Genetics and Function of the Capsules of *Burkholderia pseudomallei* and Their Potential as Therapeutic Targets

Shauna L. Reckseidler-Zenteno[1,2], Richard Moore[2] and Donald E. Woods[2,*]

[1]*Centre for Science, Athabasca University, Athabasca, Alberta, Canada T9S 9Z9;* [2]*Department of Microbiology and Infectious Diseases, University of Calgary Health Sciences Center, Calgary, Alberta, Canada T2N4N1*

Abstract: *Burkholderia pseudomallei* is the causative agent of melioidosis, a fatal disease that is endemic to Southeast Asia and northern Australia. The clinical manifestations of melioidosis may range from an acute pneumonia or acute septicemia, to chronic and latent infections. *B. pseudomallei* is inherently resistant to a number of antibiotics, and even with aggressive antibiotic therapy, the mortality rate remains high, and the incidence of relapse is common. The resistance of this organism to a number of antibiotics has created a need for the development of other therapeutic strategies, including the identification of novel therapeutic targets. *B. pseudomallei* has been shown to produce a number of capsular polysaccharides, one of which has been shown to contribute to the virulence of the organism. The structures of these polysaccharides have been determined and the genes encoding for the biosynthesis of one of the capsular polysaccharides (CPS I) have been identified. Analysis of the genome sequence of this organism has revealed the presence of three other capsule gene clusters that may encode for the chemical structures previously identified. Since one of the capsules produced by *B. pseudomallei* has been shown to be important in virulence, the genes encoding for the proteins responsible for its biosynthesis may be considered as potential targets.

Key Words: Pathogenesis, melioidosis, capsule, environment, therapeutics.

INTRODUCTION

Bacterial resistance to present day antibiotics has become a significant threat to public health. Consequently, new antibiotic development must provide new agents and novel classes to combat bacterial disease and to stay ahead of the rapid evolution of bacterial resistance mechanisms. The need for novel antibacterials has initiated a search for previously unexplored targets for chemotherapy. Several targets currently under investigation contribute to bacterial virulence. These targets are unique in that their inhibition, by definition, should interfere with the process of infection rather than with bacterial viability. If successful, virulence inhibition represents an approach to chemotherapy in which the pathogen is disarmed rather than killed outright. The principle goal of our laboratory is to study the pathogenesis of disease due to the bacterial pathogen *Burkholderia pseudomallei* in order to identify virulence determinants that might serve as targets for new therapeutics and/or vaccines.

B. pseudomallei is a gram-negative bacilli that is commonly found in soil and stagnant water in areas that lie between 20° north and south of the equator [1]. *B. pseudomallei* is the etiologic agent of melioidosis, a diseases of humans and animals that demonstrates a variety of clinical presentations. Melioidosis may be recognized as an inapparent infection, asymptomatic pulmonary infection, acute localized suppurative infection, acute pulmonary infection, acute septicemic infection or chronic suppurative infection [2]. The incubation period of the disease may range from less than 24 hours to 29 years [3, 4]. Infections by *B. pseudomallei* are due to direct inoculation into wounds and skin abrasions or to inhalation [4].

Melioidosis is recognized as an important health problem in Southeast Asia and northern Australia and it poses a concern due to increase travel and military involvement in endemic regions [1]. Isolated cases of melioidosis have been identified in a number of other regions as well, including west and east Africa, the Carribean, Central and South America, and the Middle East [3]. Melioidosis is an extremely difficult infection to treat because *B. pseudomallei* is resistant to a number of antibiotics. Although the use of antimicrobials has improved the prognosis for melioidosis, the mortality rate for the septicemic form is still 40% and relapse is common following long term prophylaxis [5]. As a result strategies for effective treatment and prevention of the disease must be explored.

The resistance of this organism to a number of antibiotics has created a need for the development of other therapeutic strategies, including the identification of novel drug targets and the development of an effective vaccine. This review will discuss the genetics and function of the capsular polysaccharides of *B. pseudomallei* and will examine one of these capsules as a vaccine component and a potential target for antimicrobial therapy.

CAPSULAR POLYSACCHARIDES OF *B. PSEUDOMALLEI*

B. pseudomallei has been shown to produce four different capsules and the structures of these have been determined [5-

*Address correspondence to this author at the Department of Microbiology and Infectious Diseases, University of Calgary Health Sciences Center, Calgary, Alberta, Canada T2N4N1; Tel: (403) 220-2564; Fax: (403) 210-8504; E-mail: woods@ucalgary.ca

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7]. The first capsule is a homopolymer of mannoheptose with the structure -3)-2-*O*-acetyl-6-deoxy-β-D-manno-heptopyranose-(1- [6]. This polysaccharide was initially characterized as a type I O-polysaccharide, but was subsequently concluded to be a capsule based on genetic homology, the importance of the capsule in virulence, and its high molecular mass [9,10]. *B. pseudomallei* has been shown to produce three other capsular polysaccharides. One is an acidic polysaccharide 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-, which is recognized by patient sera (CPS IV) [7]. The other two are: a branched 1,4-linked glucan polymer (CPS II) and a triple-branched heptasaccharide repeating unit composed of rhamnose, mannose, galatose, glucose, and glucoronic acid (CPS III) [8].

Previously our laboratory demonstrated that the capsular polysaccharide with the structure -3)-2-*O*-acetyl-6-deoxy-β-D-manno-heptopyranose-(1- was an important virulence determinant of *B. pseudomallei* [9]. The cluster containing the genes responsible for the biosynthesis and transport of the capsule was identified by subtractive hybridization and shown to demonstrate homology to the capsule clusters of other bacterial pathogens such as *Neisseria meningitidis* and *Haemophilus influenzae*. Sequencing of the capsule operon revealed the presence of 25 open reading frames involved in capsule biosynthesis and export and mutations constructed in these genes have confirmed their role in the capsule production (Fig. **1**). Mutation of the glycosyltransferase gene *wcbB* resulted in a strain that was attenuated for virulence by 10^5-fold compared to wild type. This capsule, referred to as CPS I, was found to be responsible for survival of *B. pseudomallei* in the blood of the host by reducing opsonization and phagocytosis [11]. This likely contributes to the establishment of bacteremia in the host [4]. Furthermore, this capsule was shown to be up-regulated *in vitro* in the presence of human serum and *in vivo* in the animal model. Capsule expression was shown to be induced in the presence of 30% normal human serum by measuring luminescence of a *lux* reporter fusion to the capsule gene *wcbB* [11]. Microarray analysis demonstrated that CPS I was up-regulated significantly following infection of Syrian Golden hamsters by *B. pseudomallei* (Table **1**), [11]. This capsule has been shown by sequence analysis and immunoblot analysis to be present in *B. mallei*, but not *B. thailandensis* [12]. Some of the genes are present in *B. thailandensis*, but a major portion of the *B. pseudomallei* capsule cluster has replaced an ancestral capsule cluster in *B. thailandensis* of 10 genes involved in GDP-D rhamnose biosynthesis in *Pseudomonas aeruginosa* [13]. Western blot analysis of CPS I production in clinical isolates of *B. pseudomallei* demonstrated that 52 of 55 clinical strains examined were found to produce this capsule, correlating the production of CPS I with clinical infection [14]. These clinical strains were taken from different patients and types of *B.*

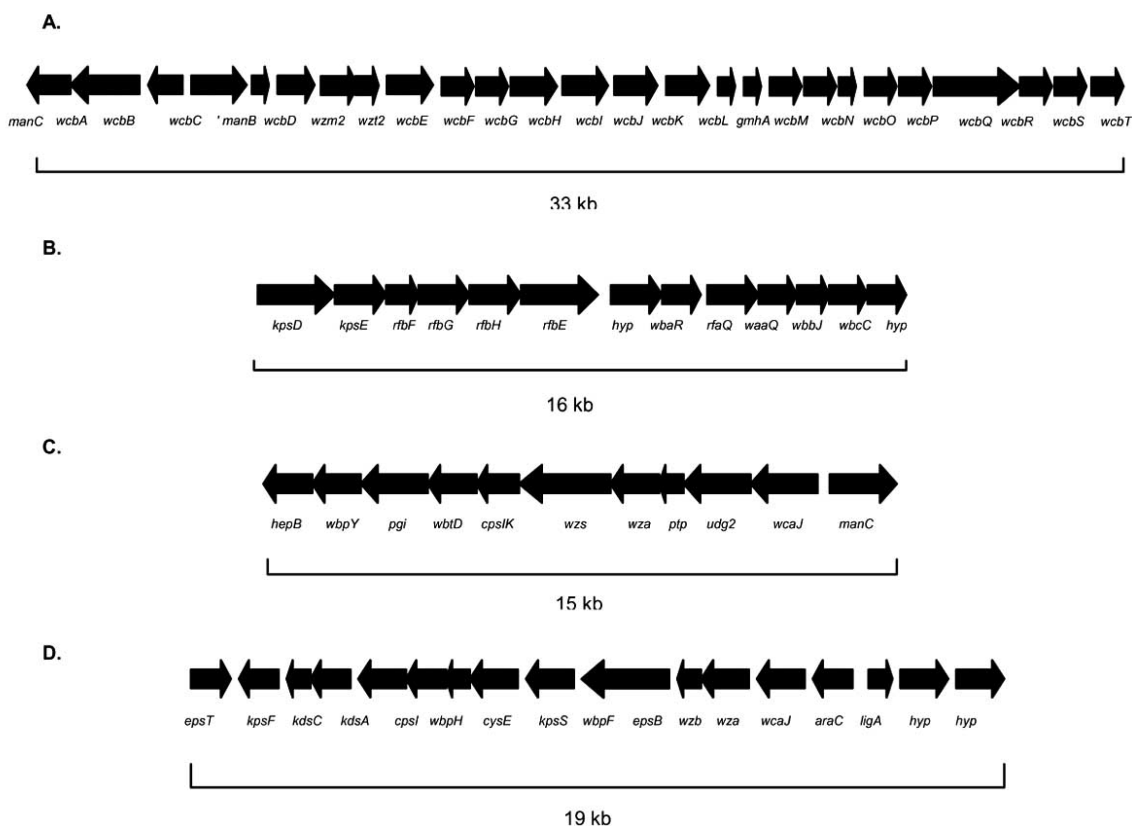

Fig. (1). Organization of the chromosomal regions containing the genes comprising the *B. pseudomallei* capsule operons. The direction of transcription is represented by arrows, and the gene names demonstrating the highest degree of homology to the *B. pseudomallei* open reading frames are indicated. The relative sizes of each locus are indicated. (A) *B. pseudomallei* capsule cluster I (CPS I). (B) *B. pseudomallei* capsule cluster II (CPS II). (C) *B. pseudomallei* capsule cluster III (CPS III). (D) *B. pseudomallei* capsule cluster IV (CPS IV). The CPS I cluster is present in *B. pseudomallei* and *B. mallei*, but not *B. thailandensis*. The other three clusters, CPS II, III, and IV are present in *B. pseudomallei* and *B. thailandensis*, but not *B. mallei*.

Table 1. Microarray Analysis of B. pseudomallei Capsule Expression Following Intraperitoneal Inoculation in the Hamster Model of Melioidosis

Gene ID	Annotation	Fold Change (*in vivo* vs. *in vitro*)	Capsule (CPS)
BPSL2786	putative acetyltransferase	13.18±1.30	I
BPSL2809	wcbA, capsule export	9.27±3.27	I
BPSL2787	wcbT, putative acyl-CoA transferase	6.10±1.93	I
BPSS0427	wbbJ, O-acetyltransferase	(-)4.82±1.44	II
BPSS0429	Hypothetical protein	(-)4.82±1.50	II
BPSS1827	pgi, glucose-6-phosphate isomerase	3.56±2.31	III
BPSS1832	ptp, capsule export, tyrosine-protein kinase	3.05±1.84	III
BPSL2770	kpsF, arabinose-5-phosphate isomerase	1.40±0.661	IV
BPSL2780	wza, capsule export	0.968±0.169	IV

pseudomallei infections, which indicates that the production and structure of CPS I is consistent over a variety of strains and infection types [14]. Although this capsule has been shown to contribute to survival in the blood of an acute infection model, there is no evidence to suggest that the absence of the capsule leads to a switch from an acute to a chronic or latent form.

Although the role of CPS I has been well characterized, the roles of the other three capsules have not been previously elucidated. Recent work has focused on genetic and functional characterization of these capsules. Sequencing of the *B. pseudomallei* genome has revealed three putative capsule operons that may correspond to the three capsule structures previously identified [15]. One of these operons was found to contain genes involved in the biosynthesis of a capsule with gene products that demonstrated homology to those of enteric capsule clusters (Fig. **1**). Based on genetic homology this cluster may contain the genes responsible for the production of the capsule composed of rhamnose, mannose, galactose, glucose, and glucuronic acid[1]. Further studies are underway to confirm whether this locus, designated CPS II, encodes for this capsule structure. Comparative genomic analysis revealed that the CPS II cluster is present in the environmental organism, *Burkholderia thailandensis*, but not in the obligate pathogen, *Burkholderia mallei*. A strain containing a mutation in this locus, SZ418, was still virulent in the hamster model and was still capable of inducing bacteremia, indicating that this capsule was not essential for virulence (Fig. **1**). SZ418 harbors a mutation in the *kpsE* gene due to insertional inactivation by the plasmid pGSV3-*lux*, a suicide vector that contains the *luxCBADE* operon as a reporter for luminscence [1]. CPS II was found to be up-regulated in water and down-regulated in human serum using a *lux*-reporter assay[1]. In addition, microarray analysis determined that this capsule was not expressed *in vivo*[1].

It was concluded that this capsule may contribute to environmental persistence rather than survival in the host. Further studies are underway to confirm whether this locus encodes for the capsule structure identified previously.

CPS III was found to contain 11 genes and was shown to be present in the genomes of *B. pseudomallei* and *B. thailandensis*, but not *B. mallei*. This cluster was found to contain four genes that demonstrated homology to glycosyltransferases and based on this may encode for the branched 1,4-linked glucan capsule described by Kawahara *et al.* [8]. CPS III was not highly expressed *in vivo* by microarray analysis and was not expressed in serum (Fig. **1**)[1]. In addition, SZ1829, which contains a mutation in the CPS III cluster, was found to be as virulent in the animal model as wild type *B. pseudomallei*. SZ1829 harbors a mutation in the *cps1K* gene due to insertional inactivation by the plasmid pGSV3-*lux* [1]. Although CPS III was not found to be required *in vivo* or for virulence, this capsule may contribute to survival of *B. pseudomallei* in the environment. CPS III expression was significantly higher in water compared to LB [1].

The fourth capsule cluster, CPS IV, is located on chromosome 1 of the *B. pseudomallei* genome and contains 17 open readings frames that demonstrated homology to gene products involved in the synthesis and export of a polysaccharide, similar to enterobacterial common antigen (Fig. **1**). These genes were also identified in *B. thailandensis*, but not *B. mallei*. CPS IV was shown to be expressed *in vivo*, but not to the extent of CPS I[1]. The CPS IV genes demonstrated homology to gene products that may encode for the Gal-Gal-Gal-KDO capsule identified and characterized previously [7, 16]. In previous experiments this capsule was shown to react with patient sera, but the role of the capsule was not determined [7]. Preliminary evidence from our laboratory suggests that this acidic exopolysaccharide may contribute to survival in the host, but it is not known if it is critical for the pathogenesis of *B. pseudomallei*. Mutants are currently being constructed in the CPS IV cluster so that the role of this capsule in virulence may be assessed[1].

B. PSEUDOMALLEI CAPSULE GENES AS POTENTIAL THERAPEUTIC TARGETS.

Targeting virulence factors with new classes of compounds can be considered an alternative to conventional an-

[1] Reckseidler-Zenteno, S.L.; Tuanyok, A.; Wong, E.; Moore, R.A.; Woods, D.E. *in preparation.*

timicrobial therapy. Targeted genes must contribute to persistence whereby a loss of function would result in a pathogen less fit for survival within the host [17]. To identify useful compounds, *in vitro* assays must be developed and this step is often challenging due to the nature of the target and the activity of the particular antimicrobial compound. For example, while antibiotics have often been developed and tested using inhibition of bacterial growth as a means of showing initial efficacy, compounds aimed at reducing adherence of uropathogenic *E.coli* (pilicides) have required sophisticated binding studies to identify useful compounds [18, 19].

The *B. pseudomallei* capsule CPS I operon consists of 25 genes and their corresponding enzymes. A number of mutations have been constructed in this operon and each has resulted in a capsule negative phenotype, as determined by western blot analysis and by an increased LD_{50} value in Syrian hamsters [9], suggesting that capsule production is required for virulence and that synthesis of a functional capsule is dependent on many and possibly every gene in the operon. Thus, the genes encoding for CPS I would appear to be obvious targets for new antimicrobials.

One possible target for new antimicrobials is the ABC transporter that is involved in capsule export. In the *B. pseudomallei* capsule operon, the *wzt2* and *wzm2* gene products likely encode an ABC transporter involved in export of capsule components across the *B. pseudomallei* inner membrane. Analysis of the predicted amino acid sequence of Wzt2 revealed a conserved ATP-binding motif and was shown, *via* mutagenesis, to be required for capsule production [9]

An approach for exploiting ABC transporters is to use them for transporting antimicrobials into the bacterial cell rather than targeting them directly. This approach takes advantage of the fact that mammalian cells do not have ABC importers [20], thus assuring target cell specificity. It also takes advantage of the observation that many antimicrobials do not work because they are unable to get into the bacterial cell. Antimicrobials linked to a natural substrate have been referred to as smugglins and one particular example involves the use of a KDO analog attached to a dipeptide [21]. The conjugate was recognized and taken up by a peptide ABC transporter and was shown to inhibit the linking of lipid A to the core polysaccharide of LPS in gram-negative bacteria. Although targeting ABC transporters in this fashion is not a new idea it should be noted that the approach has not provided a wealth of new antimicrobials thus far.

The *B. pseudomallei* CPS I operon contains 3 putative glycosyltransferases which are presumably involved in the transfer of mannoheptopyranosyl residues during capsule biosynthesis. Nucleotide glycosyltransferases typically transfer sugars from nucleotides such as UDP or GTP to a variety of acceptors. These enzymes are widespread and are involved in a large number of biological processes in addition to capsule biosynthesis. Although some progress has been made in this area, efforts to identify inhibitors of these enzymes has not been very fruitful to date. Recently, a high-throughput screen utilizing a fluorescence based substrate displacement assay has been successful in identifying active-site inhibitors of *murG*, a glycosyltranserase involved in peptidoglycan synthesis in gram-negative bacteria [22]. Presumably similar techniques could be used to identify compounds which inhibit *B. pseudomallei* capsule specific glycosyltransferases.

An initial requirement for this approach in *B. pseudomallei*, would be the cloning and expression of target capsule genes which could be used in high throughput assays to screen combinatorial libraries. To date, however, there are no reports of the cloning and expression of *B. pseudomallei* capsule enzymes which could be used for potential antimicrobial targets. Alternatively the cloning and expression of the entire *B. pseudomallei* capsule operon in a non-pathogenic organism is an approach that could be used to screen for compounds that inhibited capsule synthesis. This approach is advantageous in that it circumvents the containment difficulties surrounding these types of screens when using a level 3 pathogen such as *B. pseudomallei*, however it would still require the development of high-throughput assays that could effectively detect inhibition of capsule synthesis.

Clearly, identifying and developing compounds that would interfere with capsule synthesis in *B. pseudomallei*, whether they target ABC transporters or glycosyltransferases, would serve a niche market for small pharmaceutical companies and unfortunately it may not make economic sense for companies to invest in this type of development. Nonetheless, both targets may represent a potential means for treating a wide variety of bacterial infections and hopefully research aimed at other organisms will lead to the discovery of compounds that will also be useful for *B. pseudomallei* infections.

While antimicrobials designed to inhibit capsule synthesis offer a means of eliminating the effects of capsule, neutralizing agents, such as antibodies, may provide another way of reducing the potency of this virulence factor. Passive immunization studies have demonstrated that capsule antibody affords protection in mice. An evaluation of a panel of monoclonal antibodies (Mabs) to *B. pseudomallei* capsule (CPS I), LPS and a variety of proteins showed that the Mabs directed to the proteins provided protection when mice were challenged with 10^4 CFU but appeared to perform less well than the Mabs to capsule and LPS. At a challenge of 10^6 CFU, only the anti-capsule Mabs provided protection [23]. In another study [24] it was shown that mice vaccinated with capsular polysaccharide primarily developed an IgG2b response, which provided protection compared to unvaccinated controls when challenged intra-peritoneally. The authors also reported that mice passively immunized with either Mab or polyclonal antibody to the capsule had an increased "mean time to death" when compared to control mice receiving PBS.

In our laboratory we have examined the use of polysaccharide-protein conjugates as potential vaccines for *B. pseudomallei* and *B. mallei*. Polysaccharide-protein conjugates were chosen as it is well documented that T cell-independent type 2 (TI-2) antigens such as bacterial polysaccharides are capable of eliciting protective antibody responses in adults [9, 10] but act poorly as immunogens in the elderly, in im-

Table 2. Potential Carrier Proteins for Vaccine Glycoconjugate Synthesis

Gene	Annotation in *B. mallei*	Annotation in *B. pseudomallei*	Predicted Subcellular Localization	Expressed Protein Size
BPSS0357 / BMAA0881	Putative surface antigen protein *(SAP)*	Putative exported protein	Unknown	18.7 kDa
BPSS1548 / BMAA1548	Type III secretion system protein BsaL	Type III secretion system protein	Unknown	11.3 kDa
BPSL0976 / BMA0685	Vitamin B12 receptor, BtuB, putative	Putative outer membrane receptor protein	Outer membrane	72.4 kDa
BPSL1728 / BMA1125	Outer membrane porin, OpcP, putative	Putative outer membrane protein	Outer membrane	41.3 kDa
BPSL1984 / BMA0923	LysM domain protein	Putative exported protein	Outer membrane	48.1 kDa
BPSL2062 / BMA0841	OmpA family protein *(OAP)*	OmpA family protein	Outer membrane	25.2 kDa
BPSL2553 / BMA0477	TonB-dependent receptor	Putative siderophore receptor protein	Outer membrane	84.6 kDa
BPSL2704 / BMA2010	Outer membrane protein, OmpW family	Putative OmpW family exported protein	Outer membrane	29.4 kDa
BPSL3319 / BMA2873	Flagellin	Flagellin	Extracellular	41.7 kDa

munocompromised populations and in children less than 18 months of age [24-29]. In adult populations, TI-2 antigens typically stimulate the synthesis of antigen specific immunoglobulin M (IgM), but often fail to evoke augmented immune responses and isotype conversions following boosting with carbohydrate preparations. This phenomenon is primarily due to the inability of activated B lymphocytes to recruit $CD4^+$ T cell (Th) involvement *via* major histocompatibility complex type II (MHC II) restricted events [30, 31]. In order to remedy this situation, a number of bacterial carbohydrates (such as capsular polysaccharides or somatic O-antigens) have been covalently coupled to protein or peptide carrier molecules in order to facilitate T cell-dependent (TD) immune responses against the particular carbohydrate moieties [32-38]. The presence of T cell epitopes inherent to peptide or proteinacious carriers is believed to facilitate these events [39-41]. Thus, by conjugating *B. pseudomallei* capsule polysaccharide (CPS I) to a protein carrier we have been able to elicit an augmented immune responses against the CPS component of the vaccine while concomitantly evoking desirable immunoglobulin (Ig) class switching events [42].

Although it is possible to use carrier proteins such as tetanus toxoid which are proven to help the response to polysaccharide become T-cell dependent, it is advantageous to choose proteins that are specific to the disease being treated. The searching of sequenced genomes for DNA encoding proteins that are predicted to be surface-exposed and thus potentially protective is known as "reverse vaccinology" [43,44]. Surface-associated or outer membrane proteins are the most appropriate as antibodies raised against the carrier protein itself will target the bacterium upon challenge and may protect against development of pathology and adverse outcomes such as septicaemia [45,46].

We have initiated studies to identify the most appropriate proteins for conjugation to CPS I by scanning the annotated genome of *B. pseudomallei* K96243 for genes that encode surface exposed or associated proteins. The list generated from this scan was cross-referenced with a list of genes shared by both *B. pseudomallei* and *B. mallei*, and the predicted protein product of each was analyzed for protein homology, predicted protein localization, presence of signal peptide and location of likely cleavage site, possible transmembrane domains and predicted Class II T cell epitopes. We have cloned these genes, expressed and purified the proteins using His-tag methodologies, and conjugated them to CPS. Immunization of rats with these conjugates has identified several proteins with significant potential to induce T cell dependent immune responses resulting in high titers of antibodies developing to CPS as well as to the carrier protein. Several candidate carrier proteins including several shared by *B. mallei* and *B. pseudomallei* are described in the following table.

Two proteins in particular, a putative surface antigen protein (SAP) BPSS0357and a protein predicted to be a member of the Omp A family (OAP) BPSL2062, show significant promise as carrier proteins. It should be noted that these studies have also examined the use of oligopolysaccharide (OPS) isolated from *B. pseudomallei* LPS and have shown OPS to also be a suitable polysaccharide for these types of conjugate vaccines [42]

Proteins involved in the translocation of capsule to the exterior of the cell would also be likely vaccine candidates. Lipoprotein components of *S. pneumoniae* ABC transporters involved in manganese (PsaA) and iron uptake (PiuA and PiaA) have successfully been used to immunize mice against systemic infection [47,48]. Rather than acting as opsonins, it was presumed that the antibodies raised to the proteins diffused through capsule and cell wall and bound to their targets, and prevented biological function [48]. Thus, in addition to inducing T-cell dependent immune responses when

conjugated to capsule polysaccharide, antibodies directed to *B. pseudomallei* capsule ABC transporter proteins may effectively disrupt capsule synthesis and reduce virulence.

CONCLUSION

B. pseudomallei is the causative agent of melioidosis, a fatal disease that affects both humans and animals in many parts of the world. Because the organism is inherently resistant to a number of antibiotics new therapeutic strategies must be investigated. Ideal targets for investigation are genes encoding for proteins that are involved in survival in the host. The capsule CPS I is a major virulence factor in *B. pseudomallei* and a number of proteins involved in the production and export of this capsule may be considered to be suitable therapeutic targets. The identification of drugs that inactivate enzymes involved in polysaccharide production and export will facilitate clearance of the organism and hence, treatment of individuals with the disease. Much work remains to be done in order to identify these types of antimicrobials and license them for use. Although *B. pseudomallei* produces other capsular polysaccharides, the genes encoding for the enzymes responsible for their biosynthesis may not be ideal candidates for therapy since research thus far has shown that these capsules are not as critical for virulence.

Capsule polysaccharide used alone as a vaccine can provide protection against *B. pseudomallei* infections and, when conjugated to an appropriate protein carrier, can elicit augmented immune responses and evoke desirable immunoglobulin class switching events. Research by our laboratory has shown that when conjugated to a suitable carrier protein, CPS I has shown promise as a vaccine component. Further study is required to determine if capsule proteins involved in synthesis and export might serve as worthwhile vaccine candidates either by themselves or as a glycoconjugate.

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Received: 07 November, 2008 Revised: 01 December, 2008 Accepted: 12 December, 2008

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