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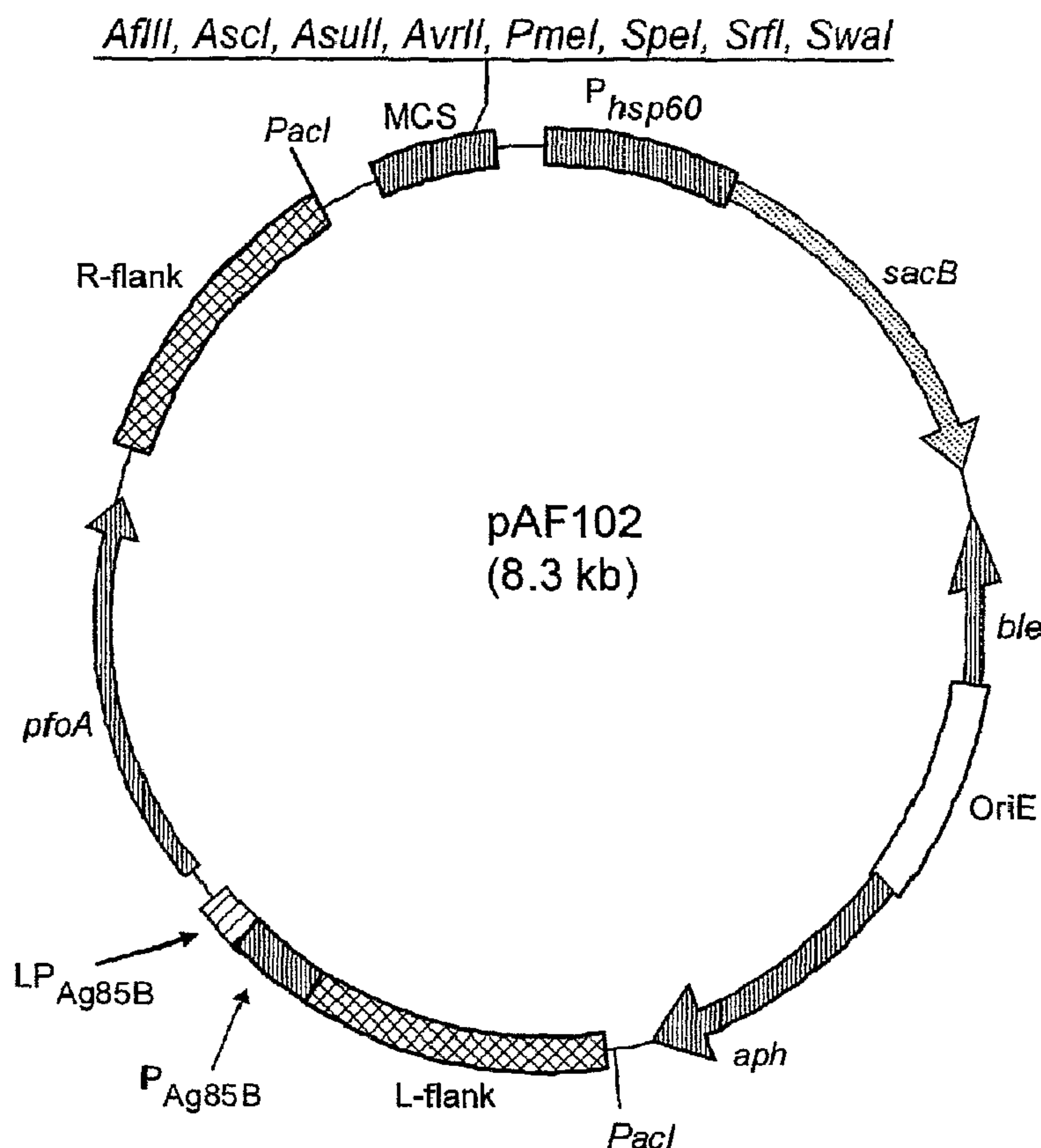
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(54) Titre : SOUCHES DE BCG RECOMBINEES POSSEDANT UNE CAPACITE ACCRUE A S'ECHAPPER DE L'ENDOSOME

(54) Title: RECOMBINANT BCG STRAINS WITH ENHANCED ABILITY TO ESCAPE THE ENDOSOME



(57) Abrégé/Abstract:

Mycobacterium strains that have an enhanced ability to elicit a Histocompatibility-Class I-restricted CD8⁺ T cell immune response are provided. The Mycobacterium strains are genetically engineered to express an endosomalytic protein that is active at neutral pH

(57) **Abrégé(suite)/Abstract(continued):**

(e.g. Perfringolysin O), permitting escape of the Mycobacterium from endosomes into the cytoplasm of the cell. The invention also provides vaccine preparations containing the Mycobacterium strains.

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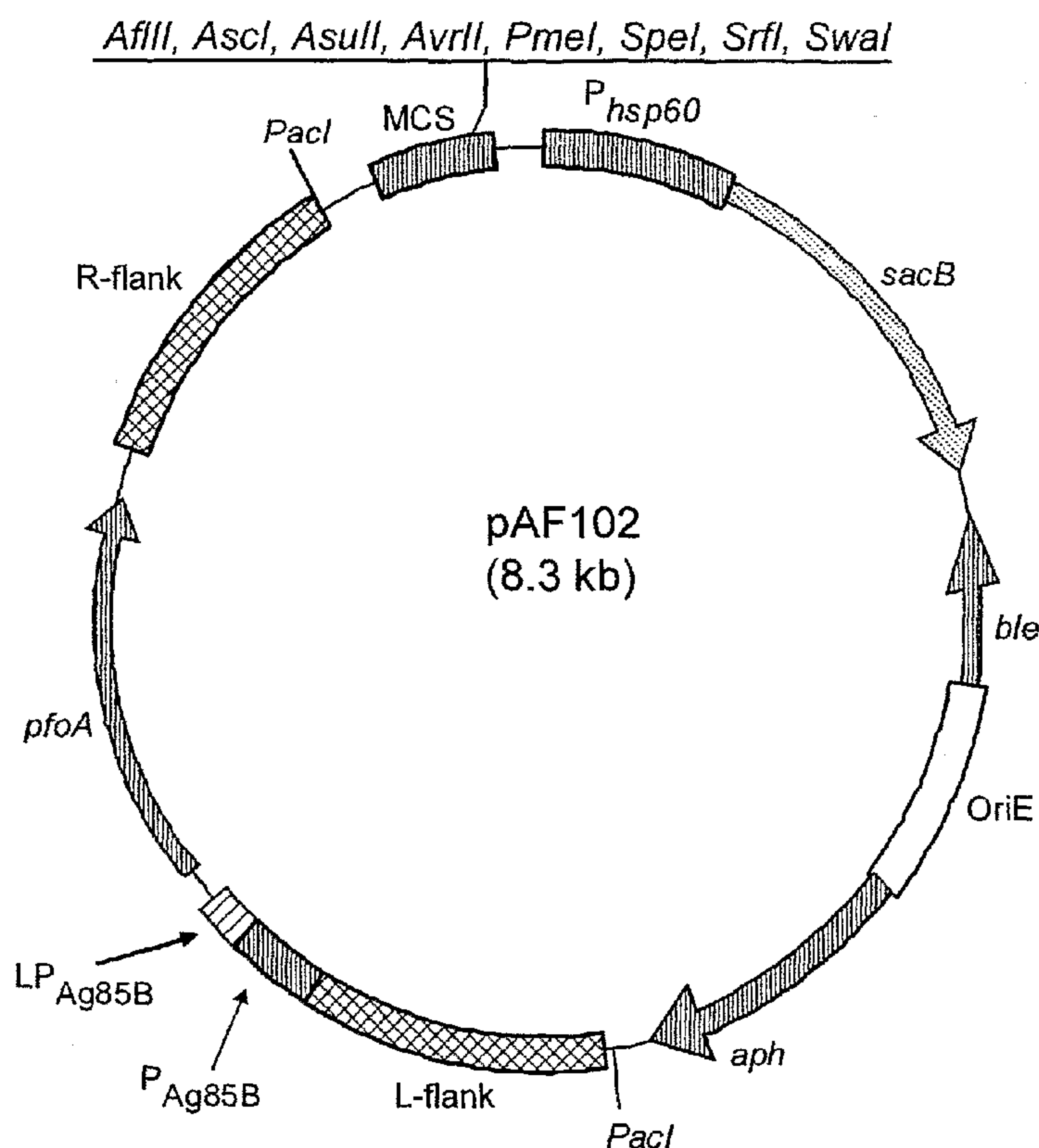
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(54) Title: RECOMBINANT BCG STRAINS WITH ENHANCED ABILITY TO ESCAPE THE ENDOSOME

(57) Abstract: *Mycobacterium* strains that have an enhanced ability to elicit a Histocompatibility-Class I-restricted CD8⁺ T cell immune response are provided. The *Mycobacterium* strains are genetically engineered to express an endosomal lytic protein that is active at neutral pH (e.g. Perfringolysin O), permitting escape of the *Mycobacterium* from endosomes into the cytoplasm of the cell. The invention also provides vaccine preparations containing the *Mycobacterium* strains.

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RECOMBINANT BCG STRAINS WITH ENHANCED ABILITY TO ESCAPE THE ENDOSOME

DESCRIPTION

BACKGROUND OF THE INVENTION

Field of the Invention

The invention provides *Mycobacterium* strains that have an enhanced ability to elicit an immune response. In vivo experiments have demonstrated, for example, a Major Histocompatibility -Class I-restricted CD8⁺ T cell immune response. In particular, the invention provides *Mycobacterium* strains that express a Perfringolysin O (PfoA) protein that permits escape of the *Mycobacterium* from endosomes, and vaccine preparations containing the *Mycobacterium* strains.

Background

Mycobacterium tuberculosis (*M. tb*) has infected one-third of the world's population, causing active disease in 8 million and killing 1.6-2.2 million individuals every year, most of whom live in the developing world. Tuberculosis (TB) is an epidemic of global proportions that is growing and becoming even more deadly as it intersects with the spread of HIV. TB is the number one killer of people with AIDS.

Bacille Calmette Guerin (BCG), an attenuated strain of *Mycobacterium bovis* and the current widely used TB vaccine, was developed over 80 years ago and when tested has had widely variable rates of efficacy against pulmonary tuberculosis, including no efficacy in the last large field trial conducted in India (Fine *et al.*, Vaccine, 16(20):1923-1928; 1998; Anonymous, Indian J Med Res., Aug;110:56-69; 1999. Nonetheless, The World Health Organization currently recommends BCG at birth or first contact with health services for all children (except those with symptoms of HIV disease/AIDS) in high TB prevalent countries. This policy is based on evidence that BCG protects against serious childhood forms of TB (Lanckriet *et al.*, Int J Epidemiol, 24(5):1042-1049; 1995; Rodrigues *et al.*, J Epidemiol Community Health 45(1): 78-80; 1991. Protection by BCG against TB beyond early childhood is a controversial subject with limited data giving mixed results. The high incidence of pediatric and adult TB in developing countries where infant BCG immunization is

widely practiced, however, indicates that BCG as currently administered is not highly efficacious over the many years when people are at risk of TB disease. Thus, BCG is considered to be an inadequate public health tool for the intervention and control of TB.

Approximately 70 percent of humans exposed to TB organisms, and who have normal immune systems, do not become infected, and of those that do become infected only about 5 percent develop disease within the first two years. The majority of infected individuals suppress the infection, which is associated with the development of robust cellular immune responses to *M. tb* antigens. An additional 5 percent later reactivate when immunity declines. Both primary and reactivation disease are much more common in people with HIV/AIDS, again emphasizing the role of immunity in preventing and controlling infection.

Because most humans are able to control TB, there is good reason to hope that by inducing long lasting immunity of the appropriate kind, it should be possible to develop effective vaccines that prevent initial infection after exposure, prevent early progression to disease, prevent reactivation from the latent state, and prevent relapse after treatment. Ultimately, it is the combination of systematic vaccine use plus chemotherapeutic intervention that will eventually eliminate *M. tb* as a human pathogen.

In light of the critical role childhood BCG vaccination is thought to play in preventing acute TB, it is difficult to replace BCG in trials to evaluate candidate TB vaccines without overwhelming evidence that the new TB vaccine is a superior product. The problem is that *M. tb* is a human-specific pathogen and animal models only mimic parts of the host-pathogen interaction. Thus, definitive evidence that a new TB vaccine possesses improved potency can only be obtained from controlled field trials in humans. This reality has led many investigators to conclude that a key step toward an improved TB vaccine will be to enhance the immunogenicity of BCG.

One example of such a strategy is to improve the capacity of BCG to induce or activate T cells for enhancing the immune response. The pivotal role of major histocompatibility complex class I-restricted CD8⁺ T cells in immunity to *M. tb* is demonstrated by the failure of β 2-microglobulin (β 2m)-deficient mice to control experimental *M. tb* infection (Flynn *et al.*, PNAS USA, 89(24): 12013-12017; 1992). The pivotal role of Histocompatibility class I-restricted CD8⁺ T cells was

convincingly demonstrated by the failure of β 2-microglobulin (β 2m) deficient mice to control experimental *M. tuberculosis* infection (Flynn *et al.*, *supra*, 1992). Because these mutant mice lack Histocompatibility class I, functional CD8⁺ T cells cannot develop. In contrast to *M. tuberculosis* infection, β 2m-deficient mice are capable of controlling certain infectious doses of the BCG vaccine strain (Flynn *et al.*, *supra*, 1992; Ladel C. H., *et al.*, Eur J Immunol, 25:377-384; 1995). Furthermore, BCG vaccination of β 2m-deficient mice only prolonged survival after *M. tuberculosis* infection, whereas BCG-immunized C57BL/6 resisted *M. tuberculosis* (Flynn *et al.*, *supra*, 1992).

This differential CD8⁺ T cell dependency between *M. tuberculosis* and BCG may be explained as follows: *M. tuberculosis* antigens gain better access to the cytoplasm than antigens from BCG leading to more pronounced Histocompatibility class I presentation (Hess and Kaufmann, FEMS Microbiol. Immunol 7:95-103; 1993). Consequently, a more effective CD8 T cell response is generated by *M. tuberculosis*. This notion was recently supported by increased Histocompatibility class I presentation of an irrelevant antigen, ovalbumin, by simultaneous *M. tuberculosis*, rather than BCG, infection of antigen presenting cells (APC) (Mazzaccaro *et al.*, Proc Natl Acad Sci USA, Oct 15:93(21):11786-91; 1996).

Thus, *M. tb* antigens access the host cell cytoplasm better than antigens from BCG, leading to increased Histocompatibility class I presentation (Hess *et al.*, *supra*, 1993) and an elevated CD8⁺ T cell response to *M. tb*. Further, *M. tb* stimulates antigen-specific Histocompatibility class II-restricted CD4⁺ T helper cells as well as Histocompatibility class I-restricted CD8⁺ cytotoxic T cells in mice and humans (Kaufmann, Annu Rev Immunol 11:129-163; 1993). By extension, this fact indicates that *M. tb* infected cells are susceptible to recognition by Histocompatibility class I-restricted CD8⁺ cytotoxic T cells. Given that 70 percent of immunocompetent humans exposed to TB organisms do not become infected, immunity induced by *M. tb* infection is highly effective in controlling this organism in the vast majority of instances. It is believed, therefore, that the efficacy of existing TB vaccine strain BCG will be improved by increasing the capacity of BCG to induce Histocompatibility class I-restricted CD8⁺ cytotoxic T cell responses (Kaufmann, Fundamental Immunology, 1997).

As a rule, antigens expressed by pathogens that remain phagosome-bound are primarily presented by Histocompatibility class II molecules to CD4⁺ T cells but are poorly recognized by CD8⁺ T cells, which normally recognize antigens presented in the context of Histocompatibility class I molecules (Kaufmann, *supra*, 1997). In contrast, intracellular bacteria, such as *Listeria monocytogenes* (e.g. ATCC # 13932), that escape the phagosome and replicate in the cytoplasm of host cells are effective at accessing the Histocompatibility class I antigen presentation pathway and at eliciting CD8⁺ T cell responses (Berche *et al.*, J Immunol, 138:2266-2276; 1987). This endosome escape function of *Listeria monocytogenes* was recently transferred into attenuated *Salmonella*, which normally resides in the phagosome, by introducing the sequences encoding listeriolysin (Llo); the resultant strains were shown to escape the endosome and were more effective at inducing CD8⁺ T cell responses (Bielecki *et al.*, Nature (London), 354:175-176; 1990; Gentschev *et al.*, Infect Immun 63(10):4202-4205; 1995; Hess *et al.*, Host Response to Intracellular Pathogens, 75-90; 1997). More recently this approach was applied to BCG; thus rBCG strains secreting Llo were constructed to improve the capacity of BCG to induce Histocompatibility class I-restricted immune responses (Hess *et al.*, PNAS USA, 95(9):5299-5304; 1998). Although early evidence suggested that a rBCG-Llo⁺ strain was more adept at eliciting CD8⁺ T cells, the strain proved to be incapable of escaping the endosome. Thus, a limitation of this approach is that the hemolytic function of Llo, which is required for endosome escape, is only fully active at pH 5.5 and almost inactive at pH 7.0. Since Mycobacteria maintain the pH of the endosome at a value of *ca.* 7.0, it is logical to surmise that Llo in rBCG-Llo strains thus far reported are dysfunctional because the environment in which they are expressed is suboptimal for hemolytic activity magnitude (Geoffroy *et al.*, Infect Immun 55(7): 1641-1646; 1987). Thus, the immune-enhancing benefit of this approach will not be realized until a strategy is developed that enables Llo to function in BCG-manipulated endosomes.

The prior art has thus far failed to provide a rBCG with an increased capacity for endosomal escape, and which can thereby increase induction of, for example, Major Histocompatibility class I-restricted CD8⁺ cytotoxic T cell responses.

SUMMARY OF THE INVENTION

An exemplary aspect of the present invention provides recombinant BCG (rBCG) strains that have an enhanced ability to elicit a Major Histocompatibility-

Class (MHC)- I-restricted CD8⁺ T-cell immune response. These novel rBCG strains have been genetically engineered to express a functional endosomalytic protein that is bioactive at pH values near neutrality (e.g. about pH 6-8 or about 6.5 to 7.5). The endosomalytic protein is therefore active within Mycobacteria-containing endosomes, which typically have an internal pH near neutrality. The activity of the endosomalytic protein produced by the rBCG results in disruption of the endosome, permitting the rBCG to escape from the endosome and into the cytoplasm of the cell. The rBCG are thus exposed to the cytoplasm, and elicit a strong T-cell response, particularly a strong MHC-I-restricted CD8⁺ cytotoxic T cell response. In one embodiment of the invention, the endosomalytic protein that is introduced into the rBCG by genetic engineering is Perfringolysin O (PfoA) from *Clostridium perfringens*.

The invention thus provides a *Mycobacterium* that is genetically engineered to express and secrete a functional endosomalytic protein that is active at neutral pH. In some embodiments, the functional endosomalytic pore-forming protein is PfoA or the mutant PfoA encoded by SEQ ID NO: 3 (herein referred to as PfoA_{G137Q}). Expression of the functional endosomalytic protein by the *Mycobacterium* permits escape of the rBCG from endosomes. The *Mycobacterium* that is so genetically engineered may be an attenuated *Mycobacterium* such as BCG. The invention also provides: a *Mycobacterium* that is genetically engineered to express and secrete PfoA; and, a *Mycobacterium* that is genetically engineered to express and secrete a PfoA_{G137Q} encoded by SEQ ID NO: 3.

The present invention further provides a method of enabling a *Mycobacterium* derivative to escape from endosomes. The method comprises the step of genetically engineering the *Mycobacterium* to contain, express and secrete a functional endosomalytic protein such as PfoA or PfoA_{G137Q} such as that encoded by SEQ ID NO: 3. In some embodiments, the *Mycobacterium* is an attenuated *Mycobacterium* such as BCG.

The invention further provides a vaccine preparation, comprising a *Mycobacterium* that is genetically engineered to express and secrete a functional endosomalytic protein that is active at neutral pH. The functional endosomalytic protein may be, for example, PfoA or PfoA_{G137Q} as encoded by SEQ ID NO:3. Expression of the functional endosomalytic enzyme by the *Mycobacterium* permits escape of the recombinant *Mycobacterium* from endosomes. In some embodiments, the *Mycobacterium* is an attenuated *Mycobacterium* such as BCG.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. The map for suicide vector pAF102. The denotation for each of the DNA segments as follow: L-flank and R-flank: left and right flanks of *ureC* gene respectively; *pfoA* is the gene encoding the mutant form of perfringolysin O (gene bank accession number: BA000016) with a single amino acid change at 137G(Q); LP_{PAg85B} is the DNA sequence encoding antigen 85B(i.e. Rv1886c) leader peptide; P_{Ag85A} is the promoter sequence of antigen 85A gene (i.e. Rv3804c); *aph* is aminoglycoside phosphotransferase gene (gene bank accession number: X06402), which confers Kanamycin resistance for the plasmid; OriE is the pUC origin of replication (gene bank accession number: AY234331); *Ble* is the gene (gene bank accession number: L36850), which confers resistance to Zeocin for the plasmid; *SacB* is the gene (gene bank accession number: Y489048) encoding levansucrase, which confers the bacteria sensitivity to sucrose; P_{hsp60} is the promoter sequence of heat shock protein gene (i.e. Rv0440); MCS is the multiple cloning sites for the indicated restriction enzymes. Note that the cassette between two *PacI* sites can be replaced with other endosomalalytic enzyme genes when applicable.

Figure 2A and B. A, The gene sequence of *PfoA* from *Clostridium perfringens* (SEQ ID NO: 1); B, the protein sequence of PfoA from *Clostridium perfringens* (SEQ ID NO: 2).

Figure 3. The gene sequence of a preferred mutant, PfoA_{G137Q} (SEQ ID NO: 3).

Figure 4. Flow chart for the main steps of allele exchange.

Figure 5. Restriction enzyme digestion for the allele exchange plasmid pAF102.

Lane 1: 1kb plus DNA ladder; Lane 2: Plasmid pAF102 digested by restriction enzyme EcoRI; Lane 3: Plasmid pAF102 undigested control; Lane 4: Vector plasmid pAF100 without PFO cassette insert digested by restriction enzyme EcoRI. Lane 5: Vector plasmid pAF100 without PFO cassette insert undigested control. From this figure, the plasmid pAF100 is linearized to produce a single band of 4.4kb in size by the restriction enzyme EcoRI as expected. The plasmid pAF102 produced two bands with size of 2.3kb and 6.0 kb respectively by restriction enzyme EcoRI as expected.

Figure 6. PCR analysis of the selected colonies for the genotype of $\Delta ureC::pfoA$. The PCR was carried as descried in Materials and Methods. The PCR product was

analyzed by gel electrophoresis in 1.2% agarose gel. The DNA ladder used was a 1Kb plus DNA standard purchased from Invitrogen. Lanes 1-6: PCR for colonies Numbered 105-1 through 105-6 respectively; lane 7 and lane 8: PCR for colonies numbered 142-7 and 142-10, respectively. Lane 9: PCR for BCG Danish1331 strain.

Figure 7. Growth and kanamycin sensitivity of AFV102. Bacteria from AFV102, BCG Danish 1331 and PfoA construct merodiploid (Pfo105 MI) were inoculated into 7H9 growth media and the growth of each strain was compared by measuring the optical density at 600nm at different time points. For the kanamycin sensitivity test, antibiotic was added to a final concentration of 50ug/ml and the growth was measured as above. Abbreviations in the legend: Ctrl: control medium without any bacterial inoculation; +Kan or -Kan: with or without kanamycin in the medium.

Figure 8. Cytotoxicity test for AFV102 in J774A.1 cells. The cells were infected with either AFV102 or BCG Danish 1331. At the indicated time points post infection, cell viability was measured as described in the Materials and Methods by comparison to the uninfected control.

Figure 9. Testing AFV102 for its survival ability in macrophages. J774A.1 monolayer cells were infected with AFV102 or BCG Danish 1331, and at the indicated time points post infection the intracellular bacteria were enumerated as described in Materials and Methods.

Figure 10. Test of AFV102 for its ability to secrete Pfo protein with pH independent hemolytic activity. The AFV102 culture was prepared as described previously and the supernatant was tested for its ability to lyse red cells at pH 7.0 and 5.5. The hemolysin activity was measured as described in Materials and Methods.

Figure 11A-D. Schematic illustration of results for Example 3. A, The bacterium (darkened oval) invades a macrophage (gray oval) within a cell (hexagon) which stimulates formation of early endosomes; B, BCG halts endosome maturation and BCG is protected inside the early endosome; C, AFV102 bacteria expressing PfoA are initially found in early endosomes after invasion; however, they will start to secrete PfoA and escape the endosome; D, AFV102 bacteria expressing PfoA can be seen emerging from the endosome or free in the cell.

Figure 12. The map for antigen over expression vector pAF105. The denotation for each of the DNA segments is as follows: P_{Rv3130} is the promoter sequence of antigen Rv3130c; P_{Ag85B} is the promoter sequence of antigen Rv1886c. The genes in the

expression cassette are Rv0288; Rv1886c and Rv3804c; *aph* is the aminoglycoside phosphotransferase gene (Gene bank accession number: X06402), which confers kanamycin resistance; OriE is the pUC origin of replication (Gene bank accession number: AY234331); *leuD* is the gene encoding 3-isopropylmalate dehydratase (i.e. Rv2987c); OriM is the origin of replication in *Mycobacterium* (Gene bank accession no: M23557).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS OF THE INVENTION

The present invention provides a rBCG that is capable of escaping from endosomes and accessing the host cell cytoplasm of cells that it infects. As a result, a superior CD8⁺ T-cell response to the rBCG is elicited in the cells.

The rBCG is genetically engineered to contain a functional endosomalysin that is expressed and secreted by the rBCG, and that mediates endosomal escape of the rBCG. The term endosomalysin herein refers to a protein that is capable of rupturing the endosomal membrane sufficiently to enable a bacterial cell to pass from the endosome into the cytosol. The term endosomalytic herein refers to a protein that has said rupturing activity. The term endosomalysis herein refers to the process of rupturing the endosomal membrane sufficiently to enable a bacterial cell to pass from the endosome into the cytosol. The endosomalytic protein is active in environments with pH values near neutrality, such as within the endosomes of cells infected with mycobacteria, and thus mediates escape of the bacteria into the cytoplasm.

In one embodiment of the present invention, the functional endosomalytic protein that is introduced into the rBCG strains of the present invention is PfoA from *Clostridium perfringens*, or a functional variant thereof. PfoA is a cytolysin secreted by *Clostridium perfringens* that is encoded by the *pfoA* gene (Genebank Accession # CPE0163). The gene and its protein sequence are shown in Figure 2 A and B, respectively. PfoA mediates bacterial escape from phagosomes, both in *Clostridium* and when expressed by *B. subtilis* (Portnoy *et al.*, Infect Immun. Jul; 60(7): 2710-7; 1992). Unlike Llo, PfoA is active at both pH 5.0 and pH 7.0 and thus remains active in the cytosol and causes damage to infected host cells (Portnoy *et al.*, *supra*, 1992). Thus, when PfoA was expressed in *L. monocytogenes* in place of Llo, it enabled the strain to escape from phagosomes (Jones and Portnoy, Infect Immun. Dec; 62(12): 5608-13; 1994). However, its expression also caused damage to the host cells. Llo

displays similar properties to PfoA, except that Llo is optimally active at pH 5.5 and displays little activity at pH 7.0. Thus, Llo mediates endosome escape once the pH of the endosome drops to pH 5.5 but does not affect the host cell because the pH of the cytosol, which is near neutrality, impedes Llo activity.

Portnoy *et al.* has further demonstrated that when PfoA is expressed in *L. monocytogenes* in a mutant form with a single amino acid change, the mutant form of PfoA is no longer toxic to the host cells, yet is still capable of mediating bacterial escape from a vacuole, and of carrying out intracellular growth (Portnoy *et al.*, *supra*, 1992). The particular strain DP-12791, with a mutation that changes Gly137 to Gln137 in Pfo (i.e. PfoA_{G137Q}), is able to escape from the phagosome in a manner similar to wild type, but without producing a toxic effect on host cells. In addition, PfoA_{G137Q} is active at both pH 5.6 and pH 7.4 with a reduced cytosolic half-life. The gene sequence of PfoA_{G137Q} is shown in Figure 3 (SEQ ID NO: 3).

The expression of PfoA allows escape from endosomes because the pore-forming activity of PfoA leads to eukaryotic cell membrane damage. PfoA forms pores in cholesterol-containing membranes by the spontaneous insertion of its domains into the bilayer. This happens via binding to cholesterol, which acts as a receptor. After binding, the toxin then forms a monomer-monomer interface (which is required for oligomer assembly), oligomerizes and partitions into the membrane by changing its structure, which is membrane binding-dependent. Formation of the membrane-bound oligomers leads to membrane damage and eventual lysis (Ramachandran *et al.*, *Nature Structure and Molecular Biology*, 11(8), 697-705 (1995). PfoA_{G137Q} is capable of mediating lysis of the vacuole in this manner. The preferable PfoA_{G137Q} has limited activity in the cytoplasm of the infected host cell, because of its sensitivity to host proteases.

In one embodiment of the invention, the functional endosomalytic enzyme that is expressed by the rBCG is PfoA that is derived or isolated from *C. perfringens*. However, those of skill in the art will recognize that other endosomalytic proteins that are active at or near neutrality also exist that would be suitable for use in the present invention. Examples of such endosomalytic proteins include but are not limited to Pneumolysin (produced by *Streptococcus pneumoniae*), Streptolysin O (produced by *Streptococcus pyogenes*), Cerolysin (produced by *Bacillus cereus*), α -hemolysin (produced by *Staphylococcus aureus*), etc., and functional variants thereof.

By “functional endosomalytic protein” or “functional form” of a protein (or the gene product of a gene that is “functionally expressed”), we mean that the form of the protein that is produced by the rBCG bacterium exhibits the characteristic magnitude of activity for which the native or natural (“wild-type”) protein is known in its native organism. The magnitude of activity of the functional form of the protein produced by the rBCG is, in general, at least about 50 percent of the usual activity of the wild-type protein when assayed under standard conditions as recognized by those of skill in the art. The magnitude of activity of the protein may be determined by measuring any physical observable, such binding to a ligand, or production of an effect, such as escape of rBCG from endosomes. Preferably, the activity magnitude is at least about 50%, 60%, 70%, 80%, 90%, 100%, or more of the standard magnitude of activity of the wild type protein when assayed under standard conditions as recognized by those of skill in the art.

By “functional variant” of a protein, we mean a polypeptide whose amino acid sequence is at least about 70% homologous to that of a wild type “reference” protein, and which retains the functional magnitude of activity (as described above) of the wild type protein. The amino acid sequence of the reference wild type protein is typically used as a starting point for mutations and alterations that are carried out by genetic engineering.

Preferably, a functional variant is a polypeptide with an amino acid sequence that displays about 75%, 80%, 85%, 90%, 95% or more identity to the reference amino acid sequence. Such functional variants include but are not limited to polypeptide sequences in which one or more conservative amino acid substitutions have been made. Conservative amino acid substitutions are well known to those of skill in the art, and include, for example, the substitution of one positively charged amino acid for another, one negatively charged amino acid for another, one hydrophobic amino acid for another, etc. Variant polypeptides may contain one or more of such substitutions, provided the resulting variant polypeptide retains the functional magnitude of activity as defined herein. “Functional variants” also encompass other changes to the primary sequence of the polypeptide of interest. Examples of changes include but are not limited to deletions and additions of amino acids, or the modification of amino acids (e.g. chemical modifications such as sulfonation, deamidation, phosphorylation, hydroxylation, etc.). Such changes may be the result of genetic engineering of a reference amino acid sequence, or may be the

result of post-translational modifications of the protein, or both. Further, functional variants of an enzyme may be the result of natural mutations such as those that occur between analogous proteins with the same or similar activities that are isolated from different strains of a species, or from different species, or from different individual organisms within a species. Proteins from such naturally occurring variants may also serve as the reference protein, and the amino acid sequence of such a natural variant may serve as the reference sequence. In any case, all such functional variants of a reference protein retain the activity magnitude of the protein, as described herein.

Sequence homologies and identities as described herein are not intended to include heterologous amino acid sequences that are derived from sources other than the reference sequence and which are attached to or included in the polypeptide sequence of a protein for various other purposes. Examples of such heterologous sequences include but are not limited to sequences that facilitate polypeptide isolation (e.g. histidine tags), sequences that facilitate secretion or localization of the polypeptide within the cell, (e.g. various leader or targeting sequences), and sequences that code for glycosylation sites (glycosylation sequences), etc.

In any case, the functional variant will retain the activity magnitude of the protein of which it is a variant to the degree that the functional variant exhibits at least about 50 percent, and preferably about 60%, 70%, 80%, 90%, 100% or more, of the activity magnitude of the protein of which it is a variant, when assayed under standard conditions as recognized by those of skill in the art.

Further, the present invention also includes nucleic acid sequences encoding proteins and functional variants of proteins utilized in the present invention. The nucleic acid sequences may be deoxyribonucleotides, ribonucleotides, or modified forms of either, and may be single- or double-stranded. The nucleic acid sequences of the present invention include any that are listed herein, and are also intended to encompass variants thereof. For example, variants of the nucleic acids may not be identical to the listed sequences but may still encode an identical amino acid sequence due to the redundancy of the genetic code. Alternatively, some changes in the sequences of the present invention may be made (e.g. substitutions, deletions or additions) that result in changes in the encoded amino acid sequence, so long as the encoded amino acid sequence is a functional variant of the reference amino acid sequence as described above. Examples include but are not limited to: changes that cause conservative amino acid substitutions in the enzyme; and changes that result in

non-conservative substitutions, or deletion or additions in the amino acid sequences. Such changes may be introduced for any reason, e.g. in order to alter post-translational modifications of the enzyme; to increase or decrease solubility; to prevent or introduce steric interactions in the translated polypeptide, etc. In general, variants of the nucleic acid sequences of the present invention will exhibit at least about 50 percent, and preferably about 60%, 70%, 80%, 90%, 95%, or 100% homology to the reference sequences, as determined by comparative procedures that are well known to those of skill in the art. Such variants are also characterized by exhibiting the ability to bind to the sequences utilized the present invention under conditions of high stringency. High stringency binding assays are well-known to those of skill in the art and can readily be applied to test potential variants of sequences of the present invention.

Sequence homologies as described herein are not intended to refer to nucleic acid sequences encoding heterologous amino acid sequences that are derived from sources other than the reference amino acid sequence, and which are attached to or included in the polypeptide sequence of a protein for various other purposes. For example, such nucleic acid sequences may encode heterologous amino acid sequences including but not limited to: sequences that facilitate polypeptide isolation (e.g. histidine tags), sequences that facilitate secretion or localization of the polypeptide within the cell, (e.g., various leader sequences or targeting sequences), and sequences that code for glycosylation sites (glycosylation sequences), etc.

Other variations of the nucleic acid sequences of the present invention that are intended to be encompassed by the present invention are sequences which have been altered for convenience or improvement in genetic engineering of the nucleic acid sequences, or in the expression of the amino acid sequences they encode. In general, such alterations will not affect the sequence of the polypeptide that is ultimately translated from the nucleic acid sequence; or the polypeptide will still fulfill the criteria set forth above for a functional variant. Examples of this type of alteration include but are not limited to: the inclusion of convenient restriction endonuclease sites in a nucleic acid sequence to facilitate manipulation of the sequence (e.g. for insertion of a sequence into a vector); the inclusion, deletion, or other change in a sequence or sequences involved in expression of the amino acid sequence (e.g. inclusion of any of various promoter and/or enhancer sequences, stop signals, super promoters, inducible promoters, and various other sequences that modify expression

of the nucleic acid sequence); the inclusion of sequences that facilitate interaction of a vector with the nucleic acid of a host organism; etc.

Further, the nucleic acid sequences of the present invention may be chemically modified or include non-traditional bases for any of many reasons that are well-known to those of skill in the art, for example, to promote stability of the nucleic acid, or to confer a desired steric conformation.

By “active at neutral pH” and “active at a pH of about 7.0” we mean that the enzyme is active at a pH value in the range of from about 6.0 to about 8.0, and preferably in a range of about 6.5 to about 7.5.

The present invention provides recombinant *Mycobacteria*. In a preferred embodiment of the invention, the *Mycobacteria* are attenuated, as exemplified by BCG. However, those of skill in the art will recognize that other attenuated and non-attenuated *Mycobacteria* exist which would also be suitable for use in the present invention. Examples of additional types of *Mycobacteria* include but are not limited to *M. tuberculosis* strain CDC1551 (See, e.g. Griffith *et al.*, *Am. J. Respir. Crit. Care Med.* Aug;152(2):808; 1995), *M. tuberculosis* strain Beijing (Soolingen *et al.*, 1995), *M. tuberculosis* strain H37Ra (ATCC#:25177), *M. tuberculosis* strain H37Rv (ATCC#:25618), *M. bovis* (ATCC#:19211 and 27291), *M. fortuitum* (ATCC#:15073), *M. smegmatis* (ATCC#:12051 and 12549), *M. intracellulare* (ATCC#:35772 and 13209), *M. kansasii* (ATCC#:21982 and 35775) *M. avium* (ATCC#:19421 and 25291), *M. gallinarum* (ATCC#:19711), *M. vaccae* (ATCC#:15483 and 23024), *M. leprae* (ATCC#), *M. marinum* (ATCC#:11566 and 11567), and *M. microti* (ATCC#:11152).

Examples of attenuated *Mycobacterium* strains include but are not restricted to *M. tuberculosis* pantothenate auxotroph strain (Sambandamurthy, *Nat. Med.* 2002 8(10):1171; 2002), *M. tuberculosis rpoV* mutant strain (Collins *et al.*, *Proc Natl Acad Sci USA.* 92(17):8036; 1995), *M. tuberculosis* leucine auxotroph strain (Hondalus *et al.*, *Infect. Immun.* 68(5):2888; 2000), BCG Danish strain (ATCC # 35733), BCG Japanese strain (ATCC # 35737), BCG, Chicago strain (ATCC # 27289), BCG Copenhagen strain (ATCC #: 27290), BCG Pasteur strain (ATCC #: 35734), BCG Glaxo strain (ATCC #: 35741), BCG Connaught strain (ATCC # 35745), BCG Montreal (ATCC # 35746).

In a further embodiment of this invention, attenuated *Mycobacterium* strains are modified to enhance apoptosis, wherein such strains induce strong cellular

immune responses. Apoptosis is programmed cell death, which differs dramatically from necrotic cell death in terms of its induction and consequences. In fact, the process by which apoptosis of antigen containing cells results in the induction of potent cellular immunity has been called cross-priming (Heath et al., *Immunol Rev* 199; 2004; Gallucci et al., *Nature Biotechnology*. 5:1249; 1999; Albert et al., *Nature* 392:86; 1998). There are several mechanisms for induction of apoptosis which lead to increased antigen specific cell mediated immunity. Caspase 8-mediated apoptosis leads to antigen specific cellular immune protection (Sheridan et al., *Science* 277:818; 1997).

Another embodiment of the present invention, therefore, provides attenuated *Mycobacterium* strains which display enhanced pro-apoptosis properties, such as but not limited to secA1 secreted SodA lacking a leader peptide from the *Salmonella enteritidis* (Genbank accession no. 1068147), *Escherichia coli* (Genbank Accession No. 1250070) or *Shigella flexneri* (Genbank accession no. 1079977) or alternatively a SodA protein that is naturally non-secreted such as the SodA from *Listeria monocytogenes* EGD-e (Genbank Accession No. 986791). Such attenuated *Mycobacterium* strains do not produce extracellular Sod and thus do not suppress host immune responses, yet they do express intracellular Sod, thereby enabling survival of said *Mycobacterium* (Edwards et al., *Am. J. Respir. Crit. Care Med.* 164(12):2213-9; 2001). Alternatively, attenuated *Mycobacterium* strains which display enhanced pro-apoptosis properties carry an inactivated Rv3238c gene.

Alternatively, expression of *Salmonella* SopE (Genbank accession # AAD54239, AAB51429 or AAC02071) or caspase-8 (Genbank accession # AAD24962 or AAH06737) in the cytoplasm of host cells by attenuated *Mycobacterium* in the present invention will impart a powerful method for inducing programmed cell death in the context of antigens expressed by said attenuated *Mycobacterium*, invoking high levels of antigen-specific cellular immunity.

Death receptor-5 (DR-5) also known as TRAIL-R2 (TRAIL receptor 2) or TNFR-SF-10B (Tumor Necrosis Factor-Superfamily member 10B) also mediates caspase 8 mediated apoptosis (Sheridan et al., 1997). Reovirus induced apoptosis is mediated by TRAIL- DR5 leading to subsequent clearance of the virus (Clarke et al., *J. Virol.* 74:8135; 2000). Expression of DR-5, such as human DR-5 (Genbank accession # BAA33723), herpesvirus-6 (HHV-6) DR-5 homologue (Genbank accession # CAA58423) etc., by attenuated *Mycobacterium* in the present invention

provides a potent adjuvant effect for induction of antigen-specific cellular immunity against Mtb antigens.

In addition, host antigen presenting cells (such as macrophages and dendritic cells) can also be induced to undergo apoptosis through Fas ligation, which is a strong stimulus for induction of antigen specific cellular immune responses (Chattergoon et al., *Nat. Biotechnol.* 18:974; 2000). Thus, attenuated *Mycobacterium* expressing Fas or Fas cytoplasmic domain/CD4 ectodomain fusion protein will induce apoptosis and augmented antigen-specific cellular immune responses.

In summary, attenuated *Mycobacterium* strains which promote the induction of apoptosis provide a powerful tool for the induction of cellular responses that lead to immune mediated cell destruction of Mtb-infected cells, with subsequent elimination, reduction or prevention of the Mtb infection.

In yet another embodiment of the present invention, the two-component TB vaccine can include attenuated *Mycobacterium* strains that over express at least one *Mycobacterium* antigen, including but not restricted to Rv0125, Rv0203, Rv0287, Rv0288, Rv0603, Rv1196, Rv1223, Rv1271c, Rv1733c, Rv1738 Rv1804c, Rv1886, Rv2031c, Rv2032, Rv2253, Rv2290, Rv2389c, Rv2626c, Rv2627c, Rv2779c, Rv2873, Rv2875, Rv3017c, Rv3407, Rv3804c, Rv3810, or Rv3841. Alternatively, the over expressed *Mycobacterium* antigens can be in the form of a fusion protein comprised of one or more said *Mycobacterium* fusion proteins, such as Mtb72f (Brandt et al., *Infect. Immun.*, 72:6622-6632; 2004; Skeiky et al., *J. Immunol.*, 172:7618-7628; 2004), Hybrid-1 (Olsen et al., *Infect. Immun.*, 72:6148-6150; 2004; Langermans et al., *Vaccine*, 23:2740-2750; 2005), Hyvac-4 (Dietrich et al., *J. Immunol.*, 174:6332-6339; 2005), etc.

This invention has utility in the development of vaccines against pathogenic *Mycobacterium* species and in the development of antigen delivery vaccine vectors. A *Mycobacterium* vector is defined herein as any *Mycobacterium* strain engineered to express at least one passenger nucleotide sequence (herein referred to as "PNS") comprised of DNA or RNA and encoding any combination of antigens, immunoregulatory factors or adjuvants, as set forth below. The PNS can be introduced into the chromosome or as part of an expression vector using compositions and methods well known in the art (Jacobs et al., *Nature* 327:532-535; 1987; Barletta et al., *Res Microbiol.* 141:931-939 ;1990; Kawahara et al., *Clin Immunol.* 105:326-331; 2002; Lim et al., *AIDS Res Hum Retroviruses.* 13:1573-1581;1997; Chujoh et

al., *Vaccine*, 20:797-804; 2001; Matsumoto et al., *Vaccine*, 14:54-60; 1996; Haeseleer et al., *Mol Biochem Parasitol.*, 57:117-126; 1993).

In the present invention, the *Mycobacterium* vector may carry a PNS encoding an immunogen, which may be either a foreign immunogen from viral, bacterial and parasitic pathogens, or an endogenous immunogen, such as but not limited to an autoimmune antigen or a tumor antigen. The immunogens may be the full-length native protein, chimeric fusions between the foreign immunogen and an endogenous protein or mimetic, a fragment or fragments thereof of an immunogen that originates from viral, bacterial and parasitic pathogens.

As used herein, "foreign immunogen" means a protein or fragment thereof, which is not normally expressed in the recipient animal cell or tissue, such as, but not limited to, viral proteins, bacterial proteins, parasite proteins, cytokines, chemokines, immunoregulatory agents, or therapeutic agents.

An "endogenous immunogen" means a protein or part thereof that is naturally present in the recipient animal cell or tissue, such as, but not limited to, an endogenous cellular protein, an immunoregulatory agent, or a therapeutic agent. Alternatively or additionally, the immunogen may be encoded by a synthetic gene and may be constructed using conventional recombinant DNA methods known to those of skill in the art.

The foreign immunogen can be any molecule that is expressed by any viral, bacterial, or parasitic pathogen prior to or during entry into, colonization of, or replication in their animal host; the *Mycobacterium* vector may express immunogens or parts thereof that originate from viral, bacterial and parasitic pathogens. These pathogens can be infectious in humans, domestic animals or wild animal hosts.

The viral pathogens, from which the viral antigens are derived, include, but are not limited to, Orthomyxoviruses, such as influenza virus (Taxonomy ID: 59771; Retroviruses, such as RSV, HTLV-1 (Taxonomy ID: 39015), and HTLV-II (Taxonomy ID: 11909), Herpes viruses such as EBV (Taxonomy ID: 10295); CMV (Taxonomy ID: 10358) or herpes simplex virus (ATCC #: VR-1487); Lentiviruses, such as HIV-1 (Taxonomy ID: 12721) and HIV-2 (Taxonomy ID: 11709); Rhabdoviruses, such as rabies; Picornoviruses, such as Poliovirus (Taxonomy ID: 12080); Poxviruses, such as vaccinia (Taxonomy ID: 10245); Rotavirus (Taxonomy ID: 10912); and Parvoviruses, such as adeno-associated virus 1 (Taxonomy ID: 85106).

Examples of viral antigens can be found in the group including but not limited to the human immunodeficiency virus antigens Nef (National Institute of Allergy and Infectious Disease HIV Repository Cat. # 183; Genbank accession # AF238278), Gag, Env (National Institute of Allergy and Infectious Disease HIV Repository Cat. # 2433; Genbank accession # U39362), Tat (National Institute of Allergy and Infectious Disease HIV Repository Cat. # 827; Genbank accession # M13137), mutant derivatives of Tat, such as Tat-Δ31-45 (Agwale *et al.*, *Proc. Natl. Acad. Sci. In press. Jul 8th*; 2002), Rev (National Institute of Allergy and Infectious Disease HIV Repository Cat. # 2088; Genbank accession # L14572), and Pol (National Institute of Allergy and Infectious Disease HIV Repository Cat. # 238; Genbank accession # AJ237568) and T and B cell epitopes of gp120 (Hanke and McMichael, *AIDS Immunol Lett.*, 66:177; 1999); (Hanke, *et al.*, *Vaccine*, 17:589; 1999); (Palker *et al.*, *J. Immunol.*, 142:3612-3619; 1989) chimeric derivatives of HIV-1 Env and gp120, such as but not restricted to fusion between gp120 and CD4 (Fouts *et al.*, *J. Virol.*, 74:11427-11436; 2000); truncated or modified derivatives of HIV-1 env, such as but not restricted to gp140 (Stamatos *et al.*, *J Virol*, 72:9656-9667; 1998) or derivatives of HIV-1 Env and/or gp140 thereof (Binley, *et al.*, *J Virol*, 76:2606-2616; 2002); (Sanders, *et al.*, *J Virol*, 74:5091-5100 ; 2000); (Binley, *et al.*, *J Virol*, 74:627-643; 2000), the hepatitis B surface antigen (Genbank accession # AF043578); (Wu *et al.*, *Proc. Natl. Acad. Sci., USA*, 86:4726-4730 ; 1989); rotavirus antigens, such as VP4 (Genbank accession # AJ293721; Mackow *et al.*, *Proc. Natl. Acad. Sci., USA*, 87:518-522; 1990) and VP7 (Genbank accession # AY003871; (Green *et al.*, *J. Virol.*, 62:1819-1823; 1988), influenza virus antigens such as hemagglutinin or (Genbank accession # AJ404627); (Pertmer and Robinson, *Virology*, 257:406; 1999); nucleoprotein (Genbank accession # AJ289872); (Lin *et al.*, *Proc. Natl. Acad. Sci.*, 97: 9654-9658; 2000) herpes simplex virus antigens such as thymidine kinase (Genbank accession # AB047378); (Whitley *et al.*, *New Generation Vaccines*, 825-854; 2004).

The bacterial pathogens, from which the bacterial antigens are derived, include but are not limited to, *Mycobacterium spp.*, *Helicobacter pylori*, *Salmonella spp.*, *Shigella spp.*, *E. coli*, *Rickettsia spp.*, *Listeria spp.*, *Legionella pneumoniae*, *Pseudomonas spp.*, *Vibrio spp.*, and *Borellia burgdorferi*.

Examples of protective antigens of bacterial pathogens include the somatic antigens of enterotoxigenic *E. coli*, such as the CFA/I fimbrial antigen (Yamamoto *et*

al., *Infect. Immun.*, 50:925-928; 1985) and the nontoxic B-subunit of the heat-labile toxin (Klipstein *et al.*, *Infect. Immun.*, 40:888-893; 1983); pertactin of *Bordetella pertussis* (Roberts *et al.*, *Vacc.*, 10:43-48; 1992), adenylate cyclase-hemolysin of *B. pertussis* (Guiso *et al.*, *Micro. Path.*, 11:423-431; 1991), fragment C of tetanus toxin of *Clostridium tetani* (Fairweather *et al.*, *Infect. Immun.*, 58:1323-1326; 1990), OspA of *Borellia burgdorferi* (Sikand, *et al.*, *Pediatrics*, 108:123-128; 2001); (Wallich, *et al.*, *Infect Immun*, 69:2130-2136; 2001), protective paracrystalline-surface-layer proteins of *Rickettsia prowazekii* and *Rickettsia typhi* (Carl, *et al.*, *Proc Natl Acad Sci USA*, 87:8237-8241; 1990), the listeriolysin (also known as "Llo" and "Hly") and/or the superoxide dismutase (also know as "SOD" and "p60") of *Listeria monocytogenes* (Hess, *et al.*, *Infect. Immun.* 65:1286-92 ; 1997; (Hess, *et al.*, *Proc. Natl. Acad. Sci.* 93:1458-1463 ; 1996) ; (Bouwer, *et al.*, *J. Exp. Med.* 175:1467-71; 1992), the urease of *Helicobacter pylori* (Gomez-Duarte, *et al.*, *Vaccine* 16, 460-71; 1998); Corthesy-Theulaz, *et al.*, *Infection & Immunity* 66, 581-6; 1998), and the receptor-binding domain of lethal toxin and/or the protective antigen of *Bacillus anthrax* (Price, *et al.*, *Infect. Immun.* 69, 4509-4515; 2001).

The parasitic pathogens, from which the parasitic antigens are derived, include but are not limited to, *Plasmodium spp.*, such as *Plasmodium falciparum* (ATCC#: 30145); *Trypanosome spp.*, such as *Trypanosoma cruzi* (ATCC#: 50797); *Giardia spp.*, such as *Giardia intestinalis* (ATCC#: 30888D); *Boophilus spp.*, *Babesia spp.*, such as *Babesia microti* (ATCC#: 30221); *Entamoeba spp.*, such as *Entamoeba histolytica* (ATCC#: 30015); *Eimeria spp.*, such as *Eimeria maxima* (ATCC# 40357); *Leishmania spp.* (Taxonomy ID: 38568); *Schistosoma spp.*, *Brugia spp.*, *Fasciola spp.*, *Dirofilaria spp.*, *Wuchereria spp.*, and *Onchocerca spp.*

Examples of protective antigens of parasitic pathogens include the circumsporozoite antigens of *Plasmodium spp.* (Sadoff *et al.*, *Science* 240:336-337; 1988), such as the circumsporozoite antigen of *P. bergerii* or the circumsporozoite antigen of *P. falciparum*; the merozoite surface antigen of *Plasmodium spp.* (Spetzler *et al.*, *Int. J. Pept. Prot. Res.*, 43:351-358; 1994); the galactose specific lectin of *Entamoeba histolytica* (Mann *et al.*, *Proc. Natl. Acad. Sci., USA*, 88:3248-3252; 1991), gp63 of *Leishmania spp.* (Russell *et al.*, *J. Immunol.*, 140:1274-1278; 1988); (Xu and Liew, *Immunol.*, 84: 173-176; 1995), gp46 of *Leishmania major* (Handman *et al.*, *Vaccine*, 18: 3011-3017; 2000), paramyosin of *Brugia malayi* (Li *et al.*, *Mol. Biochem.*

Parasitol., 49:315-323; 1991), the triose-phosphate isomerase of *Schistosoma mansoni* (Shoemaker *et al.*, *Proc. Natl. Acad. Sci.*, USA, 89:1842-1846; 1992); the secreted globin-like protein of *Trichostrongylus colubriformis* (Frenkel *et al.*, *Mol. Biochem. Parasitol.*, 50:27-36; 1992); the glutathione-S-transferase's of *Fraciola hepatica* (Hillyer *et al.*, *Exp. Parasitol.*, 75:176-186 ; 1992), *Schistosoma bovis* and *S. japonicum* (Bashir *et al.*, *Trop. Geog. Med.*, 46:255-258; 1994); and KLH of *Schistosoma bovis* and *S. japonicum* (Bashir *et al.*, *supra*, 1994).

As mentioned earlier, the *Mycobacterium* vector may carry a PNS encoding an endogenous immunogen, which may be any cellular protein, immunoregulatory agent, or therapeutic agent, or parts thereof, that may be expressed in the recipient cell, including but not limited to tumor, transplantation, and autoimmune immunogens, or fragments and derivatives of tumor, transplantation, and autoimmune immunogens thereof. Thus, in the present invention, *Mycobacterium* vector may carry a PNS encoding tumor, transplant, or autoimmune immunogens, or parts or derivatives thereof. Alternatively, the *Mycobacterium* vector may carry synthetic PNS's (as described above), which encode tumor-specific, transplant, or autoimmune antigens or parts thereof.

Examples of tumor specific antigens include prostate specific antigen (Gattuso *et al.*, *Human Pathol.*, 26:123-126; 1995), TAG-72 and CEA (Guadagni *et al.*, *Int. J. Biol. Markers*, 9:53-60; 1994), MAGE-1 and tyrosinase (Coulie *et al.*, *J. Immunothera.*, 14:104-109; 1993). Recently, it has been shown in mice that immunization with non-malignant cells expressing a tumor antigen provides a vaccine effect, and also helps the animal mount an immune response to clear malignant tumor cells displaying the same antigen (Koeppen *et al.*, *Anal. N.Y. Acad. Sci.*, 690:244-255; 1993).

Examples of transplant antigens include the CD3 molecule on T cells (Alegre *et al.*, *Digest. Dis. Sci.*, 40:58-64; 1995). Treatment with an antibody to CD3 receptor has been shown to rapidly clear circulating T cells and reverse cell-mediated transplant rejection (Alegre *et al.*, *supra*, 1995).

Examples of autoimmune antigens include IAS β chain (Topham *et al.*, *Proc. Natl. Acad. Sci.*, USA, 91:8005-8009; 1994). Vaccination of mice with an 18 amino acid peptide from IAS β chain has been demonstrated to provide protection and treatment to mice with experimental autoimmune encephalomyelitis (Topham *et al.*, *supra*, 1994).

Mycobacterium vectors which express an adjuvant

It is feasible to construct *Mycobacterium* vectors that carry PNS encoding an immunogen and an adjuvant, and are useful in eliciting augmented host responses to the vector and PNS-encoded immunogen. Alternatively, it is feasible to construct *Mycobacterium* vectors that carry PNS encoding an adjuvant, which are administered in mixtures with other *Mycobacterium* vectors that carry PNS encoding at least one immunogen to increase host responses to said immunogen encoded by the partner *Mycobacterium* vector.

The particular adjuvant encoded by PNS inserted in said *Mycobacterium* vector is not critical to the present invention and may be the A subunit of cholera toxin (i.e. CtxA; Genbank accession no. X00171, AF175708, D30053, D30052,), or parts and/or mutant derivatives thereof (E.g. the A1 domain of the A subunit of Ctx (i.e. CtxA1; Genbank accession no. K02679)), from any classical *Vibrio cholerae* (E.g. *V. cholerae* strain 395, ATCC # 39541) or El Tor *V. cholerae* (E.g. *V. cholerae* strain 2125, ATCC # 39050) strain. Alternatively, any bacterial toxin that is a member of the family of bacterial adenosine diphosphate-ribosylating exotoxins (Krueger and Barbier, Clin. Microbiol. Rev., 8:34; 1995), may be used in place of CtxA, for example the A subunit of heat-labile toxin (referred to herein as EltA) of enterotoxigenic *Escherichia coli* (Genbank accession # M35581), pertussis toxin S1 subunit (E.g. *ptxS1*, Genbank accession # AJ007364, AJ007363, AJ006159, AJ006157, etc.); as a further alternative the adjuvant may be one of the adenylate cyclase-hemolysins of *Bordetella pertussis* (ATCC # 8467), *Bordetella bronchiseptica* (ATCC # 7773) or *Bordetella parapertussis* (ATCC # 15237), E.g. the *cyaA* genes of *B. pertussis* (Genbank accession no. X14199), *B. parapertussis* (Genbank accession no. AJ249835) or *B. bronchiseptica* (Genbank accession no. Z37112).

***Mycobacterium* vector which express an immunoregulatory agent**

Yet another approach entails the use of *Mycobacterium* vector that carry at least one PNS encoding an immunogen and a cytokine, which are used to elicit augmented host responses to the PNS-encoded immunogen *Mycobacterium* vector. Alternatively, it is possible to construct a *Mycobacterium* vector that carries a PNS encoding said cytokine alone, which are used in admixtures with at least one other *Mycobacterium* vector carrying a PNS encoding an immunogen to increase host

responses to PNS-encoded immunogens expressed by the partner *Mycobacterium* vector.

The particular cytokine encoded by the *Mycobacterium* vector is not critical to the present invention includes, but not limited to, interleukin-4 (herein referred to as "IL-4"; Genbank accession no. AF352783 (Murine IL-4) or NM_000589 (Human IL-4)), IL-5 (Genbank accession no. NM_010558 (Murine IL-5) or NM_000879 (Human IL-5)), IL-6 (Genbank accession no. M20572 (Murine IL-6) or M29150 (Human IL-6)), IL-10 (Genbank accession no. NM_010548 (Murine IL-10) or AF418271 (Human IL-10)), IL-12_{p40} (Genbank accession no. NM_008352 (Murine IL-12 p40) or AY008847 (Human IL-12 p40)), IL-12_{p70} (Genbank accession no. NM_008351/NM_008352 (Murine IL-12 p35/40) or AF093065/AY008847 (Human IL-12 p35/40)), TGF β (Genbank accession no. NM_011577 (Murine TGF β 1) or M60316 (Human TGF β 1)), and TNF α (Genbank accession no. X02611 (Murine TNF α) or M26331 (Human TNF α)).

The specific method used to introduce a gene encoding a *Pfo* gene into the genome of BCG is not a critical feature of the invention and may be selected from methods well known to those skilled in the art (Parish *et al.*, *Microbiology*, 145:3497-3503; 1999). A preferred method entails targeting the *Pfo* gene to the *ureC* locus, thereby resulting in inactivation of the latter gene and creating a marker for selection of modified strains (Qadri *et al.*, *J Clin Micro.* 20(6), 1198-1199; 1984). To accomplish the same, a synthetic allelic exchange plasmid, such as the plasmid described in the Examples section below, can be modified to harbor 1 kb sequences that flank the 5-prime and 3-prime ends of the *ureC* gene (Genome Database # Mb1881). The *PfoA* gene (Genome Database # CPE0163) is then inserted in between the flanking sequences under control of Ag85B promoter. To secrete the PfoA protein, an Ag85B leader peptide sequence is used in place of the native PfoA signal sequence to ensure efficient secretion from recombinant BCG strains.

The method by which allelic exchange plasmids are introduced into target BCG strains is not a crucial feature of the present invention and can be accomplished by standard electroporation protocols for *Mycobacterium*. Similarly, the specific method to affect allelic exchange and introduce the *Pfo* allele into the *ureC* locus is not a crucial feature of the invention and can be selected from methods well known to those skilled in the art. A suicide vector such as that depicted in Figure 1 provides a

preferred method, as this plasmid contains two antibiotic selection markers, thus minimizing the selection of spontaneous antibiotic-resistant mutants. During allele exchange, the PfoA gene segment replaces the *ureC* gene as a result of homologous recombination of left and right flanking sequences, thereby resulting in stable chromosomal integration and expression of PfoA. An advantage of this approach is that no antibiotics are required to maintain the final product, and no antibiotic resistant genotype or phenotype is present in the final strain. This is the preferred embodiment for products for human use (they are "antibiotic free"). A UreC-negative phenotype will mark strains that have undergone the allelic exchange and replaced *ureC* with Pfo, it being understood that the UreC positive phenotype might also be employed in certain applications.

In the present invention, the location of the *pfoA* in the BCG is not restricted to *ureC*. Other locations include but are not limited to *pfoA* integrated into a plasmid, and the *attB* site on the chromosome. Those skilled in the art will know other locations of the chromosome that are potential sites for *pfoA* integration and expression.

The present invention also provides vaccine preparations for use in eliciting an immune response against tuberculosis. The vaccine preparations include at least one rBCG strain as described herein, and a pharmacologically suitable carrier. The preparation of such compositions for use as vaccines is well known to those of skill in the art. Typically, such compositions are prepared either as liquid solutions or suspensions, however, solid forms such as tablets, pills, powders and the like are also contemplated. Solid forms suitable for solution in, or suspension in, liquids prior to administration may also be prepared. The preparation may also be emulsified. The active ingredients may be mixed with excipients that are pharmaceutically acceptable and compatible with the active ingredients. Suitable excipients are, for example, water, saline, dextrose, raffinose, glycerol, ethanol and the like, or combinations thereof. In addition, the composition may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and the like. In addition, the composition may contain other adjuvants.

If it is desired to administer an oral form of the composition, various thickeners, flavorings, diluents, emulsifiers, dispersing aids or binders and the like may be added. The composition of the present invention may contain any such additional ingredients so as to provide the composition in a form suitable for

administration. The final amount of rBCG bacteria in the formulations may vary. However, in general, the amount in the formulations will be from about 1-99 percent. The vaccine preparations of the present invention may further comprise an adjuvant, suitable examples of which include but are not limited to Seppic, Quil A, Alhydrogel, etc. Further, the vaccine preparations of the present invention may contain a single type of rBCG. Alternatively, more than one type of rBCG may be utilized in a vaccine.

The present invention also provides methods of eliciting an immune response to tuberculosis and methods of vaccinating a mammal against tuberculosis. By eliciting an immune response, we mean that administration of the vaccine preparation of the present invention causes the synthesis of specific antibodies (at a titer in the range of 1 to 1×10^6 , preferably 1×10^3 , more preferable in the range of about 1×10^3 to about 1×10^6 , and most preferably greater than 1×10^6) and/or cellular proliferation, as measured, e.g. by ^3H thymidine incorporation. The methods involve administering a composition comprising a rBCG strain of the present invention in a pharmacologically acceptable carrier to a mammal. The vaccine preparations of the present invention may be administered by any of the many suitable means which are well known to those of skill in the art, including but not limited to by injection, orally, intranasally, by ingestion of a food product containing the rBCG, etc. In preferred embodiments, the mode of administration is subcutaneous or intramuscular.

The following examples are to be considered as exemplary of various aspects of the present invention and are not intended to be limiting with respect to the practice of the invention. Those of ordinary skill in the art will appreciate that alternative materials, conditions, and procedures may be varied and remain within the skill of the ordinarily skilled artisan without departing from the general scope of the invention as taught in the specification.

EXAMPLES

The pivotal role of MHC class I-restricted CD8⁺ T cells in TB immune protection has been demonstrated (Flynn *et al.*, *supra*, 1992). In an effort to improve the CD8 T cell response, a recombinant BCG strain has been engineered to express listeriolysin of *Listeria monocytogenes*. However, this recombinant BCG (rBCG) failed to display an increased capacity for endosomal escape, and therefore does not cause increased induction of, for example, MHC class I-restricted CD8⁺ cytotoxic T

cell responses. When examined, the strain displays less than 1% escape from the endosome. This is not surprising because listeriolysin's activity is highly pH sensitive, thus the enzyme is likely not active at the pH within the endosome. Despite this drawback, the protection and safety of this recombinant strain were both dramatically improved. Based on this observation, we have constructed a new rBCG strain by introducing PfoA_{G137Q}, derived from *Clostridium perfringens* into the chromosome of BCG. PfoA_{G137Q} has one amino acid change at position 137 from G to Q. This single amino acid change results in loss of toxicity of wild-type PfoA in mammalian cells, yet the enzyme retains its endosomolytic function. The resultant strain has been shown to be safe and immunogenic in a mouse model.

Materials and Methods: General

For each experiment described in the following sections, restriction endonucleases (herein "REs"); New England Biolabs Beverly, MA), T4 DNA ligase (New England Biolabs, Beverly, MA) and Taq polymerase (Life Technologies, Gaithersburg, MD) were used according to the manufacturers' protocols; Plasmid DNA was prepared using small-scale (Qiagen Miniprep^R kit, Santa Clarita, CA) or large-scale (Qiagen Maxiprep^R kit, Santa Clarita, CA) plasmids DNA purification kits according to the manufacturer's protocols (Qiagen, Santa Clarita, CA); Nuclease-free, molecular biology grade milli-Q water, Tris-HCl (pH 7.5), EDTA pH 8.0, 1M MgCl₂, 100% (v/v) ethanol, ultra-pure agarose, and agarose gel electrophoresis buffer were purchased from Life Technologies, Gaithersburg, MD. RE digestions, PCRs, DNA ligation reactions and agarose gel electrophoresis were conducted according to well-known procedures (Sambrook, et al., *Molecular Cloning: A Laboratory Manual*. 1, 2, 3; 1989); (Straus, et al., Proc Natl Acad Sci USA. Mar; 87(5): 1889-93; 1990). Nucleotide sequencing to verify the DNA sequence of each recombinant plasmid described in the following sections was accomplished by conventional automated DNA sequencing techniques using an Applied Biosystems automated sequencer, model 373A.

PCR primers were purchased from commercial vendors such as Sigma (St. Louis, MO) or synthesized using an Applied Biosystems DNA synthesizer (model 373A). PCR primers were used at a concentration of 150-250 µM and annealing temperatures for the PCR reactions were determined using Clone manager software version 4.1 (Scientific and Educational Software Inc., Durham, NC). PCRs were conducted in a Strategene Robocycler, model 400880 (Strategene, La Jolla, CA). The

PCR primers for the amplifications were designed using Clone Manager[®] software version 4.1 (Scientific and Educational Software Inc., Durham NC). This software enables the design of PCR primers and identifies RE sites that are compatible with the specific DNA fragments being manipulated. PCRs were conducted in a thermocycler device, such as the Strategene Robocycler, model 400880 (Strategene), and primer annealing, elongation and denaturation times in the PCRs were set according to standard procedures (Straus *et al*, *supra* 1990). The RE digestions and the PCRs were subsequently analyzed by agarose gel electrophoresis using standard procedures (Straus *et al*, *supra* 1990; and Sambrook *et al.*, *supra* 1989). A positive clone is defined as one that displays the appropriate RE pattern and/or PCR pattern. Plasmids identified through this procedure were further evaluated using standard DNA sequencing procedures, as described above.

Escherichia coli strains, such as DH5 α and Sable2^R, were purchased from Life Technologies (Bethesda, MD) and served as initial host of the recombinant plasmids. Recombinant plasmids were introduced into *E. coli* strains by electroporation using an high-voltage eletropulse device, such as the Gene Pulser (BioRad Laboratories, Hercules, CA), set at 100-200 Ω , 15-25 μ F and 1.0-2.5 kV, as described (Straus *et al*, *supra* 1990). Optimal electroporation conditions were identified by determining settings that resulted in maximum transformation rates per mcg DNA per bacterium.

Bacterial strains are typically grown on tryptic soy agar (Difco, Detroit, MI) or in tryptic soy broth (Difco, Detroit, MI), which was made according to the manufacturer's directions. Unless stated otherwise, all bacteria were grown at 37°C in 5% (v/v) CO₂ with gentle agitation. When appropriate, the media was supplemented with antibiotics (Sigma, St. Louis, MO). Bacterial strains were typically stored at -80°C suspended in (Difco) containing 30% (v/v) glycerol (Sigma, St. Louis, MO) at *ca.* 10⁹ colony-forming units (herein referred to as "cfu") per ml.

Allelic exchange in BCG. The prior art teaches methods for introducing altered alleles into *Mycobacterium* strains and those skilled in the art will be capable of interpreting and executing the methods (Parish *et al.*, *Microbiology*, 146:1969-1975; 2000). A novel method to generate an allelic exchange plasmid entails the use of synthetic DNA. The advantage of this approach is that the plasmid product will have a highly defined history and will be compliant, with federal regulations, whereas previously used methods, although effective, have poorly documented laboratory

culture records and thus are unlikely to be compliant with federal regulations. Compliance with these regulations is essential if a product is to be licensed for use in humans by United States and European regulatory authorities.

A suicide vector for allelic exchange in *Mycobacterium* is a plasmid that has the ability to replicate in *E. coli* strains but is incapable of replication in *Mycobacterium* spp., such as *M. tb* and BCG. The specific suicide vector for use in allelic exchange procedures in the current invention is not important and can be selected from those available from academic (Parish *et al.*, *supra*, 2000) and commercial sources. A preferred design of a suicide plasmid for allelic exchange is shown in Figure 1. The plasmid is comprised of the following DNA segments: an oriE sequence for the plasmid to replicate in *E. coli* (Genebank accession # L09137), a Kanamycin-resistant sequence for selection in both *E. coli* and *Mycobacterium* (Genebank accession # AAM97345), and an additional antibiotic selection marker, e.g. the zeocin-resistance gene (Genebank Accession # AAU06610), which is under the control of a *Mycobacterium* promoter (e.g. the *hsp60* promoter). The second antibiotic selection marker is not essential but may be included to enable double selection to prevent outgrowth of spontaneous kanamycin-resistant isolates during the allelic exchange process.

Construction of such suicide vectors can be accomplished using standard recombinant DNA techniques. However, current regulatory standards (e.g. federal regulations) have raised the specter of introducing prion particles acquired from products exposed to bovine products containing BSE-infected material. To avoid introducing materials (e.g. DNA sequences) into the target strain of unknown origin, therefore, it is preferable that all DNAs in the suicide vector be made synthetically by commercial sources (e.g. Picoscript, Inc.). Accordingly, a preferred method for constructing suicide vectors is to assemble a plan of the DNA sequences using DNA software (e.g. Clone Manager), and then to synthesize the DNA on a fee-for-service basis by any commercial supplier that offers such a service (e.g. Picoscript Inc.). This procedure was used to design and obtain the suicide vector utilized in the Examples section below.

The configuration suicide vector described above (Figure 1) has advantages, as this plasmid contains two antibiotic selection markers, thus minimizing selection of spontaneous mutants that display resistance to one antibiotic, which occurs at ca. $1/10^8$ per generation. Spontaneous resistance to two antibiotics is extremely rare and

only occurs at ca. $1/10^{16}$ per generation. Thus, there is less than $1/10^6$ probability of double resistant strains emerging in the cultures used to execute the allelic exchange procedure.

For negative selection during allelic exchange process, a *sacB* gene (Genbank Accession # NT01BS4354), which imparts a sucrose-sensitive phenotype, can be included to enrich cultures with strains that have undergone the final DNA recombination step and completed the allelic exchange.

Cultivation of *Mycobacterium*. Selected BCG strains are cultured in liquid media, such as Middlebrook 7H9 or Sauton Synthetic Medium, preferably at 37°C. The strains can be maintained as static or agitated cultures. In addition, the growth rate of BCG can be enhanced by the addition of oleic acid (0.06% v/v; Research Diagnostics Cat. No. 01257) and detergents such as Tyloxapol (0.05% v/v; Research Diagnostics Cat. No. 70400). The purity of BCG cultures can be evaluated by evenly spreading 100 µl aliquots of the BCG culture serially diluted (e.g. 10-fold steps from Neat – 10^{-8}) in phosphate buffered saline (herein referred to PBS) onto 3.5 inch plates containing 25-30 ml of solid media, such as Middlebrook 7H10. In addition, the purity of the culture can be further assessed using commercially available medium such as thioglycolate medium (Science Lab, catalogue number 1891) and soybean-casin medium (BD, catalogue number 211768).

BCG seed lots are stored at -80°C at a density of $0.1-2 \times 10^7$ cfu/ml. Typically, the liquid cultures are harvested at an optical density (600 nm) of 0.2 – 4.0 relative to a sterile control; the cultures are placed into centrifuge tubes of an appropriate size and the organisms are subjected to centrifugation at $8,000 \times g$ for 5-10 min. The supernatant is discarded and the organisms are resuspended in storage solution comprised of Middlebrook 7H9 containing 10-30% (v/v) glycerol at a density of $0.1-2 \times 10^7$ cfu/ml. These suspensions are dispensed into sterile 1.5 ml boron silicate freezer vials in 1 ml aliquots and then placed at -80°C .

EXAMPLE 1. Construction of rBCG-PfoA strains capable of escaping endosome

Construction of rBCG-PfoA strains capable of escaping the endosome was carried out by allelic exchange of the flanking regions of the *ureC* gene. As a result, the PfoA gene segment replaced the *ureC* gene, allowing stable chromosomal expression of PfoA. Specific details are described below.

Construction of the allelic exchange plasmid:

The allelic exchange plasmid is composed of the following DNA segments: An *oriE* sequence for the plasmid to replicate in *E. coli*, a kanamycin resistant gene sequence for selection in both *E. coli* and *Mycobacterium*, and an additional antibiotic selection marker (zeocin resistant gene), which is expressed by the Hsp60 promoter. The second marker was used to make a double selection, thus preventing spontaneous resistance to kanamycin during the process. For negative selection during the allelic exchange process, a sucrose sensitive gene was used. Finally, the left and right 1 kb flanking sequences of the *ureC* gene for the target BCG Danish 1331 strain were included with the PfoA gene in between. The PfoA gene is expressed under control of the Ag85B promoter. The Ag85B leader peptide sequence was used in place of the PfoA original secretion signal sequence for the secretion of PfoA. Finally, all these components were synthesized and assembled by Picoscript Inc (Houston, TX). The resultant plasmid is a mycobacterial suicide vector and the map for the plasmid that was obtained is as shown in Figure 1. The resultant plasmid construct was confirmed as shown in Figure 5.

Introducing the allele exchange plasmid into *Mycobacterium bovis* BCG Danish 1331 strain

The process of allele exchange is illustrated schematically in Figure 4, which outlines the major steps of the procedure. Those steps are described in detail below.

BCG Danish 1331 was cultured in 7H9 medium with 10% of OADC (oleic acid-albumin-dextrose-catalase)(BD Gibco) and 0.05% (v/v) of Tyloxapol (research and diagnostic lab) supplementation. When the culture reached log phase, the bacteria were collected and prepared as described previously (Sun *et al.*, 2004) for electroporation. Five micrograms of the allele exchange plasmid was introduced into freshly prepared electrocompetent cells using standard methodologies.

The above-constructed allelic exchange plasmid was introduced into *M. bovis* BCG Danish 1331 strain by the standard mycobacterial electroporation protocol for *Mycobacterium*. After electroporation, the cells were cultured overnight in 7H9 medium with 10% (v/v) OADC and 0.05% (v/v) of Tyloxapol supplementation. Then the cells were plated on 7H10 plates containing 50 ug/ml of both kanamycin and zeocin. The resultant colonies were picked and cultured in 7H9 medium containing 10% (v/v) of sucrose. The obtained culture was plated on 7H10 plates for cloning to obtain individual colonies, which were identified for the presence of PfoA gene in

place of ureC. A flow chart outlining the main steps of this procedure is given in Figure 4, and Table 1 describes the suicide vector, pAF 102, which is also depicted in Figure 1.

Table 1. Suicide vector used in the invention

Name	Backbone	Specific allele for allele exchange
pAF102	pAF 100	PfoA gene flanked by 1 kb flanks of ureC gene

Example 1 shows that shows that the *Mycobacterium* BCG strain is genetically engineered to express a selected endosomolytic protein that is active at neutral pH, permitting escape of the *Mycobacterium* from endosomes into the cytoplasm of the cell.

EXAMPLE 2. Validation of rBCG-PfoA Strain

Materials and Methods for Example 2.

Cultivation of *Mycobacterium*: For the following experiments, BCG strains were cultured at 37°C in Middlebrook 7H9 media (BD biosciences) with 10% OADC supplementation. Tyloxapol (0.05% v/v, Research Diagnostics Cat. No. 70400) was used to disperse the bacteria. In experiments in which growth was compared between different strains, the optical density (600 nm) was measured at different times post inoculation. For testing sensitivity to kanamycin, the culture was prepared and growth was measured as above except kanamycin was added to a final concentration of 50ug/ml. When solid medium was used to culture the bacteria, Middlebrook 7H10 agar (BD biosciences) was used. When appropriate, kanamycin was added to a final concentration of 50ug/ml, and sucrose was added to a final concentration of 3%.

Urease activity test: The resultant colonies from the sucrose plates described in Example 1 were first screened for a lack of urease activity using a urease testing kit (BD Difco) according to the manufacture's instructions. Briefly, a loop full of bacteria was resuspended in the manufacture supplied test buffer in a transparent tube. BCG Danish 1331 strain was used as a urease positive control. Buffer alone was used as the negative control. The reaction mixture was incubated at room temperature for 30 minutes and the result was judged based on the manufacture's instruction.

Genotype analysis of rBCG strains carrying Δ ureC:pfoA. PCRs with forward primer [acggctaccgtctggacat] (SEQ ID NO: 4) and reverse primer [cgatggcttcttcgatgc]

(SEQ ID NO: 5) were performed to amplify the DNA sequence of the *Pfo*-specific insertion allele and BCG genomic DNA sequences flanking the *ureC* gene. The PCR parameters were as follows: Step 1: 95°C 4 minutes one cycle; Step 2: 95°C one minute, 60°C 1 minute, and then 72°C one minute for a total 30 cycles; Step3: 72°C 10 minutes with one cycle. Step 4: 4°C storage. The resultant PCR products were analyzed by agarose gel electrophoresis and sequenced by automated dideoxynucleotide sequencing techniques, and the presence of a full-length *PfoA* gene in place of the *ureC* gene (i.e. $\Delta ureC::PfoA$) was confirmed.

Growth of AFV102 in Macrophages: The growth of the rBCG strain AFV102 *in situ* was tested in J774A.1 macrophage-like cells by determining mycobacterial colony-forming units (CFUs) in the infected macrophages. The efficacy of mycobacterial phagocytosis was determined by testing the intracellular CFU three hours after infection of J774A.1 cells. Subsequent long-term intracellular survival was performed by lysis of the cells to release the intracellular bacteria for numerating the CFU after washing with PBS five times, as previously described (Sun *et al.*, 2004).

Analysis of the hemolytic activity of the expressed Pfo by AFV102: To assess the secretion of PfoA by AFV102, the strain was grown to mid-logarithmic phase as described above. Then the culture supernatants and the bacteria were collected. The AFV102 bacterial pellet was re-suspended in 100 μ l of PBS (pH 7.0) containing 0.1% BSA in a 96 well V-bottomed plate. To test if the PfoA protein was secreted into the culture supernatant, the liquid culture was spun down and the supernatant was used for the test. For testing the expressed PfoA for its pH independent hemolysis activity, the samples were prepared as above except PBS buffer with different pH values was used as the reaction buffer. 100 μ l of 1% washed sheep erythrocytes was added to each well. The reaction was mixed gently and incubated at 37°C for 1 h with agitation. BCG Danish 1331 strain bacterium was used as the hemolysis negative control. α -hemolysin (Sigma) with known units of hemolysin activity was used in serial dilution as the hemolysis positive control. At the end of incubation, the reaction was pelleted by centrifugation at 500g for 15 minutes, and then the supernatant from the V-bottom plate was transferred into equivalent locations in a flat bottom 96-well plate and the optical density was measured (absorbance at 450 nm minus the absorbance at 540nm). The hemolytic activity of the PfoA molecule was quantified by measuring the optical density of the color change after red cell lysis. The intensity of the color measured is

in proportion to the amount of red cell lysis, which is then in proportion to the quantity of hemolysin. The sample values are then read off a standard curve through the use of known standards. Hemolytic units were defined as the dilution of the sample at which 50% of the sheep red blood cells were lysed.

Cytotoxicity of AFV102 to macrophages: The cytotoxicity of the recombinant strain on J774A.1 macrophages (ATCC No. A TIB-67) was determined by measuring the Lactate Dehydrogenase (LDH) released from infected cells using a “Cell Titer 96 Aqueous One Solution Cell Proliferation Assay” kit (Promega, cat #: G3580) according to the manufacture’s instruction. Briefly, the cells were infected with AFV102 bacteria at the multiplicity of infection of 10. At different times post infection, the supernatant was measured for the amount of LDH released from the cells, which was then compared with that of the BCG Danish 1331 strain. Uninfected normal cells were used as the negative control. The percentage of viable cells was calculated based on the amount of LDH released from the infected cells to that of the negative control cells (100% cell viability).

Results for Example 2.

AFV102 construction: During the construction of AFV102, the selected merodiploid bacteria, which harbor the entire knockout plasmid on its chromosome via allelic exchange of its homologous DNA segment with that on the knockout plasmid, were cultured on sucrose-containing Middlebrook 7H10 plates for the final allelic exchange to replace the *ureC* gene by the PfoA expression cassette. Of the colonies produced on the sucrose plates, one colony Pfo-105-5 (renamed as AFV102) was found to be urease negative, suggesting that the *ureC* gene had been replaced by the PfoA expression cassette. This bacterial colony was further subjected to genotype analysis by PCR for the genotype of $\Delta ureC::\Omega pfoA$. The resultant PCR reaction was analyzed by gel electrophoresis on a 1.2% agarose gel, and the results are presented in Figure 6. As can be seen, PCR using this bacterium as the template produced a PCR product of the expected size, which is larger than that of parental BCG Danish 1331 strain. The expected size of the DNA band for the genotype of $\Delta ureC::\Omega pfoA$ is 2180bps, while for parental BCG Danish1331 strain is the size is 1967 bps. The PCR product for colony 105-5 was further gel purified and sequenced by the commercial sequencing facility of Johns Hopkins University (Baltimore, MD). The sequencing result showed that this colony has the expected genotype of $\Delta ureC::\Omega pfoA$. In addition, PCR targeted to amplify the Kanamycin gene and the *sacB* gene from the AFV102 strain

failed to produce any PCR product (data not shown). These findings suggest that AFV102 has undergone the final allelic exchange step and has the desired genotype of $\Delta ureC::\Omega pfoA$.

Growth characterization and kanamycin sensitivity test for AFV102: Based on the urease activity test and genotyping results, clone 105-5 (renamed AFV102) exhibited the expected phenotype and desired genotype of $\Delta ureC::\Omega pfoA$.

AFV102 was then further tested for its ability to grow in 7H9 growth medium compared to that of the parental BCG Danish 1331 strain. The result is shown in Figure 7. As can be seen, the AFV102 construct has a very similar proliferation curve in 7H9 growth medium to that of the parental strain. In addition, in the presence of kanamycin, AFV102 growth declined to a similar extent as that of the parental BCG Danish 1331 strain, suggesting that, as expected, it has a similar sensitivity to kanamycin.

Cytotoxicity of AFV102: It has been reported that a single amino acid change in the PfoA protein (codon 137 substitution mutation from gga, encoding Gly, to cag, encoding Gln) results in the loss of toxicity to mammalian cells. Yet the protein retains the capability of mediating bacterial escape from a vacuole (Portnoy supra, 1996). The toxicity of the protein expressed from AFV102 was assessed by infecting J7741A cells with AFV102 bacteria. When compared with the uninfected normal cell control at different time points after infection, AFV102 did not cause any more significant cell death than the currently used BCG Danish 1331 vaccine strain (Figure 8).

Survival in an alveolar macrophage cell line: To investigate if the construct is able to survive within macrophages, mid-log phase cultures were used to infect J774A.1 alveolar macrophages. Cells were infected with a multiplicity of infection (MOI) of 1:1. Intracellular survival of the *Mycobacterium* was monitored by plate counting the bacterium at various time intervals after infection. As may be seen in Figure 9, the AFV102 construct showed a persistence phenotype similar to that of the parental strain, suggesting no defect in intracellular survival for this construct in J774A.1 cells.

Secretion of the PfoA protein by AFV102: Secretion of the PfoA protein by bacteria containing the AFV102 construct was tested by measuring the bacterial culture supernatant for enhanced hemolytic activity compared to that of the BCG Danish 1331 strain. Culture supernatants for both AFV102 and BCG Danish 1331 strain were harvested at the same optical density and compared for the ability to lyse

red blood cells. The results are shown in Figure 10. Consistent with the previous report, the BCG culture supernatant displayed a base-line level of hemolytic activity as a result of the bacteria releasing metabolites during growth, which may result in red cell lysis (Grode et al., *Journal of Clinical Investigation*, 115:2472-2479; 2005). In contrast, the AFV102 culture supernatant had a significant higher level of hemolytic activity compared to that of the BCG Danish 1331 strain, consistent with the secretion of PfoA molecules into the culture supernatant. In addition, the pH independent hemolytic activity of the PfoA was further tested and compared at both pH 5.5 and 7.0, and the results are shown in Figure 10. As can be seen, the supernatant from AFV102 has a similar hemolytic capacity at both pH 5.5 and 7.0, suggesting that the hemolytic activity secreted by the PfoA protein is pH independent, as expected.

Example 2 shows that the constructed strain has the predicted biological activities, and that the Pfo that is manufactured by the strain is secreted and has pH independent activity.

Example 3. rBCG-PfoA endosomal escape and animal immunogenicity test

A central paradigm of *Mycobacterium tuberculosis* pathogenesis is the arrest of phagosomal maturation. Armstrong and Hart (1971) established that *M. tuberculosis* phagosomes do not mix with ferritin-labelled lysosomes, referred to as the inhibition of phagosome-lysosome fusion. The vaccine strain *M. bovis* (BCG) was also found to reside in the phagosomal compartment, sequestered from the terminal endocytic organelles (Clemens and Horwitz, 1995; Hasan *et al.*, 1997; Via *et al.*, 1997). Clemens and Howitz (1995) found that the mycobacterial phagosomes persistently stain for transferrin receptor (TfR) at densities similar to those on the plasma membrane. The transferrin receptor is typically removed rapidly ($t_{1/2}$ in minutes) from endosomes and transported back to the plasma membrane; however, in mycobacterial phagosomes this process is halted and the phagosomes will contain the transferrin receptor. It is this phenomenon that allowed us to visualize phagosomes that contain mycobacteria. The phagosomes were labeled with antibodies to the transferrin receptor. At the same time, the mycobacteria were stained with a fluorescent dye that allows visual monitoring of the fate of the bacteria once inside the phagosome. The results showed that the rBCG-Pfo construct was able escape the endosome after infecting the cells.

Materials and Method for endosomal escape test: Bacteria and cells: BCG

Danish1331 and rBCG- $\Delta ureC::\Omega pfoA_{G137Q}$ (AFV102) were grown in 7H9 medium

with 10% (v/v) OADC and 0.05% (v/v) of Tyloxapol supplementation (of the growth medium) to an OD₆₀₀ of about 0.8-1.0. Before infection, the bacterial cells were labeled with Alexa Fluor 568 succinimidyl ester (Molecular Probes, Eugene, OR) in PBS at room temperature for 1-1.5 hours according to the manufacture's instruction. This dye forms very stable amide bonds to the primary amines located on proteins on the bacterial surface. Briefly, 10ml of the bacterial culture were pelleted and resuspended in 25mls of 0.625 ug/ml of Alexa Fluor 568 in PBS (pH7.2) and incubated at room temperature for 1-1.5 hours to label the bacteria. The labeled bacterial cells were then washed three times with PBS and resuspended in 7H9 growth medium and stored in the refrigerator overnight. J774A.1 cells were cultured in DMEM medium as previously described (Sun *et al*, 2004) in 6 well cell culture plates on human fibronectin coated coverslips. The cells were plated at a density of 3×10^6 cells/well and cultured for 2 days in a 37°C incubator with 5% CO₂ and humidity. During the infection, the labeled bacteria were pelleted and resuspended in DMEM+10% FBS medium and added directly to J774A.1 cells with a multiplicity of infection (MOI) of 10 for each cell. After 20 min, 8 hours and 24 hours, the cells were washed with room temperature (RT) phosphate buffered saline (PBS, pH 7.2). The cells were then fixed for 20 minutes at RT with 2% paraformaldehyde in PBS (pH 7.2). The fixed cells were then permeabilized with 0.1% Triton X-100 in PBS (pH 7.2) for 10 minutes at RT followed by washing twice with PBS (pH 7.2). Blocking was done for at least 2 hours at RT or overnight at 4°C with 3% bovine serum albumin (BSA), 5% normal goat serum (NGS), and 0.5% sodium azide in PBS (pH7.2). Blocking buffer was removed and then rat anti- mouse transferring receptor-FITC (US Biological, Swampscott, MA) was added at a dilution of 1:50 in PBS (pH 7.2) containing 1% BSA, 3% NGS, and 0.5% sodium azide followed by incubation at RT for at least 1 hour. Cells were then washed 2-3 times with PBS and mounted with vectashield mounting media on glass slides. Analysis was done at a magnification of 1500 using a Nikon TE2000 inverted microscope equipped with a Retiga EXI Mono, 12 bit cooled, IR filtered digital camera for imaging.

Result: When examined under the microscope, both BCG and rBCG-PfoA bacteria were found internalized by the host cells fifteen minutes after the infection, However, at 8 hours post infection, rBCG-PfoA bacteria were found outside the endosome, in contrast to BCG bacteria, which were found mostly located inside the host

phagosome. These results are illustrated schematically in Figures 11A-D, which illustrate bacterial invasion of a macrophage (Figure 11A), persistence of BCG within the endosome (Figure 11B), AFV102 bacterium within an early endosome (Figure 11C) and AFV102 bacteria escaping from the endosome into the cytoplasm of the cell (Figure 11D) due to secretion of recombinant PfoA. Enumeration of the bacteria showed that 100 AVF102 out of 138 had escaped the endosome (72%) after 8 hours post infection, while only 29 BCG out of 100 (26%) had escaped the phagosome. Examination of the bacteria in the 24 hours post infection sample yielded a similar result. This finding shows that expression of PfoA increases the release of AVF102 from phagosomes.

Further confirmation of this result was carried out using a different system in which a pH sensitive dye was used to label endosomes and lysosomes (Lysotracker-Red, Molecular probes, catalog number L-7528) under a similar experimental setting and procedure as above. Bacteria were visualized at wavelength emissions of 568, while phagosomes were visualized at wavelength emissions of 488. The result was consistent with the above observations.

Example 3 shows that recombinant strain AVF102 was capable of escaping the endosome while the BCG strain was much less efficient at escaping the endosome. Thus, strain AVF102 is much more likely to elicit a histocompatibility-Class I immune response than is BCG, and would be useful in vaccine applications.

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EXAMPLE 4. Formulation and vaccination strategies.

The strategy for vaccine formulation is based on studies to determine maximum viability and stability throughout the manufacturing process. This includes determination of maximum organism viability (live to dead) during culture utilizing a variety of commonly used medium for the culture of *Mycobacteria* to include the addition of glycerol, sugars, amino acids, and detergents or salts. After culture, cells are harvested by centrifugation or tangential flow filtration and resuspended in a stabilizing medium that allows for protection of cells during freezing or freeze drying. Commonly used stabilizing agents include sodium glutamate, amino acids or amino acid derivatives, glycerol, sugars and commonly used salts. The final formulation will provide sufficient viable organisms to be delivered by intradermal, percutaneous injection, perfusion or oral delivery with sufficient stability to maintain an adequate shelf-life for distribution and use.

Preclinical evaluation of TB vaccines

General safety test BALB/c mice in groups of 6 are infected intraperitoneally with 2×10^6 CFU of the rBCG strain(s) of interest and the analogous parental strains. The animals are monitored for general health and body weight for 14 days post infection. Animals that receive the BCG and rBCG strains remain healthy, and neither lose weight nor display overt signs of disease during the observation period.

Virulence of novel rBCG strains in immunocompetent mice. Groups of 15 immunocompetent BALB/c mice are infected intravenously with 2×10^6 rBCG and BCG parental strains, respectively. At day 1 post infection, three mice in each group are sacrificed and CFUs in spleen, lung and liver are analyzed to ensure that each animal has an equal infection dose. At weeks 4, 8, 12, and 16 post-infection, three mice in each group are sacrificed and CFUs in spleen, liver and lung are obtained to assess the *in vivo* growth of the rBCG strains as compared to the parental BCG strain.

Stringent safety test in immunocompromised mice. Immunocompromised mice possessing the SCID (severe combined immunodeficiency) phenotype in groups of 10 are infected intravenously with 2×10^6 cfu rBCG and the parental BCG strain. At day 1 after infection, three mice in each group are sacrificed and cfu in spleen, liver and lung are assessed to verify the inoculation doses. The remaining seven mice in each

group are monitored for general health and body weight. The survival of these mice is followed and the survival of rBCG-infected mice is compared to that of the parental strain infected animal during the entire observation period.

Guinea pig safety test. The safety of rBCG strains is also assessed in the guinea pig model in comparison to the parental BCG vaccine, which has a well-established safety profile in humans. First, the effect of the vaccine on the general health status of the animals, including weight gain, is examined. Guinea pigs are immunized intramuscularly with 10^7 (100x of vaccination dose) CFU of the recombinant and parental strains, and the animals are monitored for general health and body weight for six weeks. Post mortem examination is performed for animals that die before the six weeks period. All animals are sacrificed at the end of six weeks post infection and gross pathology is performed. For confirmation under this test, no body weight loss and no abnormal behavior is observed, and all organs appear normal at six weeks necropsy, and/or no adverse health effects are observed for rBCG-PfoA vaccine, and rBCG-PfoA-vaccinated animals gain weight at the normal rate compared to the parental strain inoculated animals.

At the same time, bacterial levels in animal organs are monitored. Guinea pigs immunized with either the parental or recombinant vaccine are euthanized at various intervals after inoculation, after which the lungs, spleens, and regional (inguinal) lymph nodes are assayed for CFU of BCG or rBCG.

Toxicity test. To evaluate the toxicity of the rBCG strains, guinea pigs (12 in each group) are vaccinated intradermally with 1 dose, four times higher than the single dose or four times lower than the single dose of human use rBCG strains, BCG parental strain or saline. At day 3 post vaccination, six animals are sacrificed to access the acute effects of the vaccine on these animals. At day 28 post vaccination, the remaining six animals are sacrificed to evaluate the chronic effects on the animals. At both time points, the body weight of each animal is obtained, and gross pathology and appearance of the injection sites are examined. Blood is taken for blood chemistry, and the histopathology of the internal organs and injection sites is performed.

Murine protection study. C57Bl/6 mice (female, 5-6 weeks of age) in groups of 13 are immunized subcutaneously with 10^6 CFU of rBCG, parental BCG or saline. Another group of mice is used as healthy controls. Eight weeks after immunization, mice are challenged with *M. tb* Erdman strain (or H37Rv Kan-resistant strain) by an aerosol generated from a 10-ml single-cell suspension containing a total of 10^7 CFU

of the challenge strain, a dose that delivers ~100 live bacteria to the lungs of each animal, as described previously (Brodin *et al.*, J Infect Dis., 190(1):115-122; 2004). The inoculated animals are monitored for survival along with unchallenged animals. Following the challenge, the animals are monitored for weight loss and general health. At day 1 after challenge, three mice in each group are sacrificed for lung CFU to confirm the challenge dose and one animal is sacrificed for spleen and lung histopathology. Five weeks after challenge, nine animals in each group are sacrificed, and histopathology and microbiology analysis of the animal is performed. Lung and spleen tissues from six mice are evaluated for CFU counts (plates with selection supplements are used to distinguish the vaccine strain from the challenge strain). If challenged with the H37Rv-kan resistant strain, Kan or TCH (thiophene-2-carboxylic acid hydrazide) is used to distinguish the challenge strain from the vaccine strain. If the *M. tb* Erdman strain is used to challenge, TCH is used to distinguish the vaccine strain from the challenge strain (BCG is susceptible, but *M. tb* is naturally resistant).

Induction of cutaneous delayed-type hypersensitivity (DTH). Specific pathogen free (SPF) guinea pigs are immunized intradermally with 10^3 rBCG or BCG parental strains. Nine weeks after immunization, the animals are shaved over the back and injected intradermally with 10 µg of PPD (protein purified derivative) in 100 µl of phosphate buffered saline. After 24 hours, the diameter of hard induration (DTH) will be measured. rBCG strains induce DTH equal to or greater than that induced by parental BCG strains.

Guinea pig challenge study. To determine the efficacy of the rBCG vaccines against *M. tb* challenge, guinea pigs (young adult SPF Hartley, 250-300 grams, male) are immunized in groups of 12, each with rBCG, parental BCG strain or saline. The vaccines and controls are administered intradermally with 10^6 cfu. At 10 weeks after immunization, the rBCG-, BCG- and sham-immunized animals are challenged by aerosol with the *M. tb* by an aerosol generated from a 10-ml single-cell suspension containing a total of 10^7 cfu of *M. tb*; this procedure delivers ~100 live bacteria to the lungs of each animal, as described previously (Brodin *et al.*, 2004). Following challenge, the animals are monitored for survival, weight loss and general health along with a healthy group of unvaccinated, unchallenged animals. Six animals in each group are sacrificed at 10 weeks post challenge and the remaining six in each group at 70 weeks post challenge for long-term evaluation. At both time points, histopathology and microbiology analysis of the animal are performed. Lung and

spleen tissues are evaluated for histopathology and CFU count (plates with selection supplements are used to distinguish the vaccine strain from the challenge strain). If challenged with H37Rv-kan resistant strain, then Kan or TCH is used to distinguish the challenge strain from the vaccine strain. If the *M. tb* Erdman strain is used to challenge, TCH is then used to distinguish the vaccine strain from the challenge strain (BCG is susceptible but *M. tb* is naturally resistant). For a successful challenge study, sham immunized animals die most rapidly after challenge, and the rBCG-immunized animals survive longer than the BCG parental strain immunized animals.

Primate safety and challenge study. More recently, non-human primates have been used for evaluation of vaccines against *M. tb*. The evolutionary relationship between humans and non-human primates and the similar clinical and pathologic manifestations of tuberculosis in these species has made the non-human primate model attractive for experimental studies of TB disease and vaccine efficacy.

This model, characterized by the development of lung cavitation, appears to be applicable to human TB. The course of infection and disease is followed by X-ray and weight loss, as well as a variety of hematological tests, including erythrocyte sedimentation rate (ESR), peripheral blood mononuclear cell (PBMC) proliferation and cytokine production, cytotoxic T lymphocyte (CTL) activity, and antibody responses. Following infection, the cynomolgus monkey develops lung pathology with characteristic lesions, and, depending on the challenge doses, death from acute respiratory infection occurs within four-to six months after infection. Lower infection doses can lead to chronic infections without disease, much like in humans.

The study directly compares varying doses of the BCG parental strain versus recombinant BCG administered either alone or followed by two subsequent boosters with the vaccine comprising sequences that are over expressed in rBCG constructs. The latter is delivered by any of several known means, including but not limited to: as a recombinant protein based in a suitable adjuvant formulation, as DNA or as an Ad35 construct.

The first study evaluates the protective efficacy of the parental BCG vs rBCG constructs without a booster. This study comprises three groups with 10 animals in each group: one group each comprising BCG, rBCG and saline. Two animals from each group are skin tested with the over expressed antigens in the rBCG constructs as well as with standard PPD and saline as controls. A positive and larger induration in the rBCG group compared with the BCG is indicative of *in vivo* vaccine take and the

elicitation of an immune response. The remaining eight animals from each group are aerosol challenged with low dose *M. tb* Erdman strain and protection is measured by reduction of bacterial burden at 16 weeks post challenge or with survival as the end point.

The follow up BCG prime protocol is essentially the same as above except that the animals are first vaccinated with BCG, rBCG and saline followed by two boosters with the over-expressed antigens.

The immunogenicity and protection study in the non-human primate model investigates immunobiological and immunopathological aspects of tuberculosis in macaques for efficacy studies on rBCG constructs. The animals are juvenile to young adults raised in captivity with an average weight of 2 to 3 kg that have been thoroughly conditioned prior to the start of the experiment. Pre-inoculation studies include baseline blood tests that include routine hematological studies and erythrocyte sedimentation rates as well as lymphocyte proliferation assays. Skin testing is done with PPD to ensure lack of sensitivity to tuberculin and chest x-rays are obtained as part of the pre-infection profile. The immunization period lasts 21 weeks in total, covering primary vaccination with BCG or rBCG at week = 0, and antigen boosts at weeks 12 and 16. Antigen-specific immunity is assessed by measuring proliferation and interferon γ (IFN γ) secretion in lymphocyte stimulation tests. The frequency of IFN γ producing lymphocytes is determined by enzyme-linked immunosorbent assay (ELISPOT) or fluorescence-activated cell sorter (FACS). To this end, blood samples are drawn at weeks 0, 4, 8, 12, 16 and 20 weeks relative to primary vaccination.

Four to six weeks after the last immunization, animals are challenged by intratracheal installation of 3 ml (1,000 cfu) of the *M. tuberculosis* Erdman strain on the same day and with the same preparation. The course of the infection is assessed for weight loss, fever, elevated erythrocyte sedimentation rate (ESR), DTH to PPD, *in vitro* proliferative response of PBMC stimulated with PPD and antigen over-expression in rBCG followed by measurements of the levels of IFN-g production. Chest x-rays are performed to detect abnormalities consistent with pulmonary TB, and finally, necropsy at 12-16 weeks post challenge.

Clinical evaluation of TB vectors and vaccines

Safety and toxicity studies Preclinical safety and toxicity studies as mandated by federal regulations are performed according to preclinical toxicology and safety

studies as described above. Following these studies, human safety studies are performed. These studies are performed initially in healthy Quantiferon negative adults, followed by age de-escalation into children and neonates.

Immunogenicity studies: Immunogenicity studies in mice and primates utilize but are not limited to standard methods of evaluating cellular immunity such as INF γ , ELISPOT, flow cytometry with short and long term antigen or peptide stimulation, etc. Similar methodologies are utilized for evaluation of human responses. Tetramer studies are employed for evaluation of CD4 and CD8 responses following vaccination of humans.

Optimization of prime-boost strategies: rBCG performs well as a stand-alone vaccine against TB or other diseases for which it has been engineered to express relevant antigens. rBCG as described here as a vaccine for TB or expressing antigens to protect against other diseases also performs extremely well to prime the immune system for booster immunization with recombinant proteins mixed with adjuvants, or viral or bacterial vectored antigens. Both in animal preclinical studies and human studies the BCG primes followed by recombinant protein/adjuvant or vector boosts are optimized in terms of regimens and doses. These prime boost strategies are the most potent means for inducing immunity in humans because of the potency of the BCG prime embodied in the present invention, followed by focusing and enhancing the booster response of the immune system by recombinant protein or vector.

Post-exposure therapeutic vaccine studies in animals: C57BL/6 mice but not limited to this animal will be used for establishing latent infection; therapeutic vaccines will be given to the mice at the point when only negligible *M. tb* specific immunity has been induced by low dose infection and a later time point when *M. tb* specific immunity induced and has subsided and is predominated by memory T cells. Then, the therapeutic benefit of the vaccines will be assessed in mice 2 and 5 months after the last therapeutic vaccine delivery by enumerating cfu counts in lungs and spleens of individual mice. The cfu counts will be analyzed by standard statistical methods in the groups of mice and the results will be used to address whether therapeutic vaccination significantly reduces latent *M. tb* infection in mice. Similar methodologies are utilized for evaluation of responses of other animals when necessary.

Clinical evaluation of BCG vectors: oral administration of rBCG vaccines. Oral vaccination of the target animal with the rBCG of the present invention is achieved

using methods previously described (Miller et al., *Can Med Assoc J.* 121(1):45-54; 1979). The amount of the rBCG of the present invention administered orally will vary depending on the species of the subject, as well as the disease or condition that is being treated. Generally, the dosage employed is about 10^3 to 10^{11} viable organisms, and preferably about 10^5 to 10^9 viable organisms.

The rBCG are generally administered along with a pharmaceutically acceptable carrier or diluent. The particular pharmaceutically acceptable carrier or diluent employed is not critical to the present invention. Examples of diluents include a phosphate buffered saline, buffer for buffering against gastric acid in the stomach, such as citrate buffer (pH 7.0) containing sucrose, bicarbonate buffer (pH 7.0) alone (Levine *et al.*, *J. Clin. Invest.*, 79:888-902; 1987; and Black *et al.*, *J. Infect. Dis.*, 155:1260-1265; 1987), or bicarbonate buffer (pH 7.0) containing ascorbic acid, lactose, and optionally aspartame (Levine *et al.*, *supra*, 1989; *Lancet*, II:467-470; 1988). Examples of carriers include proteins, e.g., as found in skim milk, sugars, e.g., sucrose, or polyvinylpyrrolidone. Typically these carriers would be used at a concentration of about 0.1-90% (w/v) but preferably at a range of 1-10% (w/v).

EXAMPLE 5. Safety in severe combined immunodeficiency (SCID) mice

As mentioned earlier, expression of Llo in BCG proved relatively ineffective at promoting endosome escape, probably due to the low activity of Llo at neutral pH of the microenvironment of the modified endosome in which BCG resides (Hess, *et al.*, *Proc Natl Acad Sci* 95:5299; 1998; Grode *et al.*, *J. Clin. Invest.*, 115(9):2472; 2005). Nonetheless, the expression of Llo apparently modified the endosome sufficiently to reduce the virulence of BCG-Llo+ in SCID mice (Grode *et al.*, 2005). This observation suggests that retargeting BCG to the cytoplasm of host cells has the potential of improving both the immunogenicity and safety of this antique live TB vaccine. The goal of this study, therefore, is to determine the safety of endosome-escape strain AFV102 which express PfoA_{G137Q} in SCID mice.

To this end, parent strains BCG Danish 1331 (BCG₁₃₃₁) and the derivative of BCG₁₃₃₁ that expresses PfoA_{G137Q}, strain AFV102 were grown in 2 L flasks with agitation (150 oscillations per minute) to late-log phase (Optical density at 540 nm of 6.5-7.5). The bacteria were harvested by centrifugation and stored at 1.5×10^9 cfu/ml at -80°C in saline + 0.05% (v/v) tyloxapol + 10% (v/v) glycerol.

Inocula were prepared by thawing the above bulk vials on ice and making sets of serial dilutions in endotoxin-free (<0.05 EU/ml) normal saline (0.85% w/v NaCl). These dilutions were used to inoculate groups of 6 SCID mice subcutaneously with the respective doses shown in Table 2 suspended in a volume of 0.1 ml.

Table 2. Design of SCID mouse safety study

Group	Vaccine	Dose
1	PBS	
2	BCG ₁₃₃₁	3×10^4
3	BCG ₁₃₃₁	3×10^5
4	BCG ₁₃₃₁	3×10^6
5	BCG ₁₃₃₁	3×10^7
6	AFV102	3×10^5
7	AFV102	3×10^6
8	AFV102	3×10^7
9	AFV102	3×10^8

After inoculation, the mice are monitored for survival over a period of 100 days post-inoculation. The results of this study show that mice inoculated with AFV102 survive longer than mice that receive the analogous dose of BCG₁₃₃₁.

The use of a heterologous booster vaccine to bolster immunity elicited by BCG has gained attention recently. Thus, BCG-primed laboratory animals and humans develop impressive cellular immune responses following a heterologous boost comprised of modified vaccinia Ankara (MVA) encoding Mtb antigen 85A (herein "Ag85A"; also known as Rv3804c; Vordemeier *et al.*, Immunol. 112(3):461; 2004; McShane *et al.*, Nature Med. 10(11):1240; 2004); in contrast, naïve individuals develop relatively unimpressive responses to the MVA-Ag85A vector (McShane *et al.*, 2004). Furthermore, there are independent studies showing that laboratory animals primed with BCG and boosted with either MVA-Ag85A (Williams *et al.*, Infect Immun. 73(6):3814; 2005) or subunit vaccine Mtb72f (Brandt *et al.*, Infect.

Immun. 72(11):6622; 2004) develop greater levels of resistance to an Mtb challenge than that achieved by vaccination with BCG alone bolstered support for this approach. Although these studies did not define the correlates of protection, it is clear that heterologous prime-boost vaccination strategies offer an effective means to invoke protection against Mtb.

The goal of this and the following example, therefore, is to evaluate endosome-escape strain AFV102 in a prime-boost vaccination regimen. The aim of the experiment in this example is to optimize the interval in a prime-boost regimen in which endosome-escape strain AFV102 is used as the prime and a replication-deficient adenovirus serotype 35 vaccine vector (Vogels *et al.*, J Virol. 77(15):8263-71; 2003; Barouch *et al.*, J. Immunol. 172(10):6290; 2004) which harbors an expression cassette encoding a fusion protein comprised of Mtb genes Rv3804c-Rv1886-Rv0288 under the control of the cytomegalovirus early promoter (Vogels *et al.*, J Virol. 77(15):8263-71; 2003) is used as the boost. The boost is administered by the intranasal (in) route, since adenoviruses expressing TB antigens are more effective by this route than by conventional parenteral routes of administration (Wang *et al.*, J. Immunol. 173(10):6357; 2004).

Accordingly, groups of 10 SPF male Hartley guinea pigs (250-300 grams) are immunized as shown in table 3 so as to evaluate 14, 18 and 21 week prime-boost intervals.

Table 3. Guinea pig regimen study design

Group	Prime I (Day 1)	Prime II (Week 3)	Prime III (Week 7)	Boost (Week 21)
1	Saline (id)	-	-	-
2	AFV102 (id)	-	-	Ad35-TBS (in)
3	-	AFV102 (id)	-	Ad35-TBS (in)
4	-	-	AFV102 (id)	Ad35-TBS (in)
5	-	-	-	Ad35-TBS (in)

Note: Ad35-TBS denotes a replication-deficient adenovirus serotype 35 vaccine vector (Vogels *et al.*, J Virol. 77(15): 8263-71; 2003; Barouche *et al.*, J. Immunol. 172(10): 6290; 2004) which harbors an expression cassette encoding a fusion protein comprised of Mtb genes Rv3804c-Rv1886-Rv0288 under the control of the cytomegalovirus early promoter (Vogels *et al.*, J Virol. 77(15): 8263-71; 2003).

The primes are administered intradermally at a dose of 10^6 cfu in 0.1 ml of 10% glycerol. Control mice are given 0.1 ml 10% glycerol intradermally alone. At 14 weeks after the prime the guinea pigs are given a boost comprised of Ad35-TBS and are administered by intranasally at a dose of 10^9 plaque forming units (i.e. Vogels *et al.*, 2003; Barouch *et al.* 2004) suspended in 10 μ l of PBS.

At 14 weeks after the boost, the animals are challenged by aerosol with Mtb strain Erdman by an aerosol generated from a 10-ml single-cell suspension containing a total of 10^7 cfu of Mtb; this procedure delivers ~ 100 live bacteria to the lungs of each animal, as described previously (Brodin *et al.*, 2004). At 5 weeks after the challenge, the animals in each group are sacrificed and the lungs and spleens are collected for histological and microbiological analysis. In the latter instance, lung and spleen tissues from the guinea pigs are evaluated for cfu counts. Since Mtb Erdman strain is used to challenge, TCH is added to the media to distinguish vaccine strain, which is sensitive to TCH, from the challenge strain.

The results of this study identify the optimal interval between the rBCG prime and the Ad35-TBS boost.

EXAMPLE 6. Immunization Challenge

To measure the potency of candidate TB vaccine strain AFV102 against Mtb challenge, groups of 8 (young-adult SPF Hartley guinea pigs (250-300 grams) are immunized as shown in Table 4.

Table 4: Guinea pig challenge study design

Group	Prime (Day 1)	Boost (Week n*)	Challenge (Boost + 14 weeks)
1	Saline (id)	-	100 cfu Erdman
2	BCG Danish 1331 (id)	-	100 cfu Erdman

3	Saline (id)	Ad35-TBS (in)	100 cfu Erdman
4	AFV102 (id)	AFV102 (id)	100 cfu Erdman
5	AFV102 (id)	Ad35-TBS (in)	100 cfu Erdman

Note:

1. n* denotes the interval between the prime and the boost will be the value defined in the preceding example.
2. Ad35-TBS denotes a replication-deficient adenovirus serotype 35 vaccine vector ((Vogels *et al.*, J Virol. 77(15): 8263-71; 2003; Barouche *et al.*, J. Immunol. 172(10): 6290; 2004) which harbors an expression cassette encoding a fusion protein comprised of Mtb genes Rv3804c-Rv1886-Rv0288 under the control of the cytomegalovirus early promoter (Vogels *et al.*, J Virol. 77(15): 8263-71; 2003).

The primes in groups 4 and 5 are administered intradermally at a dose of 10^6 cfu in 0.1 ml of 10% glycerol. Control mice in group 1 and 3 are given 0.1 ml 10% glycerol intradermally alone. Control mice in group 2, 10^6 cfu of BCG Danish 1331 in 0.1 ml of 10% glycerol.

At 14 weeks after the prime the guinea pigs are boosted. In group 5, the boost is comprised of AFV102 and is administered intradermally at a dose of 10^6 cfu in 0.1 ml of 10% glycerol. In groups 4 and 6 the boosts are comprised of Ad35-TBS and are administered by intranasally at a dose of 10^9 plaque forming units (i.e. Vogels *et al.*, 2003; Barouch *et al.* 2004) suspended in 10 μ l of PBS.

At 14 weeks after the final immunization, the animals are challenged by aerosol with the Mtb by an aerosol generated from a 10-ml single-cell suspension containing a total of 10^7 cfu of Mtb; this procedure delivers ~100 live bacteria to the lungs of each animal, as described previously (Brodin *et al.*, 2004). Following challenge, the animals are monitored for survival along with a healthy group of unvaccinated, unchallenged animals. The animals are also monitored for weight loss and general health.

The results of this study demonstrate that sham-immunized animals die most rapidly after challenge, animals vaccinated with BCG intradermally without a boost

display an intermediate mean time to death and animals immunized with AFV102 and boosted intranasally with Ad35-TBS survive the longest.

EXAMPLE 7. Apoptosis

Apoptosis is programmed cell death that differs dramatically from necrotic cell death in terms of its induction and consequences. Apoptosis of cells containing foreign antigens is a powerful known stimulus of cellular immunity against such antigens. The process by which apoptosis of antigen containing cells leads to cellular immunity has sometimes been called cross-priming.^{1,2,3} There are several mechanisms for induction of apoptosis which lead to increased antigen specific cell mediated immunity. Caspase 8 mediated apoptosis leads to antigen specific cellular immune protection.⁴ Production of Caspase 8 in the cell cytoplasm by recombinant BCG who escape the endosome will be a powerful additional method for inducing programmed cell death in the context of foreign antigens expressed by the recombinant BCG, against BCG and other tuberculosis antigens over-expressed by the recombinant BCG as well as against antigens of BCG itself leading to high levels of antigen specific cellular immunity. Death receptor-5 (DR-5) also known as TRAIL-R2 (TRAIL receptor 2) or TNFR-SF-10B (Tumor Necrosis Factor-Superfamily member 10B) also mediates caspase 8 mediated apoptosis.⁴ Reovirus induced apoptosis is mediated by TRAIL- DR5 leading to subsequent clearance of the virus.⁵ Expression of DR-5 by recombinant BCG which escape the endosome should provide a potent adjuvant effect for induction of antigen specific cellular immunity against rBCG expressed antigens. Antigen expressing cells can also be induced to undergo apoptosis through Fas ligation, which is a strong stimulus for induction of antigen specific cellular immune responses.⁶ Recombinant BCG escaping the endosome expressing Fas or Fas cytoplasmic domain/CD4 ectodomain fusion protein will induce apoptosis and antigen specific cellular immune responses.

The enhancement of cellular immunity by rBCG endosome escape strains or by rBCG endosome escape strains which produce additional enhancers of apoptosis described above is not limited to BCG antigens or antigens specifically coded for over-expression by rBCG but includes any antigen in the eukaryotic cell where the aforementioned rBCG can invade. As an example if such an rBCG is delivered to tumor cells where apoptosis is induced then cellular immunity against important tumor antigens will be induced with elimination, reduction or prevention of the tumor

and/or metastasis. This anti-tumor effect will be in addition to the general anti-tumor effect that BCG generates when given locally such as the case with bladder cancer.

In a further embodiment of this invention, rBCG with endosome escape or rBCG with endosome escape enhanced by production of specific mediators of apoptosis, delivered inside tumor or other cells wherein such rBCG also produce foreign antigens against which strong cellular immune responses will be mounted will induce the production of strong cellular responses against those tumor cells or other eukaryotic cells containing these antigens. These cellular responses will lead to immune mediated tumor cell destruction, further cross priming and induction of cellular immunity against tumor or other important antigens with subsequent elimination, reduction or prevention of the tumor and/or metastasis. An example of such a foreign antigen is an HLA antigen different from the host cell HLA against which a strong heterologous cellular response will be mounted.

rBCG with endosome escape or rBCG with endosome escape whose apoptotic induction properties are enhanced by expression of specific mediators of apoptosis that also deliver specific tumor antigens will induce strong antigen specific cellular responses against these tumor antigens, including breaking of some tolerance for these antigens leading to elimination, reduction or prevention of tumors and/or metastasis without the need for direct delivery of the rBCG into the tumor itself.

Apoptosis following DNA damage or caspase 9 induces tolerance to certain antigens. Induction of tolerance is important in controlling or preventing autoimmune diseases such as but not limited to diabetes, rheumatoid arthritis, Crohns disease, inflammatory bowel disease and multiple sclerosis. Production of caspase 9 or other apoptosis mediated tolerance inducing proteins by rBCG escaping the endosome in cells such as but not limited to β pancreatic cells, colorectal and nerve cells will produce limited apoptosis which will induce tolerance against the antigen targets of autoimmunity in those cells thereby treating or preventing the autoimmune disease condition. Identification of specific antigens involved in autoimmune reactions will allow induction of tolerance against these autoimmune target antigens through endosome escape rBCG production of both these antigens and of caspase 9 or other molecules capable of inducing apoptotic mediated tolerance. Such rBCG will treat and/or prevent these autoimmune diseases.

References for Example 7.

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EXAMPLE 8. Over-expression of vaccine antigens in an rBCG strain capable of escaping the endosome

To over-express TB antigens into rBCG strain AFV102, sequences encoding the Rv3031 promoter functionally linked to sequences encoding Rv3804c (also known as Ag85A), Rv1886 (also known as Ag85B) and Rv0288 (also known as TB10.4) were inserted into the *PacI* site of pAF100. The resulting plasmid, pAF105 (Figure 12), was subsequently digested with restriction endonuclease *NdeI* to remove the *E. coli* replicon and kanamycin-resistance gene, and re-circularized by ligation

with T4 ligase. This DNA (1-2 μ g) was introduced into rBCG strain AFV102 by electroporation. The bacteria were cultured in 8.75 cm plates containing 25-30 ml of solid media (Middlebrook 7H10). Following a prescreen by PCR to detect colonies which harbor the antigen expression plasmid, a selected rBCG colony, which is both PfoA-positive and contains the TB antigen expression cassette, is designated AFV112 and is expanded to 500 ml in agitated liquid media (Middlebrook 7H9) at 37°C. Once the culture reaches late-log phase, glycerol is added to the 500 ml culture to a final concentration of 10% (v/v) and the premaster seed is stored in 5 ml aliquots at -80°C.

The purity of BCG and rBCG cultures are evaluated by evenly spreading 100 μ l aliquots of the BCG culture serially diluted (e.g. 10-fold steps from Neat - 10^{-8}) in phosphate buffered saline (PBS) onto 8.75 cm plates containing 25-30 ml of solid media (Middlebrook 7H10). PCR and restriction endonuclease analysis of plasmid DNA is used to confirm that the desired genotype is present in each rBCG isolate. In addition, PCR-generated DNA fragments are sequenced by automated dideoxynucleotide sequencing techniques to confirm the presence of full-length genes.

To assess the secretion of PfoA by AFV102 and AFV112 harboring the TB antigen expression plasmid, both strains are grown to mid-logarithmic phase, as described above. The culture supernatants of these cultures are collected and filtered through 0.2-mm membrane filters, as previously described (Hess et al., Proc. Natl. Acad. Sci., 95:5299-304; 1998). The culture filtrate proteins then are assessed for hemolytic activity, as described above. The results show that AFV102 and AFV112 display similar levels of hemolytic activity and that AFV112 retains the $\Delta ureC::\Omega pfoA_{G137Q}$ allele and expresses a functional PfoA protein.

Finally, expression of the TB antigens is assessed in culture supernatants proteins separated on 10-15% SDS-PAGE gels. The results show increased expression of Rv3804c and Rv1886. Since Rv0288 is not expected to be over expressed in the culture supernatant, over expression of this 10 kDa protein, which is expressed on the same mRNA as Rv3804c and Rv1886, is inferred by the observation that Rv3804c and Rv1886 are over expressed.

Taken as a whole, this example demonstrates that it is possible to generate and rBCG strain which both expresses PfoA and over expresses TB antigens. Such a strain has potential to serve as a second generation TB vaccine.

The scope of the claims should not be limited by the preferred embodiments set forth in the examples, but should be given the broadest interpretation consistent with the description as a whole.

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<120> Recombinant BCG Strains with Enhanced Ability to Escape the Endosome

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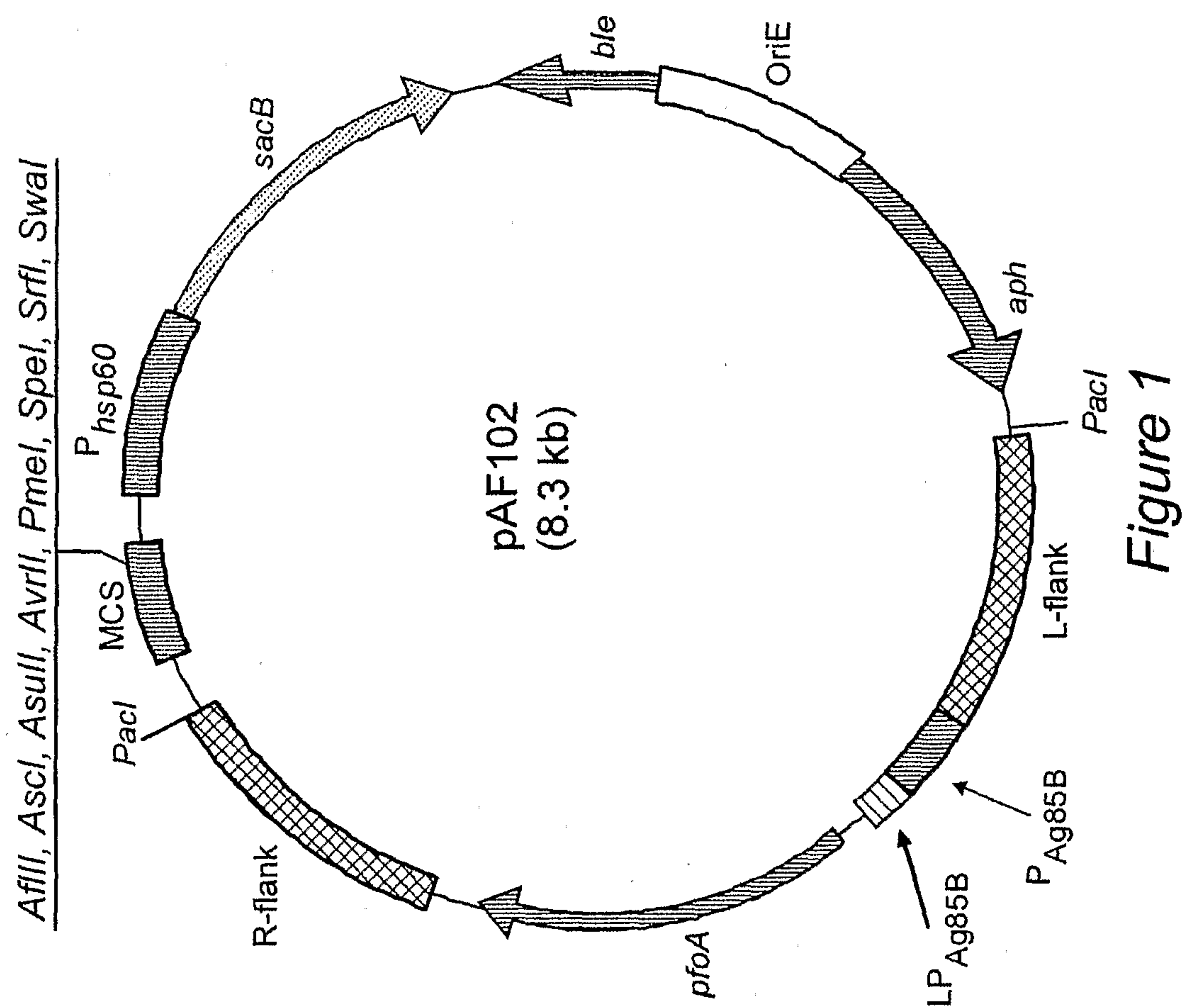
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We Claim:

1. A recombinant *Mycobacterium* that is genetically engineered to include a nucleic acid sequence that encodes an expressable and secretable Prefringolysin O (PFO) protein that is active at a pH of 6-8.
2. The recombinant *Mycobacterium* of claim 1, further comprising one or more nucleic acid sequences that encode one or more proteins of interest.
3. The recombinant *Mycobacterium* of claim 2, wherein said one or more proteins of interest include one or more *Plasmodium* antigens.
4. The recombinant *Mycobacterium* of claim 1, wherein said recombinant *Mycobacterium* is an attenuated *Mycobacterium*.
5. The recombinant *Mycobacterium* of claim 4, wherein said attenuated *Mycobacterium* is Bacille Calmette Guerin (BCG).
6. The recombinant *Mycobacterium* of claim 5, wherein said BCG is BCG Danish 1331.
7. The recombinant *Mycobacterium* of claim 1, wherein said nucleic acid sequence that encodes an expressable and secretable PFO protein is present in a chromosome of said recombinant *Mycobacterium*.
8. The recombinant *Mycobacterium* of claim 1, wherein said nucleic acid sequence that encodes an expressable and secretable PFO protein replace a urease C gene in a chromosome of said recombinant *Mycobacterium*.
9. The recombinant *Mycobacterium* of claim 2, wherein said nucleic acid sequences that encode one or more proteins of interest are present in a chromosome of said recombinant *Mycobacterium*.

10. The recombinant *Mycobacterium* of claim 2, wherein said one or more proteins of interest include *Mycobacterium tuberculosis* (Mtb) antigens.
11. The recombinant *Mycobacterium* of claim 10, wherein said Mtb antigens are selected from the group consisting of Ag85A, Ag85B, TB 10.4, Rv0125, Rv0203, Rv0287, Rv0288, Rv0603, Rv1196, Rv1223, Rv1271c, Rv1733c, Rv1738, Rv1804c, Rv1886, Rv2031c, Rv2032, Rv2253, Rv2290, Rv2389c, Rv2626c, Rv2627c, Rv2779c, Rv2873, Rv2875, Rv3017c, Rv3407, Rv3804c, Rv3810, and Rv3841.
12. The recombinant *Mycobacterium* of claim 2, wherein said nucleic acid sequences that encode one or more proteins of interest are present on a plasmid.
13. A composition comprising an aqueous solution and a recombinant *Mycobacterium* that is genetically engineered to include:
 - a nucleic acid sequence that encodes an expressable and secretable Prefringolysin O (PFO) protein that is active at a pH of 6-8; and
 - one or more nucleic acid sequences that encode one or more proteins of interest.
14. The composition of claim 13, wherein said one or more proteins of interest include *Mycobacterium tuberculosis* (Mtb) antigens.
15. The composition of claim 14, wherein said Mtb antigens are selected from the group consisting of Ag85A, Ag85B, TB 10.4, Rv0125, Rv0203, Rv0287, Rv0288, Rv0603, Rv1196, Rv1223, Rv1271c, Rv1733c, Rv1738, Rv1804c, Rv1886, Rv2031c, Rv2032, Rv2253, Rv2290, Rv2389c, Rv2626c, Rv2627c, Rv2779c, Rv2873, Rv2875, Rv3017c, Rv3407, Rv3804c, Rv3810, and Rv3841.
16. The composition of claim 13, wherein said nucleic acid sequences that encode one or more proteins of interest are present on a plasmid.
17. The composition of claim 13, wherein said recombinant *Mycobacterium* is an attenuated *Mycobacterium*.

18. The composition of claim 17, wherein said attenuated *Mycobacterium* is Bacille Calmette Guerin (BCG).
19. The composition of claim 18, wherein said BCG is BCG Danish 1331.
20. The composition of claim 13, wherein said nucleic acid sequence that encodes an expressable and secretable PFO protein is present in a chromosome of said recombinant *Mycobacterium*.
21. The composition of claim 13, wherein said nucleic acid sequence that encodes an expressable and secretable PFO protein replace a urease C gene in a chromosome of said recombinant *Mycobacterium*.
22. The composition of claim 13, wherein said nucleic acid sequences that encode one or more proteins of interest are present in a chromosome of said recombinant *Mycobacterium*.
23. The composition of claim 18, wherein said composition is a vaccine.
24. The composition of claim 23, wherein said vaccine is a vaccine for *Mycobacterium tuberculosis*.
25. The composition of claim 13, wherein said composition further comprises an adjuvant.
26. The composition of claim 13, wherein said composition further comprises a pharmacologically suitable carrier.
27. The composition of claim 13, wherein said one or more proteins of interest include *Plasmodium* antigens.



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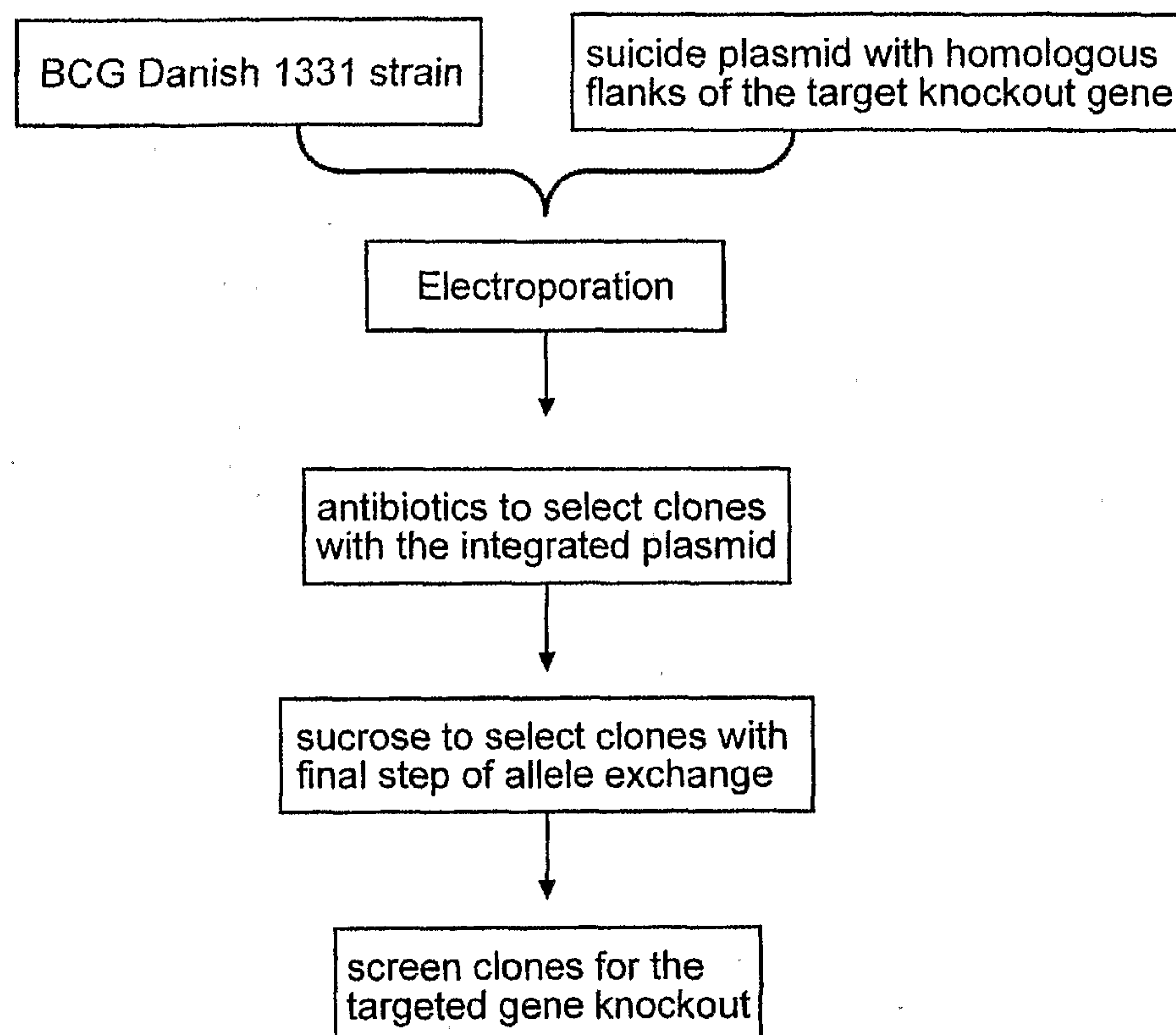
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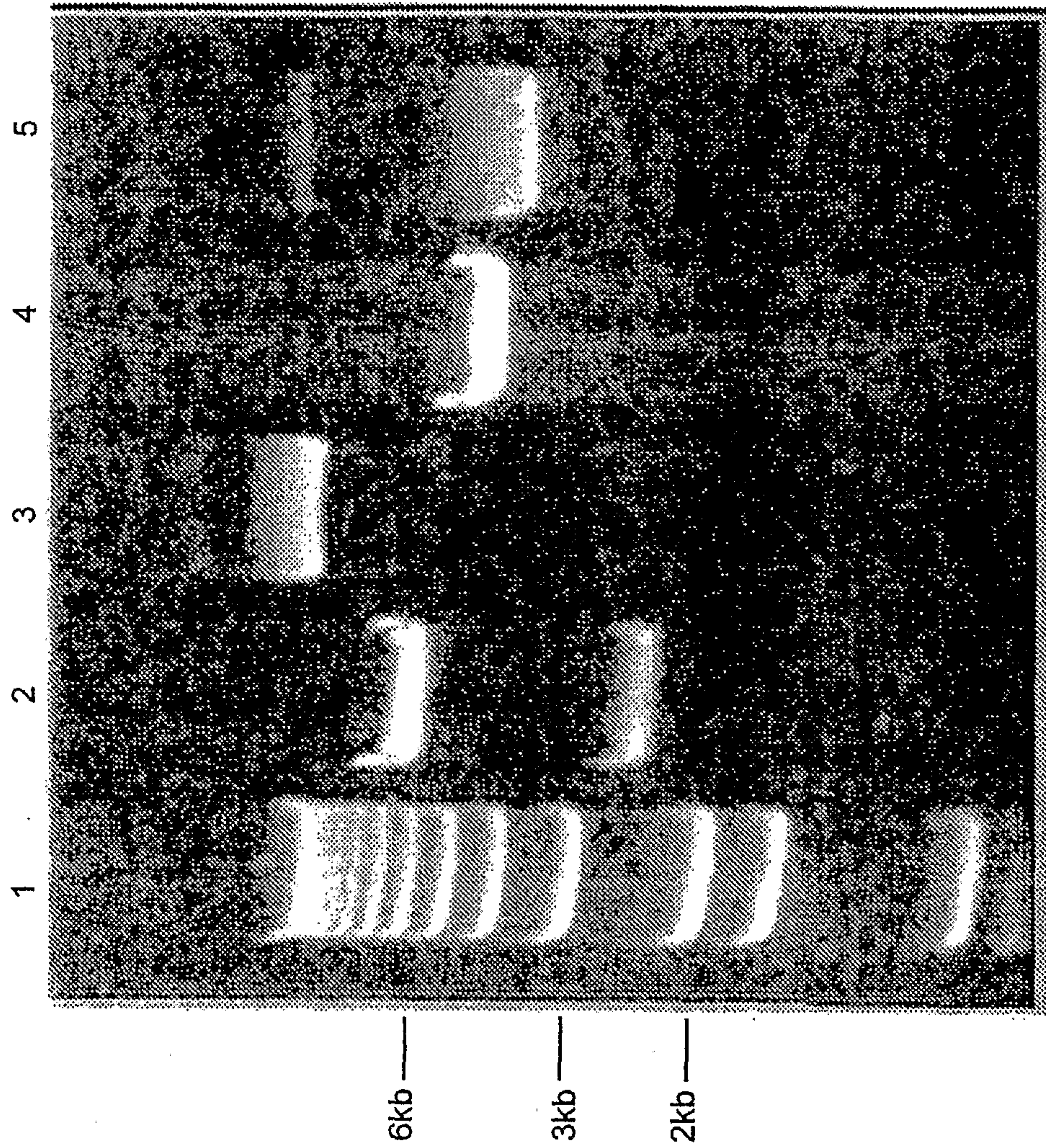
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Figure 3

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*Figure 4*

*Figure 5*

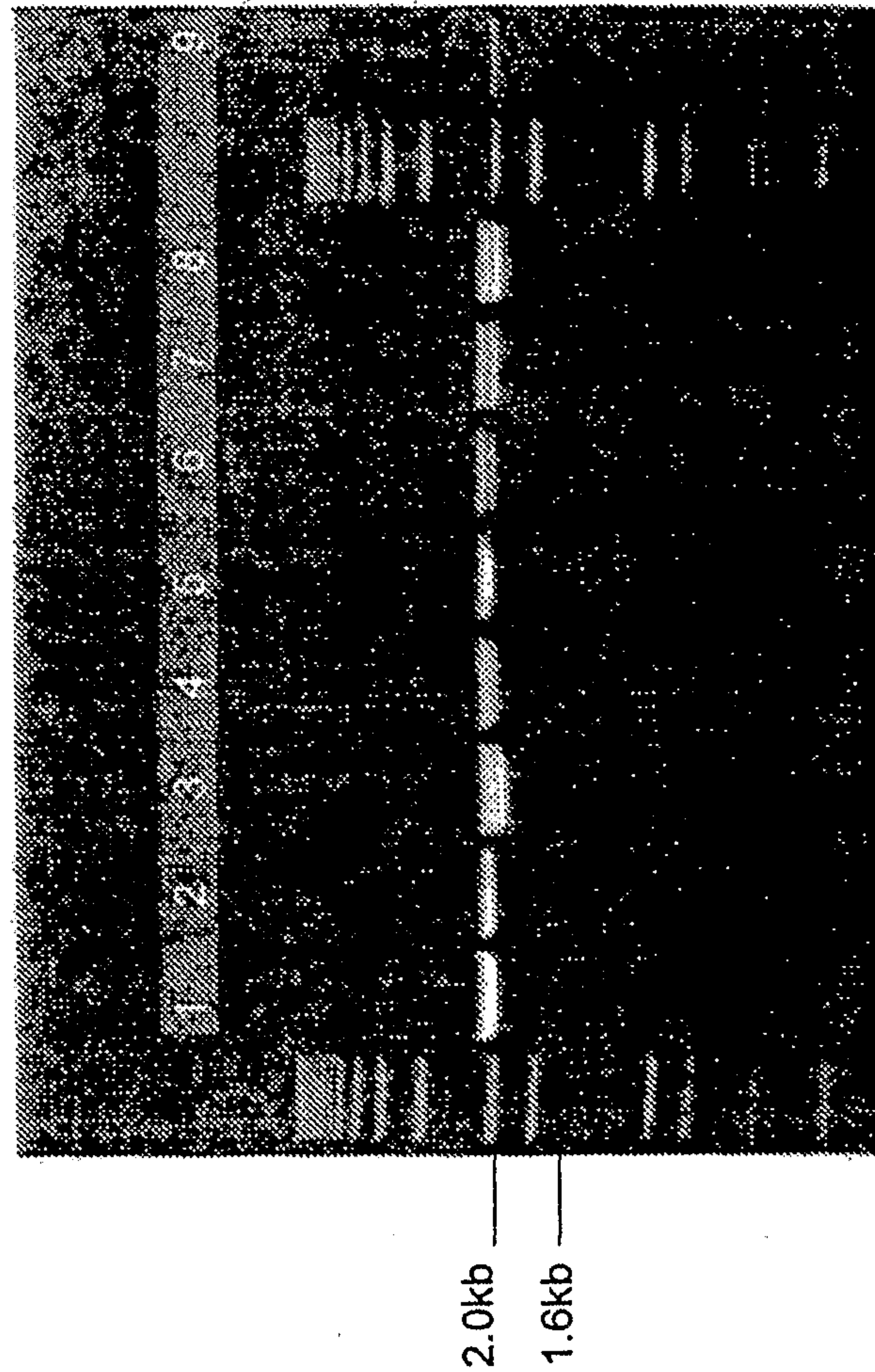


Figure 6

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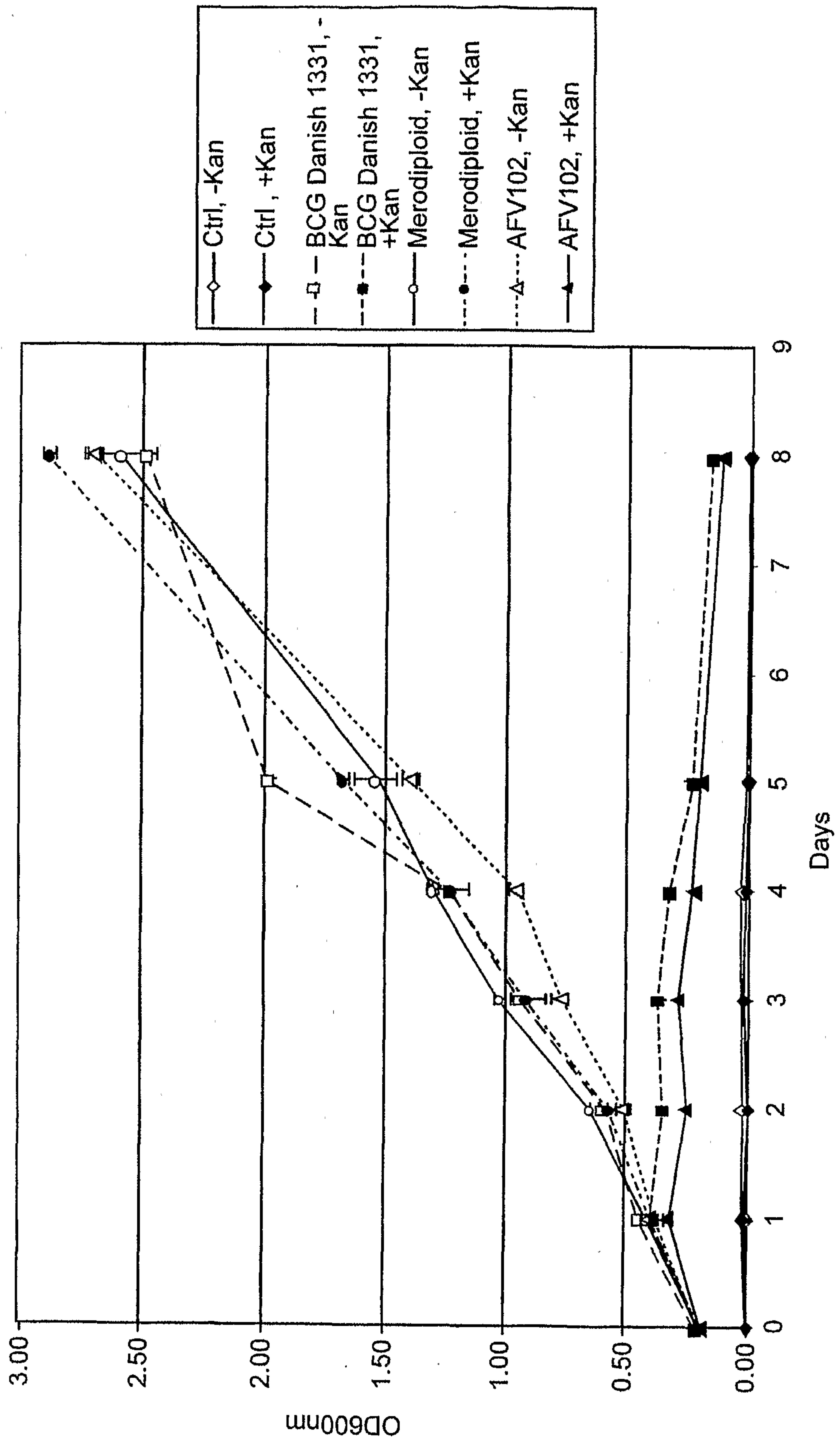


Figure 7

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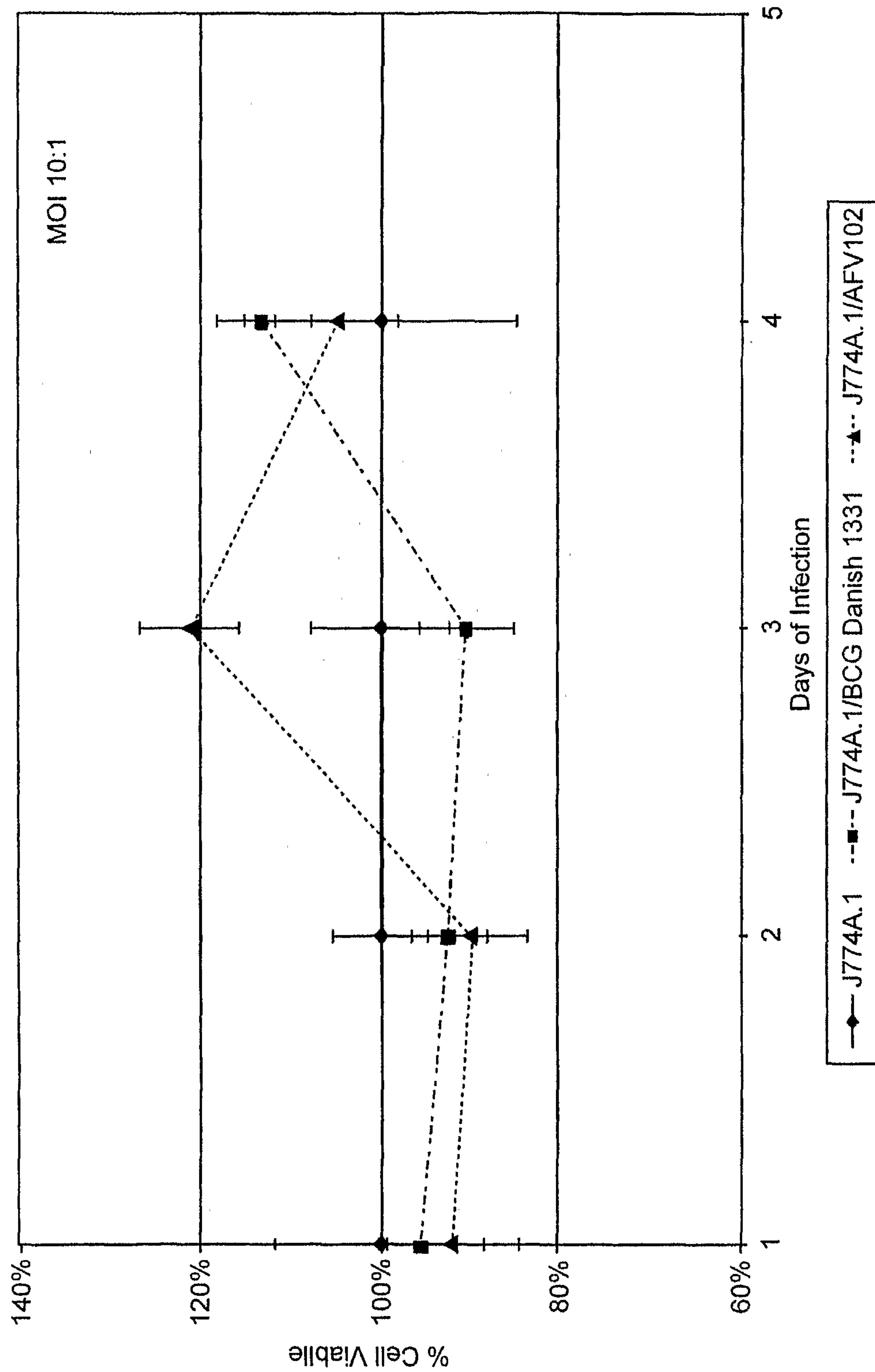


Figure 8

AFV102 Survival in Mouse Macrophage J774A.1

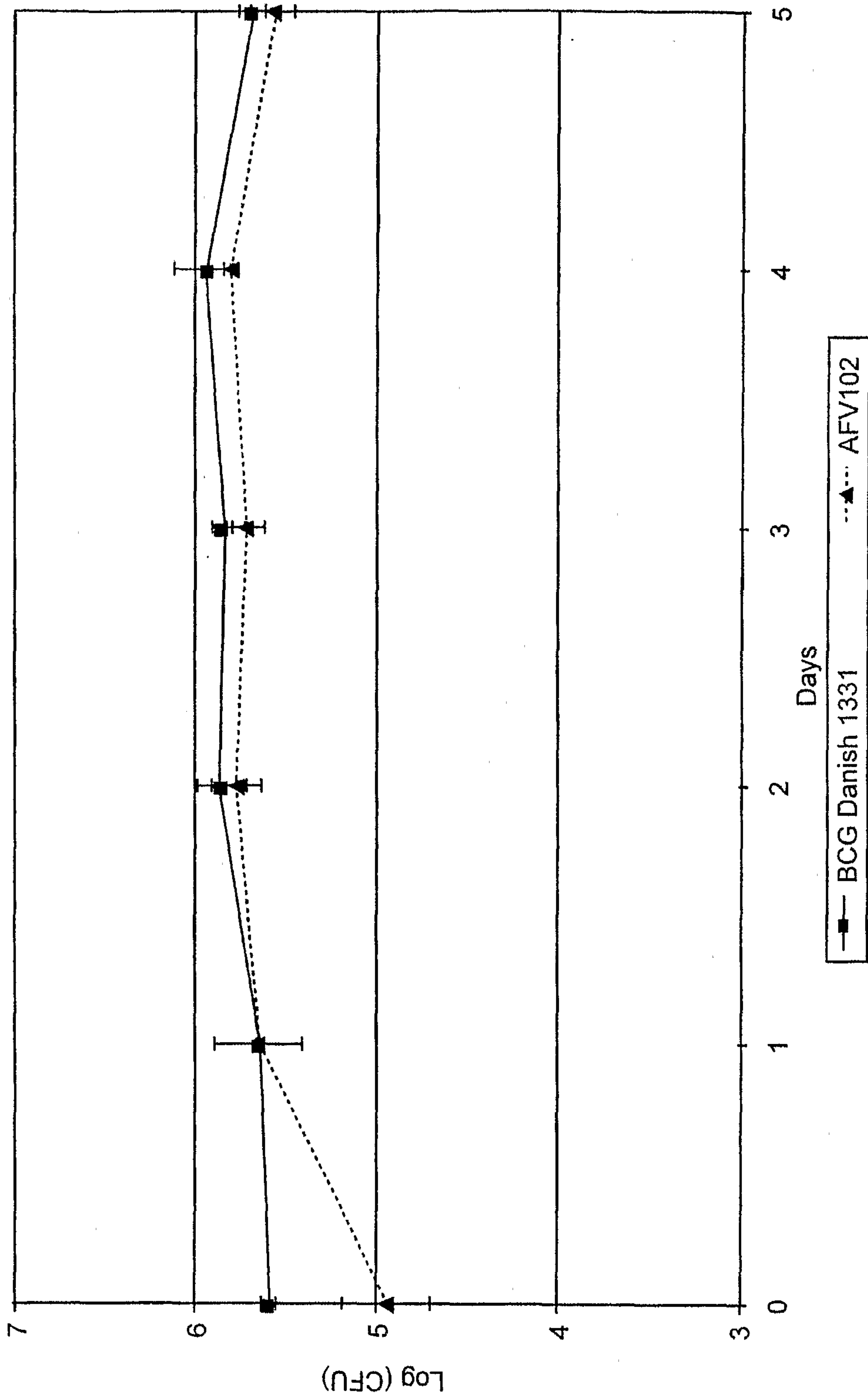


Figure 9

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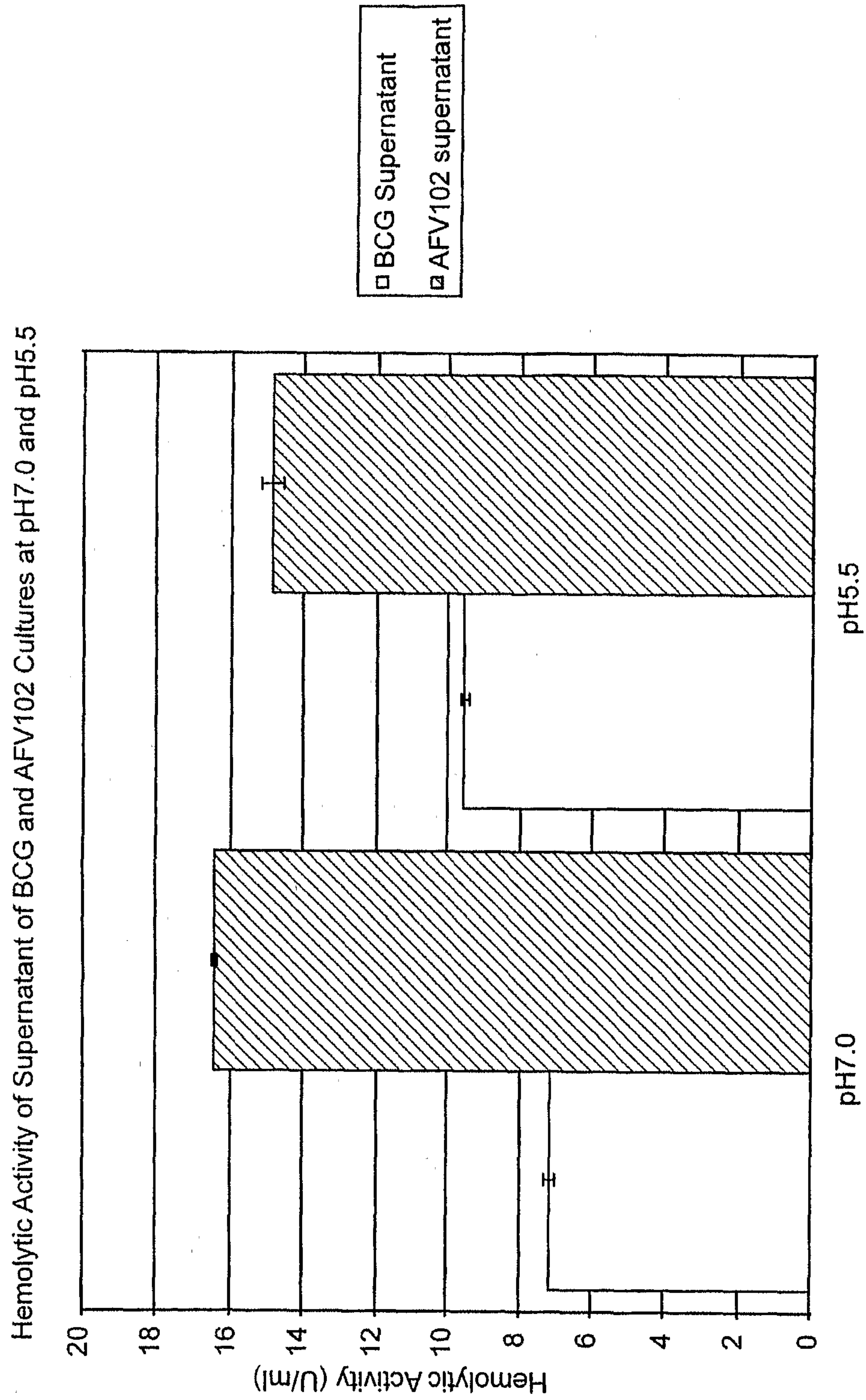
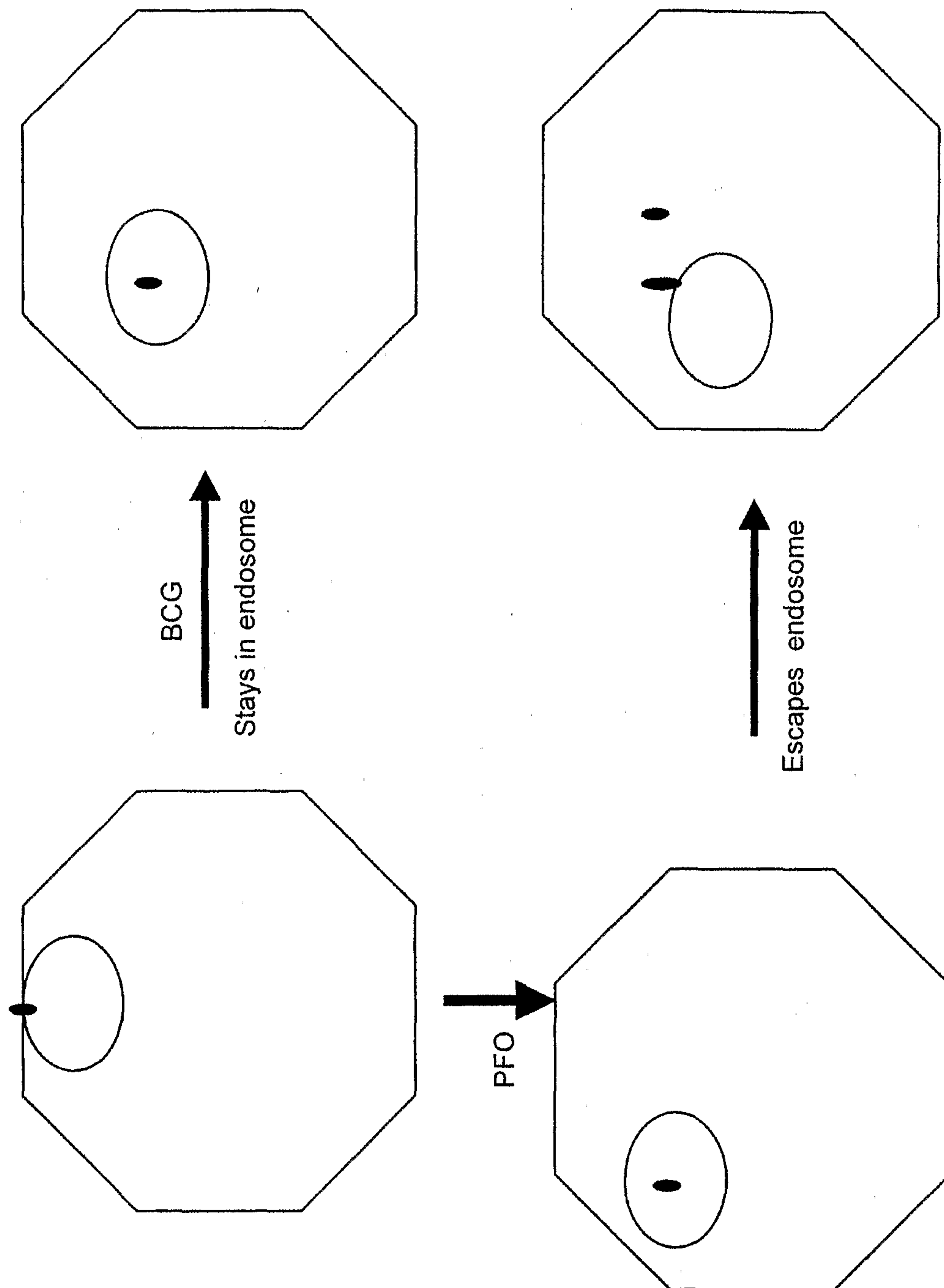
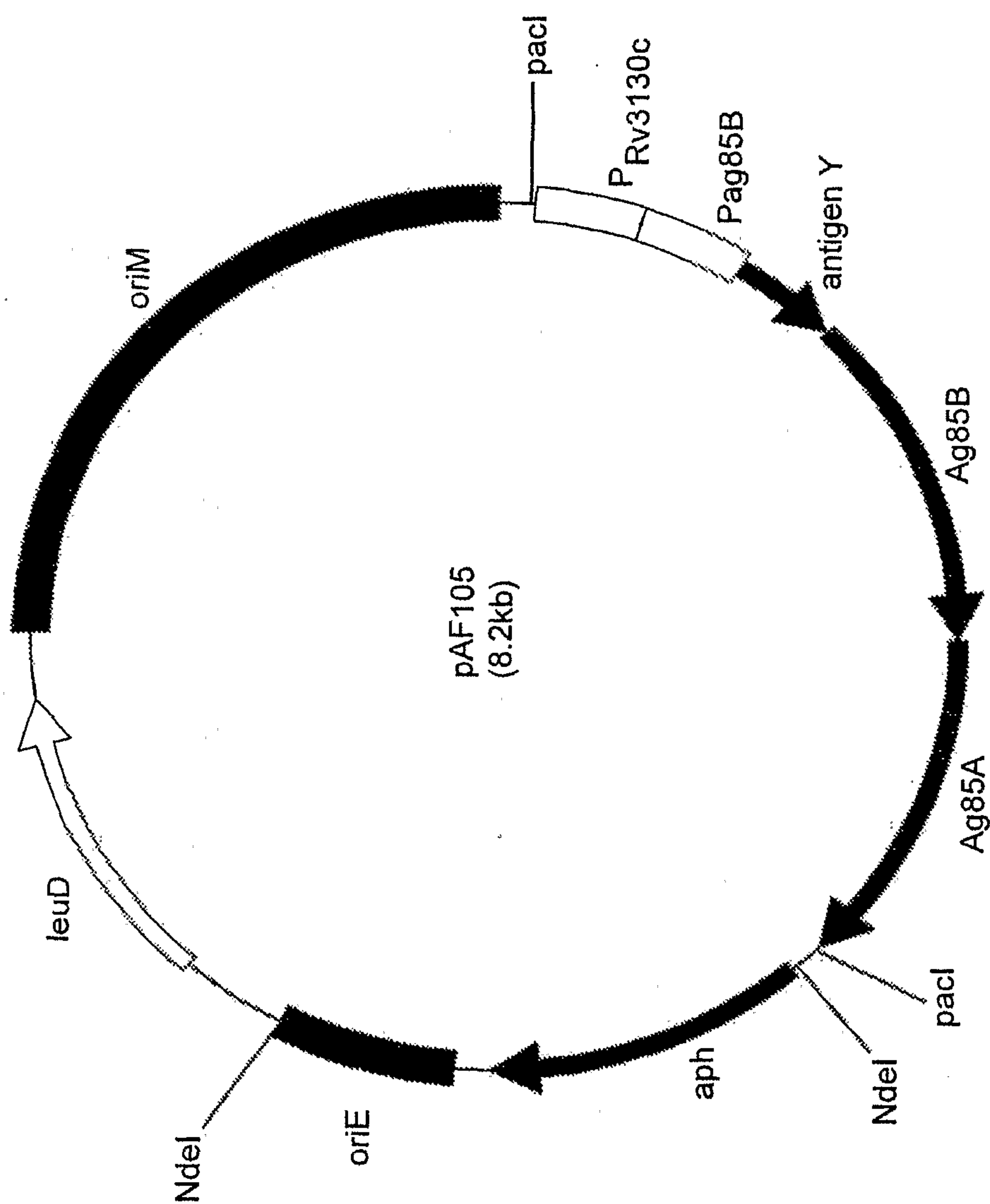


Figure 10

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*Figure 11*

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*Figure 12*

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