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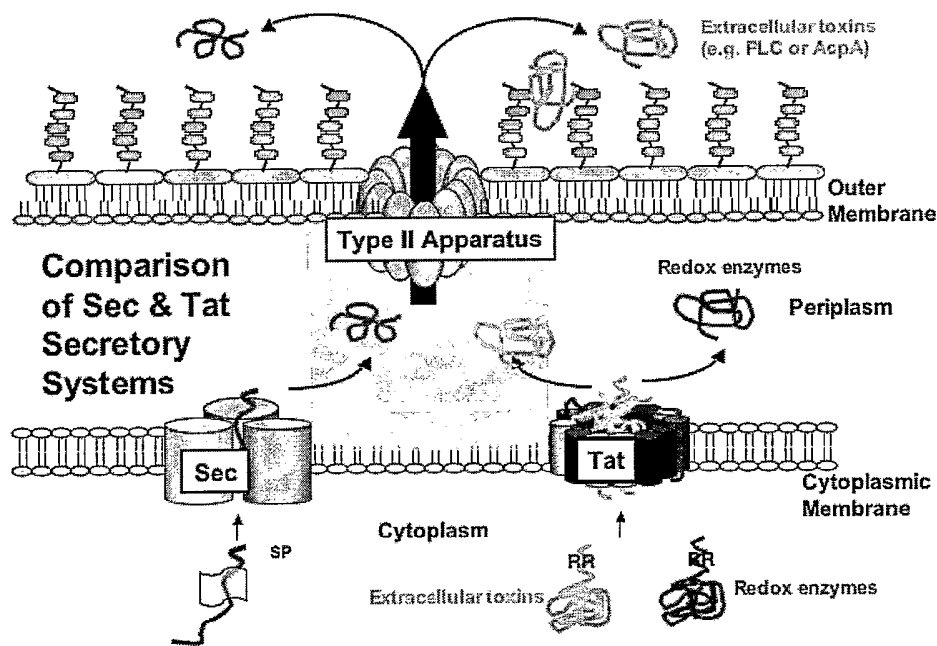
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(54) Title: TWIN ARGININE TRANSLOCASE SECRETORY APPARATUS: HIGH THROUGHPUT ASSAYS AND VACCINE VECTORS RELATED THERETO



(57) Abstract: Disclosed are high throughput assays for the identification of specific inhibitors of the twin arginine translocase secretory apparatus. Also disclosed are bacterial twin arginine translocase mutants for use as vaccines, vaccine vectors or vehicles for the delivery of therapeutic agents, and to prophylactic and therapeutic immunization and treatment methods using such vaccines, vectors and delivery vehicles.



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"Twin Arginine Translocase Secretory Apparatus: High Throughput Assays and Vaccine Vectors Related Thereto"

Field of the Invention

The present invention relates to high throughput assays for the identification of specific inhibitors of the twin arginine translocase secretory apparatus. The present invention also relates to bacterial twin arginine translocase mutants as vaccines, vaccine vectors or vehicles for the delivery of therapeutic agents, and to prophylactic and therapeutic immunization and treatment methods using such vaccines, vectors and delivery vehicles.

Background of the Invention

Secretion of proteins is a vital function of eukaryotes and prokaryotes. Until fairly recently, the Sec apparatus was thought to be the only means by which secreted proteins could be channeled through the cytoplasmic membranes of prokaryotic microbes. However, less than a decade ago, several investigative groups identified a novel Sec-independent secretion system that is able to secrete proteins from the cytoplasm of plants into the thylakoid spaces of chloroplasts (Robinson). Subsequently, in 1997 Settles et al. (Settles) reported the identification of a specific chloroplast membrane protein (Hcf106) that is required for the Sec-independent secretion of proteins into plant thylakoids. Interestingly, they found that Hcf106 is closely related to bacterial proteins. Further exploration of these findings by several research groups revealed that the Sec-independent ΔpH protein secretion apparatus is structurally and functionally very closely related to a Sec-independent, ATP independent secretion system, which is found in an increasing diverse array of bacterial genera. The bacterial counterpart of the ΔpH thylakoid secretory system is called TAT (twin arginine translocation; see Fig. 1).

A salient aspect of these novel secretory systems (e.g., TAT in plants and prokaryotes) is that the signal peptides of both essentially have all the characteristics of the Sec pathway signal peptides described above. However, there are some additional characteristics of the ΔpH /TAT secretion signal sequences that are lacking in Sec pathway signal peptides (Robinson). The most noteworthy of these is the nearly invariable presence of two consecutive Arg residues ("Twin Arginine Transport" = TAT) just before the hydrophobic core (h-region) and several additional residues between the h-region and the peptidase cleavage site. Also, the N-terminus of TAT secretion signals are usually more highly positively charged than the Sec counterpart (3-6+ vs. 1-2+). These and other

characteristics have enabled the rapid identification of potential TAT secreted proteins based on annotated genomic sequences of bacterial pathogens.

Probably the most remarkable difference between the Sec system and the Δ pH/TAT system is that this system is capable of secreting proteins that are already folded before they enter the pore apparatus in the inner membrane (Robinson). Notably, in some cases, the TAT secretory apparatus can translocate heteromeric proteins composed of one subunit with TAT secretion signal peptide and another with no signal peptide, in a piggyback fashion across the cytoplasmic membrane.

A relatively limited number of proteins secreted via the TAT system have been examined. The present inventors recently provided data indicating that the secretion of two phospholipases C produced by the opportunistic pathogen *Pseudomonas aeruginosa* is dependent on the TAT secretion system in this organism (Ochsner, 2003, PNAS; Voulhoux). The secretion signal peptides of these proteins all have the salient characteristics of TAT signal peptides described above. This was the first report that a protein secreted through the inner membrane TAT system can be further translocated through an accessory secretory apparatus (e.g. Xcp), which is required for the secretion of extracellular proteins (e.g. toxins) through the outer membrane of many gram negative organism. More recently, the present inventors' laboratory showed the direct involvement of the Tat system in the secretion of PlcH by using site-directed mutagenesis to alter the twin-arginine motif of PlcH (e.g RR to RK) (Ochsner, *ibid.*). These data demonstrated that export of PlcH occurs exclusively through the Tat secretion system. Together, these results challenge the previous vision of the general secretion pathway and suggest that, in addition to the Sec-dependent system, the Tat system feeds substrates into the Xcp pathway (See Fig. 1). Moreover, the present inventors recently discovered that a Tat secreted protein is required for the function of another secretion apparatus that directly injects toxic proteins into eukaryotic cells. This secretory apparatus, called the Type III secretion system, contributes to the virulence of many gram negative pathogens including *Y. pestis* (Cornelis). Pradel et al. recently published research showing that the TAT system of *E. coli* is an important virulence determinant of the enterohemorrhagic strains of this bacterium (Pradel et al., 2003, *Infection & Immunity* 71:4908-4916).

To further investigate the contribution of the Tat secretion system in virulence through the secretion of factors associated with pathogenesis or stress response, the present inventors characterized the phenotypes associated with a *tatC* mutation in *P. aeruginosa* PAO1 (See Ochsner et al., 2002, *supra*). In addition to the previous identification of PlcH

and PICN as Tat-secreted proteins, eighteen putative Tat-dependent secreted products were identified by screening the *P. aeruginosa* protein database using the conserved twin-arginine Tat signal sequence motif. The inventors found that the siderophore-mediated iron scavenging system, which contributes to virulence in *P. aeruginosa*, was also affected in TAT mutants. Also affected in $\Delta tatC$ mutants was the ability to form biofilms, which play an important role in pathogenesis pulmonary infections. Furthermore, analysis of the PAO1 $\Delta tatC$ strain in the chronic pulmonary rat lung model of infection illustrates the importance of the Tat system in virulence. While the lungs infected with wild-type bacteria showed multiple lesions, the lungs infected with PAO1 $\Delta tatC$ were free of visible lesions even though the colony counts retrieved from the lungs in both groups were similar. In addition, the lungs infected with wild-type cells had histological changes including congestion of blood vessels and the perivascular accumulation of densely packed mononuclear cells as well as the presence of an intense neutrophilic peribronchial inflammation. In contrast, lungs infected with PAO1 $\Delta tatC$ were intact and essentially devoid of detectable inflammation (See Fig. 2 of Ochsner et al., 2002, *supra*) despite the fact that this mutant survived for 6 days in numbers comparable to that of the parental wild type strain.

The growing emergence of pathogenic bacteria with clinically significant resistance to conventional antibiotics is a major public health concern. They are implicated in many diseases, as primary causative effects, such as in pneumonia and sepsis, and as exacerbating agents (e.g. *M. tuberculosis* in AIDS and *P. aeruginosa* and *Burkholderia cepacia* in Cystic Fibrosis). There are several paths to fighting infections, including the reliance on: (i) drugs directly toxic to the microorganisms (ii) on vaccines that stimulate the immune system, or (iii) on drugs that attenuate pathogenicity while allowing the immune system or other antimicrobial agents to ultimately clear the infectious agent.

Therefore, there is a compelling need in the art for the identification and development of novel antimicrobial agents to treat infections that are increasingly resistant to extant agents. However, the screening methods that are currently used are non-specific or, in some cases, overly specific, and do not allow evaluation of whether a candidate compound will be effective at altering the pathogenic potential of a microorganism. For example, assays that target a single enzyme may lead to the identification of an inhibitory agent for that particular enzyme, but such an agent may not be able to penetrate the live organism in order to reach the desired target, or it may be degraded by the organism. Therefore, there is a need in the art for assays that will substantially decrease the need to perform additional research to determine whether a potential inhibitor can act on a growing bacteria.

By way of example, new antibiotics are needed to fight rapidly emerging resistant strains of bacteria. Many of these bacteria are public threats. However, antibiotic development in the commercial sector has decreased significantly as the infectious disease market has become more fragmented. Many new antibiotics are variations on existing antibiotics, such as glycolcyclines. While approvals of these drugs are important public health events, development of resistant strains is virtually assured, as resistant strains already exist to the parent compounds, and the compounds target the viability of the bacterial cells as their mechanism of action. New classes of antibiotics are needed, which means new targets for antimicrobial activity need to be identified.

Vaccines are widely used to prevent disease and to treat established diseases (therapeutic vaccines). There also remains, however, an urgent need to develop safe and effective vaccines and adjuvants for a variety of diseases, including those due to infection by pathogenic agents, cancers and other disorders amenable to treatment by elicitation of an immune response.

Protein antigens (e.g. subunit vaccines, the development of which was made possible by recombinant DNA technology), when administered without adjuvants, induce weak humoral (antibody) immunity and have therefore been disappointing to date as they exhibit only limited immunogenicity. An additional disadvantage of subunit vaccines, as well as of killed virus or bacteria and recombinant bacterial and viral vaccines, is that while they appear to stimulate a strong humoral immune response when administered with adjuvants, they fail to elicit protective cellular immunity. Adjuvants are used experimentally to stimulate potent immune responses in mice, and are desirable for use in human vaccines, but few are approved for human use. Indeed, the only adjuvants approved for use in the United States are the aluminum salts, aluminum hydroxide and aluminum phosphate, neither of which stimulates cell-mediated immunity. Aluminum salt formulations cannot be frozen or lyophilized, and such adjuvants are not effective with all antigens. Moreover, most adjuvants do not lead to induction of cytotoxic T lymphocytes (CTL). CTL are needed to kill cells that are synthesizing aberrant proteins including viral proteins and mutated "self" proteins. Vaccines that stimulate CTL are being intensely studied for use against many viruses (e.g., HIV, HCV, HPV, HSV, CMV, EBV), intracellular bacteria (e.g., tuberculosis); intracellular parasites (e.g., malaria, leishmaniasis, shistosomiasis, leprosy), and all cancers (e.g., melanoma, prostate, ovarian, etc.). Thus adjuvants are needed that stimulate CTL and cell-mediated immunity in general.

~~"Live" attenuated "microorganisms"~~ (e.g., bacteria, viruses) have been used widely as effective vaccines. Live attenuated bacterial vaccines allow vaccination via the mucosal surfaces and specific targeting to professional antigen presenting cells located at the inductive sites of the immune system. Live attenuated microbial vaccines, unlike their killed vaccine counterparts or even some recombinant subunit vaccines, are more likely to elicit cellular immunity which can be critical for long term efficacy. A number of live attenuated microbial vaccines derived empirically by chemical or u.v. mutagenesis have proved to be immunogenic and protective and are still in use despite the need for repeated parenteral administration. However, the basis for the attenuation in these systems is not well defined and due to the need for repeated doses and large numbers of live microorganisms that must be administered to achieve the desired immunity, there is a need to develop more specifically designed, efficacious and safer vaccines. Genetic manipulation of microbes to produce improved live attenuated microorganisms for use as vaccines is an area of intense research. However, few of these vaccines are currently approved for human use.

For example, the best-characterized live vaccine against tularemia (Live Vaccine Strain = LVS) was derived from the original attenuated strain that was developed in the former Soviet Union prior to World War II. After initial success with this attenuated strain, it was later found that it had become so attenuated that it was no longer virulent in laboratory animals and was less immunogenic (Ellis). Further analysis of this strain revealed that it is able to segregate into two separate colony types that have distinct virulence phenotypes. One of these variants (the so called blue colony type) was shown to be more virulent and immunogenic in small animals than the alternative colonial variant (gray colony type). The current *F. tularensis* LVS was derived after five passages of the original vaccine strain through mice and appeared to be an effective vaccine that protected mice and guinea pigs against inhalation challenge with the fully virulent *F. tularensis* strain Schu 4. Several human trials were also conducted using LVS and the data suggested that LVS induced a protective response in human subjects. However, while this vaccine protected against aerosol challenge of 2000 *F. tularensis* Schu 4, protection was significantly reduced when the vaccinees were challenged with 20,000 virulent organisms. In any case, there are some features of the LVS that are a cause for concern and make future use of this particular strain problematic. First, the basis for the attenuation of LVS is not known (recent proteomic analysis of LVS and Schu 4 revealed that the vaccine strain produced barely detectable levels of AcpA in comparison to Schu 4 (Hernychova)). Moreover, the protective response induced by the vaccine has not been characterized, and LVS is fully virulent in mice if it is

delivered intraperitoneally. Finally, a recent report by Cherwonogrodzky et al. (Cherwonogrodzky) revealed that the conditions used to cultivate LVS can have a significant effect on its virulence phenotype. They demonstrated that cultivation of LVS in synthetic media increased its production of its capsule and increased its virulence for mice by about 1000 fold. Collectively, these data strongly suggest that it is possible to develop a live vaccine against tularemia, but that the LVS needs to be considerably improved, particularly with regard to its stability and safety. In this regard, Fulop et al. (Fulop) investigated the use of lipopolysaccharide (LPS) of *F. tularensis* as a component of a subunit vaccine. This LPS experimental vaccine offered some protection against LVS challenge and some protection against SchU 4 fully virulent organisms. When these investigators used LVS to boost the initial protective response, they observed a heightened protective response with the LPS subunit vaccine. They also observed that depletion of CD4⁺ or CD8⁺ cells abrogated the boosted protective response, thereby suggesting that a cell-mediated response is necessary to attain a complete protective response against fully virulent *F. tularensis*.

As another example, regarding live vaccines against plague, many of the same issues relating to LVS also exist for plague vaccines. The EV76 vaccine strain is a pigmentation mutant of *Y. pestis* that was derived from a fully virulent strain of *Y. pestis*. This vaccine has been used since the early part of the last century (Titball). Vaccination trials in mice suggest that EV76 induces an protective immune response against subcutaneous and aerosol challenge with a virulent strain of *Y. pestis*. However, the mechanisms by which this strain is attenuated are unknown. More importantly, the safety of this vaccine in humans is questionable because this strain is not fully attenuated. In challenge studies with mice, the fatality rate with this vaccine is 1% of those vaccinated (Tibball, *supra*).

Finally, the efficiency of any live microbial vector vaccine (i.e., where the microbe is used to deliver a vaccinating antigen that is foreign to the microbe) hinges on its ability to present sufficient foreign antigen to the human immune system to initiate the desired protective immune response(s). Synthesis of sufficient levels of heterologous antigen can result in an increase in metabolic burden with an accompanying decrease in the fitness of the live vector, which can ultimately lower desired immune responses to both live vector and heterologous antigen. Therefore, there is also a need in the art for the continued provision of novel live attenuated microorganisms for use as a vaccine and vaccine vector in both prophylactic and therapeutic immunization strategies.

Summary of the Invention

One method of the present invention relates to a method to identify a compound that inhibits the TAT secretory system. The method includes the steps of: (a) contacting a microorganism that has a TAT secretory system with a candidate compound; (b) measuring a level of secretion of a protein that is secreted via the TAT secretory pathway; and (c) measuring a level of expression of a gene that has increased expression when the TAT secretory pathway is inhibited. A decrease in the level of secretion of the protein in (b) in the presence of the candidate compound as compared to in the absence of the candidate compound, and an increase in the expression of the gene in (c) in the presence of the candidate compound as compared to in the absence of the candidate compound, identifies the compound as a compound that inhibits the TAT secretory system. In one aspect, the candidate compounds are putative antimicrobial compounds, such that a compound identified by the method is identified as an antimicrobial compound.

The microorganisms used in the method of the invention can include, but are not limited to, bacteria from a family selected from: Enterobacteriaceae, Micrococcaceae, Vibrionaceae, Pasteurellaceae, Mycoplasmataceae, and Rickettsiaceae. In one aspect, the microorganism is selected from: *Pseudomonas aeruginosa*, *Pseudomonas mallei*, *Pseudomonas pseudomallei*, *Bordetella pertussis*, *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Francisella tularensis*, *Vibrio cholerae*, *Bacillus anthracis*, *Salmonella enteric*, *Yersinia pestis*, *Escherichia coli* and *Bordetella bronchiseptica*.

In one aspect of this embodiment, the protein of (b) is an enzyme, and the level of secretion of the protein is measured by detecting the amount of a substrate that is converted to a detectable product by the enzyme. In one aspect, the level of secretion of the protein of (b) is measured by measuring the amount of a detectable label that directly or indirectly binds to the protein. In another aspect, the level of secretion of the protein of (b) is detected using a method selected from the group consisting of: enzyme activity, Western blot, immunoblot, enzyme-linked immunosorbant assay (ELISA), radioimmunoassay (RIA), immunoprecipitation, surface plasmon resonance, chemiluminescence, fluorescent polarization, phosphorescence, immunohistochemical analysis, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, microcytometry, microarray, microscopy, fluorescence activated cell sorting (FACS), flow cytometry, DNA binding, ligand binding, and interaction with protein binding partners. In one aspect, step (b) is performed using a colorimetric enzyme assay. In one aspect, the protein of (b) includes, but is not limited to, a protein selected from the group consisting of: phospholipase C

(*picH*), phospholipase C (*picN*), ferripyoverdine receptor (*fpvA*), pyoverdine biosynthesis protein (PA2394), pyoverdine biosynthesis protein (P2389), pyoverdine biosynthesis protein (PA2392), nitrate reductase (*napA*), ferredoxin (*napF*), nitrous oxide reductase (*nosZ*), formate dehydrogenase (*fdnG*), dehydrogenase (PA2124), dehydrogenase (PA2264), aldehyde oxidase (PA4621), aldehyde oxidase (PA1601), aldehyde oxidase (PA1880), aldehyde oxidase (PA2378), multicopper oxidase (*copA*), and protein PA0144.

A preferred protein of (b) is one or both of phospholipase C or phospholipase H. In one aspect, the level of secretion of phospholipase C or H is detected by contacting supernatant from a culture comprising the microorganism with a labeled substrate for phospholipase C or H and detecting a level of product converted from the substrate by phospholipase C or H. Such a step of detecting can include a colorimetric assay.

In one aspect of this embodiment, the level of expression of the gene in (c) is measured by detecting the expression of a protein encoded by the gene. In one aspect, the level of expression of the gene in (c) is measured by detecting the transcription of the gene. In one aspect, the level of expression of the gene in (c) is measured using a method selected from the group consisting of: detection of a reporter gene, detection of antibiotic resistance, polymerase chain reaction (PCR), reverse transcriptase-PCR (RT-PCR), *in situ* PCR, quantitative PCR (q-PCR), *in situ* hybridization, Southern blot, Northern blot, sequence analysis, and microarray analysis. In one aspect, the level of expression of the gene in (c) is measured using detection of a reporter gene, which may include using a fluorescent label or a colorimetric label. In one aspect, the gene detected in (c) is selected from the group consisting of: PA2808, PA522, PA4878, *mexA*, and *pchABCDEFGH*.

In one aspect of this embodiment, steps (a)-(c) are conducted within the same assay sample. In one aspect, steps (a)-(c) are conducted within the same well of a microtiter plate. In another aspect, the method is formatted as a high throughput assay.

In one aspect, the method further includes (or alternatively includes) a step of detecting the level of expression of a gene that has decreased expression when the TAT secretory pathway is inhibited. In another aspect, the method further includes (or alternatively includes) a step of detecting the level of expression of a reporter gene fused to the promoter of a gene that has decreased expression when the TAT secretory pathway is inhibited. For example, in one aspect, the reporter gene is a gene that confers resistance to an antibiotic onto the microorganism.

Yet another embodiment of the present invention relates to an assay kit for the identification of candidate compounds that inhibit the TAT secretory system. The kit

includes: (a) a reagent for detecting the level of a protein that is secreted by the TAT secretory system; and (b) a reagent for detecting the level of expression of a gene that is increased when the TAT secretory system is inhibited. In one aspect, the protein of (a) is an enzyme and wherein the reagent of (a) is a labeled substrate for the enzyme. In another aspect, the reagent of (a) is a detectable agent that binds to the protein. In another aspect, the protein of (a) is selected from the group consisting of: phospholipase C (*plcH*), phospholipase C (*plcN*), ferripyoverdine receptor (*fprA*), pyoverdine biosynthesis protein (PA2394), pyoverdine biosynthesis protein (P2389), pyoverdine biosynthesis protein (PA2392), nitrate reductase (*napA*), ferredoxin (*napF*), nitrous oxide reductase (*nosZ*), formate dehydrogenase (*fdnG*), dehydrogenase (PA2124), dehydrogenase (PA2264), aldehyde oxidase (PA4621), aldehyde oxidase (PA1601), aldehyde oxidase (PA1880), aldehyde oxidase (PA2378), multicopper oxidase (*copA*), and protein PA0144. In one aspect, the protein of (a) is a phospholipase C. In one aspect, the reagent of (a) is a substrate for phospholipase C. In another aspect, the reagent of (a) is used in a colorimetric assay. In another aspect, the gene in (b) is fused to a reporter gene, and the reagent of (b) is a reagent or reagents useful for detecting the expression of the reporter gene. In one aspect, the reagent of (b) comprises an oligonucleotide probe or primer that hybridizes to the gene or a transcript thereof under stringent hybridization conditions. In one aspect, the reagent of (b) is used in a fluorescent or colorimetric assay. In another aspect, the kit further includes microbial cells containing a TAT secretory system.

Another embodiment of the present invention relates to a therapeutic composition, comprising: (a) an attenuated bacterium comprising a genetic modification that reduces or abolishes the expression or biological activity of the twin arginine translocase (TAT) secretory system in the bacterium; and, (b) at least one therapeutic agent. In one aspect, at least one gene in the *tat* operon is not expressed by the bacterium. In another aspect, RNA from at least one gene in the *tat* operon is not transcribed by the bacterium. In another aspect, RNA transcribed from a genetically modified *tat* gene is not translated by the bacterium. In another aspect, the bacterium comprises a mutation in at least one gene in the *tat* operon that reduces or prevents the expression of at least one component of the twin arginine translocase (TAT) secretory system in the bacterium or that reduces or abolishes the biological activity of the TAT secretory system in the bacterium. For example, the bacterium can have at least one mutation in the coding sequence of at least one gene in the *tat* operon or at least one mutation in a regulatory region of at least one gene in the *tat* operon. In one aspect, the mutation is a partial or complete deletion of at least one gene in

the *tat* operon. In another aspect, the mutation is an insertion of a heterologous nucleic acid sequence into at least one gene in the *tat* operon sufficient to prevent expression or biological activity of at least one component of the TAT secretory system. In another aspect, the mutated *tat* gene encodes a mutated TAT secretory system component that results in a TAT secretory system with substantially no biological activity. In one aspect, the mutation prevents the expression or abolishes the biological activity of the TAT pore apparatus in the bacterium. In one aspect, the *tat* gene is selected from *tatA*, *tatB* and *tatC*. In one aspect, the bacterium lacks the ability to secrete proteins across the cytoplasmic membrane that have a TAT secretion signal sequence. In one aspect, the bacterium is from a family selected from: Enterobacteriaceae, Micrococcaceae, Vibrionaceae, Pasteurellaceae, Mycoplasmataceae, and Rickettsiaceae. In one aspect, the bacterium is a *Pseudomonas*. In one aspect, the bacterium is selected from: *Pseudomonas aeruginosa*, *Pseudomonas mallei*, *Pseudomonas pseudomallei*, *Bordetella pertussis*, *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Francisella tularensis*, *Vibrio cholerae*, *Bacillus anthracis*, *Salmonella enteric*, *Yersinia pestis*, *Escherichia coli* 0157:H7 and *Bordetella bronchiseptica*.

In one aspect, the therapeutic agent is a heterologous antigen, including, but not limited to, a peptide or a protein. In one aspect, the attenuated microorganism has been transfected with a recombinant nucleic acid molecule encoding the heterologous antigen. In another aspect, the antigen is selected from the group consisting of viral antigens, mammalian cell surface molecules, bacterial antigens, fungal antigens, protozoan antigens, helminth antigens, ectoparasite antigens, and cancer antigens. In one aspect, the composition comprises multiple antigens. In one aspect, the therapeutic agent is a biological response modifier selected from the group consisting of a cytokine, a chemokine, a hormone, a lipidic derivative, an enzyme, a small molecule drug that elicits a biological response, and a protein or peptide that regulates gene expression or cellular activity in a recipient cell. In one aspect, the therapeutic agent is a heterologous recombinant nucleic acid molecule that can be transferred into a recipient cell by the attenuated bacterium. In one aspect, the recombinant nucleic acid molecule encodes an antigen.

Another embodiment of the present invention relates to a method for stimulating an immune response against a bacterial protein, comprising administering to an animal an attenuated bacterium comprising a genetic modification that reduces or abolishes the expression or biological activity of the twin arginine translocase (TAT) secretory system in the bacterium. In one aspect, at least one gene in the *tat* operon is not expressed by the bacterium. In another aspect, RNA from at least one gene in the *tat* operon is not transcribed

by the bacterium. In another aspect, RNA transcribed from a genetically modified *tat* gene is not translated by the bacterium.

In one aspect, the bacterium comprises a mutation in at least one gene in the *tat* operon that reduces or prevents the expression of at least one component of the twin arginine translocase (TAT) secretory system in the bacterium or that reduces or abolishes the biological activity of the TAT secretory system in the bacterium. In one aspect, the bacterium comprises at least one mutation in the coding sequence of at least one gene in the *tat* operon. In another aspect, the bacterium comprises at least one mutation in a regulatory region of at least one gene in the *tat* operon. In another aspect, the mutation is a partial or complete deletion of at least one gene in the *tat* operon. In another aspect, the mutation is an insertion of a heterologous nucleic acid sequence into at least one gene in the *tat* operon sufficient to prevent expression or biological activity of at least one component of the TAT secretory system. In another aspect, the mutated *tat* gene encodes a mutated TAT secretory system component that results in a TAT secretory system with substantially no biological activity. In another aspect, the mutation prevents the expression or abolishes the biological activity of the TAT pore apparatus in the bacterium. In yet another aspect, the *tat* gene is selected from the group consisting of *tatA*, *tatB*, and *tatC*. In another aspect, the bacterium lacks the ability to secrete proteins across the cytoplasmic membrane that have a TAT secretion signal sequence. In another aspect, the bacterium is from a family selected from: Enterobacteriaceae, Micrococcaceae, Vibrionaceae, Pasteurellaceae, Mycoplasmataceae, and Rickettsiaceae. In another aspect, the bacterium is selected from: *Pseudomonas aeruginosa*, *Pseudomonas mallei*, *Pseudomonas pseudomallei*, *Bordetella pertussis*, *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Francisella tularensis*, *Vibrio cholerae*, *Bacillus anthracis*, *Salmonella enteric*, *Yersinia pestis*, *Escherichia coli* 0157:H7 and *Bordetella bronchiseptica*. In another aspect, the attenuated bacterium is from the genus *Pseudomonas* and wherein the animal has or is at risk of developing chronic or acute pulmonary infection. In yet another aspect, the attenuated bacterium is from the genus *Yersinia* and wherein the animal has or is at risk of developing plague. In another aspect, the attenuated bacterium is from the genus *Bacillus* and wherein the animal has or is at risk of developing anthrax. In another aspect, the attenuated bacterium is from the genus *Francisella* and wherein the animal has or is at risk of developing tularemia. In another aspect, the attenuated bacterium is from the genus *Salmonella*, and wherein the animal has or is at risk of developing salmonellosis. In another aspect, the attenuated bacterium is from the genus *Mycobacterium*, and wherein the animal has or is at risk of developing tuberculosis or leprosy. In yet another

aspect, the attenuated bacterium is an *Escherichia coli* 0157:H7. In yet another aspect, the attenuated bacterium is *Bordetella bronchiseptica*.

Another embodiment of the present invention relates to a method for vaccinating an animal against a disease or condition, comprising administering to the animal any therapeutic composition as described herein. In one aspect, the vaccine is administered with a pharmaceutically acceptable excipient.

Yet another embodiment of the invention relates to an attenuated bacterium comprising a genetic modification that reduces or abolishes the expression or biological activity of the *pscO* gene. In one aspect, the *pscO* gene is not expressed by the bacterium. In another aspect, RNA from the *pscO* gene is not transcribed by the bacterium. In another aspect, RNA transcribed from a genetically modified *pscO* gene is not translated by the bacterium. In another aspect, the bacterium comprises a mutation in the *pscO* gene that reduces or prevents the expression of the PscO protein in the bacterium or that reduces or abolishes the biological activity of the PscO protein in the bacterium. In yet another embodiment, the bacterium comprises at least one mutation in the coding sequence of the *pscO* gene. In another embodiment, the bacterium comprises at least one mutation in a regulatory region of the *pscO* gene.

Brief Description of the Figures of the Invention

Fig. 1 is a schematic diagram showing a comparison of the Sec and Tat secretory systems.

Fig. 2 is a schematic diagram showing homologues of the twin arginine translocase proteins (TatC) in select agents.

Fig. 3 is a schematic diagram of the experimental design for evaluating bacterial infections in a murine pulmonary model.

Fig. 4 is a diagram showing the use of a synthetic substrate to evaluate TAT function by detecting the secretion of the PLCs of *P. aeruginosa*.

Fig. 5 is a graph showing that the activity of the substrate, NPPC, is linear with the number of cells added to an assay according to the present invention, and that NPPC activity is completely inhibited when TAT is inhibited.

Fig. 6 is a graph showing the NPPC velocity in the presence and absence of inhibition of TAT.

Detailed Description of the Invention

The present invention generally relates to two important discoveries made by the present inventors that are related to the twin arginine translocase (TAT) secretory system in bacteria. First, the present invention relates to a novel high throughput assay for the identification of inhibitors that specifically target the twin arginine translocase secretory apparatus. Because inhibition of the TAT secretory pathway in bacterial pathogens (e.g., *Pseudomonas aeruginosa*) abrogates their ability to cause significant disease in animal models of infection that are directly relevant to human infections, the identification of such inhibitors is extremely valuable in the treatment and prevention of bacterial diseases. Second, the present invention relates to the use of live, attenuated bacterial strains that have at least one genetic modification (e.g., a mutation) that reduces or prevents the expression of at least one component of the twin arginine translocase (TAT) secretory system in the bacterium or reduces or abolishes the biological activity of the TAT secretory system in the bacterium. Such strains can be used as vaccine vectors or vehicles for the delivery of therapeutic agents, for prophylactic and therapeutic immunization and treatment methods.

Although the two main embodiments of the present invention are described separately below, it is to be understood that general definitions, aspects, and techniques described for one embodiment may be applicable to the other embodiment.

High Throughput Assay for the Identification of Specific Inhibitors of TAT

One embodiment of the present invention relates to a high-throughput assay for the identification of specific inhibitors of the twin arginine translocase secretory apparatus. Based on data produced in the inventors' laboratory, the present inventors have conceived and developed specific and reliable high throughput assays that can be used to identify inhibitors or antimicrobial compounds that directly affect the function of the twin arginine translocase (TAT) secretory pathway in bacteria.

More particularly, the inventors have identified positive and negative screening methods that are used in combination to identify whether a compound specifically inhibits the function of the TAT secretory pathway in a live bacterium (e.g. *Pseudomonas aeruginosa*). These screening methods are amenable to use in high throughput assays, such that a large compound library can be used to rapidly and efficiently identify specific compounds which inhibit only the TAT secretory pathway in an intact bacterial pathogen. The combined use of negative and positive screening assays will significantly increase the likelihood of identifying a particular compound from a library that is targeting the TAT secretory system.

The negative assay is based on the detection of at least one protein, such as an extracellular enzyme, that is secreted via the TAT secretory pathway in culture supernatants of organisms like *Pseudomonas aeruginosa*. These proteins are not present in culture supernatants if the TAT secretory pathway is inactivated, because they rely on the TAT apparatus to be secreted from the cell. Consequently, a compound that inhibits the secretion of such proteins is likely to be doing so by inhibiting the TAT secretory pathway.

To increase the specificity of the screening method for the function of the TAT pathway, the inventors have added to this negative assay a *positive* screening method that can be performed at the same time (e.g., in the same microtiter well) as the negative screening assay. The combination of assays significantly enhances the likelihood of identifying compounds that only target the TAT secretory pathway, since each of the assay outputs are independent. That is, there is no known condition, other than a defective TAT secretory system, that would cause the combination of the negative response and the positive response at the same time. The positive assay is based on microarray data that was generated in the inventors' laboratory that showed that inhibition of the TAT secretory pathway leads to a substantial *increase* in the expression of specific genes that can be easily assayed.

The methods described herein are believed to be the first disclosure of an assay, and particularly a high throughput assay, that is capable of identifying a compound that is a specific antimicrobial agent that can penetrate a growing bacterial pathogen and inhibit its pathogenic potential at the same time. Currently available methods for screening for antimicrobial agents are either non-specific or overly specific, and do not allow evaluation of whether a candidate compound will be effective at altering the pathogenic potential of a microorganism. For example, an assay that targets a single protein may result in the identification of an agent that specifically inhibits that protein. However, there is no immediate feedback from the assay regarding whether the agent will be able to penetrate the live organism in order to reach the desired target, or the agent may be degraded by the organism when it is investigated in an *in vivo* system. Therefore, current assays fall far short of an ideal system for the identification of useful antimicrobial agents. In contrast, the present invention provides a unique combination of positive and negative assays for the function of the TAT secretory system in live organisms, which has a significantly improved likelihood of identifying a compound that will be able to penetrate a growing bacterial pathogen and inhibit its pathogenic potential at the same time. The availability of the assays of the present invention will substantially decrease the need to perform additional research to determine whether a potential inhibitor can act on a growing bacterium.

In addition, the method of the present invention identifies antimicrobial agents that have advantages over other antimicrobial agents. First, it is noted that the TAT system is only found in plants, prokaryotes and archaea, but there are not any, even remotely homologous, TAT proteins that have been identified in animals. Homologs of TAT apparatus proteins are found in an impressive array of bacterial pathogens (e.g. *P. aeruginosa*, *M. tuberculosis*, *Staphylococcus aureus*, *E. coli*, *Legionella pneumophila*, *Bacillus anthracis*, *Yersinia pestis*, *Salmonella spp.*, *Vibrio cholerae*). Finally, as discussed above, the inventors have demonstrated that while a TAT mutant of *P. aeruginosa* was virtually nonvirulent in an infection model, it was still able to survive for a significant period of time in animals inoculated with the TAT mutant (1-2 weeks).

This information is of considerable importance for the following reasons. (i) First, the fact that TAT is not found in animals considerably reduces that the risk that a pharmaceutical product, which that blocks TAT function, would inhibit a similar system in humans and cause untoward side effects. For example, this would not be true for the Sec secretory system, since proteins comprising the Sec apparatus are found all kingdoms (i.e. prokaryotes, archaea, plants and animals). (ii) Second, since TAT is found in a wide range of prokaryotic pathogens, there is a significant chance that most compounds that inhibit TAT function as identified by the method of the present invention would very likely affect the function of TAT in other pathogens that use this translocase system. That is, a candidate anti-TAT compound would likely be a broad-spectrum therapeutic agent. In this regard, the present inventors have demonstrated that the genes encoding *E. coli* TAT proteins can function in *P. aeruginosa* and that TAT substrates from *B. pseudomallei* and *mallei* can be secreted through the *P. aeruginosa* TAT system (unpublished data). This supports the idea that it would be possible to identify a single compound that could inhibit the function of TAT in different pathogens. (iii) Finally, it is likely that because TAT is not essential in organisms that have been studied thus far, a TAT inhibitor would be less inclined to disturb the normal flora, because it would not kill these bacteria outright as do other antimicrobial compounds. TAT mutants have now been made in such diverse organisms as *P. aeruginosa*, *E. coli*, *L. pneumophila*, *Mycobacterium smegmatis*, as well as in plant pathogens and, of these, the ones that have been tested thus far are reduced in their ability to cause disease. Moreover, several independent studies have found that TAT mutants are actually hypersensitive to certain known antimicrobial compounds (e.g. β -lactams). Consequently, even if a candidate inhibitor only partially abrogates TAT function, it is possible that it would be synergistic with known antimicrobial agents.

Accordingly, one embodiment of the present invention relates to a method to identify a antimicrobial compound or a compound that inhibits the TAT secretory system in a microorganism. The method includes the steps of: (a) contacting a microorganism that has a TAT secretory system with a candidate compound; (b) measuring a level of secretion of a protein that is secreted via the TAT secretory pathway; and (c) measuring a level of expression of a gene that has increased expression when the TAT secretory pathway is inhibited. A decrease in the level of secretion of the protein in (b) in the presence of the candidate compound as compared to in the absence of the candidate compound, and an increase in the expression of the gene in (c) in the presence of the candidate compound as compared to in the absence of the candidate compound, indicates that the candidate compound is an inhibitor of the TAT secretory system and identifies the compound as an antimicrobial compound.

The first step of this method comprises contacting a microorganism that has a TAT secretory system with a candidate compound. According to the present invention, the TAT secretory system is a Sec-independent, ATP-independent secretion system, wherein the signal peptides that target the system comprise two consecutive Arg residues ("Twin Arginine Transport" = TAT) just before the hydrophobic core (h-region) and several additional residues between the h-region and the peptidase cleavage site. This system is capable of secreting proteins that are already folded before they enter the pore apparatus in the inner membrane. Three genes (*tatABC*) encode proteins that comprise the Tat secretory apparatus. In most species thus far investigated, these genes are part of an operon, although not invariably (See Fig. 2). One can readily identify a microorganism that has a TAT secretory system by identifying one or more of these genes or by identifying TAT biological activity in a microorganism. As used herein, "twin arginine translocase (TAT) biological activity" refers to any biological activity related to the secretion of proteins that have a TAT signal sequence across the cytoplasmic membrane of a microorganism. The biological function of the product of each of the *tatA*, *tatB*, and *tatC* genes is essential to the function of the TAT secretory system.

Accordingly, a microorganism that has a TAT secretory system can include any microorganism having such a system, including a microorganism that has been genetically modified to have such a system (e.g., through recombinant or other genetic engineering technology). Particularly preferred microorganisms to use in the present method include microbial strains against which the antimicrobial agent is to be directed. However, if the microorganism against which the antimicrobial agent is to be directed is pathogenic, one

advantage of the present invention is that the assay can be performed using a less pathogenic, or non-pathogenic, microorganism, and the compound is still predicted to be effective as an antimicrobial agent against the pathogenic microorganism. Furthermore, compounds that are identified using non-pathogenic microorganisms can be further tested for effectiveness as an antimicrobial agent against any microorganism, including pathogenic microorganisms. Suitable microorganisms that have a TAT secretory system can include microorganisms that have an endogenous TAT secretory system and microorganisms that are transformed to express a heterologous TAT secretory system using transformation techniques well known in the art.

Preferred families of bacterial strains that can have a TAT secretory system and are therefore useful in the invention include, but are not limited to, Enterobacteriaceae, Micrococcaceae, Vibrionaceae, Pasteurellaceae, Mycoplasmataceae, and Rickettsiaceae. Preferred genera of bacterial strains include, but are not limited to, *Pseudomonas*, *Bordetella*, *Mycobacterium*, *Vibrio*, *Bacillus*, *Salmonella*, *Francisella*, *Staphylococcus*, *Streptococcus*, *Enterococcus*, *Pasteurella*, *Yersinia*, *Shigella*, *Escherichia*, *Enterobacter*, *Serratia*, *Proteus*, *Citrobacter*, *Edwardsiella*, *Providencia*, *Klebsiella*, *Hafnia*, *Ewingella*, *Kluyvera*, *Morganella*, *Planococcus*, *Stomatococcus*, *Micrococcus*, *Aeromonas*, *Plessiomonas*, *Haemophilus*, *Actinobacillus*, *Mycoplasma*, *Ureaplasma*, *Rickettsia*, *Coxiella*, *Rochalimaea*, *Ehrlichia*, *Aerococcus*, *Gemella*, *Lactococcus*, *Leuconostoc*, *Pedococcus*, *Corynebacterium*, *Arcanobacterium*, *Actinomyces*, *Rhodococcus*, *Listeria*, *Erysipelothrix*, *Gardnerella*, *Neisseria*, *Camylobacter*, *Arcobacter*, *Wolinella*, *Heliobacter*, *Achromobacter*, *Acinetobacter*, *Agrobacterium*, *Alcaligenes*, *Chryseomonas*, *Comamonas*, *Eikenella*, *Flavimonas*, *Flavobacterium*, *Moraxella*, *Oligella*, *Skewanella*, *Weeksella*, *Xanthomonas*, *Brucella*, *Legionella*, *Afipia*, *Bartonella*, *Calymmatobacterium*, *Cardiobacterium*, *Streptobacillus*, *Spirillum*, *Peptostreptococcus*, *Peptococcus*, *Sarcinia*, *Coprococcus*, *Ruminococcus*, *Propionibacterium*, *Mobiluncus*, *Bifidobacterium*, *Eubacterium*, *Lactobacillus*, *Rothia*, *Clostridium*, *Bacteroides*, *Porphyromonas*, *Prevotella*, *Fusobacterium*, *Bilophila*, *Leptotrichia*, *Wolinella*, *Acidaminococcus*, *Megasphaera*, *Veilonella*, *Norcardia*, *Actinomadura*, *Norcardiopsis*, *Streptomyces*, *Micropolysporas*, *Thermoactinomyces*, *Treponema*, *Borrelia*, *Leptospira*, and *Chlamydiae*, with *Pseudomonas*, *Bordetella*, *Mycobacterium*, *Vibrio*, *Bacillus*, *Salmonella*, *Francisella*, *Staphylococcus*, *Streptococcus*, *Enterococcus*, *Pasteurella*, and *Yersinia* being particularly preferred.

Preferred species of bacterial strains include, but are not limited to, *Pseudomonas aeruginosa*, *Pseudomonas mallei*, *Pseudomonas pseudomallei*, *Bordetella pertussis*, *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Francisella tularensis*, *Vibrio cholerae*, *Bacillus anthracis*, *Salmonella enteric*, *Yersinia pestis*, *Escherichia coli* 0157:H7 and *Bordetella bronchiseptica*, with *Mycobacterium tuberculosis*, *Vibrio cholerae*, *Bacillus anthracis*, *Salmonella enteric* and *Yersinia pestis* being more preferred.

In another embodiment, preferred genera of bacterial strains (and preferred species indicated in parentheses) include, but are not limited to, *Pseudomonas* (*aeruginosa*, *syringae*, *putida*, *fluorescens*), *Azotobacter* (*vinelandii*), *Vibrio* (*vulnificus*, *parahaemolyticus*, *cholerae*), *Actinobacillus* (*pleuropneumoniae*), *Photobacterium* (*luminescens*), *Shewanella* (*oneidensis*), *Microbulbifer* (*degradans*), *Colwellia*, *Shigella* (*flexneri*), *Escherichia* (*coli*), *Salmonella* (*enterica*, *typhimurium*, *paratyphi*, *dublin*), *Pasteurella* (*multocida*), *Haemophilus* (*ducreyi*, *influenzae*, *somnus*), *Yersinia* (*pestis*), *Chromobacterium* (*violaceum*), *Neisseria* (*meningitidis*, *gonorrhoeae*), *Klebsiella* (*pneumoniae*), *Bordetella* (*pertussis*, *parapertussis*, *bronchiseptica*), *Burkholderia* (*pseudomallei*, *mallei*, *fungorum*), *Ralstonia* (*solanacearum*, *metallidurans*), *Nitrosomonas* (*europaea*), *Dichelobacter* (*nodosus*), *Xanthomonas* (*campestris*, *axonopodis*), *Xylella* (*fastidiosa*), *Acidithiobacillus*, *Desulfovibrio* (*vulgaris*), *Magnetococcus*, *Legionella* (*pneumophila*), *Rhodospirillum* (*rubrum*), *Desulfovibrio* (*desulfuricans*), *Novosphingobium* (*aromaticivorans*), *Ehrlichia* (*ruminantium*), *Aquifex* (*aeolicus*), *Sinorhizobium* (*meliloti*), *Brucella* (*melitensis*, *suis*), *Rhodopseudomonas* (*palustris*), *Agrobacterium* (*tumefaciens*), *Hyphomonas* (*neptunium*), *Bradyrhizobium* (*japonicum*), *Mesorhizobium* (*loti*), *Magnetospirillum* (*magnetotacticum*), *Wolinella* (*succinogenes*), *Anaplasma* (*marginale*), *Neorickettsia*, *Wolbachia*, *Deinococcus* (*radiodurans*), *Rickettsia* (*conorii*, *sibirica*, *proWazekii*), *Geobacillus*, *Helicobacter* (*pylori*, *hepaticus*), *Bacillus* (*subtilis*, *anthracis*, *cereus*, *halodurans*), *Caulobacter* (*crescentus*), *Prochlorococcus* (*marinus*), *Campylobacter* (*jejuni*), *Listeria* (*innocua*, *monocytogenes*), *Rhodobacter* (*sphaeroides*), *Silicibacter*, *Oceanobacillus* (*iheyensis*), *Mycobacterium* (*avium*, *tuberculosis*, *bovis*, *leprae*), *Synechococcus*, *Synechocystis*, *Corynebacterium* (*efficiens*, *diphtheriae*, *glutamicum*), *Nostoc* (*punctiforme*), *Thermobifida* (*fusca*), *Pyrobaculum* (*aerophilum*), *Streptomyces* (*avermitilis*), *Sulfolobus* (*solfataricus*, *tokodaii*), *Trichodesmium* (*erythraeum*), *Cytophaga* (*hutchinsonii*), *Thermosynechococcus* (*elongatus*), *Gloebacter* (*violaceus*) *Prochlorococcus* (*marinus*), *Methanosarcina* (*acetivorans*, *mazei*, *barkeri*), *Chlorobium* (*tepidum*), *Geobacter* (*metallireducens*), *Staphylococcus* (*aureus*), *Bacteroides* (*thetaitaomicron*, *fragiles*),

Dehalococcoides, *Ferroplasma* (*acidarmanus*), *Leptospira* (*interrogans*), *Tannerella*, *Thermoplasma* (*volcanium*, *acidophilum*), *Pirellula*, *Myxococcus* (*xanthus*), *Chloroflexus* (*aurantiacus*), *Archaeoglobus* (*fulgidus*), *Gemmata*, *Halobacterium*, and *Microbulbifer* (*degradans*). Regions of the genome of many of these bacteria with homology to the *tat* genes are shown in Table 1 (each accession number provided and all information represented by the accession number is incorporated herein by reference).

Table 1

Sequences producing significant alignments:	Score (bits)	E Value
ref NC_002516.1 <i>Pseudomonas aeruginosa</i> PA01, complete genome	531	e-150
ref NZ_AABQ07000004.1 <i>Pseudomonas aeruginosa</i> UCBPP-PA14 Pa...	530	e-150
gb AABQ07000004.1 <i>Pseudomonas aeruginosa</i> UCBPP-PA14 PAA.59...	530	e-150
ref NC_004578.1 <i>Pseudomonas syringae</i> pv. tomato str. DC300...	446	e-125
ref NZ_AABP02000002.1 <i>Pseudomonas syringae</i> pv. syringae B7...	446	e-124
gb AABP02000002.1 <i>Pseudomonas syringae</i> pv. syringae B728a ...	446	e-124
gnl Sanger_216595 Pflu703e10.q1c 29035 bp, 633 reads	432	e-120
gnl TIGR_220664 contig:3337:p_fluorescens <i>Pseudomonas fluor...</i>	431	e-120
ref NC_002947.3 <i>Pseudomonas putida</i> KT2440, complete genome	422	e-117
ref NZ_AAAU02000004.1 <i>Azotobacter vinelandii</i> Avin_88, whol...	388	e-107
gb AAAU02000004.1 <i>Azotobacter vinelandii</i> Avin_4, whole gen...	388	e-107
gnl TIGR_198628 contig:306:e_chrysanthemi <i>Erwinia chrysanth...</i>	311	4e-84
gnl TIGR_243233 contig:229:m_capsulatus <i>Methylococcus capsu...</i>	309	1e-83
ref NC_004459.1 <i>Vibrio vulnificus</i> CMCP6 chromosome I, comp...	307	4e-83
ref NC_005139.1 <i>Vibrio vulnificus</i> YJ016 chromosome I, comp...	307	4e-83
gnl OUACGT_44294 ap5.fasta.screen.Contig189 <i>Actinobacillus ...</i>	306	7e-83
ref NZ_AACK01000002.1 <i>Actinobacillus pleuropneumoniae</i> sero...	306	7e-83
gb AACK01000002.1 <i>Actinobacillus pleuropneumoniae</i> serovar ...	306	7e-83
gnl OUACGT_209841 ap7.fasta.screen.Contig408 <i>Actinobacillus...</i>	305	2e-82
gnl Sanger_218491 J11712Gd03.slka 1076952 bp, 19406 reads	305	2e-82
ref NC_005126.1 <i>Photorhabdus luminescens</i> subsp. laumondii ...	304	4e-82
ref NC_004347.1 <i>Shewanella oneidensis</i> MR-1, complete genome	303	6e-82
ref NZ_AABI02000008.1 <i>Microbulbifer degradans</i> 2-40 Mdeg_7,...	303	6e-82
gb AABI02000008.1 <i>Microbulbifer degradans</i> 2-40 Mdeg_7, who...	303	6e-82
gnl TIGR_167879 contig:1731:c_psychroerythraea <i>Colwellia ps...</i>	303	8e-82
ref NC_004603.1 <i>Vibrio parahaemolyticus</i> RIMD 2210633 chrom...	303	1e-81
ref NC_002505.1 <i>Vibrio cholerae</i> O1 biovar eltor str. N1696...	302	1e-81
gnl Sanger_216598 dys366d06.plk 82791 bp, 1298 reads	300	7e-81
ref NC_004741.1 <i>Shigella flexneri</i> 2a str. 2457T, complete ...	300	9e-81
gnl Sanger_216593 Epath261e06.q1k 73291 bp, 1532 reads	300	9e-81
gnl Sanger_216599 shig88d06.q1k 62983 bp, 1030 reads	300	9e-81
ref NC_002695.1 <i>Escherichia coli</i> O157:H7, complete genome	300	9e-81
gnl Sanger_216592 Eagg553b05.q1k 9830 bp, 98 reads	300	9e-81

ref NC_004337.1	Shigella flexneri 2a str. 301, complete ge...	300	9e-81
ref NC_002655.2	Escherichia coli O157:H7 EDL933, complete ...	300	9e-81
ref NC_000913.1	Escherichia coli K12, complete genome	300	9e-81
ref NC_004431.1	Escherichia coli CFT073, complete genome	298	2e-80
ref NC_004631.1	Salmonella enterica subsp. enterica serova...	296	8e-80
ref NC_003197.1	Salmonella typhimurium LT2, complete genome	296	8e-80
ref NC_003198.1	Salmonella enterica subsp. enterica serova...	296	8e-80
gnl Sanger_216597	salt10-406d09.q1k 17792 bp, 316 reads	296	8e-80
ref NC_002663.1	Pasteurella multocida, complete genome	296	1e-79
ref NC_002940.2	Haemophilus ducreyi 35000HP, complete genome	296	1e-79
gnl OUACGT_714	act.fasta.screen.Contig1 Actinobacillus acti...	296	1e-79
gnl Sanger_218493	bong453a07.plk 539452 bp, 10346 reads	295	2e-79
ref NC_004088.1	Yersinia pestis KIM, complete genome	294	5e-79
ref NC_003143.1	Yersinia pestis strain CO92, complete genome	294	5e-79
gnl SANGER_34054	Yersinia enterocolitica 8081	291	4e-78
ref NC_000907.1	Haemophilus influenzae Rd, complete genome	290	7e-78
ref NC_005085.1	Chromobacterium violaceum ATCC 12472, comp...	286	1e-76
ref NZ_AACJ01000020.1	Haemophilus somnus 2336 Haso_20, who...	282	2e-75
gb AACJ01000020.1	Haemophilus somnus 2336 Hsomnus.Contig98...	282	2e-75
ref NZ_AABO02000012.1	Haemophilus somnus 129PT Hsom_12, wh...	282	2e-75
gb AABO02000012.1	Haemophilus somnus 129PT Hsom_12, whole ...	282	2e-75
ref NC_003116.1	Neisseria meningitidis serogroup A strain ...	276	8e-74
gnl SANGER_135720	Neisseria meningitidis serogroup C FAM18	276	8e-74
ref NC_003112.1	Neisseria meningitidis serogroup B strain ...	274	5e-73
gnl WUGSC_573	kpneumo_B_KPN.Contig925 Klebsiella pneumoniae...	198	2e-72
gnl OUACGT_485	Ngon_Contig1 Neisseria gonorrhoeae unfinishe...	270	6e-72
ref NC_002929.2	Bordetella pertussis, complete genome	266	1e-70
ref NC_002928.3	Bordetella parapertussis, complete genome	266	1e-70
ref NC_002927.3	Bordetella bronchiseptica, complete genome	266	1e-70
gnl OUACGT_40325	ap1.fasta.screen.Contig255 Actinobacillus ...	264	6e-70
gnl Sanger_28450	Burkholderia pseudomallei chromosome 1	263	9e-70
gnl TIGR_243160	contig:578:b_mallei Burkholderia mallei ATC...	263	9e-70
ref NC_003295.1	Ralstonia solanacearum, complete genome	259	2e-68
ref NZ_AAAI01000354.1	Ralstonia metallidurans Reut_354, wh...	255	3e-67
gb AAAI01000354.1	Ralstonia metallidurans Reut_693, whole ...	255	3e-67
gnl Sanger_216591	BC10B5Le10.q1ka 1708961 bp, 43073 reads	254	3e-67
ref NC_004757.1	Nitrosomonas europaea ATCC 19718, complete...	253	1e-66
ref NZ_AAAJ02000069.1	Burkholderia fungorum Bcep_249, whol...	249	2e-65
gb AAAJ02000069.1	Burkholderia fungorum Bcep_402, whole ge...	249	2e-65
gnl TIGR_246195	contig:1116:d_nodosus Dichelobacter nodosus...	246	1e-64
ref NC_003902.1	Xanthomonas campestris pv. campestris str....	238	3e-62
ref NC_003919.1	Xanthomonas axonopodis pv. citri str. 306,...	238	4e-62
ref NZ_AAAL01000117.1	Xylella fastidiosa Dixon XfasA_117, ...	228	3e-59
gb AAAL01000117.1	Xylella fastidiosa Dixon XfasA_310, whol...	228	3e-59

ref NC_002488.1 <i>Xylella fastidiosa</i> 9a5c, complete genome	228	4e-59
ref NC_004556.1 <i>Xylella fastidiosa</i> Temecula1, complete genome	227	8e-59
ref NZ_AAAM01000024.1 <i>Xylella fastidiosa</i> Ann-1 XfasO_24, w...	222	2e-57
gb AAAM01000024.1 <i>Xylella fastidiosa</i> Ann-1 XfasO_123, whol...	222	2e-57
gnl Sanger_85569 stdt51e11.plk <i>Salmonella typhimurium</i> DT104...	216	3e-57
ref NZ_AAAT02000095.1 <i>Pseudomonas fluorescens</i> PfO-1 Pflu_6...	218	5e-56
gnl TIGR_243159 contig:10428:a_ferrooxidans <i>Acidithiobacill...</i>	206	1e-52
gnl TIGR_882 contig:1529:d_vulgaris <i>Desulfovibrio vulgaris</i> ...	198	4e-50
ref NZ_AAAN01000187.1 <i>Magnetococcus</i> sp. MC-1 Mmc1_187, who...	195	3e-49
gb AAAN01000187.1 <i>Magnetococcus</i> sp. MC-1 Mmc1_408, whole g...	195	3e-49
gnl CUCGC_446 45 <i>Legionella pneumophila</i> unfinished fragment...	187	5e-47
ref NZ_AAAG01000006.1 <i>Rhodospirillum rubrum</i> Rrub_6, whole ...	184	7e-46
gb AAAG01000002.1 <i>Rhodospirillum rubrum</i> Rrub_2, whole geno...	184	7e-46
ref NZ_AABN02000013.1 <i>Desulfovibrio desulfuricans</i> G20 Ddes...	180	8e-45
gb AABN02000013.1 <i>Desulfovibrio desulfuricans</i> G20 Ddes_12,...	180	8e-45
ref NZ_AAAV01000112.1 <i>Novosphingobium aromaticivorans</i> Saro...	177	5e-44
gb AAAV01000112.1 <i>Novosphingobium aromaticivorans</i> Saro_150...	177	5e-44
gnl vsunipre_254945 seq <i>Ehrlichia ruminantium</i>	176	1e-43
gnl Sanger_216596 rhiz693h02.plk 14521 bp, 220 reads	175	3e-43
ref NC_000918.1 <i>Aquifex aeolicus</i> complete genome	174	4e-43
ref NC_003047.1 <i>Sinorhizobium meliloti</i> 1021 complete genomes	174	8e-43
ref NC_003317.1 <i>Brucella melitensis</i> chromosome I, complete...	172	2e-42
ref NZ_AAAF01000001.1 <i>Rhodopseudomonas palustris</i> Rpal_1, w...	172	3e-42
gb AAAF01000014.1 <i>Rhodopseudomonas palustris</i> Rpal_58, whol...	172	3e-42
ref NC_004310.1 <i>Brucella suis</i> 1330 chromosome I, complete ...	171	4e-42
gnl TIGR_212042 contig:377:a_phagocytophila <i>Anaplasma phago...</i>	171	5e-42
ref NC_003304.1 <i>Agrobacterium tumefaciens</i> str. C58 (U. Was...	170	8e-42
ref NC_003062.1 <i>Agrobacterium tumefaciens</i> strain C58 circu...	170	8e-42
gnl TIGR_228405 contig:989:h_neptunium <i>Hyphomonas neptunium</i> ...	169	2e-41
ref NC_004463.1 <i>Bradyrhizobium japonicum</i> , complete genome	169	2e-41
ref NC_002678.1 <i>Mesorhizobium loti</i> , complete genome	168	4e-41
ref NZ_AAAP01003805.1 <i>Magnetospirillum magnetotacticum</i> Mag...	167	7e-41
gb AAAP01003805.1 <i>Magnetospirillum magnetotacticum</i> Magn_38...	167	7e-41
ref NC_005090.1 <i>Wolinella succinogenes</i> , complete genome	166	2e-40
gnl VMPBIO_234826 <i>Anaplasma marginale</i> St. Maries strain	163	1e-39
gnl WUGSC_54388 spara_B_SPA.0.17356 <i>Salmonella paratyphi</i> A ...	112	7e-39
gnl TIGR_222891 contig:36:n_sennetsu <i>Neorickettsia sennetsu</i> ...	160	1e-38
gnl TIGR_163164 contig:21352:wolbachia <i>Wolbachia endosymbio</i> ...	159	3e-38
gnl TIGR_205920 contig:378:e_chaffeensis <i>Ehrlichia chaffeensis</i> ...	157	6e-38
ref NC_001263.1 <i>Deinococcus radiodurans</i> chromosome 1, comp...	156	2e-37
ref NC_003103.1 <i>Rickettsia conorii</i> Malish 7, complete genome	155	2e-37
gnl OUACGT_1422 bstearo.fasta.screen.Contig339 <i>Geobacillus</i> ...	154	5e-37
gnl UIUC_592 senteritdis_910_10.21 <i>Salmonella enteritidis</i> u...	154	6e-37
ref NZ_AABW01000001.1 <i>Rickettsia sibirica</i> rsib_agncrt, who...	152	2e-36

gb AABW01000001.1 Rickettsia sibirica rsib_agncrt, whole g...	152	2e-36
ref NC_000921.1 Helicobacter pylori, strain J99 complete g...	152	3e-36
ref NC_000963.1 Rickettsia prowazekii strain Madrid E, com...	151	4e-36
ref NC_000964.1 Bacillus subtilis, complete genome	151	5e-36
ref NC_000915.1 Helicobacter pylori 26695, complete genome	151	5e-36
gnl TIGR_205919 contig:735:b_anthraxis-strainkrugerb Bacill...	145	2e-34
gnl TIGR_212045 contig:1488:b_anthraxis-westernna Bacillus ...	145	2e-34
ref NC_003997.3 Bacillus anthracis str. Ames, complete genome	145	2e-34
gnl TIGR_222523 contig:1754:b_cereus Bacillus cereus ATCC 1...	145	3e-34
ref NC_002696.2 Caulobacter crescentus CB15, complete genome	143	1e-33
ref NC_004722.1 Bacillus cereus ATCC 14579, complete genome	141	4e-33
ref NC_005071.1 Prochlorococcus marinus str. MIT 9313, com...	139	2e-32
ref NC_002163.1 Campylobacter jejuni, complete genome	139	3e-32
gnl TIGR_195099 contig:519:c_jejuni Campylobacter jejuni RM...	138	4e-32
gnl TIGR_195099 contig:521:c_jejuni Campylobacter jejuni RM...	138	4e-32
ref NC_002570.1 Bacillus halodurans, complete genome	136	1e-31
gnl Sanger_85569 stdt38h03.plk Salmonella typhimurium DT104...	135	2e-31
ref NZ_AAAE01000156.1 Rhodobacter sphaeroides Rsph_156, wh...	135	3e-31
gb AAAE01000156.1 Rhodobacter sphaeroides Rsph_192, whole ...	135	3e-31
gnl TIGR_246200 contig:2649:s_pomeroyi Silicibacter pomeroy...	134	9e-31
ref NC_003212.1 Listeria innocua Clip11262, complete genome	133	1e-30
ref NC_003210.1 Listeria monocytogenes strain EGD, complet...	132	2e-30
ref NC_004917.1 Helicobacter hepaticus ATCC 51449, complet...	132	3e-30
ref NC_004193.1 Oceanobacillus iheyensis HTE831, complete ...	132	3e-30
gnl TIGR_243243 contig:3273:m_avium Mycobacterium avium 104...	132	3e-30
gnl jmarq_32049 Contig051302-301 Synechococcus sp. PCC 7002...	131	4e-30
gnl CBCUMN_1770 paratb.fasta.screen.Contig254 Mycobacterium...	131	4e-30
ref NC_000911.1 Synechocystis sp. PCC 6803, complete genome	129	3e-29
ref NC_004369.1 Corynebacterium efficiens YS-314, complete...	127	1e-28
ref NZ_AAAY02000003.1 Nostoc punctiforme Npun_200, whole g...	126	2e-28
gb AAAY02000003.1 Nostoc punctiforme Npun_3, whole genome ...	126	2e-28
ref NC_002755.1 Mycobacterium tuberculosis CDC1551, comple...	125	3e-28
ref NC_002945.3 Mycobacterium bovis subsp. bovis AF2122/97...	125	3e-28
ref NC_000962.1 Mycobacterium tuberculosis H37Rv complete ...	125	3e-28
gnl TIGR_164513 mtub210_196 Mycobacterium tuberculosis stra...	125	3e-28
ref NC_003272.1 Nostoc sp. PCC 7120 complete genome	125	4e-28
gnl Sanger_216594 mar544h07.p2k1119w 217417 bp, 4010 reads	125	4e-28
gnl jmarq_32049 Contig051302-2 Synechococcus sp. PCC 7002 u...	124	5e-28
ref NZ_AAAQ01000042.1 Thermobifida fusca Tfus_42, whole ge...	122	2e-27
gb AAAQ01000042.1 Thermobifida fusca Tfus_64, whole genome...	122	2e-27
ref NC_003364.1 Pyrobaculum aerophilum, complete genome	122	2e-27
ref NC_003155.2 Streptomyces avermitilis MA-4680, complete...	122	3e-27
ref NC_002754.1 Sulfolobus solfataricus, complete genome	120	8e-27
ref NC_003888.3 Streptomyces coelicolor A3(2), complete ge...	120	1e-26

ref NZ_AABK02000029.1 Trichodesmium erythraeum IMS101 Tery...	120	1e-26
gb AABK02000029.1 Trichodesmium erythraeum IMS101 Tery_29,...	120	1e-26
gnl TIGR_243231 contig:3019:g_sulfurreducens Geobacter sulf...	119	2e-26
ref NZ_AABD02000035.1 Cytophaga hutchinsonii Chut_41, whol...	119	2e-26
gb AABD02000035.1 Cytophaga hutchinsonii Chut_140, whole g...	119	2e-26
ref NC_002677.1 Mycobacterium leprae strain TN complete ge...	119	2e-26
ref NC_005125.1 Gloeobacter violaceus, complete genome	119	2e-26
ref NC_004113.1 Thermosynechococcus elongatus BP-1, comple...	119	3e-26
ref NC_005070.1 Synechococcus sp. WH 8102, complete genome	118	4e-26
gnl TIGR_246196 contig:3439:m_smegmatis Mycobacterium smegm...	118	5e-26
ref NC_005042.1 Prochlorococcus marinus subsp. marinus str...	117	6e-26
ref NC_003552.1 Methanosarcina acetivorans str. C2A, compl...	117	8e-26
ref NZ_AAAS01000001.1 Geobacter metallireducens Gmet_1, wh...	117	1e-25
gb AAAS01000005.1 Geobacter metallireducens Gmet_5, whole ...	117	1e-25
ref NC_002932.3 Chlorobium tepidum TLS, complete genome	116	1e-25
ref NC_002745.2 Staphylococcus aureus subsp. aureus N315, ...	115	3e-25
ref NC_003106.2 Sulfolobus tokodaii, complete genome	115	3e-25
ref NC_002758.1 Staphylococcus aureus strain Mu50, complet...	115	3e-25
ref NC_004663.1 Bacteroides thetaiotaomicron VPI-5482, com...	114	5e-25
ref NC_002935.2 Corynebacterium diphtheriae, complete genome	114	5e-25
gnl OUACGT_93061 staph.fasta.screen.Contig10.comp Staphyloc...	114	9e-25
gnl TIGR_93062 S.aureus_8943 Staphylococcus aureus COL unfi...	114	9e-25
ref NZ_AABD02000073.1 Cytophaga hutchinsonii Chut_104, who...	114	9e-25
gb AABD02000073.1 Cytophaga hutchinsonii Chut_88, whole ge...	114	9e-25
ref NC_003450.2 Corynebacterium glutamicum ATCC 13032, com...	114	9e-25
gnl Sanger_159289 Staphylococcus aureus MSSA strain 476	113	1e-24
ref NC_003923.1 Staphylococcus aureus subsp. aureus MW2, c...	113	1e-24
gnl Sanger_159288 Staphylococcus aureus (EMRSA-16) chromoso...	113	2e-24
ref NC_003901.1 Methanosarcina mazei strain Goel, complete...	112	3e-24
ref NZ_AAAR01001830.1 Methanosarcina barkeri Meth_1830, wh...	112	4e-24
gb AAAR01001830.1 Methanosarcina barkeri Meth_1830, whole ...	112	4e-24
gnl UIUC_98360 sdublin_Contig3260_12.23 Salmonella dublin u...	110	1e-23
gnl TIGR_243164 contig:6871:d_ethenogenes Dehalococcoides e...	109	2e-23
gnl SANGER_817 Bacteroides fragilis NCTC9343	107	7e-23
ref NZ_AABC02000027.1 Ferroplasma acidarmanus Faci_37, who...	107	9e-23
gb AABC02000027.1 Ferroplasma acidarmanus Faci_27, whole g...	107	9e-23
ref NC_004342.1 Leptospira interrogans serovar lai str. 56...	105	4e-22
gnl TIGR_203275 contig:3853:b_forsythus Tannerella forsythe...	105	4e-22
ref NC_002689.2 Thermoplasma volcanium, complete genome	104	7e-22
gnl TIGR_29390 contig:4222:s_gordonii Streptococcus gordonii...	103	1e-21
ref NC_005027.1 Pirellula sp., complete genome	103	1e-21
ref NC_002578.1 Thermoplasma acidophilum, complete genome	102	3e-21
ref NC_005072.1 Prochlorococcus marinus subsp. pastoris st...	100	1e-20
gnl TIGR_246197 contig:425:m_xanthus Myxococcus xanthus DK ...	100	1e-20

ref NZ_AAAH01000204.1 Chloroflexus aurantiacus Chlo_204, w...	95	6e-19
gb AAAH01000972.1 Chloroflexus aurantiacus Chlo_972, whole...	95	6e-19
ref NC_000917.1 Archaeoglobus fulgidus DSM 4304, complete ...	88	7e-17
gnl TIGR_214688 contig:234:g_obscuriglobus Gemmata obscurig...	88	7e-17
ref NC_002607.1 Halobacterium sp. NRC-1, complete genome	87	1e-16
gnl TIGR_205919 contig:29534:b_anthraxis-strainkrugerb Baci...	79	3e-14
ref NZ_AABI02000594.1 Microbulbifer degradans 2-40 Mdeg_59...	75	4e-13
gb AABI02000594.1 Microbulbifer degradans 2-40 Mdeg_594, w...	75	4e-13
gnl Sanger_85569 stdt25a08.p1k Salmonella typhimurium DT104...	61	9e-09
gnl WUGSC_54388 spara_B_SPA.0.6891 Salmonella paratyphi A u...	51	1e-05

It is to be appreciated that a number of the bacterial species described above include a variety of subspecies, types, subtypes, etc. that are meant to be included within the aforementioned species.

According to the present invention, a "candidate", "putative", or "test" compound or agent refers to a compound having an unknown or previously unappreciated regulatory activity in a particular process. As such, the term "identify" with regard to methods to identify compounds is intended to include all compounds, the usefulness of which as a compound for the inhibition of the TAT secretory system or as an antimicrobial agent is determined by a method of the present invention.

Compounds to be screened in the methods of the invention include known organic compounds such as products of peptide libraries and products of chemical combinatorial libraries. Compounds may also be identified using rational drug design relying on the structure of the product of a gene. Such methods are known to those of skill in the art and involve the use of three-dimensional imaging software programs. For example, various methods of drug design, useful to design or select mimetics or other therapeutic compounds useful in the present invention are disclosed in Maulik et al., 1997, Molecular Biotechnology: Therapeutic Applications and Strategies, Wiley-Liss, Inc., which is incorporated herein by reference in its entirety.

As used herein, a mimetic refers to any peptide or non-peptide compound that is able to mimic the biological action of a naturally occurring peptide, often because the mimetic has a basic structure that mimics the basic structure of the naturally occurring peptide and/or has the salient biological properties of the naturally occurring peptide. Mimetics can include, but are not limited to: peptides that have substantial modifications from the prototype such as no side chain similarity with the naturally occurring peptide (such modifications, for example, may decrease its susceptibility to degradation); anti-idiotypic and/or catalytic antibodies, or fragments thereof; non-proteinaceous portions of an isolated

protein (e.g., carbohydrate structures); or synthetic or natural organic molecules, including nucleic acids and drugs identified through combinatorial chemistry, for example. Such mimetics can be designed, selected and/or otherwise identified using a variety of methods known in the art.

A mimetic can be obtained, for example, from molecular diversity strategies (a combination of related strategies allowing the rapid construction of large, chemically diverse molecule libraries), libraries of natural or synthetic compounds, in particular from chemical or combinatorial libraries (i.e., libraries of compounds that differ in sequence or size but that have the similar building blocks) or by rational, directed or random drug design. See for example, Maulik et al., *supra*.

In a molecular diversity strategy, large compound libraries are synthesized, for example, from peptides, oligonucleotides, carbohydrates and/or synthetic organic molecules, using biological, enzymatic and/or chemical approaches. The critical parameters in developing a molecular diversity strategy include subunit diversity, molecular size, and library diversity. The general goal of screening such libraries is to utilize sequential application of combinatorial selection to obtain high-affinity ligands for a desired target, and then to optimize the lead molecules by either random or directed design strategies. Methods of molecular diversity are described in detail in Maulik, et al., *ibid*.

Maulik et al. also disclose, for example, methods of directed design, in which the user directs the process of creating novel molecules from a fragment library of appropriately selected fragments; random design, in which the user uses a genetic or other algorithm to randomly mutate fragments and their combinations while simultaneously applying a selection criterion to evaluate the fitness of candidate ligands; and a grid-based approach in which the user calculates the interaction energy between three dimensional receptor structures and small fragment probes, followed by linking together of favorable probe sites.

Candidate compounds identified or designed by the above-described methods can be synthesized using techniques known in the art, and depending on the type of compound. Synthesis techniques for the production of non-protein compounds, including organic and inorganic compounds are well known in the art. For example, for smaller peptides, chemical synthesis methods are preferred. For example, such methods include well known chemical procedures, such as solution or solid-phase peptide synthesis, or semi-synthesis in solution beginning with protein fragments coupled through conventional solution methods. Such methods are well known in the art and may be found in general texts and articles in the area such as: Merrifield, 1997, *Methods Enzymol.* 289:3-13; Wade et al., 1993, *Australas*

Biotechnol. 3(6):332-336; Wong et al., 1991, Experientia 47(11-12):1123-1129; Carey et al., 1991, Ciba Found Symp. 158:187-203; Plaue et al., 1990, Biologicals 18(3):147-157; Bodanszky, 1985, Int. J. Pept. Protein Res. 25(5):449-474; or H. Dugas and C. Penney, BIOORGANIC CHEMISTRY, (1981) at pages 54-92, all of which are incorporated herein by reference in their entirety. For example, peptides may be synthesized by solid-phase methodology utilizing a commercially available peptide synthesizer and synthesis cycles supplied by the manufacturer. One skilled in the art recognizes that the solid phase synthesis could also be accomplished using the FMOC strategy and a TFA/scavenger cleavage mixture. A compound that is a protein or peptide can also be produced using recombinant DNA technology and methods standard in the art, particularly if larger quantities of a protein are desired.

In one example of providing a library of compounds to be screened using the method of the present invention, a peptide library can be synthesized based on the RRXFLK (SEQ ID NO:19) hexapeptide that is highly conserved in the signal sequence of TAT secreted proteins. This is a completely unbiased library based on that hexapeptide because one can address each of the six residues with all twenty known amino acids. The present inventors propose that one or more peptides in this library will bind to and inhibit the TAT machinery in *P. aeruginosa* by virtue to its similarity to the TAT signal sequence in TAT secreted proteins.

To generate libraries to screen, by way of example, approximately 203 peptides can initially be generated to screen in the method of the invention. Although there is a consensus sequence for TAT peptides as discussed above, there is some variability in three of the six residues. Hence, initially, a combinatorial peptide library would be screened based on the sequence AC-R-R-X1-F-X2-X3-NH₂, where the X's indicate residue positions in which the amino acids will be varied. Intelligent testing of mixtures will decrease considerably the number of assays that will have to be performed. Subsequently, the method of the invention will be employed to examine peptides where the twin arginine residues and the phenylalanine are altered. There is essentially no limit to the number of compounds that can be screened using the method of the invention.

Table 2 describes exemplary peptide libraries that can be used in the method of the present invention with and without permeablizing agents or treatments of the cells.

Table 2. A hexa-peptide library of 64 million peptides used for screening to select a peptide inhibitor of TAT.

Library 1	Ac-O ₁ X ₂ X ₃ X ₄ X ₅ X ₆ -amide 20 pools at position O ₁ of 3.2 million peptides/pool. This screen will define the amino acid residue at position O ₁ .
Library 2	Ac-O ₁ O ₂ X ₃ X ₄ X ₅ X ₆ -amide 20 pools at position O ₂ of 160,000 peptides/pool. This screen will define the amino acid residue at position O ₂ .
Library 3	Ac-O ₁ O ₂ O ₃ X ₄ X ₅ X ₆ -amide 20 pools at position O ₃ of 8,000 peptides/pool. This screen will define the amino acid residue at position O ₃ .
Library 4	Ac-O ₁ O ₂ O ₃ O ₄ X ₅ X ₆ -amide 20 pools at position O ₄ of 400 peptides/pool. This screen will define the amino acid residue at position O ₄ .
Library 5	Ac-O ₁ O ₂ O ₃ O ₄ O ₅ X ₆ -amide 20 pools at position O ₅ of 20 peptides/pool. This screen will define the amino acid residue at position O ₅ .
Library 6	Ac-O ₁ O ₂ O ₃ O ₄ O ₅ O ₆ -amide Screen of 20 peptides at position O ₆ will define the amino acid residue at position O ₆ .

This deconvolution approach defines one or more specific hexapeptide sequences that best inhibit of the TAT system based upon the screening assays described in this application. Once one or more inhibitory sequences are identified, these can be used in more specific assays of TAT function.

Other candidates for screening include an assortment of chemical libraries that are available through various public and commercial resources. Accordingly, the same strategy described above can be employed to select chemical libraries to screen.

The conditions under which a microbial cell (a microorganism) is exposed to or contacted with a candidate compound, such as by mixing, are any suitable culture or assay conditions. Suitable culture or assay conditions include the use of an effective medium in which the microorganism cell can be cultured or assayed in the presence and absence of a candidate compound. Microbial cells used in the present invention can be cultured in a variety of containers including, but not limited to, tissue culture flasks, test tubes, microtiter dishes, and petri plates, although a microtiter plate or similar container is preferred for high throughput assays. Culturing is carried out at a temperature, pH, nutrient level and oxygen

level that are appropriate for the particular microbial cell. Such culturing conditions are also within the skill in the art. Microbial cells are contacted with a candidate compound under conditions which take into account the number of microbial cells per container contacted (e.g., per well of a microtiter plate), the concentration of candidate compound(s) used per experimental condition (e.g., per well of a microtiter plate), and the incubation time of the candidate compound with the cells. Determination of effective protocols can be accomplished by those skilled in the art based on variables such as the size of the container, the volume of liquid in the container, conditions known to be suitable for the culture of the particular microbe used in the assay, and the chemical composition of the candidate compound(s) (i.e., size, charge etc.) being tested. A preferred amount of candidate compound(s) can comprise between about 1 nM to about 10 mM of candidate compound(s) per well of a 96-well plate.

In one embodiment of the assay, one can increase the permeability of the outer membrane of test microbial cell during the assay in order to allow better access of candidate inhibitors to the TAT machinery of the microbial cell, without compromising TAT function or growth of microbial cell. This can be accomplished in a variety of ways. First, one can use permeabilizing compounds or treatments. For example, only 250 ppm of the compound chitosan (polymeric beta-1,4-N-acetylglucosamine) at pH 5.3 induces significant uptake of the hydrophobic probe 1-N-phenylnaphthylamine in *P. aeruginosa* and other gram negative bacteria. Notably this effect was abolished by the addition of MgCl₂. Chitosan also sensitized *P. aeruginosa* to the lytic effects of ionic detergents (i.e. sodium dodecyl sulfate) and this sensitization was reversible by washing chitosan treated cells prior to exposure to detergents. Finally, it was shown that chitosan did not cause release of outer membrane LPS or other lipids from the bacterial cells, thereby maintaining viability, but increased outer membrane permeability. Other compounds that could be used include, but are not limited to, gramicidin, β -lactam antibiotics, polymyxin B, and chelating agents (e.g. EDTA or EGTA), any of which have the potential to improve access of a compound to the TAT machinery. Typically, sub-inhibitory concentrations of these compounds are used to identify the optimal concentrations that do not affect the function of TAT but which improve the access of TAT inhibitors to the TAT machinery.

Also by way of example, certain physical conditions, including brief heat shock, rapid chilling, or freezing/thawing, substantially enhance the antimicrobial activity of the polypeptide antibiotic, nisin. The access of nisin to the cytoplasmic membrane can be transient once cells are returned to lower temperatures. Accordingly, one can use different

temperatures for heat shock or chilling (e.g., using nisin as a control) to evaluate access of a known antimicrobial peptide to the inner membrane and possibly to TAT machinery. Once the optimal conditions are determined, such a protocol can be used in the screening assays of the invention, if desired.

In one embodiment of the invention, the method is carried out at a temperature from about 20°C to about 42°C, with temperatures between 24°C and 37°C being preferred. In one aspect, the method is carried out (microorganisms grown and/or tested) at 24°C. In another aspect, the method is carried out (microorganisms grown and/or tested) at 37°C.

The method of the present invention includes the negative screening step, which includes measuring a level of secretion of a protein that is secreted via the TAT secretory pathway. A decrease in the level of secretion of this protein in the presence of the candidate compound as compared to in the absence of the candidate compound indicates that the candidate compound is an inhibitor of the TAT secretory system and identifies the compound as a potential antimicrobial compound.

According to the present invention, a protein that is secreted via the TAT secretory system and is used as a "biomarker" in the present method can include any soluble protein that is secreted by this system. Such proteins can be identified by the presence of a signal peptide in the preprotein comprising two consecutive arginine (Arg) residues located just before the hydrophobic core (h-region), and several additional residues between the h-region and the peptidase cleavage site on the protein. Several proteins that are secreted by the TAT secretory system are known in the art or can be identified by this structural motif. For example, such proteins are listed in Table 3. Other such proteins can be readily identified by those of skill in the art.

Table 3. *P. aeruginosa* genes encoding proteins that are secreted by the TAT apparatus

Function	Encoded protein	Cellular location	Signal sequence*	Homologs in other bacteria**
Phospholipases				
<i>PlcH</i>	Phospholipase C	Extracellular	MTENWKFRRTFLKHGAQAATLAGLSGLFPETLRRALA SEQ ID NO:1	<i>M. tuberculosis</i> <i>Burkholderia cepacia</i> , <i>B. pseudomallei</i>
<i>PlcN</i>	Phospholipase C	Extracellular	MISKSRRSFIRLAAGTVGATVATSMPLSSIQAALA SEQ ID NO:2	
Iron acquisition				
<i>FpvA</i>	Ferripyoverdine receptor	Outer membrane	MPAPHGLSPLSKAFLMRRAFQRRILPHSLAMALSLPLAGYVQA SEQ ID NO:3	<i>M. tuberculosis</i> <i>Pseudomonas putida</i> , <i>P. syringae</i>
PA2394	Pyoverdine biosynthesis	Periplasmic	MNDRRTFLKQAGILAAGLPLLSAAQSLRAEG SEQ ID NO:4	
PA2389	Pyoverdine biosynthesis	Periplasmic	MRRTSTRALLVAVCLSPLIALA SEQ ID NO:5	
PA2392	Pyoverdine	Periplasmic	MTVSRRGFMAGLALTGAAALPVAYY	

Function	Encoded protein	Cellular location	Signal sequence*	Homologs in other bacteria**
anaerobic growth	biosynthesis		SEQ ID NO:6	
	<i>napA</i> Nitrate reductase	Periplasmic	MNLTRREFAKANAAAAIAAAAGLPILVRASNLVTEADV SEQ ID NO:7	<i>Bordetella parapertussis</i> , <i>Yersinia enterocolitica</i> ,
	<i>napF</i> Ferredoxin	Periplasmic	MSSRRELFRRLLGGHPPTRRPPWTAADFAAG SEQ ID NO:8	<i>Vibrio cholerae</i>
	<i>nosZ</i> Nitrous oxide reductase	Periplasmic	MSDDTKSPHEETHGLNRRGFLGASALTGAAALVGASA SEQ ID NO:9	<i>Salmonella enterica</i> serovar Typhi, <i>E. coli</i> , <i>Haemophilus ducreyi</i>
metabolism	<i>fdnG</i> Formate dehydrogenase	Periplasmic?	MDMNRQFFKVCIGLGGSSLAALGMAPTEAFA SEQ ID NO:10	<i>S. enterica</i> serovar Typhi, <i>E. coli</i>
	PA2124 Dehydrogenase	Periplasmic?	MHQPENPARRTLLAQTVAGSAALALGSLGGAPGVASA SEQ ID NO:11	
	PA2264 Dehydrogenase	Periplasmic?	MPDDKAVNGRRDFLRKTLTVIPAVTLAGYGVG SEQ ID NO:12	<i>Yersinia pestis</i>
	PA4621 Aldehyde oxidase	Periplasmic?	MSNRDISRRFLQGGLIAGVGVTAPLGSQAFA SEQ ID NO:13	<i>Haemophilus influenzae</i>
	PA1601 Aldehyde oxidase	Periplasmic?	MSLANPSRRGFLKAGGLLVTNLPAPLLALA SEQ ID NO:14	
	PA1880 Aldehyde oxidase	Periplasmic?	MNSKIDLSNALPGSRRGFLKGAADVGLTIGFQWSGARRALA SEQ ID NO:15	
	PA2378 Aldehyde oxidase	Periplasmic?	MKRSYPDDLIGNLSRRGFLKGVGATGVLLVAANWGWDRDALA SEQ ID NO:16	
Others	<i>copA</i> Multicopper oxidase	Periplasmic	MHRTSRRTEFVKGLAATGLLGGLWRAPAWA SEQ ID NO:17	<i>Caulobacter crescentus</i> ,
	PA0144 unknown	unknown	MSRNSGSSSRRTFLRLAALLLPAGALLGSLPGVRAAA SEQ ID NO:18	<i>B. parapertussis</i>

*The twin-arginine recognition motif (consensus RRXFLK; SEQ ID NO:19) is underlined.

**The listed species have predicted TAT-dependent homologs based on their genome sequence; however, experimental data are not available.

Secretion of a protein in this aspect of the invention can be detected or evaluated by any suitable method of detecting or measuring protein levels (amounts) or activity. Since the proteins to be measured will be secreted from the cell, such a method can include measuring the level (amount) of protein, by direct or indirect techniques, that has been secreted by the cell into the supernatant in the assay well or container. The protein can be measured directly in the assay well or container, or isolated from the assay and measured, although it is preferable to measure the protein directly in the assay well or container, particularly in a high throughput assay. Therefore, the method of protein detection or measurement is preferably suitable for use in a high throughput assay and as such, typically has an endpoint that can be evaluated rapidly and through a minimum of manipulation of the sample. Methods useful for detection of proteins in a sample include, but are not limited to: Western blot, immunoblot, enzyme-linked immunosorbant assay (ELISA), radioimmunoassay (RIA), immunoprecipitation, surface plasmon resonance, chemiluminescence, fluorescent polarization, phosphorescence, immunohistochemical analysis, matrix-assisted laser

desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, microcytometry, microarray, microscopy, fluorescence activated cell sorting (FACS), flow cytometry, and assays based on a property of the protein including but not limited to: enzyme activity, DNA binding, ligand binding, or interaction with other protein partners.

Preferably, the secretion of the protein or level (amount) of the secreted protein is detected through the use of a detectable label. Detectable labels suitable for use in the present invention include any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. For example, enzymatic labels are typically detected by providing the enzyme with a substrate and detecting the reaction product produced by the action of the enzyme on the substrate, and colorimetric labels are detected by simply visualizing the colored label. Other detection methods will be apparent to those of skill in the art.

Accordingly, in one preferred embodiment, the protein that serves as a biomarker for the negative screening portion of the method of the invention is an enzyme, whereby the protein can be detected in the assay through use of a synthetic substrate. For example, in a preferred embodiment, the negative screening portion of the assay is a colorimetric assay using a synthetic substrate that measures the quantity of an extracellular enzyme (*e.g.*, phospholipase C) that is secreted via the TAT secretory pathway. In the case of phospholipase C, substrates for this enzyme are well known in the art (*e.g.*, NPPC), as are assays for detection of the same.

For example, in one embodiment, an assay for a marker such as phospholipase C is performed as follows (see Fig. 4). Fig. 4 demonstrates the use of a synthetic substrate to evaluate TAT function by detecting the secretion of the PLCs of *P. aeruginosa*. Alteration of TAT function by deletion of the TatC gene or by mutating one of the twin arginine residues in the signal sequence of the PLC results in the failure of TAT function. The assay is initially further optimized for high throughput screening procedures. Such parameters as substrate concentrations, timing of growth and incubation with substrate, as well as growth conditions (to avoid nonspecific effects) are assessed. A microtiter plate reader that will detect absorbance at 410A will monitor the output. Growth of the microorganism will also be measured in this assay to demonstrate that a candidate inhibitor is not merely inhibiting bacterial growth. This is accomplished by monitoring the absorbance at 600A (which detects cell density) of wells with inhibitor against wells that have no inhibitor. Any compound that reduces the absorbance at 410A, but minimally affects the absorbance at

600A is considered to be a candidate inhibitor of TAT and can be evaluated in greater detail, if desired.

In addition, and as another example, one could use the evaluation of pyoverdine production in the negative screening step. TAT mutants are defective in their ability to produce the iron chelating compound pyoverdine. Pyoverdine is a yellow fluorescent compound that in the presence of another compound produced by *P. aeruginosa* (pyocyanin) imparts a green color to the culture media. Therefore, if a compound inhibits both the secretion of PLC (*e.g.*, as assessed by NPPC activity) *and* the production of pyoverdine, then there it is even more likely that it is a specific TAT inhibitor than if only one of these are affected. Pyoverdine is produced only in iron limiting media and can be easily be visually assayed, or its production can be measured by A405. Because one can optimize the growth media (*e.g.* iron levels) for the above assays, conditions can easily be established where these assays will not interfere with each other.

As an alternative or as an addition to the negative step of the assay described above, one may also use a selectable trait to provide a more definitive output in the assay. For example, one can use a gene that confers resistance to an antibiotic (*e.g.*, a gene which confers resistance to tetracycline) and fuse such gene (in the absence of its natural promoter) to the promoter of the gene encoding the protein that is secreted by the TAT system, such as a gene encoding any one of the proteins listed above or alternatively, to any gene that decreases its expression when TAT function is inhibited or abolished. For example, such genes are listed in Table 6 (see Examples). In this embodiment, the levels of the antibiotic used are optimized to obtain the cleanest output possible. The method includes detecting whether an organism became susceptible to the antibiotic in the presence of the putative inhibitor as compared to in the absence of the putative inhibitor.

Another step in the present method is the positive screening step, which includes measuring a level of expression of a gene that has increased expression when the TAT secretory pathway is inhibited (such genes are described in detail below). In this step, an increase in the level of expression of this gene in the presence of the candidate compound as compared to in the absence of the candidate compound indicates that the candidate compound is an inhibitor of the TAT secretory system and identifies the compound as a potential antimicrobial compound.

In an alternate or additional embodiment of this step of the invention, one can measure a level of expression of a gene that has *decreased* expression when the TAT secretory pathway is inhibited. The present inventors have identified that many of the genes

(e.g., *exoS1*, *pseC*, *pcrVG* & *popBDN*) involved in the Type III secretory apparatus (this is a secretory apparatus for the outer membrane) are down regulated in the TAT mutant (see Table 6), and could therefore also be evaluated in the method of the present invention. In this step, a decrease in the level of expression of the gene in the presence of the candidate compound as compared to in the absence of the candidate compound indicates that the candidate compound is an inhibitor of the TAT secretory system and identifies the compound as a potential antimicrobial compound. While the above-described positive step of detecting the expression of genes that are increased when the TAT secretory pathway is inhibited is preferred, this alternate embodiment is contemplated by the invention.

According to the present invention, a gene that is used as a "biomarker" in the present method, the expression of which is increased when the TAT secretory system is blocked or inhibited (or in the alternate embodiment, the expression of which is decreased when the TAT secretory system is blocked or inhibited), can include any gene for which such a change in expression can be detected or measured. As used herein, the term "expression", when used in connection with detecting the expression of a gene in the present invention, can refer to detecting transcription of the gene and/or to detecting translation of the gene. To detect expression of a gene refers to the act of actively determining whether a gene is expressed or not. This can include determining whether the gene expression is upregulated as compared to a control, downregulated as compared to a control, or substantially unchanged as compared to a control. Therefore, the step of detecting expression does not require that expression of the gene actually is upregulated or downregulated, but rather, can also include detecting no expression of the gene or detecting that the expression of the gene has not changed or is not different (i.e., detecting no significant expression of the gene or no significant change in expression of the gene as compared to a control). Although detection of the expression of one gene is sufficient to perform the positive screening step of the method of the present invention, the expression of additional genes (2, 3, 4, or more) whose expression is differential if the TAT secretory system is inhibited can be detected or measured, if desired.

Genes that are differentially expressed when the TAT secretory system is inhibited can be readily identified using techniques that are well known in the art. For example, the present inventors have identified several such genes through a microarray analysis. The production and application of high-density arrays in gene expression monitoring have been disclosed previously in, for example, PCT Publication No. WO 97/10365; PCT Publication No. WO 92/10588; U.S. Patent No. 6,040,138; U.S. Patent No. 5,445,934; or PCT

Publication No. WO 95/35505, all of which are incorporated herein by reference in their entireties. Also for examples of arrays, see Hacia *et al.* (1996) *Nature Genetics* 14:441-447; Lockhart *et al.* (1996) *Nature Biotechnol.* 14:1675-1680; and De Risi *et al.* (1996) *Nature Genetics* 14:457-460, each of which is incorporated by reference in its entirety. In general, in an array, an oligonucleotide, a cDNA, or genomic DNA, that is a portion of a known gene, occupies a known location on a substrate. A nucleic acid target sample is hybridized with an array of such oligonucleotides and then the amount of target nucleic acids hybridized to each probe in the array is quantified. One preferred quantifying method is to use confocal microscope and fluorescent labels. The *Affymetrix GeneChipTM Array* system (Affymetrix, Santa Clara, Calif.) and the *AtlasTM Human cDNA Expression Array* system are particularly suitable for quantifying the hybridization; however, it will be apparent to those of skill in the art that any similar systems or other effectively equivalent detection methods can also be used.

Examples of genes that are differentially expressed in microbial cells in which the TAT secretory system is inhibited, and that can serve as a biomarker in the positive screening step of the present method, include, but are not limited to: PA2808 (GenBank Accession No. AAG06196), PA3522 (GenBank Accession No. AAG06910.1), PA4878 (GenBank Accession No. AAG08263.1), PA0425-*mexA* (GenBank Accession No. AAG03814.1), *pchABCDEFGH* (GenBank Accession No. AE004839 and GenBank Accession No. AE004840). The sequences represented by all GenBank Accession numbers described herein are explicitly incorporated by reference in entirety. Table 4 shows genes, including those mentioned above, the expression of which is increased when the TAT secretory system is inhibited.

Table 4

Gene designation	Function	Fold affected
Up-regulated genes		
<i>pchABCDEFGH</i>	Siderophore (Pyochelin) Synthesis	11-76X
<i>fptAB</i>	Pyochelin Receptor	16-30X
<i>ptrA</i>	Negative regulator of Type III machinery	15X
<i>mexE</i>	Efflux	72X
PA3522	Efflux	23X
PA4878	Efflux Regulator	15X
<i>soxABDG</i>	Sarcosine Oxidase	12-18X
<i>pcoA</i>	Copper resistance	6X
<i>kdpACF</i>	Small molecule transport	10-22X
PA4218	Small molecule transport	13X

Expression of the transcripts and/or proteins encoded by the biomarker genes used the invention is measured by any of a variety of known methods in the art. In general, the nucleic acid sequence of a nucleic acid molecule (*e.g.*, DNA or RNA) in a sample can be detected by any suitable method or technique of measuring or detecting gene sequence or expression. Such methods include, but are not limited to, polymerase chain reaction (PCR), reverse transcriptase-PCR (RT-PCR), *in situ* PCR, quantitative PCR (q-PCR), *in situ* hybridization, Southern blot, Northern blot, sequence analysis, microarray analysis, detection of a reporter gene, or other DNA/RNA hybridization platforms. However, it is preferable to use a technique that is suited to a high throughput assay system and ideally, that can be used in connection with (in the same assay sample with) the negative screening step of the method of the invention. For example, in a preferred embodiment, the expression level of the gene is detected through the use of a reporter system. The reporter system can use a fluorescent output or a colorimetric assay output that will identify increased expression of the biomarker genes when the candidate compound inhibits the TAT secretory system.

By way of example, the promoters for any of the genes that are upregulated when TAT is inhibited can be fused to a gene encoding an enzyme that degrades a synthetic compound that turns a particular color when it is hydrolyzed (*e.g.* X-Gal, which turns blue), or the promoters of these genes are fused to a gene encoding a fluorescent protein (*i.e.* green fluorescent protein, or GFP) that will cause the microorganism to fluoresce green when GFP is highly expressed (*i.e.* when TAT function is altered). If a candidate compound is inhibiting TAT function, then a color or fluorescent signal is detected, while all other non-inhibitory compounds would result in no color or fluorescence change in the assay.

In an alternate embodiment, the reporter system can include the fusion of a promoter for any one or more genes that are upregulated when the TAT system is inhibited to an antibiotic resistance marker or other selectable trait marker. The present inventors have constructed strains of *P. aeruginosa* that has resistance markers for both tetracycline and gentamicin in the chromosome. These strains are clearly resistant to both tetracycline (Tc) and gentamicin (Gm). If a strain is constructed that has both resistance determinants fused to genes that are expressed when TAT is not functional, as in a TAT mutant, such a strain would be useful in the high throughput screening assays of the invention. For example, the Tc resistance marker and the Gm resistance marker could be fused to the promoters of two separate genes that are upregulated when TAT is inhibited (e.g., any gene listed in Table 4), such as ptrA (ptrA::tetA) and pchA (pchA::gm) in the wild type strain. This strain would then be susceptible to Tc and Gm under normal conditions, since the ptrA gene and the pchA gene will be expressed at low levels in the absence of inhibition of the TAT secretory pathway. To identify inhibitors of the TAT pathway, such as after contact with a putative inhibitor, one simply selects for resistance to both antibiotics at the same time, upregulation of both promoters will indicate inhibition of the TAT secretory system. If this fusion is resistant to both antibiotics in the presence of the one or more of the compounds in the libraries or individual compounds examined, then these peptides or small molecules would be excellent candidates for TAT inhibitors.

As with the detection of protein secretion in the negative screening step, detectable labels are preferably used to rapidly and easily determine differential expression of a gene in the present method. Detectable labels suitable for use in the present invention include any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Useful labels in the present invention include biotin for staining with labeled streptavidin conjugate, magnetic beads (e.g., Dynabeads.TM.), fluorescent dyes (e.g., fluorescein, texas red, rhodamine, green fluorescent protein, and the like), radiolabels (e.g., ^3H , ^{125}I , ^{35}S , ^{14}C , or ^{32}P), enzymes (e.g., horse radish peroxidase, alkaline phosphatase and others commonly used in an ELISA), and colorimetric labels such as colloidal gold or colored glass or plastic (e.g., polystyrene, polypropylene, latex, etc.) beads.

The term "quantifying" or "quantitating" when used in the context of quantifying transcription or translation levels of a gene can refer to absolute or to relative quantification. Absolute quantification may be accomplished by inclusion of known concentration(s) of one or more target nucleic acids and referencing the hybridization intensity of unknowns with the

known target nucleic acids (e.g. through generation of a standard curve). Alternatively, relative quantification can be accomplished by comparison of hybridization signals between two or more genes, or between two or more treatments to quantify the changes in hybridization intensity and, by implication, transcription level.

The positive screening step is typically performed together with the negative screening step, for example in the same microtiter well or assay sample dish/plate, so that the results most accurately identify a compound that is indicated to be an antimicrobial agent that specifically acts on the TAT secretory system. Also, this method is most amenable to use in a high throughput assay. However, the present invention is not limited to performing the negative and positive screening steps within the same assay sample. As such, if desired, the assay may be performed by running the positive screening in a separate assay sample (e.g., a separate well of a microtiter plate) before or after the negative screening or at the same timepoint as the negative screening step is run.

In the method of the present invention, the results of the positive and negative screening steps are typically performed in the presence and absence of the candidate inhibitory compound, so that a baseline or control level of protein secretion (for the negative assay) and gene expression (for the positive assay) can be used as a point of comparison. A "baseline level" is a control level of biomarker expression or activity (e.g., a negative control or a positive control) against which a test level of biomarker expression or biological activity (i.e., in the test sample) can be compared. Therefore, it can be determined, based on the control or baseline level of biomarker expression or biological activity, whether a sample to be evaluated for a given candidate inhibitory compound has a measurable increase, decrease, or substantially no change in biomarker expression or biological activity, as compared to the baseline level. Statistical significance should be at least $p < 0.05$, and more preferably, at least $p < 0.01$, and more preferably, $p < 0.005$, and even more preferably, $p < 0.001$.

The present invention also includes additional evaluation of compounds identified by the present method to confirm whether a candidate is a nonspecific inhibitor or whether it is affecting the desired target (i.e. TAT). There are a plethora of assays that enable this confirmation. For example, one can examine whether a candidate will inhibit the growth of *P. aeruginosa* (or another organism containing a TAT system), and one can examine whether the minimal bacteriocidal or bacteriostatic concentration (MBC or MIC) of the compound can be determined. The compound can also be further evaluated in combinations of assays of the invention. For example, if a compound is found to be effective in both the positive and negative screening assays and found to inhibit the production of, for example,

extracellular PLCs, then this would strongly indicate that the compound is acting on TAT. There are also an array of phenotypic assays that clearly differentiate between wild type and a TAT mutant. For example, the inventors have reported that a TAT mutant is sensitive to lower levels of copper than the wild type parent. Consequently, one can examine whether a candidate compound increases the susceptibility of *P. aeruginosa* to this ion. The inventors have also found that a TAT mutant is more sensitive to certain organic solvents (i.e. hexane) and that it cannot utilize 2-keto-gluconate as a sole carbon source. Accordingly, one can use these phenotypic characteristics of a TAT mutant and examine whether a candidate inhibitor will cause a wild type *P. aeruginosa* to exhibit some, or all of these, characteristics. One can also examine an assortment of *P. aeruginosa* strains with regard to their response to the candidate inhibitor. Similar assays can be performed with other TAT-containing microorganisms.

Moreover given the large number of genes that exhibit a significant change when TAT function is altered, including those specifically described herein, one can perform microarray experiments on *P. aeruginosa* (or other TAT containing organism) to examine the whether the pattern of gene transcription that was observed in the TAT mutant is similar to the one that is seen when the candidate TAT inhibitor is present. These experiments can be performed with various concentrations of the candidate and it can be determined with reasonable certainty whether the candidate compound affects TAT or not.

Finally, one can perform animal model studies to determine whether the candidate compounds are both safe and effective in an appropriate animal model.

The present invention also includes a kit that utilizes the screening methods of the present invention. The kit preferably contains any reagent for detecting the expression or activity of the biomarkers useful in the positive and negative screening steps of the present invention in a test sample. For example, reagents for detecting can include labeled substrate, probe, PCR primers, an antibody or antigen binding fragment thereof, or a reagent useful for detecting, measuring or visualizing labeled biomarkers that may be provided as part of the kit or constructed. The kit can include any reagent needed to perform the screening methods envisioned herein. The kit can also include suitable reagents for the detection of and/or for the labeling of positive or negative controls, wash solutions, dilution buffers, culture media and the like.

More specifically, according to one embodiment of the present invention, a reagent for detecting biomarker levels in the negative screening step of the method of the present invention includes any reagent useful for detecting the secretion of a protein that is secreted

by the TAT secretory system from a microorganism. For example, when the protein to be detected is an enzyme, the means for detecting can include a substrate, such as a synthetic substrate that is labeled for use in a colorimetric assay or other type of assay. If the protein to be detected is not an enzyme, the test kit can include an antibody, antigen binding fragment thereof, receptor, ligand, or other binding partner for the protein that can be labeled and detected according to the methods described herein. Additional reagents that may be required or useful for performing the screening step and detecting the result may also be included in the assay kit.

Similarly, a means for detecting biomarker levels in the positive screening step of the method of the present invention can include any reagent useful for detecting the expression of a gene whose expression is differentially regulated when the TAT secretory system is inhibited. For example, when the expression level of the gene is to be detected by a reporter gene, the assay kit can include test cells (e.g., bacterial cells) that are transformed with the desired reporter gene, as well as reagents needed to detect expression of the reporter gene in an assay sample. Such reagents can include reagents for detection of fluorescent or colorimetric labels on the expression product of the gene.

When the expression level of the gene is to be detected using PCR or hybridization techniques, the means for detecting can include probes or primers that hybridize to the gene of interest. According to the present invention, a probe (oligonucleotide probe) is a nucleic acid molecule that typically ranges in size from about 50-100 nucleotides to several hundred nucleotides to several thousand nucleotides in length. Therefore, a probe can be any suitable length for use in an assay described herein, including any length in the range of 50 to several thousand nucleotides, in whole number increments. Such a molecule is typically used to identify a target nucleic acid sequence in a sample by hybridizing to such target nucleic acid sequence under stringent hybridization conditions that are well known in the art. PCR primers are also nucleic acid sequences, although PCR primers are typically oligonucleotides of fairly short length (e.g., 8-30 nucleotides) that are used in polymerase chain reactions. PCR primers and hybridization probes can readily be developed and produced by those of skill in the art, using sequence information from the target sequence. (See, for example, Sambrook et al., *supra* or Glick et al., *supra*).

Additional reagents that may be required or useful for performing the positive screening step and detecting the result may also be included in the assay kit.

The reagent for detecting a biomarker and/or a control marker of the assay kit of the present invention can be conjugated to a detectable tag or detectable label. Such a tag can be

any suitable tag, which allows for detection of the reagents used to detect the biomarker or control marker and includes, but is not limited to, any composition or label detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Useful labels in the present invention include biotin for staining with labeled streptavidin conjugate, magnetic beads (e.g., Dynabeads™), fluorescent dyes (e.g., fluorescein, texas red, rhodamine, green fluorescent protein, and the like), radiolabels (e.g., ³H, ¹²⁵I, ³⁵S, ¹⁴C, or ³²P), enzymes (e.g., horse radish peroxidase, alkaline phosphatase and others commonly used in an ELISA), and colorimetric labels such as colloidal gold or colored glass or plastic (e.g., polystyrene, polypropylene, latex, etc.) beads.

As discussed above, the reagent for detecting can also include the cells (e.g., bacterial cells) containing a TAT secretory system that are useful in the present method. Such cells can be from any bacterial or other microbial strain that contains a TAT secretory system and as described in detail above. In one aspect, the microbial cells can be provided transformed with a nucleic acid molecule useful in the present invention, such as a reporter gene construct, wherein the reporter gene is ligated to the gene whose expression is differentially regulated when the TAT secretory system is inhibited. In other embodiments, the recombinant reporter construct for producing a test cell can be provided, so that the transformed host cell can be produced in the laboratory of the tester.

In addition, any one or more of the reagents for detecting of the assay kit of the present invention can be immobilized on a substrate. Such a substrate can include any suitable substrate for immobilization of a detection reagent such as would be used in any of the previously described methods of detection. Briefly, a substrate suitable for immobilization of a reagent for detecting includes any solid support, such as any solid organic, biopolymer or inorganic support that can form a bond with the reagent for detecting without significantly affecting the activity and/or ability of the detection reagent to detect the desired target molecule. Exemplary organic solid supports include polymers such as polystyrene, nylon, phenol-formaldehyde resins, acrylic copolymers (e.g., polyacrylamide), stabilized intact whole cells, and stabilized crude whole cell/membrane homogenates. Exemplary biopolymer supports include cellulose, polydextrans (e.g., Sephadex®), agarose, collagen and chitin. Exemplary inorganic supports include glass beads (porous and nonporous), stainless steel, metal oxides (e.g., porous ceramics such as ZrO₂, TiO₂, Al₂O₃, and NiO) and sand. Preferably, the various portions of the assay kit described herein are provided in a format that is useful in a high throughput assay, such as in a series of microtiter plates.

~~Compounds identified by~~ any of the methods of the present invention can be used alone or in therapeutic compositions to treat or protect an animal (including a human) from a disease or condition caused by a microorganism that has a TAT secretory system. Various aspects of composition formulation and administration methods, as well as the association of various microorganisms and diseases or conditions are described elsewhere herein, such as in the discussion of vaccines below, and can be generally applied to the use of compounds identified by the method of the present invention.

Bacterial Twin Arginine Translocase Mutants as Vaccine Vectors and Uses Thereof

One embodiment of the present invention relates to the use of live, attenuated bacterial strains that have at least one genetic modification (e.g., a mutation) that reduces or prevents the expression of at least one component of the twin arginine translocase (TAT) secretory system in the bacterium or reduces or abolishes the biological activity of the TAT secretory system in the bacterium. More particularly, this embodiment of the present invention relates to novel vaccines and therapeutic compositions containing genetically modified bacterial strains of the invention, and to therapeutic methods using such compositions and bacterial strains. For example, the genetically modified bacterial strains can be used to immunize an animal against the wild-type bacterium, which is useful for the prevention and/or treatment of a variety of diseases and condition caused by the wild-type bacteria having an intact (normal, wild-type) TAT secretory system. Alternatively, the genetically modified bacterial stains can be used as vectors for the delivery of a variety of therapeutic agents, including, but not limited to, protein or peptide antigens (e.g., by expression of a recombinant nucleic acid molecule encoding the antigen or by directly carrying the protein or peptide antigen), nucleic acid molecules, various biological response modifiers, and therapeutic drugs. Such vectors can be used in prophylactic or therapeutic vaccines or as therapeutic delivery vehicles (e.g., when a drug is to be delivered, but not necessarily in connection with the stimulation of an immune response). In a vaccine, a composition comprising the bacterial vector can induce a cellular or humoral immune response against a heterologous antigen and/or the bacterium itself, or the composition can induce tolerance to a particular antigen(s), if desired.

Prior to the present invention, the present inventors constructed a mutant of the opportunistic pathogen *Pseudomonas aeruginosa*, which has a mutation that abolishes the activity of the twin arginine translocation (TAT) secretory system. The inventors have observed that this mutant is able to replicate in the lungs of rats, but produces a minimal amount of pathology (see Ochsner et al., 2002, *Proc. Natl. Acad. Sci. USA* **99**(12):8312-

8317, incorporated herein by reference in its entirety). An assortment of virulence determinants including proteins involved in iron acquisition of *P. aeruginosa* and other pathogenic bacteria are secreted through the inner membrane via this novel secretory system. This protein translocation system was initially discovered in plants where it is used for the secretion of folded proteins into chloroplasts. However, it is now clear that it is also widely found in bacteria, including in many pathogenic bacteria. Moreover, proteins comprising this system are only found in plants and bacteria, and not in animals or any other kind of eukaryotic cell. This feature makes them especially attractive targets for novel antimicrobials. This novel secretory system, called Tat (twin-arginine translocase) was initially thought to secrete mainly redox proteins into the periplasm of bacteria. However, the present inventors' laboratory recently demonstrated that Tat also secretes an array of surface and extracellular proteins that are involved in virulence. Moreover, the inventors recently reported in that the Tat system *P. aeruginosa* is absolutely critical for the induction virulence of this opportunist bacterium in a pulmonary infection model. However, the Tat mutant was able to survive as well as the wild type parental strain for nearly a week (6 days) in the lungs of the infected animals.

The inventors have now also found that a protein designated PscO, which is required for a functional Type III secretion apparatus in *P. aeruginosa*, is secreted via Tat through the inner membrane. In a Tat mutant, PscO is not secreted and a functional Type III apparatus cannot be formed (Bergman). These experiments have led to the conclusion that the Tat secretion system may play a pivotal role in the pathogenesis *P. aeruginosa* as well as other gram negative and gram positive pathogens, all of which carry genes encoding proteins homologous to ones required for a functional TAT translocase apparatus.

In the present invention, the present inventors have discovered a novel use for the Tat mutant bacteria described above. The present inventors now disclose that this genetically modified bacterial strain can be used as a suitable vaccine against pulmonary and other infections caused by the wild-type bacterium, and can also be used as a vector to carry a variety of therapeutic agents. Moreover, many bacterial pathogens including, but not limited to, *Mycobacterium tuberculosis*, *Vibrio cholerae*, *Bacillus anthracis*, *Salmonella enteric* and *Yersinia pestis*, carry genes encoding twin-arginine secretory systems. The present invention therefore relates to the construction of twin arginine translocase mutants of any of these bacterial pathogens, and the use of these strains as vaccine candidates for diseases such as tuberculosis, cholera, anthrax, gastroenteritis, septicemia and plague, among others. The present inventors have provided evidence that the twin arginine secretory system of

Pseudomonas aeruginosa is critical in the ability of these pathogens to establish and produce infections in susceptible hosts. Twin arginine secretory system mutants as described herein are now proposed to also be safe and effective delivery systems for immunizing agents or medicines since they can replicate in a human host but will not cause disease.

One embodiment of the present invention relates to a therapeutic composition, comprising: (a) an attenuated bacterium comprising a genetic modification that reduces or abolishes the expression or biological activity of the twin arginine translocase (TAT) secretory system in the bacterium; and optionally, (b) at least one therapeutic agent. The first component of all of the therapeutic compositions described herein is an attenuated bacterium comprising a genetic modification that reduces or prevents the expression and/or reduces or abolishes the biological activity of the twin arginine translocase (TAT) secretory system in the bacterium. According to the present invention, an attenuated bacterium is generally defined as a bacterium that is less virulent, relative to the wild-type (normal, not intentionally mutated or modified) bacterium. As used herein, virulence refers to the ability of a bacterium to cause infection in a host organism and is particularly related to the invasiveness of the bacterium (e.g., adherence and/or invasion of a host cell and the ability to multiply and spread within the host) and toxicity of the bacterium (e.g., production of toxic substances that can damage a host). An attenuated bacterium has a detectably reduced virulence phenotype overall or in at least one particular virulence factor (e.g., toxicity, invasiveness as determined by adherence, invasiveness as determined by ability to proliferate) as compared to the wild-type bacterium. As discussed above, the present inventors have shown that mutation (genetic modification) of the TAT secretory system in the bacterium results in an organism that can replicate in a host, but that is unable to incite pathogenic effects (e.g., effects related to virulence).

In one embodiment of the present invention, the expression and/or biological activity of the TAT secretory system (or component thereof) is prevented or abolished. This embodiment is preferred when the bacterium is sufficiently robust to survive without a functional TAT secretory system. In another embodiment, however, the expression and/or biological activity of the TAT secretory system or component thereof is merely reduced, rather than deleted or abolished altogether. This embodiment is typically utilized in a bacterium that is not sufficiently robust to survive without a functional TAT secretory system. In this embodiment, mutants will typically be designed to be sensitive to one or more environmental factors (e.g., temperature) that allows the *tat* operon to be expressed and function in one environment but not another. For example, a temperature sensitive *tat*

mutant could be generated that would survive and replicate in the temperature of the upper respiratory tract sufficiently to be useful as a vaccine of the present invention, but which would not survive at the higher temperatures of the lower respiratory tract or bloodstream.

As discussed above, the TAT secretory system is a Sec-independent, ATP-independent secretion system, wherein the signal peptides that target the system comprise two consecutive Arg residues ("Twin Arginine Transport" = TAT) just before the hydrophobic core (h-region) and several additional residues between the h-region and the peptidase cleavage site. This system is capable of secreting proteins that are already folded before they enter the pore apparatus in the inner membrane. Three genes (*tatABC*) encode proteins that comprise the Tat secretory apparatus. In most species thus far investigated, these genes are part of an operon, although not invariably (See Fig. 2). According to the present invention, any one or more of the *tat* genes can be genetically modified to achieve the desired result of reduced or abolished TAT secretory system activity and attenuation of the microorganism. In a preferred embodiment, the *tatC* gene is genetically modified according to the invention. The *tatC* gene is usually the third gene in the operon and would therefore be a preferred target for mutagenesis, since deletion or interruption of *tatC* would not have a polar effect on the expression of *tatA* or *tatB*. Also, because the present inventors have already shown that *tatC* deletion mutants of *P. aeruginosa* are defective in secreting a large array of Tat substrates, it is likely that *tatC* deletion mutants of other bacterial strains will also be similarly defective. However, since all three genes are required to have a functional Tat secretory system, it is also an embodiment of the present invention to genetically modify either one or both of the *tatA* or *tatB* genes, in place of or in addition to genetic modification of the *tatC* gene. In addition, any other genetic modifications to the bacterium which result in a reduced expression or biological activity of the Tat secretory system in the bacterium, such as those that can be produced by classical mutagenesis (e.g., random mutation followed by directed screening), are encompassed by the present invention.

The present invention is believed to be the first disclosure of the use of bacterial strains having a mutated TAT secretory apparatus as vaccines and therapeutic vectors. Prior to the invention, as discussed above, the twin arginine translocase secretory system had only been viewed as a system for delivery of enzymes involved in oxidation reduction reactions to the periplasmic space or the cell wall of bacteria. It is an entirely a novel discovery by the present inventors that these mutants can be used as live vaccines or delivery vectors.

The present invention includes bacterial strains with one or more genetic modifications (mutations, changes) that result in decreased or substantially eliminated

~~(abolished) expression and/or biological~~ activity of a TAT secretory system as described herein. As used herein, in one embodiment, a genetically modified microorganism (or a microorganism with one or more genetic modifications) has a genome which is modified (i.e., mutated or changed) from its normal (i.e., wild-type or naturally occurring) form such that the desired result is achieved (i.e., reduction or elimination of expression or biological activity of the TAT secretory system). Alternatively or additionally, a genetically modified microorganism can be modified by recombinant techniques which may or may not delete or mutate endogenous nucleotides in the genome of the microorganism, but which can result in the insertion of heterologous nucleotides into the microorganism (e.g., via a plasmid). Genetic modification of a bacterial microorganism can be accomplished using classical strain development and/or molecular genetic techniques. Such techniques known in the art and are generally disclosed for microorganisms, for example, in Sambrook et al., 1989, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Labs Press. The reference Sambrook et al., *ibid.*, is incorporated by reference herein in its entirety. A genetically modified microorganism can include a microorganism in which nucleic acid molecules have been inserted, deleted or modified (i.e., mutated; e.g., by insertion, deletion, substitution, and/or inversion of nucleotides), in such a manner that such modifications provide the desired effect within the microorganism.

A recombinant microorganism more specifically refers to a microorganism that has a genome which is modified using recombinant technology. A recombinant microorganism according to the present invention can include a microorganism in which nucleic acid molecules have been inserted, deleted or modified (i.e., mutated; e.g., by insertion, deletion, substitution, and/or inversion of nucleotides), in such a manner that such modifications provide the desired effect within the microorganism.

As used herein, genetic modifications which result in a decrease in gene expression, in the function of the gene, or in the function of the gene product (i.e., the protein encoded by the gene) can be referred to as inactivation (complete or partial), deletion, interruption, blockage or down-regulation of a gene. For example, a genetic modification in a gene which results in a decrease in the function of the protein encoded by such gene, can be the result of a complete or partial deletion of the gene (i.e., the gene does not exist or no longer produces a protein with the same structure and/or function), a mutation in the gene which results in incomplete or no translation of the protein (e.g., the protein is not expressed), or a mutation in the gene which decreases or abolishes the natural function of the protein (e.g., a protein is expressed which has decreased or no biological activity or action). Genetic modifications

which result in an increase in gene expression or function can be referred to as amplification, overproduction, overexpression, activation, enhancement, addition, or up-regulation of a gene. Increased gene expression can be achieved, for example, by expression of a recombinant nucleic acid molecule encoding the desired protein (overexpression of a gene as compared to wild-type) or by modifying a regulatory region of an endogenous gene to increase expression of the gene. Modifications to the gene can also result in a protein product with increased, enhanced, improved biological activity as compared to wild-type.

In one embodiment, the genetically modified bacterial strains of the invention can be produced by the introduction of any genes or other nucleotide sequences or vectors into the microbe in order to inactivate or delete genes that encode one or more components of the TAT secretory system (i.e., "knock-out" or "targeted gene disruption"). In another embodiment, genetically modified strains can be produced by classical methods of random mutation, followed by specific, directed selection for the desired genotype. The present invention also includes bacterial strains that, in addition to containing an above-described genetic modification (e.g., disruption of the TAT secretory system), comprise additional genetic modifications, including but not limited to, transformation with a recombinant nucleic acid molecule encoding a therapeutic or otherwise useful heterologous protein (e.g., an antigen or other biological response modifier). In this embodiment, the bacterium is transformed with the desired heterologous nucleic acid molecule by any suitable method as well-known in the art (and described in more detail below).

In one embodiment of the invention, the genetic modification to the bacterium results in a reduction or prevention of expression of at least one gene in the *tat* operon of the bacterium (i.e., *tatA*, *tatB*, and/or *tatC*). In another embodiment, the genetic modification results in reduced or abolished *tat* RNA transcription by the bacterium. In another embodiment, RNA transcribed from the genetically modified *tat* gene is not translated, or has reduced translation, as a result of the genetic modification. In yet another embodiment, the bacterium comprises a mutation in the *tat* gene that reduces or prevents the expression or biological activity of the twin arginine translocase (TAT) secretory system in the bacterium. For example, such a mutation can include at least one mutation in the coding sequence of the *tat* gene, and/or at least one mutation in a regulatory region of the *tat* gene. The mutation can also be a partial or complete deletion of the *tat* gene. In this embodiment, the mutation can include an insertion of a heterologous nucleic acid sequence into the *tat* gene sufficient to reduce or prevent expression or biological activity of at least one component of the TAT secretion system. In one embodiment, the genetic modification results in a mutated *tat* gene

that encodes a mutated TAT secretory system or component thereof that lacks TAT biological activity. In another embodiment, the genetic modification reduces or prevents the expression or reduces or abolishes the biological activity of the TAT pore apparatus in the bacterium. Preferably, the attenuated bacterium lacks or has a reduced ability to secrete proteins across the cytoplasmic membrane that have a TAT secretion signal sequence.

In general, the biological activity or biological action of a protein refers to any function(s) exhibited or performed by the protein that is ascribed to the naturally occurring form of the protein as measured or observed *in vivo* (i.e., in the natural physiological environment of the protein) or *in vitro* (i.e., under laboratory conditions). For example, a biological activity of a the proteins comprising the twin arginine secretory system includes the ability to secrete proteins across the cytoplasmic membrane that have a TAT secretion signal sequence. Modifications of a protein, such as in a homologue or mimetic (discussed below), may result in proteins having the same biological activity as the naturally occurring protein, or in proteins having decreased or increased biological activity as compared to the naturally occurring protein. Modifications which result in a decrease in protein expression or a decrease in the activity of the protein, can be referred to as inactivation (complete or partial), down-regulation, or decreased action of a protein. Similarly, modifications which result in an increase in protein expression or an increase in the activity of the protein, can be referred to as amplification, overproduction, activation, enhancement, up-regulation or increased action of a protein.

As used herein, a protein that has "twin arginine translocase (TAT) biological activity" or that is referred to as a component of the "twin arginine translocase (TAT) secretory system" refers to a protein that participates in the secretion of proteins across the cytoplasmic membrane that have a TAT signal sequence. The components of the TAT secretory system are encoded by the *tat* genes, which can be formed as a *tat* operon, and which typically includes at least three genes: *tatA*, *tatB*, and *tatC*. The biological function of the product of each of the *tatA*, *tatB*, and *tatC* genes is essential to the function of the TAT secretory system.

According to the present invention, an isolated nucleic acid molecule or nucleic acid sequence, is a nucleic acid molecule or sequence that has been removed from its natural milieu. As such, "isolated" does not necessarily reflect the extent to which the nucleic acid molecule has been purified. An isolated nucleic acid molecule useful for transfecting bacterial cells include DNA, RNA, or derivatives of either DNA or RNA. An isolated nucleic acid molecule can be double stranded or single stranded. An isolated nucleic acid

molecule useful in the present invention includes nucleic acid molecules that encode a protein or a fragment thereof, as long as the fragment contains at least one epitope useful in a composition of the present invention. Nucleic acid molecules transformed into bacteria of the present invention can include nucleic acid sequences encoding one or more proteins, or portions thereof. Such nucleic acid molecules can comprise partial or entire coding regions, regulatory regions, or combinations thereof. A preferred number of proteins to be produced recombinantly by a bacterium of the present invention is any number of proteins that can be reasonably produced by a bacterium, and typically ranges from at least one to at least about 5, with from about 2 to about 5 compounds or more being more preferred.

A peptide or protein encoded by a nucleic acid molecule within a bacterium can be a full-length protein, or can be a functionally equivalent protein in which amino acids have been deleted (e.g., a truncated version of the protein), inserted, inverted, substituted and/or derivatized (e.g., acetylated, glycosylated, phosphorylated, tethered by a glycerophosphatidyl inositol (GPI) anchor) such that the modified protein has a biological function substantially similar to that of the natural protein, or which has enhanced or inhibited function as compared to the natural protein, if desired. Modifications can be accomplished by techniques known in the art including, but not limited to, direct modifications to the protein or modifications to the nucleic acid sequence encoding the protein using, for example, classic or recombinant DNA techniques to effect random or targeted mutagenesis. Functionally equivalent proteins can be selected using assays that measure the biological activity of the protein.

Expression of an antigen in a bacterium of the present invention is accomplished using techniques known to those skilled in the art. Briefly, a nucleic acid molecule encoding at least one desired protein (e.g., an antigen) is inserted into an expression vector in such a manner that the nucleic acid molecule is operatively linked to a transcription control sequence in order to be capable of effecting either constitutive or regulated expression of the nucleic acid molecule when transformed into a host bacterial cell. Nucleic acid molecules encoding one or more proteins can be on one or more expression vectors operatively linked to one or more transcription control sequences.

One embodiment of the present invention includes a recombinant vector to be used for transformation of a bacterial cell. According to the present invention, a recombinant vector is an engineered (i.e., artificially produced) nucleic acid molecule that is used as a tool for manipulating a nucleic acid sequence of choice and for introducing such a nucleic acid sequence into a host cell. The recombinant vector is therefore suitable for use in cloning,

sequencing, and/or otherwise manipulating the nucleic acid sequence of choice, such as by expressing and/or delivering the nucleic acid sequence of choice into a host cell to form a recombinant cell. Such a vector typically contains heterologous nucleic acid sequences, that is nucleic acid sequences that are not naturally found adjacent to nucleic acid sequence to be delivered, although the vector can also contain regulatory nucleic acid sequences (e.g., promoters, untranslated regions) which are naturally found adjacent to nucleic acid molecules that are to be expressed or transferred by the bacterial cells. The vector can be either RNA or DNA, either prokaryotic or eukaryotic, and typically is a plasmid. The vector can be maintained as an extrachromosomal element (e.g., a plasmid) or it can be integrated into the chromosome of the recombinant microorganism. The entire vector can remain in place within a host cell, or under certain conditions, the plasmid DNA can be deleted, leaving behind the nucleic acid molecule of the present invention. The integrated nucleic acid molecule can be under chromosomal promoter control, under native or plasmid promoter control, or under a combination of several promoter controls. Single or multiple copies of the nucleic acid molecule can be integrated into the chromosome. In one embodiment, a recombinant vector of the present invention contains at least one selectable marker for microorganisms according to the present invention. As used herein, the phrase "recombinant nucleic acid molecule" is used primarily to refer to a recombinant vector into which has been ligated the nucleic acid sequence to be cloned, manipulated, transformed into the host cell (i.e., the insert).

Typically, a recombinant vector, and therefore a recombinant nucleic acid molecule, includes at least one nucleic acid molecule of the present invention operatively linked to one or more expression control sequences. As used herein, the phrase "recombinant molecule" or "recombinant nucleic acid molecule" primarily refers to a nucleic acid molecule or nucleic acid sequence operatively linked to a transcription control sequence, but can be used interchangeably with the phrase "nucleic acid molecule", when such nucleic acid molecule is a recombinant molecule as discussed herein. According to the present invention, the phrase "operatively linked" refers to linking a nucleic acid molecule to an expression control sequence in a manner such that the molecule is able to be expressed when transfected (i.e., transformed, transduced, transfected, conjugated or conducted) into a host cell. Expression control sequences include transcription control sequences, which are sequences that control the initiation, elongation, or termination of transcription. Particularly important transcription control sequences are those which control transcription initiation, such as promoter, enhancer, operator and repressor sequences. Suitable transcription control sequences include

any transcription control sequence that can function in the bacterial cell being transformed. Recombinant nucleic acid molecules of the present invention can also contain additional regulatory sequences, such as translation regulatory/control sequences, origins of replication, and other regulatory sequences that are compatible with the recombinant cell.

In one embodiment, a recombinant vector of the present invention is an expression vector. As used herein, the phrase "expression vector" is used to refer to a vector that is suitable for production of an encoded product (e.g., a protein of interest). In this embodiment, a nucleic acid sequence encoding the product to be produced is inserted into the recombinant vector to produce a recombinant nucleic acid molecule. The nucleic acid sequence encoding the protein to be produced is inserted into the vector in a manner that operatively links the nucleic acid sequence to regulatory sequences in the vector (e.g., a bacterial promoter) which enables the transcription and translation of the nucleic acid sequence within the recombinant microorganism.

In another embodiment, a recombinant vector of the present invention is a targeting vector. As used herein, the phrase "targeting vector" is used to refer to a vector that is used to deliver a particular nucleic acid molecule into a recombinant cell, wherein the nucleic acid molecule is used to delete or inactivate an endogenous gene within the host cell (i.e., used for targeted gene disruption or knock-out technology). Such a vector may also be known in the art as a "knock-out" vector. In one aspect of this embodiment, a portion of the vector, but more typically, the nucleic acid molecule inserted into the vector (i.e., the insert), has a nucleic acid sequence that is homologous to a nucleic acid sequence of a target gene in the host cell (i.e., a gene which is targeted to be deleted or inactivated). The nucleic acid sequence of the vector insert is designed to bind to the target gene such that the target gene and the insert undergo homologous recombination, whereby the endogenous target gene is deleted, inactivated or attenuated (i.e., by at least a portion of the endogenous target gene being mutated or deleted).

In another embodiment of the invention, a recombinant vector is a transfer vector. As used herein, the phrase "transfer vector" refers to a vector that is used to transfer a particular nucleic acid molecule from one cell (e.g., a bacterial cell) to another cell (e.g. a host cell that is invaded by the bacterial cell). Such vectors can be used in the present invention when it is desirable to use an attenuated bacterial cell of the invention to transfer a nucleic acid molecule to a cell in the host organism (i.e., a cell in the host to which the bacterial composition is administered).

It will be appreciated by one skilled in the art that use of recombinant DNA technologies can improve control of expression of transformed nucleic acid molecules by manipulating, for example, the number of copies of the nucleic acid molecules within the host cell, the efficiency with which those nucleic acid molecules are transcribed, the efficiency with which the resultant transcripts are translated, and the efficiency of post-translational modifications. Additionally, the promoter sequence might be genetically engineered to improve the level of expression as compared to the native promoter. Recombinant techniques useful for controlling the expression of nucleic acid molecules include, but are not limited to, integration of the nucleic acid molecules into one or more host cell chromosomes, addition of vector stability sequences to plasmids, substitutions or modifications of transcription control signals (e.g., promoters, operators, enhancers), substitutions or modifications of translational control signals (e.g., ribosome binding sites, Shine-Dalgarno sequences), modification of nucleic acid molecules to correspond to the codon usage of the host cell, and deletion of sequences that destabilize transcripts.

Recombinant nucleic acid molecules of the present invention, which can be either DNA or RNA, can also contain additional regulatory sequences, such as translation regulatory sequences, origins of replication, and other regulatory sequences that are compatible with the recombinant cell. In one embodiment, a recombinant molecule of the present invention, including those which are integrated into the host cell chromosome, also contains secretory signals (i.e., signal segment nucleic acid sequences) to enable an expressed protein to be secreted from the cell that produces the protein. Suitable signal segments include a signal segment that is naturally associated with the protein to be expressed or any heterologous signal segment capable of directing the secretion of the protein according to the present invention. In another embodiment, a recombinant molecule of the present invention comprises a leader sequence to enable an expressed protein to be delivered to and inserted into the membrane of a host cell. Suitable leader sequences include a leader sequence that is naturally associated with the protein, or any heterologous leader sequence capable of directing the delivery and insertion of the protein to the membrane of a cell.

A recombinant nucleic acid molecule used for transformation of a bacterial cell as describe herein comprises any of the recombinant vectors of the present invention previously described herein, and typically includes at least one nucleic acid sequence encoding a protein to be produced by the recombinant cell or a nucleic acid sequence useful for targeted deletion or inactivation of an endogenous gene in the recombinant cell.

In one embodiment of the present invention, at least one additional recombinant nucleic acid molecule comprising a nucleic acid sequence encoding a protein to be expressed can be transformed into a bacterial cell. Alternatively, the microorganism can be transformed with at least one additional recombinant nucleic acid molecule comprising a second nucleic acid sequence that hybridizes with a target nucleic acid sequence in the microorganism such that a gene comprising the target nucleic acid sequence is mutated or inactivated by homologous recombination with the second nucleic acid sequence. In this manner, multiple proteins can be introduced into the cell, multiple genes can be inactivated, or combinations of the two are possible. The additional recombinant nucleic acid molecule can be introduced into the bacterial cell simultaneously with the first recombinant nucleic acid molecule (i.e., cotransformation), or as a subsequent transformation (e.g., for the purposes of "stacking" traits).

According to the present invention, the term "transfection" is used to refer to any method by which an exogenous nucleic acid molecule (i.e., a recombinant nucleic acid molecule) can be inserted into a cell. The term "transformation" can be used interchangeably with the term "transfection" when such term is used to refer to the introduction of nucleic acid molecules into microbial cells or plants. In microbial systems, the term "transformation" is used to describe an inherited change due to the acquisition of exogenous nucleic acids by the microorganism and is essentially synonymous with the term "transfection." However, in animal cells, transformation has acquired a second meaning which can refer to changes in the growth properties of cells in culture (described above) after they become cancerous, for example. Therefore, to avoid confusion, the term "transfection" is preferably used with regard to the introduction of exogenous nucleic acids into animal cells, and is used herein to generally encompass transfection of animal cells and transformation of plant cells and microbial cells, to the extent that the terms pertain to the introduction of exogenous nucleic acids into a cell. Therefore, transfection techniques include, but are not limited to, transformation, particle bombardment, electroporation, microinjection, lipofection, adsorption, infection and protoplast fusion.

Depending on the intended function of the vaccine or therapeutic composition of the invention or the microenvironment, proteins produced by the bacterial cell may either remain within the bacterial cell, be secreted into the extracellular milieu, be secreted into a space between two cellular membranes, or be retained on the outer surface of a cell membrane. In the event that it is desired to recover a protein from a host microorganism, proteins can be purified using a variety of standard protein purification techniques, such as, but not limited

to, affinity chromatography, ion exchange chromatography, filtration, electrophoresis, hydrophobic interaction chromatography, gel filtration chromatography, reverse phase chromatography, concanavalin A chromatography, chromatofocusing and differential solubilization.

Effective conditions for the production of recombinant bacterial strains and expression of the antigen by the bacterial cell can, in one embodiment, include an effective medium in which a bacterial strain can be cultured. An effective medium is typically an aqueous medium comprising assimilable carbohydrate, nitrogen and phosphate sources, as well as appropriate salts, minerals, metals and other nutrients, such as vitamins and growth factors. The medium may comprise complex nutrients or may be a defined minimal medium. Bacterial strains of the present invention can be cultured in a variety of containers, including, but not limited to, bioreactors, Erlenmeyer flasks, test tubes, microtiter dishes, and petri plates. Culturing is carried out at a temperature, pH and oxygen content appropriate for the bacterial strain. Such culturing conditions are well within the expertise of one of ordinary skill in the art (see, for example, Guthrie et al. (eds.), 1991, *Methods in Enzymology*, vol. 194, Academic Press, San Diego).

Any bacterial strain can be used to produce an attenuated bacterial strain of the present invention. In one embodiment, and particularly when it is desired to elicit an immune response against the bacterium itself, nonpathogenic mutants of pathogenic bacterial strains can be used in accordance with the present invention. In another embodiment nonpathogenic mutants of either pathogenic or nonpathogenic bacterial strains can be used as vectors for delivery of a therapeutic agent according to the invention. Preferred family of bacterial strains include, but are not limited to, Enterobacteriaceae, Micrococcaceae, Vibrionaceae, Pasteurellaceae, Mycoplasmataceae, and Rickettsiaceae. Particularly preferred genera, species and strains have been described in detail in the description of the high throughput assay to identify inhibitors of the TAT secretory system, and such genera, species and strains are also useful in this embodiment of the invention.

In one embodiment, a preferred bacterial strain to use in the present invention is an intracellular pathogenic bacterial strain. Such strains are preferred because they are capable of entering a cell type and, in embodiments where a therapeutic agent is carried or expressed by the bacterium, the agent is delivered intracellularly. Such strains have the advantage of effectively targeting particular cells for delivery of a therapeutic agent or for generally providing a therapeutic effect.

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A second component of a therapeutic composition of the present invention is a therapeutic agent. The therapeutic agent can be any therapeutic agent that can be combined with, attached to, loaded into, or expressed by a bacterial cell of the present invention (e.g., the attenuated bacterium or attenuated bacterial strain) and used in a composition that is expected to elicit an immune response in an animal, tolerize an animal to one or more antigens, or otherwise provide at least one therapeutic benefit to a patient. Therapeutic agents include, but are not limited to, an antigen (including heterologous antigens), a protein, a peptide, a nucleic acid molecule, a drug, a lipid, a carbohydrate, an antibody or antigen binding fragment thereof, or a biological response modifier (e.g., cytokine, a chemokine, a hormone, a lipidic derivative, an enzyme, a small molecule drug that elicits a biological response, and a protein or peptide that regulates gene expression or cellular activity in a recipient cell).

In one embodiment of the invention, a component of a therapeutic composition includes at least one antigen for vaccinating an animal (e.g., a vaccinating antigen). Most typically, the attenuated bacterium of the present invention as described above is transformed with one or more recombinant nucleic acid molecule encoding the antigen(s) and expresses the antigen(s). In another aspect of the invention, the bacterial strain is transformed with one or more transfer vectors comprising a nucleic acid sequence encoding one or more antigens, wherein the vector is transferred to a host cell upon invasion of the host cell by the bacterium. In other embodiments, the attenuated bacterium can be loaded intracellularly with, attached to, or mixed with, a protein or peptide antigen (discussed below).

The therapeutic composition can include, by presence of protein and/or nucleic acid sequence encoding a protein, one, two, a few, several or a plurality of antigens, as desired. According to the present invention, the general use herein of the term "antigen" refers: to any portion of a protein (peptide, partial protein, full-length protein), wherein the protein is naturally occurring or synthetically derived, or to a carbohydrate or other molecule, or a portion thereof, wherein the antigen elicits a humoral and/or cellular immune response (or acts as a toleragen). In one embodiment of the present invention, when it is desirable to stimulate an immune response, the term "antigen" can be used interchangeably with the term "immunogen", and is use herein to describe a protein or peptide which elicits a humoral and/or cellular immune response (i.e., is antigenic), such that administration of the immunogen to an animal (e.g., via a vaccine of the present invention) mounts an antigen-specific immune response against the same or similar proteins or peptides that are

encountered within the tissues of the animal. Therefore, to vaccinate an animal against a particular antigen means, in one embodiment, that an immune response is elicited against the antigen as a result of administration of the antigen. In another embodiment, when it is desirable to suppress an immune response against a given antigen, an antigen can include a toleragen. According to the present invention, a toleragen is used to describe a protein or peptide that is provided in a form, amount, or route of administration such that there is a reduced immune response to the antigen, and preferably substantial non-responsiveness, anergy, other inactivation, or deletion of immune system cells in response to contact with the toleragen or a cell expressing or presenting such toleragen. A "vaccinating antigen" can be an immunogen or a toleragen, but is an antigen used in a vaccine, where a biological response (elicitation of an immune response, tolerance) is to be elicited against the vaccinating antigen. An epitope is defined herein as a single immunogenic site within a given antigen that is sufficient to elicit an immune response, or a single toleragenic site within a given antigen that is sufficient to suppress, delete or render inactive an immune response. Those of skill in the art will recognize that T cell epitopes are different in size and composition from B cell epitopes, and that epitopes presented through the Class I MHC pathway differ from epitopes presented through the Class II MHC pathway. An antigen can be as small as a single epitope, or larger, and can include multiple epitopes. As such, the size of an antigen can be as small as about 5-12 amino acids (e.g., a peptide) and as large as: a full length protein, including a multimer and fusion proteins, chimeric proteins, or portions thereof. In addition, antigens include carbohydrates, such as those expressed on cancer cells, which can be loaded into the bacterium. It will be appreciated that in some embodiments (i.e., when the antigen is expressed by the bacterium from a recombinant nucleic acid molecule), the antigen is a protein, fusion protein, chimeric protein, or fragment thereof. In preferred embodiments, the antigen is selected from the group of a tumor antigen or an antigen of an infectious disease pathogen (i.e., a pathogen antigen). According to the present invention, an antigen suitable for use in the present composition or vaccine can include two or more epitopes from the same antigen, two or more epitopes or antigens from the same cell, tissue or organism, or two or more different epitopes or antigens from different cells, tissues or organisms. Preferably, the antigen is heterologous to the bacterial strain (i.e., is not protein that is naturally produced by the bacterial strain).

Vaccination preferably results in a protective or therapeutic effect, wherein subsequent exposure to the antigen (or a source of the antigen) elicits an immune response against the antigen (or source) that reduces or prevents a disease or condition in the animal,

or, when the antigen is toleragenic, subsequent exposure to the antigen results in an attenuated or substantially absent immune response against the antigen. The concept of vaccination is well known in the art. The immune response that is elicited (increased, stimulated, activated, enhanced) or suppressed (reduced, deleted, anergized, silenced, inactivated) by administration of a therapeutic composition of the present invention can be any detectable increase or decrease in any facet of the immune response (e.g., cellular response, humoral response, cytokine production, production of other biological response modifiers), as compared to in the absence of the administration of the vaccine.

In one aspect of the invention, the antigen useful in the present composition is an antigen from a pathogen (including the whole pathogen, when the attenuated bacterium of the invention is the immunizing/vaccinating agent), and particularly, from a pathogen that is associated with (e.g., causes or contributes to) an infectious disease. An antigen from an infectious disease pathogen can include antigens having epitopes that are recognized by T cells, antigens having epitopes that are recognized by B cells, antigens that are exclusively expressed by pathogens, and antigens that are expressed by pathogens and by other cells. In some instances, an antigen can include organisms or portions or individual proteins thereof which may not be ordinarily considered to be pathogenic in an animal, but against which immunization is nonetheless desired. The antigens can include one, two or a plurality of antigens that are representative of the substantially all of the antigens present in the infectious disease pathogen against which the vaccine is to be administered. In other embodiments, antigens from two or more different strains of the same pathogen or from different pathogens can be used to increase the therapeutic efficacy and/or efficiency of the vaccine.

According to the present invention, a pathogen antigen includes, but is not limited to, an antigen that is expressed by a bacterium, a mycobacterium, a virus, a parasite or a fungus (including pathogenic yeast). Preferred pathogen antigens for use in the method of the present invention include antigens from pathogens that cause a chronic infectious disease in an animal. In one embodiment, a pathogen antigen for use in the method or composition of the present invention includes an antigen from a virus. Examples of viral antigens to be used in a vaccine of the present invention include, but are not limited to, env, gag, rev, tat, nucleocapsid proteins and reverse transcriptase from immunodeficiency viruses (e.g., HIV, FIV); HBV surface antigen and core antigen; HCV antigens; influenza nucleocapsid proteins; parainfluenza nucleocapsid proteins; human papilloma type 16 E6 and E7 proteins;

Epstein-Barr virus LMP-1, LMP-2 and EBNA-2; herpes LAA and glycoprotein D; as well as similar proteins from other viruses.

In another embodiment, preferred pathogen antigens for use in the methods and compositions of the present invention include antigens from a bacterium. Examples of bacterial antigens to be used in a vaccine of the present invention include, but are not limited to, toxins, adherence factors, and surface proteins (outer membrane proteins) of bacteria.

In another embodiment, preferred pathogen antigens for use in the methods and compositions of the present invention include antigens from a mycobacterium. Examples of mycobacterial antigens to be used in a vaccine of the present invention include, but are not limited to, toxins, adherence factors and surface proteins of mycobacteria.

Tumor antigens (cancer antigens) useful in the present invention can include a tumor antigen such as a protein, glycoprotein or surface carbohydrates from a tumor cell, an epitope from a tumor antigen, and portions thereof. In one embodiment, tumor antigens useful in the present invention can be isolated or derived from an autologous tumor sample. An autologous tumor sample is derived from the animal to whom the therapeutic composition is to be administered. Therefore, such antigens will be present in the cancer against which an immune response is to be elicited. In one aspect, the tumor antigen provided in a vaccine is isolated or derived from at least two, and preferably from a plurality of allogeneic tumor samples of the same histological tumor type. According to the present invention, a plurality of allogeneic tumor samples are tumor samples of the same histological tumor type, isolated from two or more animals of the same species who differ genetically at least within the major histocompatibility complex (MHC), and typically at other genetic loci. Therefore, if administered together, the plurality of tumor antigens can be representative of the substantially all of the tumor antigens present in any of the individuals from which antigen is derived. This embodiment of the method of the present invention provides a vaccine which compensates for natural variations between individual patients in the expression of tumor antigens from tumors of the same histological tumor type. Therefore, administration of this therapeutic composition is effective to elicit an immune response against a variety of tumor antigens such that the same therapeutic composition can be administered to a variety of different individuals. In some embodiments, antigens from tumors of different histological tumor types can be administered to an animal, in order to provide a very broad vaccine.

Preferably, the tumor from which the antigen is isolated or derived is any tumor or cancer, including, but not limited to, melanomas, squamous cell carcinoma, breast cancers,

head and neck carcinomas, thyroid carcinomas, soft tissue sarcomas, bone sarcomas, testicular cancers, prostatic cancers, ovarian cancers, bladder cancers, skin cancers, brain cancers, angiosarcomas, hemangiosarcomas, mast cell tumors, primary hepatic cancers, lung cancers, pancreatic cancers, gastrointestinal cancers, renal cell carcinomas, hematopoietic neoplasias and metastatic cancers thereof.

Other preferred antigens and compounds to include in compositions (vaccines) of the present invention include antigens and compounds that are capable of suppressing an undesired, or harmful, immune response, such as is caused, for example, by allergens, autoimmune antigens, inflammatory agents, antigens involved in GVHD, certain cancers, septic shock antigens, and antigens involved in transplantation rejection. Such antigens include, but are not limited to, antihistamines, cyclosporin, corticosteroids, FK506, peptides corresponding to T cell receptors involved in the production of a harmful immune response, apoptosis-inducing proteins, suitable MHC complexes presented in such a way as to effect tolerization or anergy, T cell receptors, autoantigens (antigens against which an autoimmune response is generated), allergens, and in one embodiment, any of these antigens or compounds in combination with a biological response modifier capable of enhancing or suppressing cellular and/or humoral immunity.

Other antigens useful in the present invention and combinations of antigens will be apparent to those of skill in the art. The present invention is not restricted to the use of the antigens as described above.

Genetically modified bacterial strains can be formulated into compositions of the present invention, including preparations to be loaded into another delivery vehicle (e.g., an excipient, carrier or cell from the host) or for direct administration to a host organism, using a number of techniques known to those skilled in the art. For example, bacterial cells can be mixed with a pharmaceutically acceptable excipient, such as an isotonic buffer that is tolerated by the host organism. Examples of such excipients include water, saline, Ringer's solution, dextrose solution, Hank's solution, and other aqueous physiologically balanced salt solutions. Nonaqueous vehicles, such as fixed oils, sesame oil, ethyl oleate, or triglycerides may also be used. Other useful formulations include suspensions containing viscosity enhancing agents, such as sodium carboxymethylcellulose, sorbitol, glycerol or dextran. Excipients can also contain minor amounts of additives, such as substances that enhance isotonicity and chemical stability. Standard formulations can either be liquid injectables or solids which can be taken up in a suitable liquid as a suspension or solution for injection. Thus, in a non-liquid formulation, the excipient can comprise, for example, dextrose, human

serum albumin, and/or preservatives to which sterile water or saline can be added prior to administration.

In one embodiment of the present invention, as an alternative to expression of an antigen recombinantly in the bacterial cell, a bacterial can be loaded intracellularly with the protein or peptide antigen, or with carbohydrates or other molecules that serve as an antigen. As used herein, the term "loaded" and derivatives thereof refer to the insertion, introduction, or entry of a component (e.g., a therapeutic agent) into a cell (e.g., a bacterial cell). To load a component intracellularly refers to the insertion or introduction of the component to an intracellular compartment of the cell (e.g., through the plasma membrane and at a minimum, into the cytoplasm, a phagosome, a lysosome, or some intracellular space of the cell). To load a component into a cell references any technique by which the component is either forced to enter the cell (e.g., by electroporation) or is placed in an environment (e.g., in contact with or near to a cell) where the component will be substantially likely to enter the cell by some process (e.g., phagocytosis). Loading techniques include, but are not limited to: diffusion, active transport, liposome fusion, electroporation, phagocytosis, and bath sonication.

In another embodiment of the present invention, the antigen is physically attached to the bacterial cell. Physical attachment of the antigen to the bacterial cell can be accomplished by any method suitable in the art, including, covalent and non-covalent association methods which include, but are not limited to, chemically crosslinking the antigen to the outer surface of the bacterial cell or biologically linking the antigen to the outer surface of the bacterial cell, such as by using an antibody or other binding partner. Chemical cross-linking can be achieved, for example, by methods including glutaraldehyde linkage, photoaffinity labeling, treatment with carbodiimides, treatment with chemicals capable of linking di-sulfide bonds, and treatment with other cross-linking chemicals standard in the art. Targeting agents such as antibodies, binding peptides, soluble receptors, and other ligands may also be incorporated into an antigen as a fusion protein or otherwise associated with an antigen for binding of the antigen to the bacterial cell.

In yet another embodiment, the bacterial cell and the antigen are associated with each other by a more passive, non-specific or non-covalent binding mechanism, such as by gently mixing the bacterial cell and the antigen together in a buffer or other suitable formulation for delivery to a host organism.

As used herein, a peptide comprises an amino acid sequence of less than or equal to about 30 amino acids, while a protein comprises an amino acid sequence of more than about

30 amino acids, proteins can be multimeric. A protein or peptide useful as an antigen can be as small as a T cell epitope (i.e., greater than 5 amino acids in length) and any suitable size greater than that which comprises multiple epitopes, protein fragments, full-length proteins, chimeric proteins or fusion proteins. Peptides and proteins can be derivatized either naturally or synthetically; such modifications can include, but are not limited to, glycosylation, phosphorylation, acetylation, myristylation, prenylation, palmitoylation, amidation and/or addition of glycerophosphatidyl inositol. Peptides and proteins can be inserted directly into bacterial cells of the present invention by techniques known to those skilled in the art, such as by diffusion, active transport, liposome fusion, electroporation, phagocytosis, freeze-thaw cycles and bath sonication.

In one embodiment of the present invention, a therapeutic composition useful in the present invention can also include biological response modifier compounds, or an attenuated bacterial cell can have the ability to produce such modifiers (i.e., by transfection with nucleic acid molecules encoding such modifiers). For example, a bacterial cell can be transfected with or loaded with at least one antigen and at least one biological response modifier compound. Biological response modifiers are compounds that can modulate a biological responses (e.g., immune response, cell signal transduction, secretion of a compound, etc.). Immunomodulators are biological response modifiers that particularly modify an immune response and these terms can be used interchangeably when referring to modulation of an immune response according to the invention. Certain biological response modifiers that are immunomodulators can stimulate a protective immune response whereas others can suppress a harmful immune response. Certain biological response modifiers preferentially enhance a cell-mediated immune response whereas others preferentially enhance a humoral immune response (i.e., can stimulate an immune response in which there is an increased level of cellular compared to humoral immunity, or vice versa.). There are a number of techniques known to those skilled in the art to measure stimulation or suppression of immune responses, as well as to differentiate cellular immune responses from humoral immune responses.

Suitable biological response modifiers for use in the present invention include cytokines, hormones, lipidic derivatives, an enzyme, small molecule drugs, a protein or peptide that regulates gene expression or cellular activity in a recipient cell, and other growth modulators, such as, but not limited to, interleukin 2 (IL-2), interleukin 4 (IL-4), interleukin 10 (IL-10), interleukin 12 (IL-12), interleukin-6 (IL-6), interleukin-8 (IL-8), interferon gamma (IFN-gamma), interferon alpha (IFN-alpha), insulin-like growth factor I (IGF-I), transforming growth factor beta (TGF- β), steroids, prostaglandins and leukotrienes.

~~In one embodiment of the invention, a therapeutic composition or vaccine comprising an attenuated bacterium as described herein, optionally with a therapeutic compound (e.g., an antigen) preferably elicits an immune response in an animal such that the animal is protected from a disease that is amenable to elicitation of an immune response, including cancer or an infectious disease. In one embodiment, the therapeutic composition induces tolerance to an antigen or bacterium so that the animal is protected from a disease. As used herein, the phrase "protected from a disease" refers to reducing the symptoms of the disease; reducing the occurrence of the disease, and/or reducing the severity of the disease. Protecting an animal can refer to the ability of a therapeutic composition of the present invention, when administered to an animal, to prevent a disease from occurring and/or to cure or to alleviate disease symptoms, signs or causes. As such, to protect an animal from a disease includes both preventing disease occurrence (prophylactic treatment or vaccine) and treating an animal that has a disease or that is experiencing initial symptoms of a disease (therapeutic treatment or a therapeutic vaccine). In particular, protecting an animal from a disease is accomplished by eliciting an immune response in the animal by inducing a beneficial or protective immune response which may, in some instances, additionally suppress (e.g., reduce, inhibit or block) an overactive or harmful immune response. The term, "disease" refers to any deviation from the normal health of an animal and includes a state when disease symptoms are present, as well as conditions in which a deviation (e.g., infection, gene mutation, genetic defect, etc.) has occurred, but symptoms are not yet manifested.~~

More specifically, a vaccine as described herein, when administered to an animal by the method of the present invention, preferably produces a result which can include alleviation of the disease (e.g., reduction of at least one symptom or clinical manifestation of the disease), elimination of the disease, reduction of a tumor or lesion associated with the disease, elimination of a tumor or lesion associated with the disease, prevention or alleviation of a secondary disease resulting from the occurrence of a primary disease (e.g., metastatic cancer resulting from a primary cancer), prevention of the disease, stimulation of effector cell immunity against the disease and induction of tolerance against a given antigen(s).

For example, one embodiment of the present invention is the use of the therapeutic composition as described above to protect an animal from a disease caused by an infectious agent. An infectious agent can be any agent that can infect an animal and cause disease. Such disease may develop rapidly or after a long period of time. Suitable infectious agents

against which to protect animals using the vaccine of the present invention include, but are not limited to, viroids, prions, viruses, bacteria, fungi (including yeast), protozoa (e.g., amebas, flagellates and sporozoa), helminths and ectoparasites. It is within the scope of the present invention to protect an animal against more than one infectious agent. It should also be noted that although some infectious agents have not been definitively classified into one of these groups, such infectious agents are also included in the present invention.

Preferred compositions of the invention are capable of protecting an animal from infection by infectious agents that damage, for example, the aural, dermal, enteric, immune, neural, oral/dental, reproductive, respiratory and/or urinary systems of animals. Such infectious agents include, but are not limited to, adenoviruses, arena viruses, bunyaviruses, coronaviruses, hepadnaviruses, herpes viruses, myxoviruses, oncogenic viruses, orthomyxoviruses, papovaviruses, paramyxoviruses, parvoviruses, picornaviruses, pox viruses, rabies viruses, reoviruses, rhabdoviruses, rubella viruses, togaviruses, plant viruses, *Aspergillus*, *Bacillus*, *Brugia*, *Candida*, *Chlamydia*, *Coccidia*, *Corynebacteria*, *Cryptococcus*, *Dirofilaria*, *Francisella*, *Gonococcus*, *Histoplasma*, *Leishmania*, *Mycobacterium*, *Mycoplasma*, *Paramecium*, *Pertussis*, *Plasmodium*, *Pneumococcus*, *Pneumocystis*, *Pseudomonas*, *Rickettsia*, *Salmonella*, *Shigella*, *Staphylococcus*, *Streptococcus*, *Toxoplasma*, *Vibrio cholerae* and *Yersinia* as well as other infectious agents that cause opportunistic infections in animals that are immunodeficient or otherwise immunosuppressed. Additional preferred infectious agents include other harmful microorganisms found in brackish water, food contaminants, wounds, and biological weapons.

Preferred viruses from which to protect organisms using attenuated bacteria compositions of the present invention include Coxsackie viruses, cytomegaloviruses, Epstein-Barr viruses, flaviviruses, hepatitis viruses, herpes viruses, influenza viruses, measles viruses, mumps viruses, papilloma viruses, parainfluenza viruses, parvoviruses, rabies viruses, respiratory syncytial viruses, retroviruses and varicella viruses.

Retroviruses, herpes viruses, and hepatitis viruses are more preferred, with leukemia, lymphotropic, sarcoma and lentiviruses being even more preferred, as are other immunodeficiency or tumor viruses. Particularly preferred lymphotropic viruses from which to protect organisms include T-lymphotropic viruses, such as human T-cell lymphotropic viruses (HTLVs, such as HTLV-I and HTLV-II), bovine leukemia viruses (BLVs) and feline leukemia viruses (FLVs). Particularly preferred lentiviruses include

human (HIV), simian (SIV), feline (FIV) and canine (CIV) immunodeficiency viruses, with HIV-1 and HIV-2 being even more preferred.

Preferred bacteria from which to protect using attenuated bacteria compositions of the invention include, but are not limited to, *Pseudomonas*, *Bordetella*, *Mycobacterium*, *Vibrio*, *Bacillus*, *Salmonella*, *Francisella*, *Staphylococcus*, *Streptococcus*, *Enterococcus*, *Pasteurella*, and *Yersinia*, with *Pseudomonas aeruginosa*, *Pseudomonas mallei*, *Pseudomonas pseudomallei*, *Bordetella pertussis*, *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Francisella tularensis*, *Vibrio cholerae*, *Bacillus anthracis*, *Salmonella enteric* and *Yersinia pestis* being particularly preferred.

Cancers to be treated or prevented using the method and composition of the present invention include, but are not limited to, melanomas, squamous cell carcinoma, breast cancers, head and neck carcinomas, thyroid carcinomas, soft tissue sarcomas, bone sarcomas, testicular cancers, prostatic cancers, ovarian cancers, bladder cancers, skin cancers, brain cancers, angiosarcomas, hemangiosarcomas, mast cell tumors, primary hepatic cancers, lung cancers, pancreatic cancers, gastrointestinal cancers, renal cell carcinomas, hematopoietic neoplasias, and metastatic cancers thereof. Particularly preferred cancers to treat with a therapeutic composition of the present invention include primary lung cancers and pulmonary metastatic cancers. A therapeutic composition of the present invention is useful for eliciting an immune response in an animal to treat tumors that can form in such cancers, including malignant and benign tumors. Preferably, expression of the tumor antigen in a tissue of an animal that has cancer produces a result selected from the group of alleviation of the cancer, reduction of a tumor associated with the cancer, elimination of a tumor associated with the cancer, prevention of metastatic cancer, prevention of the cancer and stimulation of effector cell immunity against the cancer.

Additional diseases from which to protect an animal using a therapeutic composition of the present invention include, but are not limited to, allergies, autoimmune diseases (e.g., diabetes, multiple sclerosis, rheumatoid arthritis), graft versus host disease (GVHD), hematopoietic disorders, immunodeficiency diseases, immunoproliferative diseases, immunosuppressive disorders, inflammatory diseases, rejection of allografts or xenografts, septic shock, other immunological defects and combinations thereof. Many of these diseases can be acute or chronic. Examples of particular diseases from which animals can be protected using a vaccine of the present invention are disclosed herein. It is to be noted that such examples are intended only as such and do not limit the wide variety of diseases against which appropriately designed vaccines of the present invention can protect animals.

In one embodiment, a composition of the present invention can include one or more adjuvants and/or carriers. Adjuvants are typically substances that generally enhance the immune response of an animal to a specific antigen. Suitable adjuvants include, but are not limited to, Freund's adjuvant; other bacterial cell wall components; aluminum-based salts; calcium-based salts; silica; polynucleotides; toxoids; serum proteins; viral coat proteins; other bacterial-derived preparations; gamma interferon; block copolymer adjuvants, such as Hunter's Titermax adjuvant (CytRx™, Inc. Norcross, GA); Ribi adjuvants (available from Ribi ImmunoChem Research, Inc., Hamilton, MT); and saponins and their derivatives, such as Quil A (available from Superfos Biosector A/S, Denmark).

Carriers are typically compounds that increase the half-life of a therapeutic composition in the treated animal. Suitable carriers include, but are not limited to, polymeric controlled release formulations, biodegradable implants, liposomes, oils, esters, and glycols.

Therapeutic compositions of the present invention can also contain one or more pharmaceutically acceptable excipients. As used herein, a pharmaceutically acceptable excipient refers to any substance suitable for delivering a therapeutic composition useful in the method of the present invention to a suitable *in vivo* or *ex vivo* site. Preferred pharmaceutically acceptable excipients are capable of maintaining a bacterial cell in a form that, upon arrival of the bacterial cell at a target cell, tissue, or site in the body, the bacterial cell (with or without a therapeutic compound, such as an antigen), is capable of eliciting an immune response at the target site (noting that the target site can be systemic). Suitable excipients of the present invention include excipients or formularies that transport, but do not specifically target the vaccine to a site (also referred to herein as non-targeting carriers). Examples of pharmaceutically acceptable excipients include, but are not limited to water, saline, phosphate buffered saline, Ringer's solution, dextrose solution, serum-containing solutions, Hank's solution, other aqueous physiologically balanced solutions, oils, esters and glycols. Aqueous carriers can contain suitable auxiliary substances required to approximate the physiological conditions of the recipient, for example, by enhancing chemical stability and isotonicity.

Suitable auxiliary substances include, for example, sodium acetate, sodium chloride, sodium lactate, potassium chloride, calcium chloride, and other substances used to produce phosphate buffer, Tris buffer, and bicarbonate buffer. Auxiliary substances can also include preservatives, such as thimerosal, m- or o-cresol, formalin and benzol alcohol.

The present invention includes the delivery of a composition comprising attenuated bacterial cells of the invention to an animal. The administration process can be performed *ex*

~~Ex vivo or in vivo~~ administration refers to performing part of the regulatory step outside of the patient, such as administering a composition of the present invention to a population of cells (e.g., dendritic cells) removed from a patient under conditions such that the bacterial cell and antigen are loaded into the cell, and returning the cells to the patient. The therapeutic composition of the present invention can be returned to a patient, or administered to a patient, by any suitable mode of administration. Such administration can be systemic, mucosal and/or proximal to the location of the target site (e.g., near a tumor). The preferred routes of administration will be apparent to those of skill in the art, depending on the type of condition to be prevented or treated, the antigen used, and/or the target cell population or tissue. Preferred methods of administration include, but are not limited to, intravenous administration, intraperitoneal administration, intramuscular administration, intranodal administration, intracoronary administration, intraarterial administration (e.g., into a carotid artery), subcutaneous administration, transdermal delivery, intratracheal administration, subcutaneous administration, intraarticular administration, intraventricular administration, inhalation (e.g., aerosol), intracranial, intraspinal, intraocular, aural, intranasal, oral, pulmonary administration, impregnation of a catheter, and direct injection into a tissue.

Parenteral delivery can include intradermal, intramuscular, intraperitoneal, intrapleural, intrapulmonary, intravenous, subcutaneous, atrial catheter and venal catheter routes. Aural delivery can include ear drops, intranasal delivery can include nose drops or intranasal injection, and intraocular delivery can include eye drops. Aerosol (inhalation) delivery can also be performed using methods standard in the art (see, for example, Stribling et al., *Proc. Natl. Acad. Sci. USA* 189:11277-11281, 1992, which is incorporated herein by reference in its entirety). Oral delivery can include solids and liquids that can be taken through the mouth, and is useful in the development of mucosal immunity and since compositions comprising bacterial cells can be easily prepared for oral delivery, for example, as tablets or capsules, as well as being formulated into food and beverage products. Other routes of administration that modulate mucosal immunity are useful in the treatment of viral infections, epithelial cancers, immunosuppressive disorders and other diseases affecting the epithelial region. Such routes include bronchial, intradermal, intramuscular, intranasal, other inhalatory, rectal, subcutaneous, topical, transdermal, vaginal and urethral routes.

According to the present invention, an effective administration protocol (i.e., administering a vaccine or therapeutic composition in an effective manner) comprises suitable dose parameters and modes of administration that result in elicitation of an immune

response or provision of a therapeutic effect in an animal that has a disease or condition, or that is at risk of contracting a disease or condition, preferably so that the animal is protected from the disease. Effective dose parameters can be determined using methods standard in the art for a particular disease. Such methods include, for example, determination of survival rates, side effects (i.e., toxicity) and progression or regression of disease. In particular, the effectiveness of dose parameters of a therapeutic composition of the present invention when treating cancer can be determined by assessing response rates. Such response rates refer to the percentage of treated patients in a population of patients that respond with either partial or complete remission. Remission can be determined by, for example, measuring tumor size or microscopic examination for the presence of cancer cells in a tissue sample.

In accordance with the present invention, a suitable single dose size is a dose that is capable of eliciting an antigen-specific immune response in an animal when administered one or more times over a suitable time period. Doses can vary depending upon the disease or condition being treated. In the treatment of cancer, for example, a suitable single dose can be dependent upon whether the cancer being treated is a primary tumor or a metastatic form of cancer. One of skill in the art can readily determine appropriate single dose sizes for administration based on the size of an animal and the route of administration.

A suitable single dose of a therapeutic composition or vaccine of the present invention is a dose that is capable of effectively providing an attenuated bacterium and, in some embodiments, a therapeutic agent such as an antigen to a given cell type, tissue, or region of the patient body in an amount effective to elicit a bacterial antigen-specific or an antigen-specific immune response, or to otherwise provide a therapeutic benefit, when administered one or more times over a suitable time period. A preferred single dose is the lowest possible dose of cells which achieve a therapeutic or prophylactic result as discussed herein. For example, a preferred single dose of a vaccine of the present invention is from about 100 single bacterial cells per individual per administration to up to 5000 or more single bacterial cells per individual per administration, and can include any intervening number of single bacterial cells per individual per administration, in whole integers (i.e., 101, 102, 103...1000, 1001, 1002...5000, >5000 single bacterial cells per individual per administration). "Boosters" of a therapeutic composition are preferably administered when the immune response against an antigen has waned or as needed to provide an immune response or induce a memory response against a particular antigen or antigen(s). Boosters can be administered from about 2 weeks to several years after the original administration. It

will be obvious to one of skill in the art that the number of doses administered to an animal is dependent upon the extent of the disease and the response of an individual patient to the treatment.

In the method of the present invention, vaccines and therapeutic compositions can be administered to any member of the Vertebrate class, Animalia, including, without limitation, primates, rodents, livestock and domestic pets. Mammals are preferred animals to which the compositions of the present invention are administered. Preferred animals to protect include humans, dogs, cats, mice, rats, goats, sheep, cattle, horses and pigs, with humans being particularly preferred.

The following experimental results are provided for purposes of illustration and are not intended to limit the scope of the invention.

Examples

Example 1

The following example describes a high throughput assay for TAT inhibitors/antimicrobial compounds according to the present invention.

The assay is performed as schematically shown in Fig. 4. Briefly, the assay is initially optimized for high throughput screening procedures. Such parameters as substrate concentrations, timing of growth and incubation with substrate, as well as growth conditions (to avoid nonspecific effects) are assessed. A microtiter plate reader that will detect absorbance at 410A will monitor the output. Growth of the microorganism will also be measured in this assay to demonstrate that a candidate inhibitor is not merely inhibiting bacterial growth. This will be accomplished by monitoring the absorbance at 600A (which detects cell density) of wells with inhibitor against wells that have no inhibitor. Any compound that reduces the absorbance at 410A, but minimally affects the absorbance at 600A will be considered a candidate inhibitor of TAT and will be evaluated in greater detail.

Example 2

The following example describes the use of the method of the present invention to demonstrate the optimization of a prototype high throughput assay illustrating the negative screening step according to the present invention.

The inventors have performed an assay that tests for presence or absence of two proteins (PlcH and PlcN) that are secreted by the TAT secretory system. *P. aeruginosa* (wild-type and TAT mutant POA1, to represent a compound that inhibits the TAT secretory

system) were grown in microtiter plates (100 μ l of bulk liquid culture after overnight growth). Cultures were grown at 24°C and 37°C to further show the effect of temperature on the assay. The cultures were diluted in serial 3X dilutions in duplicate. To test wells, 50 μ l of substrate, *p*-nitrophenylphosphorylcholine (NPPC), were added. Further control wells contained 50 μ l media and 50 μ l NPPC. Inhibition was measured by monitoring the absorbance at 600A and 400A in the TAT mutant containing wells and the wild-type containing wells. As shown in Figs. 5 and 6, wells containing the TAT mutant were completely negative for NPPC activity, demonstrating that this assay correctly reflects TAT function in the test microorganism. Moreover, the quantitative aspects of this assay would allow the detection of a compound that might partially inhibit the function of TAT. Such a lead compound might be further modified, (e.g medicinal chemistry) to a more potent compound that was able to more effectively inhibit TAT function, as exemplified by the TAT mutant. Therefore, compound libraries can readily be added to this assay and the results detected as described here.

In addition, the results showed that growth of the organism at 24°C enhances the sensitivity of the detection of these PLC by NPPC, as compared to growth of the organism at 37°C. Consequently, it will be preferably to carry out screening assays for this organism and protein at 24°C because this temperature will afford the greatest sensitivity for detecting the function of TAT, as manifested by the secretion of PLC activity.

To add a positive screening step to this assay, one simply further detects the presence of a gene that is upregulated when TAT is inhibited, or tests for antibiotic resistance according to the detailed description of the invention as described above. This data demonstrate the ability of the method of the present invention to be used in a high throughput assay for the identification of antimicrobial agents.

Example 3

The following example describes the investigation of the ability of the avirulent *PAO1 Δ tatC* mutant to be used as a safe and effective immunogen against pulmonary infections caused by *P. aeruginosa* in rats.

Administration of free *P. aeruginosa* provides a model of acute lung infection due to the rapid clearance of the organism or acute sepsis and death. Cash *et al.* (Cash) found that embedding the *P. aeruginosa* into agarose beads establishes a model of chronic bronchopulmonary infection that has been used extensively to model CF lung infection (Beaulac; Cantin; Cheung; Iwata; Johansen; Klinger; Konstan; Lange; Pennington; Song; Thomassen; Wilderman; Woods). In the following experiments, this model will be

employed in rats (Cash) to evaluate a number of a specific parameters associated with pathogenesis of PAO1 and a *PAO1AtatC* mutant. At this point, the inventors will use some modifications to this model including the method of preparing the agarose beads (discussed below). This model has been extensively used (Ochsner, 2002, PNAS; Wilderman, 2001, Mol. Microbiol.; Wilderman, 2001, PNAS) and it is chosen over the mouse model for several reasons. The original procedure for a model of chronic bronchopulmonary infection with *P. aeruginosa* using agarose beads embedded with *P. aeruginosa* (Cash) was devised for rats and modified for use in mice by Starke *et al.* (Starke). However, for unknown reasons, this infection is more acute in mice and in fact, more mice infected with *P. aeruginosa* die in this model compared to the corresponding model in rats. The advantages of using rats for this research greatly outweighs those for using mice. For example, when determining if *P. aeruginosa* PAO1*AtatC* can be used as an effective immunogen, sterile or bacteria-laden agarose beads will be administered to the animals twice followed by administration of the challenge. This requires that three surgeries be performed on each animal involving a transtracheal incision followed by administration of the beads using an over-the-needle catheter. Furthermore, the animals will be anaesthetized each time prior to the procedure and rats will be much easier to manipulate simply due to their size.

Van Heeckeren and Schluchter (Van Heeckeren) and Nacucchio *et al.* (Nachucchio) presented evidence that free bacteria mixed with sterile agarose beads may be considered as an alternative to the use of bacteria-laden beads. Using this method, they found that the histopathological features between the two methods were similar. This new way to administer the bacteria is attractive and under consideration for several reasons. First, because the bacteria are located on the outside of the agarose beads, the variability involving the success of the bacteria to escape from the bead once inside the lung is removed. Second, this method may expose more of the antigenic proteins directly to the immune system thereby generating a more robust inflammatory response. Third, the number of bacteria either being used to infect or harvested from the lung can be more accurately quantified. Finally, although this has not been experienced with *PAO1AtatC*, for reasons not totally clear at this time, the inventors have observed that some mutants do not survive the process by which they are encased into the agarose beads.

Experimental Model

The following strategy and experimental methods is applied to characterize in-depth any differences in colonization, inflammation, and any alterations in the inflammatory

responses after infection with PAO1 wild-type and PAO1 Δ tatC over a duration of three months post-infection.

Culturing bacteria and inoculating rats. A pilot study is conducted with a few animals infected with PAO1-laden or PAO1-embedded agarose beads to determine if one method is more advantageous. Sterile agarose beads or bead embedded or associated with *P. aeruginosa* PAO1 or PAO1 Δ tatC are administered into the lungs of rats according to published protocols (Cash). Briefly, after bead preparation, the number of organisms incorporated into the beads are verified by plating serial dilutions prior to administration. A bead suspension are placed in a distal bronchus of a rat. At various time points, the lungs are harvested and evaluated for bacterial counts, gross morphology and histology, and levels of specific cytokines and chemokines.

Evaluation of Infection. The number of animals used for each time point will supply ample tissue for analysis of the lungs for colony counts, histopathology, and cytokine and chemokine levels. An overview of the experimental design for evaluating the infections in the pulmonary model is shown in Fig. 3. As shown in Fig. 3, rats will be inoculated with steril or bacteria-laden agarose beads. At various times post infection, the lungs will be harvested and analyzed for colony forming units, hitopathology and inflammation, and cytokine and chemokine levels.

Bacterial load and histopathology. Following harvesting, the lungs are immediately be examined for gross morphology and the bacterial load is determined by viable counts as previously described (Wilderman; Wilderman). In addition to determining the number of bacteria that are surviving in the lung, the location of the bacteria provides valuable information as to whether a particular environment (e.g. lobe, airways, alveoli, bronchioles) is preferentially colonized. Several techniques are available in addition to the typical staining procedures. For example, bacteria labeled with GFP are used, or the bacteria are immobilized using antibodies specific for O-antigen. Furthermore, in addition to localizing the individual bacteria, specific immune cells (e.g. neutrophils) are localized using neutrophil infiltration markers. The most specific biochemical marker for neutrophils is myeloperoxidase (MPO). This enzymatic reaction is used to globally measure neutrophil accumulation (Nick). Together this information will provide a picture of the infection relative to both the bacterium and its host.

Cytokine and Chemokine Levels. To determine if there exists a delicate balance between the induction of a protective response and that of a destructive response, the mRNA levels for numerous cytokines and chemokines in the lungs that have been infected with

PAO1 and PAO1 Δ tatC are quantified compared to those that have been treated with sterile agarose beads.

To isolate RNA from the rat lung, a method by Dr. Andres Vazques-Torres is used. In order to get a broad picture of any cytokine and response, a commercially available multiprobe nuclease protection will be employed and a variety of mRNAs of the various cytokines and chemokines will be semi-quantified. This protection approach was chosen for these studies because of its relative sensitivity and capacity to simultaneously detect several mRNA species in a single sample. Rat housekeeping genes encoding glyceraldehyde-3-phosphate dehydrogenase murine ribosomal protein (L32) (Young) are also included to ensure equal loading of total quantification. Kernacki *et al.* (Kernacki) have successfully used this method for detection chemokine levels in a corneal infection in mice caused by *P. aeruginosa*. Since the multiprobe kits available for rats do not include a probe for IL-8, a probe for IL-8 is designed and tested for independently. All negative cytokine and chemokine responses will be confirmed by RT-PCR then if necessary, quantitative RT-PCR will be performed. Additionally, ELISA will be used to directly measure the amount of specific cytokines and chemokines produced as a result of infection with PAO1 or PAO1 Δ tatC, since the level of cytokine or chemokine released from the cells may not be a direct reflection of the transcriptional activity of a cell (i.e. a cell may store the cytokine or chemokine and then release it). In this regard, ELISA will be beneficial in determining if specific cytokines or chemokines are temporally released without transcriptional upregulation. Together, these data will provide extensive data characterizing the cytokine and chemokine response to infection with PAO1 and PAO1 Δ tatC as well as demonstrating that PAO1 Δ tatC can be used as a safe and effective immunogen to protect against chronic pulmonary infections with *P. aeruginosa*.

Criteria for evaluating protective response. It is expected that, in these experiments, PAO1 Δ tatC is cleared from the rat lungs. However, even if this is not the case and PAO1 Δ tatC persists, it is expected that there will be a cytokine and chemokine response that is either minimal or not detectable. In the inventors' experiments to evaluate bacterial infection by the PAO1 Δ tatC mutant, inflammation was not detected after six days, a duration sufficient for an inflammatory or cytokine response to be generated. This is a remarkable observation, since the PAO1 Δ tatC serotype is the same as the PAO1 serotype, and since lipopolysaccharide is present in the airways for at least six days. For challenge experiments, a response is considered to be protective using the following criteria (Table 5). First, it is expected that the bacterial load of an immunized rat challenged with *P. aeruginosa* wild-type

will be decreased at least two logs. In addition, significantly reduced inflammation should be seen in the immunized animals challenged with wild-type PAO1. Finally, cytokine and chemokine levels should be reduced significantly (p value of 0.05). Despite the lack of inflammation, there is expected to be a non-inflammatory cytokine response that will be protective. Therefore, it will be informative to determine if immunization with *PAO1ΔtatC* prior to challenge with PAO1 or various *P. aeruginosa* strains will decrease any pro-inflammatory response (IL-10) or increase any anti-inflammatory response.

Table 5. Anticipated outcomes in immunized or unimmunized animals when challenged with PAO1 if PAO1 *ΔtatC* is to be considered an effective immunogen.

condition of animal	bacterial load	cellular response	cytokine/chemokine response
immunized ^a	decreased -2 logs	decreased neutrophils decrease MPO	decreased pro-inflammatory response increased anti-inflammatory response
unimmunized ^b	unaffected ^c	unaffected	unaffected

^a Animals are infected and boosted with PAO1 *ΔtatC*-laden agarose beads.

^b Animals are mock infected and mock boosted with sterile agarose beads.

^c Unaffected refers to a result as compared to that seen in PAO1-infected animals.

Example 4

The following example describes the role of the PscO, a putatively Tat secreted substrate, in type III secretion.

There are two exclusive hypotheses regarding the effect of the Tat system on the transcription of type III secretion genes. It is possible that the effect of a *tatC* mutation observed on transcription of type III secretion genes is a consequence of a nonspecific pleiotropic effect of the *tatC* mutation in *P. aeruginosa*. Alternatively, preliminary but compelling data in the present inventors' laboratory supports the hypothesis that the effects of the *tatC* mutation on type III secretion are largely or solely connected to PscO, the only type III secretion-associated protein identified in the GeneChip® analysis that is predicted to have a twin-arginine signal sequence. In addition to the remarkable phenotypes of *PAO1ΔtatC*, the inventors noticed a significant effect of the *ΔtatC* mutation on the levels of specific extracellular proteins. These observations prompted the examination of the effects of Tat on global transcription patterns in PAO1 using the GeneChip® technology. The most striking effect of a *PAO1ΔtatC* mutant compared to PAO1 was the significantly decreased expression of >15 genes encoding proteins comprising the type III secretion apparatus and its secreted proteins (Table 6). The inventors will focus specifically on one of these genes,

pscO (PA1696), which encodes a protein predicted to possess a signal sequence for Tat secretion. It is believed, without being bound by theory, that there are pleiotropic effects on the type III secretion system that could cause this down-regulation of type III secretion gene or alternatively, because PscO has a putative Tat secretion signal, PscO could play a major feedback role in regulating type III secretion genes. In these experiments, the role of the Tat system in the secretion and function of PscO, a protein of unknown function with regard to type III secretion, will be examined. Initially, a PAO1 Δ *pscO* isogenic mutant strain will be generated by allelic exchange as described (Hoang, 1998; Schweizer), and gene replacements will be verified by PCR across the region or Southern blot hybridization. The inventors will generate a non-polar mutant because *pscO* is the second gene within an eight-gene operon (PA1690 1697) encoding *pscN-pscU*. For genetic complementation of the PAO1 Δ *pscO* mutant, a fragment containing *pscO* will be cloned into mini CTX1 (Hoang, 2000) and integrated in single copy at the *attB* locus, yielding the complemented *pscO* mutant. Site-directed mutagenesis of the twin-arginine motif of *pscO* will be used by a PCR-based protocol (Turner), which the inventors have previously used to successfully to generate numerous mutants. If problems arise and the inventors are unable to generate a PAO1 Δ *pscO* strain, PscO (wild-type or mutant) can be expressed from the pVLT plasmid in wild-type PAO1. Consequently, under these circumstances, PscO will either be over-expressed, or the mutant PscO will compete with the wild-type PscO. These strains will then be assessed for their ability to maintain a functional type III secretion by inducing plaque formation in tissue culture cells (CHO). Following infection of CHO cell monolayers with *P. aeruginosa*, the monolayers are stained and examined for plaques. The ability of the organism to induce plaque formation in the monolayers correlates with the functionality of type III secretion. Using this rapid assay, the inventors already have data indicating that a PAO1 Δ *tatC* mutant is reduced in its ability to form plaques compared to PAO1 (data not shown). Furthermore, when PAO1 Δ *tatC* is complemented with a wild-type copy of *tatC*, plaque formation is restored.

Table 6. Summary of the genes that are decreased in expression in a PAO1 Δ *tatC* stain compared to that of PAO1.

fold Δ	gene #	description
22.3		<i>hasA</i> ; Heme uptake
16.5		<i>prpL</i> ; Extracellular protease (Type II secreted)
13.0	PA1708	<i>popB</i> ; translocator protein
11.0	PA2191	<i>exoY</i> ; adenylate cyclase

fold Δ	gene #	description
10.7	PA1709	<i>popD</i> ; translocator protein
8.4	PA1707	<i>pcrH</i> ; regulatory protein
7.7	PA1706	<i>pcrV</i> ; type III secretion protein
7.0	PA1698	<i>popN</i> ; outer membrane protein
6.1	PA3841	<i>exoS</i> ; exoenzyme S
5.4	PA0044	<i>exoT</i> ; exoenzyme T
5.2		<i>exsE</i> ; Type III post-translation regulation
5.1	PA1711	hypothetical protein
4.9	PA1670	conserved hypothetical protein in type III secretion
4.9	PA1700	conserved hypothetical protein in type III secretion
4.8	PA1699	conserved hypothetical protein in type III secretion
4.5	PA1712	<i>exsB</i> ; exoenzyme S synthesis protein B
4.1	PA1697	ATP synthase in type III secretion system
4.0	PA1714	hypothetical protein
3.7	PA1710	<i>exsC</i> ; exoenzyme S synthesis protein C precursor
3.4	PA1696	<i>pscO</i> ; translocation protein in type III secretion
2.6	PA1705	<i>pcrG</i> ; regulator in type III secretion
2.3		<i>toxA</i> ; Exotoxin A (Type II secreted)
2.3		<i>toxR</i> ; Exotoxin A regulation
2.1	PA1724	<i>pscK</i> ; type III export protein
1.3	PA1713	<i>exsA</i> ; transcriptional regulator

It is expected that if PscO is required for type III secretion, then a PAO1 Δ *pscO* strain will be deficient for type III secretion while the complemented strain will have a functional type III secretion system. Strains containing *pscO* with a mutated Tat secretion signal may or may not be deficient in type III secretion depending on whether mutations in the Tat secretion signal sequence redirect PscO. If by knocking out or mutating *pscO*, the type III secretion system is impaired, this indicates that PscO plays a significant role in the structure and/or synthesis of type III secretion apparatus. If these strains do not have an impaired type III secretion system, then this indicates that the effects of Tat secretion on type III secretion are indeed pleiotropic. It will also be worthwhile to determine if by modifying the signal sequence PscO, there will be a global response in transcription similar to the response seen in PAO1 Δ *tatC*. As part of these studies, it will of interest to localize PscO in PAO1, PAO1 Δ *tatC*, and strains with the mutations in the motif encoding the twin-arginine motif of

PscO. To address the localization of PscO, antibodies will be generated to use in localizing PscO and establish whether it is indeed a Tat-secreted substrate. The *pscO* gene will be cloned or amplified from *P. aeruginosa* PAO1 and ligated into an expression vector. The inventors' laboratory has over-expressed numerous proteins using both the pET vector which carries an N-terminal His•Tag® sequence (polyHis) followed by a thrombin site and three cloning sites and the pGEX vector which generates a fusion between the gene of interest and *Schistosoma japonicum* glutathione S-transferase (GST). Fusions made with either an N- or C-terminal moiety can then be purified using the appropriate matrix-containing column. After purification, PscO will be used to generate both monoclonal and polyclonal antibodies. Localization of PscO will be accomplished by analyzing the different cell fractionations. It is predicted that in *PAO1ΔtatC*, PscO will localize in the cytoplasm, however, the inventors did find that PlcH is found in the inner membrane when its Tat secretion signal is mutated. PscO and PscO mutants will then be localized with a mutated Tat signal to determine if the signal sequence is indeed a bonafide Tat secretion signal. These experiments will provide strong evidence as to if type III secretion depends on the Tat system. It is anticipated that mislocalization of PscO will result in phenotypes similar to that seen in *PAO1ΔtatC* and that mutation/deletion of PscO will lead to a down regulation of the type III secretion apparatus. Furthermore, it is expected that a mutation in the signal sequence of PscO would result in the mislocalization of PscO leading to a down-regulation in transcription of type III secretion genes analogous to the down-regulation observed in *PAO1ΔtatC*. This predicted down-regulation can be evaluated using the GeneChip® technology. In summary, through the analysis of global transcription of *PAO1ΔtatC* compare to PAO1, the inventors found that Tat secretion induces the down regulation of many type III secretion genes. The inventors found only one of the affected proteins to contain a putative Tat signal. Without being bound by theory, the hypothesis is that PscO requires the Tat system for secretion through the inner membrane, and that mislocation of PscO is responsible for significant effects that the present inventors have observed on the transcription of type III secretion genes. This suggests that PscO may have a feedback role in type III secretion. The concept that PscO is essential for the transcription of type III secretion genes has implications for using PscO as an antimicrobial target and/or to generate additional attenuated bacteria for use in any of the immunization strategies as described for the TAT mutants described herein.

Example 5

The following example demonstrates the construction of Tat mutants of *F. tularensis*, *Y. pestis*, *B. cereus* or *B. anthracis*.

Three genes (*tatABC*) encode proteins that comprise the Tat secretory apparatus. In most species thus far investigated these genes are part of an operon, although not invariably (See Fig. 2). The *tatC* gene is usually the third gene in the operon and would therefore be a better target for mutagenesis since deletion or interruption of *tatC* would not have a polar effect on the expression of *tatA* or *tatB*. Also, because the inventors have already shown that *tatC* deletion mutants of *P. aeruginosa* are defective in secreting a large array of Tat substrates, it is preferred to first construct *tatC* mutants of *F. tularensis*. Moreover, in a tBLASTn search for Tat homologs in *F. tularensis*, the most significant similarity for Tat apparatus proteins was TatC (see Fig. 2).

The entire *tatC* gene will be cloned either by PCR or standard cloning methods. The entire gene will be amplified using PCR and primers based on the *F. tularensis* genomic sequence that encodes a protein homologous to TatC. A polymerase with a reduced error rate will be used or alternatively, a fragment of the *tatC* gene will be amplified and used as a probe to identify genomic fragments that will be ligated into plasmid vectors. The intact TatC gene will ultimately be used for complementation studies once a *tatC* mutant is produced. In order to construct this mutant, sequences flanking *tatC* will be individually cloned so that we can construct mutants that have the entire *tatC* gene deleted. The individual flanking sequences will be combined with an antibiotic resistance cassette (i.e. Em^r), which replaces the *tatC* gene in a vector that cannot replicate in *F. tularensis* (Baron). This plasmid will be transformed or electroporated into *F. tularensis* and Em^r transformants will be selected as previously described (Baron, *ibid.*). These transformants will be screened by PCR and Southern hybridization methods for the double crossover (i.e. replacement of the *tatC* gene by the Em^r cassette) and loss of vector plasmid sequences. The present inventors have successfully used this approach to construct many deletion/replacement mutants of *P. aeruginosa* (Ochsner; Ochsner). In the unlikely event that a replacement mutant is not found among the Em^r transformants, this could suggest that the *tatC* gene may be essential under the conditions used for selection. For example, *tat* mutants of *P. aeruginosa* do not tolerate the relatively high salt concentrations that are found in standard Luria media, but they do tolerate this media if it does not contain NaCl (Ochsner, 2002, PNAS). Consequently a variety of selection media will be used to screen for allelic replacement mutants. Also, different plasmids may behave differently with regard to cointegrate resolution. To overcome such problems, it may be necessary to provide a selective pressure to assist in the isolation of an allelic replacement mutant. The use of the Sac selection system that is now commonly used to select for allelic replacement mutants in

a wide variety of organisms including *M. tuberculosis* (Raynaud) and *Y. pestis* (Bobrov) can be used. Finally, if difficulties are encountered in the construction of a *tatC* allelic replacement mutant by the above methods, it will be determined whether it is possible to mutate the *tatC* gene. This will be done by inserting an internal fragment of the *tatC* gene from *F. tularensis* into a plasmid that cannot replicate in this organism (Baron). This plasmid will be transformed into *F. tularensis* and transformants will be selected based on the resistance genes on the plasmid. In this case, any transformants will be *tatC* insertion mutants. If such mutants are isolated (i.e. Em^r transformants), these data will indicate that *tacC* is **not** an essential gene in *F. tularensis* and that it is simply a matter of finding the right conditions or vectors to use to construct an allelic replacement mutant for *tatC*. If in the unlikely event that deletion of the *tatC* is lethal, deletion mutations in the *tatA* or *tatB* genes will be produced using the same approaches. Similar approaches will be used in the construction of *tat* mutants with *Y. pestis* and with *B. cereus*.

Similar, but more effective and developed protocols will be used to construct Tat mutant of *Y. pestis* or *B. anthracis*. The genetic systems are considerably more tractable for these organisms and the Sac selection system has been used for the isolation of unmarked deletion mutants in *Y. pestis* (Bobrov). Because the TAT secretory system is an auxillary secretory system that translocates only a subset of all proteins that are exported, including ones that are involved in virulence, it is not likely that TAT is essential in all of these organisms. Consequently, Tat mutants of these bacteria will be readily constructed for use in safe and effective vaccines.

Tat mutants of these organisms will first be constructed in attenuated strains. This will not compromise the ability to evaluate these mutants in experimental models. For example, the LVS (live vaccine) strain of *F. tularensis* is attenuated when it is injected in mice via the intradermal route, but it is fully virulent when delivered via the intraperitoneal route (Ellis). Consequently, a Tat mutant will first be constructed in the LVS strain and then its virulence will be examined in comparison to the wild type parent in mice infected via the intraperitoneal route. Once it has been determined that the Tat mutant of LVS is more attenuated than its parental counterpart, construction of a Tat mutant in the fully virulent Schu 4 strain will be initiated. Alternatively, a Tat mutant of *Francisella novicida* will be constructed. This species is very closely related to *F. tularensis* and it is highly virulent in mice, but it does not infect humans, making it a useful experimental model for the mutant construction system. Also it is more easily manipulated genetically and it has less fastidious growth requirements than *F. tularensis* (Baron). Again, once it has been demonstrated that a

~~Tat mutant of *F. novicida* is less~~ virulent than its parental wild type, a Tat mutant of *F. tularensis* Schu 4 will be constructed with the goal of constructing a live vaccine strain that can be used in humans. Regarding this issue for *Y. pestis*, the EV76 live vaccine strain (a pigmentation mutant) will first be used, which is still virulent in mice (Titball). Once the attenuation of *Y. pestis* by introduction of deletion mutations in *tat* genes has been demonstrated, Tat mutants of fully virulent *Y. pestis* strains will be constructed and tested those in experimental models for their safety and efficacy as live vaccines. Also to be evaluated is whether the Tat mutants show some of the phenotypic variation (i.e. blue colony form) that has been noted in certain instances with the LVS strain and it will be determined whether growth conditions of the mutants affect their attenuation in mice as has been observed for the LVS strain (Cherwonogrodzky). Finally selection of antimicrobial resistant genes introduced into *F. tularensis* and *Y. pestis* will be controlled to obviate any issues relating the construction of stains that are resistant to antimicrobial agents used in treatment of tularemia or plague (Navas). For example, cassettes encoding resistance to erythromycin for *F. tularensis* and kanamycin resistance cassettes for *Y. pestis* will be used when allelic replacement mutants are constructed for these organisms because erythromycin and kanamycin are not typically used to treat tularemia and plague, respectively.

Example 6

The following example describes *in vitro* characterization of Tat mutants.

Although the construction of either an insertion or deletion Tat mutant of various bacterial strains as described in the above Examples will be confirmed through comparison of the mutants and wild type parental strains by PCR or Southern blot analysis of their genomic DNA, it will be useful to have a clear picture of how mutation of the *tat* gene(s) affects the overall phenotypic characteristics of these pathogens. For example, the present inventors have found that TatC mutants of *P. aeruginosa* are affected in: their ability to scavenge iron (loss of siderophore biosynthesis because the siderophore receptor is a Tat substrate), their fimbriae-mediated twitching motility, their swimming motility, their ability to form biofilms, and their susceptibility to aldehydes, which are found in natural substances (skin of apples), and to certain divalent metals (i.e. copper) (Ochsner, 2002, PNAS). While it is not necessarily expected that Tat mutants of *F. tularensis* and *Y. pestis* will have the same phenotypic characteristics as *P. aeruginosa* Tat mutants, the present inventors have found that many of the proteins that are secreted by the Tat apparatus (i.e. Tat substrates) are highly conserved in many different species (Yen). Consequently, Tat mutants of the various organisms will be evaluated for their susceptibility to the agents mentioned above, for their

motility, for their ability to grow on various carbon sources (Note: *P. aeruginosa* Tat mutants are unable to use 2-keto-gluconate, but not other carbohydrates, as a sole carbon source) and for their ability to secrete outer membrane and extracellular proteins (e.g. AcpA) that are associated with virulence. These studies will be done using well-established techniques, and the information garnered will be potentially valuable if these Tat mutants are eventually to be considered as live vaccines for humans. One of the problems with current live vaccines is that virtually nothing is known about how they are attenuated. In contrast, the present inventors will precisely know how the Tat mutant vaccines are attenuated.

As part of the *in vitro* analysis of *F. tularensis* and *Y. pestis*, the ability of the Tat mutants to survive in phagocytic cells (i.e. macrophage) (Baron; Fortier; Fortier; Polsinelli) will be evaluated. In contrast to *P. aeruginosa*, which is an extracellular pathogen, *F. tularensis* and *Y. pestis* are facultative intracellular bacteria, which have both intracellular and extracellular growth phases in infected hosts. Accordingly, the ability of the Tat mutants of *F. tularensis* and *Y. pestis* to enter and multiply in macrophages will be evaluated. The results of these experiments will contribute to the evaluation of the immune response generated to the live Tat vaccines and to *F. tularensis* and *Y. pestis*. For example, if Tat mutants are unable to survive in macrophages, these vaccines could still stimulate a B-cell mediated response that would be protective against extracellular organisms that the host will initially encounter, or against extracellular bacteria during the blood borne phase of infection. Alternatively, if the Tat mutants are able to survive in macrophage without killing them, they would be more likely to induce a T-cell response that could be protective against intracellular organisms in an infected host that would be sequestered from a protective antibody response. In any case, these initial studies will be conducted using previously described murine macrophage cell lines (e.g. J774A.1) and methods (Baron; Fortier; Fortier; Polsinelli). The uptake of the mutant and parental wild type strains and the intracellular growth of these strains as well as their ability to kill these macrophage cell lines will be examined. It is expected that Tat mutants will be attenuated in their ability to survive in macrophage. As observed with Tat mutants of *P. aeruginosa*, it is expected that Tat mutants of *F. tularensis* will be able to survive in macrophages for a period of time, but will not be cytotoxic to these cells.

The methods for evaluating intracellular growth and virulence of *F. tularensis* and *Y. pestis* have been well described (Baron; Fortier; Fortier; Polsinelli; Young). However, if no differences are observed between the growth and virulence of the Tat mutants for J774 macrophage the use of alternative cell lines will be explored. For example, the ability of the

~~Tat mutants to survive in murine~~ Tat mutants to survive in murine alveolar macrophages as described by Polsinelli et al. (Polsinelli) will be investigated. More specific parameters such as cytotoxicity and induction of apoptosis in macrophage (Lai) will also be examined. Alternatively, the growth and virulence of the Tat mutants of *F. tularensis* and *Y. pestis* will be tested in a human monocytic cell line that can be converted to a macrophage-like cell by treatment with phorbol esters as previously described (Miller). If no differences between the Tat mutant and the wild type strain in macrophage are observed, the ability of these strains to induce a respiratory burst in neutrophils will be examined, since it was previously reported that the AcpA of *F. tularensis* inhibits the respiratory burst in these cells (Reilly). In this regard, a potential pitfall the inventors will avoid is the construction of Tat mutants that may produce a partially functional TatC protein. Recently, Baron et al. reported that an AcpA mutant of *F. novicida* was not attenuated for growth in macrophage (Baron). However, the mutation that these investigators constructed led to the production of a truncated protein that was nearly as large as the wild type AcpA. Because the present inventors have found that the enzymatically active site is in the first half of proteins that are homologous to PlcH, it is very likely that their mutant still produces a fully active enzyme (Stonehouse). Consequently all of the mutants produced in the present invention will have at least the entire TatC gene deleted.

Example 7

The following example demonstrates the safety of Tat mutants as live vaccines in murine models of tularemia and plague.

Most studies that examine the virulence of wild type strains, live vaccines and mutants of *F. tularensis* and *Y. pestis* have employed mice as a model host for tularemia and plague (Ellis; Welkos). These mouse models for tularemia and plague have been well described. Accordingly this model will be used to evaluate the present inventors' hypothesis that Tat mutants of *F. tularensis* and *Y. pestis* can be used as safe live vaccines against tularemia and plague. For *F. tularensis* infections, C57BL/Cr1BR or C57BL/10N are typically used. There are several major routes of challenge: aerosol, oral, subcutaneous (SC) and intraperitoneal (IP). Eventually the Tat mutants will be compared to the wild type parental strain by all of these routes. Of particular interest is challenge by the aerosol route. If Tat mutants derived from LVS or the EV76 strains are used, infection will be performed via the intraperitoneal route since these live vaccine strains are most virulent via this route (Ellis; Welkos). However, for mutants derived from fully virulent *F. tularensis* (e.g. Schu 4) and *Y. pestis* (e.g. KIM) strains, mice will be challenged by all routes. Several parameters

will be evaluated including: (i) dosage; (ii) lethality; and (iii) survival of wild type and mutant in the blood, lungs and spleens of infected animals. These methods are well described in many publications. If it is determined that the Tat mutants can still survive in mice, despite evidence of reduced lethality or histopathology, histochemical and immunological localization studies of the Tat mutants will be performed in infected animals to determine whether the mutants are located in a different tissue or cellular compartment of the infected animals than the wild type parental strains. Also, competition studies can be performed between the Tat mutants and the parental wild type strains to evaluate whether the mutant can survive in the host in the presence of the wild type parental strain (Gort; Wilderman; Wilderman). Such studies have implications regarding the survival of the Tat mutant vaccines in immunized hosts. Finally, the cytokine profiles (e.g. INF- γ , TNF- α Il-1, Il-8, etc.) of tissues (e.g. lungs) from mice infected with the Tat mutants and the wild type parental strains (Kovarova) will be compared. Following these studies, assuming that as expected, the Tat mutants of *F. tularensis* and *Y. pestis* are significantly attenuated and are still able to survive in these model hosts, the efficacy studies outlined below will be performed.

To obtain highly statistically significant data demonstrating the safety of our Tat live vaccine, side by side comparisons of our Tat vaccines will be performed with the extant live vaccines in order to demonstrate whether the Tat vaccines are safer and have fewer side effects than the currently available vaccines. For such studies the experiments will be performed at least five times with large groups of animals (50-100) in each category to ensure that the Tat mutants of the present invention are safe live vaccines. Data will be analyzed by appropriate statistical methods (e.g. Mann-Whitney test (Nelson)).

Example 7

The following example demonstrates efficacy of Tat mutants as live vaccines in murine models of tularemia and plague.

Once it has been established that Tat mutants of *F. tularensis* and *Y. pestis* are less virulent in the infection models described above (see Example 3), an evaluation will be performed to determine whether the Tat mutants will induce an immune response that will protect the immunized mice against experimental tularemia and plague. Immunization protocols will be evaluated using various modes of challenge, including subcutaneous challenge, intraperitoneal challenge and most importantly aerosol challenge. An assortment of vaccination regimens and challenge doses will be used and the Tat mutant performance will be evaluated in comparison to the extant vaccines, LCV and EV76. For example, it will

be determined whether vaccination by different routes (e.g. IP, SC, oral aerosol) will provide the best protective response. It will also be determined whether a single boost will provide protection or whether multiple re-inoculations will be required to provide a significant protective response. The Tat vaccine strain will be evaluated for protection against different virulent isolates of these organisms including ones of different serotypes, and longevity of the protective response will be evaluated from several months to 2 years. A protective response will be evaluated as follows. Animals (~20-25) will be vaccinated with the Tat vaccine strain along with the appropriate extant live vaccine (e.g. LCV or EV76). Unvaccinated animals will be used as controls and in some cases, animals immunized with an unrelated vaccine (e.g. Tat mutants of *P. aeruginosa* or *E. coli*) will be used as controls. All groups will be challenged with the appropriate virulent parental strain and eventually other virulent strains of the same organism. The vaccinated and unvaccinated controls will be examined for survival, spleen counts and many of the same parameters described above that were used to evaluate the safety of the Tat vaccines. It is expected that the Tat vaccines will provide a better or comparable protective response than the extant live vaccine strains.

To boost the protective response, the use of adjuvants will also be explored. One approach for the induction of mucosal antibody responses to a protein antigen has been to administer the target antigen in conjunction with cholera toxin (CT) or the heat-labile toxin from *Escherichia coli* (LT). Both molecules have remarkable adjuvant properties that can facilitate the induction of mucosal immune responses to antigens, which normally are not highly immunogenic. There are various strategies that will be explored for the use of CT or LT as adjuvants, particularly in the event that a weak protective immune response is achieved with the Tat vaccines alone. The adjuvants can be administered at the same time as the live vaccine or CT or LT or chimeric variants of these adjuvants can be expressed in the live Tat vaccines using techniques previously described (Arrington; Sultan). For example, a domain of AcpA associated with the CT B-subunit can be expressed in the Tat vaccines, which is expected to provide an enhanced response against this virulence determinant.

Example 8

The following example describes the expression of a foreign antigen in a bacterial strain of the invention for use in a vaccine.

Because the Sec secretory system is still intact in a TAT mutant it will be possible to still secrete proteins from TAT mutants to the outer membrane or extracellularly. For example it might be possible to secrete mutant forms of the Protective Antigen (PA) of *Bacillus anthracis* in a TAT mutant via the intact Sec system so that the mutant PA would

induce a protective immunological response in a vaccinee carrying this strain. This strategy offers some important advantages over the use of PA toxoids that would be directly administered to persons for vaccination against anthrax. That is, the mutant PA would be expressed in the vaccinees for an extended period of time so that a more prolonged protective response would be realized. The gene encoding candidate mutant PA proteins would be moved into either a TAT mutant in an avirulent *B. anthracis* strain or a closely related strain (e.g. *Bacillus cereus*). These strains which would express the mutant PA would provide long term protection against anthrax. The gene encoding the mutant PA could either carry its own Sec-dependent signal sequence or it could be preceded by an alternative Sec-dependent signal sequence that would allow more efficient secretion of the mutant PA. The gene encoding the mutant PA can either be located on a plasmid in the above strains or they may be integrated into their chromosomes to increase their stability.

Each reference cited and described herein is incorporated herein by reference in its entirety.

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While various embodiments of the present invention have been described in detail, it is apparent that modifications and adaptations of those embodiments will occur to those skilled in the art. It is to be expressly understood, however, that such modifications and adaptations are within the scope of the present invention, as set forth in the following claims:

What is claimed is:

1. A method to identify a compound that inhibits the TAT secretory system, comprising:

- a) contacting a microorganism that has a TAT secretory system with a candidate compound;
- b) measuring a level of secretion of a protein that is secreted via the TAT secretory pathway; and
- c) measuring a level of expression of a gene that has increased expression when the TAT secretory pathway is inhibited;

wherein a decrease in the level of secretion of the protein in (b) in the presence of the candidate compound as compared to in the absence of the candidate compound, and an increase in the expression of the gene in (c) in the presence of the candidate compound as compared to in the absence of the candidate compound, identifies the compound as a compound that inhibits the TAT secretory system.

2. The method of Claim 1, wherein the candidate compounds are putative antimicrobial compounds, and wherein a compound identified by the method is identified as an antimicrobial compound.

3. The method of Claim 1, wherein the microorganism is a bacteria from a family selected from the group consisting of: Enterobacteriaceae, Micrococcaceae, Vibrionaceae, Pasteurellaceae, Mycoplasmataceae, and Rickettsiaceae.

4. The method of Claim 1, wherein the microorganism is selected from the group consisting of: *Pseudomonas aeruginosa*, *Pseudomonas mallei*, *Pseudomonas pseudomallei*, *Bordetella pertussis*, *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Francisella tularensis*, *Vibrio cholerae*, *Bacillus anthracis*, *Salmonella enteric*, *Yersinia pestis*, *Escherichia coli* and *Bordetella bronchiseptica*.

5. The method of Claim 1, wherein the protein of (b) is an enzyme, and wherein the level of secretion of the protein is measured by detecting the amount of a substrate that is converted to a detectable product by the enzyme.

6. The method of Claim 1, wherein the level of secretion of the protein of (b) is measured by measuring the amount of a detectable label that directly or indirectly binds to the protein.

7. The method of Claim 1, wherein the level of secretion of the protein of (b) is detected using a method selected from the group consisting of: enzyme activity, Western blot, immunoblot, enzyme-linked immunosorbant assay (ELISA), radioimmunoassay (RIA), immunoprecipitation, surface plasmon resonance, chemiluminescence, fluorescent polarization, phosphorescence, immunohistochemical analysis, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, microcytometry, microarray, microscopy, fluorescence activated cell sorting (FACS), flow cytometry, DNA binding, ligand binding, and interaction with protein binding partners.

8. The method of Claim 1, wherein step (b) is performed using a colorimetric enzyme assay.

9. The method of Claim 1, wherein the protein of (b) is a protein selected from the group consisting of: phospholipase C (*plcH*), phospholipase C (*plcN*), ferripyoverdine receptor (*fpvA*), pyoverdine biosynthesis protein (PA2394), pyoverdine biosynthesis protein (P2389), pyoverdine biosynthesis protein (PA2392), nitrate reductase (*napA*), ferredoxin (*napF*), nitrous oxide reductase (*nosZ*), formate dehydrogenase (*fdnG*), dehydrogenase (PA2124), dehydrogenase (PA2264), aldehyde oxidase (PA4621), aldehyde oxidase (PA1601), aldehyde oxidase (PA1880), aldehyde oxidase (PA2378), multicopper oxidase (*copA*), and protein PA0144.

10. The method of Claim 1, wherein the protein of (b) is one or both of phospholipase C or phospholipase H.

11. The method of Claim 10, wherein the level of secretion of phospholipase C or phospholipase H is detected by contacting supernatant from a culture comprising the microorganism with a labeled substrate for phospholipase C or phospholipase H and detecting a level of product converted from the substrate by phospholipase C or phospholipase H.

12. The method of Claim 11, wherein the step of detecting is a colorimetric assay.

13. The method of Claim 1, wherein the level of expression of the gene in (c) is measured by detecting the expression of a protein encoded by the gene.

14. The method of Claim 1, wherein the level of expression of the gene in (c) is measured by detecting the transcription of the gene.

15. The method of Claim 1, wherein the level of expression of the gene in (c) is measured using a method selected from the group consisting of: detection of a reporter gene, detection of antibiotic resistance, polymerase chain reaction (PCR), reverse transcriptase-PCR (RT-PCR), *in situ* PCR, quantitative PCR (q-PCR), *in situ* hybridization, Southern blot, Northern blot, sequence analysis, and microarray analysis.

16. The method of Claim 1, wherein the level of expression of the gene in (c) is measured using detection of a reporter gene.

17. The method of Claim 16, wherein the reporter gene is detected using a fluorescent label or a colorimetric label.

18. The method of Claim 1, wherein the gene detected in (c) is selected from the group consisting of: PA2808, PA522, PA4878, *mexA*, and *pchABCDEFGH*.

19. The method of Claim 1, wherein steps (a)-(c) are conducted within the same assay sample.

20. The method of Claim 1, wherein steps (a)-(c) are conducted within the same well of a microtiter plate.

21. The method of Claim 1, wherein the method is formatted as a high throughput assay.

22. The method of Claim 1, further comprising a step of detecting the level of expression of a gene that has decreased expression when the TAT secretory pathway is inhibited.

23. The method of Claim 1, further comprising a step of detecting the level of expression of a reporter gene fused to the promoter of a gene that has decreased expression when the TAT secretory pathway is inhibited.

24. The method of Claim 23, wherein the reporter gene is a gene that confers resistance to an antibiotic onto the microorganism.

25. An assay kit for the identification of candidate compounds that inhibit the TAT secretory system, comprising:

a) a reagent for detecting the level of a protein that is secreted by the TAT secretory system; and

b) a reagent for detecting the level of expression of a gene that is increased when the TAT secretory system is inhibited.

26. The assay kit of Claim 25, wherein the protein of (a) is an enzyme and wherein the reagent of (a) is a labeled substrate for the enzyme.

27. The assay kit of Claim 25, wherein the reagent of (a) is a detectable agent that binds to the protein.

28. The assay kit of Claim 25, wherein the protein of (a) is selected from the group consisting of: phospholipase C (*plcH*), phospholipase C (*plcN*), ferripyoverdine receptor (*fpvA*), pyoverdine biosynthesis protein (PA2394), pyoverdine biosynthesis protein (P2389), pyoverdine biosynthesis protein (PA2392), nitrate reductase (*napA*), ferredoxin (*napF*), nitrous oxide reductase (*nosZ*), formate dehydrogenase (*fdnG*), dehydrogenase (PA2124), dehydrogenase (PA2264), aldehyde oxidase (PA4621), aldehyde oxidase (PA1601), aldehyde oxidase (PA1880), aldehyde oxidase (PA2378), multicopper oxidase (*copA*), and protein PA0144.

29. The assay kit of Claim 25, wherein the protein of (a) is a phospholipase C.

30. The assay kit of Claim 25, wherein the reagent of (a) is a substrate for phospholipase C.

31. The assay kit of Claim 25, wherein the reagent of (a) is used in a colorimetric assay.

32. The assay kit of Claim 25, wherein the gene in (b) is fused to a reporter gene, and wherein the reagent of (b) is a reagent or reagents useful for detecting the expression of the reporter gene.

33. The assay kit of Claim 25, wherein the reagent of (b) comprises an oligonucleotide probe or primer that hybridizes to the gene or a transcript thereof under stringent hybridization conditions.

34. The assay kit of Claim 25, wherein the reagent of (b) is used in a fluorescent or colorimetric assay.

35. The assay kit of Claim 25, further comprising microbial cells containing a TAT secretory system.

36. A therapeutic composition, comprising:

a) an attenuated bacterium comprising a genetic modification that reduces or abolishes the expression or biological activity of the twin arginine translocase (TAT) secretory system in the bacterium; and,

b) at least one therapeutic agent.

37. The therapeutic composition of Claim 36, wherein at least one gene in the *tat* operon is not expressed by the bacterium.

38. The therapeutic composition of Claim 36, wherein RNA from at least one gene in the *tat* operon is not transcribed by the bacterium.

39. The therapeutic composition of Claim 36, wherein RNA transcribed from a genetically modified *tat* gene is not translated by the bacterium.

40. The therapeutic composition of Claim 36, wherein the bacterium comprises a mutation in at least one gene in the *tat* operon that reduces or prevents the expression of at least one component of the twin arginine translocase (TAT) secretory system in the bacterium or that reduces or abolishes the biological activity of the TAT secretory system in the bacterium.

41. The therapeutic composition of Claim 40, wherein the bacterium comprises at least one mutation in the coding sequence of at least one gene in the *tat* operon.

42. The therapeutic composition of Claim 40, wherein the bacterium comprises at least one mutation in a regulatory region of at least one gene in the *tat* operon.

43. The therapeutic composition of Claim 40, wherein the mutation is a partial or complete deletion of at least one gene in the *tat* operon.

44. The therapeutic composition of Claim 40, wherein the mutation is an insertion of a heterologous nucleic acid sequence into at least one gene in the *tat* operon sufficient to prevent expression or biological activity of at least one component of the TAT secretory system.

45. The therapeutic composition of Claim 40, wherein the mutated *tat* gene encodes a mutated TAT secretory system component that results in a TAT secretory system with substantially no biological activity.

46. The therapeutic composition of Claim 40, wherein the mutation prevents the expression or abolishes the biological activity of the TAT pore apparatus in the bacterium.

47. The therapeutic composition of Claim 40, wherein the *tat* gene is selected from the group consisting of *tatA*, *tatB* and *tatC*.

48. The therapeutic composition of Claim 36, wherein the bacterium lacks the ability to secrete proteins across the cytoplasmic membrane that have a TAT secretion signal sequence.

49. The therapeutic composition of Claim 36, wherein the bacterium is from a family selected from the group consisting of: Enterobacteriaceae, Micrococcaceae, Vibrionaceae, Pasteurellaceae, Mycoplasmataceae, and Rickettsiaceae.

50. The therapeutic composition of Claim 36, wherein the bacterium is a *Pseudomonas*.

51. The therapeutic composition of Claim 36, wherein the bacterium is selected from the group consisting of: *Pseudomonas aeruginosa*, *Pseudomonas mallei*, *Pseudomonas pseudomallei*, *Bordetella pertussis*, *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Francisella tularensis*, *Vibrio cholerae*, *Bacillus anthracis*, *Salmonella enteric*, *Yersinia pestis*, *Escherichia coli* 0157:H7 and *Bordetella bronchiseptica*.

52. The therapeutic composition of Claim 36, wherein the therapeutic agent is a heterologous antigen.

53. The therapeutic composition of Claim 52, wherein the heterologous antigen is a peptide or a protein.

54. The therapeutic composition of Claim 52, wherein the attenuated microorganism has been transfected with a recombinant nucleic acid molecule encoding the heterologous antigen.

55. The therapeutic composition of Claim 52, wherein the antigen is selected from the group consisting of viral antigens, mammalian cell surface molecules, bacterial antigens, fungal antigens, protozoan antigens, helminth antigens, ectoparasite antigens, and cancer antigens.

56. The therapeutic composition of Claim 52, wherein the composition comprises multiple antigens.

57. The therapeutic composition of Claim 36, wherein the therapeutic agent is a biological response modifier selected from the group consisting of a cytokine, a chemokine, a hormone, a lipidic derivative, an enzyme, a small molecule drug that elicits a biological response, and a protein or peptide that regulates gene expression or cellular activity in a recipient cell.

58. The therapeutic composition of Claim 36, wherein the therapeutic agent is a heterologous recombinant nucleic acid molecule that can be transferred into a recipient cell by the attenuated bacterium.

59. The therapeutic composition of Claim 58, wherein the recombinant nucleic acid molecule encodes an antigen.

60. A method for stimulating an immune response against a bacterial protein, comprising administering to an animal an attenuated bacterium comprising a genetic modification that reduces or abolishes the expression or biological activity of the twin arginine translocase (TAT) secretory system in the bacterium.

61. The method of Claim 60, wherein at least one gene in the *tat* operon is not expressed by the bacterium.

62. The method of Claim 60, wherein RNA from at least one gene in the *tat* operon is not transcribed by the bacterium.

63. The method of Claim 60, wherein RNA transcribed from a genetically modified *tat* gene is not translated by the bacterium.

64. The method of Claim 60, wherein the bacterium comprises a mutation in at least one gene in the *tat* operon that reduces or prevents the expression of at least one component of the twin arginine translocase (TAT) secretory system in the bacterium or that reduces or abolishes the biological activity of the TAT secretory system in the bacterium.

65. The method of Claim 64, wherein the bacterium comprises at least one mutation in the coding sequence of at least one gene in the *tat* operon.

66. The method of Claim 64, wherein the bacterium comprises at least one mutation in a regulatory region of at least one gene in the *tat* operon.

67. The method of Claim 64, wherein the mutation is a partial or complete deletion of at least one gene in the *tat* operon.

68. The method of Claim 64, wherein the mutation is an insertion of a heterologous nucleic acid sequence into at least one gene in the *tat* operon sufficient to prevent expression or biological activity of at least one component of the TAT secretory system.

69. The method of Claim 64, wherein the mutated *tat* gene encodes a mutated TAT secretory system component that results in a TAT secretory system with substantially no biological activity.

70. The method of Claim 64, wherein the mutation prevents the expression or abolishes the biological activity of the TAT pore apparatus in the bacterium.

71. The method of Claim 64, wherein the *tat* gene is selected from the group consisting of *tatA*, *tatB*, and *tatC*.

72. The method of Claim 64, wherein the bacterium lacks the ability to secrete proteins across the cytoplasmic membrane that have a TAT secretion signal sequence.

73. The method of Claim 60, wherein the bacterium is from a family selected from the group consisting of: Enterobacteriaceae, Micrococcaceae, Vibrionaceae, Pasteurellaceae, Mycoplasmataceae, and Rickettsiaceae.

74. The method of Claim 60, wherein the bacterium is selected from the group consisting of: *Pseudomonas aeruginosa*, *Pseudomonas mallei*, *Pseudomonas pseudomallei*, *Bordetella pertussis*, *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Francisella tularensis*, *Vibrio cholerae*, *Bacillus anthracis*, *Salmonella enteric*, *Yersinia pestis*, *Escherichia coli* 0157:H7 and *Bordetella bronchiseptica*.

75. The method of Claim 60, wherein the attenuated bacterium is from the genus *Pseudomonas* and wherein the animal has or is at risk of developing chronic or acute pulmonary infection.

76. The method of Claim 60, wherein the attenuated bacterium is from the genus *Yersinia* and wherein the animal has or is at risk of developing plague.

77. The method of Claim 60, wherein the attenuated bacterium is from the genus *Bacillus* and wherein the animal has or is at risk of developing anthrax.

78. The method of Claim 60, wherein the attenuated bacterium is from the genus *Francisella* and wherein the animal has or is at risk of developing tularemia.

79. The method of Claim 60, wherein the attenuated bacterium is from the genus *Salmonella*, and wherein the animal has or is at risk of developing salmonellosis.

80. The method of Claim 60, wherein the attenuated bacterium is from the genus *Mycobacterium*, and wherein the animal has or is at risk of developing tuberculosis or leprosy.

81. The method of Claim 60, wherein the attenuated bacterium is an *Escherichia coli* 0157:H7.

82. The method of Claim 60, wherein the attenuated bacterium is *Bordetella bronchiseptica*.

83. A method for vaccinating an animal against a disease or condition, comprising administering to the animal a therapeutic composition according to any one of Claims 36-59.

84. The method of Claim 83, wherein said vaccine is administered with a pharmaceutically acceptable excipient.

85. An attenuated bacterium comprising a genetic modification that reduces or abolishes the expression or biological activity of the *pscO* gene.

86. The attenuated bacterium of Claim 85, wherein the *pscO* gene is not expressed by the bacterium.

87. The attenuated bacterium of Claim 85, wherein RNA from the *pscO* gene is not transcribed by the bacterium.

88. The attenuated bacterium of Claim 85, wherein RNA transcribed from a genetically modified *pscO* gene is not translated by the bacterium.

89. The attenuated bacterium of Claim 85, wherein the bacterium comprises a mutation in the *pscO* gene that reduces or prevents the expression of the PscO protein in the bacterium or that reduces or abolishes the biological activity of the PscO protein in the bacterium.

90. The attenuated bacterium of Claim 85, wherein the bacterium comprises at least one mutation in the coding sequence of the *pscO* gene.

91. The attenuated bacterium of Claim 85, wherein the bacterium comprises at least one mutation in a regulatory region of the *pscO* gene.

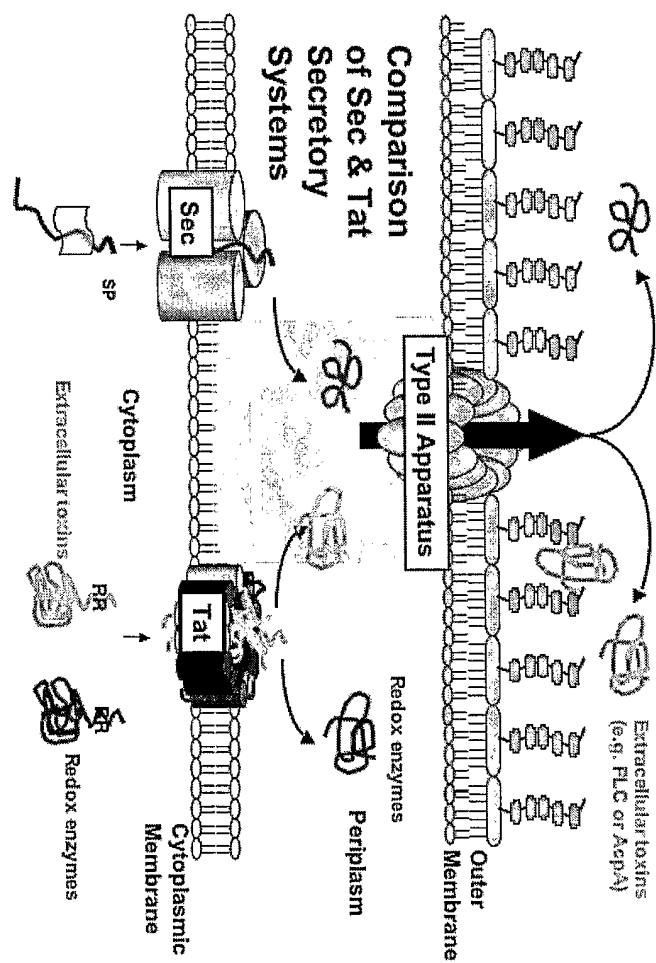


FIG. 1

FIG. 2

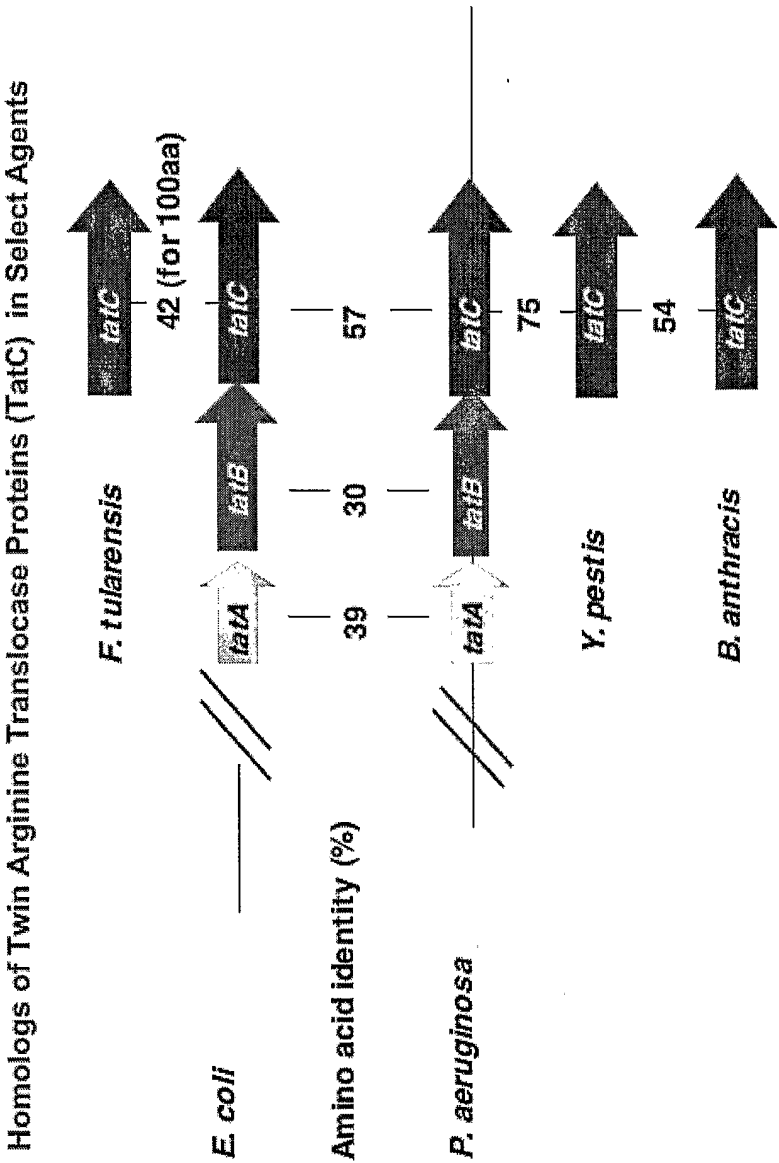
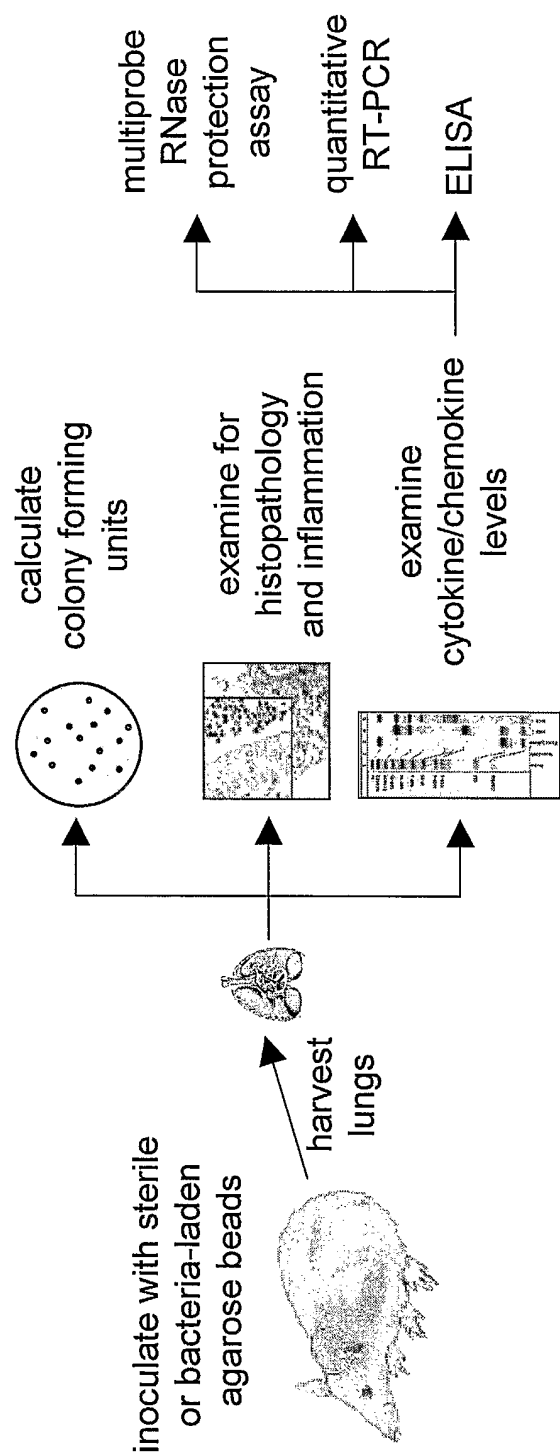


FIG. 3



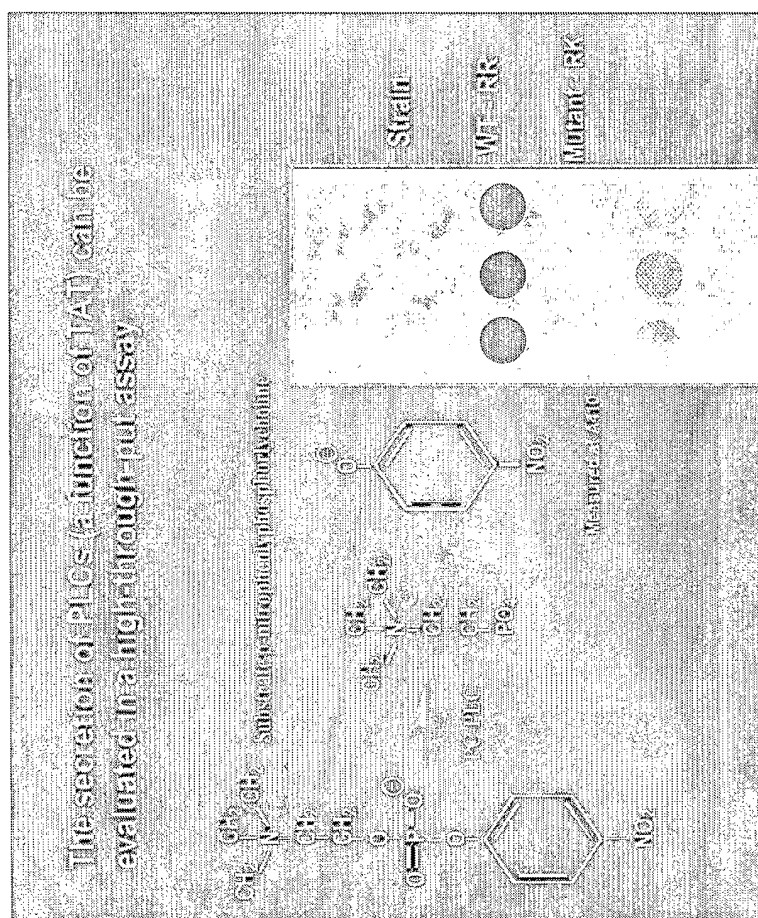


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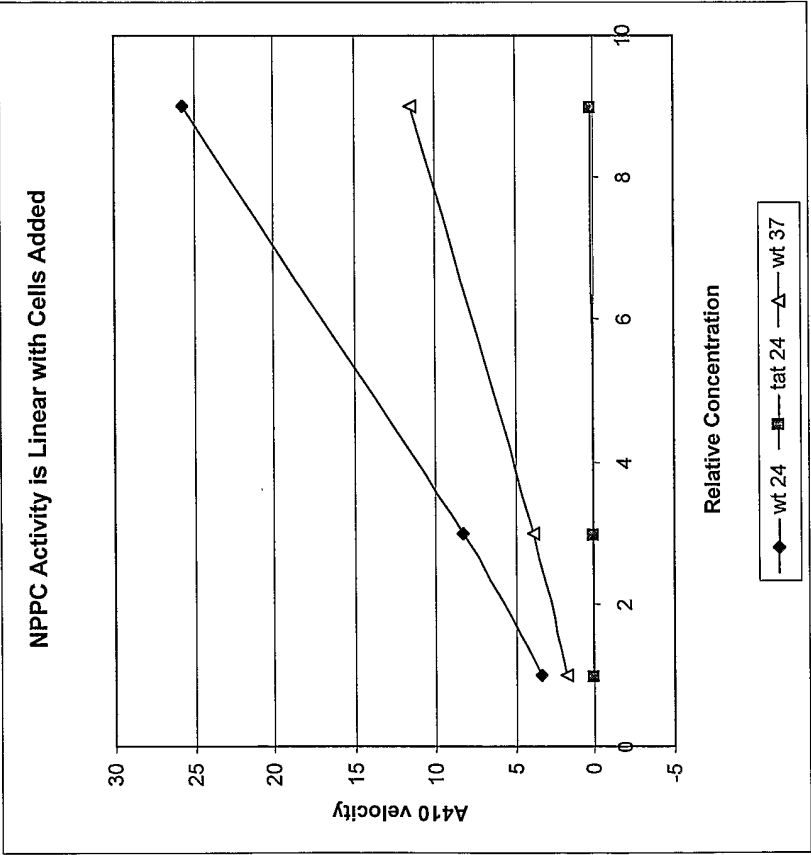


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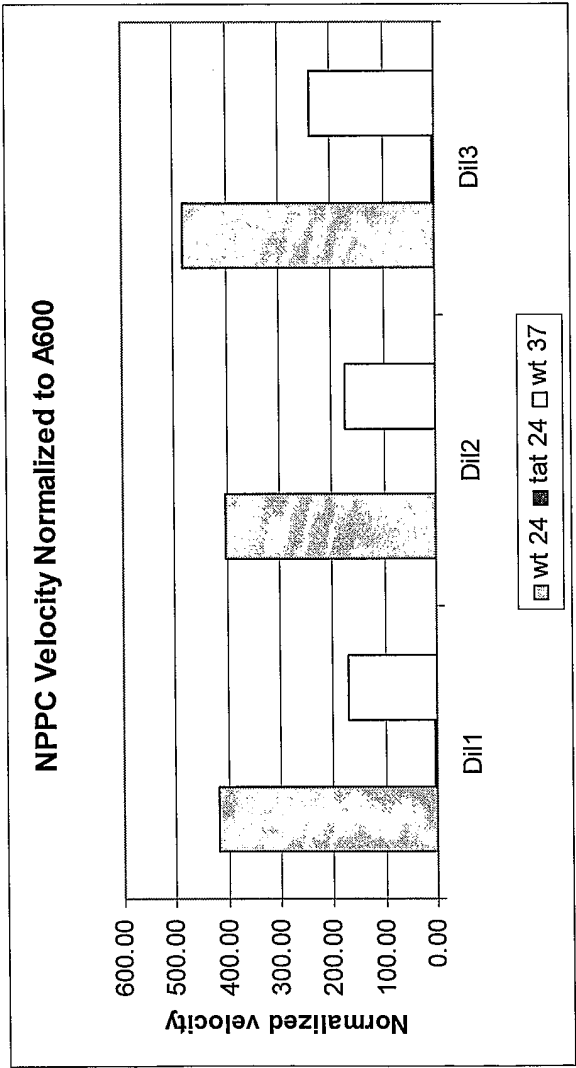


FIG. 6

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Vasil, Michael
Vasil, Adriana
Ochsner, Urs
Snyder, Aleksandra

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<222> (3)..(3)

<223> Xaa can be any naturally occurring amino acid

<400> 19

Arg Arg Xaa Phe Leu Lys
1 5