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PRINCIPAL INVESTIGATOR: Donald E. Woods, Ph.D.

CONTRACTING ORGANIZATION: University of Calgary
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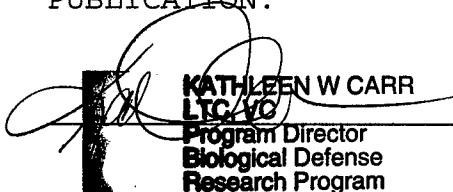
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FOREWORD

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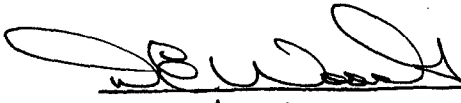
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INTRODUCTION

Burkholderia mallei is the causative agent of glanders which is primarily a disease of the horse, mule or donkey, although goats, sheep, cats and dogs sometimes naturally contract the disease. The mode of infection in animals remains controversial; considerations include inhalation, ingestion or inoculation through breaks in the skin. Glanders in humans has never been common, but it gains tremendous importance from the serious nature of the infection. Our knowledge of the pathogenesis of disease due to *B. mallei* is lacking. It is recognized from earlier studies that *B. mallei* is an organism with tremendous infectivity and poses a significant hazard to humans exposed to aerosols containing this organism. At present, no effective vaccines are available against this organism, and information on the treatment of this organism with antibiotic therapy is also not available.

The basic studies which we have proposed on the pathogenesis of disease due to *B. mallei* are acutely needed, and the information gained from these studies will provide a knowledge base which is required to rationally design new modes of therapy directed against this organism. The long-term objective of our research is to define at a molecular level the pathogenesis of disease due to *B. mallei* and to develop immunoprotective vaccines against these organisms for use in humans.

Since glanders is of military significance as a biological warfare agent, the development of an effective vaccine and treatments are of particular concern. Our understanding of the disease caused by *B. mallei* is minimal, and we must move forward with these studies in order to develop new and effective vaccines and/or therapies against this organism. There is considerable dual use potential, since this disease is important in various areas of the world. Development of vaccines and treatments can, therefore, provide important items to assist the World Health Organization and to assist signatories of the Biological and Toxin Weapons Convention under Article X of the Convention.

BODY

GENETIC STUDIES

Characterization of the *B. mallei* Genome.

Pulsed field examination of chromosomal DNA from the 10 *B. mallei* strains has provided us with the observation that a single replicon exists in this organism.

Search for Genes in *B. mallei* Homologous to Genes in *B. pseudomallei*.

Genomic DNA from *B. mallei* and *B. pseudomallei* isolates were used as templates to PCR-amplify the *fliC* (flagellin structural gene) genes from these strains. The **Flagella-right** (5'-TTATTGCAGGAGCTTCAGCAC-3') and **Flagella-left** (5'-ATGCTCGGAATCAACAGCAAC-3') oligodeoxyribonucleotide primers used in the PCR were designed based upon the previously published *B. pseudomallei* 1026b *fliC* sequence. The *fliC* loci were PCR-amplified in a 100 µl reaction mix containing 10 ng of chromosomal DNA, 1X PCR buffer II, 1.5 mM MgCl₂, 200 µM of each dNTP, 0.5 µM of each primer and 0.5 U of taq DNA Polymerase (Gibco Canada Inc.). The PCR mixture was transferred to a GeneAmp PCR System 9600 (Perkin Elmer Cetus) thermal cycler and subjected to the following conditions: 97C for 4 minutes; 30 cycles of 97C for 45 sec, 55C for 45 sec, 72C for 90 sec. The size and purity of the predicted 1.2 kb PCR product was assessed by electrophoresis of 20 µl of the reaction mixture through a 1.5% agarose gel followed by visualization of ethidium bromide-stained fragments with a UV light source.

PCR products were separately digested with the restriction enzymes *Pst* I and *Msp* I and subjected to agarose gel electrophoresis on a 2.5% gel. The resulting banding patterns were identical for all strains examined including the *B. mallei* isolate analyzed in these studies.

These are very exciting results as *B. mallei* has always been classified as non-motile; however, it is now clear that it contains the genetic information for the flagellin structural gene. We are probing *B. mallei* chromosomal DNA with *B. pseudomallei* flagellin synthesis genes (we have isolated over 30 of these) in an attempt to determine why the organism is non-motile.

Transposon Mutagenesis of *Burkholderia mallei*.

To determine the role of the putative virulence determinants such as cytotoxin, protease, motility and invasion capacity, in disease due to *B. mallei* we will perform studies to fulfill the molecular form of Koch's postulates. These postulates as defined by Falkow can be summarized as follows: (1) the virulence property should always be associated with pathogenic strains; (2) specific inactivation of the gene that specifies the virulence factor should cause a measurable loss in virulence; (3) reversion of the mutated gene or allelic replacement by recombination or by complementation should restore pathogenicity. A series of mutants each singly deficient in the

production of a specific virulence determinant will be obtained by transposon mutagenesis and compared to the parental strain in appropriate animal models of *B. mallei* infection.

We tested ten strains of *B. mallei* for susceptibility to transposon mutagenesis procedures. We determined that *B. mallei* strain GB6 could be mutagenized with a Tn5 derivative, Tn5-OT182. We have used this system to mutagenize *B. pseudomallei* with great success. Tn5-OT182 contains *bla* and *tetAR*, the genes encoding ApR and TcR, respectively. Most *B. mallei* isolates are inhibited by Tc at a concentration of 12.5 ug/ml; thus, the use of tetracycline at a concentration of 50 ug/ml efficiently selects for those cells that have obtained a copy of Tn5-OT182 in the chromosome. Tn5-OT182 is a self-cloning, promoter probe transposon. It contains a ColE1 origin of replication, allowing for the rapid cloning of flanking DNA from the desired mutant simply by digesting chromosomal DNA with the appropriate enzyme, ligating, and transforming into *E. coli* with selection for TcR and/or ApR. In addition, Tn5-OT182 contains a promoterless *lacZ* at one end, allowing for the formation of transcriptional fusions when the transposon inserts downstream of a promoter. This feature is useful for determining the orientation of a mutated gene as well as identifying physical and/or chemical stimuli that may affect expression of the mutated gene.

B. mallei Gb6 TcRSmR transconjugants were obtained at a frequency of 10^{-5} to 10^{-4} per donor cell. Ten TcRSmR transconjugants were chosen at random. Chromosomal DNA was isolated from these strains and digested with *EcoRI*, which recognizes a single site in Tn5-OT182. These chromosomal fragments were separated by electrophoresis, blotted to a nylon membrane, and probed with pOT182. All contained Tn5-OT182, whereas GB6 did not. In addition, Tn5-OT182 has inserted in a unique location in all of these, suggesting that Tn5-OT182 inserts randomly in the *B. mallei* chromosome. The Tn5-OT182 system should prove to be an extremely valuable tool in isolating isogenic mutants of *B. mallei* as well as in the isolation and characterization of genes containing a transposon insertion.

To confirm the association between a transposon insertion and a change in virulence phenotype, certain control experiments will be performed. The first three controls will ensure that the altered phenotype is not caused by a second mutation. (1) Southern hybridization analysis will be performed using transposon DNA as a probe, to ensure that there is only one copy of the transposon in the chromosome. (2) Linkage between the transposon and the mutant phenotype will be confirmed by transduction analysis. Selection for the drug resistance marker associated with the transposon in a genetic cross should result in 100% inheritance of the altered virulence phenotype. (3) We will ensure that reversion of the mutant phenotype occurs when the transposon is lost by precise excision or by allelic replacement with wild-type DNA. (4) Functional copies of cloned virulence determinant genes will be used for these two final controls as they become available. As insertion mutations can cause polar effects on the expression of downstream genes in operons, we will control for this by performing complementation tests with a recombinant plasmid expressing a functional copy of the gene that has been inactivated by the transposon in the chromosome. These complementation tests will be particularly important if the phenotype of the mutant is pleiotropic or if genetic and molecular analysis suggests that the virulence factor gene is not monocistronic. As expression of virulence factors in pathogenic bacteria is often controlled by global regulators, inactivation of the regulatory gene would cause

pleiotropic changes affecting the expression of several virulence factors simultaneously. We will ensure by Southern blotting, using the virulence factor structural gene DNA as a probe, that the coding sequence for the gene under study has indeed been inactivated.

Novel Procedure for the Detection of Bacterial Virulence Genes.

Dr. David DeShazer, Ms. Shauna Reckseidler and I developed a Novel Procedure for the Detection of Bacterial Virulence Genes. A patent application has been filed in the US PTO, and a copy of the Patent Application is attached as Appendix 1.

The detection of virulence genes in bacteria is of great biologic interest. We have described a method combining PCR-based subtractive hybridization, insertional mutagenesis and an animal infection model for efficiently detecting such virulence genes, and we have applied the method to the pathogens *Burkholderia pseudomallei* and *B. mallei*. Virulence genes specific to the pathogens *B. pseudomallei* and *B. mallei* were obtained by subtractive hybridization against a related nonpathogenic species, *B. thailandensis*. An internal A-T rich fragment of one of these genes was cloned into a mobilizable suicide vector that allowed the site-specific insertion of this fragment into the wild-type gene in *B. pseudomallei* creating a specific knock-out mutant which rendered this strain avirulent in a hamster infection model. This mutant, SR1015, demonstrated a phenotype similar to that of the nonpathogenic organism, *B. thailandensis*. Specifically, neither *B. pseudomallei* SR1015 nor *B. thailandensis* produces a major surface antigen identified as lipopolysaccharide I, and both organisms have attenuated virulence in hamsters relative to *B. pseudomallei* wild type. The method described should help identify previously unknown virulence genes in pathogens and provide new insights into microbial genetic diversity and evolution.

Two subtractive hybridization libraries were constructed using the PCR-Select Bacterial Genome Subtraction Kit (Clontech). In the first subtractive hybridization library *B. pseudomallei* genomic DNA was used as the "tester" and *B. thailandensis* genomic DNA was used as the "driver" (*B. pseudomallei* subtraction library). The secondary PCR products obtained were cloned into pZerO-2.1 (Invitrogen) and were enriched for *B. pseudomallei*-specific sequences. Sixteen distinct plasmid inserts from this library were sequenced. The DNA inserts were 250 bp to 1000 bp in length and contained a G + C content of approximately 45-55%, which is considerably lower than the 68% G + C content of the *B. pseudomallei* genome. This suggests that the DNA may have been acquired by horizontal transfer relatively recently in evolution. The DNA sequences were analyzed with the program BLASTX and only three of the sequences predicted proteins with homology to proteins present in the Genbank database and these proteins were not involved in virulence.

In the second subtractive hybridization library *B. mallei* genomic DNA was used as the "tester" and *B. thailandensis* genomic DNA was used as the "driver" (*B. mallei* subtraction library). The secondary PCR products obtained were cloned into pCR2.1-TOPO (Invitrogen) and were enriched for *B. mallei*-specific sequences. Seven distinct plasmid inserts from this library were sequenced. The DNA inserts were 125 bp to 700 bp in length and contained a G + C content of approximately 45-55%, which is considerably lower than the 68% G + C content of the *B. mallei*

genome. This suggests that the DNA may have been acquired by horizontal transfer relatively recently in evolution. The DNA sequences were analyzed with the program BLASTX, and only one of the sequences predicted a protein with homology to a protein present in the Genbank database. This protein was similar to ABC-2 transporters involved in polysaccharide biosynthesis.

We identified a plasmid in the *B. pseudomallei* subtraction library (pDD1015) that contained a DNA insert that was 99% identical to an insert from a plasmid in the *B. mallei* subtraction library (pDD3005). The DNA inserts were 373 bp in length and there were 4 nucleotide differences between them. The DNA insert from pDD1015 was cloned into the mobilizable suicide vector pSKM11, creating pSR1015. This plasmid was mobilized into *B. pseudomallei* 1026b and Tc^R transconjugates were isolated following a single homologous recombination between the cloned DNA and the corresponding chromosomal DNA. The integration of the plasmid pSR1015 should disrupt the chromosomal copy of the gene. One of the Tc^R transconjugates was selected and designated SR1015.

B. pseudomallei and *B. mallei* both produce type I O-PS and EPS, but *B. thailandensis* does not produce these surface polysaccharides. An ELISA was performed with a monoclonal antibody specific for EPS and *B. pseudomallei* 1026b (parental strain), and SR1015 contained EPS. Rabbit polyclonal sera specific for flagellin and type I O-PS was used in immunoelectron microscopy studies on 1026b and SR1015. *B. pseudomallei* 1026b reacted with both flagellin and type I O-PS antibodies (Figure 4A), but SR1015 only reacted with the flagellin antibodies. The LD₅₀ of 1026b in the hamster model of melioidosis was < 10 bacteria. The LD₅₀ for SR1015, on the other hand, was found to be > 10⁵ bacteria. This demonstrates that SR1015 is severely attenuated for virulence in this animal model of infection and that type I O-PS is an important virulence determinant of *B. pseudomallei* (and probably *B. mallei*).

The method for identification of virulence genes described herein should be applicable to a broad range of pathogenic bacteria. The combination of PCR-based subtractive hybridization, insertional mutagenesis and an animal infection model provides for the efficient detection of virulence genes. While we have applied the method to the pathogens *Burkholderia pseudomallei* and *B. mallei* in our current studies, it is obvious that the methodology could be applied to any species and for which only a few basic prerequisites are in place. These prerequisites include related virulent and avirulent strains, suitable suicide vectors for use in the species and an infection model for differentiation of virulent and avirulent strains. With these tools in hand, and using the method that we have described, our understanding of molecular pathogenesis could proceed in an exponential fashion.

Studies on *Burkholderia mallei* Macrophage Interactions.

Nucleoside diphosphate kinase (Ndk) is an important enzyme that generates nucleoside triphosphates (NTPs) or their deoxy derivatives by terminal phosphotransfer from a NTP such as ATP or GTP to any nucleoside diphosphate or its deoxy derivative. Since NTPs, particularly GTP, are important for cellular macromolecular synthesis and signaling mechanisms, Ndk plays an important role in bacterial growth, signal transduction and pathogenesis.

Burkholderia mallei strain GB6 secretes nucleotide diphosphate kinase (Ndk). Aside the mechanism of secretion, an important question would be the physiological implication of such secretion on pathogenesis. Why should *B. mallei* secrete an enzyme that uses ATP as a substrate? There is very little ATP in most environments except perhaps in host tissues. In macrophages, apoptosis and phagosome-lysosome fusions are believed to be mediated by surface-associated receptors called P2Z receptors whose activation is solely dependent on the availability of mM concentrations of ATP. Thus, an important experiment has been to examine the effect of concentrated supernatants of GB6 on macrophage cell death as measured by release of LDH enzyme. We also needed to purify the secreted Ndk to examine its size and some properties.

We have fractionated ammonium sulfate fractions through blue sepharose and mono-Q columns and have separated the ATPase and Ndk activities. We have purified the Ndk and ATPase from the culture filtrate of *Mycobacterium bovis* BCG. We have shown that a concentrated version of this culture filtrate will greatly reduce ATP-induced macrophage cell death, presumably by removing the ATP from P2Z receptors. This is important for mycobacteria because they grow in macrophages, and must prevent macrophage cell death (ATP-induced) in order to avoid their own destruction. We have also noticed that mucoid *Pseudomonas aeruginosa* secretes a good amount of Ndk while the nonmucoid *P. aeruginosa*, that normally secretes protein virulence factors, secretes very little Ndk. Is Ndk a virulence factor whose secretion is facilitated by alginate regulatory gene (or alginate secretion machinery) so that mucoid cells can survive better in the CF lung tissues? To find out the role of Ndk in virulence, we isolated a Ndk knock-out mutant (Alg-minus in phenotype since Ndk is needed for extensive GTP synthesis). Of course, there is no NTP synthesizing enzyme (meaning Ndk) in the growth supernatant of this mutant. But when such supernatant is incubated in presence of γ -³²P-ATP and a mixture of UDP/GDP/CDP, instead of producing the NTPs, the supernatant produces a single spot from ATP that runs between CTP and UTP on a TLC plate. Interestingly, when we did a macrophage cell death assay with concentrated supernatants of WT mucoid *P. aeruginosa* and its Ndk-deleted mutant, the WT supernatant inhibited the ATP-induced macrophage death (by removing ATP), but the supernatant growth medium of the ndk-deleted strain more than doubled macrophage cell death.

During purification of the *Burkholderia mallei* Ndk/ATPase, we have observed the presence of the same spot only when Ndk is separated during fractionation. Or in other words, it appears that both *P. aeruginosa* and *B. mallei* secrete another enzyme that converts ATP (this reaction is detectable in absence of Ndk because ³²P-CTP masks its detection). We do not know what this spot is, but it must be a form of ATP (or another NTP derived from ATP) that has a different mobility, and, more importantly, this compound is likely a better agonist of the P2Z receptor so that the secreted enzyme converts ATP to this product which greatly activates P2Z receptor which in turn enhances macrophage death. We have never detected this spot in secreted mycobacterial preparations. Our suspicion is that mycobacteria do not secrete this enzyme because they want the macrophages for growth and they die if the macrophages die. In contrast, *P. aeruginosa* and *B. mallei* secrete this enzyme to enhance macrophage death to evade the host defense. We will proceed to purify this secreted enzyme from both *B. mallei* and *P. aeruginosa*.

and find out what they convert ATP into. Then we will purify the product and test its ability to act as an agonist of P2Z receptors and enhance macrophage cell death. Then we will make knock-out mutations in this gene in *B. mallei* and check the virulence of the WT, the Ndk knock-out and this gene knock-out mutants in appropriate animal models.

Our current working hypothesis is that pathogens secrete ATP-grabbing enzymes to keep P2Z type of receptors in check so that macrophage death can either be prevented (mycobacteria, *B. pseudomallei*) or accelerated (*P. aeruginosa*/*B. mallei*), depending upon their mode of action. Our continuing studies designed to test this hypothesis should provide important information on the pathogenesis of disease due to *B. mallei*.

Characterization of Cytotoxic Activity.

We have examined the ability of the cell-free supernatants from ten *B. mallei* strains to kill a variety of cell types including HeLa cells, Chinese Hamster Ovary cells, Vero cells, A549 cells and alveolar macrophages; and we have examined their ability to act as a leucocidin (i.e., cytotoxic for PMNs) as this might explain the propensity of this organism to cause septicemic infections. HeLa cells, CHO cells, Vero cells and A549 cells are cell lines in continuous use in my laboratory. Alveolar macrophages were obtained from rats by bronchoalveolar lavage. Rats were anesthetized with pentobarbital, exsanguinated, the diaphragm punctured, the neck entered and a tracheostomy performed. Flexible sialistic tubing was introduced into the trachea and a suture tied around it to secure it in place. Lavage with normal saline was performed in 5 ml aliquots to a total of 50 ml and collected in a container on ice. A 100 ul aliquot of the cell suspension was used to derive a total cell count. A second 100 ul aliquot was cytocentrifuged directly onto a glass slide, air dried and stained with the LeukoStat staining kit to obtain a differential cell count. PMNs were obtained from heparinized whole human blood by dextran sedimentation of erythrocytes by previously described techniques.

Cytotoxicity for tissue culture cell lines was assessed by the MTT assay as described. Briefly, 5,000 cells per well were plated in 96 well plates and allowed to grow overnight. The cells were washed in serum-free media and the cytotoxin added in 100 ul serum-free media. The cells were allowed to grow 1-2 days, at which time 25 ul 5 mg/ml MTT (Sigma, St. Louis, MO) in PBS (pH 7.4) is added. After a 3 hr incubation, the cells were lysed with 100 ul 20% SDS in 50% dimethyl formamide. Following overnight incubation, the absorbance at 570 nm was determined on a microtiter plate reader. Cytotoxicity for alveolar macrophages and PMNs was assessed by the ability of the toxin to inhibit 3H-thymidine incorporation.

The results from the testing of the cell-free supernatants were uniformly negative when unconcentrated supernatants were tested on all of the cells described above. When 10x concentrated supernatants were tested on the cells, it became apparent that cytotoxic activity is present in the supernatants from all of the 10 *B. mallei* strains tested. Our current plans are to repeat the isolation of the cell-free supernatant fluids from the 10 *B. mallei* strains from a variety of growth conditions. We intend to harvest supernatants from cells grown under conditions varied for temperature, energy source and osmolality. We anticipate that we will be able to increase the yields of cytotoxic activity by varying these environmental conditions.

We have tested all of the concentrated supernatants and other cellular fractions from *B. mallei* for toxicity in hamsters. Results from these studies indicate that the interperitoneal injection of concentrated supernatants leads to hamster death. These are ongoing studies, and we would anticipate that we will identify and isolate this toxic activity in the next year of the contract period.

Goals for the Coming Year:

1. We will obtain cell-free supernatants from *B. mallei* strains grown under a variety of environmental conditions. These supernatants will be tested for cytotoxicity. These studies should provide us information regarding the optimal growth conditions for harvesting significant amounts of cytotoxic activity. Following the determination of optimal growth conditions for harvesting cytotoxic activity, we will proceed to the purification and characterization of this activity.
2. We will continue our studies on *B. mallei*-macrophage interactions. We will proceed to purify the secreted enzyme from *B. mallei* which transforms ATP and find out what it converts ATP into. Then we will purify the product and test its ability to act as an agonist of P2Z receptors and enhance macrophage cell death. Then we will make knock-out mutations in this gene in *B. mallei* and check the virulence of the WT, the Ndk knock-out and this gene knock-out mutants in appropriate animal models.
3. We will continue to probe the *B. mallei* DNA with *B. pseudomallei* genes to look for homologous sequences, particularly those related to flagellin synthesis and secretion. These are important studies as if we are able to demonstrate that *B. mallei* contains all of the genes necessary for flagellin structure and synthesis, we will initiate studies to determine under which conditions these organisms may indeed be motile. If it is indeed found that *B. mallei* produces flagellin under certain conditions, it may be possible to design a flagellin-based vaccine for this organism as we have done for *B. pseudomallei*.
4. Having discovered what we believe is a "pathogenicity island" that is present in *Burkholderia pseudomallei*, *B. mallei* and *B. cepacia*, we are proceeding with the cloning and characterization of this chromosomal segment.
5. We believe that we will have the pathogenicity island cloned and sequenced by the next quarterly report, and we will have submitted the completed manuscript detailing this important discovery to Science. We are tremendously excited about our recent discovery, and we are proceeding with great haste in our studies.
6. We believe that an important antigen encoded by the pathogenicity island is Type I lipopolysaccharide present in *B. pseudomallei*. We will proceed to determine if this antigen is present in *B. mallei* as we have recently discovered that this antigen is present in certain *B. cepacia* strains. We are also proceeding with the purification of this antigen with an eye towards

the development of scale-up production methods for this antigen that may be an important vaccine candidate.

Administrative Issues:

We have completed the construction of the Level III Biocontainment Facility at the University of Calgary. This facility has been site-visited by Dr. Robert Hawley of USAMRIID, and recommendations made by Dr. Hawley have been implemented. We anticipate that we will begin work in the facility within the next week. We have submitted the Application to Import Animal Pathogens to the Canadian Food Inspection Agency, and we anticipate that we will begin our studies with live organisms shortly.

We will initiate discussions with LTC Carr in reference to the possibility of altering the budget expenditures. It has become clear that DNA sequencing costs will increase as a result of our exciting findings regarding a pathogenicity island in *B. mallei*. In combination with studies from other laboratories that have demonstrated that hamsters and guinea pigs serve as excellent animal hosts to study glanders, we would submit that the animal studies budget could be reduced as a result of not having to determine the most appropriate host for our animal studies. Thus, it would seem appropriate to transfer funds from the animal studies budget to a line item for DNA sequencing costs.

CONCLUSIONS:

We have obtained information regarding the production of cytotoxic activity by *B. mallei*. Following the determination of optimal growth conditions for harvesting cytotoxic activity, we will proceed to the purification and characterization of this activity. In relation to *B. mallei*-macrophage interactions, our current working hypothesis is that pathogens secrete ATP-grabbing enzymes to keep P2Z type of receptors in check so that macrophage death can either be prevented (mycobacteria, *B. pseudomallei*) or accelerated (*P. aeruginosa*/*B. mallei*), depending upon their mode of action. Our continuing studies designed to test this hypothesis should provide important information on the pathogenesis of disease due to *B. mallei*. If we are able to demonstrate that *B. mallei* contains all of the genes necessary for flagellin structure and synthesis, we will initiate studies to determine under which conditions these organisms may indeed be motile. If it is indeed found that *B. mallei* produces flagellin under certain conditions, it may be possible to design a flagellin-based vaccine for this organism as we have done for *B. pseudomallei*. Having discovered what we believe is a "pathogenicity island" that is present in *Burkholderis pseudomallei*, *B. mallei* and *B. cepacia*, we are proceeding with the cloning and characterization of this chromosomal segment. We are tremendously excited about our recent discovery, and we are proceeding with great haste in our studies. We believe that an important antigen encoded by the pathogenicity island is Type I lipopolysaccharide present in *B. pseudomallei*. We will proceed to determine if this antigen is present in *B. mallei* as we have recently discovered that this antigen is present in certain *B. cepacia* strains. We are also proceeding with the purification of this antigen with an eye towards the development of scale-up production methods for this antigen that may be an important vaccine candidate.

APPENDIX 1

PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (b) (2)

DETECTION OF PHENOTYPIC GENES

REPORT OF INVENTIONS AND SUBCONTRACTS (Pursuant to "Patent Rights" Contract Clause) (See Instructions on back)				Form Approved GSA No. 9000-0088 Expires July 31, 1998	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (9000-0088), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. PLEASE DO NOT RETURN YOUR COMPLETED FORM TO THIS ADDRESS. RETURN COMPLETED FORM TO THE CONTRACTING OFFICER.					
1. NAME OF CONTRACTOR/SUBCONTRACTOR Canadian Commercial Corp. / University of Calgary Ottawa, Ontario Canada K1A 0S5		2. NAME OF GOVERNMENT PRIME CONTRACTOR US Army Medical Research Acquisition Act. 820 Chandler Street Ft. Detrick, MD 21702-5014		3. TYPE OF REPORT (If any) a. INTERIM ☒ b. FINAL 4. REPORTING PERIOD (YYMMDD) a. FROM 97/12/15 b. TO 2000/05/14	
5. NAME OF CONTRACTOR/SUBCONTRACTOR University of Calgary		6. CONTRACT NUMBER DAMD17-C-8002		7. AWARD DATE (YYMMDD) 97/12/15	
8. ADDRESS (Include ZIP Code)		9. AWARD DATE (YYMMDD)		10. FROM 97/12/15	
11. TO 2000/05/14		12. TO 2000/05/14		13. TO 2000/05/14	
SECTION I - SUBJECT INVENTIONS					
1. NAME OF INVENTOR(S) (Last, First, Middle Initial)		2. TITLE OF INVENTION(S)		3. CONFIRMATORY INSTRUMENT OR ASSIGNMENT FORWARDED TO CONTRACTING OFFICER (If)	
Woods, Donald E. DeShazer, David Reckseidler, Shauna L.		Detection of Phenotypic Genes		NO	
4. EMPLOYER OF INVENTOR(S) NOT EMPLOYED BY CONTRACTOR/SUBCONTRACTOR		5. ELECTED FOREIGN COUNTRIES IN WHICH A PATENT APPLICATION WILL BE FILED		6. ELECTED FOREIGN COUNTRIES IN WHICH A PATENT APPLICATION WILL BE FILED	
(1) (a) NAME OF INVENTOR (Last, First, Middle Initial)		(2) (a) NAME OF INVENTOR (Last, First, Middle Initial)		(3) FOREIGN COUNTRIES OF PATENT APPLICATION	
(b) NAME OF EMPLOYER		(b) NAME OF EMPLOYER		All PCT Countries Thailand, Malaysia, Taiwan, Philippines	
(c) ADDRESS OF EMPLOYER (Include ZIP Code)		(c) ADDRESS OF EMPLOYER (Include ZIP Code)			
SECTION II - SUBCONTRACTS (Containing a "Patent Rights" clause)					
1. NAME OF SUBCONTRACTOR(S)		2. ADDRESS (Include ZIP Code)		3. SUBCONTRACT NUMBER(S)	
University of Calgary		2500 University Dr. Calgary, Alberta T2N 1N4		SFUSA-7-M156/001/SV	
4. TITLE		5. DESCRIPTION OF WORK TO BE PERFORMED UNDER SUBCONTRACT(S)		6. SUBCONTRACT DATES (YYMMDD)	
Preparation of a Burkholderia mallei Vaccine		1,2		97/12/15 2000/05/14	
SECTION III - CERTIFICATION					
7. CERTIFICATION OF REPORT BY CONTRACTOR/SUBCONTRACTOR (Not required if: (X) as appropriate)		8. SMALL BUSINESS or		9. NONPROFIT ORGANIZATION	
I certify that the reporting party has procedures for prompt identification and timely disclosure of "Subject Inventions," that such procedures have been followed and that all "Subject Inventions" have been reported.					
10. NAME OF AUTHORIZED CONTRACTOR/SUBCONTRACTOR OFFICIAL (Last, First, Middle Initial)		11. TITLE		12. SIGNATURE	
Jessop, Emlyn L.		University of Calgary Director, Research Services		L. F. Foster	
DD FORM 882, FEB 96		PREVIOUS EDITION IS OBSOLETE.		LORI FOSTER Associate Director External Awards Research Services	

DD FORM 882, FEB 96

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (b)(2).

Docket Number: 09561/002001 Type a plus sign (+) inside this box →

INVENTOR(S)/APPLICANT(S)			
LAST NAME	FIRST NAME	MIDDLE NAME/INITIAL	RESIDENCE/CITY AND EITHER STATE OR FOREIGN COUNTRY
Woods	Donald	E.	Alberta, Canada
DeShazer	David		Alberta, Canada
Reckseidler	Shauna	L.	Alberta, Canada

TITLE OF THE INVENTION (250 characters max)

DETECTION OF PHENOTYPIC GENES

*Express Mail mailing label number EL163701325US
Date of Deposit 11/20/98
I hereby certify that this paper or fee is being deposited with the United States Postal Service *Express Mail Post Office to addressee's service under 37 CFR 1.10 on the date indicated above and is addressed to the Commissioner of Patents and Trademarks, Washington, D.C. 20231

CORRESPONDENCE ADDRESS (including Addressee's service under 37 CFR 1.10 on the date indicated above and is addressed to the Commissioner of Patents and Trademarks, Washington, D.C. 20231)

John R. Wetherell Jr., Ph.D.
FISH & RICHARDSON P.C.
4225 Executive Square, Suite 1400
La Jolla, California 92037

ENCLOSED APPLICATION PARTS (Check all that apply)

☒ Specification	Number of Pages	62	☒ Small Entity Statement
☒ Drawing(s)	Number of Sheets	4	☒ Other (specify) 1 page Abstract

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT (check one)

☒ A check or money order is enclosed to cover the filing fees	FILING FEE AMOUNT (\$)	\$150.00
☐ The Commissioner is hereby authorized to charge filing fees and credit Deposit Account Number		

The invention was made by or agency of the United States Government or under a contract with an agency of the United States Government

☒ Yes

☐ Yes, the name of the U.S. Government agency and the Government contract number are

Respectfully submitted,

SIGNATURE

John R. Wetherell Jr. Reg. No. 40900

Date 11/20/98

TYPED or PRINTED NAME John R. Wetherell Jr., Ph.D.

REGISTRATION NO. 31,678

☐ Additional inventors are being named on separately numbered sheets attached hereto

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

Burden Hour Statement: This form is estimated to take 0.2 hours to complete. Time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, DC 20231

ATTORNEY DOCKET NO. 095617

Applicant or Patentee: Donald Woods et al.

Serial or Patent No.:

Filed or Issued:

For:

11/20/98

DETECTION OF PHENOTYPIC GENES

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(j)) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

Name of Organization: Canadian Microbiology Consortium, Inc.
Address of Organization: Room 282 - Heritage Medical Research Building, 3330 Hospital Drive NW
Calgary, AB T2N 4N1
Canada

Type of Organization:

- ☒ UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION
☐ TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501(c)(3))
☐ NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA
(NAME OF STATE:)
(CITATION OF STATUTE:)
☐ WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501(c)(3)) IF
LOCATED IN THE UNITED STATES OF AMERICA
☐ WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF
AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA
(NAME OF STATE:)
(CITATION OF STATUTE:)

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code with regard to the invention entitled DETECTION OF PHENOTYPIC GENES by inventor(s) Donald E. Woods, David Deshazer and Shauna L. Reckseidler described in

- ☒ the specification filed herewith.
☐ application serial no. , filed 11/20/98.
☐ patent no. , issued .

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business: concern under 37 CFR 1.9(c) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

Full Name:

Address:

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status when any new rule 53 application is filed or prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name: Karen G. Corzini

Title: Chief Executive Officer

Address: Room 282 - Heritage Medical Research Building, 3330 Hospital Drive NW, Calgary, AB T2N 4N1, CANADA

Signature:

Date:

Nov. 20/98

~~CONFIDENTIAL~~

PROVISIONAL APPLICATION

UNDER 37 CFR 1.53(b)(2)

TITLE: DETECTION OF PHENOTYPIC GENES

APPLICANT: DONALD E. WOODS, DAVID DeSHAZER, SHAUNA RECKSEIDLER

"EXPRESS MAIL" Mailing Label Number EL163701325US

Date of Deposit November 20, 1998

I hereby certify under 37 CFR 1.10 that this correspondence is being deposited with the United States Postal Service as "Express Mail Post Office To Addressee" with sufficient postage on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.



Scott H. Sebbo

DETECTION OF PHENOTYPIC GENES

TECHNICAL FIELD

The invention relates to compositions and methods of producing and identifying a polynucleotide sequence encoding a phenotypic gene.

BACKGROUND

The study of infection is being revolutionized by the development of technology that allows the sequencing and analysis of entire microbial genomes (Jenks, *J. Med. Microbiol.* 47:375 (1998)). The genomes of a number of important human pathogens have already been or are in the process of being sequenced. The continuing release of data from these projects, combined with advances in techniques used to investigate molecular pathogenesis, provides a framework that will facilitate our understanding of these organisms, and ultimately, lead to new anti-infective strategies.

The identification of bacterial virulence genes has traditionally relied upon empirical predictions of putative virulence determinants, inactivation of the genes encoding for these putative virulence determinants by any number of methods, followed by comparisons of virulence between mutant and wild-type in infection models (Falkow, *Rev. Infect. Dis.*, 10(2):S274, 1988). IVET (*in vivo* expression technology), STM (signature-tagged transposon method) and DFI (differential fluorescence induction) have been developed to facilitate identification of expressed sequences or genes important under a given set of circumstances within a test host; however, these approaches do not necessarily elucidate roles of the gene products in pathogenesis (Strauss, *Science*. 276:707, 1997).

Burkholderia pseudomallei and *B. mallei* are bacterial pathogens that cause the diseases melioidosis and glanders, respectively (Sanford, In *Principles and Practice of Infectious Diseases* (Eds, Mandell *et al.*) Churchill Livingstone, New York, pp. 1692, 1990). *B. thailandensis* is a non-pathogenic soil organism originally isolated in Thailand (Brett *et al.*,

Epidemiol. Infect. 118:137, 1997). Based on biochemical, immunological and genetic data, *B. pseudomallei*, *B. mallei* and *B. thailandensis* are relatively closely related species in the genus *Burkholderia*.

Syrian hamsters are highly susceptible to infection by *B. pseudomallei* and *B. mallei* and are commonly employed to study the relative virulence of these organisms. The 50% lethal dose (LD₅₀) of *B. pseudomallei* and *B. mallei* for Syrian hamsters is approximately 10 bacteria. In contrast, Syrian hamsters are highly resistant to infection by *B. thailandensis*, and the LD₅₀ is approximately 10⁶ bacteria (Brett *et al.*, *Epidemiol. Infect.* 118:137, 1997). However, in spite of their pathogenic importance, relatively little is known about the virulence determinants of *B. pseudomallei* and *B. mallei*.

SUMMARY OF THE INVENTION

Thus, it is an object of the present invention to provide a method of identifying a polynucleotide sequence associated with a specific phenotype in an organism, by obtaining a first nucleic acid population from a first organism, a second nucleic acid population from a second organism, comparing the first nucleic acid population and the second nucleic acid population to obtain a third nucleic acid population, transforming a host cell with a selected polynucleotide from the third nucleic acid population, and detecting a phenotypic change in the host cell. The third nucleic acid population comprises one or more unique polynucleotide sequences in the first or second population.

In another embodiment, the invention provides a method of identifying a polynucleotide sequence associated with a specific phenotype in an organism, by obtaining a first nucleic acid population from a first organism, a second nucleic acid population from a second organism, comparing the first nucleic acid population and the second nucleic acid population to obtain a third nucleic acid population, cloning a selected polynucleotide from the third population into a vector, transforming a host cell with the vector, and detecting a phenotypic change in the host cell. The vector can be any vector capable of transforming a host cell, for example expression, viral, and suicide vectors.

In yet another embodiment, the invention provides a method of identifying a polynucleotide sequence associated with a pathogenic phenotype in an organism by obtaining a first nucleic acid population from a first organism, a second nucleic acid population from a second organism, processing the first nucleic acid population and the second nucleic acid population to obtain a third nucleic acid population, cloning a selected polynucleotide from the third population into a vector, transforming a host cell with the vector, and detecting a change in pathogenicity of the host cell.

In another embodiment, the invention provides a method of identifying a polynucleotide sequence associated with a pathogenic phenotype in an organism by obtaining a first nucleic acid population from a first organism, obtaining a second nucleic acid population from a second organism, comparing the first nucleic acid population and the second nucleic acid population to obtain a third nucleic acid population, cloning a selected polynucleotide from the third nucleic acid population into a vector, transforming a host cell with the vector, inoculating a third organism with the transformed host cell, and measuring pathogenesis of the host cell in the third organism compared to a control.

In yet another embodiment, the invention provides an immunostimulatory composition comprising a polypeptide encoded by a polynucleotide sequence associated with a pathogenic phenotype in an organism by obtaining a first nucleic acid population from a first organism, a second nucleic acid population from a second organism, processing the first nucleic acid population and the second nucleic acid population to obtain a third nucleic acid population, cloning a selected polynucleotide from the third population into a vector, transforming a host cell with the vector, detecting a change in pathogenicity of the host cell, isolating the selected polynucleotide and expressing a polypeptide encoded by the select polynucleotide.

In another embodiment, the invention provides a therapeutic composition including an antibody to a polypeptide encoded by a polynucleotide sequence associated with a pathogenic phenotype in an organism, by obtaining a first nucleic acid population from a first organism, obtaining a second nucleic acid population from a second organism, comparing the first

nucleic acid population and the second nucleic acid population to obtain a third nucleic acid population, cloning a select polynucleotide from the third population into a vector, transforming a host cell with the vector, detecting a change in pathogenicity of the host cell, isolating the selected polynucleotide and expressing a polypeptide encoded by the select polynucleotide, and inoculating an organism with the polypeptide to obtain an immune response.

In another embodiment, the invention provides a polynucleotide selected from the group associated with *B.pseudomallei* virulence consisting of the polynucleotide of SEQ ID NO:1, SEQ ID NO: 1, wherein T is U, and nucleic acid sequences complementary to thereto.

In another embodiment, the invention provides a polynucleotide selected from the group associated with *B.mallei* virulence consisting of the polynucleotide of SEQ ID NO:2, SEQ ID NO: 2, wherein T is U, and nucleic acid sequences complementary to thereto.

In a further embodiment, the present invention provides a method for detecting a virulent organism in a sample, comprising contacting the sample with a polynucleotide having a sequence of SEQ ID NO:1 or 2 and detecting hybridization of the probe with a polynucleotide in the sample, wherein the detection of hybridization is indicative of a virulent organism in the sample.

In yet another embodiment, the invention provides a method for identifying a compound which modulates pathogenesis of an organism by incubating components comprising the compound and a polypeptide encoded by a polynucleotide identified by the process of obtaining a first nucleic acid population from a first organism, obtaining a second nucleic acid population from a second organism, comparing the first nucleic acid population and the second nucleic acid population to obtain a third nucleic acid population, cloning a select polynucleotide from the third population into a vector, transforming a host cell with the vector, detecting a change in pathogenicity of the host cell, isolating the selected

polynucleotide and expressing a polypeptide encoded by the select polynucleotide, and measuring modulation of the organism pathogenicity.

5 In another embodiment, the present invention provides a method of immunization comprising delivering an effective amount of a plasmid expression vector to transform differentiated somatic cell tissue of a living organisms wherein said plasmid expression vector contains a virulence-associated polynucleotide sequence identified by the process of obtaining a first nucleic acid population from a first organism, obtaining a second nucleic acid population from a second organism, comparing the first nucleic acid population and the second nucleic acid population to obtain a third nucleic acid population, cloning a select polynucleotide from the third population into a vector, transforming a host cell with the vector, detecting a change in pathogenicity of the host cell, isolating the selected polynucleotide.

15 In another embodiment, provided is a method for identifying a compound which regulates expression of a specific phenotype in an organism or activity of a polypeptide conferring a specific phenotype comprising, incubating components comprising the compound and the organism under conditions sufficient to allow the components to interact, and measuring the effect of the compound on phenotypic expression.

20 In another embodiment, the invention provides a method for inducing a protective immune response in a subject by administering an immunostimulatory composition to a subject thereby inducing a protective immune response. The composition comprises a polypeptide encoded by a polynucleotide identified by the methods of the invention.

25 In another embodiment, the invention provides a method for providing a protective immune response to a subject, by administering a therapeutic composition to the subject thereby providing a protective immune response. The therapeutic composition comprises an antibody to a polypeptide encoded by a polynucleotide identified by the methods of the invention.

Also provided are immunostimulatory compositions comprising products produced by a polypeptide encoded by a polynucleotide identified by the methods of the invention. Further provided are methods for inducing a protective immunity by administering the product to a subject.

5 In another embodiment, the invention provides a pharmaceutical composition comprising a therapeutic agent that interacts with a product produced by a polypeptide encoded by a polynucleotide identified by the method of the invention. Further, therapeutic agent includes antibodies to provide protective immunity by administering the therapeutic agent to a subject.

10 In order to identify the genetic determinant(s) that confer enhanced virulence in *B. pseudomallei* and *B. mallei*, subtractive hybridization technology was used to isolate DNA fragments found in *B. pseudomallei* and *B. mallei* but not *B. thailandensis*.

15 The method for identification of virulence genes described herein is applicable to a broad range of pathogenic bacteria. The combination of PCR-based subtractive hybridization, insertional mutagenesis and an animal infection model provides for the efficient detection of virulence genes. While the Examples below have applied the method to the pathogens *Burkholderia pseudomallei* and *B. mallei*, the methodology is applicable to any species and for which only a few basic prerequisites are in place. These prerequisites include related virulent and avirulent strains, suitable suicide vectors for use in the species and an infection model for differentiation of virulent and avirulent strains. With these tools in hand, and using
20 the method described herein, an understanding of molecular pathogenesis could proceed in an exponential fashion.

25 The detection of virulence genes in bacteria is of great biologic interest. Disclosed is a method combining PCR-based subtractive hybridization, insertional mutagenesis and an animal infection model for efficiently detecting such virulence genes, and we have applied the method to the pathogens *Burkholderia pseudomallei* and *B. mallei*. Virulence genes specific to the pathogens *B. pseudomallei* and *B. mallei* were obtained by subtractive

hybridization against a related nonpathogenic species, *B. thailandensis*. An internal A-T rich fragment of one of these genes was cloned into a mobilizable suicide vector that allowed the site-specific insertion of this fragment into the wild-type gene in *B. pseudomallei* creating a specific knock-out mutant which rendered this strain avirulent in a hamster infection model. This mutant, SR1015, demonstrated a phenotype similar to that of the nonpathogenic organism, *B. thailandensis*. Specifically, neither *B. pseudomallei* SR1015 nor *B. thailandensis* produces a major surface antigen identified as lipopolysaccharide I, and both organisms have attenuated virulence in hamsters relative to *B. pseudomallei* wild type. The method described should help identify previously unknown virulence genes in pathogens and provide new insights into microbial genetic diversity and evolution.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a phylogenetic analysis of *Ralstonia* and *Burkholderia* species based on 16S rRNA gene sequences. DNA sequences were analyzed with GeneJockey version 1.20 software for the Macintosh and the University of Wisconsin Genetics Computer Group package. The nucleotide sequences of the 16S rRNA genes were aligned with the program PILEUP using the default parameters. The aligned sequences were edited so that there were no gaps, and the program DISTANCES was used to calculate a matrix of the pairwise evolutionary distances between the aligned sequences. The Tajima-Nei method was used to correct the distances for multiple substitutions at a site. GROWTREE was used to reconstruct a phylogenetic tree from the distance matrix using the neighbor-joining method. The scale bar indicates distance in substitutions per nucleotide.

Figure 2 shows an alignment of the 373-bp fragments from pDD1015 (*B. pseudomallei*) and pDD3005 (*B. mallei*). The location of nucleotide differences between the two sequences are indicated by asterisks.

Figure 3 shows a schematic representation of the construction of *B. pseudomallei* SR1015. The cointegrate strain SR1015 was created by integration of the suicide vector pSR1015 (Table 1) into the chromosome of *B. pseudomallei* 1026b. The X represents the location of homologous recombination between pSR1015 and the 1026b chromosome. The integration of this plasmid results in the disruption of an open reading frame required for synthesis of type I O-PS and hamster virulence.

Figure 4 shows immunogold electron microscopy of *B. pseudomallei* strains. Bacteria were reacted with polyclonal rabbit serum directed against type I O-PS and flagella, washed and reacted with a goat anti-rabbit I.G. gold conjugate (5 nm). A. 1026b. B. SR1015.

Magnification, X 30,000

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

DETAILED DESCRIPTION OF THE INVENTION

Bacterial sepsis and related septic shock

Bacterial sepsis and related septic shock are frequently lethal conditions caused by infections which can result from certain types of surgery, abdominal trauma and immune suppression related to cancer, transplantation therapy or other disease states. It is estimated that over 700,000 patients become susceptible to septic shock-causing bacterial infections each year in the United States alone. Of these, 160,000 actually develop septic shock, resulting in 50,000 deaths annually.

Gram negative bacterial infections comprise the most serious infectious disease problem seen in modern hospitals. Two decades ago, most sepsis contracted in hospitals was attributable to

more acute gram positive bacterial pathogens such as *Staphylococcus* and *Streptococcus*. By contrast, the recent incidence of infection due to gram negative bacteria, such as *Escherichia coli* and *Pseudomonas aeruginosa*, has increased.

5 Gram negative bacteria now account for some 200,000 cases of hospital-acquired infections yearly in the United States, with an overall mortality rate in the range of 20% to 60%. The majority of these hospital-acquired infections are due to such gram negative bacilli as *E. coli* (most common pathogen isolated from patients with gram negative sepsis), followed in frequency by *Klebsiella pneumoniae* and *P. aeruginosa*.

10 Gram negative sepsis is a disease syndrome resulting from the systemic invasion of gram negative rods and subsequent endotoxemia. The severity of the disease ranges from a transient, self-limiting episode of bacteremia to a fulminant, life-threatening illness often complicated by organ failure and shock. The disease is often the result of invasion from a localized infection site, or may result from trauma, wounds, ulcerations or gastrointestinal obstructions. The symptoms of gram negative sepsis include fever, chills, pulmonary failure and septic shock (severe hypotension).

15 Gram negative infections are particularly common among patients receiving anti-cancer chemotherapy and immunosuppressive treatment. Infections in such immuno-compromised hosts characteristically exhibit resistance to many antibiotics, or develop resistance over the long course of the infection, making conventional treatment difficult. The ever increasing use of cytotoxic and immunosuppressive therapy and the natural selection for drug resistant bacteria by the extensive use of antibiotics have contributed to gram negative bacteria evolving into pathogens of major clinical significance.

20 Fortunately, more than a decade ago, investigators in the United States and Germany demonstrated that gram negative endotoxins of many different bacterial genera have a "common core structure." In other words, while many infectious gram negative organisms contain individual capsule and surface polysaccharide, there is a core lipopolysaccharide

(LPS) structure that is widely shared among the diverse gram negative bacterial genera and their endotoxins.

This core structure contains material identified as "lipid A" that is felt to be responsible for all of the biologic properties of "endotoxin," including pyrogenicity, activation of the complement and clotting systems, hypotension and death in experimental animals. This core or LPS structure is therefore significant for at least two reasons; its association with endotoxicity and its conservation in gram negative bacterial genera.

Because antibiotic treatment remains largely suboptimal against gram negative sepsis, particularly that associated with *P. aeruginosa* bacterial infection, (antibiotics are only effective in treating the bacteria and not in reducing the effects of microbial endotoxins) attention has increasingly focused on immunologic methods to prevent and control such infections. Immunotherapy involves the administration of immunoglobulins (antibodies or active fragments thereof) to bolster the host's native defenses against the toxic effects of the bacteria, for example, by enhancing opsonization and phagocytosis of the infecting bacterial cells, or by neutralization of the biological effects of LPS. Antibodies, or active fragments thereof, that bind with the core structure or lipid A, *i.e.*, LPS, could have a broad reactivity with a number of gram negative endotoxins.

Antibodies directed against epitopes or antigenic determinants on the O-specific side chains of smooth gram negative bacteria have limited utility for use in immunotherapy. This is because they are effective against only those strains of bacteria having complementary or cross-reactive antigenic determinants. Such strain-specific antibodies are of only limited utility. While the core oligosaccharide and lipid A of all strains are thought to share antigenic determinants, the few previous attempts to produce and utilize monoclonal antibodies reactive with these regions in *Pseudomonas* have been largely unsuccessful.

Immunoglobulins that bind most of the clinically significant gram negative pathogens are essential to the success of immunotherapy. *P. aeruginosa* organisms, which account for 5%

to 15% of bloodstream infections, have at least 16 different serotypes (O-antigenic types). *Klebsiella* organisms have more than 80 capsular types, and *E. coli* organisms, which are far more common, have more than 130 serotypes.

Subjects with bacteremia often do not have a confirmed specific diagnosis as to the type of bacterial infection until bacteriologic results are available, which may take several days. Therapy often must be started based on an empirical diagnosis in order to prevent a patient's condition from rapidly deteriorating during the critical first 24 to 48 hours of illness.

The Gram-negative bacteria are a diverse group of organisms and include Spirochaetes such as *Treponema* and *Borrelia*, Gram-negative bacilli including the *Pseudomonadaceae*, *Legionellaceae*, *Enterobacteriaceae*, *Vibrionaceae*, *Pasteurellaceae*, Gram-negative cocci such as *Neisseriaceae*, anaerobic Bacteroides, and other Gram-negative bacteria including *Rickettsia*, *Chlamydia*, and *Mycoplasma*.

Gram-negative bacilli (rods) are important in clinical medicine. They include (1) the Enterobacteriaceae, a family which comprises many important pathogenic genera, (2) *Vibrio*, *Campylobacter* and *Helicobacter* genera, (3) opportunistic organisms (e.g., *Pseudomonas*, *Flavobacterium*, and others) and (4) *Haemophilus* and *Bordetella* genera. The Gram-negative bacilli are the principal organisms found in infections of the abdominal viscera, peritoneum, and urinary tract, as well secondary invaders of the respiratory tracts, burned or traumatized skin, and sites of decreased host resistance. Currently, they are the most frequent cause of life-threatening bacteremia. Examples of pathogenic Gram-negative bacilli are *E. coli* (diarrhea, urinary tract infection, meningitis in the newborn), *Shigella* species (dysentery), *Salmonella typhi* (typhoid fever), *Salmonella typhimurium* (gastroenteritis), *Yersinia enterocolitica* (enterocolitis), *Yersinia pestis* (black plague), *Vibrio cholerae* (cholera), *Campylobacter jejuni* (enterocolitis), *Helicobacter jejuni* (gastritis, peptic ulcer), *Pseudomonas aeruginosa* (opportunistic infections including burns, urinary tract, respiratory tract, wound infections, and primary infections of the skin, eye and ear), *Haemophilus influenzae* (meningitis in children, epiglottitis, otitis media, sinusitis, and bronchitis), and

Bordetella pertussis (whooping cough). *Vibrio* is a genus of motile, Gram-negative rod-shaped bacteria (family *Vibrionaceae*). *Vibrio cholerae* causes cholera in humans; other species of *Vibrio* cause animal diseases. *E. coli* colonize the intestines of humans and warm blooded animals, where they are part of the commensal flora, but there are types of *E. coli* that cause human and animal intestinal diseases. They include the enteroaggregative *E. coli* (EaggEC), enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC) and enterotoxigenic *E. coli* (ETEC). Uropathogenic *E. coli* (UPEC) cause urinary tract infections. There are also neonatal meningitis *E. coli* (NMEC). Apart from causing similar infections in animals as some of the human ones, there are specific animal diseases including: calf septicaemia, bovine mastitis, porcine oedema disease, and air sac disease in poultry.

The pathogenic bacteria in the Gram-negative aerobic cocci group include *Neisseria*, *Moraxella* (*Branhamella*), and the *Acinetobacter*. The genus *Neisseria* includes two important human pathogens, *Neisseria gonorrhoeae* (urethritis, cervicitis, salpingitis, proctitis, pharyngitis, conjunctivitis, pharyngitis, pelvic inflammatory disease, arthritis, disseminated disease) and *Neisseria meningitidis* (meningitis, septicemia, pneumonia, arthritis, urethritis). Other Gram-negative aerobic cocci that were previously considered harmless include *Moraxella* (*Branhamella*) *catarrhalis* (bronchitis and bronchopneumonia in patients with chronic pulmonary disease, sinusitis, otitis media) has recently been shown to be an common cause of human infections.

The *Neisseria* species include *N. cinerea*, *N. gonorrhoeae*, *N. gonorrhoeae* subsp. *kochii*, *N. lactamica*, *N. meningitidis*, *N. polysaccharea*, *N. mucosa*, *N. sicca*, *N. subflava*, the asaccharolytic species *N. flavescens*, *N. caviae*, *N. cuniculi* and *N. ovis*. The strains of *Moraxella* (*Branhamella*) *catarrhalis* are also considered by some taxonomists to be *Neisseria*. Other related species include *Kingella*, *Eikenella*, *Simonsiella*, *Alysiella*, CDC group EF-4, and CDC group M-5. *Veillonella* are Gram-negative cocci that are the anaerobic counterpart of *Neisseria*. These non-motile diplococci are part of the normal flora of the mouth.

As used herein, the term "amplifying" refers to increasing the number of copies of a specific polynucleotide. For example, polymerase chain reaction (PCR) is method for amplifying a polynucleotide sequence using a polymerase and two oligonucleotide primers, one complementary to one of two polynucleotide strands at one end of the sequence to be amplified and the other complementary to the other of two polynucleotide strands at the other end. Because the newly synthesized DNA strands can subsequently serve as additional templates for the same primer sequences, successive rounds of primer annealing, strand elongation, and dissociation produce rapid and highly specific amplification of the desired sequence. PCR also can be used to detect the existence of the defined sequence in a DNA sample.

"Corresponding" means homologous to or substantially equivalent to the designated sequence.

"Genetic material" is a material containing any DNA sequence or sequences either purified or in a native state such as a fragment of a chromosome or a whole chromosome, either naturally occurring or synthetically or partially synthetically prepared DNA sequences, DNA sequences which constitute a gene or genes and gene chimeras, *e.g.*, created by ligation of different DNA sequences.

"Exogenous genetic material" is a genetic material not obtained from or does not naturally form a part of the specific target organism.

"DNA sequence" is a linear sequence comprised of any combination of the four DNA monomers, *i.e.*, nucleotides of adenine, guanine, cytosine and thymine, which codes for genetic information, such as a code for an amino acid, a promoter, a control or a gene product. A specific DNA sequence is one which has a known specific function, *e.g.*, codes for a particular polypeptide, a particular genetic trait, or affects the expression of a particular phenotype.

"Gene" is the smallest, independently functional unit of genetic material which codes for a protein product or controls or affects transcription and comprises at least one DNA sequence.

5 A "vector" is a polynucleotide in which another polynucleotide segment is attached, so as to bring about a particular desired result with the attached segment. Such vectors include replicons (*i.e.*, replication competent vectors) and non-replicating vectors such as suicide vectors.

A "coding sequence" is a polynucleotide sequence which is transcribed and/or translated into a polypeptide.

10 A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase and initiating transcription of a downstream (*i.e.*, in the 3' direction) coding sequence.

A coding sequence is "under the control" of the promoter sequence in a cell when transcription of the coding sequence results from the binding of RNA polymerase to the promoter sequence; translation of the resulting mRNA then results.

15 "Operably linked" refers to a juxtaposition wherein the components are configured so as to perform their usual function. Thus, control sequences operably linked to a coding sequence are capable of effecting the expression of the coding sequence.

20 "Control sequences" refers to those sequences which control the transcription or translation of a coding sequence; these may include, but are not limited to, promoter sequences, transcriptional initiation and termination sequences and translational initiation and termination sequences. In addition, "control sequences" refers to sequences which control the processing of the polypeptide encoded within the coding sequence; these may include, but are not limited to sequences controlling secretion, protease cleavage, and glycosylation of the polypeptide.

5 "Transformation" is the insertion of an exogenous polynucleotide (*i.e.*, the transgene) into a host cell. The exogenous polynucleotide is integrated within the host genome. In the present invention transformation generally means insertion of exogenous genetic material into the host genome. "Genome", as used herein, refers to the genetic material found in the host arranged in chromosomes.

10 "Transfection" is the method whereby an exogenous polynucleotide is transferred into a host cell. The methods include, for example, electroporation, CaCl_2 and other methods well known to those of skill in the art. Transfection may result in stable transformation of the host cell, transient transformation of the host cell, or any temporal period therebetween in which the exogenous polynucleotide is present in the host cell or the host cell's progeny.

15 As used herein, "nucleic acid construct" refers to a plasmid, vector, virus or other vehicle known in the art that has been manipulated to include nucleic acid sequences. Generally, such constructs contain a promoter sequence which facilitates the efficient transcription of the inserted genetic sequence or transcription of selectable markers. Methods which are well known to those skilled in the art can be used to produce nucleic acid constructs containing a coding sequence and appropriate transcriptional/translational control signals. These methods include *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* recombination/genetic techniques. (See, for example, the techniques described in Maniatis *et al.*, 1989 Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y.)

20

25 The invention provides a method for creating a mutation in a target gene or polynucleotide sequence. As used herein, the term "mutation" refers to a change in the nucleotide sequence of a gene or polynucleotide sequence. The invention provides a method for producing a nonpathogenic organism, by generating a mutation in a gene or polynucleotide sequence associated with a virulence phenotype. Such a polynucleotide may encode a virulent associated polypeptide. The method includes inserting a nucleic acid sequence encoding a selectable marker into the site of the mutation; introducing the mutated polynucleotide into a

chromosomal gene of an organism to produce a mutation in the chromosomal gene; and selecting organisms having the mutation.

In another embodiment, a chromosomal gene encoding a polypeptide associated with a specific phenotype in the genome of an organism is disrupted by cloning into the gene a selectable marker. In this way, the chromosomal gene is disrupted thus providing a knockout mutation. In another embodiment, a chromosomal gene encoding a polypeptide associated with a specific phenotype in the genome of an organism is disrupted by cloning into a gene a polynucleotide capable of hybridizing to the chromosomal gene. A vector containing the polynucleotide may be used. The vector can be any vector, for example a suicide vector. The suicide vector containing the cloned polynucleotide is capable of undergoing homologous recombination with the chromosomal gene.

In one embodiment, the nucleic acid sequence encoding a selectable marker encodes resistance to kanamycin, however, it will be recognized that any number of selectable markers can be used including for example, ampicillin resistance, kanamycin resistance, tetracycline resistance and others well known to those skilled in the art. For example, a mobilized suicide vector, encoding a gene for resistance to tetracycline (*tet*), may be cloned into the polynucleotides encoding the polypeptide for selection of the mutated polynucleotide on tetracycline plates to produce a knockout mutation.

"Genotype" is the genetic constitution of an organism. "Phenotypic expression" is the expression of the code of a DNA sequence or sequences which results in the production of a product, *e.g.*, a polypeptide or protein, or alters the expression of the organism's natural phenotype. As used herein, the term "specific phenotype" refers to an organism's phenotype as a result of expression of its genotype. For example, phenotype encompasses physical traits displayed by an individual. These include both visible traits such as size, structure, colour, and also invisible traits, such as the ability to synthesize a particular enzyme. The phenotype is determined both by the organism's genotype and by its environment. For example, a specific phenotype may be associated with bacterial pathogenicity. Examples of phenotypes

associated with bacterial pathogenicity include a molecular mechanisms by which bacteria cause disease. Many bacteria can infect humans or animals, sustain themselves, and multiply on or in host tissues. The pathogenicity of bacteria is complex and multifactorial, often involving a series of biochemical mechanisms acting in concert to produce disease. The genes encoding the various virulence determinants involved may be located on the bacterial chromosome, but are frequently carried on extrachromosomal polynucleotides (e.g., plasmids), for example the toxins produced by many pathogenic *Escherichia coli*, or on bacteriophages, for example diphtheria toxin in *Corynebacterium diphtheriae*. Bacterial virulence factors can be divided broadly into those that assist colonization of the host (e.g. adherence to tissue surfaces and invasion of host cells) and those that assist survival in the hostile environment therein (e.g. resistance to host defenses and the production of toxins).

Specific *E. coli* phenotypes have been associated with intestinal diseases, notably diarrhoea, and extraintestinal conditions including urinary tract infections and meningitis in the newborn. Like many pathogens, *E. coli* strains produce adhesins structures that mediate attachment to eukaryotic cells and which can be distinguished by their specificity for receptors on the target cell. Adhesins can represent the filamentous, hair-like structures known as fimbriae or pili, or they may be nonfilamentous components of the cell surface. Common F1A (type 1) fimbrial adhesins recognize the sugar α -mannose in glycoproteins, whereas mannose-resistant (MR) adhesins bind to eukaryotic receptors other than mannose.

A wide range of filamentous adhesins are produced by different *E. coli* strains with specificities for various receptors on human and animal tissues. Pathogenic strains may contain sets of genes encoding one or more types of fimbriae, sometimes in combination with nonfimbrial adhesins.

P pili of *E. coli*, which represent the main group of MR fimbriae associated with urinary tract infections, possess G-adhesins, which mediate the binding of uropathogenic *E. coli* to a digalactoside receptor on the epithelial surface. The biosynthesis and expression of P pili are encoded by the pap gene cluster present on the chromosome of P fimbriate *E. coli*. Disruption of one or more of these genes would effect the pathogenicity of this organism. This complex

structure can be viewed as a means of increasing the chances of successful contact between the adhesin molecule and its receptor on the host epithelial surface.

Many bacteria are surrounded in a layer of capsular polysaccharide which lies outside the cell envelope. The capsule protects the bacterium against desiccation, but also assists in resistance to phagocytosis and the lethal effects of serum. Bacteria of the same species may produce chemically distinct capsular polysaccharide, for example >70 types can be produced in *E. coli*. Blocks of genes encoding translocation of polysaccharide across the inner and outer membranes to the cell surface are conserved and flank a central variable group of genes responsible for biosynthesis of the specific capsule concerned.

In addition, phenotypic changes in toxin synthesis are included in the invention. For example, enterotoxigenic *E. coli* elaborate at least two types of enterotoxin which induce secretory diarrhoea in humans and animals: a high molecular weight heat-labile toxin (LT), and a low molecular weight heat-stable toxin (ST). LTs belong to a family of cholera-like protein.

Many pathogenic bacteria also exhibit qualitative changes in phenotype in the form of antigenic variation of their dominant exposed surface components. This assists the pathogen to avoid antibodies elicited in the host by previous infections of the same type. An example of antigenic variation is provided by the related pathogenic bacterium *N. gonorrhoeae*, the causative agent of gonorrhoea, and *N. meningitidis*, the causative agent of meningococcal meningitis. Disruption or mutation of genes associated with such antigenic variation are also encompassed by the methods of the present invention.

When the vector is a plasmid, it generally contains a variety of components including promoters, signal sequences, phenotypic selection genes, origins of replication sites, and other necessary components as are known to those of skill in the art. Promoters most commonly used in prokaryotic vectors include the *lacZ* promoter system, the alkaline phosphatase *phoA* promoter, the bacteriophage λ PL promoter (a temperature sensitive promoter), the *tac* promoter (a hybrid *trp-lac* promoter regulated by the *lac* repressor), the

tryptophan promoter, and the bacteriophage T7 promoter. For example, the low-copy vector pMW118 is under the control of the *lacZ* promoter. The vector may also include mobilized suicide vectors. Such suicide vectors are useful for generating mutants (in both bacteria and eukaryotes) by nonspecific insertion of mobile genetic elements (transposons) into the DNA required to be mutated. This vector not only cause insertion mutations which disrupt gene function, but, because of the presence of an identifiable marker facilitates identification and cloning of the disrupted gene. Transposon mutagenesis is typically accomplished by conjugal transfer or transfection of a plasmid vector carrying a transposon to a recipient cell in which the plasmid will not replicate. Once the transposon-loaded vector is introduced into a recipient cell, the transposon inserts a copy of itself into the recipient DNA. Because the plasmid cannot replicate it is soon diluted out and lost. Potential mutants are isolated by their ability to grow in the presence of a particular antibiotic (*i.e.*, selectable marker) to which resistance has been conferred by the transposon.

As used herein, "host cells" are cells in which a vector can be harbored and its DNA expressed, recombined with the host DNA, or propagated. The term also includes any progeny of the subject host cell. It is understood that all progeny may not be identical to the parental cell since there may be mutations that occur during replication. However, such progeny are included when the term "host cell" is used. Methods of stable transfer, meaning that the foreign DNA is continuously maintained in the host, are known in the art.

Transformation of a host cell with recombinant DNA may be carried out by conventional techniques as are well known to those skilled in the art. Where the host is prokaryotic, such as *E. coli*, competent cells which are capable of DNA uptake can be prepared from cells harvested after exponential growth phase and subsequently treated by the CaCl_2 method using procedures well known in the art. Alternatively, MgCl_2 or RbCl can be used.

Transformation can also be performed after forming a protoplast of the host cell if desired. For another example, triparental conjugation may be used to genetically introduce vector into *E. coli*. The transformed cells are selected by growth on an antibiotic, commonly tetracycline

(*tet*) or ampicillin (*amp*), to which they are rendered resistant due to the presence of *tet* or *amp* resistance genes on the vector.

Among the prokaryotic organisms which may serve as host cells are *E. coli* strain JM101, *E. coli* K12 strain 294 (ATCC number 31,446), *E. coli* strain W3110 (ATCC number 27,325),
5 *E. coli* X1776 (ATCC number 31, 537), *E. coli* XL-1Blue (Stratagene), and *E. coli* B; however, many other strains of *E. coli*, such as HB101, NM522, NM538, NM539 and many other species and genera of prokaryotes can be used as well. Besides the *E. coli* strains listed above, bacilli such as *Bacillus subtilis*, other enterobacteriaceae such as *Salmonella typhimurium* or *Serratia marcesans* and various *Pseudomonas* species can all be used as
10 hosts. In one specific embodiment, the prokaryotic host cell is enteropathogenic *E. coli*.

The nucleic acid construct of the invention comprises a suitable detectable marker expressed under the control of a promoter that is active in a targeted cell. "Detectable marker" or "selectable marker", as used herein, refers to any identifiable composition useful for distinguishing cells containing a nucleic acid construct of the present invention from those
15 cells that do not contain such a construct.

As previously noted, the nucleic acid construct can contain a promoter for expression of the detectable marker. "Promoter", as used herein, refers to a minimal sequence sufficient to direct transcription. Depending on the host/vector system utilized, any of a number of suitable transcription and translation elements, including constitutive and inducible
20 promoters, transcription enhancer elements, transcription terminators, *etc.* may be used in the nucleic acid construct (see *e.g.*, Bitter *et al.*, *Methods in Enzymology* 153:516, 1987).

"Transformed", as used herein, refers to a cell into which (or into an ancestor of which) has been introduced, by means of recombinant nucleic acid techniques, a heterologous nucleic acid molecule. Random integration of heterologous DNA into the genome may disrupt
25 endogenous genes which are necessary for the maturation, differentiation and/or viability of

the cells or organism. Homologous recombination or integration is encompassed by this term.

5 The homology-dependent recombination between exogenous DNA and chromosomal DNA sequences is sometimes referred to as "gene targeting". Gene targeting allows the transfer of genetic alterations/mutations created *in vitro* to precise sites within the host cellular genome. Thus, the present invention provides a method for facilitating homologous recombination events between DNA sequences residing in the genome of a cell or organism and newly introduced DNA sequences. This provides a means for systematically altering the genome of the cell or organism. "Homologous recombination" is defined herein as recombination
10 between related or identical DNA sequences; "non-homologous recombination" is defined as recombination between unrelated DNA sequences.

As used herein, a "target DNA sequence" is a region within the genome of a cell which is targeted for modification by the nucleic acid construct of the invention. Target DNA sequences can include, for example, structural genes (*i.e.*, DNA sequences encoding
15 polypeptides), regulatory sequences such as enhancers sequences, promoters and the like and other regions within the genome of interest. A target DNA sequence may also be a sequence which, when targeted by a construct of the invention, has no effect on the function of the host genome. Generally, the target DNA contains at least first and second regions. Each region contains a homologous sequence portion which is used to design the construct of the
20 invention. The homologous portions of the target DNA are homologous to sequence portions contained in the nucleic acid construct of the invention.

The precise procedure used to introduce the altered genetic material into the host cell is not critical. Any of the well known procedures for introducing foreign nucleotide sequences into host cells may be used. These include use of plasmid vectors, viral vectors and any other
25 well known methods for introducing cloned genomic DNA, cDNA, synthetic DNA or other foreign genetic material into the host cell. It is only necessary that the particular genetic engineering procedure utilized be capable of successfully introducing the nucleic acid

construct of the invention into the host cell which is then capable of expressing the desired selectable marker.

Detection of integration

As previously described, a nucleic acid construct of the present invention is useful for
5 introducing exogenous nucleic acid sequence into a host cell. The integration of a construct of the invention can be detected by PCR techniques. Such a technique is particularly useful when the integrated construct contains unique sequences not endogenous to the genome of the host cell.

In general, the primers used for PCR amplification according to the method of the invention
10 embrace oligonucleotides of sufficient length and appropriate sequence which provides specific initiation of polymerization of a significant number of nucleic acid molecules containing the target nucleic acid under the conditions of stringency for the reaction utilizing the primers. In this manner, it is possible to selectively amplify a specific nucleic acid. Specifically, the term "primer" as used herein refers to a sequence comprising two or more
15 deoxyribonucleotides or ribonucleotides, preferably at least eight, which sequence is capable of initiating synthesis of a primer extension product that is substantially complementary to a target nucleic acid strand. The oligonucleotide primer typically contains 15-22 or more nucleotides, although it may contain fewer nucleotides as long as the primer is of sufficient specificity to allow essentially only the amplification of the specifically desired target
20 nucleotide sequence (*i.e.*, the primer is substantially complementary).

Experimental conditions conducive to synthesis include the presence of nucleoside triphosphates and an agent for polymerization, such as DNA polymerase, and a suitable temperature and pH. The primer is preferably single stranded for maximum efficiency in amplification, but may be double stranded. If double stranded, the primer is first treated to
25 separate its strands before being used to prepare extension products. Preferably, the primer is an oligodeoxyribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the inducing agent for polymerization. The exact

length of primer will depend on many factors, including temperature, buffer, and nucleotide compound.

Primers used according to the method of the invention are designed to be "substantially" complementary to each strand of a target nucleotide sequence to be amplified. Substantially complementary means that the primers must be sufficiently complementary to hybridize with their respective strands under conditions which allow the agent for polymerization to function. In other words, the primers should have sufficient complementarity with the flanking sequences to hybridize therewith and permit amplification of the nucleotide sequence. Preferably, the 3' terminus of the primer that is extended has perfectly base paired complementarity with the complementary flanking strand.

Oligonucleotide primers used according to the invention are employed in any amplification process that produces increased quantities of target nucleic acid. Typically, one primer is complementary to the negative (-) strand of the mutant nucleotide sequence and the other is complementary to the positive (+) strand. Annealing the primers to denatured nucleic acid followed by extension with an enzyme, such as the large fragment of DNA Polymerase I (Klenow) or Taq DNA polymerase and nucleotides or ligases, results in newly synthesized + and -strands containing the target nucleic acid. Because these newly synthesized nucleic acids are also templates, repeated cycles of denaturing, primer annealing, and extension results in exponential production of the region (*i.e.*, the target mutant nucleotide sequence) defined by the primer. The product of the amplification reaction is a discrete nucleic acid duplex with termini corresponding to the ends of the specific primers employed. Those of skill in the art will know of other amplification methodologies which can also be utilized to increase the copy number of target nucleic acid.

The amplified product may be detected by Southern blot analysis, with or without using radioactive probes. In such a process, for example, a small sample of DNA containing a very low level of a heterologous nucleic acid sequence is amplified, and analyzed via a Southern blotting technique. The use of non-radioactive probes or labels is facilitated by the high level

of the amplified signal. Other methods of detection include, for example, detecting expression of a selectable marker, such as a resistance to an antibiotic.

The present invention provides a method and compositions useful in identifying and modulating a specific phenotype of an organism. Specifically, the present invention can be used to identify genes encoding a specific phenotype. More specifically, the present invention provide a method of identifying a gene associated with pathogenesis of a particular organism.

According to the methods of the invention, nucleic acids are isolated from a first organism. The nucleic acids can be isolated by any number of means well known to those of skill in the art. Such isolation methods will depend upon the quantity of nucleic acid desired as well as the type of organism. For example, RNA can be isolated using guanidinium isothiocyanate techniques. Such techniques are the basis of a number of commercial products. DNA can be obtained from prokaryotes as well as eukaryotes by a number of technique. For example, there are a number of commercially available kits for performing minipreps, midipreps and maxipreps of DNA from bacteria (see also, Wilson, K. 1987. Preparation of genomic DNA from bacteria, p.2.4.1-2.4.5 In F.M. Ausubel *et al.*, Current Protocols in Molecular Biology. John Wiley & Sons, New York, N.Y.).

A nucleic acid can be isolated from any sample or organism, including, but not limited to, eukaryotic cells, prokaryotic cells, biological samples (e.g., blood, serum, sputum, saliva, urine). Such samples or organisms can be obtained from *in vitro* cell cultures, including agar plates or broth. Alternatively, readily made genomic or cDNA libraries can be purchased and used as a source of the nucleic acid.

A nucleic acid obtained by the techniques described herein are obtained from more than one source. For example, a nucleic acid (*i.e.*, DNA or RNA) is isolated from both a first organism and a second organism. Alternatively, the nucleic acid may be obtained from a first nucleic acid library and a second nucleic acid library or a combination of an organism and a

library. The first organism or first library and second organism or second library, contain nucleic acids from phylogenetically related organisms. By "phylogenetically related" is meant organisms falling within the same phylogenetic family. Preferably the organisms are of the same genus. More preferably, the organisms are of the same species.

5 Once the nucleic acids are obtained the nucleic acids are processed and compared. Methods of comparing the nucleic acids from one organism or library to a second organism or library are well known in the art. For example, such techniques include plus and minus screening techniques. These techniques are particularly suitable for isolating tissue-specific or developmentally regulated cDNA sequence or clones derived from mRNAs that are induced
10 by particular treatments. Additionally, these techniques are especially suited to determining specific genotypic differences between related organism, and more particularly to identifying specific phenotypes related to the genotype. Such techniques are described in Old and Primrose, Principles of Gene Manipulation, Blackwell Scientific Publications (1989). Such techniques include colony hybridization techniques. For example, clones of a nucleic acid population isolated from a first organism and cloned into an unrelated host cell are probed
15 with a second nucleic acid population from a second organism. In this instance the probe is labeled. The probe can be labeled with any number of markers including, for example, fluorescent markers, radioactive markers or other markers well known to those of skill in the art. Using this technique, clones that are not labeled represent clones containing nucleic acid sequences which are not found in the second nucleic acid population (*i.e.*, the nucleic acid
20 obtained from the second organism).

In another technique, subtractive hybridization techniques are utilized. This technique involves comparing a first nucleic acid population obtained from a first organism or library to that of a second nucleic acid population obtained from a second organism or library.

25 Comparing the two populations can be performed by combining the nucleic acid populations under conditions to allow hybridization of the nucleic acids. For example, in nucleic acid hybridization reactions, the conditions used to achieve a particular level of stringency will vary, depending on the nature of the nucleic acids being hybridized. For example, the length,

degree of complementarity, nucleotide sequence composition (*e.g.*, GC v. AT content), and nucleic acid type (*e.g.*, RNA v. DNA) of the hybridizing regions of the nucleic acids can be considered in selecting hybridization conditions. An additional consideration is whether one of the nucleic acids is immobilized, for example, on a filter. An example of progressively
5 higher stringency conditions is as follows: 0.2 x SSC/0.1% SDS at about room temperature (low stringency conditions); 0.2 x SSC/0.1% SDS at about 42°C (moderate stringency conditions); and 0.1 x SSC at about 68°C (high stringency conditions). Washing can be carried out using only one of these conditions, *e.g.*, high stringency conditions, or each of the conditions can be used, *e.g.*, for 10-15 minutes each, in the order listed above, repeating any
10 or all of the steps listed. However, as mentioned above, optimal conditions will vary, depending on the particular hybridization reaction involved, and can be determined empirically.

Following hybridization, a third population of nucleic acids representing non-hybridized nucleic acids are obtained. This third population can be further processed by any number of
15 molecular biology techniques including for example, cloning into expression vectors, cloning into suicide vectors, PCR amplifying, reverse transcribing or other techniques well known to those of skill in the art. Commercially available subtractive hybridization kits are available from a number of companies, for example, PCR-Select Bacterial Genome Subtraction Kit (Clontech Laboratories, Inc.).

The first nucleic acid population and the second nucleic acid population are obtained from
20 different organisms. In one embodiment the organisms are related. In another embodiment, the first organism is a virulent organism and the second organism is a non-virulent organism. In a preferred embodiment, the third nucleic acid population represents at least one unique polynucleotide sequence, which is unique to the first organism (*e.g.*, the virulent organism).
25 For example, upon comparison at least one nucleic acid sequence will be obtained that is not contained in the second nucleic acid population (*i.e.*, unique to the first nucleic acid population).

The third nucleic acid population can be cloned into an expression vector and transfected into a host cell. Such host-expression systems represent vehicles by which the coding sequences of interest may be produced and subsequently purified, but also represent cells which, when transformed or transfected with the appropriate nucleotide coding sequences, exhibit the phenotype coded by the gene product of the invention *in situ*. These hosts include, but are not limited to, microorganisms such as bacteria (*e.g.*, *E. coli*, *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing a selected polynucleotide from the third nucleic acid population; yeast (*e.g.* *Saccharomyces*, *Pichia*) transformed with recombinant yeast expression vectors containing a selected polynucleotide from the third nucleic acid population; insect cell systems infected with recombinant virus expression vectors (*e.g.*, baculovirus) containing a selected polynucleotide from the third nucleic acid population; plant cell systems infected with recombinant virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing a selected polynucleotide from the third nucleic acid population; or mammalian cell systems (*e.g.*, COS, SHO, BHK, 293, 3T3) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the adenovirus late promoter; the vaccinia virus 7.5K promoter).

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for a selected polynucleotide from the third nucleic acid population being expressed. For example, when a large quantity of such a polypeptide expressed from the polynucleotide is to be produced, for the generation of pharmaceutical compositions or for raising antibodies to the polypeptide, for example, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited to the *E. coli* expression vector pUR278 (Ruther *et al.*, 1983, *EMBO J.* 2:1791), in which a selected polynucleotide from the third nucleic acid population may be ligated individually into the vector in frame with the *lac z* coding region that a fusion protein is produced; pIN vectors (Inouye & Inouye, 1985, *Nucleic Acids Res.* 13:3101-3109); and the like. pGEX vectors may also be used to express foreign

polypeptide as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

In an insect system, *Autographa colifornica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperday* cells. A selected polynucleotide from the third nucleic acid population may be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under the control of an AcNPV promoter. Successful insertion of a selected polynucleotide from the third nucleic acid population will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus. These recombinant viruses are then used to infect *S. frugiperda* cells in which the inserted gene is expressed.

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, a selected polynucleotide from the third nucleic acid population of interest may be ligated to an adenovirus transcription/translation control complex, *e.g.*, the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (*e.g.*, region E1 or E3) will result in a recombinant virus that is viable and capable of expressing a selected polynucleotide from the third nucleic acid population in infected hosts (See Logan & Shenk, 1984, *Proc. Natl. Acad. Sci. USA* 81:3655-3659). Specific initiation signals may also be required for efficient translation of a selected polynucleotide from the third nucleic acid population. These signals include the ATG initiation codon and adjacent sequences. In cases where an entire gene, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translation control signals may be needed. However, in cases where only a portion of a coding sequences is inserted,

exogenous translational control signals, including, the ATG initiation codon must be provided.

Alternatively, suicide vectors may be used as described above. In this embodiment, a selected polynucleotide from the third nucleic acid population is cloned into the suicide vector. The polynucleotide is then transfected into a host cell. The host cell can be phylogenetically related or non-related. The suicide vector then undergoes homologous recombination with the genome of the host cell. In a preferred embodiment, the host cell is selected from a phylogenetically related organism, the first organism, or the second organism.

Transfection via retroviral vectors, naked DNA methods and mechanical methods including micro injection and electroporation may be used to provide either stably transfected host cells (*i.e.*, host cells that do not lose the exogenous DNA over time) or transient transfected host cells (*i.e.*, host cells that lose the exogenous DNA during cell replication and growth).

Screen for compounds which modulate virulence

In another embodiment, the invention provides a method for identifying a compound which modulates the activity of a gene associated with a specific phenotype, for example a virulence gene and the protein product encoded therefrom. The method includes: a) incubating components comprising the compound and the encoded product under conditions sufficient to allow the components to interact; and b) determining the effect of the compound on the product activity before and after incubating in the presence of the compound. Compounds that affect the product activity include peptides, peptidomimetics, polypeptides, chemical compounds and biologic agents.

Incubating includes conditions which allow contact between the test compound and the product of the selected polynucleotide or its related gene. Contacting includes in solution and in solid phase, or in a cell. The test compound may optionally be a combinatorial library for screening a plurality of compounds. Compounds identified in the method of the invention can be further evaluated, detected, cloned, sequenced, and the like, either in solution or after binding to a solid

support, by any method usually applied to the detection of a specific DNA sequence such as PCR, oligomer restriction (Saiki, *et al.*, *Bio-Technology*, 3:1008-1012, 1985), allele-specific oligonucleotide (ASO) probe analysis (Conner, *et al.*, *Proc. Natl. Acad. Sci. USA*, 80:278, 1983), oligonucleotide ligation assays (OLAs) (Landegren, *et al.*, *Science*, 241:1077, 1988), and the like. Molecular techniques for DNA analysis have been reviewed (Landegren, *et al.*, *Science*, 242:229-237, 1988).

As used herein, "gene product" means a polypeptide encoded by a gene identified using the methods of the invention. Gene product also encompasses products encoded by polynucleotide sequence which may be fragments of a full length gene. For example, gene product encompasses virulence associated polypeptide sequence encoded by a virulence gene or gene fragment (*e.g.*, a polynucleotide sequence encoding less than the full gene).

As previously noted, included in the screening method of the invention are combinatorial chemistry methods for identifying chemical compounds that bind to a product encoded by a gene associated with a specific phenotype, for example a virulence gene. See, for example, Plunkett and Ellman, "*Combinatorial Chemistry and New Drugs*", *Scientific American*, April, p.69, (1997).

It is envisioned that the method of the invention can be used to identify ligands or substrates that bind to, modulate or mimic the action of the gene product. The gene product may have the biological activity associated with the wild-type gene product, or may have a loss of function mutation due to a point mutation in the coding sequence, substitution, insertion, deletion or scanning mutations.

Areas of investigation for combinatorial chemistry are the development of therapeutic treatments. Drug screening identifies agents or compounds that provide a replacement, enhancement or regulation of protein's function in affected cells. Of particular interest are screening assays for agents that have a low toxicity for human cells. A wide variety of assays may be used for this purpose, including labeled *in vitro* protein-protein binding assays, protein-

DNA binding assays, electrophoretic mobility shift assays, immunoassays for protein binding, and the like. The purified protein may also be used for determination of three-dimensional crystal structure, which can be used for modeling intermolecular interactions, transcriptional regulation, *etc.*

5 The term "agent" or "compound" as used herein describes any molecule, *e.g.* protein or pharmaceutical, with the capability of altering or mimicking the physiological function of a virulence protein. Generally a plurality of assay mixtures are run in parallel with different agent concentrations to obtain a differential response to the various concentrations. Typically, one of these concentrations serves as a negative control, *i.e.* at zero concentration or below the level of
10 detection.

Candidate agents encompass numerous chemical classes, though typically they are organic molecules, preferably small organic compounds having a molecular weight of more than 50 and less than about 2,500 daltons. Candidate agents comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least
15 an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The candidate agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Candidate agents are also found among biomolecules including, but not limited to: peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs
20 or combinations thereof.

Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides and oligopeptides. Alternatively, libraries of natural compounds
25 in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means, and may be used to produce

combinatorial libraries. Known pharmacological agents may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification or amidification to produce structural analogs.

Where the screening assay is a binding assay, one or more of the molecules may be joined to a label, where the label can directly or indirectly provide a detectable signal. Various labels include radioisotopes, fluorescers, chemiluminescers, enzymes, specific binding molecules, particles, *e.g.* magnetic particles, and the like. Specific binding molecules include pairs, such as biotin and streptavidin, digoxin and antidigoxin. For the specific binding members, the complementary member would normally be labeled with a molecule that provides for detection, in accordance with known procedures.

A variety of other reagents may be included in the screening assay. These include reagents like salts, neutral proteins, *e.g.* albumin, detergents, etc that are used to facilitate optimal protein-protein binding and/or reduce non-specific or background interactions. Reagents that improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors or anti-microbial agents may be used. The mixture of components are added in any order that provides for the requisite binding. Incubations are performed at any suitable temperature, typically between 4 and 40°C. Incubation periods are selected for optimum activity, but may also be optimized to facilitate rapid high-throughput screening. Typically between 0.1 and 1 hours will be sufficient.

The compounds having the desired pharmacological activity may be administered in a physiologically acceptable carrier to a host for treatment of disease attributable to a virulence gene or gene product. The therapeutic agents may be administered in a variety of ways, orally, topically, parenterally *e.g.* subcutaneously, intraperitoneally, by viral infection, intravascularly, *etc.* Depending upon the manner of introduction, the compounds may be formulated in a variety of ways. The concentration of therapeutically active compound in the formulation may vary from about 0.1-100 wt.%.

The pharmaceutical compositions can be prepared in various forms, such as granules, tablets, pills, suppositories, capsules, suspensions, salves, lotions and the like. Pharmaceutical grade organic or inorganic carriers and/or diluents suitable for oral and topical use can be used to make up compositions containing the therapeutically-active compounds. Diluents known to the art include aqueous media, vegetable and animal oils and fats. Stabilizing agents, wetting and emulsifying agents, salts for varying the osmotic pressure or buffers for securing an adequate pH value, and skin penetration enhancers can be used as auxiliary agents.

Therapeutic Compositions

In another embodiment, the present invention provides therapeutic compositions useful in treating infections associated with bacterial pathogenesis or associated with a virulence phenotype. Such compositions include but are not limited to both active vaccines and passive vaccines.

By "passive vaccine" is meant the administration of antibodies against a particular virulent associated polypeptide to a subject. For example, in neonatal rats passive immunity is established by the transcytosis of maternal immunoglobulin across gut epithelia. In the low pH conditions of the gut, immunoglobulin binds to receptors on the apical membrane of duodenal epithelial cells. The immunoglobulin Nreceptor complexes are internalized through coated pits, delivered to apical endosomes, and subsequently sorted into vesicles (transcytotic vesicles) which deliver them, either directly or via the basolateral endosome system, to the basolateral membrane. In the neutral pH conditions of the basolateral surface the immunoglobulin dissociates from the receptor. In contrast, IgA is captured from the blood by IgA receptors at the sinusoidal membrane of, for example hepatocytes, the complex is transported via the endosomal system to the bile canaliculus (apical membrane) and released by proteolytic cleavage of the IgA receptor.

Such vaccines can be developed using the methods of the present invention. As discussed above, it is possible to identify genes and gene products related to particular virulent phenotypes. For example, upon identifying a loss of pathogenicity or virulence by homologous recombination,

the polynucleotide contained in the vector associated with the loss of a virulent phenotype can be identified by techniques known in the art, for example by duplicate plating techniques. The vector containing the polynucleotide of interest can then be obtained and cloned into an appropriate vector for expression of the polynucleotide product. Examples of expression vectors
5 have been disclosed above, however, any vector capable of transcribing and/or translating the polynucleotide are encompassed by the present invention. Once expressed the resulting product can be obtained. The product may be further purified by techniques known in the art, for example, by chromatography and/or gel electrophoresis. Alternatively, the polynucleotide can be used as a probe to probe a genomic library.

10 Antibodies can be developed against the product by any number of means well known to those skilled in the art. For example, such antibodies may include, but are not limited to, polyclonal antibodies, monoclonal antibodies, humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab')₂ fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above. Such antibodies may
15 be used, for example, in the detection of a gene product in a biological sample, including, but not limited to, blood, plasma, and serum. Alternatively, the antibodies may be used as a method for the inhibition of a gene product's activity. Thus, such antibodies may be utilized as part of treatment for disease associated with the product of the selected polynucleotide, and may be used as part of diagnostic techniques whereby patients may be tested for abnormal levels of a gene
20 products, or for the presence of abnormal forms of such proteins.

For the production of antibodies against a selected polynucleotide product of the third nucleic acid population or its related gene product, various host animals may be immunized by injection with a polypeptide encoded by a polynucleotide of interest, or a portion thereof. Such host animals may include but are not limited to rabbits, mice, and rats, to name but a few. Various
25 adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides,

oil emulsion, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG, interferon and other cytokines effecting immunological response.

Polyclonal antibodies are a heterogenous population of antibody molecules derived from the sera of animals immunized with an antigen, such as a product of the polynucleotide of interest, or an antigenic functional derivative thereof. In general, for the production of polyclonal antibodies, host animals such as those described above, may be immunized by injection with a product encoded by a polynucleotide supplemented with adjuvants as also described above.

Monoclonal antibodies (mAbs), which are homogenous population of antibodies to a particular antigen, may be obtained by any technique which provides for the production of antibody molecules by continuous cell lines in culture. These techniques include, but are not limited to, the hybridoma technique of Kohler and Milstein, (1975, Nature 256:495-497; and U.S. Patent No. 4,376,110), human B-cell hybridoma technique (Kosbor *et al.*, 1983, Immunology Today 4:72; Cole *et al.*, 1983, Proc. Natl. Acad. Sci. USA 80:2026-2030), and the EBV-hybridoma technique (Cole *et al.*, 1985, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Such antibodies may be of any immunoglobulin class including I.G., IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the mAb of this invention may be cultivated *in vitro* or *in vivo*. Production of high titers of mAbs *in vivo* makes this the presently preferred method of production.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison *et al.*, 1984, Proc. Natl. Acad. Sci. 81:6851-6855; Neuberger *et al.*, 1984, Nature, 312:604-608; Takeda *et al.*, 1985, Nature, 314:452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. A chimeric antibody is molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region.

Alternatively, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778; Bird, 1988, Science 242:423-426; Huston *et al.*, 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; and Ward *et al.*, 1989, Nature 334:544-546) can be adapted to produce single chain antibodies against a product encoded by a polynucleotide. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

Antibody fragments which recognize specific epitopes may be generated by known techniques. For example, such fragments include but are not limited to: the F(ab')₂ fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab')₂ fragments. Alternatively, Fab expression libraries may be constructed to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

The antibodies describe above, are capable of interacting with the antigenic determinant or a polypeptide. By "interact" is meant, for example, the ability of the antibody to bind, recognize, contact or modulate the activity or effect of the antigen or polypeptide.

By "active vaccine" is meant the process of immunizing a subject with an attenuated pathogen or antigenic determinant in order to raise an immune response in a host. The process provides a direct immunostimulatory response as the result of immunization with an antigenic determinant. The antigenic determinant may be any agent that creates a immune response. For example, attenuated pathogen (*e.g.*, knockout organism), surface antigens, toxins, polypeptides and fragments thereof identified, purified or developed using the methods of the invention.

DNA Vaccines

This invention further relates to a method of providing protective immunity to vertebrates, including humans, against disease caused by a pathogenic bacteria. Protective immunity of the invention elicits humoral and/or cell-mediated immune responses which interfere with the infectivity or activity of a pathogenic organism, or which limit its spread or growth, resulting in

protection against subsequent challenge by the pathogen. According to the present invention, a nucleic acid construct containing a DNA transcription unit encoding an antigen identified by the method of the invention is administered to an individual in whom immunization and protection is desired.

5 A DNA transcription unit is a polynucleotide sequence, bounded by an initiation site and a termination site, that is transcribed to produce a primary transcript. As used herein, a "DNA transcription unit" includes at least two components: (1) antigen-encoding DNA, and (2) a transcriptional promoter element or elements operatively linked for expression of the antigen-encoding DNA. Antigen-encoding DNA can encode one or multiple antigens, such as
10 antigens from two or more different rotavirus proteins. The DNA transcription unit can additionally be inserted into a vector which includes sequences for expression of the DNA transcription unit.

A DNA transcription unit can optionally include additional sequences such as enhancer elements, splicing signals, termination and polyadenylation signals, viral replicons, and bacterial plasmid
15 sequences. In the present method, a DNA transcription unit (*i.e.*, one type of transcription unit) can be administered individually or in combination with one or more other types of DNA transcription units.

DNA transcription units can be produced by a number of known methods. For example, DNA encoding the desired antigen can be inserted into an expression vector (see, for example,
20 Sambrook *et al.*, Molecular Cloning. A Laboratory Manual, 2d, Cold Spring Harbor Laboratory Press (1989)). With the availability of automated nucleic acid synthesis equipment, DNA can be synthesized directly when the nucleotide sequence is known, or by a combination of polymerase chain reaction (PCR), cloning, and fermentation. Moreover, when the sequence of the desired polypeptide is known, a suitable coding sequence for the polynucleotide can be
25 inferred.

The DNA transcription unit can be administered to an individual, or inoculated, in the presence of adjuvants or other substances that have the capability of promoting DNA uptake or recruiting immune system cells to the site of the inoculation. It should be understood that the DNA transcription unit itself is expressed in the host cell by transcription factors provided by the host cell, or provided by a DNA transcription unit.

The "desired antigen" can be any antigen or combination of antigens identified by the method of the invention. Preferably, the antigen is encoded by a virulence gene identified from a pathogenic organism by the method of the invention. The antigen or antigens can be naturally occurring, or can be mutated or specially modified. These antigens may or may not be structural components of a pathogenic organism. The encoded antigens can be translation products or polypeptides. The polypeptides can be of various lengths, and can undergo normal host cell modifications such as glycosylation, myristoylation, or phosphorylation. In addition, they can be designated to undergo intracellular, extracellular, or cell-surface expression. Furthermore, they can be designed to undergo assembly and release from cells.

Potential pathogens for which the DNA transcription unit can be used include DNA encoding antigens derived from any nucleic acid from a pathogenic organism. It is to be understood that this list includes all potential pathogens against which a protective immune response can be generated according to the methods herein described.

A subject can be inoculated through any parenteral route. For example, a subject can be inoculated by intravenous, intraperitoneal, intradermal, subcutaneous, inhalation, or intramuscular routes, or by particle bombardment using a gene gun. Muscle is a useful site for the delivery and expression of DNA transcription unit-encoded polynucleotides, because animals have a proportionately large muscle mass which is conveniently accessed by direct injection through the skin. A comparatively large dose of polynucleotides can be deposited into muscle by multiple and/or repetitive injections, for example, to extend therapy over long periods of time. Muscle cells are injected with polynucleotides encoding immunogenic polypeptides, and these polypeptides are presented by muscle cells in the context of antigens of the major

histocompatibility complex to provoke a selected immune response against the immunogen (see, e.g., Felgner, *et al.* WO90/11092, herein incorporated by reference).

The epidermis is another useful site for the delivery and expression of polynucleotides, because it is conveniently accessed by direct injection or particle bombardment. A comparatively large dose of polynucleotides can be deposited in the epidermis by multiple injections or bombardments to extend therapy over long periods of time. In immunization strategies of the invention, skin cells are injected with polynucleotides coding for immunogenic polypeptides, and these polypeptides are presented by skin cells in the context of antigens of the major histocompatibility complex to provoke a selected immune response against the immunogen.

In addition, a subject can be inoculated by a mucosal route. The DNA transcription unit can be administered to a mucosal surface by a variety of methods including DNA-containing nose-drops, inhalants, suppositories, microsphere encapsulated DNA, or by bombardment with DNA coated gold particles. For example, the DNA transcription unit can be administered to a respiratory mucosal surface, such as the nares or the trachea. Any appropriate physiologically compatible medium, such as saline for injection, or gold particles for particle bombardment, is suitable for introducing the DNA transcription unit into an individual.

Virulence Related Polynucleotides

In another embodiment, the invention discloses virulence related polynucleotide sequences and polypeptides. A virulence associated *B.pseudomallei* polynucleotide sequence (Fig. 2) derived from *B. pseudomallei* is provided (SEQ ID NO:1). Also provided is a virulence-associated *B.mallei* polynucleotide sequence (SEQ ID NO:2). As used herein, "*B.pseudomallei* virulent polynucleotide sequence" and "*B.mallei* virulent polynucleotide sequence" refers to (a) a gene containing the DNA sequence shown in Fig. 2; (b) any DNA sequence that hybridizes to the sequences shown in Fig. 2, SEQ ID NO's: 1 or 2, under stringent conditions, e.g., hybridization to filter-bound DNA in 0.5 M NaHPO₄, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C, and washing in 0.1xSSC/0.1% SDS at 68°C (Ausubel F.M. *et al.*, eds., 1989, *Current Protocols in Molecular Biology*, Vol. 1, Green Publishing Associates, Inc., and John Wiley & Sons, Inc.,

New York, at p. 2.10.3) and polypeptide product functionally equivalent to a polypeptide product encoded by sequences shown in Fig. 2; and/or (c) any DNA sequence that hybridizes to the complement of the sequences disclosed herein (as shown in Fig. 2), under less stringent conditions, such as moderately stringent conditions, *e.g.*, washing in 0.2% SSC/0.1% SDS at 42°C (Ausubel *et al.*, 1989, *supra*), and encodes a gene product functionally equivalent to a polypeptide product encoded by sequences shown in Fig. 2.

The invention also includes nucleic acid molecules, preferably DNA molecules, that hybridize to, and are therefore the complements of, the DNA sequences (a) through (c), in the preceding paragraph. Such hybridization conditions may be highly stringent or less highly stringent, as described above. In instances wherein the nucleic acid molecules are deoxyoligonucleotides ("oligos"), highly stringent conditions may refer, *e.g.*, to washing in 6xSSC/0.05% sodium pyrophosphate at 37 °C (for 14-base oligos), 48° C (for 17-base oligos), 55 °C (for 20-base oligos), and 60 °C (for 23-base oligos). These nucleic acid molecules may act at gene regulation and/or as antisense primers in amplification reactions of virulence-associated nucleic acid sequences of Figure 2. Further, such sequences may be used as part of ribozyme and/or triple helix sequences, also useful for gene regulation. Still further, such molecules may be used as components of diagnostic methods whereby the presence of a pathogen or metastatic tumor cell may be detected.

The invention also encompasses (a) DNA vectors that contain any of the foregoing polynucleotide sequences and/or their complements (*e.g.*, antisense); (b) DNA expression vectors that contain any of the foregoing coding sequences operatively associated with a regulatory element that directs the expression of the coding sequences; and (c) genetically engineered host cells that contain any of the foregoing polynucleotide sequences operatively associated with a regulatory element that directs the expression of the coding sequences in the host cell. As used herein, regulatory elements include, but are not limited to, inducible and non-inducible promoters, enhancers, operators and other elements known to those skilled in the art that drive and regulate expression.

The invention includes fragments of any of the DNA sequences disclosed herein. Fragments of the polynucleotide sequence encoding the *B.pseudomallei* and *B.mallei* virulent polynucleotide sequence are useful. Such fragments may encode truncated products that retain a biological activity of the product encoded by the *B.pseudomallei* or *B.mallei* virulent polynucleotide, such as activity or immunogenicity.

In addition to the sequences described above, homologs of such sequences, as may, for example, be present in other species, including humans, may be identified and may be readily isolated, without undue experimentation, by molecular biological techniques well known in the art. Further, there may exist genes at other genetic loci within the genome that encode proteins which have extensive homology to one or more domains of such gene products. These genes may also be identified via similar techniques.

The virulent polynucleotides of the invention and its homologs can be obtained from other organisms thought to contain similar or related pathogenic activity. For obtaining cDNA, tissues and cells in which similar virulent activity is expressed are optimal. For example, the isolated virulent polynucleotide sequences may be labeled and used to screen a cDNA library constructed from mRNA obtained from an organism of interest. The hybridization conditions used should be of a lower stringency when the cDNA library is derived from an organism different from the type of organism from which the labeled sequence was derived. Alternatively, the labeled fragment may be used to screen a genomic library derived from the organism of interest, again, using appropriately stringent condition. Low stringency conditions are well known in the art, and will vary predictably depending on the specific organism from which the library and the labeled sequences are derived. For guidance regarding such conditions see, for example, Sambrook *et al.*, 1989, *Molecular Cloning, a Laboratory Manual*, Cold Springs Harbor Press, N.Y.; and Ausubel *et al.*, 1989, *Current Protocols in Molecular Biology*, Green Publishing Associates and Wiley Interscience, N.Y.

In cases where the polynucleotide identified is the normal, or wild type, gene, this gene may be used to isolate mutant alleles of the gene. Mutant alleles may be isolated from cells or subjects

either known or proposed to have a genotype which contributes to a disease or pathogenic phenotype.

Methods of Identifying Products

5 The polypeptides encoded by the polynucleotide identified by the method of the invention can encode enzymes and enzymatically active fragment thereof. These polypeptides can be used in the production of enzymatic products. Such enzymatic products include toxins, lipopolysaccharides to name a few. These product have research and diagnostic utility in the development of assays to detect or treat pathologies associated with the product or organism.

10 The enzymatic products may be produced by any number of methods. Standard enzymology techniques well known in the art may be utilized to develop systems to provide enzymatic products (see for example the *Methods in Enzymology*, volume series published by Academic Press; and Tim Bugg, "An Introduction to Enzyme and Coenzyme Chemistry", 1997, Blackwell Sciences, Inc.).

15 "Substrate", as used herein, means any material or combinations of different materials, that may be acted upon by the polypeptide of the invention to give rise to an enzymatic product.

Cells containing and cell-free systems may be used to produce the enzymatic products of the present invention. Cells containing and cell-free systems will be better understood in the description and examples that follow. Such systems are useful in the development of enzymatic products such as toxins, and lipopolysaccharides.

20 The present invention provides a method for synthesizing enzymatic products by reacting substrates in the presence of a polypeptide encoding an enzyme or enzyme fragments, capable of catalyzing the formation of the products from the substrates.

The polypeptide may be used regardless of its origin so long as it is capable of producing the enzymatic product from the substrates. The source of the polypeptide may be derived according

to the methods and compositions as described herein, for example, through the methods of identifying a virulence related polynucleotide, and expressing the product of the polynucleotide.

5 The substrates are allowed to react with the polypeptide under suitable conditions to allow formation of the enzymatic product. Suitable conditions can be easily determined by one skilled in the art. For example, suitable conditions will include contacting the substrate and polypeptide for a sufficient time and under sufficient conditions to allow formation of the enzymatic product. These conditions will vary depending upon the amounts and purity of the substrate and enzyme, whether the system is a cell-free or cellular based system. These variables will be easily adjusted by those skilled in the art. For example, the period of exposure of the enzyme to the substrate
10 will be longer at lower temperatures, *e.g.*, 4 °C rather than at higher temperatures.

In the case of cell containing systems the host cell is contacted with the substrate, under conditions and for sufficient time to produce the oligosaccharide. The time and conditions will vary depending upon the host cell type and culture conditions and can be easily determined by those of skill in the art.

15 The invention provides a gene expression system for producing the polypeptide encoded by the polynucleotide identified by the method of the invention.

The method involves culturing a gene expression system created according to the methods described above under conditions sufficient to produce the polypeptide. The gene expression system comprises a host cell which has been recombinantly modified with a polynucleotide
20 encoding a polypeptide or a portion thereof.

The method is also directed to harvesting the polypeptide. A further step of the method involves substantially purifying the harvested polypeptide. The purified polypeptide may be used in the synthesis of enzymatic product or the preparation of antibodies as described above.

As used herein, the term "harvesting" means collecting or separating from the gene expression system the product produced by the inserted polynucleotide.

The method of the invention produces a polypeptide which are substantially pure. As used herein, the term "substantially pure" refers to a protein which is free of other proteins, lipids, carbohydrates or other materials with which it is normally associated. One skilled in the art can purify the polypeptide using standard techniques for protein purification including preparative chromatography and immunological separations involving monoclonal or polyclonal antibodies. For example, the substantially pure protein will yield a single major band of approximately on a non-reducing polyacrylamide gel. The purity of the polypeptide can also be determined by amino-terminal amino acid sequence analysis.

The enzymatic product or "product" produced by the polypeptide encoded by the polynucleotide identified by the method of the invention can then be used in any number of ways, including for example, as an immunostimulatory composition in the production of both active and passive vaccine compositions.

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The following examples are to be considered illustrative and thus are not limiting of the remainder of the disclosure in any way whatsoever.

EXAMPLE 1

Materials and Methods

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are described in Table 1. *B. pseudomallei*, *B. thailandensis*, and *E. coli* were grown at 37°C on Luria-Bertani (LB) broth base (Becton Dickinson) agar plates or in LB broth. For animal studies, *B. pseudomallei* and *B. thailandensis* cultures were grown at 37°C in TSBDC medium (Brett *et al.*, *Epidemiol. Infect.* 118:137, 1997). When appropriate, antibiotics were added at the following concentrations: 50 µg tetracycline per

ml for *B. pseudomallei* and 100 µg ampicillin, 50 µg kanamycin, and 15 µg tetracycline per ml for *E.coli*.

TABLE 1. Bacterial strains and plasmids used in this study.

	Strain or plasmid	Description	Reference or source
	Strains		
5	<i>E. coli</i> SM10	Mobilizing strain, transfer genes of RP4 integrated in chromosome; Km ^r Sm ^r F ⁻ ϕ 80dlacZ Δ M15 Δ (lacZYA-argF) U169	5 Bethesda Research Laboratories
10		<i>endA1 recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1</i>	
		F ⁻ , <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ lacZ Δ M15	Invitrogen
15		Δ lacX74 <i>deoR recA1 araD139</i> Δ (<i>ara-leu</i>) 7697	
		<i>galU galK rpsL endA1 nupG</i>	
	<i>B. pseudomallei</i>		
	1026b	Clinical isolate; Sm ^r Tc ^s	6
20	SR1015	1026b:pSR1015 Sm ^r ; Tc ^r	This study
	<i>B. thailandensis</i>		
	E264	Environmental isolate Sm ^r Tc ^s	
	Plasmids		
25	pSKM11	Positive selection cloning vector; IncP mob; ColE1 ori; Ap ^r Tc ^s	5
	PZErO-2.1	Positive selection cloning vector; ColE1; Km ^r	Invitrogen
30	PCR2.1-TOPO	Topoisomerase-mediated cloning vector; Ap ^r Km ^r	Invitrogen
	pDD1015	<i>B. pseudomallei</i> subtractive hybridization PCR product cloned into pZErO-2.1 Km ^r	This study
35	pSR1015	<i>Kpn1-Xho1</i> fragment from pDD1015 cloned into pSKM11 Ap ^r Tc ^r	This study
	pDD3005	<i>B. mallei</i> subtractive hybridization PCR product cloned into pCR2.1-TOPO; Ap ^r Km ^r	This study
40			

Construction and screening of subtractive hybridization libraries

Subtractive hybridization was carried out between virulent species *B. pseudomallei* and *B. mallei* and the avirulent *B. thailandensis*. Two subtractive hybridization libraries were constructed using the PCR-Select Bacterial Genome Subtraction Kit (Clontech). The procedure was followed as is outlined in the manual except the hybridization temperature was increased from 63°C to 73°C to allow for the high GC content in the genomes of these species. In the first subtractive hybridization library *B. pseudomallei* genomic DNA was used as the "tester" and *B. thailandensis* genomic DNA was used as the "driver" (*B. pseudomallei* subtraction library). The secondary PCR products obtained were cloned into pZerO-2.1 (Invitrogen) and were enriched for *B. pseudomallei*-specific sequences. In the second subtractive hybridization library *B. mallei* genomic DNA was used as the "tester" and *B. thailandensis* genomic DNA was used as the "driver" (*B. mallei* subtraction library). The secondary PCR products obtained were cloned into pCR2.1-TOPO (Invitrogen) and were enriched for *B. mallei*-specific sequences. Chromosomal DNA used in the subtractive hybridization experiments was isolated from *B. pseudomallei*, *B. mallei* and *B. thailandensis* using a previously described protocol (Wilson, *Preparation of genomic DNA from bacteria*, p.2.4.1-2.4.5. In *Current protocols in molecular biology*, F.M. Ausubel *et al.*, ed., John Wiley & Sons, New York, N.Y., 1987).

DNA sequencing

Automated DNA sequencing was performed by the University Core DNA Services (University of Calgary) with the ABI PRISM DyeDeoxy Termination Cycle Sequencing System and AmpliTaq DNA polymerase (Perkin-Elmer). DNA sequencing reactions were analyzed with an ABI100 DNA Sequencer. The M13 primer was used to initiate sequence reactions with the subtractive hybridization clones. The DNA sequences were analyzed through GenBank at NCBI with the BLASTX program.

Cloning of a subtractive hybridization product and mobilization into wild type *B. pseudomallei*

The DNA insert from pDD1015 (*B. pseudomallei* subtraction library) was cloned as a Kpn I-Xho I fragment into a mobilizable suicide vector pSKM11 (Mongkolsuk *et al.*, *Gene* **143**:145, 1994;

Simon *et al.*, *Biotechnology*, 1:784, 1993). The 373 bp fragment was ligated to pSKM11 digested with the same enzymes to create pSR1015. The ligation was carried out in a 25 µl reaction volume at 16°C overnight. The ligation mix (2 µl) was electroporated into *E. coli*, using a GenePulser II/Pulse Controller Plus apparatus (BioRad) as instructed by the manufacturer.

5 Restriction enzymes and T4 DNA ligase were purchased from Gibco BRL and were used according to the manufacturer's instructions. DNA fragments used in cloning procedures were excised from agarose gels and purified with a GeneClean II kit (Bio 101). Plasmids were purified from overnight cultures by using Wizard Plus Minipreps (Promega).

SM10(pSR1015) was conjugated to *B. pseudomallei* 1026b by using a previously described protocol (DeShazer *et al.*, *J. Bacteriol.* 179:2116, 1997). Briefly, 100 µl of overnight cultures of SM10(pSR1015) and *B. pseudomallei* 1026b were combined in 3 ml of sterile 10 mM MgSO₄, mixed, and filtered through a 0.45-µm-pore-size nitrocellulose filter, using a 25 mm Swinnex filter apparatus (Millipore). Negative control tubes contained either the donor strain or the recipient strain alone. Filters were placed on LB plates supplemented with 2 M MgSO₄ and

10 incubated at 37°C for 8 hours. The filters were then washed in 2 ml of 0.85% NaCl and the bacterial suspension plated on LB plates containing 50 µg/ml tetracycline. The tetracycline resistant colonies were identified after 24-36 hours of incubation. One of the TcR colonies was selected and designated SR1015.

15

Animal studies

20 The animal models of acute *B. pseudomallei* infection have been previously described (Brett *et al.*, *Epidemiol. Infect.* 118:137, 1997). Syrian Golden hamsters (females, 6 to 8 weeks) were injected intraperitoneally with 100 µl of one of a number of serial dilutions of logarithmic-phase cultures adjusted appropriately with sterile phosphate-buffered saline. The control group (5 animals) were inoculated with 10¹ organisms of wild-type *B. pseudomallei*. The test animals (5

25 per dilution) were inoculated with either 10¹, 10², or 10³ organisms of the mutant strain, SR1015. Blood from two of the test animals was diluted and plated on Ashdown Media with and without 50 µg/ml tetracycline to verify stability of pSR1015 (8). For determination of LD₅₀ of SR1015,

hamsters were inoculated with 10^3 , 10^4 , 10^5 , and 10^6 organisms (5 hamsters per dilution). After 48 hours the LD₅₀ was calculated (9).

Immunogold electron microscopy

Immunogold electron microscopy was performed as previously described. Bacteria were grown in LB broth to early to middle logarithmic phase and placed on a Formvar-coated nickel grid (400 mesh) for 2 min. The grid was blocked for 30 min with 0.5% bovine serum albumin (BSA) in PBS, and a 1:100 dilution of primary antibody was added for 30 min. The primary antibody was polyclonal rabbit antiserum raised against a *B. pseudomallei* O-PS-flagellin protein conjugate preparation in which adipic acid dihydrazide was used as a spacer molecule (Brett and Woods, *Infect. Immun.* 64:2824, 1996). The polyclonal antiserum was absorbed with formalin-fixed *B. thailandensis* E264 to remove the antibodies directed against type II O-PS. The grid was washed three times with PBS/BSA, and a 1:50 dilution of a goat anti-rabbit IgG gold conjugate (5 nm; Sigma) was added for 30 min. The grid was washed three times with PBS/BSA, three times with dH₂O and examined on a Hitachi H-7000 electron microscope.

Construction and screening of subtractive hybridization libraries

Two subtractive hybridization libraries were constructed using the PCR-Select Bacterial Genome Subtraction Kit (Clontech). In the first subtractive hybridization library *B. pseudomallei* genomic DNA was used as the "tester" and *B. thailandensis* genomic DNA was used as the "driver" (*B. pseudomallei* subtraction library). The secondary PCR products obtained were cloned into pZER0-2.1 (Invitrogen) and were enriched for *B. pseudomallei*-specific sequences. Sixteen distinct plasmid inserts from this library were sequenced. The DNA inserts were 250 bp to 1000 bp in length and contained a G + C content of approximately 45-55%, which is considerably lower than the 68% G + C content of the *B. pseudomallei* genome. This suggests that the DNA may have been acquired by horizontal transfer relatively recently in evolution. The DNA sequences were analyzed with the program BLASTX and only three of the sequences predicted proteins with homology to proteins present in the Genbank database and these proteins were not involved in virulence.

In the second subtractive hybridization library *B. mallei* genomic DNA was used as the "tester" and *B. thailandensis* genomic DNA was used as the "driver" (*B. mallei* subtraction library). The secondary PCR products obtained were cloned into pCR2.1-TOPO (Invitrogen) and were enriched for *B. mallei*-specific sequences. Seven distinct plasmid inserts from this library were sequenced. The DNA inserts were 125 bp to 700 bp in length and contained a G + C content of approximately 45-55%, which is considerably lower than the 68% G + C content of the *B. mallei* genome. This suggests that the DNA may have been acquired by horizontal transfer relatively recently in evolution. The DNA sequences were analyzed with the program BLASTX, and only one of the sequences predicted a protein with homology to a protein present in the Genbank database. This protein was similar to ABC-2 transporters involved in polysaccharide biosynthesis.

Use of reverse genetics to characterize a novel DNA fragment isolated from both libraries.

A plasmid was identified in the *B. pseudomallei* subtraction library (pDD1015) that contained a DNA insert that was 99% identical to an insert from a plasmid in the *B. mallei* subtraction library (pDD3005) (Figure 2). The DNA inserts were 373 bp in length and there were 4 nucleotide differences between them (Figure 2, asterisks). The DNA insert from pDD1015 was cloned into the mobilizable suicide vector pSKM11, creating pSR1015 (Figure 3). This plasmid was mobilized into *B. pseudomallei* 1026b and Tc^R transconjugates were isolated following a single homologous recombination between the cloned DNA and the corresponding chromosomal DNA. As depicted in Figure 3, the integration of the plasmid pSR1015 should disrupt the chromosomal copy of the gene. One of the Tc^R transconjugates was selected and designated SR1015.

SR1015 is deficient in type I O-PS and is avirulent in the hamster model of melioidosis.

B. pseudomallei and *B. mallei* both produce type I O-PS and EPS, but *B. thailandensis* does not produce these surface polysaccharide. An ELISA was performed with a monoclonal antibody specific for EPS and *B. pseudomallei* 1026b (parental strain), and SR1015 contained EPS (data not shown). Rabbit polyclonal sera specific for flagellin and type I O-PS was used in immunoelectron microscopy studies on 1026b and SR1015 (Figure 4). *B. pseudomallei* 1026b

reacted with both flagellin and type I O-PS antibodies (Figure 4A), but SR1015 only reacted with the flagellin antibodies (Figure 4B). The LD₅₀ of 1026b in the hamster model of melioidosis was < 10 bacteria. The LD₅₀ for SR1015, on the other hand, was found to be > 10⁵ bacteria. This demonstrates that SR1015 is severely attenuated for virulence in this animal model of infection and that type I O-PS is an important virulence determinant of *B. pseudomallei* (and probably *B. mallei*).

It will be apparent to those skilled in the art that various modifications and variations can be made to the compounds and processes of this invention. Thus, it is intended that the present invention cover such modifications and variations, provided they come within the scope of the appended claims and their equivalents. Accordingly, the invention is limited only by the following claims.

WHAT IS CLAIMED IS:

1. A method of identifying a polynucleotide sequence associated with a specific phenotype in an organism, comprising:

- obtaining a first nucleic acid population from a first organism;
- obtaining a second nucleic acid population from a second organism;
- comparing the first nucleic acid population and the second nucleic acid population to obtain a third nucleic acid population;
- transforming a host cell with a selected polynucleotide from the third nucleic acid population; and
- detecting a phenotypic change in the host cell.

2. The method of claim 1, wherein the first organism and the second organism are prokaryotes.

3. The method of claim 1 or 2, wherein the first and second organism are from the same phylogenetic family.

4. The method of claim 1 or 2, wherein the first and second organism are from the same phylogenetic genus.

5. The method of claim 1 or 2, wherein the first and second organism are from the same phylogenetic species.

6. The method of claim 2, wherein the prokaryotes are bacteria.

7. The method of claim 1, wherein the nucleic acid is DNA.

8. The method of claim 1, wherein the nucleic acid is RNA.

9. The method of claim 8, wherein the RNA is reverse transcribed.

10. The method of claim 1, wherein the comparing is by hybridization.
11. The method of claim 10, wherein the comparing is by plus-minus hybridization.
12. The method of claim 1, wherein the comparing is by subtractive hybridization.
13. The method of claim 1, further comprising cloning the third nucleic acid population into a vector.
14. The method of claim 1, wherein the host cell is a prokaryote.
15. The method of claim 1, wherein the host cell is phylogenetically the same species as the first organism or the second organism.
16. The method of claim 1, wherein the phenotypic change is the result of homologous recombination.
17. The method of claim 1, wherein the phenotypic change is a change in enzymatic activity.
18. The method of claim 1, wherein the phenotypic change is a change in virulence.
19. The method of claim 1, wherein the detecting of a phenotypic change is by identifying an organism specific marker.
20. The method of claim 19, where in the organism specific marker is a surface antigen.
21. A method of identifying a polynucleotide sequence associated with a specific phenotype in an organism, comprising:
- obtaining a first nucleic acid population from a first organism;
 - obtaining a second nucleic acid population from a second organism;

1 comparing the first nucleic acid population and the second nucleic acid population to
2 obtain a third nucleic acid population;
3 cloning a selected polynucleotide from the third population into a vector;
4 transforming a host cell with the vector; and
5 detecting a phenotypic change in the host cell.

6 22. The method of claim 21, wherein the first organism and the second organism are
7 prokaryotes.

8 23. The method of claim 21 or 22, wherein the first and second organism are from the same
9 phylogenetic family.

10 24. The method of claim 21 or 22, wherein the first and second organism are from the same
11 phylogenetic genus.

12 25. The method of claim 21 or 22, wherein the first and second organism are from the same
13 phylogenetic species.

14 26. The method of claim 22, wherein the prokaryotes are bacteria.

15 27. The method of claim 21, wherein the nucleic acid is DNA.

16 28. The method of claim 21, wherein the nucleic acid is RNA.

17 29. The method of claim 28, wherein the RNA is reverse transcribed.

18 30. The method of claim 21, wherein the comparing is by hybridization.

19 31. The method of claim 30, wherein the comparing is by plus-minus hybridization.

32. The method of claim 21, wherein the comparing is by subtractive hybridization.
33. The method of claim 21, further comprising cloning the third nucleic acid population into a suicide vector.
34. The method of claim 21, wherein the host cell is a prokaryote.
35. The method of claim 21, wherein the host cell is phylogenetically the same species as the first organism or the second organism.
36. The method of claim 21, wherein the phenotypic change is the result of homologous recombination.
37. The method of claim 21, wherein the phenotypic change is a change in enzymatic activity.
38. The method of claim 21, wherein the phenotypic change is a change in virulence.
39. The method of claim 21, wherein the detecting of a phenotypic change is by identifying an organism specific marker.
40. The method of claim 39, where in the organism specific marker is a surface antigen.
41. A method of identifying a polynucleotide sequence associated with a pathogenic phenotype in an organism, comprising:
- obtaining a first nucleic acid population from a first organism;
 - obtaining a second nucleic acid population from a second organism;
 - comparing the first nucleic acid population and the second nucleic acid population to obtain a third nucleic acid population;
 - cloning a selected polynucleotide from the third population into a vector;

1 transforming a host cell with the vector; and
2 detecting a change in pathogenicity of the host cell.

3 42. The method of claim 41, wherein the first organism and the second organism are
4 prokaryotes.

5 43. The method of claim 41, wherein the first and second organism are from the same
6 phylogenetic family.

7 44. The method of claim 41, wherein the first and second organism are from the same
8 phylogenetic genus.

9 45. The method of claim 41, wherein the first and second organism are from the same
10 phylogenetic species.

11 46. The method of claim 42, wherein the prokaryotes are bacteria.

12 47. The method of claim 41, wherein the nucleic acid is DNA.

13 48. The method of claim 41, wherein the nucleic acid is RNA.

14 49. The method of claim 48, wherein the RNA is reverse transcribed.

15 50. The method of claim 41, wherein the comparing is by hybridization.

16 51. The method of claim 50, wherein the comparing is by plus-minus hybridization.

17 52. The method of claim 41, wherein the comparing is by subtractive hybridization.

18 53. The method of claim 41, further comprising cloning the third nucleic acid population into

1 a suicide vector.

2 54. The method of claim 41, wherein the host cell is a prokaryote.

3 55. The method of claim 41, wherein the host cell is phylogenetically the same as the first
4 organism or the second organism.

5 56. The method of claim 41, wherein the phenotypic change is the result of homologous
6 recombination.

7 57. The method of claim 41, wherein the phenotypic change is a change in enzymatic
8 activity.

9 58. The method of claim 41, wherein the phenotypic change is a change in virulence.

10 59. The method of claim 41, wherein the detecting of a phenotypic change is by identifying
11 an organism specific marker.

12 60. The method of claim 59, wherein the organism specific marker is a surface antigen.

13 61. The method of claim 41, wherein the phenotypic change is detected *in vitro*.

14 62. The method of claim 41, wherein the phenotypic change is detected *in vivo*.

15 63. The method of claim 41, further comprising inoculating an organism with the
16 transformed host cell and measuring pathogenesis of the transformed host cell.

17 64. A method of identifying a polynucleotide sequence associated with a pathogenic
18 phenotype in an organism, comprising:

19 obtaining a first nucleic acid population from a first organism;

obtaining a second nucleic acid population from a second organism;
comparing the first nucleic acid population and the second nucleic acid population to
obtain a third nucleic acid population;
cloning a selected polynucleotide from the third nucleic acid population into a vector;
transforming a host cell with the vector;
inoculating a third organism with the transformed host cell; and
measuring pathogenesis of the third organism compared to a control.

65. An immunostimulatory composition comprising:
a polypeptide encoded by a polynucleotide identified by a method as in any of claims 1,
21, 41, or 64.

66. A therapeutic composition comprising an antibody that interacts with a polypeptide
encoded by a polynucleotide identified by a method as in any of claims 1, 21, 41, or 64.

67. The therapeutic composition of claim 66, wherein the antibody is polyclonal.

68. The therapeutic composition of claim 67, wherein the antibody is monoclonal.

69. A polynucleotide selected from the group consisting of:
a) SEQ ID NO:1;
b) SEQ ID NO:1, wherein T is U; and
c) nucleic acid sequences complementary to a) or b).

70. A vector containing a polynucleotide of claim 69.

71. A host cell containing a vector of claim 70.

72. A polynucleotide selected from the group consisting of:
a) SEQ ID NO:2;

- b) SEQ ID NO:2, wherein T is U; and
- c) nucleic acid sequences complementary to a) or b).

73. A vector containing a polynucleotide of claim 72.

74. A host cell containing a vector of claim 73.

75. A method for detecting a virulent organism in a sample, comprising:

- a) contacting the sample with a polynucleotide probe having a sequence of SEQ ID NO:1 or 2; and
- b) detecting hybridization of the probe with a polynucleotide in the sample, wherein the detection of hybridization is indicative of the presence of a virulent organism in the sample.

76. The method of claim 75, wherein the sample is tissue.

77. The method of claim 75, wherein the sample is a biological fluid.

78. A method for identifying a compound which modulates a phenotype of an organism comprising:

- a) incubating components comprising the compound and a polypeptide encoded by a polynucleotide identified by the method of claim 1, 21, 41, or 64; and
- b) measuring modulation of the organism's phenotype.

79. The method of claim 78, wherein the polynucleotide is SEQ ID NO:1 or 2.

80. The method of claim 78, wherein the phenotype is a pathogenic phenotype.

81. The method of claim 78, wherein the compound modulates the organism's pathogenesis.

82. A method of inducing a protective immune response in an organism comprising delivering an effective amount of a plasmid expression vector to transform differentiated somatic cell tissue of the organism wherein said plasmid expression vector contains a virulence-associated polynucleotide sequence identified by a method as in any one of claims 1, 21, 41, or 64.

83. The method of claim 82, wherein the polynucleotide comprises SEQ ID NO:1 or 2.

84. A method for identifying a compound which regulates expression of a specific phenotype in an organism or activity of a polypeptide conferring a specific phenotype comprising:

- a) incubating components comprising the compound and the organism under conditions sufficient to allow the components to interact; and
- b) measuring the effect of the compound on phenotypic expression.

85. The method of claim 84, wherein said compound is a peptide.

86. The method of claim 84, wherein said compound is a peptidomimetic.

87. The method of claim 84, wherein said compound is a nucleic acid.

88. The method of claim 84, wherein said compound is an antibody or fragment thereof.

89. A method for inducing a protective immune response in a subject, comprising administering the immunostimulatory composition of claim 65 to the subject thereby inducing a protective immune response.

90. A method for providing a protective immune response to a subject, comprising administering the therapeutic composition of claim 66 to the subject thereby providing a protective immune response.

25 91. An immunostimulatory composition comprising:
26 a product which results from the activity of a polypeptide encoded by a polynucleotide
27 identified by a method as in any of claims 1, 21, 41, or 64.

28
29 92. The immunostimulatory composition of claim 91, wherein the product is selected from
30 the group consisting of surface antigens, lipopolysaccharides, and toxins.

31
32 93. A method for inducing a protective immune response in a subject, comprising
33 administering the immunostimulatory composition of claim 91 to the subject thereby inducing
34 a protective immune response.

35
36 94. A pharmaceutical composition comprising a therapeutic agent that interacts with a
37 product which results from the activity of the polypeptide encoded by a polynucleotide identified
38 by a method as in any of claim 1, 21, 41, or 64.

39
40 95. The pharmaceutical composition of claim 94, wherein the therapeutic agent is a
41 polyclonal antibody.

42
43 96. The pharmaceutical composition of claim 94, wherein the therapeutic agent is a
44 monoclonal antibody.

45
46 97. The pharmaceutical composition of claim 94, wherein the product is selected from the
47 group consisting of surface antigens, lipopolysaccharides, and toxins.

48
49 98. A method for providing a therapeutic treatment to a subject having a disease, comprising
50 administering the pharmaceutical composition of claim 94 to the subject thereby treating the
51 disease.

52
53 99. A method of identifying a polynucleotide sequence associated with a specific phenotype
54 in an organism, comprising:

55 obtaining a first nucleic acid population from a first organism;
56 obtaining a second nucleic acid population from a second organism;
57 comparing the first nucleic acid population and the second nucleic acid population to
58 obtain at least one nucleic acid sequence not contained within the second nucleic acid
59 population;
60 transforming a host cell with the nucleic acid sequence; and
61 detecting a phenotypic change in the host cell.
62
63 100. A therapeutic compound identified by the method of claim 78 or 84.

ABSTRACT

The invention relates to compositions and methods of rapidly identifying previously unknown virulence genes in pathogens. The method combines PCR-based subtractive hybridization, insertional mutagenesis and an infection model for efficient detection of virulence genes.

74665.LJ1

Figure 1

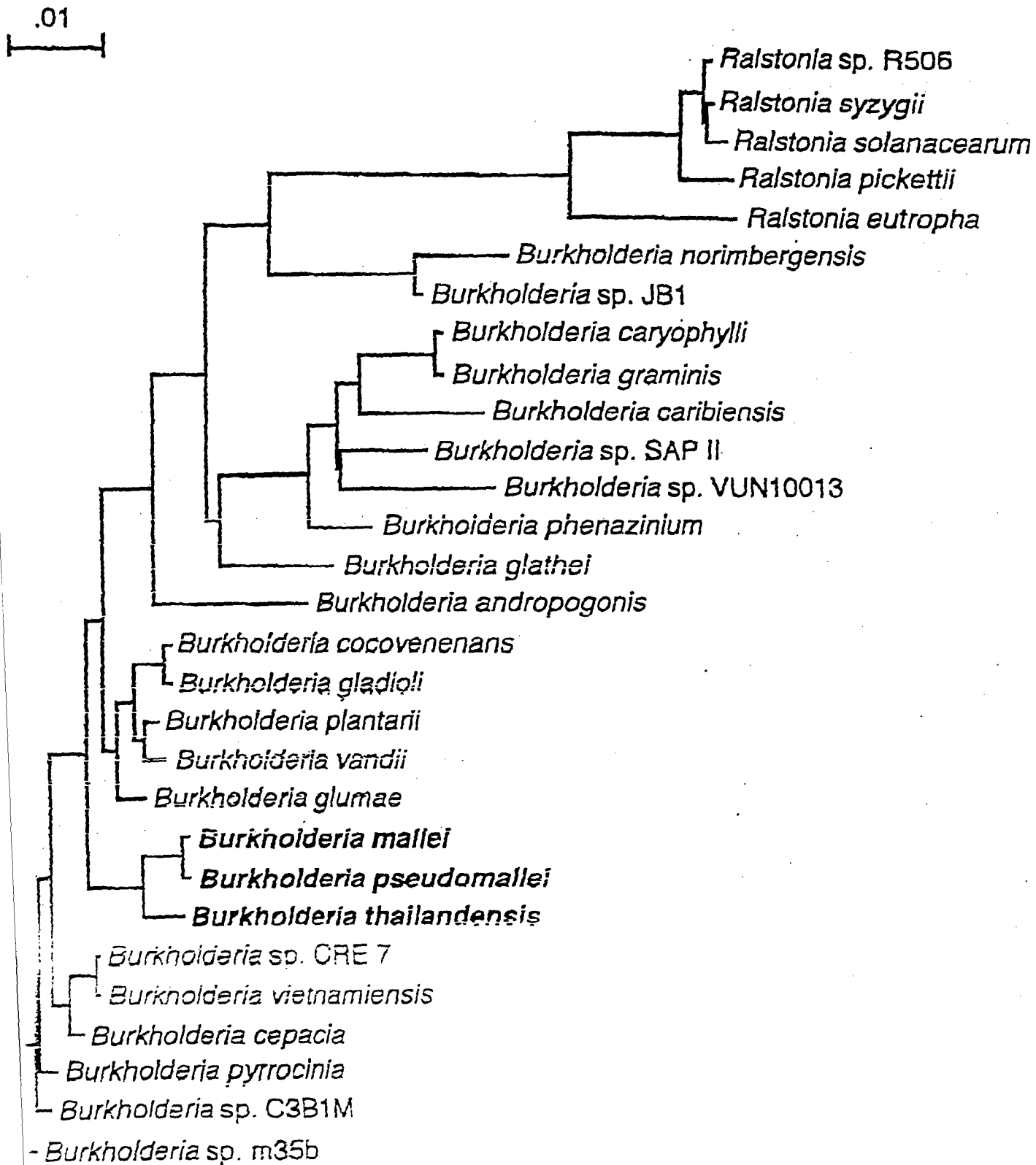


Figure 2

<i>B. pseudomallei</i>	GTACATCCGG	CACATATGGCA	GCCGAGCGCG	CGCCGTGCTC	AGCGAGCGTG	50
<i>B. mallei</i>	GTACATCCGG	CACATATGGCA	GCCGAGCGCG	CGCCGTGCTC	AGCGAGCGTG	50
<i>B. pseudomallei</i>	GCTTTTTCGC	CATTCTGTGG	GAAAGAGATT	CGGCTCTTGT	TTTCGCGGTG	100
<i>B. mallei</i>	GCTTTTTCGC	CATTCTGTGG	GAAAGAGATT	CGGCTCTTGT	TTTCGCGGTG	100
<i>B. pseudomallei</i>	CTTACGTCAT	CAATTGGTAA	CAAAATGCG	ATATTCAGGC	TAGCCGCGCA	150
<i>B. mallei</i>	CTTACGTCAT	CAATTGGTAA	CAAAATGCG	ATATTCAGGC	TAGCCGCGCA	150
<i>B. pseudomallei</i>	TGCTTGCCCTG	AGATCGATAT	TCAACACAAAG	TTTCCCAAAAT	GGCATATTGT	200
<i>B. mallei</i>	TGCCTGCCCTG	AGATCGATAT	TCAACGCAAG	TTTCCCAAAAT	GGCATATTGT	200
<i>B. pseudomallei</i>	TGCATACAAAG	CCACAGCGGA	ATGGAAATTC	CACGCTATTAA	CAAGAAACTG	250
<i>B. mallei</i>	TGCATACAAAG	CCACAGCGGA	ATGGAAATTC	CACGCTATTAA	CAAGAAACTG	250
<i>B. pseudomallei</i>	GCGAGCCCTCG	GTATTAAATC	GGTATTCCCTC	ATCCACGACC	TCATCCCGCT	300
<i>B. mallei</i>	GCGAGCCCTCG	GTATTAAATC	GGTATTCCCTC	ATCCATGACC	TCATCCCGCT	300
<i>B. pseudomallei</i>	GACTCATGCCC	GAATACACCC	GCCCGGGAGT	CGAGCACACG	CATCGCCGGC	350
<i>B. mallei</i>	GACTCATGCCC	GAATACACCC	GCCCGGGAGT	CGAGCACACG	CATCGCCGGC	350
<i>B. pseudomallei</i>	GAATTCATAC	GGCGTTAGGG	TAC	373		
<i>B. mallei</i>	GAATTCATAC	GGCGTTAGGG	TAC	373		

Figure 3

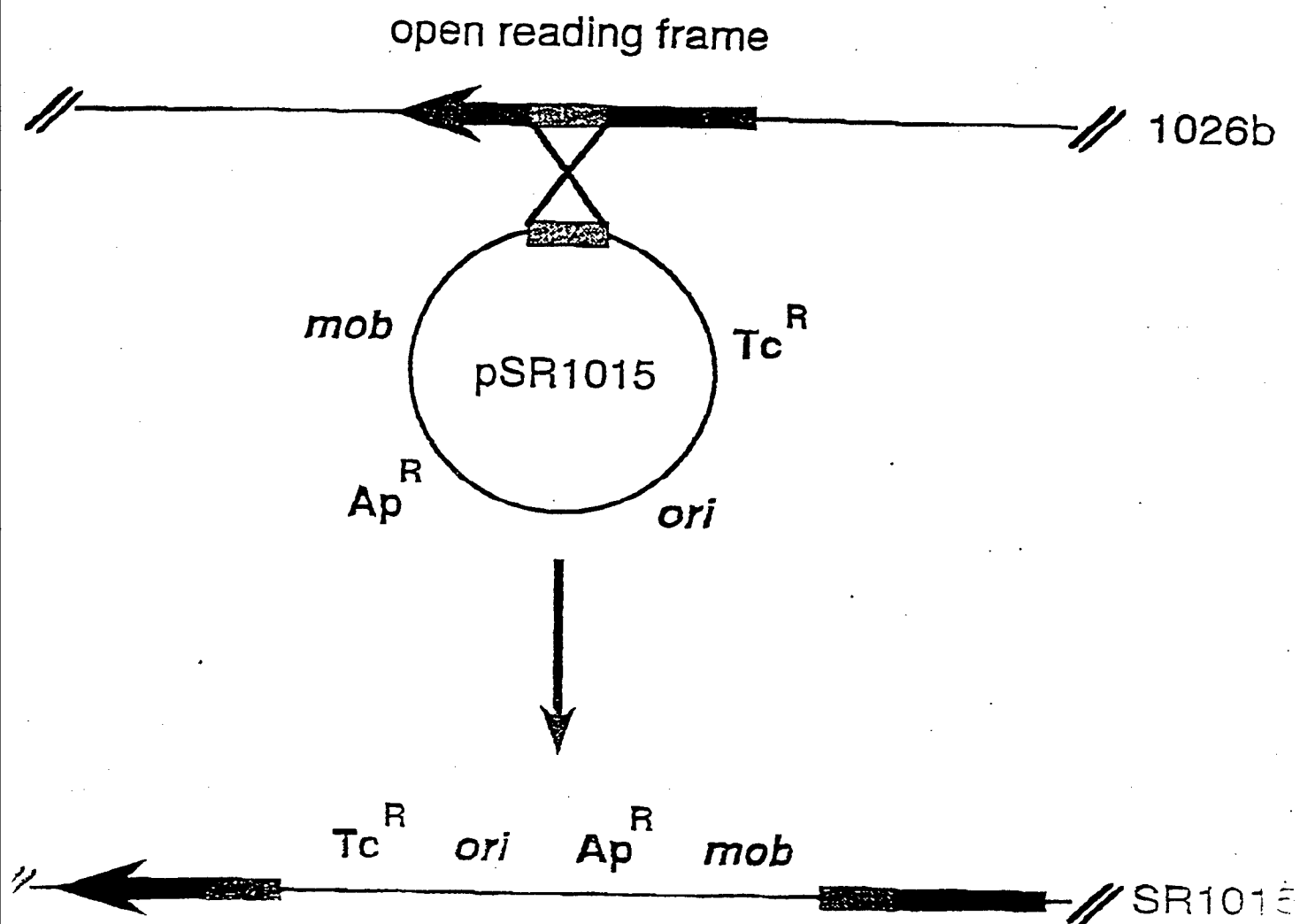
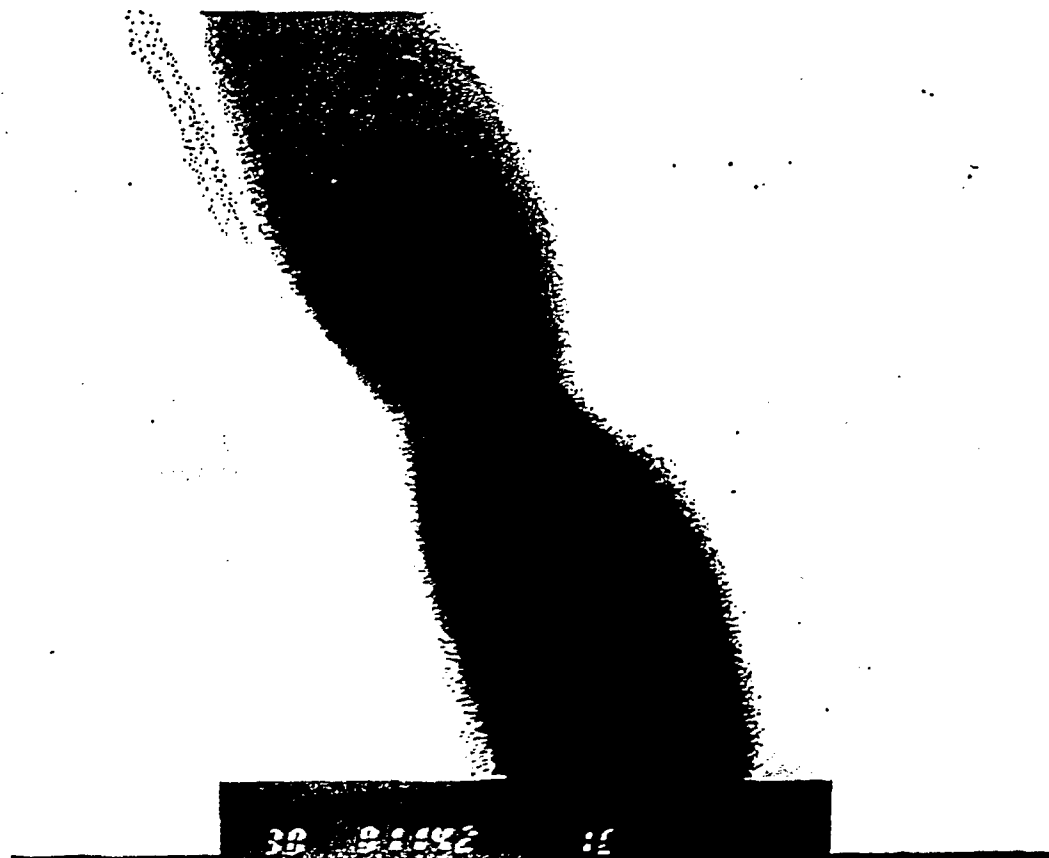


Figure 4





DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

Rec'd
7/23/2001

REPLY TO
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Encl

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