RELATIONSHIP BETWEEN CAPSULATION AND CELL SURFACE PHYSIOLOGY IN *BURKHOLDERIA MULTIVORANS*

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BURKHOLDERIA MULTIVORANS

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CHAPTER I

INTRODUCTION

Burkholderia multivorans is an obligately aerobic gram-negative bacillus found in soil. It is one of nine species comprising the *Burkholderia cepacia* complex (Bcc). Five of the Bcc members were transferred as genomovars from the genus *Pseudomonas* to *Burkholderia* in 1992 on the basis of their distinct 16S rRNA sequence charateristics, cell envelope lipid composition, DNA-DNA homology, and physiologic properties (Mahenthrialingam *et al.,* 2005). These phenotypically similar yet genotypically disparate genomovars were given numeral designations until the assignment of species names in 1997 (Vandamme *et al*., 1997). After thorough genomic DNA analysis, it was proposed that genomovar II be named *B. multivorans* due to its nutritional versatility (from the Latin *multus* meaning much and *vorans* meaning devouring or digesting).

B. multivorans is the etiologic agent of opportunistic pulmonary infections in cystic fibrosis (CF), chronic granulomatous disease (CGD), and in otherwise immunocompromised patients. It is able to penetrate human airway epithelial cells, thereby causing pulmonary deterioration leading to tissue necrosis and death within weeks to months (Zahariadis, 2003). The organism is thought to be transmitted by either infectious aerosols or direct physical contact and is intrinsically resistant to many antibiotics and disinfectants. There is no simple antibiotic regimen for *B. multivorans*; therefore, prevention is the key to infection control (Coenye *et al*., 2001).

There have been an increasing number of infections attributed to members of the Bcc over the past twenty years (Mahenthiralingam *et al*., 2005). It has therefore become necessary to better characterize the physiologic properties of each genomovar in order to better understand the mechanisms underlying their virulence. The purpose of the present study was to characterize the outer cell envelope physiology of an American Type Culture Collection (ATCC) type strain, as well as seven clinical phenotypic variants of *B. multivorans* which have been shown to exhibit disparate *in vitro* and *in vivo* virulence properties. Emphasis was placed on extracellular polysaccharide (EPS) expression in *B. multivorans*. A paucity of information about EPS expression exists in the literature despite a previous report suggesting that EPS production may be an important factor in the disease process (Chung *et al*., 2003).

It was hypothesized that the mucoid colonial phenotype is a function of the degree to which *B. multivorans* cells are capsulated with EPS, thereby masking the cell surface proper and influencing the ability of the organism to adhere to host cell surfaces. This property might also be reflected physiologically with regard to factors such as outer cell envelope permeability to nonpolar antimicrobial agents, cell surface hydrophobicity, the ability to form biofilms, and ultimately, virulence. This hypothesis was tested by characterizing an ATCC cell type strain and seven clinical phenotypic variants of *B. multivorans* which have been shown to exhibit disparate *in vivo* and *in vitro* virulence properties (Zelazny *et al.,* 2008). Parameters examined included ultrastructural and morphological characteristics, batch cultural growth kinetics, degree of capsulation, accessibility of the cell surface to nonpolar compounds, outer cell envelope permeability for nonpolar antimicrobial agents having disparate mechanistic targets, cell surface

hydrophobicity properties, and the ability to form biofilms. Reference *Pseudomonas aeruginosa* and *Pasteurella multocida* strains were included in some assays for control purposes due to their markedly disparate outer cell envelope permeability properties for nonpolar molecules (Ellison and Champlin 2007; Ellison *et al.,* 2007).

CHAPTER II

REVIEW OF LITERATURE

In the late 1940s Walter H. Burkholder isolated four bacterial cultures during an outbreak of soft onion rot in New York and called them “cepacia” meaning “of or like onion” (Moore and Elborn, 2001; Mahenthiralingam *et al.,* 2005). The organism was named as *Pseudomonas cepacia,* but has also been referred to as *Pseudomonas multivorans* or *Pseudomonas kingii.* In 1992 seven *Pseudomonas* species including *cepacia* were transferred to the new genus *Burkholderia* on the basis of 16S r RNA sequencing, DNA-DNA homology, cellular lipid and fatty acid composition, and other phenotypic characteristics. Vandamme *et al.* (1997) subsequently divided *Burkholderia cepacia* into five phenotypically similar, yet genotypically distinct genomovars. They are collectively referred to as the *Burkholderia cepacia* complex (Bcc) which has recently been expanded to include nine genomovars (Mahenthiralingam *et al.,* 2005).

Members of the Bcc have been employed to produce antimicrobial compounds which prevent molding of various fruits and “damping off” diseases in seedlings, colonize plant roots to potentiate nitrogen fixation, thereby promoting plant growth and crop production, and bio-degrade synthetic organic compounds found contaminating groundwater (Moore and Elborn, 2001; Mahenthiralingam *et al.,* 2005). Prior to the

1980s, Bcc member pathogenicity was limited primarily to contaminated IV solutions and disinfectant misuse resulting in nosocomial pneumonia and septicemia. Bcc members have more recently become increasingly associated with other types of infections.

Bcc genomovar II was eventually named *Burkholderia multivorans* by virtue of its nutritional versatility and similarities to other Bcc strains (Vandamme *et al.*, 1997). *B. multivorans* is misidentifie d as *B. cepacia* when using various biochemical identification systems including the API 20NE, MicroScan, Vitek GNI, and Vitek NFC, thereby precluding clinical laboratory personnel from accurately differentiating it from other Bcc species (van Pelt *et al.,* 1999). Identification of *B. multivorans* is critically important because of its role as an opportunistic pathogen in CF, CGD, and in otherwise immmunocompromised patients (Coenye *et al.,* 2001).

Cystic fibrosis is an autosomal recessive disease caused by a mutation on chromosome 7 (Stern, 1997). This mutation causes a defect in the secretory processes of the chloride channels in epithelial cells resulting in mucous accumulation initially in the lungs, and eventually in the pancreas (Widmaier *et al.,* 2006). Patients with CF typically contract chronic *Pseudomonas aeruginosa* infections which since the 1980s have become increasingly associated with Bcc infections. The combination of these two organisms can cause infection with no change in pulmonary status, an accelerated decline of pulmonary function, or necrotizing pneumonia with fatal clinical deterioration within 1-6 months (Vandamme *et al.,* 1997; van Pelt *et al.,* 1999; Zahariadis *et al.,* 2003). The most severe presentation has come to be called “cepacia syndrome” with *B. cenocepacia* and *B.*

multivorans accounting for 45 and 39% respectively of all Bcc organisms isolated from CF patients (Bertot *et al.,* 2007).

Chronic granulomatous disease is an autosomal recessive or X-linked recessive disease that causes a defect in the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme (Johnston 2001; Rosenzweig and Holland 2004). This enzymatic defect prevents phagocytic cells from killing certain bacteria after ingestion (Winkelstein *et al.,* 2000). Bcc species are among the few organisms responsible for septicemia and pneumonia in patients with CGD and are the second most common cause of death in these patients.

B. multivorans is transmitted by patient-to-patient contact and is highly resistant to antibiotics (Coenye *et al.,* 2001; Sist *et al.,* 2003). Limiting patient contact in outpatient settings and in shared hospital rooms are suggested components of infection control plans designed to mitigate patient exposure to this organism. It has been shown that even when infected with the same clonal strain, patients may experience disparate clinical outcomes (Zlosnik *et al.,* 2008). Little is known with regard to humoral immune responses to *B. multivorans* in particular and the Bcc in general (Bertot *et al.,* 2007). Because of its prevalence and clinical diversity in CF and CGD patients, it is critical to better characterize *B. multivorans* to better understand the mechanisms underlying its virulence (Chung, *et al.,* 2003).

The literature contains many reports regarding the EPS produced by Bcc species and its roles in infective processes, including protection of cells from phagocytosis, opsonisation, and dehydration (Sist *et al.,* 2003; Herasimenka *et al.,* 2007). It has also been suggested that EPS may play an additional role in the pathogenesis of *B.*

multivorans, similar to that seen in *P. aeruginosa* (Chung *et al.,* 2003; Zlosnik *et al.,* 2008) in which patients are typically infected with a non-mucoid strain that subsequently converts to a highly mucoid phenotype expressing EPS composed of alginate. The production of alginate contributes to biofilm formation, thereby enabling establishment of a chronic infection. It has been demonstrated in a murine model that variant mucoid *B. cenocepacia* strains have the ability to persist in the lungs longer than parental strains. In addition, EPS inhibits neutrophil chemotaxis while able to scavenge reactive oxygen species (Herasimenka *et al.,* 2007). Work with another mouse model showed that if *B. cenocepacia* EPS is overproduced, it could enhance the persistence and virulence of an infection (Conway *et al.,* 2004).

Little is known regarding the chemistry, biochemistry, and biological activities of Bcc EPS (Sist *et al.,* 2003; Zlosnik *et al.,* 2008). These bacteria produce at least four types of EPS (Figure 1), the most common of which is known as cepacian (Herasimenka *et al.,* 2007). Cepacian was first identified in *B. cepacia* and is composed of a polymer backbone that includes acetyl substituents which differ in number, depending on culture conditions (Figure 1A). It has been shown that other Bcc organisms including *B. multivorans* can produce this type of EPS. The remaining three types of EPS (Figures 1B, 1C, and 1D) are rarely seen, and type PS1 (Figure 1D) has been found to be produced by a clinical strain of *B. multivorans.*

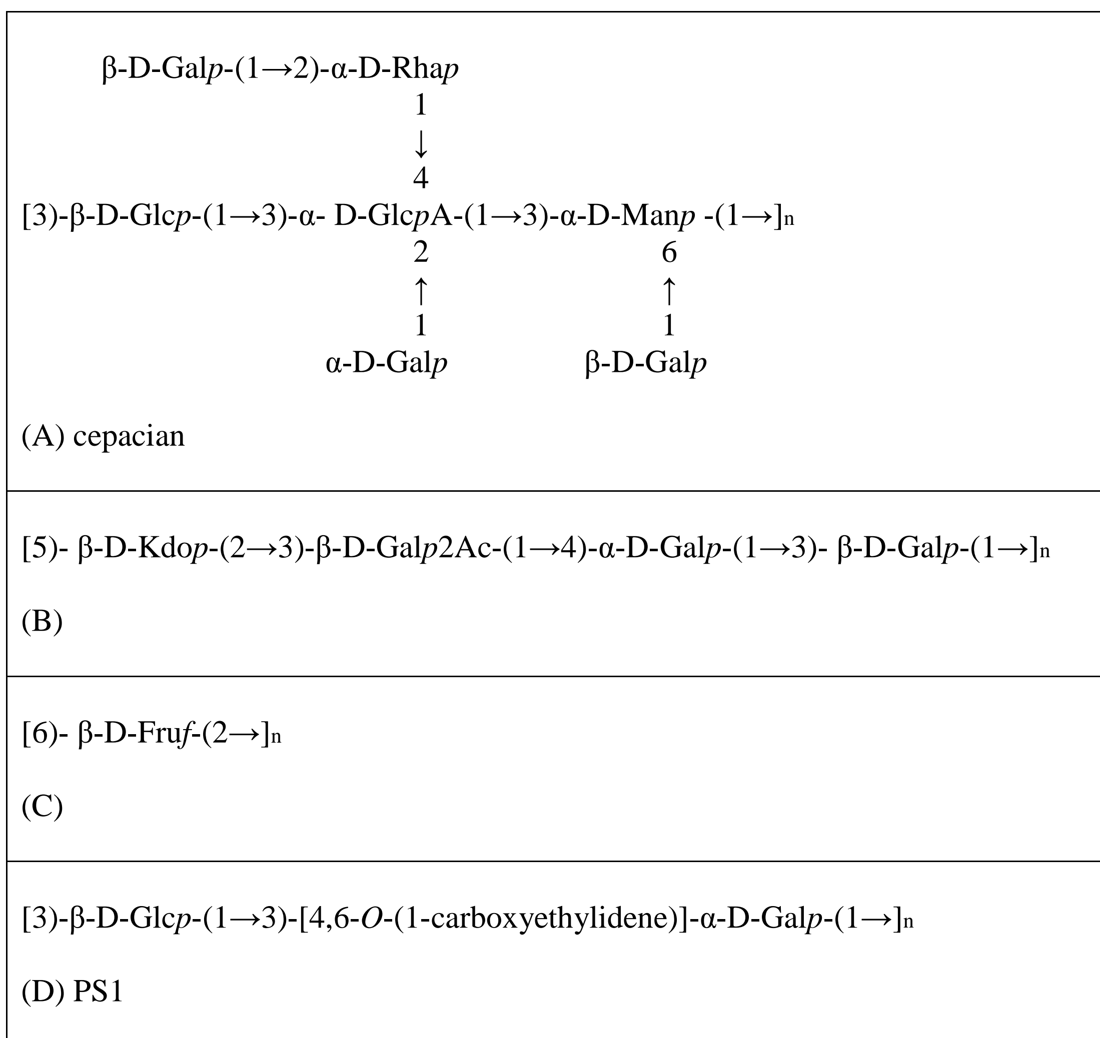

Figure 1. Chemical structures of the four types of EPS produced by the Bcc (Herasimenka *et al.,* 2007).

The ability to form biofilms has been shown to be an important virulence factor in certain bacteria (Cunha *et al.,* 2004). *P. aeruginosa* biofilms consist of cells which are more resistant to antibiotics and better able to evade the host immune system than are planktonic cells. *B. multivorans* and *B. cenocepacia* are the most clinically relevant Bcc species and have been shown to form *in vitro* biofilms as has Bcc species *B. dolosa* (Caraher *et al.,* 2007). Cell-to-cell signaling molecules involved in the process of biofilm formation have been reported in these *in vitro* studies (Mahenthiralingam, *et al.,* 2005).

B. cepacia and *P. aeruginosa* have been shown to coexist as a mixed biofilm in the lungs of CF patients, moreover, the Bcc organisms are able to recognize quorum-sensing signals produced by *P. aeruginosa,* thereby allowing cross-communication between the two species (Cunha *et al.,* 2004; Mahenthiralingam *et al.,* 2005). Biofilm formation may then provide a means by which the Bcc organisms are protected from antibiotics and the host immune system as is seen in *P. aeruginosa*.

CHAPTER III

METHODOLOGY

Bacterial Strains

P. aeruginosa PAO1 and *P. multocida* ATCC 11039 are maintained as capsulated reference organisms in this laboratory while *B. multivorans* BAA-247 (type strain) was purchased from the American Type Culture Collection (ATCC; Manassas, VA). Clinical isolates were obtained from Dr. Adrian Zelazny (NIH-NIAID) and included *P. aeruginosa* PA1211 as well as seven strains of *B. multivorans* (designated AZ01 through AZ07 in this laboratory). All cultures were maintained under cryoprotective conditions at -80 ° C as described previously (Darnell *et al*., 1987). *P. aeruginosa* PAO1, *P. aeruginosa* PA1211, and *P. multocida* ATCC 11039 were employed in this study for references purposes due to the disparate outer membrane permeability properties of the two organisms (Ellison and Champlin 2007; Ellison *et al*., 2007).

Cultural Growth Conditions

Inocula were obtained from working cultures which were prepared by inoculating Mueller-Hinton Agar (MHA; Difco Laboratories, Detroit, MI) or Luria Agar Base Miller (LAB; Difco Laboratories) plates with cells from cryopreserved stock cultures, incubating at 37° C for approximately 18 h, and storing at 4° C for no longer than 7 to 10 days (Ellison and Champlin 2007; Ellison *et al*., 2007). Starter cultures

for all experiments were prepared by inoculating approximately 20 ml of Mueller-Hinton Broth (MHB; Difco Laboratories), Luria Broth Base (LB; Difco Laboratories), or Luria Bertani Broth (LBB; Difco Laboratories) in 125-ml growth flasks with cells from working cultures and incubating for 15 to 18 h at 37° C with rotary aeration at 180 rpm (Excella E24 Incubater Shaker Series; New Brunswick Scientific, Edison, NJ). The use of starter cultures provided stationary-phase inocula acclimated to each experimental growth environment.

Physiological Characterization

Cells from working cultures were streak inoculated on Trypticase Soy Agar plates containing 5% sheep blood (Becton, Dickinson, & Co., Sparks, MD) for isolated colonies and incubated at 37° C for approximately 18 h. Colonial and cellular morphology, gram reactivity, and hemolysis were assessed using conventional methods. The identity of each strain was confirmed using the API 20NE Identification System (bioMérieux, Hazelwood, MO) (data not shown).

Total Cell Cultural Growth Kinetics

Total cell density growth kinetics were assessed turbidmetrically using a modified version of the method outlined by Champlin *et al*. (2005). Test cultures each consisting of 50 ml of LB in a 125-ml growth flask were inoculated with stationary-phase cells from LB starter cultures to an optical density at 620nm (OD_{620}) of 0.025 (Spectronic 20D+; Thermo Scientific, Waltham, MA) and incubated at 37° C with rotary aeration at 180 rpm while monitoring the OD_{620} at 30-min intervals for a total of 6 h. Mean values obtained

from no fewer than three independent turbidimetric measurements for each test culture were plotted as a function of time on semi-logarithmic graph paper in order to document cultural growth.

Capsulation Assessment

The degree of cellular capsulation was assessed microscopically with the aid of an India ink negative stain protocol (Duguid 1951; Beveridge *et al.,* 2007). Cells from working cultures were used to streak inoculate Luria Bertani Agar (LBA; Difco Laboratories) plates for isolated colonies prior to incubating at 37° C for approximately 18 h. Representative individual colonies were each suspended in a solution consisting of one drop of India Ink (Becton, Dickinson and Company, Franklin Lakes, NJ) and one drop of distilled water in a 13 x 100-mm disposable borosilicate culture tube. One drop of the mixture was placed on a glass microscope slide and covered with a cover slip overlayed with a bibulous paper strip. Slight manual pressure was applied until a light, gray brown color was observed. Cells were observed microscopically at 1000x under oil immersion and the degree to which cells were capsulated was graded as follows: 0, none; +, slight; ++, moderate; and +++, heavy (see Figure 2 as examples of none and heavy capsulation).

Macrobroth Dilution Bioassay

In order to ascertain the relative propensities of *B. multivorans* variant strain surfaces to associate with moderately hyrophobic compounds, minimal inhibitory concentrations (MICs) were determined for three nonpolar antimicrobial agents having

disparate mechanistic targets (Champlin *et al.*, 2005; Ellison and Champlin 2007; Ellison *et al.*, 2007) using a standard macrobroth dilution bioassay. Novobiocin and rifamycin SV were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO), and triclosan (Irgasan DP 300) was obtained from Ciba Speciality Chemical Corp. (High Point, NC). Novobiocin (1024 *u*g/ml), rifamycin SV (512 *u*g/ml), and triclosan (64 *u*g/ml) stock solutions were prepared in MHB and filter sterilized (Fisherbrand 0.22-µm mixed cellulose ester syringe filter assemblies; Fisher Scientific, Pittsburgh, PA). Triclosan was initially dissolved in ethanol (95%) and diluted such that the final solvent concentration never exceeded 0.4%. Inocula were prepared using early exponential-phase cells from MHB batch cultures incubated until an OD_{620} of 0.1 was obtained (approximately 2 h), and diluted by transferring 0.1 ml of cell suspension to 31.1ml of MHB, thereby yielding a final viable cell density of approximately 5.0 x 10^5 CFU/ml. Antimicrobial agent stock solutions were diluted serially in a two-fold manner using 1.0 ml of sterile MHB as diluent. Each resulting solution was then inoculated with 1.0 ml of standardized cell suspension, thereby yielding a final viable cell density of 2.5 x 10^5 CFU/ml. The MIC was defined as the lowest concentration of antimicrobial agent that inhibited the initiation of visible growth after incubation with rotary aeration at 180 rpm at 37° C for 24 h.

Gentian Violet Uptake Assay

The gentian violet uptake assay described by Bhaduri *et al.* (1987) was performed to qualitatively assess relative cell surface hydrophobicity properties of *B. multivorans* strains at the colonial level. Cells from working cultures were used to streak inoculate

LBA plates for isolated colonies. After incubating at 37° C for 18 or 24 h, each plate was flooded with 8.0 ml of gentian violet (Riedel-De Haen Ag Seelze, Hannover, Germany) solution (0.085 mg/ml in deionized H_2O) for 4 min at ambient temperature and decanted. The degree to which the hydrophobic gentian violet was taken up by the colonies was graded visually on the basis of color intensity as follows: 0, none; +, slight; ++, moderate; and +++, heavy.

Hydrocarbon Adherence Assay

In order to further measure cell surface hydrophobicity properties in a more quantitative faction, the degree to which cells were able to associate with n-hexadecane was assessed using the modified hydrocarbon adherence method (Rosenberg *et al.,* 1980) as modified in our laboratory (Darnell *et al.,* 1987; Theis and Champlin 1989; Watt *et al*., 2003). Test cultures each consisting of 100 ml of LBB in a 250-ml growth flask were inoculated with stationary-phase cells from LBB starter cultures to an initial OD_{620} of 0.025 and incubated at 37° C with rotary aeration at 180 rpm until late-exponential phase (approximately 3h). Cells were harvested by centrifuging at 12,000 x G and 4˚C for 15 minutes (Avanti J-25 Superspeed centrifuge, Beckman Coulter, Inc., Fullerton, CA), washed with cold PPMS buffer (6.97 g of K_2HPO_4, 2.99 g of KH_2PO_4, and 0.2 g of $MgSO_4 \cdot 7H_2O$ per liter of deionized water at pH 7.2), and resuspended in ambient temperature PPMS buffer to an OD_{620} of 0.50 (as determined using 13 x 100-mm borosilicate disposable sample holders). Precisely 4.0 ml of each cell suspension were dispensed into each of four 20 x 150-mm borosilicate disposable culture tubes, three of which were treated with 1000 µl of *n*-hexadecane while the fourth served as an untreated

control. Each sample was vortex agitated with 15- second bursts for a total of 1.0 min, after which phase separation was allowed to occur undisturbed for 15 minutes at ambient temperature. Turbidity of the lower phase aqueous cell suspensions was measured spectrophotometrically as before and cell surface hydrophobicity determined on the basis of the portion of cells that partitioned into the n-hexadecane phase as compared to control cells and reported as percent adherence.

Biofilm Formation Assessment

The potential to form biofilms was accessed based on the ability of the bacteria to adhere to polystyrene microtiter plates (Costar 96-well sterile microtiter plates; Corning Inc., Lowell, MA) using previously described procedures (O'Toole and Kolter 1998; Caraher *et al.,* 2007). Test cultures each consisting of 50 ml of LBB in a 125-ml growth flask were inoculated with stationary-phase cells from LBB starter cultures to an initial OD_{620} of 0.025 and incubated at 37° C with rotary aeration at 180 rpm until late-exponential phase (approximately 3h). An Eppendorf pipette (Eppendorf AG, Hamburg, Germany) was used to asceptically transfer 100 µl of late-exponential-phase cells from LBB test cultures to each microtiter well. Each plate was incubated at 37° C for 3h (late exponential phase), 24 h (early stationary phase), or 48 h (late stationary phase) in a sealed plastic container which included moisten paper towels. After washing thoroughly with distilled water and air drying completely, 100 µl of a 1% crystal violet solution (10 mg/ml in deionized H_2O) was added to each well and allowed to stain adherent biomass for 30 minutes. After washing and drying as before, 200 µl of ethanol (95%) was added to dissolve the crystal violet-stained biofilm and 125 µl of the solution was transferred to

clean wells. A Spectra Max 384 Plus Absorbance Microplate Reader (Molecular Devices Corp., Toronto, Canada) was used to measure the absorbance at 600nm (A_{600}) of each sample. Polystyrene plastic is known to have hydrophobic properties (Corning Life Science, 2009), therefore the 3 h reading was seen as resultant propensity of each strain to adhere nonspecifically to the polystyrene plastic. Biofilm formation was defined as a continuous increase in absorbance over time. Initial elevated absorbance values were taken to indicate nonspecific adherence of cells to polystyrene mediated by nonspecific hydrophobic interactions.

CHAPTER IV

FINDINGS

Total Cell Cultural Growth Kinetics

Turbidimetric growth curves were constructed for all *B. multivorans* strains cultivated under batch test culture conditions in LB under standard growth conditions for the present study. As can been seen in Figure 2, no significant differences are evident among the type or clinical strains with regard to overall cultural growth kinetics including both generation time and total biomass. These data suggest that all strains are phenotypically similar with regard to their overall physiology under standard experimental cultivation conditions and are confirmatory of isolate identity.

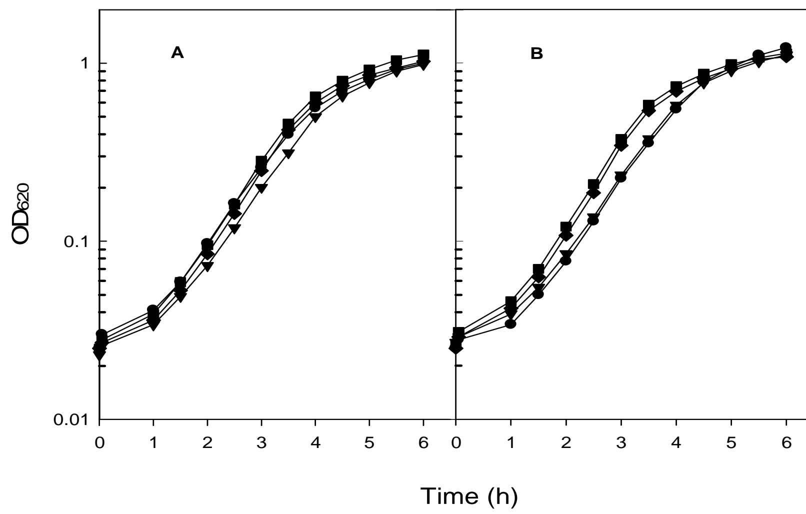

Figure 2. Total cell density growth kinetics of *B. multivorans* variant strains cultured in LB. Symbols: (A) ●, ATCC BAA-247; ▼, AZ01; ■, AZ02; ♦, AZ03 and (B) ●, AZ04; ▼, AZ05; ■, AZ06; ♦, AZ07.

Colonial Morphology and Capsulation Assessment

Colonial morphology was assessed after cultivating the *B. multivorans* type and clinical strains on LBA for 3, 24, and 48 h at 37° C. The type strain and all but one of the clinical strains exhibited the butyrous colonial phenotype, while strain AZ03 produced highly mucoid colonies (Table 1, Figure 3). Microscopic assessments of EPS capsulation were performed on cells after incubation for 24 and 48 h (Table 1). Only strains AZ03 and AZ07 express EPS. All others, including the type strain, failed to do so. All of strain AZ03 cells were moderately to heavily capsulated in a manner consistent with its highly mucoid colonial morphology. It should be noted that while approximately 30% of butyrous strain AZ07 cells were heavily capsulated at 24 h, 10% of the cells were only slightly capsulated at 48 h. A direct microscopic comparison of the butyrous noncapsulated strain AZ04 and the mucoid capsulated strain AZ03 can be seen in Figure 4. These findings strongly suggest the mucoid colonial phenotype is a function of the degree to which the cell surfaces of each *B. multivorans* strain are capsulated with EPS.

Table 1. Microscopic capsulation and cell surface hydrophobicity analyses of *B. multivorans* variant strains

Organism	Colonial consistency	India ink stain for capsulation[a]		Gentian violet uptake[c]	
		24	48	18	24
Burkholderia multivorans					
ATCC BAA-247	Butyrous	0	0	0	0
AZ01	Butyrous	0	0	0	0
AZ02	Butyrous	0	0	++	0
AZ03	Highly mucoid	+++	++	0	0
AZ04	Butyrous	0	0	+	++
AZ05	Butyrous	0	0	0	0
AZ06	Butyrous	0	0	0	0
AZ07	Butyrous	+++[b]	+	0	0

[a]Capsulation was graded as follows: 0, none; +, slight; ++, moderate; +++, heavy. Cultures were cultivated on LBA for 24 and 48 h.
[b]Only 30% and 10 % were capsulated at 24 and 48 h respectfully.
[c]Cultures were grown on LBA for 18 and 24 h. Colonial uptake of gentian violet was graded as follows: 0, white; +, slight; ++, moderate; +++, heavy.

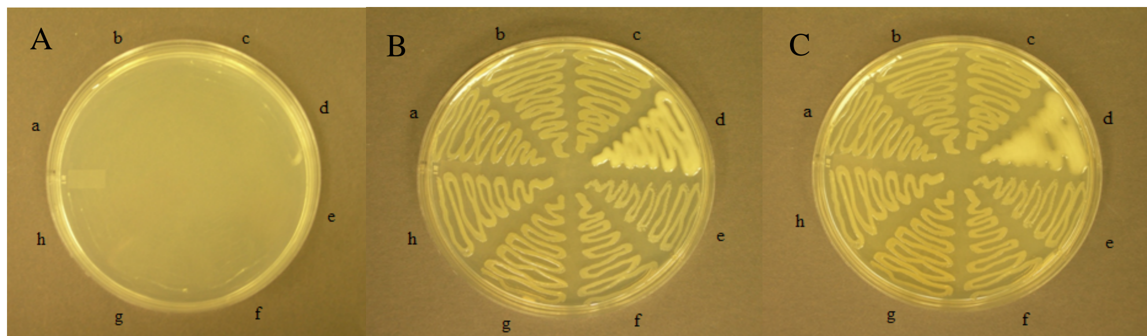

Figure 3. Cultural growth of *B. multivorans* variant strains on LBA at 37°C for 3 h (A), 24 h (B), and 48 h (C). Strains: a, ATCC BAA-247; b, AZ01; c, AZ02; d, AZ03; e, AZ04; f, AZ05; g, AZ06; h, AZ07.

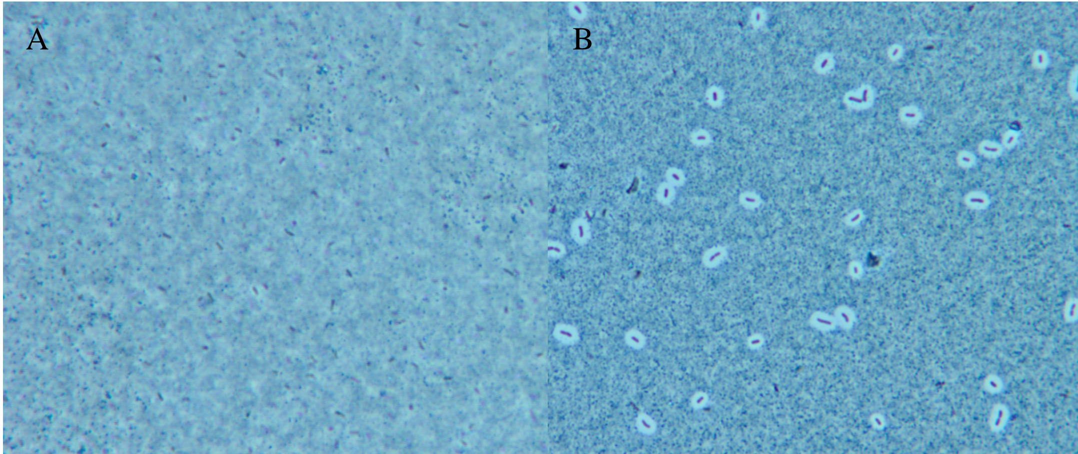

Figure 4. Microscopic observation of the capsular phenotype with the aid of an India ink negative stain protocol. Panels: (A) strain AZ04 and (B) strain AZ03.

Susceptibility to Antimicrobial Agents

Minimum inhibitory concentrations of nonpolar antimicrobial agents having disparate mechanistic targets were obtained in order to assess surface accessibility and outer membrane permeability properties for hydrophobic molecules (Table 2). *P. aeruginosa* PA01 and PA1211 strains were employed as refractory controls for each of the antimicrobial agents while *P. multocida* strain ATCC 11039 served as a susceptible control. Because the macrobroth dilution bioassay is generally considered to involve inherent experimental error of plus or minus one two-fold dilution, these data reveal no significant MIC differences among the three nonpolar antimicrobial agents for the *B. multivorans* type and clinical strains. All *B. multivorans* strains are relatively resistant to the hydrophobic molecules novobiocin, rifamycin SV, and triclosan in a manner consistant with that of the refractory control organism, regardless of the degree to which they are capsulated.

Table 2. Minimal inhibitory concentrations of nonpolar antimicrobial agents for *B. multivorans* variant strains

Organism	MIC (μg/ml)[a]		
	Novobiocin	Rifamycin SV	Triclosan[b]
Pseudomonas aeruginosa			
PAO1	>512	64	>32
PA1211	>512	128	>32
Pasteurella multocida			
ATCC 11039	4	1	0.0625
Burkholderia multivorans			
ATCC BAA-247	32	>256	>32
AZ01	32	>256	>32
AZ02	16	64	32
AZ03	16	128	>32
AZ04	16	64	>32
AZ05	16	64	32
AZ06	32	64	32
AZ07	32	128	>32

[a]Each value was obtained from three to four independent twofold serial dilutions.
[b]Ethanol (<0.4% final concentration) was employed to facilitate triclosan solubility and exhibited no effect on control growth (data not shown).

Cell Surface Hydrophobicity

The uptake of the hydrophobic dye gentian violet was used to assess relative cell surface hydrophobicity properties on a preliminary basis (Table 1). After 18 h of growth, the AZ02 strain appeared moderately hydrophobic, as indicated by the moderate dye uptake, while AZ04 appeared only slightly hydrophobic. The remaining strains failed to take up the stain. After 24 h of growth, the hydrophobicity of strain AZ04 increased to a moderate level while all other strains appeared hydrophilic.

In order to more quantitatively assess relative cell surface hydrophobicity properties, the ability to partition into n-hexadecane was measured using a conventional hydrocarbon adherence method (Rosenberg *et al.,* 1980) as modified by our laboratory (Darnell *et al.,* 1987; Theis and Champlin 1989; Watt *et al*., 2003). All *B. multivorans* strains may be considered hydrophilic to moderately hydrophilic when compared to other gram-negative organisms (Darnell *et al.,* 1987; Theis and Champlin 1989; Watt *et al*., 2003). When compared with each other, however, statistically significant differences can be seen with the most capsulated strains AZ03, and AZ07 being the most hydrophilic (Figure 5). In contrast, noncapsulated strain AZ04 (Table 1, Figure 4) was the most hydrophobic of the strains in a manner consistent with the ability of its colonies to take up gentian violet (Table 1). The remaining type and clinical strains all exhibited cell surface hydrophobicity properties intermediate between the extremes. These results reveal strain AZ04 to be the most hydrophobic tested while strains AZ03 and AZ07 are the most hydrophilic, thereby suggesting an inverse relationship between cell surface hydrophobicity properties and EPS capsulation, based pm the degree to which colonial

biomass absorbed the hydrophobic dye gentian violet and cells partitioned into the hydrocarbon n-hexadecane.

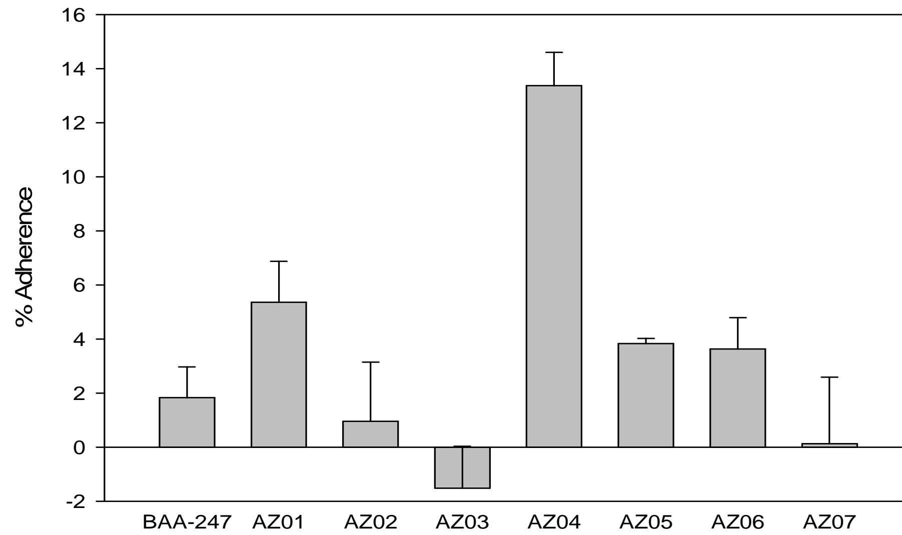

Figure 5. Cell surface hydrophobicity properties of *B. multivorans* variant strains cultured in LBB as determined using the hydrocarbon adherence method. Each value represents the mean of three to four independent determinations ± standard error.

Potential to form Biofilms

The ability of EPS phenotypically variant *B. multivorans* strains to form biofilms in polystyrene microtiter wells was assessed after incubation for 3 h (late exponential phase), 24 h (early stationary phase), and 48 h (late stationary phase) using a conventional *in vitro* assay (Figure 6). Strains AZ02 and AZ06 initially adhered to the polystyrene wells presumably due to their lack of capsulation and slightly hydrophobic cell surfaces; however, the decrease in adhesion over time during stationary phase is indicative of an inability to form true biofilms. Strains BAA-247 and AZ01also exhibited initial adhesion due to their slightly hydrophobic cell surfaces, however the apparent slight biofilm production seen at 24 h failed to continue after 48 h of incubation. Hydrophobic noncapsulated strain AZ04 exhibited the greatest initial adherence at 3h yet failed to produce a biofilm over time. Strain AZ05 initially adhered to the plastic at a very low level and appeared to form a significant amount of biofilm by 24 h. However, this apparent biofilm production declined by 48 h.

Highly mucoid strain AZ03, which exhibited both the greatest EPS expression and hydrophilic cell surface properties, exhibited no initial adhesion to polystyrene. Moreover, it increasingly adhered in a time-dependent manner at 24 h and 48 h, making it the only strain to continuously produce a biofilm. The lesser capsulated yet also hydrophilic strain AZ07 both adhered initially and exhibited a significant amount of biofilm production at 24 h. However, the biofilm decreased by 48 h. These data are consistent with the notion that EPS precludes initial nonspecific adhesion to the hydrophobic plastic seen by less capsulated and noncapsulated strains which possess

relatively nonpolar surfaces, while the possible down-regulation of EPS allows for controlled adhesion and subsequent biofilm production over time.

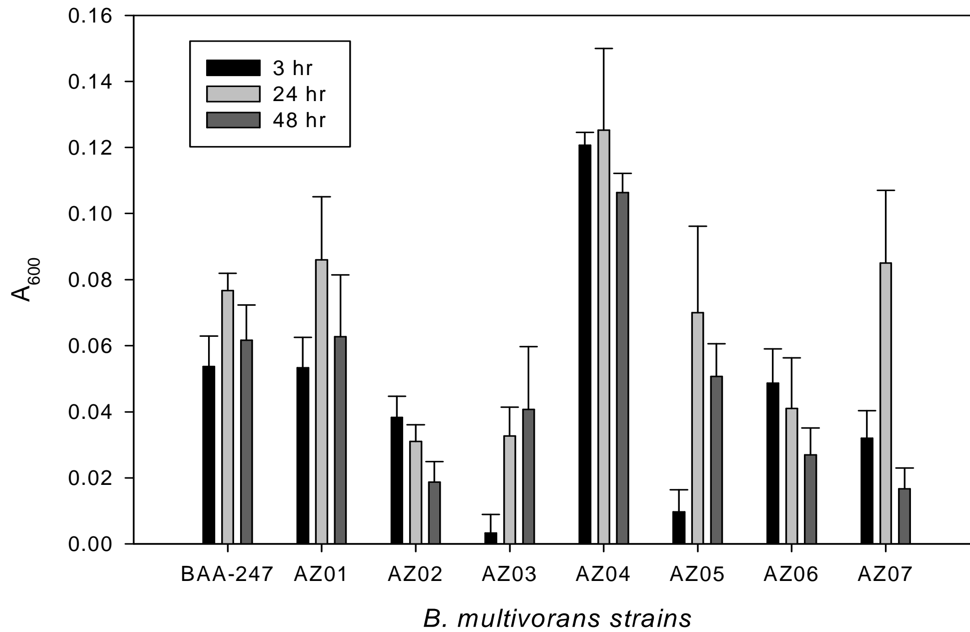

Figure 6. Biofilm formation potential of *B. multivorans* variant strains. LBB cultures were cultivated at 37°C in polystyrene microtiter wells for 3, 24, or 48 h. Each value represents the mean of three independent determinations ± standard error.

CHAPTER V

CONCLUSION

B. multivorans is an opportunistic etiological agent in CF and CGD patients for which its physiological properties and virulence factors are poorly understood. The purpose of the present study was to characterize and compare the outer cell envelope physiology of a *B. multivorans* ATCC type strain, with seven clinical phenotypic variants which exhibit disparate *in vitro* and *in vivo* virulence properties (Zelazny *et al.,* 2008).

No significant differences were noted among *B. multivorans* strains with regard to batch cultural growth kinetics, thereby suggesting phenotypic similarity with regard to overall physiology and unity with regard to identity. The mucoid cell surface phenotype was found to be a function of the degree to which each *B. multivorans* strain is capsulated with EPS. All *B. multivorans* strains are relatively resistant to the hydrophobic molecules novobiocin, rifamycin SV, and triclosan, regardless of the degree to which they were capsulated. These data indicate that the cell envelopes of all strains are uniformly accessible and permeable to nonpolar compounds which have been solubilized in aqueous medium. Cellular capsulation was inversely related to cell surface hydrophobic properties as judged both on the basis of the degree to which colonial biomass absorbed the hydrophobic dye gentian violet and the degree to which cells were able to partition into the hydrocarbon n-hexadecane. The ability to form biofilms did not appear to be directly related to the degree to which the *B. multivorans* strains were

capsulated. However, regulation of EPS expression may play a role in growing and maintaining biofilms as observed in the most highly capsulated strain AZ03. These data support the hypothesis that while EPS production may affect the ability of *B. multivorans* to bind to host cells, it does not hinder the accessibility of the outer cell surface to nonpolar antimicrobial agents or presumably, host cell surfaces.

The highly capsulated and mucoid strain AZ03 was the most hydrophilic and exhibited the greatest propensity for biofilm formation of all the strains examined in the present study. We speculate that the AZ03 EPS plays a vital role in avoiding the body's immune system enabling a biofilm to be established in pulmonary tissues, thereby setting up conditions which potentiate chronic infection as seen in *P. aeruginosa* models. EPS expression may be down regulated concomitantly with biofilm formation (Figure 7) as preliminary microscopic capsule assessments in this laboratory suggest (preliminary data). This model is consistent with present data in which the *in vitro* and *in vivo* virulence properties of AZ03 in CGD where mortality occurs 100% with 10 days of infection (Zelazny *et al.,* 2008). In contrast, the five strains which failed to initiate *in vitro* biofilm formation might employ *in vivo* cell-to-cell signaling with *P. aeruginosa* biofilms to facilitate establishment of a mixed biofilm as seen with *B. cepacia* (Mahenthiralingam *et al.,* 2005).

Future extensions of the present research will involve more detailed comparative analyze of the mucoid hydrophilic strain AZ03 and the most hydrophobic strain AZ04 with regard to colonial morphology, microscopic EPS production, cell surface hydrophobicity properties, and biofilm production at different points in the *in vitro* growth curve (e.g. 3, 24, and 48 h post inoculation). The fluorescent probe 1-N-

phenylnaphthylamine will be employed to more accurately assess the availability of the outer cell envelope to hydrophobic substrates such as host tissue. It is anticipated that such data will contribute to a better understanding of the basic physiology of *B. multivorans* with regards in the molecular mechanisms and virulence factor underlying its opportunistic pathogenicity in diseases such as CF and CGD. Characterization of EPS and cell envelope lipid composition will also provide additional needed information. Molecular approaches featuring PCR and rtPCR will allow further insight pertaining to the regulation of EPS expression.

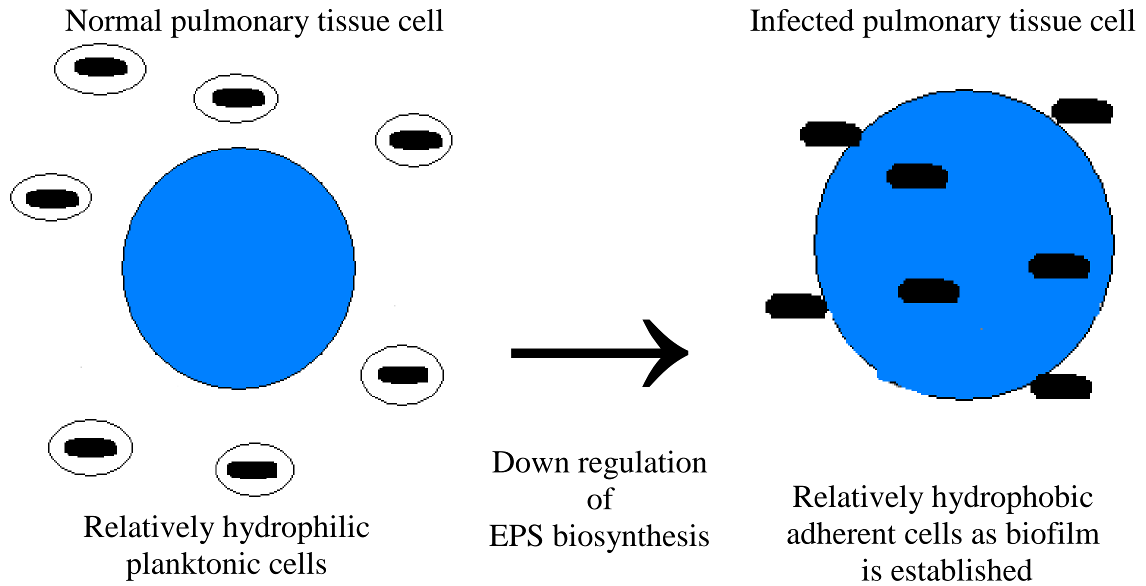

Figure 7. Proposed paradigm to explain possible relationship between EPS capsulation, cell surface hydrophobicity, and the propensity of *in vitro* biofilm formation in *B. multivorans*. While capsular material is produced in copious amounts during the exponential phase of growth, the cessation of active growth is accompanied by the concomitant down regulation of EPS expression, thereby allowing for adhesion to host tissues and initiation of biofilm establishment.

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VITA

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The purpose of the present study was to better characterize the cell surface physiology of a type reference strain and seven clinical isolates of *Burkholderia multivorans* which represent virulence and colonial phenotypic variants. Microscopic observation, standard macrobroth dilution susceptibility, cell surface hydrophobicity, and biofilm formation analyses were employed to assess pertinent aspects of outer cell surface physiology among strains.

Findings and Conclusions:

The cell surface of the mucoid phenotype was found to be a function of extracellular polysaccharide expression and appeared to facilitate initiation of biofilm production, while being inversely related to cell surface hydrophobic properties. However, the outer cell envelopes of all strains were uniformly permeable to hydrophobic antimicrobial agents as suggested by their uniform minimal inhibitory concentrations. These data support the hypothesis that while extracellular polysaccharide production may affect the ability of *B. multivorans* to bind to host cells, it does not influence the accessibility of the outer cell surface to nonpolar antimicrobial agents.

ADVISER'S APPROVAL: Franklin R. Champlin, Ph.D.