marker suitable for said *Escherichia coli*, said first region supporting replication in *Escherichia coli* comprising the minimal

5 functional component of said origin of replication for said *Escherichia coli*, wherein said vector is capable of transforming bacteria at a transformation efficiency rate of greater than or equal to 10^7 transformants per microgram of vector DNA.



103. The vector according to claim 102, wherein said minimal functional component of said origin of replication of said *Escherichia coli* essentially consisting of the sequence provided in SEQ ID No:1.

104. A vector for carrying and expressing selected genes in *Escherichia coli* comprising a first region supporting replication in *Escherichia coli* and a second coding region for a selection marker suitable for said *Escherichia coli* strain, said second coding region for a selection marker suitable for said
5 *Escherichia coli* comprising the minimal functional component of said selection marker suitable for said *Escherichia coli*, wherein said vector is capable of transforming bacteria at a transformation efficiency rate of greater than or equal to 10^7 transformants per microgram of vector DNA.

105. The vector according to claim 104, wherein said minimal functional component of said selection marker suitable for said *Escherichia coli* comprising a kanamycin selection marker for said *Escherichia coli* essentially consisting of the sequence provided in SEQ ID No:2.

106. A method of administering to a human or animal an effective amount of the pharmaceutical composition according to claim 1 to stimulate cellular immunity in said human or animal.

107. The method according to claim 106, further comprising administration of a cytokine associated with cellular immunity.

108. The method according to claim 106, further comprising administration of a chemotherapeutic agent.

109. A method of administering to a human or animal an effective amount of the pharmaceutical composition according to claim 11 to stimulate cellular immunity in said human or animal.

110. The method according to claim 109, further comprising administration of a cytokine associated with cellular immunity.

111. The method according to claim 109, further comprising administration of a chemotherapeutic agent.

112. A method of administering to a human or animal an effective amount of the pharmaceutical composition according to claim 20 to stimulate cellular immunity in said human or animal.

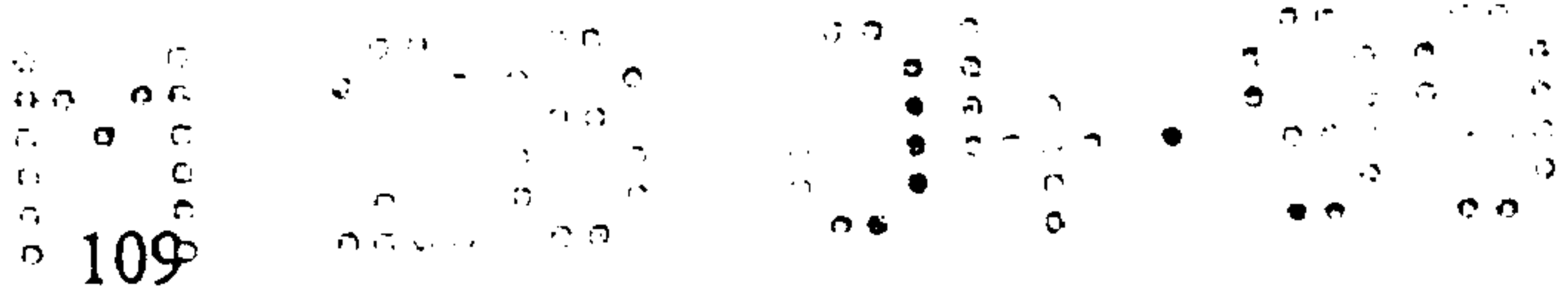
113. The method according to claim 112, further comprising administration of a cytokine associated with cellular immunity.

114. The method according to claim 112, further comprising administration of a chemotherapeutic agent.

115. A method of administering to a human or animal an effective amount of the pharmaceutical composition according to claim 27 to stimulate cellular immunity in said human or animal.

116. The method according to claim 115, further comprising administration of a cytokine associated with cellular immunity.

117. The method according to claim 115, further comprising administration of a chemotherapeutic agent.



118. A method of administering to a human or animal an effective amount of the pharmaceutical composition according to claim 36 to stimulate cellular immunity in said human or animal.

119. The method according to claim 118, further comprising administration of a cytokine associated with cellular immunity.

120. The method according to claim 118, further comprising administration of a chemotherapeutic agent.

121. A method of administering to a human or animal an effective amount of the pharmaceutical composition according to claim 44 to stimulate cellular immunity in said human or animal.

122. The method according to claim 121, further comprising administration of a cytokine associated with cellular immunity.

123. The method according to claim 121, further comprising administration of a chemotherapeutic agent.

124. A method of administering to a human or animal an effective amount of the pharmaceutical composition according to claim 51 to stimulate cellular immunity in said human or animal.

125. The method according to claim 124, further comprising administration of a cytokine associated with cellular immunity.

126. The method according to claim 124, further comprising administration of a chemotherapeutic agent.

127. A method of administering to a human or animal an effective amount of the pharmaceutical composition according to claim 55 to stimulate cellular immunity in said human or animal.

128. The method according to claim 127, further comprising administration of a cytokine associated with cellular immunity.

129. The method according to claim 127, further comprising administration of a chemotherapeutic agent.

130. A culture medium comprising about 0.25% proteose peptone; about 0.2% nutrient broth; about 0.075% pyruvic acid; about 0.05% sodium glutamate; about 0.5% albumin fraction V; about 0.7% dextrose; about 0.0004% catalase; about 0.005% oleic acid; $L_{(-)}$ amino-acid complex (about 0.126% alanine; about
5 0.097% leucine; about 0.089% glycine; about 0.086% valine; about 0.074% arginine; about 0.06% threonine; about 0.059% aspartic acid; about 0.057% serine; about 0.056% proline; about 0.05% glutamic acid; about 0.044% isoleucine; about 0.033% glutamine; 0.029% phenylalanine; about 0.025% asparagine; about 0.024% lysine; about 0.023% histidine; about 0.021% tyrosine; about 0.02%
10 methionine; about 0.014% tryptophan; and about 0.01% cysteine); about 0.306% Na_2HPO_4; about 0.055% KH_2PO_4; about 0.05% NH_4Cl; about 0.335% NaCl; about 0.0001% $ZnSO_4$; about 0.0001% $CuSO_4$; about 0.0001% $FeCl_3$; about 0.012% $MgSO_4$; and about 0.05% Tween 80; wherein the pH of said medium is about 7.

131. The culture medium according to claim 130, further comprising about 0.8% glycerol.

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5′ ....GTT**T**TTCCATAGGCTCCGCCCCCCTGACGAGCATCAC
AAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGA
TACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACC
CTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCG
CTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGC
TCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCC
TTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCG
CCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGC
GGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGG
ACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGA
GTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTT
TTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGAT
CCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGT
TAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGA........... 3′

Fig. 1

5′ .... G**TTGTGT**CTCAAAATCTCTGATGTT**ACATTG**CACAAGATAAAAATATA
TCATCATGAACAATAAAACTGTCTGCTTACATAAACAGTAATA**CAAGGGGT**GTT**ATG**AGCCATAT
TCAACGGGAAACGTCTTGCTCGAGGCCGCGATTAAATTCCAACATGGATG
CTGATTTATATGGGTATAAATGGGCTCGCGATAATGTCGGGCAATCAGGT
GCGACAATCTATCGATTGTATGGGAAGCCCGATGCGCCAGAGTTGTTTCT
GAAACATGGCAAAGGTAGCGTTGCCAATGATGTTACAGATGAGATGGTCA
GACTAAACTGGCTGACGGAATTTATGCCTCTTCCGACCATCAAGCATTTT
ATCCGTACTCCTGATGATGCATGGTTACTCACCACTGCGATCCCCGGGAA
AACAGCATTCCAGGTATTAGAAGAATATCCTGATTCAGGTGAAAATATTG
TTGATGCGCTGGCAGTGTTCCTGCGCCGGTTGCATTCGATTCCTGTTTGT
AATTGTCCTTTTAACAGCGATCGCGTATTTCGTCTCGCTCAGGCGCAATC
ACGAATGAATAACGGTTTGGTTGATGCGAGTGATTTTGATGACGAGCGTA
ATGGCTGGCCTGTTGAACAAGTCTGGAAAGAAATGCATAAGCTTTTGCCA
TTCTCACCGGATTCAGTCGTCACTCATGGTGATTTCTCACTTGATAACCT
TATTTTTGACGAGGGGAAATTAATAGGTTGTATTGATGTTGGACGAGTCG
GAATCGCAGACCGATACCAGGATCTTGCCATCCTATGGAACTGCCTCGGT
GAGTTTTCTCCTTCATTACAGAAACGGCTTTTTCAAAAATATGGTATTGA
TAATCCTGATATGAATAAATTGCAGTTTCATTTGATGCTCGATGAGTTTT
TCTAATCAGAATTGGT**TAA**......................... 3′

Fig. 2

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5′ ....**T**^{4439}**GTTCCTCCTGGTTGGT**^{4455}ACAGGTGGTTGG**G**^{4468}**GGT**
GCTCGGCTGTCGC^{4484}GGTTGTTCCACCACCAGGGCTCGACGGGAGAGCG**G**^{4519}**GGGAGTG**
TGCAGTT^{4533}GTGGGGTGGCCCCTCAGCGAAATATCTGACTTGGAG**C**^{4570}**TCGTGTCGGACC**
AT^{4584}ACA^{4587}CCGGTGATTAATCGTGGTCTACTACCAAGCGTGAGCCACGT
CGCCGACGAATTTGAGCAGCTCTGGCTGCCGTACTGGCCGCTGGCAAGCG
ACGATCTGCTCGAGGGGATCTACCGCCAAAGCCGCGCGTCGGCCCTAGGC
CGCCGGTACATCGAGGCGAACCCAACAGCGCTGGCAAACCTGCTGGTCGT
GGACGTAGACCATCCAGACGCAGCGCTCCGAGCGCTCAGCGCCC^{4822}G^{1}GG
GGTCCCATCCGCTGCCCAACGCGATCGTGGGCAATCGCGCCAACGGCCAC
GCACACGCAGTGTGGGCACTCAACGCCCCTGTTCCACGCACCGAATACGC
GCGGCGTAAGCCGCTCGCATACATGGCGGCGTGCGCCGAAGGCCTTCGGC
GCGCCGTCGACGGCGACCGCAGTTACTCAGGCCTCATGACCAAAAACCCC
GGCCACATCGCCTGGGAAACGGAATGGCTCCACTCAGATCTCTACACACT
CAGCCACATCGAGGCCGAGCTCGGCGCGAACATGCCACCGCCGCGCTGGC
GTCAGCAGACCACGTACAAAGCGGCTCCGACGCCGCTAGGGCGGAATTGC
GCACTGTTCGATTCCGTCAGGTTGTGGCCTATCGTCCCGCCCTCATGCG
GATCTACCTGCCGACCCGGAACGTGGACGGACTCGGCCGCGCGATCTATG
CCGAGTGCCACGCGCGAAACGCCGAATTCCCGTGCAACGACGTGTGTCCC
GGACCGCTACCGGACAGCGAGGTCCGCGCCATCGCCAACAGCATTTGGCG
TTGGATCACAACCAAGTCGCGCATTTGGGCGGACGGGATCGTGGTCTACG
AGGCCACACTCAGTGCGCGCCAGTCGGCCATCTCGCGGAAGGGCGCAGCA
GCGCGCACGGCGGCGAGCACAGTTGCGCGGCGCGCAAAGTCCGCGTCAGC
CATGGAGGCATTGCTATGAGCGACGGCTACAGCGACGGCTACAGCGACGG
CTACAACCGGCAGCCGACTGTCCGCAAAAAGCCGTGACGCGCCGAAGGCG
CTCGAATCACCGGACTATCCGAACGCCACGTCGTCCGGCTCGTGGCGCAG
GAACGCAGCG^{863}**A**^{864}**GTGGCTCGCCGAG**^{877}CAGGCTGCACGCGCGCGAAGCATCCGCGC
CT**A**^{909}**TCACGACGACGAG**^{922}GGCCACTCTTGGCCGCAAACGGCCAAACATTTCGGG**C**^{959}**TGC**
ATCTGGACAC^{972}CGTTAAGCGACTCGGCTATCGGGCGAGGAAAGAGCGTGCGG**C**^{1014}**AGAACA**
GGAAGCG^{1027}GCTCAAAAGGCCCACAACGAAGCCGACAATCCACCGC**T**^{1065}**GTTCTAACGCA**
AT^{1078}T^{1079}..........................3′

Fig. 3A

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5′ ……………………**G^{4468}GGTGCTCGGCTGTCGC**^{4484}GGTTGTTCCACCACCAGGGCTCGA
CGGGAGAGCG**G^{4519}GGGAGTGTGCAGTT**^{4533}GTGGGGTGGCCCCTCAGCGAAATATCTGACTTG
GAG**C^{4570}TCGTGTCGGACCAT**^{4584}ACA^{4587}CCGGTGATTAATCGTGGTCTACT
ACCAAGCGTGAGCCACGTCGCCGACGAATTTGAGCAGCTCTGGCTGCCGT
ACTGGCCGCTGGCAAGCGACGATCTGCTCGAGGGGATCTACCGCCAAAGC
CGCGCGTCGGCCCTAGGCCGCCGGTACATCGAGGCGAACCCAACAGCGCT
GGCAAACCTGCTGGTCGTGGACGTAGACCATCCAGACGCAGCGCTCCGAG
CGCTCAGCGCCC^{4822}G^{1}GGGGTCCCATCCGCTGCCCAACGCGATCGTGGGC
AATCGCGCCAACGGCCACGCACACGCAGTGTGGGCACTCAACGCCCCTGT
TCCACGCACCGAATACGCGCGGCGTAAGCCGCTCGCATACATGGCGGCGT
GCGCCGAAGGCCTTCGGCGCGCCGTCGACGGCGACCGCAGTTACTCAGGC
CTCATGACCAAAAACCCCGGCCACATCGCCTGGGAAACGGAATGGCTCCA
CTCAGATCTCTACACACTCAGCCACATCGAGGCCGAGCTCGGCGCGAACA
TGCCACCGCCGCGCTGGCGTCAGCAGACCACGTACAAAGCGGCTCCGACG
CCGCTAGGGCGGAATTGCGCACTGTTCGATTCCGTCAGGTTGTGGGCCTA
TCGTCCCGCCCTCATGCGGATCTACCTGCCGACCCGGAACGTGGACGGAC
TCGGCCGCGCGATCTATGCCGAGTGCCACGCGCGAAACGCCGAATTCCCG
TGCAACGACGTGTGTCCCGGACCGCTACCGGACAGCGAGGTCCGCGCCAT
CGCCAACAGCATTTGGCGTTGGATCACAACCAAGTCGCGCATTTGGGCGG
ACGGGATCGTGGTCTACGAGGCCACACTCAGTGCGCGCCAGTCGGCCATC
TCGCGGAAGGGCGCAGCAGCGCGCACGGCGGCGAGCACAGTTGCGCGGCG
CGCAAAGTCCGCGTCAGCCATGGAGGCATTGCTATGAGCGACGGCTACAG
CGACGGCTACAGCGACGGCTACAACCGGCAGCCGACTGTCCGCAAAAAGC
CGTGACGCGCCGAAGGCGCTCGAATCACCGGACTATCCGAACGCCACGTC
GTCCGGCTCGTGGCGCAGGAACGCAGCG^{863}**A^{864}GTGGCTCGCCGAG**^{877}CAGG
CTGCACGCGCGCGAAGCATCCGCGCCT**A^{909}TCACGACGACGAG**^{922}GGCCACTCTTGGCCGCAAA
CGGCCAAACATTTCGGG**C^{959}TGCATCTGGACAC**^{972}CGTTAAGCGACTCGGCTATCGGGCGAGGA
AAGAGCGTGCGG**C^{1014}AGAACAGGAAGCG**^{1027}…………………………………………… 3′

Fig. 3B

5/10

5′ ………………………………… **G^{1}TGAGAGAATCTTCA**^{15}CTGCACCAGCTCCGATCTGGTGT
A^{39}CCGCCCCTCGTCTG^{53}TTGCAGCAGGCGGGGGGCTTTCTTCGTCTGTCGG**A^{88}GGTCG
AAGGTAGCA**^{102}GATGTGTCGCTGTATC**C^{119}GGGCAGCATAAATG**^{133}CAGGTCATTAG
TGTCGCTCTAAGG**T^{158}CGCGGCCCCCTCTC**^{172}GGGG^{176}ATCCGGTCCTCGGGCT
AAAAACCACCTCTGACCTGTG**GAGCGGGCGACGGGAATCGAACCCGCGT
AGCTAGTTTGGAAGT**AAGGGGGTCGGCGTGTCACATTCTCCCAGCTCAG
ACCCTGTTTTTAGCTCTGACCCTGTGCGACC**TTGAAG**TGGACAAAAATG
CCTG**TTCAC**GGACACGCAAAGACGTCT**GAAGG**TCGCAATAAGGTCGCAT
TCCGGTAGCCTGTTTCGC**ATG**GCAGCAAGACGGAGAGGATGGGGATCGC
TGCGGACCCAGCGCAGCGGTCGAGTGCAAGCGTCGTACGTCAGCCCGAT
CGACGGGCAGCGGTACTTCGGGCCGAGGAACTACGACAACCGGATGGAC
GCCGAAGCGTGGCTCGCGTCTGAGAAGCGGCTGATCGACAACGAGGAGT
GGACCCCGCCGGCCGAGCGCGAGAAGAAGGCTGCGGCGAGTGCCATCAC
GGTCGAGGAGTACACCAAGAAGTGGATCGCCGAGCGAGACCTCGCTGGC
GGCACCAAGGATCTCTACAGCACGCACGCTCGCAAGCGGATCTACCCGG
TGTTGGGCGACACCCCGGTCGCCGAGATGACCCCCGCCCTTGTCCGGGC
GTGGTGGGCCGGGATGGGTAAGCAGTACCCGACGGCACGGCGGCACGCC
TACAACGTACTCCGGGCGGTCATGAATACCGCTGTAGAGGACAAGCTGG
TGTCGGAGAACCCGTGCCGGATCGAGCAGAAGGCACCCGCTGAGCGCGA
CGTGGAAGCCCTCACACCGGAGGAGCTGGACGTAGTGGCCGGGGAGGTG
TTCGAGCACTACCGCGTGGCCGTCTACATCCTGGCGTGGACCAGCCTGC
GGTTCGGTGAGCTGATCGAGATCCGCCGCAAGGACATCGTGGATGACGG
CGAGACGATGAAGCTCCGCGTGCGCCGGGGCGCGGCCCGCGTCGGCGAG
AAGATCGTCGTCGGCAACACCAAGACCGTCAGGTCCAAGCGGCCGGTGA
CCGTGCCGCCTCACGTCGCGGCGATGATCCGCGAGCACATGGCTGACCG
GACGAAGATGAACAAGGGGCCGGAAGCTCTCCTGGTGACCACCACGCGG
GGGCAGCGGCTGTCGAAGTCTGCGTTCACTCGCTCGCTGAAGAAGGGCT
ACGCCAAGATCGGTCGACCGGACCTCCGCATCCACGACCTCCGGGCCGT
GGGAGCCACGCTGGCGGCTCAGGCCGGTGCGACGACCAAGGAGCTGATG
GTGCGCCTCGGGCACACGACTCCGCGCATGG**C^{1449}GATGAAGTACCAGA
TG**^{1465}GCCTCAGCAGCCCGTGACG**A^{1485}GGAGATAGCGAGGCGA**^{1501}ATGT
CGGAGCTGGCAGG**G^{1519}ATTACCCCCTGA**^{1531}AACGCAAAAAGCCCCCCTCCCAAGG
CCATACAGCC**T^{1567}CAAGAGGGGGGTTTCT**^{1583}TGTCACTCAGTCCACACGGTCCATTGGA
TC**T^{1614}TGGGCGTGTAGACGAT**^{1630}C^{1631}…………………………………… 3′

Fig. 4A

5′ ………………… **C**^{119}**GGGCAGCATAAATG**^{133}CAGGTCATTAGTGTCGCTCTAAGG**T**^{158}**C**
GCGGCCCCCTCTC^{172}GGGG^{176}ATCCGGTCCTCGGGCTAAAAACCACCTCT
GACCTGTG**GAGCGGGCGACGGGAATCGAACCCGCGTAGCTAGTTTGGAA**
GTAAGGGGGTCGGCGTGTCACATTCTCCCAGCTCAGACCCTGTTTTTAG
CTCTGACCCTGTGCGACC**TTGAAG**TGGACAAAAATGCCTG**TTCAC**GGAC
ACGCAAAGACGTCT**GAAGG**TCGCAATAAGGTCGCATTCCGGTAGCCTGT
TTCGC**ATG**GCAGCAAGACGGAGAGGATGGGGATCGCTGCGGACCCAGCG
CAGCGGTCGAGTGCAAGCGTCGTACGTCAGCCCGATCGACGGGCAGCGG
TACTTCGGGCCGAGGAACTACGACAACCGGATGGACGCCGAAGCGTGGC
TCGCGTCTGAGAAGCGGCTGATCGACAACGAGGAGTGGACCCCGCCGGC
CGAGCGCGAGAAGAAGGCTGCGGCGAGTGCCATCACGGTCGAGGAGTAC
ACCAAGAAGTGGATCGCCGAGCGAGACCTCGCTGGCGGCACCAAGGATC
TCTACAGCACGCACGCTCGCAAGCGGATCTACCCGGTGTTGGGCGACAC
CCCGGTCGCCGAGATGACCCCCGCCCTTGTCCGGGCGTGGTGGGCCGGG
ATGGGTAAGCAGTACCCGACGGCACGGCGGCACGCCTACAACGTACTCC
GGGCGGTCATGAATACCGCTGTAGAGGACAAGCTGGTGTCGGAGAACCC
GTGCCGGATCGAGCAGAAGGCACCCGCTGAGCGCGACGTGGAAGCCCTC
ACACCGGAGGAGCTGGACGTAGTGGCCGGGGAGGTGTTCGAGCACTACC
GCGTGGCCGTCTACATCCTGGCGTGGACCAGCCTGCGGTTCGGTGAGCT
GATCGAGATCCGCCGCAAGGACATCGTGGATGACGGCGAGACGATGAAG
CTCCGCGTGCGCCGGGGCGCGGCCCGCGTCGGCGAGAAGATCGTCGTCG
GCAACACCAAGACCGTCAGGTCCAAGCGGCCGGTGACCGTGCCGCCTCA
CGTCGCGGCGATGATCCGCGAGCACATGGCTGACCGGACGAAGATGAAC
AAGGGGCCGGAAGCTCTCCTGGTGACCACCACGCGGGGGCAGCGGCTGT
CGAAGTCTGCGTTCACTCGCTCGCTGAAGAAGGGCTACGCCAAGATCGG
TCGACCGGACCTCCGCATCCACGACCTCCGGGCCGTGGGAGCCACGCTG
GCGGCTCAGGCCGGTGCGACGACCAAGGAGCTGATGGTGCGCCTCGGGC
ACACGACTCCGCGCATGG**C**^{1449}**GATGAAGTACCAGATG**^{1465}GCCTCAGCA
GCCCGTGACG**A**^{1485}**GGAGATAGCGAGGCGA**^{1501}ATGTCGGAGCTGGCAGG
G^{1519}**ATTACCCCCTGA**^{1531} ……………………… 3′

Fig. 4B

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5'...................................**T^{158}CGCGGCCCCCTCTC**^{172}GGGG^{176}ATCCGGTCC
TCGGGCTAAAAACCACCTCTGACCTGTG**GAGCGGGCGACGGGAATCGAA
CCCGCGTAGCTAGTTTGGAAGT**AAGGGGTCGGCGTGTCACATTCTCCC
AGCTCAGACCCTGTTTTTAGCTCTGACCCTGTGCGACC**TTGAAG**TGGAC
AAAAATGCCTG**TTCAC**GGACACGCAAAGACGTCT**GAAGG**TCGCAATAAG
GTCGCATTCCGGTAGCCTGTTTCGC**ATG**GCAGCAAGACGGAGAGGATGG
GGATCGCTGCGGACCCAGCGCAGCGGTCGAGTGCAAGCGTCGTACGTCA
GCCCGATCGACGGGCAGCGGTACTTCGGGCCGAGGAACTACGACAACCG
GATGGACGCCGAAGCGTGGCTCGCGTCTGAGAAGCGGCTGATCGACAAC
GAGGAGTGGACCCCGCCGGCCGAGCGCGAGAAGAAGGCTGCGGCGAGTG
CCATCACGGTCGAGGAGTACACCAAGAAGTGGATCGCCGAGCGAGACCT
CGCTGGCGGCACCAAGGATCTCTACAGCACGCACGCTCGCAAGCGGATC
TACCCGGTGTTGGGCGACACCCCGGTCGCCGAGATGACCCCCGCCCTTG
TCCGGGCGTGGTGGGCCGGGATGGGTAAGCAGTACCCGACGGCACGGCG
GCACGCCTACAACGTACTCCGGGCGGTCATGAATACCGCTGTAGAGGAC
AAGCTGGTGTCGGAGAACCCGTGCCGGATCGAGCAGAAGGCACCCGCTG
AGCGCGACGTGGAAGCCCTCACACCGGAGGAGCTGGACGTAGTGGCCGG
GGAGGTGTTCGAGCACTACCGCGTGGCCGTCTACATCCTGGCGTGGACC
AGCCTGCGGTTCGGTGAGCTGATCGAGATCCGCCGCAAGGACATCGTGG
ATGACGGCGAGACGATGAAGCTCCGCGTGCGCCGGGGCGCGGCCCGCGT
CGGCGAGAAGATCGTCGTCGGCAACACCAAGACCGTCAGGTCCAAGCGG
CCGGTGACCGTGCCGCCTCACGTCGCGGCGATGATCCGCGAGCACATGG
CTGACCGGACGAAGATGAACAAGGGGCCGGAAGCTCTCCTGGTGACCAC
CACGCGGGGGCAGCGGCTGTCGAAGTCTGCGTTCACTCGCTCGCTGAAG
AAGGGCTACGCCAAGATCGGTCGACCGGACCTCCGCATCCACGACCTCC
GGGCCGTGGGAGCCACGCTGGCGGCTCAGGCCGGTGCGACGACCAAGGA
GCTGATGGTGCGCCTCGGGCACACGACTCCGCGCATGG**C^{1449}GATGAAG
TACCAGATG**^{1465}GCCTCAGCAGCCCGTGACG**A^{1485}GGAGATAGCGAGGCG
A**^{1501}ATGTCGGAGCTGGCAGG**G^{1519}ATTACCCCCTGA**^{1531}................. 3'

Fig. 4C

5'...GTTGTGTCTCAAAAT
CTCTGATGTTACATTGCACAAGATAAAAATATATCATCATGAA
CAATAAAACTGTCTGCTTACATAAACAGTAATACAAGGGGTG
TTATG.. **3'**

Fig. 5

5′ ....G^{2078}GTCACCTGCGATCACACCGAGCGTGCAGGTAGCGAAGTCC
TCATCACCACCAGGACGGGCCTGGGCGATACCAGCGCCGGGGGCGATCCCGCCAGGAAA
TGCCGTCCAATCGGTGTCCGCGACTGCGGCGGAGCGGACACTCCGACCAACACAACAAC
CAACGTCGTCATAGCGACGACGAACCACGATCGGATGATCCGAATCACTGCGCTGTCCA
TACAGGCGGCCACCCCTCGAACTCACCAGCTTCAATGCGCGTCTGCAAAGACTGCCATG
GAGCGCTACTCGGGCCGGTCTCAACGCACTGCTCGAAGAAATCGACAGCGGCCAGTGCA
CCGAACTCCTTGTGCTGCTCGGCTTGCAGCTCGGCGCTCCACGTCTTCACCTCGGGCGC
GGACAATTCGACGACCTTGTTAGCGATCGACGCATTGGTCGCCGCAGCAATGCCCGCCA
CATCCCAGTCCCCTGGATCGAGGTCGGCGCGGCACAACAGCTCCGCGATCCGACCCCGA
TCCAGCGCCTGCCTCACCACTTTTCGTCGTCGCGGGGCTCACCCGGGTACTGAACCGGA
TCGCCACTATCGAAACGGCTACGCGCGGCGGCAGCGGCGGCGCTGGCGGCGGCACGTTC
ATCACCACCGGACCGGGAACCAGCGTCGATTCATCGATGGCCGGCTGAATCGGCCGGCG
TTCGTCGGGCAGCAGGTCCGCGAGCTCGTCGGCATCGATGTACTGCCGGCCGGCGGATC
GTCGTCACGCAGAATGTGGGACACCAGCGCCTTGTCGCGGGCCTCTTCGCCGGTGA**GGA**
TCCGCTCGGAGGCGCGGTCGCGGCGCGGCTGTGGCATGTCGGGGCGTGCCGCTCCCCCG
GCGCCGCCCATCGGCCCGCCCATTGGCATTCCGCCCATGCCGCCCATCATTCCTGTGGA
GCCAGCTGGCCCGGTCTTCAATGGAGGCAGGCCCGCTGACGGCGACGTGGAGGCGGTGC
GCCCCGAAATCTGGGCCGGATCAACTCGGCCACCGGTCACGGTCGGATTGGCGGCCGGT
GTTGTCGGTGCGACAACACCGCCGACAACGCCGCGCCCCGCCATCGCCGAACCACGGGG
TGGTGGGTGCGTCCGACCTGCCAGAATCGTCCCGGCGTCGCGGCTGCTGCTGAACACCG
CCGAGCCCGCCGCCAGTCGGGAAAGCGCTGGGCATCATGGTCGGGCCGGGGGCCATCGG
AGCGGGTGCACCTGTCGGGGCTGGTGGCGGCGTCAGCGCCGTCGCCTGCACCATCGGCC
GTGGGCCGCCGACACCTCCGTGGTCGCACCGCCGCCGCCGACGATCGTGTCGTCAGCGC
CGCCGCCGACGATGGTGTCGTCCCAACCGTCGCGCGGCTGGAGGTCGCGGGGCGACCGG
AAAATGCCTTTATCGTGGCCGGACACCTTGGAATCGGTGTCCGGCTCGTCGGGCAGGCC
TTCCGTCGCTGACGTGCACGCGCGCTCCAATCGCTCCAGCGCCGCCTGGACCTCGGGAT
CGGCAGCCGTCCCGCCCCGAATGACCGGGCGGCCGCGGCCGGCCTCTCCCACCGCACGC
AGGGCCGTCGGCGATTTTCAGCAGGTCGCCGCCCATTTCCGACATCTTTTCCTCGGCGG
CCGATCGCCGCACCGGACCCAATGTCGTCCGGAAACGGCTCGGCCGCGATCGACTCCAG
CAACGCGGCCATGTCGATGCGCTCCTGAAACTCGGCCTCGTTGGTCAGCGAATCGCCGT
CATAACGGATGGCGCCCGGGCCGCCGCGATATCGAGCCGAGAACGTTATCGAAGTTG
GTCATGTGTAATCCCCTCGTTTGAACTTTGGATTAAGCGTAGATACACCCTTGGACAAG
CCAGTTGGATTCGGAGACAAGCAAATTCAGCCTTAAAAAGGGCGAGGCCCTGCGGTGGT
GGAACACCGCAGGGCCTCTAACCGCTCGACGCGCTGCACCAACCAGCCCGCGAACGGCT
GGCAGCCAGCGTAAGGCGCGGCTCATCGGGCGGCGTTCGCCACGATGTCCTGCACTTCG
AGCCAAGCCTCGAACACCTGCTGGTGTGCACGACTCACCCGGTTGTTGA^{4173}.............. 3′

Fig. 6

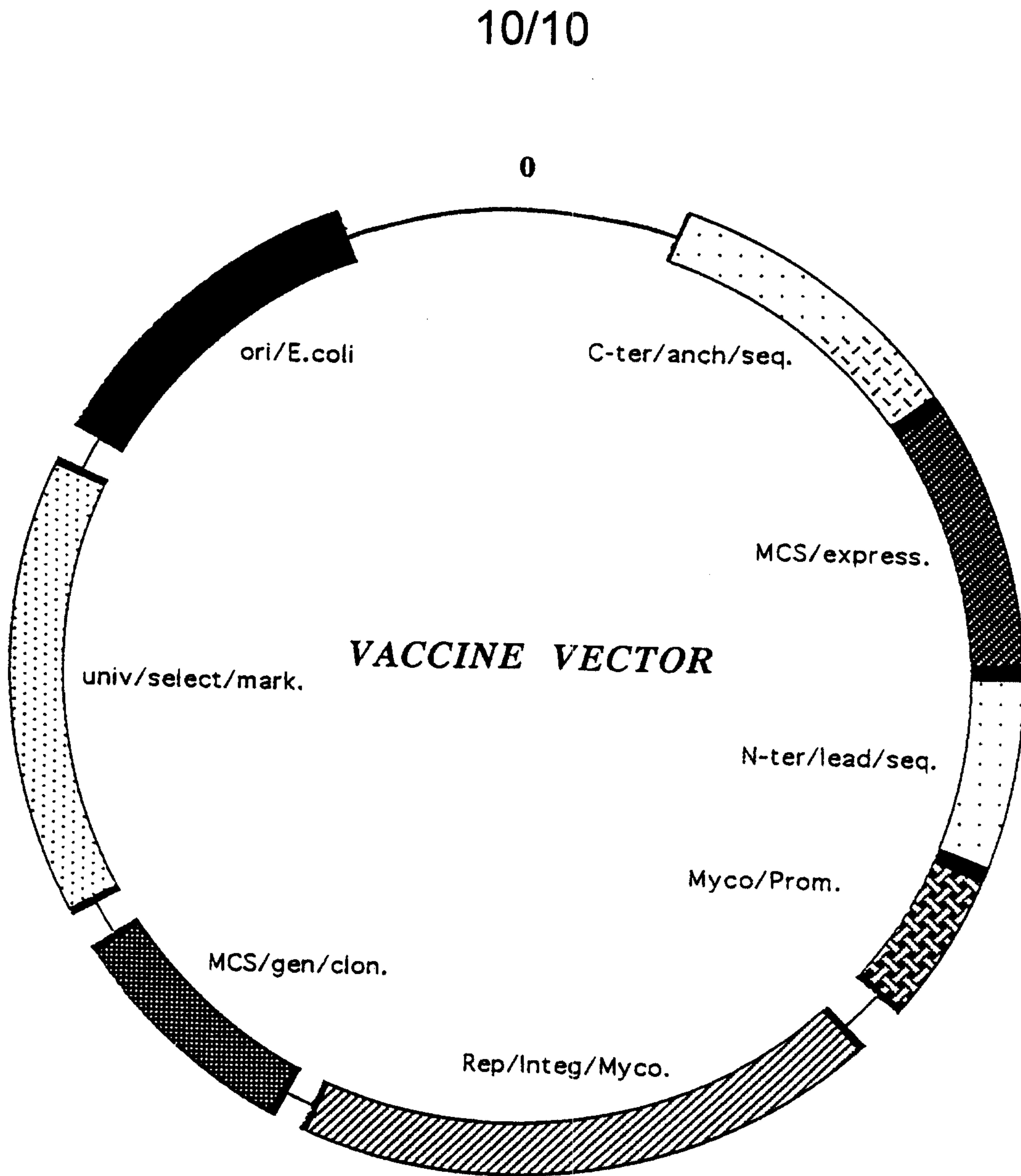

Fig. 7