AD NUMBER

ADB267159

NEW LIMITATION CHANGE

TO

Approved for public release, distribution unlimited

FROM

Distribution authorized to U.S. Gov't. agencies only; Proprietary Info.; Jan 2000. Other requests shall be referred to US Army Medical Research and Materiel Comd., Fort Detrick, MD 21702-5012.

AUTHORITY

U.S. Army Medical Research and Materiel Command ltr., dtd July 23, 2001.

AD_____

Award Number: DAMD17-98-C-8003

TITLE: Preparation of a **Burkholderia Mallei Vaccine**

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

CONTRACTING ORGANIZATION: **Canadian Commercial Corporation**
Ottawa, Ontario, Canada K1A OS6

REPORT DATE: January 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

NOTICE

USING GOVERNMENT DRAWINGS, SPECIFICATIONS, OR OTHER DATA INCLUDED IN THIS DOCUMENT FOR ANY PURPOSE OTHER THAN GOVERNMENT PROCUREMENT DOES NOT IN ANY WAY OBLIGATE THE U.S. GOVERNMENT. THE FACT THAT THE GOVERNMENT FORMULATED OR SUPPLIED THE DRAWINGS, SPECIFICATIONS, OR OTHER DATA DOES NOT LICENSE THE HOLDER OR ANY OTHER PERSON OR CORPORATION; OR CONVEY ANY RIGHTS OR PERMISSION TO MANUFACTURE, USE, OR SELL ANY PATENTED INVENTION THAT MAY RELATE TO THEM.

LIMITED RIGHTS LEGEND

Award Number: DAMD17-98-C-8003
Organization: Canadian Commercial Corporation
Location of Limited Rights Data (Pages):

Those portions of the technical data contained in this report marked as limited rights data shall not, without the written permission of the above contractor, be (a) released or disclosed outside the government, (b) used by the Government for manufacture or, in the case of computer software documentation, for preparing the same or similar computer software, or (c) used by a party other than the Government, except that the Government may release or disclose technical data to persons outside the Government, or permit the use of technical data by such persons, if (i) such release, disclosure, or use is necessary for emergency repair or overhaul or (ii) is a release or disclosure of technical data (other than detailed manufacturing or process data) to, or use of such data by, a foreign government that is in the interest of the Government and is required for evaluational or informational purposes, provided in either case that such release, disclosure or use is made subject to a prohibition that the person to whom the data is released or disclosed may not further use, release or disclose such data, and the contractor or subcontractor or subcontractor asserting the restriction is notified of such release, disclosure or use. This legend, together with the indications of the portions of this data which are subject to such limitations, shall be included on any reproduction hereof which includes any part of the portions subject to such limitations.

THIS TECHNICAL REPORT HAS BEEN REVIEWED AND IS APPROVED FOR PUBLICATION.

_____ _____

_____ _____

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	January 2000	Annual (15 Dec 98 - 14 Dec 99)

4. TITLE AND SUBTITLE
Preparation of a Burkholderia Mallei Vaccine

5. FUNDING NUMBERS
DAMD17-98-C-8003

6. AUTHOR(S)
Donald E. Woods, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Canadian Commercial Corporation
Ottawa, Ontario, Canada K1A OS6

E-MAIL: woods@acs.ucalgary.ca

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Distribution authorized to U.S. Government agencies only (proprietary information, Jan 00). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

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 are performing 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.

14. SUBJECT TERMS
Burkholderia Mallei Vaccine, BD

15. NUMBER OF PAGES
68

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Limited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

___ Where copyrighted material is quoted, permission has been obtained to use such material.

___ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

___ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

X In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

8 Jan 2000

PI - Signature Date

TABLE OF CONTENTS

INTRODUCTION

Glanders, caused by *Burkholderia mallei*, is a significant disease for humans due to the serious nature of the infection. It is recognized that *B. mallei* is an organism with tremendous infectivity which poses a significant hazard to humans exposed to aerosols containing this organism. Our knowledge of the pathogenesis of disease due to *B. mallei* is lacking. 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 are performing 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

Detection of Bacterial Virulence Genes by Subtractive Hybridization

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*. *B. pseudomallei*, the etiologic agent of melioidosis, is responsible for a broad spectrum of illnesses in humans and animals particularly in Southeast Asia and northern Australia, where it is endemic. *Burkholderia thailandensis* is an environmental organism closely related to *B. pseudomallei*, but is non-pathogenic. Subtractive hybridization was carried out between these two species in order to identify genes encoding for virulence determinants in *B. pseudomallei*. Screening of the library revealed A-T rich DNA sequences unique to *B. pseudomallei*, suggesting that they may have been acquired by horizontal transfer. One of the subtraction clones, pDD1015, encoded a protein with homology to a glycosyltransferase from *Pseudomonas aeruginosa*. This gene was insertionally inactivated in wild type *B. pseudomallei* to create SR1015. It was determined by ELISA and immunoelectron microscopy that the inactivated gene was involved in the production of a major surface polysaccharide. SR1015 was found to be severely attenuated for virulence in the Syrian hamster model of infection. The LD_{50} for wild type *B. pseudomallei* is <10. The LD_{50} for SR1015 was determined to be 3.5×10^5, similar to that of *B. thailandensis* (6.8×10^5). DNA sequencing of the region flanking the glycosyltransferase gene revealed open reading frames similar in sequence to capsular polysaccharide genes in *Haemophilus influenzae*, *E. coli*, and *Neisseria meningitidis*. In addition, *Burkholderia mallei* and *Burkholderia cepacia* showed reactivity in Southern blot analysis using the glycosyltransferase fragment as a probe, and a capsular structure was identified on the surface of *B. cepacia* via immunoelectron microscopy. The combination of PCR-based subtractive hybridization, insertional inactivation and animal virulence studies should facilitate the identification of relevant virulence determinants from a number of bacterial species.

A manuscript describing the studies described above is attached as Appendix 1.

TnphoA Mutagenesis of *Burkholderia mallei*

The objective of this work was to develop a system that facilitates the isolation of defined mutations in virulence genes of *B. mallei*. One such system is the *TnphoA* fusion vector. The *pho*A gene fusion approach takes advantage of the fact that for the normally periplasmic protein bacterial alkaline phosphatase (PhoA) to be active, it must be localised extracytoplasmically. The *TnphoA* system is composed of a truncated *phoA* gene (lacking the signal sequence) at one end of Tn5 forming a transposon, which can randomly generate gene fusions to *phoA* upon insertion into the recipient chromosome. The hybrid proteins expressed by such gene fusions exhibit PhoA activity only if the target gene encodes a membrane, periplasmic, outer membrane, or extracellular protein. Because exported proteins represent the most frequent classes of proteins involved in pathogenesis; the use of *TnphoA* provides a strong enrichment for insertion

into virulence genes. Selection of mutants carrying active *phoA* gene fusions is a simple procedure; colonies expressing alkaline phosphatase appear blue on agar containing the indicator 5-bromo-4-chloro-3-indolyl phosphate (XP).

Isolation and cloning of the *B. mallei* acid phosphatase gene. In order for the *TnphoA* procedure to be effective, the recipient organism must be acid phosphatase (AP) negative. Primers constructed to the AP gene of *B. pseudomallei* strain 1026b were used to amplify the AP gene from *B. mallei* GB8. The resulting PCR product was cloned into the TOPO cloning vector and sequenced; the DNA sequence was at least 94 % identical to the AP gene of 1026b.

Construction of a *B. mallei* acid phosphatase mutant. The pKAS46 allelic exchange vector (Km^R and RpsL) has been used successfully in the *B. pseudomallei* strain 1026b. We have previously used this vector to construct a plasmid (p463EZ) which contained an insertion inactivation in the 1026b AP gene utilising a zeocin cassette. Because of the high DNA homology (>94 %) between GB8 and 1026b AP genes an attempt was made to use (p463EZ) to inactivate the GB8 AP gene. One requirement of the pKAS46 vector is that recipient organisms are streptomycin (Sm) resistant. Unfortunately, spontaneous Sm^R *B. mallei* mutants were repeatedly also Sm dependent, and therefore would not grow in the absence of antibiotic. To overcome this problem, transconjugates from a GB8 and SM10λpir (p463EZ) mutagenesis were selected on XP, zeocin plates. 64/1500 transconjugates had lost AP activity, and 1/64 was Km sensitive, indicating a double crossover event had occurred and vector had been lost. The GB8 AP mutant (Ze^R, Km^S) mutant was designated G8P. *B. mallei* is resistant to Polymixin B at 15ug/ml; this concentration was routinely used to kill the *E. coli* donor strain following mutagenesis experiments. Unfortunately, due to the very low number of transconjugates obtained from the *TnphoA* mutagenesis, a high background of Polymixin B resistant (15ug/ml) *E. coli* occurred. To counteract this problem, a spontaneous Nalidixic acid resistant (75ug/ml) G8P was selected, designated G8PN.

***TnphoA* mutagenesis utilising pRT733(*TnphoA*).** Upon transfer of the suicide vector pRT733 carrying the *TnphoA* into G8PN, approximately 2% of transconjugants expressed PhoA activity (PhoA+), unfortunately, initial experiments with this system yielded very few transconjugates, 10-20 per mutagenesis. Mutagenesis conditions were optimized and the final procedure enabled ten mutagenesis' to be performed on one agar plate without the possibility of cross contamination; one selection plate was used per mutagenesis resulting in 100 to 200 transconjugates per mate. To prevent duplication of clones, generally one PhoA+ cfu was picked per mating. Transconjugates were selected on TSAG-DC agar (iron chelated media) and TSAG supplemented with 10%horse serum containing Nalidixic acid (75ug/ml), zeocin (5 ug/ml), Kanamycin (5 ug/ml) and XP (80 ug/ml).

Limitation of the pRT733(*TnphoA*) system in *B. mallei*. The optimised protocol did work; however, it was not considered efficient enough for our purposes. Although the frequency of PhoA+ mutants was 2 %, in practice, when a large number of matings were performed simultaneously (30), not all matings resulted in PhoA+ mutants. Whereas some matings would result in up to eight PhoA+ mutants, these mutants were considered to be identical. Some PhoA+ mutants would also turn white and lose PhoA activity, which suggested instability of the

transposon and/or PhoA fusion proteins. The isolation of flanking clones was found to be an extremely inefficient process. Approximately 10 % or less of the mutants resulted in the isolation of flanking clones. The isolation of flanking clones involved digesting the chromosomal DNA isolated from the PhoA+ mutant with either SalI or BamHI and ligating the resulting digest into the appropriately digested pBR322. This would result in an insert containing the *TnphoA* (7kb) and the DNA upstream of the *TnphoA* insertion. However, pBR322 has a limit of the size of DNA that can be efficiently inserted into it of approximately 7 kb (the size of the *TnphoA*). Therefore, the combination of limited restriction sites and the large size of the *TnphoA* probably contribute to the inefficiency of this system.

Development of an Alternative Transposon Delivered Phoa Fusion System

The transposon system, which appeared to have the most potential, was the minitransposon self-cloning vector *Tn5mod-OGm*. Minitransposons are specialized transposons which arrange the cognate transposase outside of the transposon's inverted repeats. This arrangement allows the minitransposon to integrate into target DNA without its transposase and prevents further transposition and DNA rearrangements. These synthetic transposons are small and stable and exhibit virtually no preference for specific target DNA sequence (unlike the *TnphoA* system). The *Tn5Mod-0Gm* is constructed with a conditional origin of replication within the transposon and allows the rapid cloning of the DNA adjacent to the transposon's site of insertion. Furthermore, rare restriction endonuclease sites are incorporated near the inverted repeats containing a total of 18 restriction sites. Analysis of the pmini-*Tn5Mod-OGm* DNA sequence indicated that insertion of the *phoA* gene without a signal sequence into the last restriction site (*KpnI*) of one of the multiple cloning sites in the correct orientation could result in a fusion protein when the transposon inserted into the recipient chromosome. As a result of the insertion of *phoA* into the pmini-*Tn5Mod-OGm*, two restriction sites would no longer be available for cloning (*DraI* and *KpnI*) due to there presence in the *phoA* insert; however, sixteen cloning sites would still be available.

Preliminary mutagenesis experiments with GB8 and *Tn5Mod-OGm* resulted in approximately 1500 transconjugates per mating, a frequency 10-fold higher than the pRT733(*TnphoA*).

Construction of *phoAMod-OGm*. Primers were constructed containing *KpnI* sites to the alkaline phosphatase gene (*phoA*) of *E. coli* (without the signal sequence). The *phoA* gene was amplified using PCR with pRT733 (*TnphoA*) as the template. The resulting PCR fragment and the pmini-*Tn5Mod-OGm* were digested with *KpnI* and the vector was dephosphorylated with CIP. Following ligation and subsequent transformation into *E. coli* DH5α, 9/50 transformants contained the *phoA* gene. Digestion of the plasmids with *SphI* determined that 3 plasmids contained the *phoA* gene in the correct orientation. The resulting plasmid is preliminarily named pmini-*Tn5phoAMod-OGm*. pmini-*Tn5phoAMod-OGm* was subsequently transformed into *E.coli* SM10.

***TnphoA* mutagenesis utilising pmini-*Tn5phoAMod-OGm*.** Transfer of *phoA-Mod-0Gm* into G8PN utilised the protocol developed for pRT733(*TnphoA*) with the exception that gentamicin

(5ug/ml) was incorporated into the selection agar instead of kanamycin. Preliminary experiments resulted in approximately 1000 to 2000 transconjugates per mating, with a frequency of PhoA+ mutants of 1.5 %. Flanking clones have been isolated from the preliminary mutagenesis experiment with a success rate of 100% (6/6). At present, plasmids are being isolated, and sequencing will determine the effectiveness of this system. However, results appear promising because flanking clones possess PhoA activity indicating that they contain a phoA fusion protein. Therefore, preliminary experiments indicate that this system appears more efficient than the pRT733(*TnphoA*).

Analysis of PhoA+ Mutants Derived From pRT733(*TnphoA*)

PhoA+ G8PN mutants were screened for loss of α-haemolytic activity and lipase activity. One mutant (AJB53) did not appear to possess lipase activity; sequencing of the *TnphoA* gene fusion is in process Computer searches of the DNA sequence isolated from the flanking clone of the PhoA+ mutant AJB34 indicated that the *TnphoA* had formed a fusion with a protein exhibiting high homology to the dipeptide transport system permease protein (DPPB) of *E. coli*. The search also indicated the presence of a second open reading frame immediately upstream from the DPPB homologue. The second orf exhibited high homology to an *E. coli* periplasmic dipeptide-binding protein (DBP), which also serves as a chemoreceptor in *E. coli*, and as a haemin-binding protein (HbpA) in *Haemophilus influenzae*. The *B. mallei* DBP/HbpA homologue will be further investigated.

Invasion and Intracellular Survival of *Burkholderia mallei*

Little is known about the virulence factors and pathogenesis of *B. mallei*, although the chronic and highly invasive forms of infection suggest that this pathogen possesses mechanisms for both cellular invasion and evasion of the host immune response. The objective of this work is to analyze the invasion and intracellular survival of *B. mallei* for human alveolar epithelial cell line (A549) and a human monocyte cell line (U937) which differentiates into macrophage-like cells when treated with phorbol 12-myristate 13-acetate (PMA).

A549 invasion assays. Invasion assays involved infecting confluent monolayers of eucaryotic cells (10^6 cells) with 10^7 organisms for 2 h at 37°C in 5 % CO_2. Samples were assayed in triplicate. Following a 1 h incubation with 150 ug/ml kanamycin and subsequent washes, cells were lysed with triton X-100. Intracellular bacteria were quantitated by plating serial dilution of the lysate. Routinely, the invasive *B. pseudomallei* strain 1026b was used as a positive control and the non-invasive *E. coli* HB101 was used as a negative control.

Preliminary experiments indicated that *B. mallei* strain GB8 only invaded A549 cells at low levels, 10 fold greater than *E. coli* HB101, and 100 fold less than 1026b. Cells did not appear to be killed by the bacteria as determined by a MTT assay. Centrifugation appeared to increase the recoveries of GB8 (a non-motile strain) but not 1026b (a motile strain). The growth conditions also appeared to affect invasion, for example, *B. mallei* grown in media supplemented with either 5 % normal human serum (NHS) or heat inactivated horse serum, appeared to be approximately 50 fold more invasive than grown without serum. However, it has been shown with other

invasive pathogens that growth phase itself can affect the invasion of eucaryotic cells. To determine that the preliminary data was not due to growth phase alone, an invasion assay was performed using cultures of *E. coli* HB 101, *B. pseudomallei* 1026b and *B. mallei* GB8 at different phases of growth.

Table 1.

Strain	Growth Media	Growth Conditions (agitation/time)	Growth Phase	Mean % invasion	SD
HB101	LB	agitated (3h)	mid log	0.003	0.001
1026b	LB	agitated (3h)	mid log	7.069	3.02
GB8	TSAG	agitated (5h20')	mid log	0.033	0.005
GB8	TSAG+NHS	non-agitated (4h)	lag	1.217	0.151
GB8	TSAG+NHS	agitated (3h)	lag	1.263	0.169
GB8	TSAG+NHS	agitated (5h)	mid log	0.323	0.048
GB8	TSAG+NHS	agitated (6.5h)	late log	0.155	0.045
GB8	TSAG+NHS	agitated (o/n)	stationary	2.653	0.468

Note:
TSAG: Tryptic soy broth supplemented with 4 % glycerol.

NHS: Normal human serum (final concentration 5%)

Table 1 shows that all cultures of GB8 supplemented with NHS were more invasive than TSAG alone. This may represent that *B. mallei* contains a receptor for a serum component that acts as a signal transducer (switching on genes involved in invasion). Alternatively, acquisition of a serum factor by *B. mallei* may be required to attach to epithelial cells prior to invasion (as is found with the fibronectin binding protein of *Streptococcus pyogenes*). Unexpectedly, it appeared that lag and stationary cultures were more invasive than the log phase cultures; active growth appears to diminish invasion, an observation that is in contrast to other pathogens. The non-agitated culture (oxygen limited) appeared to be more invasive than the agitated log cultures; this finding is consistent with the invasion of Salmonella, where growth of this pathogen under oxygen limited conditions promotes invasion.

Intracellular survival of B. mallei within the U937 cell line. GB8 was grown under different growth conditions and differentiated U937 cells (10^6 cells) were infected with 10^7 organisms for 2 h at 37°C in 5 % CO_2. Following a 1 h incubation with 150 ug/ml kanamycin and subsequent washes, cells were either lysed with triton X-100 (Time 0) or incubated for 18 h with fresh media containing 30ug/ml kanamycin (time 18h). Intracellular bacteria were quantitated by plating serial dilution of the lysate. Routinely, the invasive *B. pseudomallei* strain 1026b was used as a positive control and the non-invasive *E. coli* HB101 was used as a negative control.

Table 2.

Strain	Growth Media	% inv Time 0	% inv 18 h
HB101	LB	12.5	8.19
1026b	LB	14.87	65.5
GB8	TSBG-DC	1.63	0.14
GB8	TSAG+NHS	0.91	0.06
GB8	TSAG+HS	1.72	0.1
GB8	TSAG	0.77	0.07

Note:
LB: Luria broth
TSBG-DC: Tryptic soy broth supplemented with 4 % glycerol and chelated with Chelex-100 (6 h), dialysed overnight, autoclaved and supplemented with 0.05M monosodium glutamate.
TSAG: Tryptic soy broth supplemented with 4 % glycerol
HS: heat inactivated horse serum (final concentration of 5 %)
NHS: Normal human serum (final concentration of 5 %)
% inv: % invasion relative to the initial inoculum.

Low numbers of viable GB8 were recovered from U937 cells at time 0, and over 18 h, a log decrease in the number of viable organisms located intracellulary occurred. The growth conditions did not appear to affect intracellular survival of GB8. However, the negative control, HB101 was not killed efficiently, indicating these cells are not very bactericidal. A similar result has been observed with the J774 cell line. These cells should efficiently kill HB101. One possibility is that most researchers supplement the tissue culture medium with fetal calf serum (fcs), however, we have been using a cheaper product 'Fetal Clone', a bovine serum product which is not actually fcs. This may affect the bactericidal properties of these cells. If these cells are defective in the bactericidal pathway, these results could suggest that GB8 resists phagocytosis and/or escapes from the macrophage once ingested. Also another possibility is that GB8 may kill the U937 cells. Experiments designed to test each of these potential scenarios will be performed.

ATPase and NDK Production By *Burkholderia Spp.*

Intracellular pathogens such as *B. pseudomallei* has been shown to survive inside phagocytic cells rendering it able to evade the humoral immune response and the specific cell-mediated response. How this organism survives inside the macrophage is not well documented. Studies of other *Burkholderia* spp. are also limited, especially for *B. mallei*. Studies from *Mycobacterium tuberculosis*, *M. bovis*, and other less virulent mycobacterium strains have shown that virulent strains can survive intracellularly, while non-virulent ones cannot. Recently, a new mechanism for killing of intracellular mycobacteria by apoptosis has been shown. This mechanism involves an activation of specific receptors, called P2Z, on the macrophage cell membrane by extracellular ATP. The activated receptors trigger the macrophages to go into programmed cell death, or apoptosis, and the intracellular bacteria die with the macrophages. The difference between virulent and avirulent mycobacteria has also been elucidated recently in that the secretion of exoenzymes, such as ATPase and NDK, appear to promote survival inside of macrophages. Upon production of ATPase or NDK, the extracellular ATP is used up, and the macrophages cannot go on to apoptosis; hence, virulent mycobacteria will survive in those macrophages. To assess any similarity between *Mycobacterium* and *Burkholderia*, ATPase, NDK, and cytotoxicity assays were performed in four different species of *Burkholderia*. The conclusions from our studies are as follows:

1. ATPase activity is present in the cell supernatant fluids from all *Burkholderia* spp. However, the ATPase in the supernatant obtained from E264 released radioactive ^{32}P, Pi, which is different from Pi* formed by ATPase extracted from GB8, 1026b, and K56-2. Also, E264 did not show any UTP, CTP, or GTP production indicating that there was no NDK activity.
2. The production of ATPase from E264 may be responsible for the increase in macrophage survival upon apoptosis stimulation by ATP.
3. The concentrated culture supernatants from GB8, 1026b, and K56-2 did not increase the survival rate of macrophages after they had been stimulated with LPS and ATP.
4. It remains to be seen there is any correlation between intracellular survival and an inhibition of the apoptosis in E264. Unlike *Mycobacterium bovis* BCG, avirulent *Burkholderia thailandensis* prevents apoptosis upon ATP activation, but the successful inhibition of macrophage apoptosis may not be involved in *Burkholderia* survival.
5. The preliminary results obtained here did not demonstrate any obvious differences among *Burkholderia mallei* GB8, *B. pseudomallei* 1026b, and *B. cepacia* K56-2.

Immunological Studies of *Burkholderia mallei* Infection in Syrian Hamsters and Major Histocompatibility Complex - Gene Knock Out C57BL/6 Mice

Introduction

B. mallei infection in golden Syrian hamsters always leads to acute infection and death, usually within 5 days, before any specific immune response can be measured or detected. This observation indicates that an innate response, such as the phagocytic activity from polymorphonuclear cells, complement, bactericidal peptides, etc. cannot kill all the bacteria. It is difficult to predict whether the hamster would be able to mount protective immunity against a *B. mallei* infection if the hamster could survive long enough to produce specific immunity. The hamster can however be challenged with *B. mallei* antigens without causing a lethal infection. One can use formalin-killed bacteria as immunogens to study the specific immune response 7-10 days after the challenge. As *B. mallei* is a facultative intracellular organism, and evidence exists to demonstrate that the humoral response might not be completely protective against *B. mallei* infections, we have begun to focus on the cell-mediated response in animal models.

Cell-mediated immune responses are dependent upon different sets of T cells. The cytotoxic response is dependent upon CD8+ cytotoxic T cells, which require the MHC class I molecule for antigen presentation. The cell-mediated immune response dependent upon CD4+ delayed-type hypersensivity T cells (T_{DTH}), requires the MHC class II. It is possible that *B. mallei* can invade and survive inside certain host cells, e.g. epithelial cells. Cytotoxic T cells can only kill infected cells, but not the bacteria. As a result, the intracellular bacteria are released to the extracellular compartment. However, the cytotoxic activity probably plays a significant role in eliminating the hiding places for these bacteria, and the professional phagocytic cells are provided an opportunity to remove the extracellular bacteria. The professional phagocytic cells that have the best chance to kill *B.* mallei are likely to be activated macrophages, which are effector cells of T_{DTH}.

Macrophages require γ-interferon (γ-IFN) from T_{DTH} to be activated, and macrophage activation results in a delayed type hypersensitivity (DTH) response. The goals of the present studies were to measure the DTH response in susceptible animals challenged with *B. mallei* antigens, and to determine whether the hamsters survived a subsequent challenge with wild type *B. mallei* after the specific immune response had been produced. To accomplish these goals, we developed the following specific aims:

1. To determine whether specific immunity improves the outcome of *B. mallei* infection.
2. To demonstrate the role of the specific cell-mediated response via activated macrophages in *B. mallei* infection in susceptible animals.

Materials and Methods

Syrian hamsters model. To determine whether hamsters were able to produce a specific immune response against *B. mallei* the following experiment was performed. Hamsters were divided into 3 groups of 3 animals; the first group served as a control and was treated with PBS; the second group was treated with 5 % formalin-killed *B. mallei* strain GB8; and the third group

was treated with tuberculin solution. The DTH response was determined at two different sites: the subcutaneous injection site of the abdomen and the hind foot pad. Finally, the hamsters were challenged with a lethal dose of GB8 (approximately 2000 CFU), and the progression of the disease in the different groups of hamsters was compared.

DTH response. Hamsters were challenged with 5 % formalin-killed whole bacterial cells. *B. mallei* GB8 were grown over night to about 10^8 CFU/ml. The cells were harvested and resuspended in 5 % formalin in PBS for 30 minutes. The cells were centrifuged and washed three times with PBS. The final pellet was resuspended in 2 ml PBS, to a density of about 5 $\times 10^8$ cells/ml. The initial challenge dose was 50 µl of the cell suspension injected subcutaneously at each of the lower two quadrants of the abdomen. The DTH response was determined at two weeks after the initial challenge by injecting 50 µl of the 5 % formalin-killed cell suspension subcutaneously into the upper two quadrants of the abdomen and also in the left hind foot pad. The diameter of the induration at the abdomen and foot pad thickness were determined at 24 hr after the second injection.

MHC gene knock out mouse model. Different strains of MHC knock out (C57BL/6) mice obtained from Taconic were used to determine the type of cell-mediated response most important in protection against *B. mallei* infection. The MHC class I knock out strain, β_2m gene knock out, lacks class I MHC molecules that are necessary for CD8+ cytotoxic activity. The MHC class II knock out strain, Abb gene knock out, lacks class II MHC molecules that are necessary for CD4+ T cell function. The double MHC class I and II knock out, which lacks both classes of MHC molecules, served as a control. There were two groups of two mice per strain; the first group was challenged with *B. mallei* ~10^4 CFU; the second group was challenged with ~10^5 CFU.

Results

Lethal dose challenge in hamsters after stimulation with 5 % formalin-killed *B. mallei* GB8. Two hamsters from each group (PBS, 5 % formalin-killed cell, and tuberculin treated) were challenged intraperitoneally with ~2,000 CFU of *B. mallei* GB8 that had been passaged through hamsters three times. All hamsters from the PBS and tuberculin-treated groups were dead on day three, while the two hamsters in the 5 % formalin-killed cell treated group were still alive. These two hamsters died on day four.

DTH response in hamsters.

1) Primary challenge.

- PBS-injected group: The group injected with PBS showed no sign of inflammation at the PBS injection sites.
- 5% formalin killed GB8 group: All of the hamsters injected with 5 % formalin-killed GB8 showed some degree of inflammation as indicated by red lesions of approximately 3-4 mm induration at the injection sites 24 hr after the injection.
- Tuberculin-injected group: One of the hamsters injected with tuberculin solution showed about 1 mm induration at the site of injection, but the other two showed no signs of inflammation.

2) Secondary challenge. The induration at the abdominal sites was larger than that seen after primary challenge in the 5 % formalin-killed cell injected group, and the groups injected with PBS or tuberculin did not show any induration. A change in foot pad thickness change also occurred in the 5 % formalin-killed cell injected group and not in the PBS or tuberculin injected groups. Table 1 shows the size of induration at the abdominal injected sites and the changes in foot pad thickness at 24 hr post secondary challenge. The results at 48 and 72 hr post secondary challenge were similar to those noted at 24 hr in all animals.

Table 1. The DTH response to *B. mallei* GB8 in hamster.

		Abdomen	Hind foot pads		
	number	induration diameter (mm.)	Left foot (mm.)	Right foot (mm.)	% thickness change
PBS group	1	0	3	3	100
	2	0	3.05	2.9	105
	3	0	2.8	2.85	98
5% formalin killed GB8 group	1	9.7	5.3	2.9	183
	2	7.4	4.9	2.9	169
	3	8.4	4.9	2.9	169
Tuberculin group	1	0	2.9	2.9	100
	2	0	3.1	3.1	100
	3	0	3.2	2.9	110

Susceptibility of class I MHC gene knock out C57BL/6 mice. The mice in all groups of class I MHC knockout mice survived for two weeks. At the end of the second week, one mouse from each group was sacrificed, and internal organs were examined. The spleen, liver, and lungs were harvested for bacterial culture and PCR analysis using primers specific for *B. mallei* 16S ribosomal DNA. The gross pathology of all organs were normal, the bacterial cultures showed no growth, and PCR anslyses were negative for *B. mallei* DNA in all animals.

Susceptibility of class II MHC and double MHC gene knock out mice. Two months after *B. mallei* GB8 challenge, the last mouse in each group was sacrificed, the internal organs were examined and bacterial cultures were performed on these. PCR analysis is in progress. The class II knock out mouse showed spleen enlargement, approximately 4 times larger than normal, with miliary nodules covering the spleen. The liver was approximately 1.5 times larger than normal. The double gene knock out mouse also showed spleen and liver enlargement to the same degree as in the class II knock out mouse. Furthermore, the double gene knock out mouse demonstrated signs of infection in the pleural cavities, as there was blood collected in both sides of the pleural cavity. Bacterial culture of homogenized organs of these two knock out mice, class II and double knock out, resulted in bacterial growth on LB agar plates characteristic of *B. mallei*.

Summary

Formalin-treated whole bacterial cells do protect against lethal infection in the hamster model. This failure may be due to the inability of the killed bacteria to up-regulate the cell-mediated response. It is most likely that the hamsters can mount specific immune response to *B. mallei*,

but the phagocytic cells, such as activated macrophages, were not highly responsive, or the bacterial killing mechanism is defective in hamsters (this also includes the defect in polymorphonuclear cell killing activity).

The hamster clearly produces a DTH response to *B. mallei* antigens, as shown by an increase in the size of the indurations at the sites of secondary antigen challenge and the increase in foot pad thickness. In future experiments we will use separate, purified antigens, and the dose will be adjusted, so that the degree of DTH stimulation can be measured for each separate antigen. We expect that particular antigens will best serve to specifically enhance the DTH response.

We are very excited about using the class II MHC gene knock out mice as an animal model for chronic *B. mallei* infections. These animals will be of significant practical use in our immunological studies due to the availability of mouse reagents for use in these types of studies.

Aminoglycoside And Macrolide Susceptibility In *Burkholderia Mallei*

Unlike *Burkholderia pseudomallei*, *B. mallei* is sensitive to aminoglycoside antibiotics such as streptomycin, gentamycin and tobramycin and macrolide antibiotics such as azithromycin and clarithromycin. Resistance to both aminoglycosides and macrolides in *B. pseudomallei* is due to an efflux-mediated system. Mutants of *B. pseudomallei* lacking the efflux system display an 8 - 128 fold increase in aminoglycoside susceptibility. We were interested in determining whether *B. mallei* was susceptible to aminoglycoside and macrolides due to loss of efflux genes. Southern hybridizations were performed using an internal region of the efflux operon gene *amrA* from *B. pseudomallei* as a probe for homologous *amrA* sequences in *B. mallei* chromosomal DNA. Hybridization results indicated the presence of homologous *amrA* sequences in six of nine *B. mallei* strains examined suggesting the presence of an efflux system in these strains. We are currently trying to complement these strains with cloned regions of the *amr* locus from *B. pseudomallei* in order to determine if *B. mallei* lacks certain components of the efflux system.

Goals for the Coming Year

1. We will continue our studies on the on the genes encoded in pathogenicity island that we have identified in *B. mallei* and *B. pseudomallei*. In particular, we are tremendously excited about the identification of an extracellular polysaccharide component present on these organisms that may very well serve as an ideal vaccine candidate. We will continue our studies on the purification and characterization of this polysaccharide.

2. We will continue our studies on *phoA* mutagenesis of *B. mallei* relative to the identification of mutants deficient in the production of extracellular proteins involved in the virulence of these organisms. Now that we have developed the minitransposon-*phoA* system, it is anticipated that these studies will proceed rapidly.

3. We will continue our studies on the use of the class II MHC knockout mice as an animal model for chronic *B. mallei* infections. These studies should prove to be tremendously important in defining the immunological parameters of glanders as well as providing us an opportunity to test vaccines and chemotherapeutic agents for treating the disease.

4. We will continue our studies on *B. mallei*-macrophage interactions. Using a genetic approach, we will construct mutants deficient in macrophage uptake and/or survival in an effort to define the parameters associated with *B. mallei*-macrophage interactions.

5. We will continue our studies on antibiotic resistance in *B. mallei*. In particular, we will complement *B. mallei* strains with cloned regions of the *amr* locus from *B. pseudomallei* in order to determine if *B. mallei* lacks certain components of the efflux system.

Administrative Issues

We have completed our first year in our Level III Biocontainment Facility at the University of Calgary. As a result of the delay in our ability to work with live organisms, the first phase of U.S. Army Contract DAMD17-98-C-8003 has been extended for one year as per information received from Ms. Sacelia Heller. This report thus represents an **ANNUAL REPORT** and NOT a Final Report.

Key Research Accomplishments

- We have described a number of genes encoded in pathogenicity island in *B. mallei* and *B. pseudomallei*. We are tremendously excited about the identification of an extracellular polysaccharide component present on these organisms that may very well serve as an ideal vaccine candidate. We are continuing our studies on the purification and characterization of this polysaccharide.

- We have developed a *phoA* mutagenesis of *B. mallei* procedure which should prove to be tremendously useful for the identification of mutants deficient in the production of extracellular proteins involved in the virulence of these organisms. Now that we have developed the minitransposon-*phoA* system, it is anticipated that these studies will proceed rapidly.

- We have developed class II MHC knockout mice as an animal model for chronic *B. mallei* infections. These studies should prove to be tremendously important in defining the immunological parameters of glanders as well as providing us an opportunity to test vaccines and chemotherapeutic agents for treating the disease.

- We have describe a number of parameters important in *B. mallei*-macrophage interactions. Using a genetic approach, we will construct mutants deficient in macrophage uptake and/or survival in an effort to define the parameters associated with *B. mallei*-macrophage interactions.

- We have initiated studies on antibiotic resistance in *B. mallei*. We are in the process of complementing *B. mallei* strains with cloned regions of the *amr* locus from *B. pseudomallei* in order to determine if *B. mallei* lacks certain components of the efflux system.

Reportable Outcomes

- We have completed one manuscript describing our studies on the identification of a pathogenicity island in *B. mallei* and *B. pseudomallei*. This manscript is attached as Appendix 1 and will be submitted to Molecular Microbiology.

- We are collaborating with Dr. David DeShazer on a manuscript describing the details of the genes present in the pathogenicity island in *B. mallei*.

- Dr. David Deshazer's postdoctoral training was supported in part by this contract. Dr. Alex Bolton and Dr. Chanwit Tribuddharat are postdoctoral fellows currently supported by this contract.

- We successfully obtained a Medical Research Council Operating Grant based on work supported in part by this contract.

- We have developed an animal model of chronic *B. mallei* infection in Class II MHC knockout mice.

Conclusions

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*. The combination of PCR-based subtractive hybridization, insertional inactivation and animal virulence studies should facilitate the identification of relevant virulence determinants from a number of bacterial species; however, we will continue to concentrate on the genes present in the pathogenicity island that we have identified in *B. mallei* and *B. pseudomallei*. In particular, we are tremendously excited about the identification of an extracellular polysaccharide component present on these organisms that may very well serve as an ideal vaccine candidate. We will continue our studies on the purification and characterization of this polysaccharide. We have developed *TnphoA* mutagenesis utilizing pmini-*Tn5phoAMod-OGm*. Preliminary experiments indicate that this system appears more efficient than the pRT733(*TnphoA*), and this system should prove tremendously useful in generating mutants deficient in the production of extracellular proteins important in virulence. Our studies on the use of the class II MHC knockout mice as an animal model for chronic *B. mallei* infection are tremendously exciting ones. These studies should prove to be significantly important in defining the immunological parameters of glanders as well as providing us an opportunity to test vaccines and chemotherapeutic agents for treating the disease. The overall significance of the work resides in the realization that we are beginning to understand the virulence of *B. mallei*, and we are progressing towards the development of a vaccine that will protect against disease due to this organism.

Detection of Bacterial Virulence Genes by Subtractive Hybridization: Identification of the Capsular Polysaccharide of *Burkholderia pseudomallei* as a Major Virulence Determinant

Shauna L. Reckseidler[1], David DeShazer[2], Pamela A. Sokol[1], and Donald E. Woods[1]*

[1]Department of Microbiology and Infectious Diseases

University of Calgary Health Sciences Center

3330 Hospital Drive NW

Calgary, Alberta, Canada T2N 4N1

Telephone: (403) 220-2564

Fax: (403) 283-5241

e-mail: woods@ucalgary.ca

[2]Bacteriology Division

U.S. Army Medical Research Institute of Infectious Diseases

Fort Detrick, MD 21702

Running title: Detection of Virulence Genes in *Burkholderia pseudomallei*

*Corresponding author

Abstract

Burkholderia pseudomallei, the etiologic agent of melioidosis, is responsible for a broad spectrum of illnesses in humans and animals particularly in Southeast Asia and northern Australia, where it is endemic. *Burkholderia thailandensis* is an environmental organism closely related to *B. pseudomallei*, but is non-pathogenic. Subtractive hybridization was carried out between these two species in order to identify genes encoding for virulence determinants in *B. pseudomallei*. Screening of the library revealed A-T rich DNA sequences unique to *B. pseudomallei*, suggesting that they may have been acquired by horizontal transfer. One of the subtraction clones, pDD1015, encoded a protein with homology to a glycosyltransferase from *Pseudomonas aeruginosa*. This gene was insertionally inactivated in wild type *B. pseudomallei* to create SR1015. It was determined by ELISA and immunoelectron microscopy that the inactivated gene was involved in the production of a major surface polysaccharide. SR1015 was found to be severely attenuated for virulence in the Syrian hamster model of infection. The LD_{50} for wild type *B. pseudomallei* is <10. The LD_{50} for SR1015 was determined to be 3.5×10^5, similar to that of *B. thailandensis* (6.8×10^5). DNA sequencing of the region flanking the glycosyltransferase gene revealed open reading frames similar in sequence to capsular polysaccharide genes in *Haemophilus influenzae*, *E. coli*, and *Neisseria meningitidis*. In addition, *Burkholderia mallei* and *Burkholderia cepacia* showed reactivity in Southern blot analysis using the glycosyltransferase fragment as a probe, and a capsular structure was identified on the surface of *B. cepacia* via immunoelectron microscopy. The combination of PCR-based subtractive hybridization, insertional inactivation and animal virulence studies should facilitate the identification of relevant virulence determinants from a number of bacterial species.

Introduction

Burkholderia pseudomallei, the causative agent of melioidosis, is a gram-negative, facultatively anaerobic, motile bacillus that is commonly found in the soil and stagnant waters in Southeast Asia and northern Australia. Infection by *B. pseudomallei* is often due to either direct inoculation into wounds and skin abrasions, or by inhalation of contaminated material (Chaowagul *et al.*, 1989, Howe *et al.*, 1971, Leelarasamee and Bovornkitti, 1989). This would explain the prevalence of the disease among rice farmers and the incidence of infected helicopter pilots in the Vietnam War who developed melioidosis due to inhalation of contaminated dust (Howe *et al.*, 1971, Sanford, 1990). Melioidosis may present as an acute pneumonia or an acute septicemia, which is the most severe form of the disease. The disease may also manifest as a chronic infection involving long-lasting suppurative abscesses in numerous sites in the body. Infection with *B. pseudomallei* may even result in a sub-clinical infection and remain undetected for a number of years. Both the chronic and sub-clinical forms generally remain undiagnosed until activated by a traumatic event or a decrease in immunocompetence (Ip *et al.*, 1995).

Both secreted and cell-associated antigens have been identified in *B. pseudomallei.* Cell associated antigens include exopolysaccharide (EPS) and lipopolysaccharide (LPS) (Bryan *et al.*, 1994, Steinmetz *et al.*, 1995, Brett and Woods, 1996). The EPS produced by *B. pseudomallei* is an unbranched polymer of repeating tetrasaccharide units with the structure –3)-2-*O*-acetyl-β-D-Gal*p*-(1-4)-α-D-Gal*p*-(1-3)-β-D-Gal*p*-(1-5)-β-D-KDO*p*-(2- (Masoud *et al.*, 1997, Nimtz *et al.*, 1997). The role of EPS in virulence is unknown, but sera from patients with melioidosis have been shown to contain antibodies against EPS (Steinmetz *et al.*, 1995). The LPS of *B. pseudomallei* has been reported to contain two types of O-polysaccharide moieties termed type I O-PS and type II O-PS (Knirel *et al.*, 1992, Perry *et al.*, 1995). Type II O-PS is an unbranched

heteropolymer with repeating D-glucose and L-talose residues with the structure –3)-β-D-glucopyranose-(1-3)-6-deoxy-α-L-talopyranose-(1-, in which approximately 33% of the talose residues contain 2-*O*-methyl and 4-*O*-acetyl substituents, while the other L-talose residues contain only 2-*O*-acetyl substituents. Type II O-PS has been shown to be involved in serum resistance (DeShazer *et al.*, 1998). Mutants lacking in type II O-PS were found to be sensitive to the bacteriocidal activities of 30% normal human serum. Type II O-PS mutants also demonstrated reduced virulence in three animal models of *B. pseudomallei* infection (DeShazer *et al.*, 1998). Type I O-PS is an unbranched homopolymer with the structure –3)-2-*O*-acetyl-6-deoxy-β-D-*manno*-heptopyranose-(1-. The role for this polysaccharide in infection is still undefined.

Burkholderia thailandensis is a non-pathogenic soil organism originally isolated in Thailand (Brett *et al.*, 1997). Based on biochemical, immunological, and genetic data, *B. pseudomallei* and *B. thailandensis* are closely related species. However, these two organisms differ in a number of ways and have been classified into two different species based on these differences (Brett *et al.*, 1998). The rRNA sequence of *B. thailandensis* differs from that of *B. pseudomallei* by fifteen nucleotides and there are significant differences in genomic macrorestriction patterns between these organisms (Chaiyaroj *et al.*, 1999). Second, the biochemical profiles of these two species differ in that *B. thailandensis* can utilize L-arabinose, while *B. pseudomallei* does not (Brett *et al.*, 1998, Wuthiekanun *et al.*, 1996). *B. thailandensis* is known to lack type I O-polysaccharide, EPS, and insertion sequences that are present in *B. pseudomallei* (Brett *et al.*, 1998, Mack and Titball, 1998, Steinmetz et al., 1999). The most distinct difference between these two species, however, is their relative virulence. The 50% lethal dose (LD_{50}) for *B. pseudomallei* in the Syrian Golden hamster model of acute melioidosis

is <10 organisms whereas the LD_{50} for *B. thailandensis* is approximately 10^6 organisms (Brett *et al.*, 1998). It has also been shown that the two species can be differentiated based on their propensity to cause disease in humans. Environmental strains isolated in Thailand that are able to assimilate L-arabinose are not associated with human infection whereas clinical isolates are not able to utilize L-arabinose (Trakulsomboon *et al.*, 1997).

In order to identify the genetic determinants that confer enhanced virulence in *B. pseudomallei*, a method combining subtractive hybridization, insertional mutagenesis, and animal virulence studies was developed and the results of these studies are presented here. The described method should aid in the identification of virulence factors in pathogenic bacteria and provide insights into microbial diversity and evolution.

Results

Construction and screening of the *B. pseudomallei* subtraction library

Subtractive hybridization was carried out between the virulent *B. pseudomallei* and the avirulent *B. thailandensis* in order to isolate DNA sequences encoding for virulence determinants unique to *B. pseudomallei*. Screening of the subtraction library revealed a number of DNA sequences unique to *B. pseudomallei*. Twenty-two distinct plasmid inserts from the library were sequenced (Table 1). The DNA inserts ranged from 100 to 1000 bp in length and were found to contain a G+C content of approximately 45-55%, which is considerably lower that the 68% G+C content of the *B. pseudomallei* chromosome. This suggests that the DNA may been acquired by horizontal transfer recently in evolution. The DNA sequences were analyzed using the BLASTX program and only three of the sequences predicted proteins present in the GenBank database. One of the plasmid inserts, pDD1000, was found to demonstrate homology to DprA, a protein required for chromosomal DNA transformation in *Haemophilus influenzae*

(Karudapuram *et al.*, 1995). Another insert, pDD1005, was shown to demonstrate homology to a mobilization protein found in small plasmids. The third, pDD1015, was found to share limited homology with WbpX, a glycosyltransferase, from *Pseudomonas aeruginosa* (Rocchetta *et al.*, 1998).

Insertional inactivation of the glycosyltransferase gene in wild type *B. pseudomallei*

A plasmid was identified in the *B. pseudomallei* subtraction library (pDD1015) containing a DNA insert of 373 bp that demonstrated homology to a glycosyltransferase from *P. aeruginosa.* The DNA insert from pDD1015 was cloned into a mobilizable suicide vector, pSKM11 (Mongkolsuk *et al.*, 1994). The resulting plasmid, pSR1015, was mobilized into wild type *B. pseudomallei* 1026b to create the mutant strain, SR1015. Since the insert from pDD1015 was found to demonstrate homology to a glycosyltransferase, it was postulated that we might have located a gene involved in carbohydrate synthesis. It had been previously shown that *B. pseudomallei* produces both type I O-polysaccharide and exopolysaccharide, but *B. thailandensis* does not (Brett *et al.*, 1998, Steinmetz *et al.*, 1999). To define the phenotype of SR1015, an ELISA was performed with the EPS-specific monoclonal antibody 3015, and *B. pseudomallei* 1026b and SR1015 were both found to contain EPS (data not shown) (Steinmetz *et al.*, 1995). SR1015 was also shown to contain type II O-PS and was found to be serum resistant (data not shown). Immunogold electron microscopy studies using rabbit polyclonal sera specific for flagellin and type I O-PS was performed on the parent strain, 1026b and SR1015 (Fig.1). *B. pseudomallei* 1026b reacted with both flagellin and type I O-PS (Fig. 1A), but SR1015 only reacted with the antibodies to flagellin (Fig. 1B). *B. thailandensis*, the negative control, did not react with either the antibodies to flagellin or to type I O-PS (Fig. 1C). Western blot analysis of proteinase K digested whole cells from *B. pseudomallei* 1026b, *B. thailandensis*

E264, and *B. pseudomallei* SR1015 using rabbit polyclonal sera specific for type I and type II O-PS confirmed the lack of type I O-PS in SR1015 (Fig. 2). *B. pseudomallei* 1026b reacted to both type I and type II O-PS, while *B. pseudomallei* SR1015, like *B. thailandensis*, reacted only to type II O-PS antibodies. From these results it was determined that we had identified and insertionally inactivated a gene involved in the synthesis of a major surface polysaccharide of *B. pseudomallei.*

SR1015 is avirulent in the animal model of infection

SR1015 was tested for virulence in the Syrian Golden hamster model of acute septicemic melioidosis. After 48 hours the animals inoculated with 10^1 cfu of wild type *B. pseudomallei* 1026b had succumbed to infection and died while the animals inoculated with 10^3 cfu of SR1015 were still alive. The LD_{50} of SR1015 was then determined in the Syrian hamster. The LD_{50} for SR1015 after 48 hours was calculated to be 3.5×10^5, while the LD_{50} of the parent strain, 1026b, has been calculated to be <10 (Table 2). The LD_{50} value for SR1015 was noted to be similar to that for the avirulent *B. thailandensis* (6.8×10^5). This demonstrates that SR1015 is severely attenuated for virulence in the animal model.

Cloning and sequencing of the genetic loci required for type I O-PS production and export

Two methods were used to clone the genes involved in the production and export of this surface polysaccharide. The DNA flanking the insertion of pSR1015 was cloned from SR1015 to yield pSR1015Bg, a *Bgl II* fragment which contains approximately 8 kb of flanking DNA. We also employed transposon mutagenesis and identified mutants as a means to clone the genes involved in the production of the polysaccharide. This was carried out in order to obtain any unlinked genes that may be involved in polysaccharide production. Approximately 1300 transposon

mutants were screened for loss of type I O-PS by ELISA. Six mutants were identified and the DNA flanking the transposon insertion was cloned and sequenced for each. The Tn5-OT182 integrations in five of the mutants, SLR5, SLR8, SLR13, SLR18, and SLR19 mapped to the same region of the chromosome (Fig.3). The DNA flanking the insertion of Tn5-OT182 in each of the mutant strains was cloned. Sequence analysis of the cloned fragments revealed the presence of nineteen potential open reading frames (ORFs) involved in the synthesis and export of type I O-PS (Fig.3). The reading frames that predicted proteins involved in polysaccharide biosynthesis were found to demonstrate homology to proteins involved in the synthesis of a polysaccharide structure composed primarily of mannose. The other reading frames in the locus predicted proteins involved in the transport of capsular polysaccharides in a variety of bacteria, particularly those that produce group 2 and group 3 capsular polysaccharides (Whitfield and Roberts, 1999). The organization of the genes responsible for the production of type I O-PS was found to be similar to other loci encoding for capsular polysaccharides in that the genes are divergently transcribed (Fig. 3) (Roberts, 1996). The gene cluster involved in the production of this polysaccharide is similar to group 3 capsule gene clusters in that there are no genes encoding for KpsF and KpsU (Whitfield and Roberts, 1999). However, the organization of the *B. pseudomallei* type I O-PS gene cluster does not appear to contain two export regions flanking a single biosynthetic region as seen in other group 3 capsule polysaccharide clusters (Clarke *et al.*, 1999). The biosynthetic genes identified thus far are not organized into one continuous transcriptional unit, instead *wcbB*, *manC*, and *wcbP* are separated from the rest of the biosynthetic genes that lie between the transport genes, *wzt2*, and *wcbO*. Another interesting feature is that *kpsC* is usually found next to *kpsS* in other group 2 and 3 clusters and this is not the case with *wcbA* and *wcbO* in *B. pseudomallei*. The promoter sequences of these transcriptional regions have yet to be identified. The overall G+C content of this region is about

58%, lower than the G+C content of the rest of the chromosome (68% G+C). The low G+C content in these clusters suggests that polysaccharide genes have a common origin and may have been transferred horizontally between species (Frosch *et al.*, 1992).

The genes involved in the production of the type I O-PS have been named according to the bacterial polysaccharide gene nomenclature (BPGN) scheme (Reeves *et al.*, 1996). The gene products associated with the type I O-PS cluster that have been identified are listed in Table 3. The gene *wcbA* predicts a protein that exhibits sequence homology to the LipA protein of *N. meningitidis* (Table 3). The gene *wcbO* predicts a protein with homology to the LipB protein of *N. meningitidis*. The LipA and LipB proteins are involved in the modification of the capsular polysaccharide of *N. meningitidis*. The LipA protein, along with LipB, is thought to be responsible for the addition of a phospholipid-anchoring group onto the reducing ends of the capsule polysaccharide chain (Frosch *et al.*, 1993). The *wcbA* and *wcbO* gene products also demonstrate homology to the KpsC and KpsS proteins, respectively, of *E. coli*. These proteins, are thought to be involved in the attachment of a phosphotidyl-KDO moeity to the polysaccharide prior to export across the cytoplasmic membrane by KpsT and KpsM (Roberts, 1996). It is therefore probable that *wcbA* and *wcbO* are involved in the processing and export of the polysaccharide, however, further genetic and biochemical analysis is necessary to prove this. The *wcbA* gene was inactivated by the insertion of a trimethoprim resistance cassette (Fig.3). The resulting strain, SR202::Tp, demonstrated attenuated virulence in the hamster model, similar to SR1015 (data not shown). Although the exact role of the *wcbA* gene in *B. pseudomallei* has yet to be determined, it is evident that this gene is required for polysaccharide production and necessary for virulence.

The *wcbC*, *wcbD*, *wzm2*, and *wzt2* genes encode for proteins that demonstrate strong homology to proteins involved in the transport of capsular polysaccharides. The *wcbC* gene

predicts a protein that shares homology with KpsD, an outer memebrane protein involved in the final stages of capsule polysaccharide export in *E. coli* (Frosch et al., 1992, Kroll et al., 1990). An isogenic mutant was constructed in this gene by the insertion of a trimethoprim cassette. The resulting strain, SR201::Tp, was still virulent in the hamster and was detected on Western blots (data not shown). Since the KpsD protein is involved in the export of capsular polysaccharide across the outer membrane, it may be that a mutation in the *wcbC* gene was not enough to abolish production of the polysaccharide. The *wcbD* gene product was found to demonstrate homology to CtrB from *N. meningitidis*, BexC from *H. influenzae*, and KpsE from *E. coli*. All of these are inner membrane proteins that have been implicated in the export of capsular polysaccharide (Table 3) (Frosch *et al.*, 1991, Kroll *et al.*, 1990, Rosenow *et al.*, 1995a).

The gene products encoded for by *wzm2* and *wzt2* are homologous to the KpsM and KpsT proteins of *E. coli*, CtrC and CtrD of *N. meningitidis*, and BexA and BexB of *H. influenzae*. These proteins are ATP-binding cassette (ABC) transporters that comprise an inner-membrane polysaccharide export system (Smith *et al.*, 1990). ABC-2 transporter systems are composed of an inner membrane protein (Wzm), and a cytoplasmic ATP-binding protein (Wzt). In *E. coli* the ABC-2 transport complex is proposed to consist of two molecules of KpsM that form an inner-membrane spanning pore, associated with two molecules of KpsT that catalyze ATP and energize the transport process (Roberts, 1996). The *wzm2* and *wzt2* gene products of *B. pseudomallei* likely comprise an ABC-transporter system that is involved in the transport of the type I O-polysaccharide across the cytoplasmic membrane. These genes have been designated *wzm2* and *wzt2* since *wzm* and *wzt* have already been identified that are associated with the type II O-PS gene cluster (DeShazer *et al.*, 1998). A hydrophobicity plot of the predicted *wzm2* gene product has revealed a hydrophobic protein with multiple membrane spanning domains, like KpsM, that may act as an integral membrane protein for the export of

polysaccharide (Kyte and Doolittle, 1982). Analysis of the primary amino acid sequence of the predicted Wzt2 protein from *B. pseudomallei* has shown that this protein contains a conserved ATP-binding motif, including an A site (GGNGAGKST) and a B site (DCFLIDE) (Walker *et al.*, 1982). The *wzt2* gene was found to be necessary for the production of type I O-PS in *B. pseudomallei*. In SLR18, the insertion of Tn5-OT182 in the *wzt2* gene resulted in a loss of type I O-PS, however, the exact roles of the *wzm2* and *wzt2* gene products in the production of this polysaccharide have yet to be proven. The termination codon of the *wzm2* gene overlaps the initiation codon of the *wzt2* gene, which suggests that these two genes are translationally coupled. The *kpsM* and *kpsT* genes of *E. coli* are known to be organized into a single transcriptional unit and both genes are translationally coupled (Roberts *et al.*, 1996).

The *wcbB* gene encodes for a protein that shares homology to a glycosyltransferase, WbpX, from *P. aeruginosa* as well as to mannosyltransferases from a variety of bacteria (Table 3). The function of glycosyltransferases is to catalyze the sequential transfer of sugar residues from nucleotide precursors to the membrane-bound acceptor, und-P-P-Glc*p*NAc (Whitfield, 1995). Since the structure of type I O-PS is known to be a homopolymer of mannoheptopyanosyl residues, it is probable that the *wcpA* gene product is involved in the transfer of mannose residues in its biosynthesis. This gene was determined to be required for the synthesis of type I O-PS based on two lines of evidence: the insertional inactivation of this gene using pSKM11 rendered the mutant strain, SR1015, negative for type I O-PS production; a transposon mutant, SLR5, lacked type I O-PS due to the insertion of Tn5-OT182 in the *wcbB* gene.

The *wcbE* and *wcbH* genes are two distinct reading frames encoding for proteins that demonstrate homology to different mannosyltranferases or glycosyltransferases from a variety of bacterial species (Table 3). Since type I O-PS is a homopolymer of mannoheptopyranosyl

residues, it is likely that these two genes are involved in the biosynthesis of this polysaccharide. This is supported by the fact that the insertion of Tn5-OT182 in both the *manA* and *manB* genes (SLR19 and SLR18, respectively), resulted in mutant strains lacking type I O-PS (Fig. 3). Furthermore, an internal fragment of the *wcbE* gene was cloned into pSKM11 and used to insertionally inactivate this gene in *B. pseudomallei*. The resulting strain, SR1016, was found to lack type I O-PS and demonstrated attenuated virulence in the animal model (data not shown).

Another biosynthetic gene, *manC* has been identified that encodes for a putative mannose-1-phosphate guanyltransferase (Fig 3., Table 3). In order to confirm the role of this gene in the production of type I O-PS and to define the limits of this gene cluster, we have constructed an allelic exchange mutant by insertion of a trimethoprim resistance cassette. The resulting strain, SR204::Tp will be tested for capsule production and for virulence in animals. Sequencing revealed the presence of an incomplete *manB* gene in the polysaccharide cluster. This open reading frame has been designated *'manB*. It shares sequence homology with the C-terminal region of the manB protein of a number of bacterial species (Table 3).

Other biosynthetic genes that have been identified are shown in Figure 3 and their homologues are listed in Table 3. The functions of all of these genes have yet to be determined. One of these genes, *wcbP* predicts a protein that shares homology to the YooP protein of M. tuberculosis (Table 3). The YooP protein has been characterized as a putative oxidoreductase based on sequence comparisons (Cole et al., 1998). The role of the predicted *wcbP* gene product in *B. pseudomallei* is unclear, however, the insertion of Tn5-OT182 into this gene in the mutant strain SLR13 rendered the organism negative for the production of type I O-PS (Fig. 3). Sequence data of the region downstream of the *wcbP* gene has revealed the presence of an open reading frame encoding for a type I polyketide synthase from *Streptomyces* species (data not

shown). We are currently constructing an allelic exchange mutant in this gene to investigate the role of the polyketide synthase gene in the production of type I O-PS in *B. pseudomallei.*

There are three open reading frames in the polysaccharide cluster that do not demonstrate significant homology to any proteins in the GenBank (Fig. 3, Table 3). *wcbF* shares sequence homology with a heparin-sulfate sulfotransferase as well as limited homology to a En/Spm transposon protein. *wcbG* predicts a protein that shares homology with a phenylalanyl-tRNA synthetase. *wcbI* predicts a protein that demonstrates weak homology to the NifQ protein of *Enterobacter agglomerans* (Siddavattam *et al.*, 1993). More analysis is needed in order to determine what these open reading frames encode for and the functions of their proteins. It is interesting, however, that *wcbF* and *wcbG* demonstrate homology to a transposon protein and a tRNA synthetase protein since pathogenicity islands are often associated with these types of genes (Hacker *et al.*, 1997). It may be that the proteins encoded by these genes are not involved in the synthesis of the polysaccharide, but rather act as sites of recombination (*wcbG*) or exist as cryptic genes that have been carried in on transposable elements (*wcbF*).

The *yafJ* gene encodes for a protein of 278 amino acids that demonstrates homology to the YafJ protein from *H. influenzae* and *E. coli* (Table 3). The YafJ protein is a putative amidotransferase in these organisms. The *yggB* gene encodes for a protein of 235 amino acids that shares homology with the YggB protein of *E. coli* (Table 3). The function of this protein is unclear, but has been defined as a hypothetical 30.9 kDa protein in an intergenic region. The G+C content of these genes is 65.7% G+C for *yafJ* and 65.4% G+C for *yggB*, much higher than the rest of the polysaccharide cluster and consistent with the G+C content of the *B. pseudomallei* chromosome. Southern blot analysis using these genes as probes has demonstrated that these genes are present in *B. thailandensis* (data not shown). For this reason, it is likely that these genes represent the end of the type I O-PS gene cluster (Fig. 3). An allelic exchange mutant,

SR203::Tp, was constructed that contains an insertion of a trimethoprim resistance cassette in the *yafJ* gene (Fig.3). This mutant will be tested for the production of type I O-PS as well as for virulence in the Syrian hamster.

The type I O-polysaccharide is also present in *B. mallei* and *B. cepacia*

Southern blot analysis using the 373 bp A-T rich glycosyltransferase fragment from pDD1015 as a probe confirmed that this fragment was present in *B. pseudomallei* 1026b and SR1015, but not in *B. thailandensis* (Fig.4). *Burkholderia mallei* and *Burkholderia cepacia* were also tested for reactivity to this A-T rich probe. It was found that this fragment is present in *B. mallei* and the *B. cepacia* Genomovar IV strain FCO362. None of the other *B. cepacia* genomovars appear to contain this DNA fragment. Southern blot analysis was carried out on six other Genomovar IV strains: CEP0717, CEP0467, CEP0485, CEP0726, FCO473, and FCO367. All of the Genomovar IV strains tested showed reactivity to the 373 bp probe (data not shown). The presence of type I O-PS was confirmed in the *B. cepacia* Genomovar IV strain FCO362 by immunoelectron microscopy. As seen in Figure 1D, *B. cepacia* Fco362 showed reactivity to the type I O-PS antibodies, but lacked a uniform distribution of the polysaccharide on the cell surface, and therefore appears to produce less of this polysaccharide compared to *B. pseudomallei* or *B. mallei.*

Discussion

Burkholderia pseudomallei is the causative agent of melioidosis, a disease that is emerging as an important cause of morbidity and mortality, particularly in Southeast Asia and northern Australia. Although the disease is less common outside of this area, it may be underdiagnosed in other regions and it poses a concern due to increased travel and military involvement in

endemic regions (Dance, 1990). Recently our attention has been focused on the identification of genetic determinants that contribute to the pathogenesis of *B. pseudomallei* infection. In order to obtain virulence determinants unique to *B. pseudomallei*, we employed subtractive hybridization between this organism and a related non-pathogenic organism, *B. thailandensis*.

Analysis of the subtractive hybridization library revealed that *B. pseudomallei* contains a number of DNA sequences that are not found in *B. thailandensis* (Table 1). All of the DNA fragments were found to be A-T rich and many of the DNA fragments demonstrated little homology to proteins in the GenBank database. The G+C content of the *B. pseudomallei* chromosome is 68% G+C, whereas the G+C contents of the subtraction clones ranged from 44% to 52% G+C. This suggests that *B. pseudomallei* acquired this DNA through a horizontal transfer event. Further screening of the subtraction library should reveal more A-T rich sequences and potential virulence determinants.

One of the subtraction clones, pDD1015, demonstrated weak homology to a glycosyltransferase, WbpX, from *Pseudomonas aeruginosa* (Rocchetta *et al.*, 1998). The insert from pDD1015 was cloned into a mobilizable suicide vector, pSKM11, for insertional inactivation of the glycosyltransferase gene in wild type *B. pseudomallei*. The resulting strain, SR1015, was tested in the animal model for virulence compared to the parent strain. The LD_{50} value for wild type *B. pseudomallei* 1026b in the Syrian hamster has been shown to be <10, while the LD_{50} value for SR1015 was determined to be 3.5×10^5, a 10,000 fold reduction in virulence (Table 2). The LD_{50} for *B. thailandensis* was found to be 6.8×10^5, similar to that for SR1015. This demonstrated that *B. pseudomallei* contains DNA sequences encoding for virulence determinants that are not found in *B. thailandensis*, and that the glycosyltransferase gene may encode for an important virulence determinant in *B. pseudomallei*.

Since glycosyltransferases are involved in the production of polysaccharides, we assumed that we had insertionally inactivated a gene involved in the production of a polysaccharide in *B. pseudomallei*. Using antibodies to type I O-PS we determined that SR1015 harboured a mutation in a glycosyltransferase gene involved in the production of type I O-PS.

Sequence analysis of the DNA flanking the glycosyltransferase gene revealed the presence of at least nineteen open reading frames involved in the synthesis and export of type I O-PS (Fig.3, Table 3). The genes identified encode for proteins that are similar to proteins involved in the biosynthesis and export of capsular polysaccharides, particularly those involved in the production of group 3 capsular polysaccharides. Group 3 capsules include the *E. coli* K10 capsule, and may also include the *H. influenzae* group b capsule, and the capsule produced by *N. meningitidis* serogroup B (Whitfield and Roberts, 1999). Group 3 capsules are often co-expressed with O serogroups, are not thermoregulated, are transported by an ABC-2 exporter system, do not contain the *kpsU* and *kpsF* genes, and usually the gene clusters map near the *serA* locus (Whitfield and Roberts, 1999). Thus far, a *serA* locus has not been identified that is associated with the type I O-PS cluster, but this polysaccharide is co-expressed with O antigen, lacks the *kpsU* and *kpsF* genes, and genes encoding for a putative ABC-2 transporter have been identified. The genes involved in the production of group 3 capsules are organized into regions and are divergently transcribed. Regions 1 and 3 are generally conserved and contain genes involved in export of the polysaccharide. These regions flank region 2, which contains the biosynthetic genes and is not conserved between serotypes (Roberts, 1996). The genetic organization of the type I O-PS is also similar to that of other capsule gene clusters in that the genes are organized into transcriptional units and appear to be divergently transcribed. However, the organization of the *B. pseudomallei* type I O-PS cluster differs in that the biosynthetic genes identified thus far are not organized into one biosynthetic region, they are, in

fact, separated by the four export genes, *wz2t*, *wzm2*, *wcbD*, and *wcbC* and the modification genes, *wcbA* and *wcbO*. The Tn5-OT182 integrations in SLR5, SLR8, SLR13, SLR18, and SLR19 were all physically mapped to the same genetic locus. All of these transposon mutants were found to lack type I O-PS. An allelic exchange mutant constructed in the *wcbA* gene abolished polysaccharide production and resulted in attenuated virulence in the animal model. We are currently investigating isogenic mutants in the *manC* and *yafJ* genes to define the limit of the gene cluster. Preliminary evidence suggests that *yafJ* and *yggB* may not be involved in the production of type I O-PS since they have a high G+C content (62-65%) and Southern blots have demonstrated that they are present in *B. thailandensis* (data not shown). We are also currently constructing a mutant in the polyketide synthase gene that lies downstream of the *wcbP* gene in order to define the other end of the type I O-PS cluster.

The polysaccharide with the structure –3)-2-*O*-acetyl-6-deoxy-β-D-*manno*heptopyranose-(1- was originally isolated and characterized as an O-PS component of LPS in *B. pseudomallei* and designated type I O-PS (Perry *et al.*, 1995). However, our results suggest that this polysaccharide is a capsule rather than an O-PS moiety. The genes involved in the production of this capsule demonstrate strong homology to the genes involved in the production of capsular polysaccharides in many organisms, including *N. meningitidis*, *H. influenzae*, and *E. coli*. In addition, the export genes associated with this cluster are not associated with the previously characterized O-PS gene cluster (DeShazer *et al.*, 1998). Western blot analysis of proteinase K cell extracts (Fig. 2) and silver staining (data not shown) have shown that this polysaccharide has a high molecular weight (200 kDa) and lacks the banding pattern seen with O-PS moieties. Studies by our laboratory have indicated that mutants in the production of the core oligosaccharide of the LPS are still capable of producing this polysaccharide (Burtnick *et al.*, 1999). Furthermore, this polysacchride has been shown to be

important for the virulence of *B. pseudomallei*. Capsule production has been correlated with virulence in many bacteria, particularly those causing serious invasive infections of man (Boulnois and Roberts, 1990). Based on the above criteria and the genetic similarity to group 3 capsules, we propose that this polysaccharide is a group 3 capsule.

These studies have demonstrated that this capsule is critical for the virulence of *B. pseudomallei*. However, its role in infection has yet to be elucidated. A number of functions have been suggested for polysaccharide capsules. These include: prevention of desiccation for transmission and survival, adherence for colonization, resistance to complement-mediated phagocytosis and complement-mediated killing, and resistance to specific host immunity due to a poor antibody response to the capsule (Roberts, 1996). Preliminary studies have shown that type I O-PS is not involved in serum resistance. SR1015 was tested for resistance to killing by 30% normal human serum and was found to be resistant to killing (data not shown). Studies are currently underway to define the role of the capsule in infection.

Burkholderia mallei 10260 as well as representative strains from the five genomovars of *B. cepacia* were tested for reactivity to the A-T rich probe from pDD1015 by Southern hybridization. The DNA from *B. mallei* 10260 and *B. cepacia* FCO362, a Genomovar IV strain hybridized to the probe (Fig.4). Six other Genomovar IV strains were tested and demonstrated reactivity to the probe by Southern hybridization (data not shown), however, none of the other *B. cepacia* genomovars reacted to the probe and therefore likely do not produce this polysaccharide (Fig.4). Immunoelectron microscopy using antibodies to this polysaccharide was carried out and *B. cepacia* FCO362 was found to contain this capsule(Fig.1). Interestingly, *B. cepacia* FCO362 was noted to produce less of this polysaccharide than *B. pseudomallei*. Unlike *B. pseudomallei*, *B. cepacia* FCO362 lacked a uniform distribution of the polysaccharide on the cell surface. The importance of the capsule in *B. cepacia* Genomovar IV strains has yet to be elucidated.

Virulence genes of a number of pathogenic bacteria have been recently located on pathogenicity islands. Pathogenicity islands (Pais) are regions on the bacterial chromosome that are present in in the genome of pathogenic strains, but absent or rarely present in those of non-pathogenic strains. The Pais may range in size from about 30 kb to 200 kb in size, the G+C contents of which often differ from that of the remaining bacterial genome, and are often associated with the carriage of many virulence genes. These genetic units are often flanked by direct repeats and may be associated with tRNA genes or insertion sequence (IS) elements at their boundaries. They may also be associated with the presence of "mobility" genes, such as IS elements, integrases, transposases, and origins of plasmid replication. These DNA regions are considered to be unstable in that they may delete with high frequencies or undergo duplications and amplifications (Hacker *et al.*, 1997). A number of Pais have been described for both gram-positive and gram-negative bacteria, and the application of subtraction hybridization has been used to successfully identify such genetic elements (Hacker *et al.*, 1997, Mahairas *et al.*, 1996). The subtractive hybridization that was carried out between *B. pseudomallei* and *B. thailandensis* led to the identification of a number of sequences that were found to be A-T rich compared to the rest of the *B. pseudomallei* chromosome. One of these clones, pDD1005, contained an insert that was found to share homology with a plasmid mobility gene and one, pDD1000, was found to contain an insert that demonstrated homology to a protein, DprA, that is involved in DNA transfer (Karudapuram *et al.*, 1995). This combined with the fact that insertional mutagenesis of the glycosyltransferase gene identified by this method resulted in an avirulent strain, suggests that we may have identified DNA sequences from a putative pathogenicity island and that the capsular polysaccharide gene cluster may be located on this island. It is possible that *B. pseudomallei*, and even *B. mallei*, and *B. cepacia* Genomovar IV strains acquired DNA encoding for capsule as well as other potential, yet unidentified virulence factors by horizontal

transfer recently in evolution. *B. pseudomallei* is known to contain IS elements that are present in *B. cepacia*, but not in *B. thailandensis* (Mack and Titball, 1998). However, IS elements have not yet been identified in association with the capsule gene cluster. Further studies are currently underway to determine whether a pathogenicity island exists in these organisms and whether the capsule gene cluster is located on such a genetic element.

The identification of bacterial virulence genes has traditionally relied upon empirical predictions of putative virulence determinants and 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 infection models (Falkow *et al.*, 1988). Technologies such as IVET (in vivo expression technology), signature-tagged mutagenesis, and DFI (differential fluorescence technology) have been developed to facilitate the identification of expressed sequences under a given set of circumstances within a test host; however, these approaches do not necessarily lead to the identification of virulence determinants (Strass and Falkow, 1997). 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 pathogen *Burkholderia pseudomallei* in our current studies, this methodology could be applied to any species and for which only a few 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. The described method should lead to the identification of relevant virulence determinants for a number of bacterial species and further the understanding of molecular pathogenesis.

Experimental Procedures

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are described in Table 4. *B. pseudomallei*, *B. thailandensis*, *B. cepacia* and *E. coli* were grown at 37°C on Luria-Bertani (LB) broth base (Becton Dickinson) agar plates or in LB broth. *B. mallei* was grown at 37°C on LB plates or in LB broth supplemented with 4% glycerol and at pH 6.8. For animal studies, *B. pseudomallei* and *B. thailandensis* cultures were grown at 37°C in TSBDC medium (Brett *et al.*, 1997). When appropriate, antibiotics were added at the following concentrations: 50 μg tetracycline, 100 μg streptomycin, 100 μg polymyxin B, 100 μg trimethoprim, 25 μg gentamicin, and 25 μg kanamycin per ml for *B. pseudomallei* and 100 μg ampicillin, 25 and 50 μg kanamycin, 15 μg tetracycline, and 1.5 mg trimethoprim per ml for *E. coli*.

Construction and screening of subtractive hybridization libraries

Subtractive hybridization was carried out between *B. pseudomallei* and *B. thailandensis* using the PCR-Select Bacterial Genome Subtraction Kit (Clonetech). 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 G+C content in the genomes of these species. In the construction of the *B. pseudomallei* subtractive hybridization library, *B. pseudomallei* genomic DNA was used as the "tester" and *B. thailandensis* genomic DNA was used as the "driver". The secondary PCR products obtained were cloned into pZErO-2.1 (Invitrogen) and were enriched for *B. pseudomallei* specific sequences. The subtraction library was screened by sequencing of the tester-specific DNA fragments. The library containing random clones was diluted in sterile PBS to 10^{-6} and 100 μl was plated on LB plates containing 50 μg/ml kanamycin and 1 mM IPTG.

Individual colonies were picked and grown overnight at 37°C in LB with kanamycin (50 μg/ml). Plasmid DNA was isolated using a mini-prep plasmid isolation kit (Qiagen).

DNA sequencing and analysis

Automated DNA sequencing was performed by ACGT (Northbrook, Illinois) and the University of Calgary Core DNA Services (University of Calgary). The M13 primer was used to initiate sequence reactions with the subtractive hybridization clones. DNA flanking the Tn5-OT182 insertions was sequenced using the previously described primers OT182-LT and OT182-RT (DeShazer et al., 1997). The DNA flanking the insertion of pSR1015 was sequenced using the pSKM11 primer. DNA and protein sequences were analyzed with DNASIS version for IBM and with the ORF finder program at NCBI. DNA sequences were analyzed for homology using the BLASTX program through GenBank at NCBI.

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

The DNA insert from pDD1015 was cloned as a *Kpn I-Xho I* fragment into a mobilizable suicide vector, pSKM11 (Mongkolsuk *et al.*, 1994). The 373 bp fragment was ligated to pSKM11 digested with the same enzymes to create pSR1015. SM10(pSR1015) was conjugated to *B. pseudomallei* 1026b using a previously described protocol (DeShazer et al., 1997).

Animal studies

The animal models of acute *B. pseudomallei* infection have been previously described (DeShazer, 1999). 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 PBS. The control group (5 animals) were inoculated with 10^1 cfu/ml of wild type *B. pseudomallei*. The test animals (5 per dilution) were inoculated with either 10^1, 10^2, or 10^3 cfu/ml of the mutant strain, SR1015. Blood from two of the test animals was diluted and plated on Ashdown media with and without the addition of 50 μg/ml tetracycline to verify the stability of pSR1015 (Ashdown, 1979). For determination of LD_{50} for SR1015, hamster were inoculated with 10^3, 10^4, 10^5, and 10^6 cfu/ml (5 hamsters per dilution). After 48 hours the LD_{50} was calculated (Reed, 1938).

Immunogold electron microscopy

Immunogold electron microscopy was performed as previously described ((DeShazer, 1998).

Western blot analysis

Preparation of the samples for Western blot analysis was carried out as previously described (Bryan, 1994). Immunoassay was performed as outlined using a 1:250 dilution of the primary antibody, polyclonal rabbit antiserum raised to a *B. pseudomallei* O-PS-flagellin protein conjugate (Bryan, 1994, Brett, 1996). The secondary antibody used was horseradish peroxidase conjugated goat anti-rabbit IgG (Sigma).

Southern hybridization

Southern hybridization was performed according to the procedure outlined in the GeneScreen and GeneScreen *Plus* manual (NEN Research Products, Du Pont). The 373 bp A-T rich fragment from pDD1015 was labelled with ^{32}P according to the manual for use as a probe. Chromosomal DNA from *B. pseudomallei*, *B. thailandensis*, *B. mallei*, and *B. cepacia* was digested with *Bam HI* or *Sst 1* for Southern blot analysis.

Tn5-OT182 mutagenesis and screening for type I O-PS mutants

In order to screen for mutants deficient in type I O-PS, it was first necessary to create a strain that produced only type I O-PS. This is because the antiserum available for use to screen by ELISA was polyclonal antiserum to a flagellin-O-PS conjugate that contains antibodies to both type I and type II O-PS. Therefore a strain was constucted that is lacking in type II O-PS and flagella, SR1001. Allelic exchange was carried out using two strains that were previously constructed in the laboratory. The donor strain, SM10λ*pir*(pPB611::Gm) has a plasmid containing a copy of the *wbiE* gene, involved in the synthesis of type II O-PS, which has been mutated by the insertion of a gentamicin resistance cassette (DeShazer, 1998). The recipient strain, PB401, is a *B. pseudomallei* strain that has a deletion in the *fliC* gene. SM10λ*pir* (pPB611::Gm) was conjugated to PB401 and transconjugants were selected for by plating on LB containing gentamycin (25 μg/ml), kanamycin (25 μg/ml), and polymyxin B (100 μg/ml). Transconjugants that were Sm^R, and Gm^R, but not Km^R were selected (to select for loss of the vector, pKAS46) and one was designated SR1001. Transposon mutagenesis of SR1001 was performed with Tn5-OT182 according to a previously described protocol (DeShazer et al., 1997) except that transconjugants were selected for on plates containing 25 μg/ml gentamicin as well as 50 μg/ml tetracycline. Transposon mutants were picked into 96-well plates containing 200 μl of LB with gentamicin (25 μg/ml) and tetracycline (50 μg/ml) and grown overnight at 37°C and 250 rpm. A negative growth control well was included for each plate. For the ELISA, the wells of a 96-well ELISA plate were coated with 10 μl of bacteria and 90 μl of coating buffer (0.05 M carbonate buffer, pH 9.6), and the plate was incubated at 37°C for 1 h. The wells were washed 2X with PBS plus 0.05% Tween-20 and blocked with a 3% solution of skim milk in PBS-Tween

for 1h. The wells were washed with PBS-Tween and a 1:1000 dilution of the primary antibody, polyclonal rabbit antiserum to a *B. pseudomallei* O-PS-flagellin protein conjugate, was added and the plate incubated for 1h at 37°C (Brett, 1996). The wells were washed and a 1:1000 dilution of a goat anti-rabbit IgG peroxidase conjugate (Sigma) was added to each well. The plate was incubated for 1h at 37°C, washed and developed with an ABTS substrate (Kirkegaard and Perry Laboratories) for 30 min. The optical density at 405 nm (OD_{405}) was determined using an ELISA reader. *B. pseudomallei* 1026b was included as a positive control and *E. coli* DH5α was included as a negative control. Transposon mutants that had OD_{405} readings comparable to the negative control ($OD_{405} = < 0.100$) were chosen for further analysis.

Construction of allelic exchange mutants

Allelic exchange was carried out as previously described (DeShazer *et al.*, 1998). The allelic exchange vector used in these experiments was pKAS46, an allelic exchange vector based on *rpsL* for counterselection (Skorupski and Taylor, 1996). *B. pseudomallei* DD503, a double mutant that contains the *ΔamrR-oprA* and *rpsL* mutations was the recipient strain used for all allelic exchange experiments (DeShazer *et al.*, 1998; Moore *et al.*, 1999). All of the genes in these experiments were mutated by the insertion of a self-cloning trimethoprim resistance from p34EoriTp (P.J. Brett, D. DeShazer and D.E. Woods, unpublished). For each allelic exchange experiment, SM10λpir transformed with pKAS46 containing the mutated allele was conjugated to *B. pseudomallei* DD503 according to the method described above for the construction of SR1001 except the transconjugants were plated on Pm, Km, and Tp. The Pm^R, Km^R, Tp^R transconjugants were subsequently transferred to plates containing Sm to select for the loss of pKAS46. Mutant alleles were confirmed by self-cloning and sequencing.

DNA manipulation

Restriction enzymes and T4 DNA ligase were purchased from Gibco BRL and New England Biolabs and used according to the manufacturer's instructions. DNA fragments used in cloning procedures were excised from agarose gels and purified using the GeneClean II kit (Bio 101) or the Qiagen Gel Extraction kit. Chromosomal DNA was isolated from *B. pseudomallei*, *B. thailandensis*, *B. cepacia*, and *B. mallei* using a previously described protocol (Wilson, 1987). The self-cloning of *B. pseudomallei* flanking DNA from Tn5-OT182 mutants and from SR1015 was performed as described previously (DeShazer et al., 1997).

Nucleotide sequence accession number

The nucleotide sequence reported in this paper has been deposited in the GenBank database under the accession number?

Acknowledgements

This work was supported by the Department of Defense Contract DAMD 17-98-C-8003 and the Canadian Bacterial Diseases Network of Centers of Excellence. D.D. is the recipient of an Alberta Heritage Foundation for Medical Research (AHFMR) Fellowship award. We would like to thank Trish Darling and Amie White for excellent technical assistance. We would also like to thank Ivo Steinmetz for the EPS-specific monoclonal antibody 3015 and Dr. Malcolm Perry for many helpful discussions.

References

Boulnois, G.J., and Roberts, I.S. (1990) Genetics of capsular polysaccharide production in bacteria. *Curr Top Microbiol Immunol* **150**: 1-18.

Brett, P.J. and Woods, D.E. (1996) Structural and immunologic characterization of *Burkholderia pseudomallei* O-polysaccharide-flagellin protein conjugates. *Infect Immun* **64**: 2824-2828.

Brett, P.J., DeShazer, D., and Woods, D.E. (1997) Characterization of *Burkholderia pseudomallei* and *Burkholderia pseudomallei*-like strains. *Epidemiol Infect* **118**: 137-148.

Brett, P.J., DeShazer, D., and Woods, D.E. (1998) *Burkholderia thailandensis* sp. nov., description of a *Burkholderia pseudomallei*-like species. *Int J Syst Bacteriol* **48**: 317-320.

Bryan, L.E., Wong, S., Woods, D.E., Dance, D.A., and Chaowagul, W. (1994) Passive protection of diabetic rats with antisera specific for the polysaccharide portion of the lipopolysaccharide isolated from *Pseudomonas pseudomallei*. *Can J Infect Dis* **5**(4): 170-178.

Burtnick, M.N. and Woods, D.E. (1999) Isolation of polymyxin B-susceptible mutants of *Burkholderia pseudomallei* and molecular characterization of genetic loci involved in polymyxin B resistance. *Antimicrob Agents Chemother* **43**: 2648-2656.

Chaiyaroj, S.C., Kotrnon, K., Koonpaew, S., Anantagool, N., White, N.J., and Sirisinha, S. (1999) Differences in genomic macrorestriction patterns of arabinose-positive (*Burkholderia thailandensis*) and arabinose-negative *Burkholderia pseudomallei*. *Microbiol Immunol* **43**(7): 625-30.

Chaowagul, W., White, N.J., Dance, D.A., Wattanagoon, Y., Naigowit, P., Davis, T.M., Looareesuwan, S., and Pitakwatchara, N. (1989) Melioidosis: a major cause of community-acquired septicemia in northeastern Thailand. *J Infect Dis* **159**: 890-899.

Clarke, B.R., Pearce, R., Roberts, I.S. (1999) Genetic organization of the *Escherichia coli* K10 capsule gene cluster: identification and characterization of two conserved regions in group III capsule gene clusters encoding polysaccharide transport functions. *J Bacteriol* **181**(7): 2279-2285.

Dance, D.A.B. (1990) Melioidosis. *Rev Med Microbiol* **1**: 143-150.

DeShazer, D., Brett, P.J., Carylon, R., and Woods, D.E. (1997) Mutagenesis of *Burkholderia pseudomallei* with Tn5-OT182: isolation of motility mutants and molecular characterization of the flagellin structural gene. *J Bacteriol* **179**: 2116-2125.

DeShazer, D., Brett, P.J., and Woods, D.E. (1998) The type II O-antigenic polysaccharide moeity of *Burkholderia pseudomallei* lipopolysaccharide is required for serum resistance and virulence. *Mol Microbiol*

DeShazer, D., and Woods, D.E. (1999) Animal models of melioidosis. In *Handbook of Animal Models of Infection*. O.Zak, M. Sande (ed). Academic Press, London.

Falkow, S. (1988) Molecular Koch's postulates. *Rev Infect Dis* **10**(2): S274-278.

Frosch, M., Edwards, U., Bousset, K., Krausse, B., and Weisgerber, C. (1991). Evidence for a common molecular origin of capsular gene loci in gram-negative bacteria expressing group II capsular polysaccharides. *Mol Microbiol* **5**: 1251-1263.

Frosch, M., Müller, D., Bousset, K., Müller, A. (1992) Conserved outer membrane protein of *Neisseria meningitidis* involved in capsule expression. *Infect Immun* **60**: 798-803.

Frosch, M. and Müller, A. (1993) Phospholipid substitution of capsular polysaccharide and mechanisms of capsule formation in *Neisseria meningitidis*. *Mol Microbiol* **8**: 483-493.

Hacker, J., Blum-Oehler, G., Mühldorfer, I., and Tschäpe, H. (1997) Pathogenicity islands of virulent bacteria: structure, function and impact on microbial evolution. *Mol Microbiol* **23**: (6) 1089-1097.

Howe, C., Sampath, A., and Spotnitz, M. (1971) The pseudomallei group: a review. *J Infect Dis* **124**: 598-606.

Ip, M., Osterberg, L.G., Chau, P.Y., and Raffin, T.A. (1995) Pulmonary melioidosis. *Chest* **108**(5): 1420-1424.

Karudapuram, S., Zhao, X., and Barcak, G.J. (1995) DNA sequence and characterization of *Haemophilus influenzae dprA*+, a gene required for chromosomal but not plasmid transformation.

Knirel, Y.A., Paramonov, N.A., Shashkov, A.S., Kochetkov, N.K., Yarullin, R.G., Farber, S.M., and Efremenko, V.I. (1992) Structure of the polysaccharide chains of *Pseudomonas pseudomallei* lipopolysaccharides. *Carbohydr Res* **233**: 185-193.

Kroll, S., Loynds, B., Brophy, L., and Moxon, E. (1990) The *bex* locus in encapsulated *Haemophilus influenzae*: a chromosomal region involved in capsule polysaccharide export. *Mol Microbiol* **4**: 1853-1862.

Kyte, J. and Doolittle, R.F. (1982) A simple method for displaying the hydropathic character of a protein. *J Mol Biol* **157**: 105-132.

Leelarasamee, A., and Bovornkitti, S. (1989) Melioidosis: review and update. *Rev Infect Dis* **11**: 413-425.

Mack, K., and Titball, R.W. (1998) The detection of insertion sequences within the human pathogen *Burkholderia pseudomallei* which have been identified previously in *Burkholderia cepacia*. *FEMS Microbiol Let* **162**: 69-74.

Mahairas, G.G., Sabo, P.J., Hickey, M.J., Singh, D.V., and Stover, C.K. (1996) Molecular analysis of genetic differences between *Mycobacterium bovis* BCG and virulent *M. bovis*. *J Bacteriol* **178**: 1274-1284.

Masoud, H., Ho, M., Schollaardt, T., and Perry, M.B. (1997) Characterization of the capsular polysaccharide of *Burkholderia pseudomallei* 304b. *J Bacteriol* **179**: 5663-5669.

Merriman, T.R., and Lamont, I.L. (1993) Construction and use of a self-cloning promoter probe vector for Gram-negative bacteria. *Gene* **126**: 17-23.

Miller, V.L., and Mekalanos, J.J. (1988) A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. *J Bacteriol* **170**: 2575-2583.

Mongkolsuk, S., Rabibhadana, S., Vattanaviboon, P., and Loprasert, S. (1994) Generalized and mobilizable positive-selective cloning vectors. *Gene* **143**: 145-146.

Moore, R.A., DeShazer, D., Reckseidler, S., Weissman, A., and Woods, D.E. (1999) Efflux-mediated aminoglycoside and macrolide resistance in *Burkholderia pseudomallei*. *Antimicrob Agents Chemother* **43**(3): 465-470.

Nimtz, M., Wray, V., Domke, T., Brenneke, B., Haussler, S., and Steinmetz, I. (1997) Structure of an acidic exopolysaccharide of *Burkholderia pseudomallei*. *Eur J Biochem* **250**: 608-616.

Perry, M.B., MacLean, L.L., Schollaardt, T., Bryan, L.E., and Ho, M. (1995) Structural characterization of the lipopolysaccharide O antigens of *Burkholderia pseudomallei*. *Infect Immun* **63**: 3348-3352.

Petit, C., Rigg, G., Pazzani, C., Smith, A., Sieberth, V., Stevens, M., Boulnois, G., Jann, K., and Roberts, I.S. (1995) Region 2 of the *Escherichia coli* K5 capsule gene cluster encoding proteins for the biosynthesis of the K5 polysaccharide. *Mol Microbiol* **17**(4): 611-620.

Reed, L.J., and Muench, H. (1938) A simple method of estimating fifty percent endpoints. *Am J Hyg* **27**: 493-497.

Reeves, P.R., Hobbs, M., Valvano, M.A., Skurnik, M., Whitfield, C., Coplin, D., *et al.* (1996) Bacterial polysaccharide synthesis and gene nomenclature. *Trends Microbiol* **498**: 495-503.

Roberts, I.S. (1996) The biochemistry and genetics of capsular polysaccharide production in bacteria. *Annu Rev Microbiol* **50**: 285-315.

Rocchetta, H.L., Burrows, L.L., Pacan, J.C., and Lam, J.S. (1998) Three rhamnosyltransferases responsible for assembly of the A-band D-rhamnan polysaccharide in *Pseudomonas aeruginosa*: a fourth transferase, WbpL, is required for the initiation of both A-band and B-band lipopolysaccharide synthesis. *Mol Microbiol* **28**(6):1103-19.

Rosenow, C., Esumeh, F., Roberts, I.S., and Jann, K. (1995) Characterization and localisation of the KpsE protein from *Escherichia coli* K5, which is involved in polysaccharide export. *J Bacteriol* **177**: 1137-1143.

Sanford, J.P. (1990) In *Principles and Practice of Infectious Diseases*. Mandell, G.L., Douglas Jr., R.G., and Bennett, J.E. (eds). Churchill Livingstone, New York, pp. 1692-1696.

Siddavattam, D., Singh, M., and Klingmuller, W. (1993) Structure of the nifQ gene from *Enterobacter agglomerans* 333 and its overexpression in *Escherichia coli. Mol Gen Genet* **239**(3): 435-40.

Simon R., Priefer, U., and Puhler, A. (1983) A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. *Bio/Technology* **1**: 784-791.

Skorupski, K., and Taylor, R.K. (1996) Positive selection vectors for allelic exchange. *Gene* **169**: 47-52.

Smith, A.N., Boulnois, G.J., Roberts, I.S. (1990) Molecular analysis of the *Escherichia coli K5* kps locus: identification and characterization of an inner-membrane capsular polysaccharide transport system. *Mol Microbiol* **4**: 1863-69.

Steinmetz, I., Reganzerowski, A., Brenneke, B., Haussler, S., Simpson, A., and White, N.J. Rapid identification of *Burkholderia pseudomallei* by latex agglutination based on an exopolysaccharide-specific monoclonal antibody. *J Clin Microbiol* **37**(1): 225-8.

Steinmetz, I., Rohde, M., and Brenneke, B. (1995) Purification and characterization of an exopolysaccharide of *Burkholderia (Pseudomonas) pseudomallei. Infect Immun* **63**: 3959-3965.

Strauss, E.J., and Falkow, S. (1997) Microbial pathogenesis: genomics and beyond. *Science* **276**: 707-712.

Trakulsomboon, S., Dance, D.A.B., Smith, M.D., White, N.J., and Pitt, T.L. (1997) Ribotype differences between clinical and environmental isolates of *Burkholderia pseudomallei. J Med Microbiol* **46**: 565-570.

Walker, J.E., Saraste, M., Runswick, M.J., and Gay, N.J. (1982) Distantly related sequences in the α- and β-subunits of ATP synthase, myosin, kinases, and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J* **1**: 945-951.

Whitfield, C. (1995) Biosynthesis of lipopolysaccharide O antigens. *Trends Microbiol* **3**: 178-185.

Whitfield, C., and Roberts, I.S. (1999) Structure, assembly and regulation of expression of capsules in *Escherichia coli. Mol Microbiol* **31**(5): 1307-1319.

Wilson, K. (1987) Preparation of genomic DNA from bacteria. In *Current Protocols in Molecular Biology*. Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., *et al.* (eds). New York: John Wiley & Sons, pp. 2.4.1-2.4.5.

Wuthiekanun, V., Smith, M.D., Dance, D.A., Walsh, A.L., Pitt, T.L., and White, N.J. (1996) Biochemical characteristics of clinical and environmental isolates of *Burkholderia pseudomallei*. *J Med Microbiol* **45**(6): 408-12.

Figure Legends

Figure 1. Immunogold electron microscopy of *B. pseudomallei* 1026b, SR1015, *B. thailandensis* E264, and *B. cepacia* FCO362. Bacteria were reacted with polyclonal rabbit antiserum directed against an O-PS-flagellin protein conjugate absorbed with *B. thailandensis* E264 to remove the antibodies directed against type II O-PS, washed, and reacted with a goat anti-rabbit IgG gold conjugate (5nm). A. *B. pseudomallei* 1026b; B. *B. pseudomallei* SR1015; C. *B. thailandensis* E264; D. *B. cepacia* FCO362. Magnification, X 30,000.

Figure 2. Western blot analysis of LPS isolated from *B. pseudomallei* 1026b, SR1015, and *B. thailandensis* E264. Bacteria were reacted with proteinase K, subjected to SDS-polyacrylamide gel electrophoresis, electroblotted, and reacted with polyclonal rabbit antiserum raised to an O-PS-flagellin protein conjugate from *B. pseudomallei*. Lane M, prestained protein molecular weight standards (New England Biolabs), Lane 1, *B. pseudomallei* 1026b; Lane 2, *B. pseudomallei* SR1015; Lane 3, *B. thailandensis* E264. The apparent molecular weights in kDa, of the prestained proteins are indicated.

Figure 3. Organization of the chromosomal region containing the genes responsible for the synthesis and export of type I O-PS in *B. pseudomallei*. A. Genetic map. The location of the genes and direction of transcription is represented by arrows and the gene names are indicated. The locations of Tn5-OT182 insertions are represented by triangles. Mutants constructed by allelic exchange are shown. The straight line indicates the insertion of the trimethoprim cassette into the gene of interest. B. Physical map. The horizontal line represents *B. pseudomallei* chromosomal DNA, and the locations of relevant restriction endonuclease recognition sites are shown. Bg, *Bgl II*; E, *Eco R1*; H, *Hind III*; K, *Kpn I*; B, *Bam H1*.

Figure 4. Southern hybridization analysis of *B. pseudomallei*, *B. thailandensis*, *B. mallei*, and *B. cepacia*. The 373 bp A-T rich fragment with homology to a glycosyltransferase from pDD1015 was used as a probe. Lane 1, *B. mallei* 10260; Lane 2, *B. pseudomallei* 1026b; Lane 3, *B. thailandensis* E264; Lane 4, *B. pseudomallei* SR1015; Lane 5, *B. cepacia* Genomovar I; Lane 6, *B. cepacia* Genomovar II; Lane 7, *B. cepacia* Genomovar IV; Lane 8, *B. cepacia* Genomovar III; Lane 9, *B. cepacia* Genomovar V.

Table 1. Description of recombinant plasmids in the *B. pseudomallei -B. thailandensis* subtraction library.

Plasmid	Vector	Insert Size	%G+C	Homologue*
pDD1000	pPCR	326 bp	50.6	DprA
pDD1001	pPCR	800 bp	44	none
pDD1002	pPCR	434	49.5	GuaA
pDD1003	pPCR	346	50.8	none
pDD1004	pPCR	800	44	none
pDD1005	pPCR	531	46	Mob protein
pDD1006	pPCR	353	48.2	none
pDD1007	pZErO-2.1	325	51	none
pDD1008	pZErO-2.1	250	44	none
pDD1009	pZErO-2.1	350	52	none
pDD1012	pZErO-2.1	505	46.7	none
pDD1015	pZErO-2.1	373	52	WbpX
pDD1016	pZErO-2.1	259	46	none
pDD1017	pZErO-2.1	100	50	none
pDD1018	pZErO-2.1	433	49.6	none

* Homologue and reference are as follows: *Haemophilus influenzae* DprA, Karudapuram *et al.*, 1995; *Bacillus subtilis* GuaA, Mantsala *et al.*, 1992; *Pseudomonas aeruginosa* WbpX, Rocchetta *et al.*, 1998.

Table 2. LD_{50} values for *B. pseudomallei* 1026b, SR1015, and *B. thailandensis* E264 in the Syrian Golden hamster.

Strain	LD_{50} value
B. pseudomallei 1026b	<10
B. pseudomallei SR1015	3.5×10^5
B. thailandensis E264	6.8×10^5

Table 3. Genes involved in the production and export of type I O-PS in *B. pseudomallei* and homologous proteins located in the non-redundant sequence database.

Gene	Size (bp)	Homologous proteins	Putative Function
manC	1428	ManC *E. coli*	mannose-1-phosphate guanyltransferase
		ManC *S. typhimurium*	mannose-1-phosphate guanyltransferase
		ManC *K. pneumoniae*	mannose-1-phosphate guanyltransferase
wcbA	1524	KpsC *E. coli*	capsule polysaccharide export protein
		LipA *N. meningitidis*	capsule polysaccharide modification protein
		PhyA *P. multocida*	capsule polysaccharide export protein
wcbB	1049	WbpX *P.aeruginosa*	glycosyltransferase
		ManB *A. aeolicus*	mannosyltransferase
		MtfA *A. fulgidis*	mannosyltransferase
wcbC	1164	KpsD *E. coli*	capsule export outer membrane protein
		CtrA *N. meningitidis*	capsule export outer membrane protein

		BexD *H. influenzae*	capsule export outer membrane protein
'manB	162	XanA *X. campestris*	phosphomannomutase
		ManB *K. pneumoniae*	phosphomannomutase
		Rfk9 *E. coli*	phosphomannomutase
wcbD	1236	BexC *H.influenzae*	capsule export inner membrane protein
		CtrB *N. meningitidis*	capsule export inner membrane protein
		KpsE *E. coli*	capsule export inner membrane protein
wzm2	411	CtrC *N. meningitidis*	capsule export inner membrane protein
		BexB *H. influenzae*	capsule export inner membrane protein
		KpsM *E. coli*	capsule export inner membrane protein
wzt2	750	BexA *H. influenzae*	ATP-binding protein
		CtrD *N.meningitidis*	ATP-binding protein
		KpsT *E. coli*	ATP-binding protein

wcbE	1524	MtfB *A. aeolicus*	mannosyltransferase
		WbpX *P.aeruginosa*	glycosyltransferase
		ManB *Synechocystis spp.*	mannosyltransferase
wcbF	1380	hyp. protein *H. sapiens*	heparan-sulfate 6-sulfotransferase
		putative *A. thaliana*	En/Spm transposon protein
wcbG	942	SyfB *H. pylori*	phenylalanyl-tRNA synthetase
wcbH	1797	MtfA *Archaeoglobus*	mannosyltransferase
		hypothetical protein *Synechocystis spp.*	unknown
		putative glycosyltransferase *S. coelicolor*	glycosyltransferase
wcbI	1188	NifQ *E. agglomerans*	nitrogen fixation
wcbJ	843	Rbd1 *E. coli*	dTDP-4-dehydrorhamnose reductase
		RmlD *M. tuberculosis*	dTDP-4-dehydrorhamnose reductase

wcbK	1014	Gm4D *E. coli*	GDP-mannose-dehydratase
		Gm4D *Y. enterocolitica*	GDP-mannose-dehydratase
		Gm4D *V. cholerae*	GDP-mannose-dehydratase
wcbL	1041	LmbP *Synechocystis sp.*	lincomycin production
		Rv0115 *M. tuberculosis*	lincomycin production
		GalK *E. coli*	galactose utilization
gmhA	594	GmhA *M. tuberculosis*	phosphoheptose isomerase
		LpcA *E. coli*	phosphoheptose isomerase
		LpcA *H. influenzae*	phosphoheptose isomerase
wcbM	693	RmlA2 *M. tuberculosis*	mannose-1-phosphate guanyltransferase
wcbN	1230	YaeD *E.coli*	hypothetical protein
			intergenic
wcbO	1203	KpsS *E. coli*	capsule polysaccharide export protein
		PhyB *P.multocida*	capsule polysaccharide export protein
		LipB *N. meningitidis*	capsule polysaccharide modification protein

wcbP	1950	YooP *M. tuberculosis*	oxidoreductase
		HetN *Anabaena sp.*	oxidoreductase
yafJ	837	YafJ *E. coli*	amidotransferase
		YafJ *H. influenzae*	putative amidotransferase
yggB	708	YggB *E. coli*	hypothetical protein
			intergenic

Table 4. Bacterial strains and plasmids used in this study.

Strain or plasmid	Description	Reference
Strains		
E. coli		
SM10	Mobilizing strain, transfer genes of RP4 integrated in chromosome; Km^R, Sm^S	Simon *et al.*, (1983)
SM10λ*pir*	SM10 with a λ prophage carrying the gene encoding the π protein	Miller and Mekalanos (1988)
DH5α	High efficiency transformation	Bethesda Research Laboratories
TOP10	High efficiency transformation	Invitrogen
XL10-Gold	High efficiency transformation	Stratagene
B. pseudomallei		
1026b	Clinical isolate; Km^R, Gm^R, Sm^R, Pm^R, Tc^S, Tp^S	DeShazer *et al.*, (1997)
SR1015	1026b: pSR1015; Sm^R, Tc^R	This study
SR1016	1026b: pSR1016; Sm^R, Tc^R	This study
DD503	1026b derivative; allelic exchange strain; Δ (*amrR-oprA*)(Km^S Gm^S Sm^S); *rpsL* (Sm^R)	Moore *et al.*, (1999)
PB401	DD503 derivative; Δ *fliC*	Brett *et al.*, (unpublished)
SR1001	DD503 derivative; Δ *fliC*, *wbiE*::*aacC1*; Gm^R	This study
SLR5	SR1001 derivative; *wbpX*::Tn5-OT182; Tc^R	This study

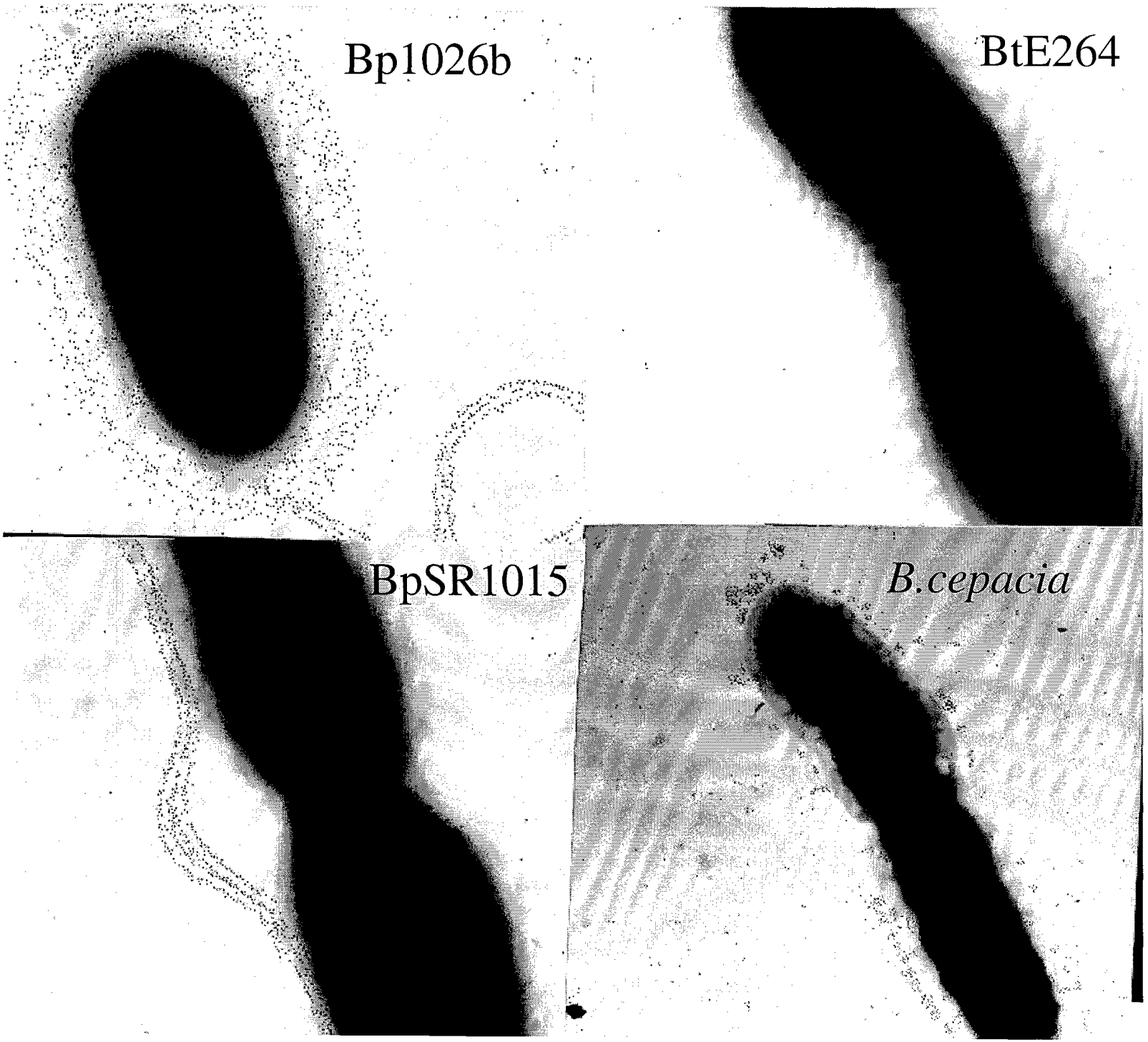
SLR8	SR1001 derivative; *wzt*::Tn5-OT182; Tc^R	This study
SLR 13	SR1001 derivative; *kpsS*::Tn5-OT182; Tc^R	This study
SLR18	SR1001 derivative; *manA1*::Tn5-OT182; Tc^R	This study
SLR19	SR1001 derivative; *manA2*::Tn5-OT182; Tc^R	This study
SR201::Tp	DD503 derivative; *kspD*::Tp	This study
SR202::Tp	DD503 derivative; *kpsC*::Tp	This study
SR203::Tp	DD503 derivative; *yafJ*::Tp	This study
SR204::Tp	DD503 derivative; *manC*::Tp	This study
B. thailandensis		
E264	Soil isolate; LPS contains only type II O-PS	Brett *et al.*, (1998)
B. mallei ATCC 10260		
B. cepacia FCO362		

Plasmids

pSKM11	Positive selection cloning vector; IncP mob; ColE1 ori; Ap^R, Tc^S	Mongolsuk *et al.*, (1994)
pZErO-2.1	Positive selection cloning vector; ColE1; Km^R	Invitrogen
pPCR2.1-TOPO	Topoisomerase-mediated cloning vector; Ap^R Km^R	Invitrogen
pDD1015	Subtractive hybridization product cloned into pZErO-2.1 Km^R	This study

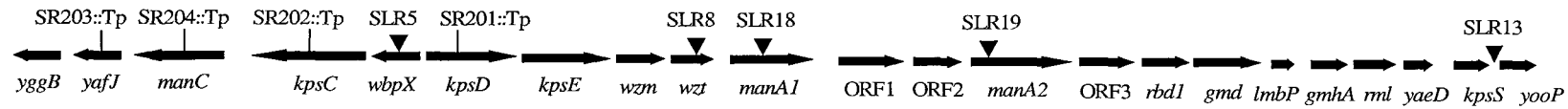
pDD1016	Subtractive hybridization product cloned into pZErO-2.1 Km^R	This study
pSR1015	*Kpn I-Xho I* fragment from pDD1015 cloned into pSKM11 Ap^R Tc^R	This study
pSR1016	*Kpn 1-Xho 1* fragment from pDD1016 cloned into pSKM11 Ap^R Tc^R	This study
pSR1015Bg	8 kb *Bgl II* fragment from SR1015 obtained by self-cloning; Ap^R Tc^R	This study
pOT182	pSUP102(Gm)::Tn5-OT182; Cm^R Gm^R Ap^R Tc^R	Merriman and Lamont (1993)
pSLR5B	8 kb *BamHI* fragment from SLR5 obtained by self-cloning; Ap^R	This study
pSLR5H	10 kb *Hind III* fragment from SLR5 obtained by self-cloning; Ap^R	This study
pSLR13H	9 kb *Hind III* fragment from SLR13 obtained by self-cloning; Ap^R	This study
p34E-oriTp	Vector containing self-cloning Tp cassette; *dhfrllb*-p15A oriV	Brett *et al.*, (unpublished)

Strain	**No PMN**	**+ PMN**	**+ opsonin**	**+ PMN + opsonin**	**+ PMN + opsonin + Type I O-PS**
B. pseudomallei 1026b	$5.1x10^5$				

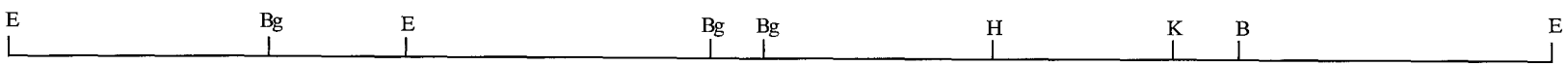

Bp1026b
BtE264
BpSR1015
B.cepacia

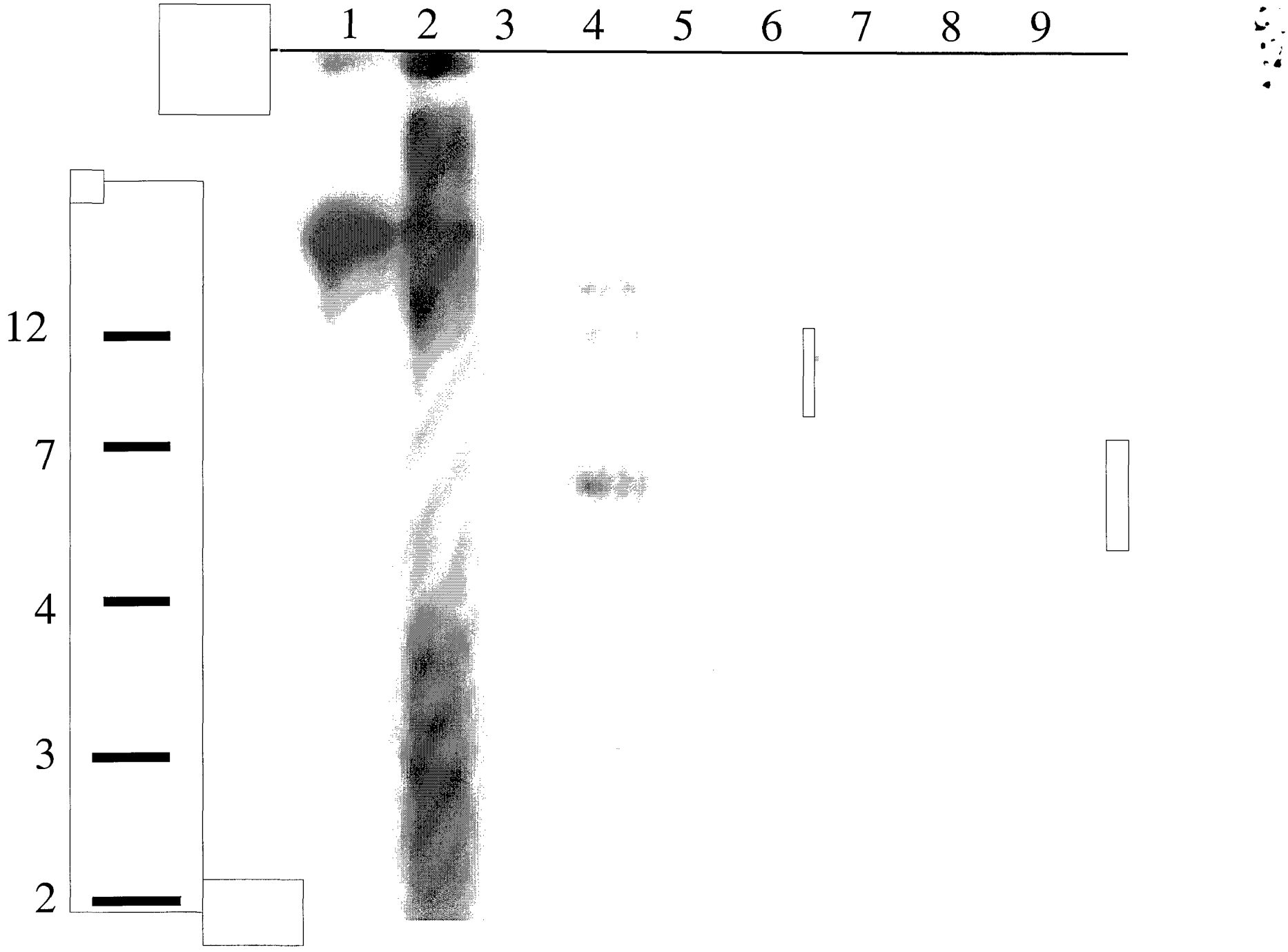
Exopolysaccharide Operon

A.



B.




1 2 3 4 5 6 7 8 9
12
7
4
3
2

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

Received 7/23/2001

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports. Request the limited distribution statement for reports on the enclosed list be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

Encl