IDENTIFICATION AND PARTIAL CHARACTERIZATION OF A

TRANSPOSON INSERTION MUTANT OF

BURKHOLDERIA MULTIVORANS WITH REDUCED RESISTANCE

TO POLYMYXIN B

by

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Abstract

Burkholderia multivorans is an antibiotic resistant opportunistic pathogen that is being isolated with an increased frequency from immunocompromised patients. *B. multivorans* can cause serious lung infections in patients with cystic fibrosis but the mechanism of pathogenesis is not understood. As well, *B. multivorans* causes lung infections in chronic granulomatous disease patients. Neutrophils from these patients are unable to elicit an oxidative burst and are therefore dependent on nonoxidative killing mechanisms for destroying phagocytosed microbes. *B. multivorans* is resistant to *in vitro* killing by chronic granulomatous disease neutrophils and is resistant to killing by cationic peptides which are a major component of neutrophil nonoxidative killing. The purpose of these studies was to understand how *B. multivorans* resists killing by cationic peptides.

A transposon insertion mutant of *B. multivorans* (26D7) was created that had increased susceptibility to polymyxin B, colistin and the cationic peptide CP26 (a cecropin-melittin hybrid). In order to determine the genetic nature of this mutation, a cosmid library of *B. multivorans* strain ATCC 17616 was constructed and screened by Southern analysis using a probe specific for the DNA adjacent to the transposon insertion site. Sequence analysis of the DNA at the site of transposon insertion in mutant 26D7 showed four open reading frames (ORFs). ORF2 showed protein sequence similarity to several glucosyltransferases. ORF3 had protein sequence similarity to methyltransferases and was the site of the transposon insertion in mutant 26D7. Phenotypic analysis of mutant 26D7 showed that the outer membrane of this mutant was more permeable to 1-*N*-phenylnaphthylamine in the presence of polymyxin than the parent strain; however, like the parent strain, the mutant remained incapable of binding

dansylated polymyxin. No difference was seen in the lipopolysaccharide ladders. TLC analysis revealed one fewer glycolipid and a possible shift in one of the phosphatidyl ethanolamine spots in the mutant. The results presented here suggested that a group of genes were identified that encoded for proteins possibly involved in lipid modification, important for resistance to cationic peptides. Further studies may identify the role of lipid modification in this important phenotype of the *Burkholderia cepacia* complex.

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Dedication

I would like to dedicate this thesis to my daughter Katherine who has been patiently playing beside me whilst I studied and to my parents who have helped to make it possible for me to be a mother and a PhD student.

List of Abbreviations

Ara4N   4-amino-4-deoxyarabinosame

ATP   adenosine triphosphate

BLAST   Basic Local Alignment Search Tool

BPI   bacterial permeability increasing

CCCP   carbonyl cyanide-*m*-chlorophenylhydrazone

CEME   cecropin-melittin hybrid

CF   cystic fibrosis

CFTR   cystic fibrosis transmembrane regulator

CGD   chronic granulomatous disease

CL   cardiolipin

CP-26   cecropin-melittin hybrid with modifications

CSA   surface carbohydrate antigen

C-terminal carboxy-terminal

CTP   cytosine triphosphate

Dansyl   1-dimethylaminonaphthalene-5-sulfonyl chloride

DMSO   dimethylsulfoxide

DNA   deoxyribonucleic acid

DPX   dansyl-polymyxin

dNTP   dexoynucleotide triphosphate (N can be A, T, C or G)

EDTA   ethylenediaminetetraacetic acid

ELISA   enzyme linked immunosorbant assay

ETC   extracellular toxic complex

EMBL   European Molecular Biology Laboratory

gHBSS   gel Hank's balanced salt solution

GTP   guanosine triphosphate

HEPES   *N*-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid

IFN-γ   interferon gamma

iNOS   inducible nitric oxide synthetase

IS   insertion sequence

Kb   kilobase

kDa   kilodalton

KDO   3-deoxyoct-2-ulosonic acid

LB   Luria Britani

LPS   lipopolysaccharide

MH   Meuller Hinton

MIC   minimal inhibitory concentration

NO   nitric oxide

NPN   1-*N*-phenylnaphthylamine

N-terminal   amino terminal

OD   optical density

OF   oxidative fermentative

ORF   open reading frame

PCR   polymerase chain reaction

PE   phosphatidyl ethanolamine

PEG   polyethyleneglycol

PG   Phosphatidyl glycerol

PNPG   *p*-nitrophenyl-β-D-galactopyranoside

RAPD-PCR   random amplified polymorphic DNA polymerase chain reaction

rRNA   ribosomal ribonucleic acid

SDS   sodium dodecyl sulphate

SDS-PAGE   sodium dodecyl sulphate polyacrylamide gel electrophoresis

TLC   thin layer chromatography

Chapter 1   **Introduction**

1.1 *Burkholderia multivorans* and the *Burkholderia cepacia* complex

Burkholderia cepacia complex organisms are Gram-negative, non-spore-forming, aerobic bacillae. They are motile with peritrichous pili, 0.5-1.0 μm in width and 1.5-5.0 μm in length with rounded ends. They are weakly oxidase positive and typically catalase positive although some catalase negative strains exist. The optimal growth temperature is considered to range between 30-35°C although some strains are found to grow at temperatures as high as 47°C.

B. cepacia was first isolated and described by Burkholder in 1950 as a phytopathogen that causes sour skin rot in onions (Burkholder 1950). Based on the description in this paper it was placed in the genus *Pseudomonas* as *Pseudomonas cepacia* (cepia is Latin for onion). In 1973 the genus *Pseudomonas* was divided into five distinct groups based on ribosomal ribonucleic acid (rRNA) homologies, and *P. cepacia* was placed into rRNA homology group II (Palleroni et al. 1973). In 1992, on the basis of 16S rRNA sequence homology, DNA-DNA homology values, cellular lipid and fatty acid composition and phenotypic characteristics, Yabuuchi et al, proposed that *P. cepacia* and other members of the RNA homology group II be transferred to a new genus *Burkholderia*. *Burkholderia cepacia* was proposed as the type strain for this new genus (Yabuuchi et al. 1992). Vandamme et al, in 1997, studied the taxonomic structure of *Burkholderia cepacia* and its relationship with other *Burkholderia* species. Their data showed that the *B. cepacia* strains studied belonged to at least five distinct genomic species. This group of five phenotypically similar but genetically distinct species was referred to as the *B. cepacia* complex (Vandamme et al. 1997). Each group within this complex is called a genomovar. Genomovars share a low level of DNA hybridization (50-70% reassociation) and represent distinct species however, until a differential diagnostic test is available a nomenspecies is not proposed (Ursing et al. 1995). The name *Burkholderia multivorans* has been given to the *B.*

cepacia genomovar II strains and the genomovar V strains correspond to the previously described new species *Burkholderia vietnamiensis* (Gillis et al. 1995 and Vandamme et al. 1997). The remaining *B. cepacia* groups are referred to as genomovars I, III, and IV. The role and pathogenic potential of each of these genomovars is under investigation. Genomovar I strains are mainly environmental isolates. *B. multivorans* (genomovar II) strains cause clinical infections in both cystic fibrosis (CF) and chronic granulomatous diseased (CGD) patients. Genomovar III strains seem to be the cause of most of the fatal and epidemic outbreaks in CF centers and cause what is often referred to as 'cepacia syndrome' (Vandamme et al. 1997, Mahenthiralingam et al. 1997). *B. cepacia* is no longer considered an authentic pseudomonad. It belongs to the β-subclass of *Proteobacteria* rather than the γ-subclass, where the genus *Pseudomonas* has been placed (Palleroni et al. 1981 and Palleroni, 1993). Bacteria of the β-subclass generally share more than 86% similarity in 16S rRNA nucleotide sequences. The extent of 16S rRNA similarity between members of the β-subdivision and members of the γ-subdivision of *Proteobacteria* is in the range of 81-83%. Most of the published work on organisms from the *B. cepacia* complex occurred prior to the identification of the different genomovars. For simplicity I will refer to *B. cepacia* when reviewing published work. The strain used for this study was a genomovar II strain *B. multivorans* ATCC 17616.

1.2 The unusual genome of *B. cepacia*

The *B. cepacia* genome consists of two to five chromosomes (replicons) and an overall genome size ranging from 5 to 9 Mb with the majority of strains closer in size to 9 Mb. Many insertion sequence (IS) elements are located throughout the *B. cepacia* genome and have been identified based on their ability to promote genomic rearrangements. Insertion sequences are small elements of DNA flanked by inverted repeat sequences and contain an open reading frame

(ORF) which spans its entire length and codes for a transposase. This transposase is essential for the transposition of the insertion sequence from one DNA site to another. In *B. cepacia*, IS elements play a role in mediating the fusion of plasmids (Barsomian et al. 1986), rearranging plasmids (Gaffney et al. 1987), increasing the expression of neighboring genes (Scordalis et al. 1987) and activating the *lac* genes on a broad host range plasmid (Wood et al. 1990). As well, these IS elements have been shown to play a role in the recruitment of genes by transposition of adjacent genes specifically related to novel catabolic functions such as degradation of the herbicide 2,4,5-Trichlorophenoxyacetic acid (2,4,5-T) (Haugland et al. 1990, Daubaras et al. 1995). IS elements play a central role in transcription of this gene and likely have stimulated a rapid evolution of this degradative pathway (Hubner et al. 1997). A detailed study of a population of *Burkholderia cepacia* (213 isolates) suggests genetic recombination events do occur frequently in the environment (Wise et al. 1995). In *B. cepacia*, the presence of multiple chromosomes and IS elements, a wide variation in genome size, and its extraordinary genetic plasticity is reflected by the organisms nutritional versatility and ability to colonize both plants and animals.

1.3 Significance of *B. cepacia*

1.3.1 Environmental

Due to the nutritional versatility of *B. cepacia*, it has possibilities as an agent for both bioremediation and agricultural disease control. *B. cepacia* is capable of degrading highly chlorinated compounds, which are toxic and found in complex pesticides and herbicides, some being carcinogenic. One example is 2,4,5-T, which is a potent herbicide, extensively used in many countries for weed control. This compound is not easily degraded and persists for long periods in the environment. This herbicide can act as the sole carbon and energy source for *B.*

cepacia and several experiments have demonstrated that *B. cepacia* strain AC1100 is capable of removing significant amounts of 2,4,5-T from contaminated soil (Daubaras et al. 1996). *B. cepacia* has also been shown to grow on fluorene and a wide variety of other polycyclic aromatic compounds, which are pollutants of concern because they are toxic and potent carcinogens (Grifoll et al. 1995). Other compounds *B. cepacia* is capable of degrading are catechols (Oh et al. 1997), phthalates, wastes discharged into the environment by plastics, paper and paint industries (Saint et al. 1996) and the herbicide diclofop-methyl (Smith-Greeier et al. 1996).

B. cepacia is known to antagonize and repress many soilborne plant pathogens. It is capable of preventing blight caused by the fungus *Altermaria* by inhibiting spore germination (Holmes et al. 1998) and can kill the fungus *Aphanomyces euteiches*, which causes root rot in peas (Bowers et at. 1993, King et al. 1996). Stem rot of poinsettia is caused by *Rhizobium solani* and can also be controlled if poinsettia cuttings are rooted with *B. cepacia* (Cartwright et al. 1995). *B. cepacia* appears to be an attractive alternative to toxic fungicides (which cannot be broken down in the environment) for prevention of plant diseases. At this time, many patents are being sought in the hopes of using *B. cepacia* for agricultural applications and decontamination of soils. This poses a potential problem due to the emergence of *B. cepacia* as a human pathogen. The possibility of *B. cepacia* being released in large quantities in the environment for both bioremediation and agricultural uses is alarming for those individuals susceptible to infections caused by this same organism.

1.3.2 Clinical

Prior to 1980, reports of *B. cepacia* infections in humans were few and restricted to patients exposed to contaminated disinfectant and anaesthetic solutions in which *B. cepacia* is capable of surviving for long periods of time (Govan et al. 1996). The incidence of *B. cepacia* infection was on the rise in the early 1980s and analysis of the USA databases of nosocomial infections confirmed a large increase in clinically significant *B. cepacia* isolations at that time (Jarvis et al.1987, Martone et al. 1987). The investigations showed that the main source of nosocomial outbreaks of *B. cepacia* were contaminated liquid reservoirs and moist environmental surfaces. Apart from being a nosocomial pathogen, *B. cepacia* has also emerged as an important pathogen in two congenital diseases: cystic fibrosis (Isles et al. 1984, Thomassen et al. 1985) and chronic granulomatous disease (O'Neil et al. 1986).

Cystic fibrosis (CF) is the most common inherited lethal disorder of Caucasian populations with 50,000 individuals affected, a frequency of 1 in 2,500 live births and a carrier frequency of 1 in 25 (Bye et al. 1994). In 1989, the gene responsible for CF was isolated by the groups of Lap-Chee Tsui in Toronto, Canada and Francis Collins in Michigan, USA (Kerem et al. 1989, Riordan et al. 1989, and Rommers et al. 1989). The CF gene encodes a complex 1480 amino acid protein called the cystic fibrosis transmembrane conductance regulator (CFTR). CFTR is able to conduct chloride at a high rate (Bear et al. 1992) and has been localized to the apical surface of epithelial cells in the intestinal and respiratory tract (Trezise et al. 1991). A mutation in CFTR results in the failure of cAMP regulated chloride ion transport on the surface of epithelial cells (Knowles et al.1983, Quinton et al. 1986). In normal cells, the osmotic movement of water linked to chloride secretion probably represents one way of hydrating mucosal surfaces. A salt imbalance is caused by defective chloride ion transport in CF effecting the epithelial cells lining internal organs. This disturbance in chloride secretion combined with an increase in

sodium absorption and water influx into cells causes dehydration of the mucosal surfaces. This leads to a buildup of sticky dehydrated mucus in male sex ducts, ducts of the pancreas and the airways of the lung. In the lungs, this impairs mucociliary clearance and probably contributes to the persistent microbial colonization of the CF lung often resulting in episodes of debilitating and ultimately fatal infection (Stern et al. 1996). By far the most common pathogen in CF is mucoid *Pseudomonas aeruginosa* followed by what is considered the controversial new opportunistic pathogen *B. cepacia*.

Due to aggressive antibiotic therapy, today the prognosis for newly diagnosed patients is good with a median life expectancy of 30 years. *P. aeruginosa* lung infections in CF are characterized by early colonization and associated with an increase in the host immune response, followed by episodes of persistent, increasingly severe lung disease. *B. cepacia* infections rose significantly in CF clinics during the 1980s stabilizing in the last 10 years. The prevalence of *B. cepacia* in different CF clinics ranges from 0-40% patient colonization (Sajjan et al. 1992a). Infection of CF patients with *B. cepacia* is associated with clinical outcomes ranging from essentially asymptomatic to fatal necrotizing pneumonia (Govan et al. 1996). There is strong evidence that this heterogeneity in clinical outcomes may be related to the fact that *B. cepacia* is a complex of five genomovars (Vandamme et al. 1997). Genomovar III strains seem to be associated with cases of epidemic spread and high mortality rate in the Vancouver, B.C. CF clinic. *B. multivorans* strains are also common in CF patients although there is little evidence for patient to patient spread and it is found mainly in the pediatric population (Mahenthiralingam et al. 1997). However, an outbreak among pediatric patients of *B. multivorans* associated with significant mortality has been reported (Whiteford et al. 1995).

Another serious problem with *B. cepacia* is its resistance to potent anti-pseudomonal drugs. This has led to a lack of effective antimicrobial therapy for patients with *B. cepacia* infections

and the prevalence of this organism may be in part a result of aggressive antibiotic therapy and selection of this opportunist (Simpson et al. 1994). Even when a particular *B. cepacia* isolate shows susceptibility to antimicrobial agents *in vitro*, aggressive therapy rarely results in clinical improvement. Many studies are underway to try and understand the pathogenic and virulence properties of *B. cepacia* in relation to CF but it is still not clear how *B. cepacia* is able to establish infection in the lungs of these patients. A clue to understanding the interaction of *B. cepacia* with the immune system of the human host comes from a second genetic disorder, chronic granulomatous disease (CGD).

CGD is a rare disease with an estimated frequency of 1 in 500,000 births. CGD results from a mutation that renders the phagocytic NADPH oxidase enzyme dysfunctional. Phagocytic cells (neutrophils, eosinophils, monocytes and macrophages) kill ingested micro-organisms by releasing microbicidal proteins from cytoplasmic granules and by generating superoxide (O_2^-) and other reactive oxygen species in the intracellular phagosomal compartment that contains the ingested microorganism as depicted in figure 1. The enzyme that catalyzes the formation of superoxide is NADPH oxidase. This is a multi-component

Figure 1. Phagocytosis, degranulation and oxidative radical generation. Unopsonized and microbes opsonized with IgG antibodies and complement fragments attach to the phagocytic cell. This causes induction of phagocytosis, followed by fusion of granules with the phagosomal membrane and activation of the NADPH oxidase. (From Roos, 1994)

enzyme, consisting of five subunits. Two of these subunits are integral membrane proteins and the other three subunits are localized in the cytosol of resting phagocytes (Babior, 1991). In activated phagocytes, the three cytosolic proteins are translocated to the two membrane proteins

(Clark et al. 1990) and together these five proteins form a functional oxidase capable of generating superoxide (Rotrosen et al. 1993). Without a functioning NADPH oxidase enzyme the phagocytes from CGD patients are dependent on nonoxidative killing mechanisms for destroying invading microbes and as a result are unable to kill certain bacteria and fungi. These patients suffer from severe recurrent bacterial and fungal infections of the subcutaneous tissues, the lungs and the lymph nodes (Forrest et al. 1988). *B. cepacia* is an important pathogen in CGD children and has been reported to cause significant morbidity (Bottone, et al. 1975, O'Neil et al. 1986, Lacy et al. 1993). Studies by Speert et al, 1994 showed that neutrophils from CGD patients are unable to kill *B. cepacia in vitro* but are still capable of killing *P. aeruginosa* where normal neutrophils were able to kill both organisms equally efficiently. From these studies it was concluded that *B. cepacia* is resistant to the nonoxidative killing mechanisms of CGD neutrophils and this resistance may be a major contributor to the ability of *B. cepacia* to thwart the immune system of immunocompromised hosts and cause serious lung infections in CGD. Virulence of *B. cepacia* in CGD does not appear to have the variation that is seen in CF nor is it dependent on genomovar designation. An example of two strains recovered from CGD patients with severe infections were from genomovar II (*B. multivorans*) and genomovar V (*B. vietnamiensis*) (Speert et al. 1999).

1.4 Virulence Factors of *B. cepacia*

Most of the studies done on virulence of *B. cepacia* are related to its pathogenesis in the CF lung. The ability of a potential pathogen to adhere to the host mucosal or epithelial cells is essential in the establishment of an infection. Airway mucins (mucus glycoproteins) are synthesized and secreted by mucous cells of the submucosal glands in the respiratory mucosa. Piliated *B. cepacia* from sputa of CF patients are able to bind to purified mucin, a 55-kDa

receptor and a membrane glycolipid on epithelial cells (Sajjan et al. 1993). The *B. cepacia* adhesin was identified as a 22-kDa protein localized to the pili present over the entire surface of the cell, not seen in non-mucin binding strains (Sajjan et al. 1992b). The major subunit of the 22-kDa pilin associated adhesin was purified and immunolocalized to peritrichously arranged pili and, due to their unusual appearance as large intertwined fibers, they were referred to as cable pili (Sajjan et al. 1995). A study by Butler et al in Great Britain of 38 strains of *B. cepacia* showed only six were capable of mucin binding but it was the epidemic isolate that had the highest affinity for mucin (Butler et al. 1992). Invasion of epithelial cells by *B. cepacia* has been demonstrated and electron microscopy showed intracytoplasmic bacteria enclosed by membrane-bound vacuoles with no lysosomal fusion (Burns et al. 1996a).

Production of siderophores enables bacteria to compete with host iron-binding proteins, transferrin and lactoferrin, chelating essential iron required for growth and promoting establishment and maintenance of infection. *B. cepacia* produces at least four different iron-binding siderophores: 1. Pyochelin not related to the *Pseudomonas* siderophore (Sokol et al. 1986), 2. Salicylic acid (Visca et al. 1993, Sokol et al. 1992), 3. Cepabactin (Meyer et al. 1989) and 4. Ornibactins (Stephan et al. 1993). In a study of 43 *B. cepacia* isolates from CF patients, 49% produced pyochelin and those pyochelin positive strains were isolated from patients with severe pulmonary infections (Sokol et al. 1986). Using a rat model of chronic lung infection involving transtracheal inoculation of rats with agar beads, Sokol et al, 1988 showed that exogenous pyochelin supplied to the rats, increased the virulence of *B. cepacia.* Darling et al, in 1998 studied 61 CF isolates; 62% produced pyochelin, 93% produced salicylic acid, 11% produced cepabactin and 91% isolates tested produced ornibactin (Darling et al. 1998). From these data, it is suggested that the siderophores in *B. cepacia* are important in chelating iron from host iron binding proteins and that pyochelin may increase virulence of *B. cepacia.*

Extracellular bacterial products have been identified as important virulence factors in other organisms including *P. aeruginosa* and may be important in *B. cepacia*. In 1984, McKevitt and Woods identified the only protease that has been characterized for *B. cepacia*. This protease is a 37-kDa extracellular metalloprotease (Abe et al. 1996) and, upon instillation into rat lungs, causes pneumonia exhibited by neutrophil infiltration and proteinaceous exudation in the large airways (McKevitt et al. 1989). A hemolysin from a highly transmissible strain of *B. cepacia* was identified and partially characterized as a lipopeptide toxin. At low concentrations, this hemolysin induces nucleosomal degradation and apoptosis in human neutrophils and mouse macrophages (Hutchison et al. 1998). As well, the lipase activity of *B. cepacia* is well documented in the literature and was first described in *Pseudomonas sp.* in 1941 by Starr and Burkholder (Starr et al. 1941). Purified lipase from *B. cepacia* is not cytotoxic but is capable of reducing the phagocytic function of rat alveolar macrophages (Straus et al. 1992). An extracellular toxic complex (ETC) consisting of a surface carbohydrate antigen (CSA), lipopolysaccharide (LPS) and protein (Straus et al. 1989), appears to be responsible for the lethality and extensive pulmonary necrosis associated with lung infections caused by *B. cepacia* (Straus et al. 1988). Toxicity of the ETC was considered to be associated with the LPS portion of the complex because there was no effect on activity after boiling and proteolysis and LPS is resistant to both of these treatments (Straus et al. 1988). These studies suggest that *B. cepacia* can produce a protease, a hemolysin, a lipase and an ETC that act as virulence factors important in assisting the bacteria in evasion of host defenses.

1.5 The Gram-negative outer membrane

1.5.1 General structure

The envelope of Gram-negative bacteria consists of two membranes separated by the periplasmic space and a layer of peptidoglycan. The cytoplasmic membrane is considered a typical phospholipid bilayer with the negatively charged phosphate head groups on the two outer surfaces of the membrane and the hydrophobic fatty acid chains extending into the center of the membrane perpendicular to its plane. Integral and peripheral membrane proteins are found throughout the inner membrane and their main function is in cellular energy (electron transport), export of toxic cellular byproducts and transport of nutrients (Cronan et al. 1987). The peptidoglycan layer is closely associated with the inner leaflet of the outer membrane and provides structural integrity to the bacterial cell. The outer membrane, is an asymmetrical bilayer in which the inner leaflet is composed of phospholipids and the outer leaflet contains LPS. The most abundant phospholipid components of *B. cepacia* are, in order of abundance, phosphatidyl ethanolamine, diphosphatidyl glycerol (cardiolipin) and phosphatidyl glycerol similar to that found for *P. aeruginosa* (Kawai et al. 1988).

1.5.2 Lipopolysaccharide

LPS found in the outer leaflet of the outer membrane of Gram-negative bacteria, plays an important role in the interaction of the organism with its environment. The host defense system recognizes bacterial LPS and in response produces antibodies that specifically recognize LPS structure. LPS is released into the environment and once released LPS (also called endotoxin) can cause fever, hypotension and toxic shock syndrome in the host organism. LPS has been shown to activate B-lymphocytes, neutrophils and macrophages to release immune activating cytokines.

LPS consists of a three distinct regions:

1) **Lipid A** is the hydrophobic membrane anchoring region of LPS and rather than carrying two fatty acid residues typical of phospholipids, it carries five to seven attached to a phosphorylated glucosamine dimer. All the fatty acids in lipid A are saturated or hydroxylated (unlike the unsaturated fatty acids found in phospholipids). 2) **The rough core** oligosaccharide is divided into the outer core and inner core regions. The inner core is a short chain of sugars, including several 2-keto-3-deoxyoctonoic acid (KDO) and heptose residues (see Figure 2 as an example of LPS from *Salmonella*). The outer core consists of common hexoses (Glucose, D-galactose, N-

Figure 2. Lipid A and inner core region of LPS from *Salmonella*. The numbers in circles represent the number of carbon atoms present in each acyl chain. Residues R1 constitute Ara4N and R2 constitutes 2-aminoethylpyrophosphate substitutions. (From Rietschel et al. 1993)

acetyl-D-glucosamine and N-acetyl galactosamine) and has also been termed the hexose region. This region of LPS contains phosphate residues and 16 residues can be found per LPS molecule in *P. aeruginosa*. 3) Attached to the rough core is the hydrophilic O side chain **(O-Antigen)**, composed of many repeating tetra- or pentasaccharide units (Rietschel et al. 1993 for a review). This O-antigen polysaccharide, the immunodominant portion of LPS, is only present on approximately 10% of all LPS molecules in one bacterium (Hancock et al. 1994). Strains lacking the O-side chain are referred to as having rough colony morphology and are sensitive to

killing by human serum and strains carrying an O-side chain on their LPS are referred to as having smooth colony morphology and are resistant to killing by human serum.

The distribution and structure of LPS gives the outer membrane many of its barrier properties. The presence of the phosphate residues in core region of LPS results in a strong negative charge on the surface of *P. aeruginosa*. This strong negative charge across the surface of *P. aeruginosa* results in a layer of water molecules that restrict the uptake of hydrophobic compounds through the outer membrane. As well, these phosphate residues stabilize the outer membrane by forming non-covalent cross-bridges with adjacent LPS molecules with the help of divalent cations (Mg^{2+} or Ca^{2+}) (Rottem et al. 1977). The interaction of the hydrophobic lipid A component of LPS with membrane proteins also aids in stabilization of the membrane (Nikaido et al. 1985).

54% of *B. cepacia* CF isolates possess rough LPS (McKevitt et al. 1984) and a highly transmissible virulent strain was shown to be serum sensitive (Butler et al. 1994) suggesting that the O-antigen is not important in virulence of *B. cepacia*. Western blotting and absorption studies have shown that a large proportion of serum antibodies from CF patients infected with *B. cepacia* reacted with the core LPS of *B. cepacia*. These antibodies do not cross react consistently with different strains of *B. cepacia* or with the core region of *P. aeruginosa* LPS (Nelson et al. 1993). This strongly suggests structural differences exist not only between *B. cepacia* and *P. aeruginosa* but also amongst strains of *B. cepacia*. The LPS from *B. cepacia* contains relatively low amounts of phosphorus and more heptose, with glucose and rhamnose being the major saccharide components (Maniello et al. 1979). Only a single KDO and two orthophosphate residues were found in the core region of *B. cepacia* LPS. A 4-amino-4-deoxyarabinose (Ara4N) was produced upon dephosphorylation of what was a phosphate ester (Cox et al. 1991). By analogy to other LPS molecules it is likely that the Ara4N is

phosphodiester linked to the 4-position of a glucosamine residue of lipid A (see R1 in Figure 2, Meyer et al. 1989). The low number of phosphate residues found in the LPS of *B. cepacia* and the presence of arabinosamine substituted phosphates reduces the amount of negative charges found in the outer leaflet of the outer membrane of this organism.

1.5.3 Membrane proteins

B. cepacia produces five major outer membrane proteins: 40-kDa, 36-kDa, 24.5-kDa, 17-kDa and 14.5-kDa (Anwar, H. et al. 1983). The major outer membrane protein is 81-kDa (OpcPO) and dissociates into a 36-kDa (OpcP1) and a 27-kDa (OpcP2, same as 24.5 in Anwar's study) protein, forming a noncovalent association to make up the 81-kDa protein (Gotoh et al. 1994). It has been suggested that this is a pore forming protein (porin) and reduced expression of these proteins is associated with higher resistance to β-lactams (Aranoff et al. 1988). Porins serve as diffusion channels that facilitate the passage of hydrophilic molecules across the outer membrane. If this truly is a porin, it is unique in that most Gram-negative porins are trimers not dimers (Benz et al. 1988). Parr et al, 1987 showed that the outer membrane permeability coefficient for *B. cepacia* to the β-lactam nitrocefin was 10-fold less that that for *E. coli*. Antibiotic resistance and specifically β-lactam resistance in *B. cepacia* is due to the poor permeability of its outer membrane. These studies of *B. cepacia* outer membrane proteins suggest that porins in the outer membrane of *B. cepacia* have very small pores compared to *E. coli* and *P. aeruginosa* making diffusion of hydrophilic compounds difficult.

1.6 *B. cepacia* LPS and host interaction

LPS causes activation of host immune cells resulting in the synthesis and secretion of cytokines that are required for the regulation of immune responses (Rietschel et al. 1993). The

consequence of overproduction of cytokines is severe as observed in endotoxic shock, which is associated with elevated levels of TNF-α in the host (Mathison et al. 1988). It has been suggested that elevated levels of TNF-α may play a role in 'cepacia syndrome' (Govan et al. 1996). *B. cepacia* LPS is four to five times more toxic than *P. aeruginosa* LPS (Shaw et al. 1995). *B. cepacia* LPS can stimulate IL-8 release by monocytes. IL-8 is a cytokine important for the recruitment of neutrophils during an infection (Palfreyman et al. 1997). As well, LPS from *B. cepacia* causes increased expression of complement receptor 3 (CR3) on neutrophils and primes the respiratory burst, in contrast with *P. aeruginosa*, which shows little or no neutrophil priming activity. This increased surface expression of CR3 facilitates migration of neutrophils to the lung circulation and is involved in adhesion, phagocytosis and activation of neutrophils (Hughes et al. 1997). The LPS purified from CF isolates of *B. cepacia* induces a concentration of TNF-α similar to that of *E. coli* LPS (considered the most biologically active LPS), four times the activity of *P. aeruginosa* and eight times the activity of *S. maltophilia*. *B. cepacia* also induces the production of more superoxide anion than *P. aeruginosa* and *S. maltophilia* (Zughaier et al. 1999a). In addition, certain epidemic strains of *B. cepacia* produce a melanin-like pigment that acts as a scavenger for superoxide anion and may therefore protect *B. cepacia* from oxidative killing by phagocytic cells (Zughaier et al. 1999b). Recently, it has been reported that inducible nitric oxide sythetase (iNOS) may not be expressed in the bronchial epithelium of CF patients. Since nitric oxide (NO) has been shown to contribute to the antimicrobial activity of reactive oxygen species in the normal lung, evidence suggests that this lack of iNOS may contribute to the survival of *B. cepacia* in the CF lung (Kelley et al. 1998). *B. cepacia* cannot be killed by either superoxide anion or NO alone rather their combined activity is required (Smith et al. 1999). Interestingly, the treatment of CGD patients has been successful with the use of interferon-γ (IFN-γ) therapy (International Chronic Granulomatous Cooperative Study Group,

1991). IFN-γ is a cytokine, which is known to induce NO production in neutrophils (Evans et al. 1996). These properties of *B. cepacia* LPS provide some of the first insights into the virulence of this organism.

1.7 Antibiotic Resistance of *B. cepacia*

Bacteria have evolved a variety of mechanisms to escape the action of antibiotics such as membrane permeability changes causing restricted entry into the periplasm and/or cytoplasm, enhanced export of a drug via specific transport proteins, production of enzymes that modify and inactivate the antibiotic and modification of the target itself. Antibiotic resistance probably had its earliest origins in the soil organisms that produce antibiotics in order to protect themselves against the antibiotics they produce themselves. In general, *B. cepacia* is resistant to a wide range of antibiotics. Those resistance mechanisms that have been identified in *B. cepacia* are reviewed here.

1.7.1 Antibiotic uptake across the outer membrane

Three possible pathways exist for the uptake of antibiotics across the outer membrane of Gram-negative bacteria and the path of uptake is dependent on the physical properties of the particular antibiotic. Hydrophobic antibiotics such as some β-lactams, quinolones, rifampicin and macrolides are usually excluded by Gram-negatives, due to the high negative charge of LPS (Nikaido, 1983). Hydrophilic antibiotics such as most β-lactams, tetracycline and chloramphenicol pass across the Gram-negative outer membrane through the water filled channels formed by porins. For example, porin deficient mutants have significantly increased MICs for some β-lactams (Hancock et al. 1988). As mentioned, the putative porin proteins identified for *B. cepacia* are considered to have extremely small channels inhibiting the entry of

these hydrophilic antibiotics. The 'self-promoted-uptake' pathway has been proposed as the route of uptake for polycationic antibiotics like polymyxin, aminoglycosides and cationic peptides (Hancock, 1981). This 'self-promoted uptake' model involves the interaction of a polycationic molecule with the negatively charged LPS molecule. The LPS molecules are stabilized in the membrane by Mg^{2+} or Ca^{2+} crossbridging of the negatively charged phosphate residues found on LPS. Polycationic antimicrobials have a higher affinity for LPS, displacing the Mg^{2+} or Ca^{2+}. Due to the bulky size of the cationic peptides, they disrupt the outer membrane and cause a permeability change, promoting their own uptake and the uptake of otherwise excluded molecules into the cell (Hancock et al. 1984, Hancock et al. 1991). It has been clearly demonstrated that *B. cepacia* does not have a 'self-promoted-uptake' pathway (Moore et al. 1986) inhibiting the ability of aminoglycosides or cationic antimicrobial agents to penetrate the cell. This is thought to be due to the low number of negatively charged phosphate residues on *B. cepacia* LPS. One of these phosphates is attached to the lipid A portion of LPS and is linked to an Ara4N sugar (Cox et al. 1991). This Ara4N may form a bridge with the adjacent phosphate on the next LPS molecule such that divalent crossbridging is not required for the outer membrane of *B. cepacia*.

1.7.2 Antibiotic efflux pumps

Multidrug efflux pumps that traverse both the inner and outer membranes of Gram-negative bacteria make a major contribution to intrinsic resistance of these bacteria. These pumps are composed of at least three components, are energized by the proton-motive force and can pump out a wide variety of antibiotics (Nikaido, 1998). An efflux system involving three proteins was identified in *Pseudomonas aeruginosa* (Poole et al.1993). These proteins were designated MexA, MexB and OprM (same as OprK) and are critical for the resistance of *P.*

aeruginosa to quinolones, β-lactams (other than imipenem), tetracycline and chloramphenicol which was demonstrated by studying knock out mutants of all three genes involved. An efflux pump with similarity to this system has been identified in *B. cepacia* that involves an outer membrane protein designated OpcK, similar to OprM, shown to be responsible for intrinsic resistance to tetracycline, chloramphenicol and ciprofoxacin (Burns et al. 1996b).

1.7.3   Antibiotic modifying enzymes

Enzymes that modify and inactivate β-lactam antibiotics have been identified in *B. cepacia*. In 1979, Beckman and Lessie discovered that *B. cepacia* is able to utilize penicillin G as the sole carbon source and extracts of the bacteria show high levels of β-lactamase activity. Resistance to β-lactam antibiotics has been attributed to inducible β-lactamases in many Gram-negative bacteria. Inducible β-lactamases are chromosomally encoded and are normally repressed; organisms can be induced to express high levels of these β-lactamases after exposure to certain β-lactam compounds. An inducible chromosomal β-lactamases has been identified in *B. cepacia* encoded by the *penA* gene (Prince et al. 1988) which is regulated by an upstream open reading frame called *penR* (Proenca et al. 1993, Trepanier et al. 1997) and can be induced by imipenem (Trepanier et al. 1997). Carbapenems such as imipenem and meropenem are currently the most potent and broad spectrum β-lactam antibiotics available and are resistant to most β-lactamases (Livermore et al. 1992) but can be hydrolyzed by *B. cepacia* (Simpson et al. 1993). A zinc-dependent carbapenemase (PCM-1) has been identified and partially purified. PCM-1 is capable of hydrolyzing a wide range of β-lactams including imipenem, meropenem, biapenem, cephaloridine, ceftazidime, penzylpenicillin, ampicillin and carbenicillin (Baxter et al.

1994). The high level of resistance to β-lactam antibiotics in *B. cepacia* is due to a combination of poor entry into the cell and the presence of these inactivating enzymes.

1.8 Cationic peptides

With the rapid emergence of antibiotic resistance and some bacteria having developed resistance to all antibiotics available (Neu, 1992), the development of a new class of antibiotics has become more important. Antimicrobial peptides have attracted increasing interest due to their unique properties. They are important components of non-specific host defense mechanisms, encoded by host genes and found throughout the animal kingdom (Boman, 1995). These antimicrobial peptides are 12-45 amino acids in length and are amphipathic with both hydrophobic and cationic surfaces. They are cationic because of the presence of excess lysine and arginine residues resulting in a net charge of at least +2 at neutral pH. It is the cationic nature of these peptides that accounts for their ability to target the negatively charged membranes of microbes.

In Gram-negative bacteria cationic peptides like polymyxin and aminoglycosides use the 'self-promoted-uptake' pathway to cross the outer membrane and reach their proposed target, the cytoplasmic membrane. These peptides, like polymyxin, interact electrostatically with the negatively charged headgroups of bacterial phospholipids found in the inner membrane, insert into the membrane forming channels that cause leakage of cell contents leading to cell death (Christensen et al., 1988, Lehrer et al. 1989, Wimley et al. 1994). The amphipathic nature of these peptides appears to be important in their interaction with membranes and their formation of channels within these membranes (Saberwal, 1994).

Advantages of cationic peptides for use in antimicrobial therapy are 1. They are able to kill rapidly. 2. They have a broad spectrum of activity (against Gram-positive and Gram-

negative bacteria, fungi and viruses). 3. It is difficult to select for spontaneously resistant mutants *in vitro*. 4. These peptides show activity against most antibiotic resistant pathogens (Hancock et al. 1998). However, many *in vitro* studies have shown that *B. cepacia* is resistant to all cationic peptides.

1.8.1 Cationic peptides of neutrophils

Neutrophils constitute 50-70% of the total white blood cells in humans. They play a vital role in the host immune response by ingesting and killing invading microorganisms. These invading microbes are destroyed by two possible mechanisms called the "oxygen-dependent" and the "oxygen-independent". The microbicidal-cytotoxic proteins of nonoxidative killing are found in neutrophil cytoplasmic granules (primary and secondary) and are discharged into the phagocytic vacuole containing the microbe as shown in Figure 1 on page 7.

Defensins are the most abundant cationic peptide found in neutrophils comprising 10-18% of their total protein (Lehrer et al. 1993). Defensins are classified into three groups, the classical α-defensins, β-defensins and insect/plant defensins (thionins). α-Defensins are found primarily in neutrophils and paneth cells where as the β-defensins are more widely distributed. β-Defensins have been found in the epithelial cells of many human (Zhao et al. 1996) and murine (Huttner et al. 1997) organs, human skin (Harder et al. 1997), bovine neutrophils (Selsted et al. 1993), tongue (Schonwetter et al. 1995) and trachea (Diamond et al. 1991). The plant defensins (now called α and β thionins) are compact, L-shaped molecules containing two long disulphide linked α-helices and two short antiparallel β-sheets (Bohlmann et al. 1991). Different from the insect defensins, the structure of α and β-defensins are dominated by antiparallel β-sheets (no α-helices), stabilized by disulfide cross bridges and contain six cysteine residues. They consist of three regions of β-sheet structure and a protruding β-hairpin hydrophobic loop.

Defensins are active against a wide variety of microbes including Gram-positive and negative bacteria, fungi, spirochetes, mycobacteria and enveloped (not naked) viruses (Lehrer et al. 1993). Defensins permeabilize the outer and inner membrane of *E. coli* sequentially, with cell death occurring upon inner membrane disruption (Lehrer et al. 1989). Defensins are capable of disrupting negatively charged but not neutral membranes (Cociacich et al. 1993) and form multimers within the membrane resulting in voltage-dependant ion channels (Kagan et al. 1990, Pardi et al. 1992, Wimley et al. 1994).

Also found in neutrophils are the high molecular weight cationic proteins that show similar activity to the cationic peptides, probably due to their positive charges. These are bacterial permeability increasing protein (BPI) and the serprocidins: proteinase 3, azurocidin and cathepsin G. In addition, a cleavage product of lactoferrin (also found in neutrophil granules) has bacterial lysis activity. BPI is a 50-kDa cationic protein that contains 456 amino acid residues. BPI is cytotoxic to a wide range of Gram-negative bacteria, however, for *S. typhimurium* and *E. coli,* bacterial susceptibility varies and is determined by the structure of LPS and the length of the O-antigen (Weiss et al. 1986). This is not true for *P. aeruginosa* where all strains, regardless of O-antigen length, are equally susceptible (Siefferman et al. 1991). Binding of BPI to live bacteria is shown to increase the permeability of the outer membrane (Weiss et al. 1992). Longer O-antigen chains impede the access of BPI to the core region of LPS, the main site of the negative charges to which BPI binds (Kastowsky et al. 1992).

Lactoferrin is an 80-kDa iron-binding glycoprotein that is closely related in structure to transferrin. There is strong evidence indicating a direct killing effect of lactoferrin, unrelated to iron binding, which is mediated by binding to the surface of susceptible organisms (Arnold et al. 1982). Gram-negative bacteria exposed to lactoferrin release LPS (Ellison et al. 1988) and this antimicrobial activity is mediated by the acid-pepsin cleavage product lactoferricin, the cationic

N-terminal region of lactoferrin (Bellamy et al. 1992). Lactoferricin is highly basic, has a single disulfide bond and no iron-binding capacity and it is active against bacteria, yeast and fungi (Bellamy et al. 1992b, Bellamy et al. 1993, Yamauch et al. 1993).

These peptides and proteins are the major components of neutrophil nonoxidative killing. *B. cepacia* is resistant to nonoxidative killing components of neutrophils as seen in killing assays using CGD and normal neutrophils (Speert et al. 1994).

1.8.2 α-Helical cationic peptides

The α-helical cationic peptides exist in a random-coil configuration in aqueous solutions (Steiner, 1982) but form a helix-turn-helix structure when interacting with membranes (Demsey et al. 1990). α–Helical peptides are characterized by one amphipathic α-helix and one hydrophobic α-helix. Cecropins were first isolated from the pupae of silkmoth, *Hyalophora cecropia* (Steiner et al. 1981) and similar molecules have since been isolated from many insect orders (Merrifield et al. 1994), pig intestine (Lee et al. 1989) and marine protochordates (Zhoa et al. 1997). Cecropins cause instant lysis of bacterial cells by destruction of the cytoplasmic membrane (Boman et al. 1993) and only lyse negative or neutral charged membranes forming ion channels. It is the cationic N-terminus that aids the binding of cecropins to the bacterial membrane (Wade et al. 1990, Christensen et al. 1988). Melittin, isolated from bee venom toxin, has a similar helix-turn-helix structure, however, opposite to cecropins, it has a hydrophobic C-terminus and a cationic N-terminus (Piers et al. 1994). Magainins were isolated from the skin of the African frog, *Xenopus laevis* (Zasloff et al. 1987) and form pores in the cell membranes of susceptible microorganisms but not eukaryotic membranes (Bechinger et al. 1997, Ludke et al. 1996). Bactenecins are produced in bovine neutrophils and exist in an extended loop form. These peptides bind to the outer membrane of Gram-negative bacteria and increase outer

membrane permeability followed by an increase in permeability of the inner membrane and inhibition of the electron transport chain (Skerlavaj et al. 1990).

1.8.3 Mechanism of action

The overall mechanism of killing Gram-negative bacteria for cationic peptides and polymyxin has been proposed to be disruption of the cytoplasmic membrane of bacteria (Hancock et al. 1998). The mechanism of action has been proposed to involve three steps. First binding of the peptide to the cell surface, probably through displacement of the Mg^{2+} or Ca^{2+} cations cross-linking the negative charges on LPS. Second permeabilization of the outer membrane occurs via the 'self-promoted-uptake' pathway described earlier in section 1.7.1. This is followed by permeabilization of the cytoplasmic membrane resulting in loss of viability of the cell by lysis and possible DNA damage (Lichtenstein et al. 1988). In model membrane systems, cationic peptides aggregate to form multimers that result in channels across the inner membrane with the hydrophobic face of the peptides positioned outward interacting with the membrane and the hydrophilic faces oriented towards the inside of the formed channels. This results in leakage of protons, loss of the proton motive force, loss of membrane potential and leakage of other compounds causing cell death (Lehrer et al. 1989). Selectivity of cationic peptides for bacterial membranes over eukaryotic cell membranes is due to their compositional and charge differences. Bacterial membranes are composed of mostly negatively charged lipids such as LPS, phosphatidyl glycerol and cardiolipin. Eukaryotic cell membranes are composed of zwitterionic lipids such as phosphatidyl choline and sphingomyelin (a glycolipid for which ceramide is the lipid precursor). As well, cholesterol, abundant in eukaryotic membranes, is thought to inhibit insertion of cationic peptides into the membrane. Bacterial cells have a large negative transmembrane potential of approximately –140 mV whereas the membrane potential of

eukaryotic cells is approximately to –20 mV. This large membrane potential is thought to aid in the insertion of these peptides into the membrane of bacterial but not eukaryotic cells (Hancock, 1997). Spontaneous resistance to cationic peptides has not been observed and this is probably due to the fact that an alteration in membrane structure to prevent insertion and channel formation is difficult to achieve.

1.8.4 Bacterial resistance to cationic peptides and polymyxin

Polymyxin B, produced by *Bacillus polymyxa* is considered a cationic antibiotic with a positively charged ring structure and a hydrophobic lipid tail. Like cationic peptides, polymyxin binds to the outer membrane (Hancock et al. 1981), induces sloughing of the outer membrane (Peterson et al. 1985) and increases the permeability of the outer membrane to proteins and hydrophobic compounds (Vaara et al. 1981, 1983). In addition to this, polymyxin binds to and disrupts the packing arrangement of isolated LPS (Peterson et al. 1985). Resistance of Gram-negative bacteria to polymyxin has been achieved through changes in the LPS structure. Resistant mutants of *S. typhimurium* have increased levels of arabinosamine (and ethanolamine) esterified to the lipid A phosphate residues (see Figure 2 on page 12). Esterification of a phosphate residue reduces the negative charge of that phosphate, reducing the overall negative charge of the LPS molecule. Such mutants and their isolated LPS bind less polymyxin B than the wild-type does (Vaara et al. 1981). Similar observations were seen for several strains of *E. coli* and *S. typhimurium* (Peterson et al. 1987). One of the major control points of *Salmonella* sp. pathogenesis is the PhoP-PhoQ two-component regulatory system (Miller et al. 1989) that induces the expression of resistance to cationic peptides and polymyxin. The ability of *S. typhimurium* to survive within macrophages is due in part to activation of PhoP-PhoQ resulting in expression of resistance to antimicrobial peptides and this expression of resistance to

antimicrobial peptides correlates with virulence in mice (Miller et al. 1989). This supports the hypothesis that resistance to antimicrobial cationic peptides is potentially a virulence mechanism as was suggested by Speert et al, 1994 where *B. cepacia* was shown to be resistant to nonoxidative killing of CGD neutrophils. Other *S. typhimurium* mutants that are resistant to polymyxin map to a locus that has been designated *pmr*A (polymyxin resistance) (Makela et al. 1978). DNA sequence analysis of this locus revealed an operon including *orf1, pmrA,* and *pmrB* (Roland et al. 1993) and PmrA-PmrB is a two component regulatory system controlling resistance to antimicrobial peptides that is regulated by the PhoP-PhoQ system (Gunn et al. 1996). Mutants of *pmr*A are resistant to polymyxin and show an Ara4N ester linked to a phosphate of lipid A (Helander et al. 1994). In addition to these studies, an operon, *pmr*F has been shown to be under the control of PmrA-PmrB and is directly involved in the substitution of the 4' phosphate on the glucosamine of lipid A shown in Figure 2 on page 12 (Helander et al. 1994, Gunn et al, 1998). There is also evidence to support that increased acylation of lipid A in *S. typhimurium* results in resistance to cationic antimicrobial agents (Guo et al. 1998).

Most of the other genetic studies to date on resistance to cationic peptides and polymyxin have mapped to the LPS biosynthesis operon as seen for *Ralstonia solanacearum* (Titarenkoe et al. 1997), *Brucella bronchisetica* (Baremanns et al. 1998) and *S. typhimurium* (Macias et al. 1990). Organisms that are inherently resistant to polymyxin have been shown to carry LPS, which has the 4'-phosphate on lipid A completely substituted with 4AraN such as *Proteus mirabilis* (Sidorczyk et al. 1983) and *Chromobacterium violaceum* (Hase et al. 1977).

A two component regulatory system as seen in *Salmonella* has not been identified in *B. cepacia*. Resistance of *B. cepacia* is most likely due in part to the inability of polymyxin to bind to the LPS and the absence of the 'self-promoted-uptake' pathway. The constitutive presence of 4AraN ester linked to a phosphate of lipid A in the LPS of *B. cepacia*, by analogy to the *pmr*A

mutants of *S. typhimurium*, probably plays a role in the resistance of *B. cepacia* to cationic peptides. This 4AraN linked to *B. cepacia* LPS would reduce its ability to bind cationic peptides or polymyxin. The actual target for bacterial killing by polymyxin and cationic peptides is the cytoplasmic membrane where interaction causes cell lysis. One study by Manniello et al. 1978 showed that spheroplasts of *B. cepacia* are resistant to lysis by polymyxin suggesting that resistance to polymyxin B may be related to a resistant cytoplasmic membrane. Although cationic peptides and antibiotics appear to be unable to kill *B. cepacia*, several studies have shown that cationic peptides and polymyxin are capable of reducing the MICs for other antibiotics to *B. cepacia* (Rajyaguru et al. 1997, 1998).

1.9 Thesis Objective

Burkholderia cepacia is an important opportunistic pathogen that infects immunocompromised individuals and causes fatal infections for both CF and CGD patients. Virulence factors have been studied in some detail in relation to its pathogenicity in CF; however, a strong correlation between the studied virulence factors and *B. cepacia* pathogenicity in CF does not exist. *B. cepacia* is extremely resistant to a large number of antibiotics, probably due to its impermeable outer membrane. Of particular interest is the resistance of *B. cepacia* to killing by nonoxidative mechanisms of neutrophils. The main components of nonoxidative killing are the cationic peptides and proteins to which *B. cepacia* is resistant. For *Salmonella* a strong correlation exists between resistance to defensins and virulence in a mouse model (Fields et al. 1989). By understanding why *B. cepacia* is so resistant to cationic peptides one might gain insight into a possible virulence mechanism for this organism. The aim of this study was to identify a transposon insertion mutant of *B. cepacia* that had reduced susceptibility to cationic peptides. The strain used in this study is a genomovar II strain, *B. multivorans* ATCC 17616, and the

phenotype of cationic peptide resistance is shared by all members of the *B. cepacia* complex. Characterization of the transposon insertion mutant was carried out as follows:

1. DNA sequence analysis of the region of DNA surrounding the site of transposon insertion to identify genes that may be involved in *B. multivorans* cationic peptide resistance.

2. Comparison of outer membrane protein profiles, LPS ladders, phospholipids by TLC, membrane permeability and LPS binding of polymyxin were compared for the parent and mutant strains.

Both the genetic and phenotypic characterization of this mutant in this study has lead to the identification of a novel mechanism of resistance that *B. cepacia* complex organisms may require in order to resist killing by cationic peptides.

Chapter 2   **Materials and Methods**

2.1 Strains, Plasmids and Growth Conditions

The bacterial strains and plasmids used in this study are listed in Table 1. For routine bacterial growth, Luria Britani (LB) broth was used. Mueller-Hinton (MH) broth (Difco Laboratories, Detroit, Mich, see appendix A for recipes) was used for minimal inhibitory concentration (MIC) determinations. All cultures were incubated at 37°C overnight with aeration unless otherwise stated. Plasmids were maintained by growth in the presence of the appropriate antibiotic. Concentrations of antibiotics were: tetracycline and kanamycin sulfate (Boerhinger Mannheim, GmbH) at 20 μg/ml, ampicillin (Novopharm) at 100 μg/ml for *E. coli,* tetracycline at 100 μg/ml for *B. multivorans,* trimethoprim (ICN Biomedical Inc.) at 100 μg/ml for both *E. coli* and *B. multivorans.* All strains were stored at −70°C in MH broth with 8.0% dimethylsulfoxide (DMSO) (v/v). *B. multivorans* cultures were inoculated directly from the freezer stock when used.

2.2 Construction of a Tn*5-751S* Insertion Mutant Library of B. *multivorans.*

pTGL166 (carrying transposon Tn*5-751S*) was transferred from *E. coli* DH5α to *B. multivorans* ATCC 17616 by conjugation. Equal amounts of donor and recipient bacteria were plated on LB agar for four hours at 30°C. Resistant *B. multivorans* transconjugants were selected at 30°C on LB agar containing tetracycline and trimethoprim and 31 μg/ml polymyxin B sulfate (ICN Biochemicals Inc., 7640 Units/mg). This low concentration of polymyxin B acted to counterselect the *E. coli* donor strain. The selected pTGLI66 containing derivatives of strain 17616 were propagated for 60 hours at 47°C in LB broth so that pTGL166 was lost. Appropriate dilutions were then plated on supplemented minimal salts agar (see appendix A

TABLE 1. Strains and plasmids. All the strains and plasmids pertinent to this study and the relevant references are listed:

Strain or plasmid	Pertinent characteristics	Source
Strains		
B. multivorans		
ATCC 17616	B. cepacia genomovar II type strain	ATCC
26D7	Derivative of ATCC 17616 with Tn*5-751S* inserted in its chromosome. Reduced resistance to polymyxin.	This study
JTC	CGD isolate	Speert et al. 1994
P. aeruginosa		
M2	isolated from mouse intestine	Stieritz et al, 1975
E. coli		
DH5α	F-, φ80dlacZΔM15 Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(r_K^-,m_K^+) phoA supE44 λ⁻ thi-1 gyrA96 relA1.	Gibco BRL
XL1-Blue MR	Cosmid library host strain	Stratagene
HB1005	cationic peptide sensitive strain	Hancock
Plasmids		
pBluescript II SK+	high copy cloning vector	Stratagene
SuperCos 1	high copy cosmid cloning vector	Stratagene
pDN18	Broad-host-range low copy Tet^R cloning vector	Nunn et al, 1990.
pRK2013	Kan^R transfer plasmid	Figurski, 1979
pTGL166	53 kb broad-host-range *Inc* P1 plasmid; temperature sensitive with respect to its replication due to a mutation in *trf* A; contains Tn*5-751S*, which confers resistance to both kanamycin and trimethoprim, as well as the nontransposable penicillin and tetracycline resistance markers. The *Spe*I site within Tn*5-751S* was replaced by a *Swa*I site.	Cheng et al, 1994.
pBS1	pBluescript II KS(+) derivative carrying the Kan^R gene from Tn*5-751* and flanking DNA from mutant 26D7.	This study
5G6, 5H11, 2F6, 2F9, 1G3	*E. coli* XL-1Blue MR carrying SuperCos 1 derivatives with 35 kb *Sau*3AI partially digested ATCC 17616 chromosomal DNA corresponding to the region of DNA adjacent to the transposon insertion in mutant 26D7	This study
pBS15	pBluescript II KS (+) carrying the 3.8 kb *Sal*I chromosomal fragment from ATCC 17616.	This study
pBS16	pBluescript II KS (+) carrying the 3.8 kb *Sal*I chromosomal fragment in opposite orientation to pBS15.	This study
pBS4	pBluescript II KS (+) carrying the 4.0 kb *Bam*HI chromosomal fragment from ATCC 17616.	This study
pBSP1	pBluescript II KS (+) carrying the 7.0 kb *Pst*I chromosomal fragment from ATCC 17616.	This study
pDBS6	pDN18 carrying the 4.0 kb *Bam*HI fragment from pBS4.	This study
pDBS9	pDN18 carrying the 4.0 kb *Bam*HI fragment from pBS4 in the opposite orientation to pDBS6.	This study
pDBS1	pDN18 carrying *Kpn*I-*Pst*I subclone from pBS15.	This study
pDBS2	pDN18 carrying *Kpn*I-*Pst*I subclone from pBS16.	This study

ATCC- American Type Culture Collection (Rockville, Md.)

for recipe) containing trimethoprim. Trimethoprim resistant clones (carrying Tn*5-751S*) were screened for sensitivity to tetracycline to ensure they had lost pTGLI66. Chromosomal DNA from randomly selected mutants was digested completely with each of *Sal*I, *Bam*HI or *Eco*RI. A polymerase chain reaction (PCR) product was made using the primers N725-5 and N727-3 (see table of primers, appendix B). These primers are specific for the kanamycin cassette found in transposon Tn*5-751S* and the resulting PCR product was used for Southern hybridization (probe 1) to identify digested chromosomal fragments carrying the transposon.

2.3 Polymerase Chain Reaction (PCR)

A single PCR cycle consisted of denaturation at 94°C for one min, annealing at 60°C for 1 min and extension at 72°C for two min. This was repeated for 30 cycles. PCR was performed in a Perkin Elmer DNA Thermal Cycler using one unit of Taq DNA polymerase, 20 ng of template DNA, 250 μM deoxynucleoside triphosphates, 15 pmoles primers and 3 mM $MgCl_2$ in a total volume of 20 μl per reaction. For making radiolabeled probes, the PCR product was gel purified using Prep-A-Gene® DNA purification Kit (Bio-Rad). After a second round of PCR and gel purification, the PCR product was radiolabeled as described in section 2.4. RAPD-PCR was done as per Mahenthiralingam et al. 1996.

2.4 Southern Analysis

DNA fragments were resolved on a 1.0% agarose gel and transferred to a positively charged nylon membrane (Nytran®Plus, Boehringer Mannheim) by alkali transfer. The gel was soaked in the alkaline buffer (0.5 N NaOH, 1.5 M NaCl) for 30 minutes and then placed on a sheet of saran wrap. A piece of nylon membrane equal in dimension to the gel was placed carefully on top of the gel. A piece of Whatman filter paper, followed by a stack of paper towels

(all cut to the size of the gel), a glass plate and finally a weight were placed on top of the gel so that the DNA was pulled by capillary action upward onto the nylon membrane. The membrane was then hybridized with radiolabeled DNA at 55°C, overnight in hybridization buffer (0.6X SSC (3 M NaCl, 0.3 M sodium citrate), 5X Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), 0.02 mg/ml salmon sperm DNA). Probes were labeled with [α-^{32}P]-dGTP by random priming for 30 minutes at 37°C using Klenow fragment of DNA polymerase I of *E. coli* (Gibco BRL). The reaction was set up in 20µl total with 20µCi of [α-^{32}P]-dGTP, 1 µl of 5mM dATP, dTTP, dCTP and 1 Unit of Klenow. Unincorporated nucleotides were removed by using an S-400HR spin column (Pharmacia, Biotech.). After hybridization, the membranes were washed twice under low stringency (2x SSC, 0. 1% SDS) once at 55°C, secondly at 23°C, for 15 minutes each and twice under high stringency 0.2x SSC, 0.1% SDS for 5 minutes at 23°C. The blots were exposed to X-ray film (X-OMAT AR film, Eastman Kodak Co., Rochester, N.Y.) at –70°C with intensifying screens.

2.5 Screening for a Mutant Susceptible to Polymyxin

Putative mutants from the random insertion library (section 2.2) were picked. 6,000 were gridded into 96 well ELISA plates containing 200 µl of LB broth and trimethoprim. These were incubated at 37°C for 16 hours followed by replica plating on each of: supplemented minimal salts media without antibiotic or plates containing either 600 or 1200 Units of polymyxin. Clones that did not grow on the two concentrations of polymyxin were selected off the master plate as potential polymyxin sensitive mutants.

2.6 Determination of Minimal Inhibitory Concentrations (MICs)

The MICs for all antibiotics and antimicrobial peptides were done by the standard microtitre broth dilution method as described in The National Committee for Clinical Laboratory

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Standards Manual. These were conducted in polypropylene ELISA plates (Falcon) using MH broth without cation supplementation. The first well contained a final concentration of antibiotic at 528µg/ml followed by serial dilutions (by ½) in each adjacent well down to 0.5 µg/ml. The last well contained no antibiotic. Bacteria were added to each well at a concentration of 10^5 bacteria per ml. Microtitre plates were read after 24 and 48 hours of incubation at 37°C and the lowest concentration of antibiotic where no growth was observed (by eye) was designated the MIC for that antibiotic.

2.7 Isolation of Cosmid Clones

2.7.1 Construction of a Cosmid Library of *B. multivorans* Genomic DNA

A 17616 genomic DNA library was constructed in SuperCos I cosmid according to manufacturer instructions (Stratagene, La Jolla, CA). To obtain chromosomal DNA from *B. multivorans* ATCC 17616, 2.0 ml of overnight culture was harvested by centrifugation at 7,000xg for 10 minutes. Pellets were resuspended in 200 µl GET buffer (5 mM glucose, 10 mM EDTA, 25 mM Tris, pH 8.0) followed by the addition of 2.8 ml of lysis buffer [1.0% SDS, 50 mM Tris-Cl pH 8.0, 50 mM EDTA pH 8.0, 60 µg/ml RNAse (Pharmacia) and 30 µg/ml Proteinase K (Boehringer Mannheim)]. This was tumbled in a 15.0 ml screw capped tube at 37°C for one hour. After the addition of 1.0 ml of saturated ammonium acetate, the lysate was shaken vigorously and left at room temperature for one hour to precipitate proteins and polysaccharides. The debris was removed by centrifugation. The DNA was ethanol precipitated and chloroform extracted. This DNA was partially digested with the restriction enzyme *Sau*3AI to give fragments in the size range of 30 to 40 kb. Fragments of this size were obtained by digesting 10µg of chromosomal DNA with 0.015 Units of *Sau*3A for 5 minutes (see appendix C for details). These were ligated to *Bam*Hl restricted SuperCos 1 with a ratio of 3.28 insert to 1

vector at 16°C for 4 hours. The ligated DNA was packaged using the Gigapack®II-XL packaging extract according to the manufacturer instructions and transfected into the host strain *E. coli* XLI-Blue MR (Stratagene, La Jolla, CA). The host strain was grown in LB broth supplemented with 0.2% (w/v) maltose and 10mM $MgCl_2$. Random ampicillin resistant transfectants were selected and restriction digested with *Bam* HI to ensure the cosmid library contained cosmid clones with random inserts (see Appendix D). Clones were then picked and gridded into 96 well ELISA plates.

2.7.2 Construction of a Probe Specific to Chromosomal DNA at the Transposon Insertion Site

Chromosomal DNA was isolated from mutant 26D7 and digested with the restriction enzyme *Sal*I with the intention of leaving the kanamycin resistance cassette intact in the transposon. The entire *Sal*I 26D7 chromosomal digest was ligated to *Sal*I restricted pBluescript II SK+. *E. coli* DH5-α was electroplated using a gene pulser (BioRAD) at 1900 V, 25 μF capacitance and 200 ohm resistance, revived for one hour in SOB (appendix A) and plated onto LB agar containing kanamycin. Kanamycin resistant clones were isolated. Restriction mapping and Southern analysis (using probe 1, described in section 2.2) confirmed that a clone (pBS1) contained 2.3 kb of transposon DNA as well as 1.45 kb of flanking chromosomal DNA. DNA sequence from the flanking chromosomal fragment was obtained using primer T3 and tn5-1. This chromosomal sequence was used to design the primers SeqlR and SeqlL (probe 2). For primer sequences see appendix B. These primers gave a 1.2 kb PCR product specific for the chromosomal DNA adjacent to the transposon insertion site.

2.7.3 Screening of the *B. multivorans* 17616 Genomic Library

Briefly, colonies were grown overnight at 37°C on nylon membrane (Nytran® Plus) placed on top of LB agar containing ampicillin. The membrane was washed by placing on Whatman paper soaked in 0. 5 N NaOH, 1. 5 M NaCl, 0. 1% SDS for 15 minutes followed by neutralization on 1.0 M Tris-HCl, pH 7.5, 1.5 M NaCl for 5 minutes and 2x SSC for 15 minutes. Cellular debris was removed by gently rubbing the surface of the membrane immersed in 2x SSC by hand using a clean glove. The blots were processed as described in section 2.4 using radiolabeled probe 2.

2.8 Cloning of the *Bam*HI, *Sal* I and *Pst*I Fragments

The five cosmids isolated from the 17616 chromosomal library were digested with several different restriction enzymes. Southern analysis (using probe 2) was done to identify enzyme fragments from the cosmid clones carrying the DNA of interest. A 4.0 kb *Bam* HI, a 3.8 kb *Sal* I and a 9.0 kb *Pst*I fragment were identified and gel purified using Prep-A-Genes DNA purification Kit (Bio-Rad). These fragments were subcloned in pBluescript II KS+ and subsequently pDN18 (see Table 1).

2.9 Complementation of Mutant 26D7

pDBS1, pDBS2, pDBS6, pDBS9 and pDN18 were transferred into mutant 26D7 by triparental matings using pRK2013 as the helper plasmid. MICs were performed (described in section 2.6) in the absence of vector selection. To ensure the vector was not lost during the procedure after the MIC was determined, the bacteria were plated in the presence and absence of tetracycline.

2.10 Nucleotide Sequencing and Analysis

pBS15 plasmid DNA was purified by polyethyleneglycol (PEG) precipitation as per the PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit Protocol. Sequencing was performed by automated PCR sequencing using an Applied Biosystems 377 Automated DNA Sequencer and Ampli Taq Dye Deoxy ™ Terminator Cycle Sequencing. All reactions were done in the presence of 5.0% DMSO. Complete sequence of the complementing DNA was obtained by chromosome walking using custom primers (see appendix C). Primers were synthesized on a Perkin-Elmer-ABI 394-8 synthesizer. Sequences were assembled using Lasergene for Windows, DNASTAR Inc., Madison, WI. DNA sequencing and primer synthesis was performed with the assistance of the Nucleic Acid - Protein Service Unit at the University of British Columbia. Open reading frames were identified using GeneMark (http://genemark.biology.gatech.edu/GeneMark). Sequence similarity searches were done using the National Center for Biotechnology Information Basic Local Alignment Search Tool (NCBI-BLAST, Altschul et al. 1990, http://www.ncbi.nlm.nih.gov/blast). Sequence alignments were done using Multiple Sequence Alignments accessed through the Bayor College of Medicine Search Launcher web site using CLUSTALW (http://www.hgsc.bcm.tmc.edu/SearchLauncher/). The FASTA format from these alignments was entered into BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html). Protein predictions were determined using PSORT (http://www.psort.nibb.ac.jb:8800/) and EMBL "protein predict" (http://www.embl-heidelberg.de/services/index.html#5) programs. Protein pIs and molecular weights were predicted using pI/MW tools at http://www.expasy.ch/tools/pi_tool.html. For the phylogenetic analysis the FASTA format of the sequence alignment from CLUSTALW were entered into the PHYLIP package (Felsentein, 1993).

2.11 Biochemical Analysis

The API Rapid NFT system (PML Microbiologicals, Richmond, BC, CANADA) was used followed by glucose, maltose, lactose, mannitol, xylose and sucrose oxidation-fermentation (OF) reactions, and Moeller lysine decarboxylase test (Difco). All incubations for these tests were performed at 32°C in ambient air. OF sugars were incubated for 3 days. Key positive biochemical reactions for *B. multivorans* complex are: (i) oxidation of glucose and xylose and at least one positive reaction among oxidation of maltose, lactose, sucrose and/or lysine decarboxylase; (ii) weakly positive oxidase reaction, defined as a faint purple color occurring between 20 and 60 seconds with the Pathotec cytochrome oxidase strip (Remel, Lenexa, Kans) (a strong oxidase reaction was considered a deep purple color developing within 10 seconds; (iii) *p*-nitrophenyl-β-D-galactopyranoside (PNPG) positively and no nitrogen gas production from the reduction of nitrate (both read from the API Rapid NFT strip).

2.12 Gram Stains

Bacteria were heat fixed to glass slides and stained for one minute with crystal violet, one minute with Gram's iodine, 15-30 seconds with acetone-ethyl alcohol (1:1), followed by counterstain of 10 seconds with Safranin. Slides were rinsed with tap water between each step.

2.13 Electron Microscopy

Negative staining for electron microscopy was done by Dr. Terry Beveridge in the Department of Microbiology at the University of Guelph, as described in Martin et al., 1986.

2.14 Neutrophil Killing Assay

Previously described by Speert et al. 1994. R5 serum, complement component 5 deficient serum that has been adsorbed to sheep red blood cells, was used because *B. multivorans* is susceptible to complement-mediated lysis. Adsorption to sheep red blood cells was done previously for an unrelated experiment in the lab and had no effect on this procedure. Neutrophils were isolated from human blood by first diluting heparinized blood 1:1 with 0.9% saline followed by sedimentation in 6.0% Dextran 70 in 0.9% saline (Macrodex-Saline Pharmacia) 3:1 Dextran to blood by volume. Neutrophils were sedimented out of the resulting plasma by Ficoll-Hypaque density gradient sedimentation at 750xg for 30 minutes. The pellet was washed in Hank's Balanced Salt Solution with 0.1% gelatin (gHBSS) at 160xg for 10 minutes. The remaining red blood cells in the pellet were lysed by resuspending the washed pellet in 0.87% ammonium chloride and incubated at 37°C (rotating) for 10 minutes. The cells were then washed twice in gHBSS and counted on a hemocytometer and resuspended in gHBSS at $5x10^6$ cells/ml. Bacterial cultures were adjusted to an OD_{620} of 0.600 (or an optical density corresponding to 10^9 bacteria/ml). The bacterial cells are diluted 1/27 in gHBSS to a concentration of $2.5x10^7$ bacteria/ml. Neutrophils and bacteria were added (1:1) to a total volume of 500 µl gHBSS with 10% R5 serum in 12x75 mm polypropylene snap cap tubes and tumbled for 120 minutes at 37°C. Samples of 50 µl were removed at 0, 30 and 120 minutes, neutrophils lysed for 10 minutes in 0.1% gelatin HBSS and viable bacterial counts were made. For controls bacteria in the absence of neutrophils and a sterile control of neutrophils only were plated out.

2.15 Permeabilization of whole cells of *B. multivorans* to 1-*N*-Phenylnaphthylamine

1-*N*-Phenylnaphthylamine (NPN) assays were performed as described (Moore, 1986, Loh, 1984, Hancock, 1984). NPN was dissolved in acetone at a concentration of 500 μM. Cells were grown to log phase (OD_{600} of 0.600) and were resuspended to OD_{600} of 0.600 in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.2) containing 5 μM CCCP. CCCP stops any energy dependent efflux of NPN out of the cells so that any increase in fluorescence observed (only seen in the mutant) remains at a maximum. 10 μM NPN was added to 1.0 ml of CCCP treated *B. multivorans* cells plus polymyxin in a quartz cuvette. Enhancement in permeability of cells to NPN in the presence of increasing concentrations of polymyxin was measured as increased fluorescence with a Perkin-Elmer fluorescence spectrophotometer attached to a Perkin-Elmer Coleman 165 strip-chart recorder. Excitation and emission wavelengths for NPN were set at 350 and 420 nm, respectively, with slit widths of 5 nm.

2.16 Dansyl-polymyxin binding assay

A fluorescent derivative of polymyxin was prepared by coupling 1-dimethylaminonaphthalene-5-sulfonyl chloride (dansyl chloride) to polymyxin sulfate to give dansyl-polymyxin as described by Schindler and Teuber 1975. Binding of dansyl-polymyxin to whole cells of *Burkholderia multivorans* was monitored by measuring fluorescence intensity with a Perkin-Elmer 650-10S fluorescent spectrophotometer set with an excitation, wave-length of 340 nm and an emission wavelength of 485 nm as described (Moore et al, 1984). Cells were grown to mid log phase (OD_{600} 0.5), harvested by centrifugation and resuspended in 5 mM HEPES pH 7.2 of 5 μM CCCP. Background fluorescence was measured by adding 500 pmoles

of dansy-polymyxin to 1.0 ml of buffer 5-10 times in the same cuvette and the associated

increase in fluorescence was monitored. This was then done with whole *B. multivorans* cells.

2.17 Isolation of Membrane Proteins

2.0 liters of bacterial culture were grown into late log phase to OD_{600} of 0.800. Cells

were harvested by centrifugation at 5,930xg for 10 minutes at 4°C. Pellets were resuspended in

0.01 volumes of 20% (w/v) sucrose in 10 mM Tris pH 8.0, 50 µg/ml DNase I (Boerhinger

Mannheim). The cells were frozen to enhance breakage and passed twice at 15,000psi through a

French Pressure cell. Debris was removed by centrifugation at 2.000xg for 10 minutes at 4°C. A

two step sucrose gradient was done to separate inner and outer membranes using 14 ml 70%

sucrose, 14 ml 50% sucrose and 12 ml of sample in 20% sucrose. Centrifugation was done at

4°C overnight at 23,000 rpms (89,000xg) in a SW 27/8 rotor. Inner (between the 20% and 50%

sucrose steps) and outer (between the 50% and 70% sucrose steps) membrane bands were

isolated. At least 2 volumes of distilled water was added to the collected samples to dilute out

the sucrose to below 20% (sucrose interferes with the protein assay). These samples were

centrifuged at 47,000 rpm (228,000xg) in 60Ti tubes for one hour. Using a syringe the pellets

were resuspended in 1.0 ml of distilled water. The BioRad protein (Bradford) assay was

performed according to manufacturer's instructions, to determine the protein concentration in

each sample. Approximately 10 µg of each sample was analyzed by SDS-PAGE (section 2.18).

Gels were stained with Coomassie brilliant blue in 25% (v/v) isopropanol-7%(v/v) acetic acid.

2.18 Whole Cell Lysates and Proteinase K Digestion

This method was originally described in Hitchcock et al., 1983. Bacteria were grown

overnight on solid LB media containing the appropriate antibiotic. Cells were harvested with a

sterile swab and suspended in 10.0 ml of cold Hank's Balanced Salt Solution (HBSS) pH 7.2 to a turbidity of OD_{600} 0.200. 1.5 ml of this was microfuged for 1.5 minutes. The pellet was resuspended in 50 µl of lysine buffer (2% SDS, 4% 2-mercaptoethanol, 10% glycerol, 1 M Tris (pH 6.8) and bromophenol blue. These were heated at $100^{\circ}C$ for 10 min. To digest protein, 25 µg of proteinase K (Boehringer Mannheim GmBH) solubilized in 10 µl of lysing buffer was added to the boiled lysates and incubated at $60^{\circ}C$ for 60 minutes. 1µl of each sample was analyzed by SDS-PAGE (section 2.19) and silver stained (section 2.20).

2.19 SDS-PAGE

Preparations of both the whole cell lysates and the membrane proteins were analyzed by SDS-PAGE (Laemmli, 1970). A 4.5% stacking with a 12.5% separating gel containing SDS was used for both protein and LPS ladder separation. For a 12.5% gel the protein standards included: Myosin 200-kDa, β-galactosidase 116-kDa, BSA 97.4-kDa, ovalbumin 66-kDa, carbonic anhydrase 45-kDa, soybean trypsin inhibitor 31-kDa, lysozyme 21.5-kDa, aprotinin 6.5-kDa (Bio-RAD).

2.20 Silver Staining of Lipopolysaccharide

This method was originally described in Hitchcock et al., 1983 to stain LPS ladders from whole cell lysates (section 2.18). All glassware and electrophoresis plates were cleaned with detergent, warm water and rinsed thoroughly with tap water followed by milliQ water (15 ohms) Polyacrylamide minigels were fixed overnight in 200 ml 25% isopropanol in 7% acetic acid. Oxidization was performed for 5 minutes in 150 ml milliQ water, 1.05 g periodic acid, 4.0 ml isopropanol, and 7% acetic acid. The gels were then washed 8 times in milliQ water for 30 minutes each time. Silver staining was done for 10 minutes using 1.0 ml concentrated

ammonium hydroxide, 28 ml 0.1 N NaOH, 115 ml milliQ water and 5.0 ml 20% silver nitrate. When making the silver stain the ammonium hydroxide was fresh and the silver nitrate was added slowly drop by drop so that the solution cleared with each drop. If a brown precipitate started to form less silver nitrate was added (e.g. 3.5 mls was often sufficient). The gels were then washed four times in milliQ water for 10 minutes each. Development of the gels was done in a water bath at 25°C to reduce staining of proteins. This was done for 10-20 minutes in 50 mg citric acid, 0.5 ml 37.0% formaldehyde (fresh), milliQ water added to get 1.0 Litres. The reaction was stopped just before the bands were the correct intensity because darkening still occurred in the Stop bath. The Stop bath was added quickly and contained 1.0 ml 7.0% acetic acid and 200 ml milliQ water and allowed to sit for one hour. The gel was then stored in milliQ water. The same standards were used as for the membrane protein gels.

2.21 Extraction of Lipids

This method was originally described by Kawai et al, 1978. 10.0 Liters of bacteria were grown to log phase (OD_{600} 0.600). Cells were harvested by centrifugation at 7,000xg for 10 minutes and resuspended in 0.9% sodium chloride solution. Cells were washed three times in 0.9% sodium chloride. Pellets were frozen overnight at −70°C and freeze dried. One gram of cells was homogenized by stirring for 30 minutes with 20 ml of 0.9% sodium chloride, 50 ml of methanol and 25 ml chloroform (TLC grade chemicals were used). After another 25 mls of chloroform was added the solution was stirred overnight. 25 mls of sodium chloride was then added and stirred 10 minutes. The suspension was centrifuged at 7,000xg for 10 minutes to separate out the chloroform layer (bottom phase). The chloroform layer was removed and evaporated under nitrogen for one hour. The lipid extract was resuspended in chloroform-

methanol (2:1 v/v) and then passed through a glass filter to remove insoluble materials. This filtrate was evaporated, weighed and resuspended in 2:1 chloroform-methanol.

2.22 Thin-Layer Chromatography (TLC)

Two dimensional thin-layer chromatography of total extractable cellular lipids was performed on an aluminum backed TLC plate (Merck 5554) with a two solvent system. In the vertical direction, chloroform/ methanol/water (65:25:4 by volume) and in the horizontal direction chloroform/methanol/acetic acid (65:25:10 by volume). The developed plates were allowed to air dry for 30 minutes. For visualization of all lipids, plates were sprayed with molybdophosphoric acid (5.0% in 95% ethanol). Control lipid cardiolipin, phosphatidyl ethanolamine and phosphatidyl glycerol were purchased from Sigma Chemical Co., St. Louis, Mo.

Chapter 3 Isolation and Partial Characterization of a Tn*5-751S* Insertion Mutant of *B. multivorans* with Reduced Resistance to Polymyxin B.

3.1 Objective

To identify a mutant of *B. multivorans* rendered sensitive to killing by polymyxin B and cationic peptides, a random transposon insertion library of the *B. multivorans* strain ATCC 17616 was constructed using the temperature sensitive plasmid carrying Tn*5-751S*. This library was then screened for mutants susceptible to polymyxin B. Mutant 26D7 was isolated.

3.2 Transposition of Tn*5-751S* in *B. multivorans*

While constructing the random transposon insertion library, it was determined that many strains of the *B. cepacia* complex were poor recipients for plasmid DNA. Therefore, when suicide plasmids were unsuccessful in delivering transposons to these strains, it was difficult to know whether the conjugal transfer or the transposition had failed. The use of a temperature sensitive plasmid allowed for separation of these two steps. A second important consideration with *B. multivorans* was its natural resistance to most antibiotics and substances (including mercury) used for selection of genetic cassettes. *B. multivorans* ATCC 17616 showed some susceptibility to trimethoprim and tetracycline, and the temperature sensitive plasmid pTGL166 shown in Figure 3 has shown previous success in transposition of Tn*5-751S* in *B. multivorans* (Cheng et al. 1994) and this system was thus employed here. This plasmid carries a tetracycline resistance cassette and the transposon is Tn*5* with the trimethoprim cassette inserted into the *Bam* HI site on the transposon. Conjugal transfer of the plasmid from *E. coli* DH5α to *B. multivorans* ATCC 17616 was achieved on solid media at low temperature (30°C) with a donor to recipient ratio of 1:1. Recipient *B. multivorans* cells, immediately following conjugal transfer, were placed in LB broth at 47°C for 60 hours. At temperatures lower than 47°C *B. multivorans*
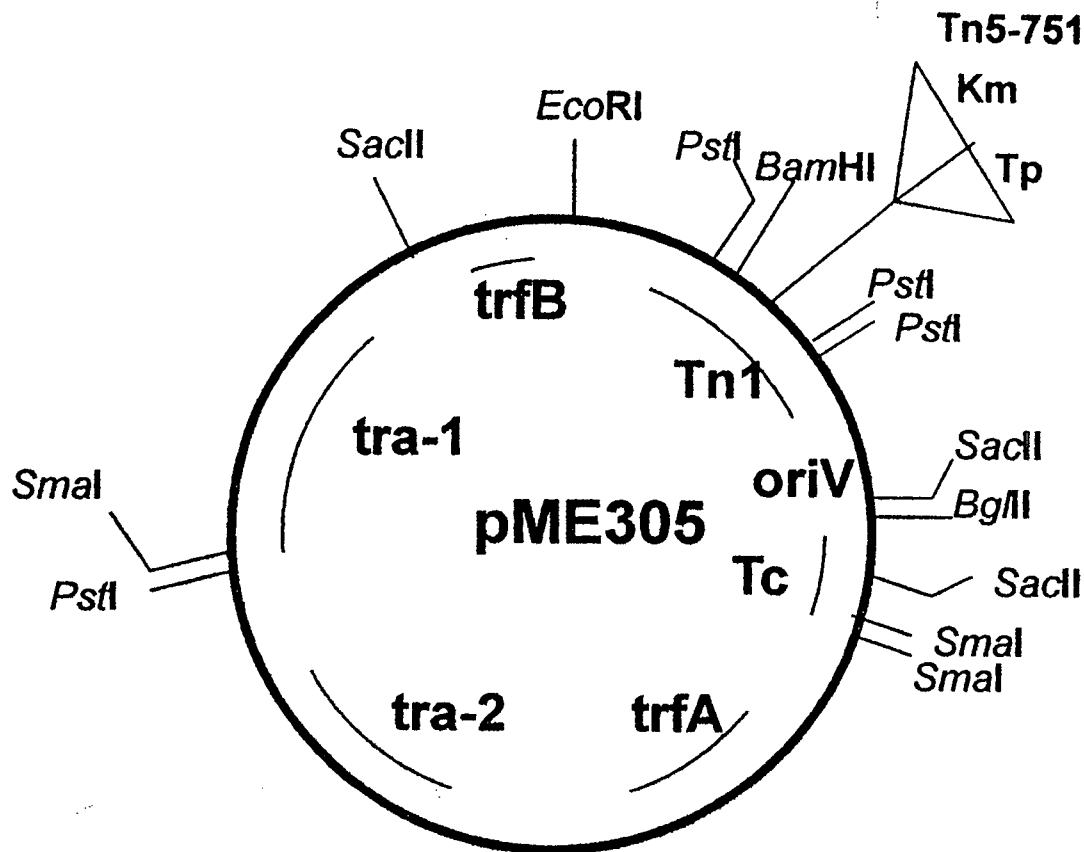
pME9 (carries tn5-751)
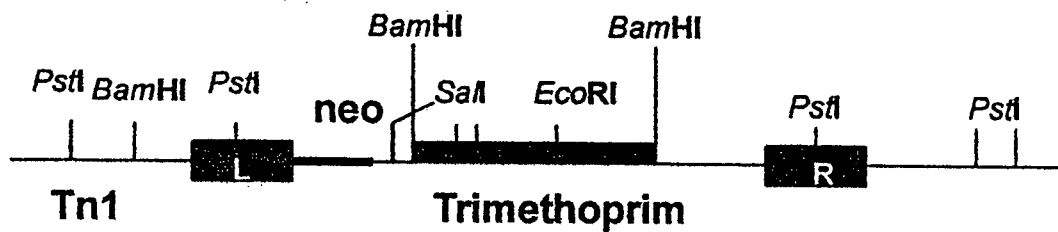
pTGL166 - *Spe*I site within Tn5-751S replaced with *Swa*I

Figure 3. Transposon-donor plasmid pTGL166. pTGL166 is a pME9 derivative in which the *Spe* I site within Tn5-751 was replaced by a *Swa* I site to make Tn-751S (Cheng et al. 1994). The vector pME9 is pME305::Tn5-751. The vector pME305 is a derivative of RP1 (Thomas, 1981) with a temperature sensitive mutation in the *trf*A gene. The primase genes (Lanka et al. 1984), the kanamycin resistance gene and IS21 of RP1 were removed by a single 12 kb deletion between the transfer gene regions *tra-1* and *tra-2*. Transposition of Tn5-751 into pME305 is described in Rella et al. 1985.

never lost the plasmid. This may be due to the fact that the temperature sensitive *trf*A mutant plasmid pTGL166 was selected for in *E. coli* (Thomas et al, 1981) and temperature sensitivity was presumably differently expressed in *B. multivorans*.

Putative mutants were randomly selected and subjected to PCR with primers specific for the kanamycin cassette found in Tn*5-751S*. The resulting PCR product of 375 bp corresponded to the presence of the transposon within the selected putative mutant clones as shown in Figure 4. These same mutants were replica plated onto tetracycline and absence of growth showed that the vector had been lost. Southern analysis of randomly selected mutants using a probe specific for the transposon indicated that selected clones from the *B. multivorans* insertion library carried Tn*5-751S* inserted into different sites in the chromosome. This is demonstrated in Figure 5 where the transposon specific probe hybridizes with a different sized *Sal* I DNA fragment for each mutant. To ensure that the transposon mutants were *B. multivorans*, Random Amplified Polymorphic DNA (RAPD) PCR was done on the parent strain and randomly selected mutants. RAPD-PCR uses random priming of DNA to create PCR products of varying sizes and when resolved on agarose gels, results in a pattern that is unique and strain dependant. The pattern depicted by *B. multivorans* ATCC 17616 is true to RAPD type 30 found amongst the *B. multivorans* strains (genomovar II of the *B. cepacia* complex) and the random mutants showed the same pattern as shown in figure 6.

3.3 Isolation of Mutant 26D7

Transposon mutants were replica plated on supplemented minimal salts media with either 600 or 1200 Units of polymyxin. Minimal salts media was used because it reduced the background growth of spontaneously resistant clones of *B. multivorans*. Six thousand colonies were screened and clones that did not grow on either 600 or 1200 Units of polymyxin were
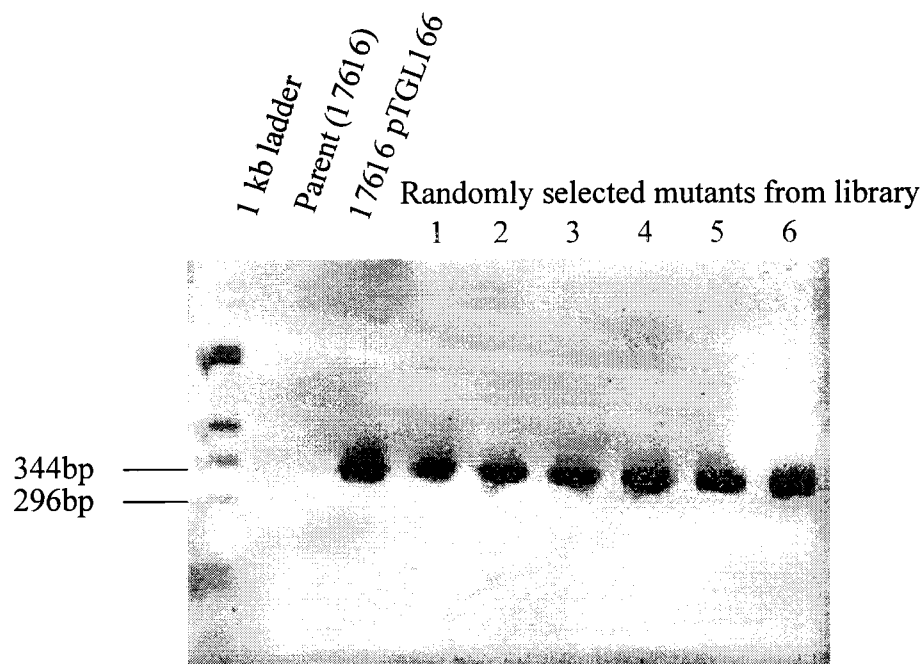
Figure 4. PCR of putative mutants using primers specific for the transposon.
Tetracycline sensitive mutants were selected from the Tn*5-751S* insertion library
of *B. multivorans* strain ATCC 17616. PCR was performed on DNA extracted from
these strains using primers N725-5 and N727-3 (see appendix B). These primers are
specific for the kanamycin cassette within the transposon Tn*5-751S*. A PCR product
of 375 bp indicated the presence of the transposon within the chromosome of the
selected putative mutant. PCR products were resolved on a 1.0% agarose gel
buffer and DNA was stained with EtBr (represented by dark bands). ATCC 17616
was used as a negative control and 17616 pTGL166 as a positive control.
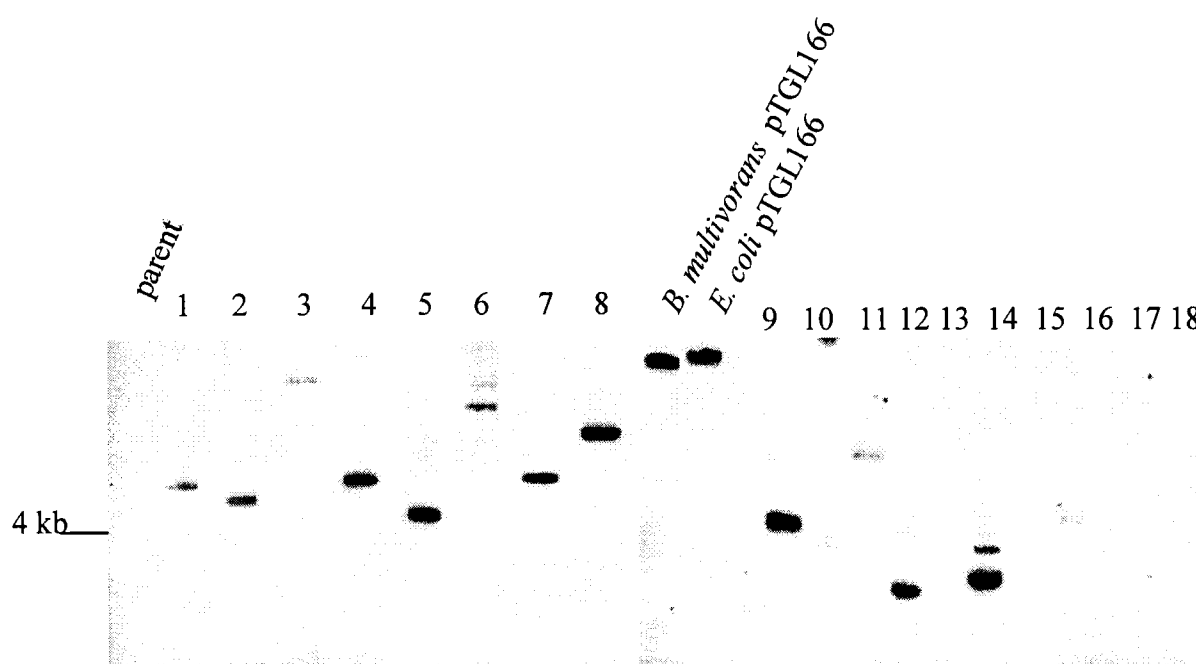
Figure 5. Southern Blot Analysis of Putative Mutants Using a ^{32}P-labeled probe specific for the transposon. Chromosomal DNA was isolated from 18 random transposon insertion mutants, the parent strain ATCC 17616, ATCC 17616 pTGL166 and *E. coli* pTGL166. These were restriction digested using the restriction endonuclease *Sal*1. Digested DNA was resolved on a 1.0% agarose gel and transferred by Southern blot to a nylon membrane. A ^{32}P-labeled probe, specific for the kanamycin resistance gene on Tn*5-751S*, was used to determine the size of the *Sal* 1 fragment carrying part of the transposon insertion for each mutant. Hybridization was done at high stringency as described in section 2.4 of the Materials and Methods.

1.0 kb —
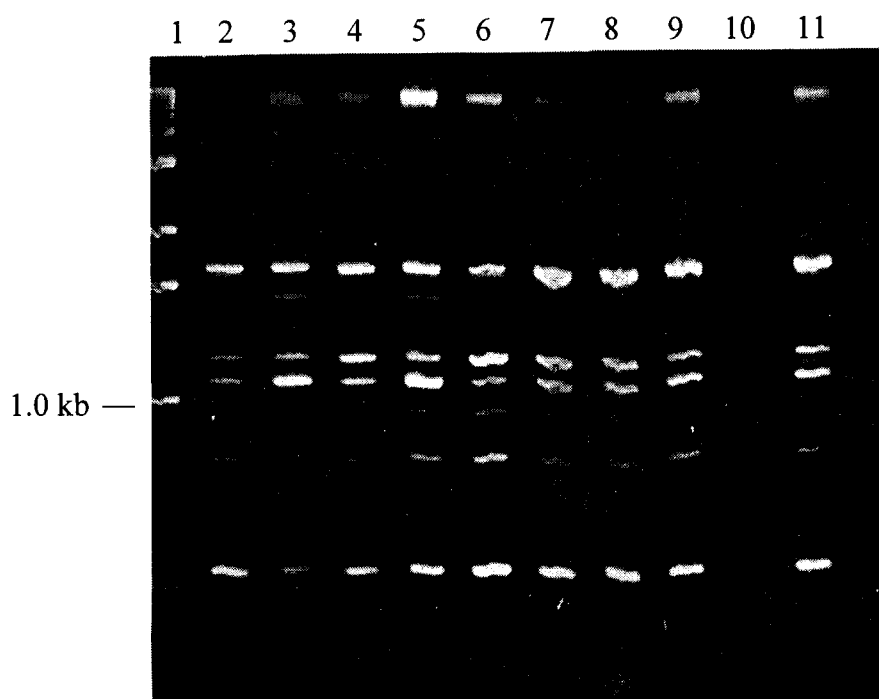
Figure 6. RAPD-PCR of mutants compared with the parent _B. multivorans_ strain ATCC 17616. RAPD-PCR was performed on randomly selected mutant colonies from the Tn*5-751S B. multivorans* ATCC 17616 insertion library using the random primer (appendix B). PCR products were resolved on a 1.0% agarose gel followed by staining with EtBr. The RAPD-PCR profiles for all mutants tested were the same as the parent strain profile. Lane 1, 1.0 Kb ladder; Lane 2, *B. multivorans* ATCC 17616; Lanes 3-11, randomly selected mutants.

selected off the master plate as putative polymyxin susceptible mutants. These mutants were then tested three separate times in a standard MIC experiment to determine if a reduction in the MIC to polymyxin of any of the mutants had occurred. Southern analysis of *Sal* I digested DNA of these selected mutants, using the probe specific for the transposon kanamycin cassette, showed that a single copy of the transposon was present in each mutant chromosome (Figure 7). Figure 7 also demonstrated that some of these mutants might have insertions in the same site on the chromosome or that siblings were present in the insertion library. For example, 46C8, 46D3 and 46G2 have the same MICs to polymyxin B in Table 2 and the probe hybridizes to a *Sal* I fragment that appears to be approximately 12kb in size. The probe hybridizes to a 4.2 kb fragment for both 45D6 and 45G3 (Figure 7), however their MICs to polymyxin B were different as shown in Table 2. Mutant 26D7 had the largest reduction in MIC of 8-fold when compared with the parent strain as shown in table 2 and the transposon specific probe hybridized to a 3.75 kb *Sal* I fragment (Figure 7). Mutant 26D7 was selected for further characterization.

3.4 Initial Characterization of Mutant 26D7

Mutant 26D7 was shown to have a reduced MIC to polymyxin B. Susceptibility to other cationic peptides was also tested and the results are shown in table 3. These peptides were CP26 and CEME, two synthetic cecropin-melittin hybrid α-helical peptides, and colistin (polymyxin E). A significant reduction in MIC from >500 μg/ml to 31 μg/ml was observed for CP26. However, surprisingly, no difference in MIC was seen for CEME. The MIC to colistin was reduced 8-fold from 64 μg/ml to 8 μg/ml. To ensure that the mutation in mutant 26D7 was not a general permeability mutation, MICs of other families of antibiotics were also determined for the parent and mutant strain. No differences were observed for rifampin, tetracycline, erythromycin, ciprofloxacin, ceftazidime, ticarcillin and ampicillin (Table 4).
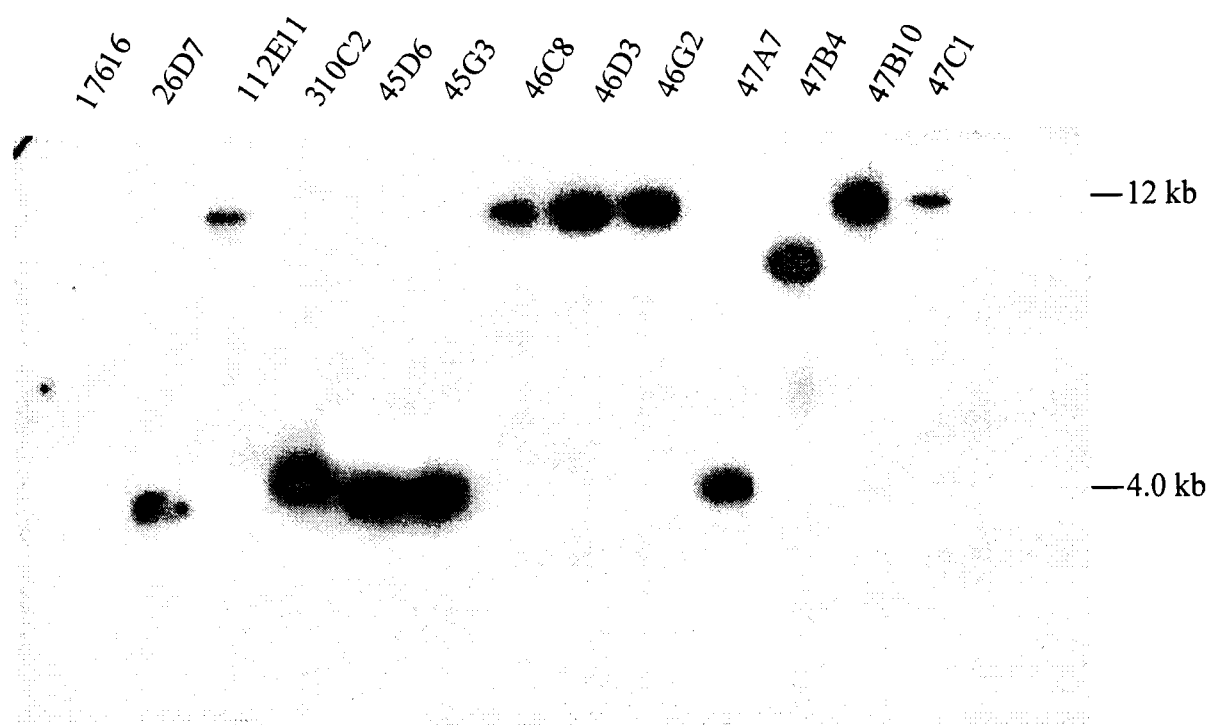
Figure 7. Southern blot analysis of selected putative polymyxin B sensitive mutants using a probe specific for the transposon.
Chromosomal DNA for *B. multivorans* parent strain ATCC 17616 and twelve mutants was digested with the restriction enzyme *Sal* I. Digested DNA was separated by gel electrophoresis and transferred to a nylon membrane. A ^{32}P-labeled PCR product specific for the kanamycin resistance cassette in the transposon, Tn*5-751S,* was used for Southern blot analysis. The probe hybridized to a 3.75 kb *Sal* 1 fragment in mutant 26D7.

Table 2. MICs of polymyxin B and gentamicin for selected mutants. The parent strain ATCC 17616 and the putative mutants isolated were incubated at 37°C for 24 and 48 hours in the presence of increasing concentrations of gentamicin and polymyxin. The lowest concentration of antibiotic that inhibited growth of the strains tested at 48 hours was recorded. MICs experiments were done by broth dilution as described in the National Committee for Clinical Standards Manual. Results from one of three replicate experiments are shown.

B. multivorans	Gentamicin (μg/ml)	Polymyxin (μg/ml)*
parent strain	256	64
26D7	128	8
112E11	100	16
310C2	200	16
45D6	256	16
45G3	256	64
46C8	256	32
46D3	256	32
46G2	256	32
47A7	256	64
47B4	256	64
47B10	>256	16
47C1	150	32

*7640 Units per ml

Table 3. MICs of cationic antimicrobial agents for the parent *B. multivorans* ATCC 17616 and mutant 26D7. The parent strain (ATCC 17616) and mutant 26D7 were incubated for 24 hours at 37°C in the presence of increasing concentrations of the cationic antimicrobials: polymyxin B, colistin, a cecropin-melittin hybrid (CEME) and a modified cecropin-melittin hybrid (CP26). MICs were done by broth dilution as described in the National Committee for Clinical Laboratory Standards Manual. Results from one of three replicate experiments are shown.

Strain	polymyxin B (μg/ml)	colistin (μg/ml)	CP26 (μg/ml)	CEME (μg/ml)
B. multivorans				
17616	64	64	>500	64
26D7	8	8	31	64

Table 4. MICs of different families of antibiotics for the parent *B. multivorans* ATCC 17616 and mutant 26D7. The parent strain (ATCC 17616) and mutant 26D7 were incubated at 37°C for 24 hours in the presence of increasing concentrations of the antibiotic indicated. The lowest concentration of antibiotic that inhibited growth of the strains tested was recorded. Results indicated that the transposon insertion in mutant 26D7 had no effect on MICs to other antibiotics. MICs were done by broth dilution as described in the National Committee for Clinical Laboratory Standards Manual. Results from one of three replicate experiments are shown.

Antibiotic tested	Parent (µg/ml)	Mutant 26D7 (µg/ml)
Rifampin	8	8
Tetracycline	0.25	0.25
Erythromycin	32	32
Ciprofloxacin	2	2
Ceftazidime	2	2
Ticarcillin	128	128
Ampicillin	>256	>256

3.5 Discussion

B. multivorans and other members of the family *Burkholderia* are known to have an extremely high level of resistance to cationic peptides and resistance to polymyxin is often used in clinical laboratories to aid in identification of the *B. cepacia* complex. A transposon insertion mutant of *B. multivorans* strain ATCC 17616 was isolated and showed reduced resistance to polymyxin B, polymyxin E and the cecropin-melittin hybrid CP26. The mutant identified (26D7) had MICs for polymyxin B and E of 8 μg/ml, reduced by 8-fold when compared to the MIC of the parent strain as shown in table 3. In *E. coli*, *Salmonella typhi* or *P. aeruginosa* an MIC of 8 μg/ml to polymyxin is considered fully resistant. This suggested that *B. multivorans* may have more than one mechanism or phenotype that causes resistance to cationic antimicrobial agents and a second mutation would be required for mutant 26D7 to become fully susceptible to polymyxin B and colistin.

Since aminoglycosides and cationic peptides enter Gram-negative bacterial cells by the same 'self promoted uptake' pathway, (Hancock et al, 1981, Nicas et al, 1983), MICs to gentamicin for the various mutants identified were also determined. None of these mutants showed a significant (i.e. greater than two-fold) reduction in MIC to gentamicin. The most likely explanation for this is the above mentioned possibility that more than one mechanism is involved in *B. multivorans* resistance to cationic antimicrobial agents. However, the presence of aminoglycoside modifying enzymes or ribosomes resistant to aminoglycoside binding in *B. multivorans* cannot be ignored, although neither has been identified to date.

Cecropin and melittin are alpha helical cationic peptides that take on a helix-turn-helix configuration upon interaction with membranes. Both peptides have one amphipathic α-helix and one hydrophobic α-helix and the order of these two helices is reversed in the two peptides. Cecropin-melittin hybrids consist of the amphipathic N-terminal α-helix of cecropin A and the

hydrophobic N-terminal α-helix of melittin (Piers et al. 1994). The CEME peptide consists of residues 1 to 8 of cecropin followed by residues 1 to 18 of melittin (**KWKLFKKI**GIGAVLKVLTTGLPALIS). CP26 is a modified form of CEME (KWKSFIKKLTSAAKKVVTTAKPLISS) such that all the hydrophobic residues are found on one face of the α-helix and all the hydrophilic residues are found on the opposite side of the helix (Hancock et al. 1998). These structural differences between CP26 and CEME are important for the activity of these peptides on Gram-negative bacteria. Despite their similarities in sequence and structure, these peptides have different spectra of activity (e.g. CP26 is Gram-negative selective, whereas CEME is more broad spectrum), different susceptibilities to salt and different mechanisms of interaction with membranes (Friedrich et al, 1999, Scott et al, 1999). *B. multivorans* strain ATCC 17616 was resistant to both CP26 and CEME although, CEME could be considered marginally more active against *B. multivorans* than CP26 in that an MIC could not be determined for the former as shown in table 3. The mutant 26D7 showed a >16 fold reduced MIC to CP26 and no change in MIC to CEME. Thus the sequence differences between CP26 and CEME appeared to be significant in terms of the activity of these two peptides on *B. multivorans* and mutant 26D7.

Once it had been established that a mutant susceptible to cationic peptides had been isolated it was important to ensure that this was a mutation specific to cationic peptide activity and that a general outer membrane permeability barrier mutant had not been isolated. Determination of the MICs for different families of antibiotics that use different pathways to enter into bacterial cells was performed and the mutant and parent strain compared. Rifampin and the macrolide, erythromycin showed no change in MIC confirming that permeability to hydrophobic antibiotics and efflux of these substances was not affected by this mutation. Tetracycline, the penicillins, ticarcillin and ampicillin, the cephalosporin, ceftazidime and the

fluoroquinolone, ciprofloxacin, also showed no change in MIC confirming that the hydrophilic (porin mediated) uptake pathway had not been affected by this mutation. Overall, it was confirmed that a *B. multivorans* mutant was created using transposon insertional mutagenesis. This mutant was partially characterized as having a reduced resistance to specific cationic peptides and no change in the MIC for the aminoglycoside, gentamicin or other families of antibiotics. This absence of change in MIC to gentamicin, even though aminoglycosides are known to use the same self-promoted-uptake pathway as polymyxin B, may be due to differences in the preferred interaction sites of polymyxin versus aminoglycosides (Hancock et al., 1994). Perhaps the mutation in mutant 26D7 had not affected the self-promoted-uptake pathway but rather another step in the mechanism of action of cationic peptides.

Chapter 4 Identification of Putative Genes Involved in Resistance to Polymyxin

4.1 Objective

To identify the genes responsible for the phenotypic change in mutant 26D7, the DNA

adjacent to the transposon insertion site was cloned from the parent strain, *B. multivorans* ATCC

17616. DNA sequence analysis of both strands identified four complete open reading frames

(ORFs) and one truncated ORF.

4.2 Cloning of the Locus at the Transposon Insertion Site

To identify the genes responsible for the phenotype of mutant 26D7, it was necessary to

clone the DNA from wild-type *B. multivorans* ATCC 17616 corresponding to the site of the Tn*5-*

751S insertion in mutant 26D7. A *Sal* I mini library of the chromosomal DNA from mutant

26D7 was constructed. *Sal* I digested Tn*5-751S* so that the kanamycin resistance cassette found

on the transposon was left intact. A kanamycin resistant clone, pBS1, was isolated from this

library by selection on agar plates containing kanamycin. pBS1 contained a 3.75 Kb fragment of

DNA. As shown in figure 8 this DNA corresponded to 2.3 Kb of transposon (with the

kanamycin cassette) plus 1.45 Kb of chromosomal DNA flanking the transposon at the 5' end.

DNA sequence data from either end of this 1.45 Kb fragment of chromosomal DNA was

obtained (described in section 2.72). PCR primers were designed from this sequence data,

Seq1L and Seq1R. The location of the priming sites for Seq1L and Seq1R are shown in figure 8.

The resulting PCR product was labeled and used as a radioactive probe to identify any

hybridizing clones from a *B. multivorans* ATCC 17616 cosmid library made using the cosmid

vector pSuperCos 1. The cosmid library, maintained in *E. coli* XL-1 Blue MR, was gridded onto

nylon membranes as described in section 2.73. Five clones 5G6, 5H11, 2F6, 2F9 and 1G3 were

identified by colony hybridization as shown and circled in Figure 9. The same PCR product

Figure 8. Restriction enzyme map of the 3.75 Kb *Sal* I fragment from mutant 26D7 that hybridized the probe specific for the transposon. Chromosomal DNA from mutant strain 26D7 was restriction digested completely with the restriction enzyme *Sal* I. The entire chromosomal digest from this mutant was cloned into the *Sal* I cloning site on the vector pBluescript SK+ and clones were selected for kanamycin resistance. The restriction enzyme *Sal* I digested Tn*5-751S* such that the kanamycin resistance cassette was left intact. Only those clones carrying the kanamycin cassette plus flanking DNA were able to grow. The *Sal* I fragment that contained this piece of Tn*5-751S* had the second *Sal* I site approximately 1.45 Kb 5' to the transposon insertion in mutant 26D7.
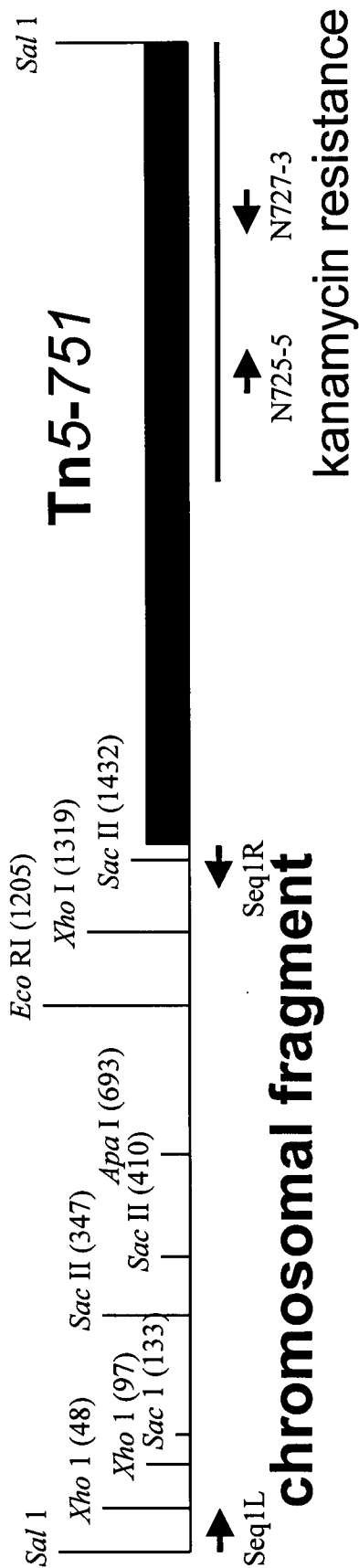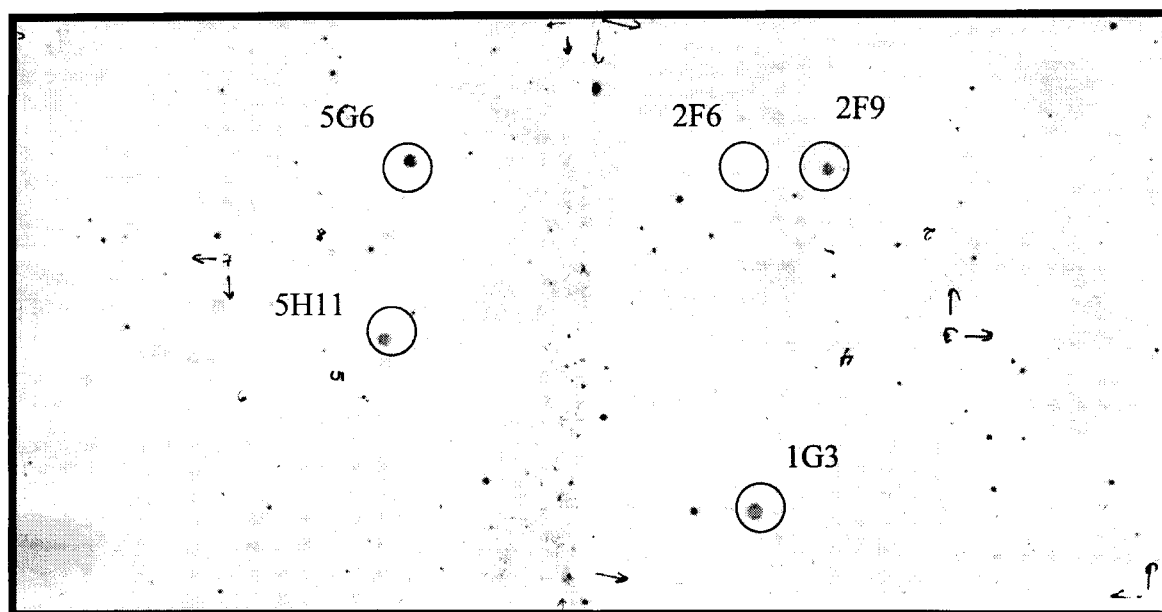
**Figure 9. Southern blot analysis of colony dot blots of the *B. multivorans*
strain ATCC 17616 cosmid library.** 800 *E. coli* XL-1 Blue MR cosmid clones
from the *B. multivorans* cosmid library were selected and gridded into 96 well
ELISA plates. These clones were replica plated onto a nylon membrane placed
on L-agar. After overnight growth, Southern blot analysis was performed under
stringent conditions using a random labeled ^{32}P-probe specific for the DNA
adjacent to the transposon insertion site in mutant 26D7. Positive clones are
circled and labeled.

from primer Seq1R and Seq1L was used as a probe for Southern hybridization of the *Bam* HI,

Sal I and *Pst* I digested cosmid clones identified. *Pst* I and *Bam* I (*Sal* I not shown) digests of the

cosmid clones identified are shown in figure 10A. Southern hybridization (Figure 10B) resulted

in the identification of 4.0 Kb *Bam* HI, 3.8 Kb *Sal* I and 8.0 Kb *Pst* I fragments that hybridized to

the PCR product made from primers Seq1R and Seq2R. All three fragments were cloned from

the cosmid clone 2F6 into pBluescript II KS+. The clone pBS15 contained the 3.6 Kb *Sal* I

fragment, pBS4 contained the 4.0 Kb *Bam* HI fragment and pBSP1 contained the 8.0 Kb *Pst* I

fragment.

4.3 Sequence Analysis of the region of DNA near the transposon insertion site

DNA sequence of both strands of DNA of the 4.0 Kb *Bam* HI and the overlapping 3.6

Kb *Sal* I fragment for an entire region of 6,029-bp was determined as described in the materials

and methods. The DNA sequence of this region is shown in Figure 11. The overall GC content

for this region of DNA was 72%. Four complete open reading frames (ORFs) and one truncated

ORF were identified. Two ORFs were identified on the 4.0 Kb *Bam* HI, neither of which was

the site of the transposon insertion found in mutant 26D7. The first ORF (ORF1) started at base

1138 and ended at base 139. ORF1 was in the opposing orientation and 632 base pairs upstream

of the start site of ORF2. The second ORF (ORF2) started at base 1770 and ended at base 2948.

The largest ORF identified in the intergenic region between ORF1 and ORF2 contained only 177

bp. There might not be a functional ORF between ORF1 and ORF2 and this large intergenic

distance may be required to accommodate for the presence of two promoters. A putative

promoter region for ORF2 was identified and is underlined with the −35 and −10 regions labeled

in figure 11. Sequencing of the overlapping *Sal* I fragment identified two more complete ORFs

and one truncated ORF. The first ORF on this fragment (ORF3) started at base 3120 and ended
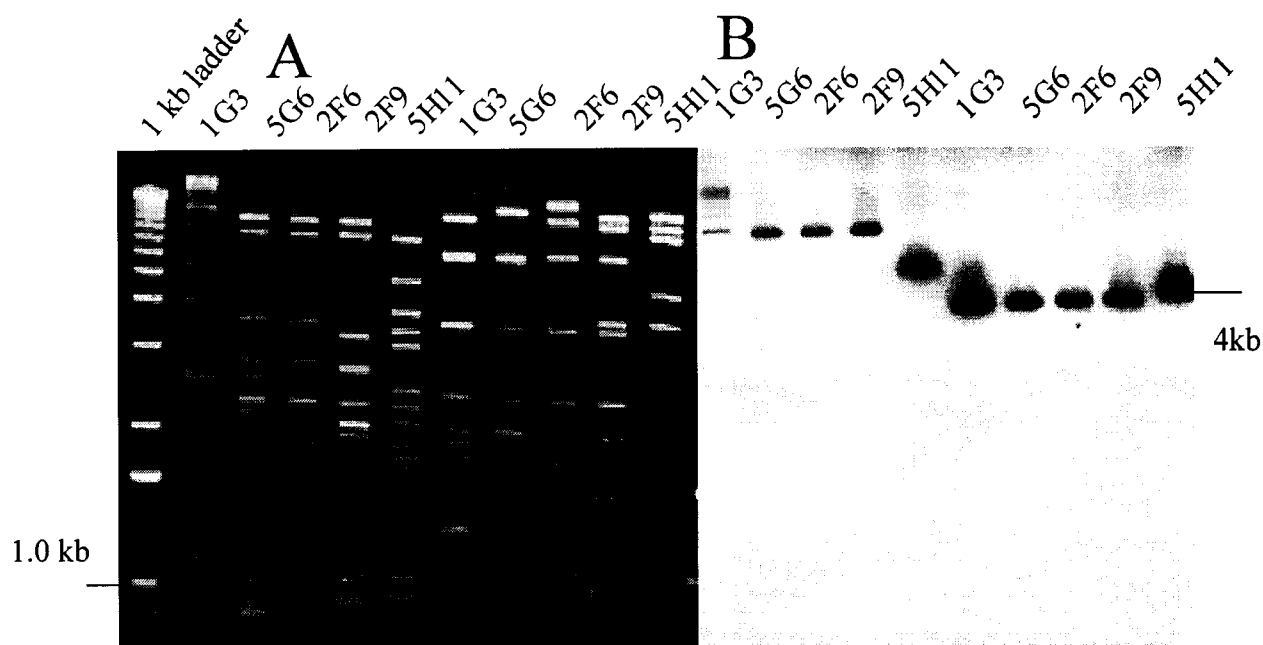
Figure 10. Identification of restriction enzyme digested cosmid fragments that hybridized to the DNA probe specific for the DNA adjacent to the transposon insertion in mutant 26D7. The five clones selected from the cosmid library were digested with *Pst* I (shown as the first 5 digests in panel A), *Bam* HI (shown as the last five lanes in panel A) and *Sal* I (not shown). The fragments were resolved on a 1.0% agarose gel and stained with EtBr (panel A). Southern analysis was performed using the probe specific for the DNA at the transposon insertion site (panel B). 8.0 Kb *Pst* I, a 4.0 Kb *Bam* HI and a 3.8 Kb *Sal* I fragments were identified as hybridizing to the probe specific to the transposon insertion site.

Figure 11. Nucleotide sequence of the 6,029 bp DNA region from *B. multivorans* ATCC 17616 near the transposon insertion site in mutant 26D7. The deduced amino acid sequence for the ORFs belonging to the putative operon is listed below the first nucleotide of the corresponding codon. The potential ribosomal binding region (underlined and labeled as RB) for the putative ORFs are indicated. ORFs are marked at their start codon, which is indicated in, bold. The *Sal* I site labeled within ORF2 shows where the cut site is for the *Sal* I fragment that did not complement the mutant phenotype. The *Bam* HI cut site is labeled within ORF3 corresponding to the 3' end of the *Bam* HI fragment that phenotypically suppressed the mutant phenotype. The transposon inserted between the boldfaced C and T bases and is marked as a downward arrow. A putative promoter sequence was identified upstream of ORF2 and the –35 and –10 regions are labeled. A potential rho-independent terminator is marked as inverted arrows between ORF2 and ORF3. The deduced amino acid sequence for ORF1 is presented in Figure 12.

Bam HI
```
   1 GGATCCGGACGCCTTCCACAAGGCGCTGCTCGACGGGCTCGACGCGGTGCGCACGCCGTAACGCGCAGCCGGGCGCGCGCGGCATGCGGC
  91 GGCGCGCATTGGGCCGCGCGCATCTCCGCAGCGGCGCCCGGCGCCGTTGCTATCGCCGCTCGCTCGCGAGCTCGTCGCGGATCTGCGCGG
 181 CCAGTTCGAACGAGCGCAGCCGCGCCGCGTGATCGTAGATCTGCGCGGTGATGATCAGCTCGTCCGCGCCCGTCTGCGCGATCCGGTCGC
 271 GCAGCTTGTCGCGCACCGTGTCGCGCGAGCCGACCGCCGCGAACGACAGCGAATGCGCGACGTTCGCGAGTTCGAGTTCGTTCGCCTCGA
 361 GCACGTCGACGGGCGGCGGCAGCTTGCCCGGCGTGCCGCGCCGCAGGTTGATGAACTGCTGCTGCAGCGACGTGAAGAGGCGCCGCGCCT
 451 CGTCGTCGGTGTCGGCCGCGAACACGTTGACACCTACCATCGCATACGGCTTCGGCCACGCGGCCGACGGCCGGTACTGCGCGCGGTACA
 540 CCTCGAGCGCCCGCATCAGATAGTCGGGCGCGAAGTGCGACGCGAACGCGAACGGCAGGCCGAGCATCGCGGCGAGCTGCGCGCTGAACA
 631 GGCTCGAGCCGAGCAGCCATACGGGCACGTCGAGCCCCGCGCCGGGCACCGCCACGCACGCGCTGGCCGGGCGCCGGCTCGGCGAAGTAGC
 721 GCAGCAGTTCGGCGACGTCGTCGGGGAATGAATCGGCGCTGCCGATCAGGTCGCGGCGCAGCGCACGCGACGTCGTCTGATCGGTGCCCG
 811 GCGCGCGGCCGAGGCCGAGATCGATGCGCCCCGGATACAGTGACGCCAGCGTGCCGAACTGCTCTGCGATCACGAGCGGCGCGTGGTTCG
 901 GCAGCATGATGCCGCCGGAGCCGACGCGGATCGTCTTCGTGCCGCCCGCGACGTGGCCGATCACGACCGCGGTGGCCGCGCTCGCGATGC
 991 CGGGCATGTTGTGGTGTTCGGCGAGCCAGTAACGTCGATAGCCCCAGCGCTCCGCATGTTGCGCGAGATCGAGCGTATTGCGGAAGGCCT
1080 GCGCGGCGTCGGCGCCGGCGGGAATGGGGGCGAGGTCAAGAACGGAAAACGGTGTCATCGGGTGCCTTCGAAGGGGGGAGGGGCGCGGTGA
                                                               ←orf1        RB
1171 TGTGCGCACGCAACAGATCCGTCGATTTTGCCAAAGGGTTCCGGAACGCGCTGGCGCCGCCGCGATACCCATGCGTGTAGCAGGGCCGCG
1261 CGCGACGTGCTCGCGATTGTTGCAAAAATCAATCCAATCGTCGTTTTGTTACGAATCTTTACCGCGATTGGCGGCGAATCTGCATCGGCA
1351 TACAAATCTGCACGAAAGGTTTGATTTTCAAGACTGCAATACGCCTGCAAAGCGCTTACGCTAACGTGAACGTGGCTGCGCCGGATCGTT
1441 TCGTCGGCTGCCCGGCTTTGCGGGAATCGCACCAGTCCGTTTCGCGCGGTGCCACGCGCGGGCGCGTGCAGACTGTTAAAAATCCGGCGCC
1531 TGTCCATCTCGTGCCGAAGGCAATCATCCGATTTTTCCGCGCCGCACGGCATGCGGGAAACGCCACGCTGAACGGGGCACGGCCGCCTTG
1621 CGCGGACCGGGCCGTGCCGCCCCAAATACTGAGGTCGCTGGAACAAGGAGTCGGGTCGTGCCCGCGTTTGTCGCTGTCGGAATATCCCAT
                                                                          -35
1711 CATCTGCCGGGCGGCCGGATCGGCCGCCACGCGGCCGGCCTCGTCGGAGGGCGCCGCCAATGACGACGCCCGCCGTGTCGCTGCTCGACT
      -10                                          RB     m  t  t  p  a  v  s  l  l  d  w
                                                                 orf2
1801 GGCTGCTGATCGCGTTCGCCGTGGCGGCGGCCGCTTACGCGCTGCTCGCGGCATTCGCGCCGCGTCCGCGCGTGCCGCGCACGGCCGCGC
      l  l  i  a  f  a  v  a  a  a  a  y  a  l  l  a  a  f  a  p  r  p  r  v  p  r  t  a  a  r

1891 GCGACGGCGCCGAACCGGTCAGCGTGCTCAAGCCGCTGTGCGGCGCCGAGCCGCATCTGTACGAGAACCTCGCGACTTTCTGCGAACAGC
      d  g  a  e  p  v  s  v  l  k  p  l  c  g  a  e  p  h  l  y  e  n  l  a  t  f  c  e  q  h

1980 ATCATCCGCGTTACGAGGTGCTGTTCGGCGTCGCGTCGGCCGGAGATCCCGCGATTGCGGTCGTCGAGCGGCTGCGCGCCGCGTATCCGG
      h  p  r  y  e  v  l  f  g  v  a  s  a  g  d  p  a  i  a  v  v  e  r  l  r  a  a  y  p  d

2071 ATCGCGATATCGCGCTCGTCGTCGACGCGCGCGTGCACGGCACGAATCTGAAGGTCAGCAATCTGATCAATCTCGCCGACCGCGCGAAAT
      r  d  i  a  l  v  v  d  a  r  v  h  g  t  n  l  k  v  s  n  l  I  n  l  a  d  r  a  k  y

2161 ACGATCGGATCGTGATCGCGGACAGCGACATCGCGGTCGCGCCCGACTACCTCGAGCGCGTGACGGCGCCGCTGGCCGACGCGTCGGTCG
      d  r  i  v  i  a  d  s  d  i  a  v  a  p  d  y  l  e  r  v  t  a  p  l  a  d  a  s  v  g

2251 GCGTCGTCACGTGTCTGTATCATGCGCGCAGCGTCGGCGGGGTTCTGGACGCGCATCGGCGCGCAGTTCGTCGACGCATGGTTCGCGCCGT
      v  v  t  c  l  y  h  a  r  s  v  g  g  f  w  t  r  i  g  a  q  f  v  d  a  w  f  a  p  s
                                                                     *Sal* I
2341 CGGTGCGGATCACGCATCTCGGCGGCTCGAGCCGCTTCGGCTTCGGCGCGACGCTCGCGCTCACGCGCGACACGCTCGAGCGGATCGGCG
      v  r  I  t  h  l  g  g  s  s  r  f  g  f  g  a  t  l  a  l  t  r  d  t  l  e  r  i  g  g

2431 GCCTGCGCGTGCTGAGGGACGAGCTCGCCGACGATTTCTGGCTCGCCGAGCTGCCGCGCCGCCTCGGGCGGCGCACCGTGCTGTCCGAAG
      l  r  v  l  r  d  e  l  a  d  d  f  w  l  a  e  l  p  r  r  l  g  r  r  t  v  l  s  e  v

2521 TCGAGGTCGCAACCGACGTGATCGAGGCGTCGTTCGGCCCGCTCTGGCATCGCGAGACGCGCTGGCTGCGCACGATCCGGTCGCTGAATC
      e  v  a  t  d  v  i  e  a  s  f  g  p  l  w  h  r  e  t  r  w  l  r  t  i  r  s  l  n  p

2611 CGGCCGGCTTCGCGTTCCTGTTCATCACGTTCACCGCGCCGTGGCTCGCGATCGCCGCGGCGCTCGCGCTGAAGCTCGACGGCACCGTCG
      a  g  f  a  f  l  f  i  t  f  t  a  p  w  l  a  i  a  a  a  l  a  l  k  l  d  g  t  v  a

2701 CCGGCACGGTGGCCGGCGTGGCGGCGGCCGCGGGCACGTTCGGCCGGCTCGTGCTGCACGCGCGCGGCGAAGACGGCTGGCGCGCGTACT
      g  t  v  a  g  v  a  a  a  a  g  t  f  g  r  l  v  l  h  a  r  g  e  d  g  w  r  a  y  w

2791 GGCGCGACCTGCCGCTCGTCGCGGTGCGCGACACGCTGCTCGCGCTCGAATGGCTCGTCGCCGCGTTCGGTACGCAGGTCGTCTGGCGCG
      r  d  l  p  l  v  a  v  r  d  t  l  l  a  l  e  w  l  v  a  a  f  g  t  q  v  v  w  r  g

2881 GCGCACGCATGACGGTGGTCGGCGGCGGCAACGCGCGGCGGCGGCCGTGGAAGGCGGCGACGGTCGCTAGCGCCGCCTCGGACACTGGCAGG
      a  r  m  t  v  v  g  g  e  r  a  a  a  a  v  e  g  g  d  g  r  end

2971 CGCGCGCGACCCGCGTGCCGACGGAATACGGCCCGGCAGCGGGCCCATGCGCGGCGAGCGAAGCTGCCGCCGCGCGACATCACGGAGTAG
                                                                                      --
3061 CGGGCAGCCGGTACGGCGGCCCGCCGTTTTTTTTGACTGAATCGTTGAGACGAATCGAACTATGCAGGCTACCGGAGCATTCATGAAAACG
      ------→       ←------------                                  RB    m  q  a  t  g  a  f  m  k  t
                                                                                 orf3
3151 CTGTTCTTGCAGGCCCCGTCCTACGACGGCTTCGACGGCGGAGCCGGCTCGCGCTATCAGGCCAAGCGTGAAATCCGTTCCTTCTGGTAT
      l  f  l  q  a  p  s  y  d  g  f  d  g  g  a  g  s  r  y  q  a  k  r  e  i  r  s  f  w  y

3241 CCGACCTGGCTCGCGCAGCCGGCCGCGCTCGTGCCGGGCAGCCGCGTCGTCGATGCGCCGGCCGACGGCCTGTCGGTCGAGGAAACGCTG
      p  t  w  l  a  q  p  a  a  l  v  p  g  s  r  v  v  d  a  p  a  d  g  l  s  v  e  e  t  l
```

```
3331 AAGATCGCGAACGACTACGACCTCGTGATCATCCATACGAGCACGCCGTCGTTCCCGACCGACGCGATGTTCGCGCAGGACCTGAAGAAG
      k  i  a  n  d  y  d  l  v  i  i  h  t  s  t  p  s  f  p  t  d  a  m  f  a  q  d  l  k  k

3421 ATGAAGCCGTCGATGCTGGTCGGCATGGTGGGTGCGAAGGTGATGGTCGATCCGCACAACTCGCTGACGGCGAGCGACGCGATCGACTTC
      m  k  p  s  m  l  v  g  m  v  g  a  k  v  m  v  d  p  h  n  s  l  t  a  s  d  a  i  d  f

3510 GTCTGCCGCGAGGAATTCGACTACACCTGCAAGGAACTCGCCGAAGGCAAGCCGTTCGCCGAGATCAAGGGCTTGAGCTGGCGCGCGAAG
      v  c  r  e  e  f  d  y  t  c  k  e  l  a  e  g  k  p  f  a  e  i  k  g  l  s  w  r  a  k

3601 GACGGCTCGATCGAGCATAACGAAGCGCGTCCGATCCTCGAGAACATGGACGAACTGCCGTTCGTCGCGCCCGTCTACAAGCGAGATCTG
      d  g  s  i  e  h  n  e  a  r  p  i  l  e  n  m  d  e  l  p  f  v  a  p  v  y  k  r  d  l
                                                                      ↓
3691 AAGATCGAGAACTACTTCATCGGCTATCTGAACTATCCGTACGTGTCGATCTACACGGGCCGCGGCTGCAAGTCGCGCTGCACGTTCTGC
      k  i  e  n  y  f  i  g  y  l  n  y  p  y  v  s  i  y  t  g  r  g  c  k  s  r  c  t  f  c

3781 CTGTGGCCGCAGACGGTCAGCGGCCACCGCTATCGCACGCGCTCGGTCGAGAACGTGCTCGCGGAAGCGAAGT**GGATCC**GCGACAACATG
      l  w  p  q  t  v  s  g  h  r  y  r  t  r  s  v  e  n  v  l  a  e  a  k  w  i  r  d  n  m
                                                                              *Bam* HI

3871 CCGGAAGTGAAGGAACTGATGTTCGACGACGACACCTTCACCGACGATCTGCCGCGCGCGGAAGCGATCGCCATCGGCCTCGGCAAGCTC
      p  e  v  k  e  l  m  f  d  d  d  t  f  t  d  d  l  p  r  a  e  a  i  a  i  g  l  g  k  l

3961 GGCATCACGTGGTCGTGCAACGCGAAGGCGAACGTGCCGTACAAGACGCTGAAGGTGATGAAGGAAAACGGGCTGCGCCTGCTGCTCGTC
      g  i  t  w  s  c  n  a  k  a  n  v  p  y  k  t  l  k  v  m  k  e  n  g  l  r  l  l  l  v

4051 GGCTTCGAATCCGGCGACGACCAGATCCTCGTGAACATCAAGAAGGGCGTGCGCACCGATTTCGCGCGCCGCTTCAGCGCGGACTGCAAG
      g  f  e  s  g  d  d  q  i  l  v  n  I  k  k  g  v  r  t  d  f  a  r  r  f  s  a  d  c  k

4141 AAGCTCGGCATCAAGATCCACGGCACCTTCATCCTCGGCCTGCCGGGCGAGACGAAAGAGACGATCAAGAAGACGATCCAGTACGCGAAG
      k  l  g  i  k  i  h  g  t  f  i  l  g  l  p  g  e  t  k  e  t  i  k  k  t  i  q  y  a  k

4231 GAAATCAATCCGCACACGATCCAGGTGTCGCTCGCCGCGCCGTATCCGGGCACGACGCTCTACAAGCAGGCCATCGAAAACGGCTGGATG
      e  i  n  p  h  t  i  q  v  s  l  a  a  p  y  p  g  t  t  l  y  k  q  a  i  e  n  g  w  m

4321 GAAGAGAACAAGACGATCAACCTCGTCAGCAAGGAAGGCGTGCAGCTCGCCGCGATCGGCTATCCGCACCTGTCGCGCGACGAGATCTAT
      e  e  n  k  t  i  n  l  v  s  k  e  g  v  q  l  a  a  i  g  y  p  h  l  s  r  d  e  i  y

4410 CACCATCTCGAGCAGTTCTATCGCGAGTTCTACTTCCGTCCGTCGAAGATCTGGGAGATCGTGCGCGAGATGCTGACGAGCTGGGACATG
      h  h  l  e  q  f  y  r  e  f  y  f  r  p  s  k  i  w  e  i  v  r  e  m  l  t  s  w  d  m

4501 ATGAAGCGCCGTCTGCGCGAGGGCGTCGAATTCTTCCGTTTCCTGCGCGCGCAC**GAGG**CC**TGA**TT**CGTG**ACGACGCAGCGGGCGGCGCGG
      m  k  r  r  l  r  e  g  v  e  f  f  r  f  l  r  a  h  e  a  end  v  t  t  q  r  a  a  r
                                                          RB      orf4

4591 GCGCTCATCTTCACCGCGGACGACTTCGGGCTGCACGAGCGTGTGAATGCGGCGGTCGAGCGCGCGCATCGCGACGGCGTGCTGAACGCC
      a  l  i  f  t  a  d  d  f  g  l  h  e  r  v  n  a  a  v  e  r  a  h  r  d  g  v  l  n  a

4681 GCGAGCCTGATGGTCGGCGCGCCGGCCGCGCAGGATGCGATCGCACGCGCGCGGCGGCTGCCGTCGCTCGCGGTCGGGTTGCATCTGGTG
      a  s  l  m  v  g  a  p  a  a  q  d  a  i  a  r  a  r  r  l  p  s  l  a  v  g  l  h  l  v

4771 CTCGCGGACGGCCCCGCGACGCTGCCCGCGCGCGAGATTCCGGCGCTGGTCGGCGCCGACGGCCGCTTCGGCGGCGCGATGGCGAAGGAC
      l  a  d  g  p  a  t  l  p  a  r  e  i  p  a  l  v  g  a  d  g  r  f  g  g  a  m  a  k  d

4861 GGCTGCCGGTTCTTCTTTCTGCCGCACGTGCGCGCGCAACTGCGGCGCGAGATCCGTGCGCAGTTCGAAGCGTTCGCGGCGACGGGCCTG
      g  c  r  f  f  f  l  p  h  v  r  a  q  l  r  r  e  i  r  a  q  f  e  a  f  a  a  t  g  l

4950 CCGCTCGATCACGTGAACGCGCACAAGCACTTCCATCTGCATCCGACCGTGCTGTCGATGATCGTCGAGATCGGGCGCGACTACGGTCTG
      p  l  d  h  v  n  a  h  k  h  f  h  l  h  p  t  v  l  s  m  i  v  e  i  g  r  d  y  g  l

5041 CGCGCGGTGCGGCTGCCGTACGAGACGACCGCGCCCGTGTGGCTGCGGCCGTGGATCGCGCTCGTGCGCGCGCAGCTCGACCGCGCGGGG
      r  a  v  r  l  p  y  e  t  t  a  p  v  w  l  r  p  w  i  a  l  v  r  a  q  l  d  r  a  g

5131 CTCGCGCATAACGACTACGTGGTCGGCATCGCGCAGACGGGCCGGATGGACGAGGCCGTGCTGCTCGATGCGCTCGCGGCGCTGCCGCCC
      l  a  h  n  d  y  v  v  g  i  a  q  t  g  r  m  d  e  a  v  l  l  d  a  l  a  a  l  p  p

5221 GGTGTCGGCGAGATCTACTGTCATCCGGCCGAAGCGGGCGACGGGCCGATCACGCCGACGATGGCCGACTACCGTCCCGCCGACGAACTC
      g  v  g  e  I  y  c  h  p  a  e  a  g  d  g  p  I  t  p  t  m  a  d  y  r  p  a  d  e  l

5311 GACGCGCTGCTGTCGCCGCGCGTCGCCGCGGCGCTCGCGCGCGCGGGCGTCGCGACCGGCGGCTTCGCGGCCGTGTTCGGCACGCGCGAC
      d  a  l  l  s  p  r  v  a  a  a  l  a  r  a  g  v  a  t  g  g  f  a  a  v  f  g  t  r  d

5401 GCGCAGCGCGGCGCACCCGCGGCGCGCAGACCG**GGAG**CGCAGCCGTC**ATGA**GCAAATGGTTCAAATGGCTCGGCTGGCCGATCGGCATCG
      a  q  r  g  a  p  a  a  r  r  p  g  a  q  p  s  end
                                    RB                m  s  k  w  f  k  w  l  g  w  p  i  g  i  g
                                                       orf5
5491 GCATCCTGCTCGCGCTGGGGCTGCACGAGGGCATCGGCGACGTGTCGCAGATGCTCGCGCGAGCCGGTTACGCGCTGCTGTGGCTCGTGC
      i  l  l  a  l  g  l  h  e  g  i  g  d  v  s  q  m  l  a  r  a  g  y  a  l  l  w  l  v  p
```

65

```
5581 CGTTCCACGCGCTGCCGCTGCTGCTCGATGCGTACGCATGGCACGTGCTGCTCGACCGGCGCGGCTCGCTGCCGTTTCTGTGGTGGATCG
      f  h  a  l  p  l  l  l  d  a  y  a  w  h  v  l  l  d  r  r  g  s  l  p  f  l  w  w  i  a

5671 CAACCGTGCGCGAGGCCGTGAACCGGCTGCTGCCGGTCGTCGGGATCGGCGGCGAGCTCGTCGGGATCCGGCTCGCGCGCTGGCAGGTCG
      t  v  r  e  a  v  n  r  l  l  p  v  v  g  i  g  g  e  l  v  g  i  r  l  a  r  w  q  v  a

5761 CCGATGCGAGTCGCGTGACGGCTTCGGTGATCGTCGAGGTGCTGGTGACGATCGTCGTCCAGTACGCGTTTGCCGCGCTCGGCCTCGTGC
      d  a  s  r  v  t  a  s  v  i  v  e  v  l  v  t  i  v  v  q  y  a  f  a  a  l  g  l  v  l

5851 TGCTGCTCGCGATGACGAACGCGATGGGCGGCGGCACGATCGGCGTCGCGCTGCTGCTGACGCTGCCGTTGCCCGTGCTCGGCGTCGTGC
      l  l  a  m  t  n  a  m  g  g  g  t  I  g  v  a  l  l  l  t  l  p  l  p  v  l  g  v  v  l

5941 TGATGCGGCGCGGCGGGATCTTCCATGCGATCGAGCGCTTCGCCGGGCGGCTGCTCGGCGATTCGCACCGGCTGTTGCAAGGC**GTCGAC**
      m  r  r  g  g  I  f  h  a  I  e  r  f  a  g  r  l  l  g  d  s  h  r  l  l  q  g  v  d
                                                                                      *Sal* I
```

at base 4563 and contained the transposon insertion in mutant 26D7 which is marked as a downward arrow in figure 11. A terminator sequence was identified immediately upstream of ORF3. The second ORF (ORF4) on this fragment started at base 4566 (only 3 base pairs are between ORF3 and ORF4) and ended at base 5453. A fifth ORF (ORF5) started at base 5448 and no stop codon was identified because ORF5 was truncated by the *Sal* I site at base 6029. The coding regions for ORF1, ORF2, ORF3 and ORF5 all used ATG codons to initiate transcription, ORF4 used GTG.

4.4 Identification of Putative Genes on the Complementing Fragment

4.4.1   ORF 1

The nucleotide sequence and corresponding amino acid sequence of ORF1 are found in figure 12. PSORT and PredictProtein programs suggested that this protein might be located in the inner membrane, however further studies would be required to prove this. ORF1 contained 999 base and the GC content of this sequence was 72% as expected for *Burkholderia sp.* The ORF1 start codon is labeled in Figure 11 and was down stream of a putative ribosome binding site (RBS). This start site was found 632 base pairs upstream and in the opposite orientation from the start site of the ORF2.

ORF1 encoded a predicted protein of 332 amino acid 36.0-kDa protein with a predicted pI of 6.46. A gapped-BLASTP search of the nonredundant database of NCBI was performed using ORF1 as the query sequence. A BLASTP search uses protein sequence as the query sequence and protein sequence as the subject sequence. The three sequences from this search that gave the lowest expect values are listed in Table 5. The lowest expect value of 1e-101 belonged to a hypothetical protein of *E. coli* found in the *yhb*W-*mtr* intergenic region. The next two genes identified were from *B. subtilis,* one similar to alkanal monooxygenase and one

```
5' -ATGACACCGTTTTCCGTTCTTGACCTCGCCCCCATTCCCGCCGGCGCCGACGCCGCGCAGGCCTTCCGCAATACGCTCGATCTCGCGCAA  90
      m  t  p  f  s  v  l  d  l  a  p  i  p  a  g  a  d  a  a  q  a  f  r  n  t  l  d  l  a  q    30

 91 CATGCGGAGCGCTGGGGCTATCGACGTTACTGGCTCGCCGAACACCACAACATGCCCGGCATCGCGAGCGCGGCCACCGCGGTCGTGATC 180
 31 h  a  e  r  w  g  y  r  r  y  w  l  a  e  h  h  n  m  p  g  i  a  s  a  a  t  a  v  v  I    60

181 GGCCACGTCGCGGGCGGCACGAAGACGATCCGCGTCGGCTCCGGCGGCATCATGCTGCCGAACCACGCGCCGCTCGTGATCGCAGAGCAG 270
 61 g  h  v  a  g  g  t  k  t  i  r  v  g  s  g  g  i  m  l  p  n  h  a  p  l  v  i  a  e  q    90

271 TTCGGCACGCTGGCGTCACTGTATCCGGGGCGCATCGATCTCGGCCTCGGCCGCGCGCCGGGCACCGATCAGACGACGTCGCGTGCGCTG 360
 91 f  g  t  l  a  s  l  y  p  g  r  i  d  l  g  l  g  r  a  p  g  t  d  q  t  t  s  r  a  l   120

361 CGCCGCGACCTGATCGGCAGCGCCGATTCATTCCCCGACGACGTCGCCGAACTGCTGCGCTACTTCGCCGAGCCGGCGCCCGGCCAGCGC 450
121 r  r  d  l  i  g  s  a  d  s  f  p  d  d  v  a  e  l  l  r  y  f  a  e  p  a  p  g  q  r   150

451 GTGCGTGCGGTGCCCGGCGCGGGGCTCGACGTGCCCGTATGGCTGCTCGGCTCGAGCCTGTTCAGCGCGCAGCTCGCCGCGATGCTCGGC 540
151 v  r  a  v  p  g  a  g  l  d  v  p  v  w  l  l  g  s  s  l  f  s  a  q  l  a  a  m  l  g   180

541 CTGCCGTTCGCGTTCGCGTCGCACTTCGCGCCCGACTATCTGATGCGGGCGCTCGAGGTGTACCGCGCGCAGTACCGGCCGTCGGCCGCG 630
181 l  p  f  a  f  a  s  h  f  a  p  d  y  l  m  r  a  l  e  v  y  r  a  q  y  r  p  s  a  a   210

631 TGGCCGAAGCCGTATGCGATGGTAGGTGTCAACGTGTTCGCGGCCGACACCGACGACGAGGCGCGGCGCCTCTTCACGTCGCTGCAGCAG 720
211 w  p  k  p  y  a  m  v  g  v  n  v  f  a  a  d  t  d  d  e  a  r  r  l  f  t  s  l  q  q   240

721 CAGTTCATCAACCTGCGGCGCGGCACGCCGGGCAAGCTGCCGCCGCCCGTCGACGTGCTCGAGGCGAACGAACTCGAACTCGCGAACGTC 810
241 q  f  i  n  l  r  r  g  t  p  g  k  l  p  p  p  v  d  v  l  e  a  n  e  l  e  l  a  n  v   270

811 GCGCATTCGCTGTCGTTCGCGGCGGTCGGCTCGCGCGACACGGTGCGCGACAAGCTGCGCGACCGGATCGCGCAGACGGGCGCGGACGAG 900
271 a  h  s  l  s  f  a  a  v  g  s  r  d  t  v  r  d  k  l  r  d  r  i  a  q  t  g  a  d  e   300

901 CTGATCATCACCGCGCAGATCTACGATCACGCGGCGCGGCTGCGCTCGTTCGAACTGGCCGCGCAGATCCGCGACGAGCTCGCGAGCGAG 990
301 l  i  i  t  a  q  i  y  d  h  a  a  r  l  r  s  f  e  l  a  a  q  i  r  d  e  l  a  s  e   330

991 CGGCGATAG -3'
331 r  r  end
```

Figure 12. DNA and corresponding amino acid sequence for the identified *B. multivorans* putative ORF1. This putative ORF1 contained 999 base pairs, encoded for 332 amino acids, a 36.0-kDa protein. The GC content for this ORF was 72%. Within the 6029 base pair region that was sequenced, this gene was found to start at base 1138 and end at base139. ORF1 was found 632 base pairs upstream of the putative ORF2 start site in the opposite orientation as shown in figure 11.

Table 5. Genes and expect values from the results of a gapped-BLASTP search using *B. multivorans* ORF1 predicted protein sequence as the query sequence. The first three genes or hits from a gapped-BLASTP search and their respective expect values are listed. The last gene listed was the most closely related sequence that has been functionally studied. The expect values is the number of hits one can "expect" to see by chance when doing a BLAST search on a database of a particular size (in this case it is the nonredundant database of NCBI). % Similarity is the % of amino acid residues that are identical or have similar properties to the residues in the query sequence. Similar properties refer to polarity, acidic or basic properties or similar charges at the same pH and amino acid substitutions like these do not change the property of the protein.

Organism	Description	%similarity	Expect Value
Esherichia coli; Eubacteria; *Proteobacter*; gamma subdivision; *Enterobacteriaceae*; *Escherichia*	hypothetical 37.1 kDa protein, ORF found in *yhb*W-*mtr* intergenic region	75%	1e-101
Bacillus subtilus; Eubacteria; firmicutes; *Bacillaceae*; *Bacillus*	Similar to alkanal monooxygenase	75%	2e-97
Bacillus subtilus; Eubacteria; firmicutes; *Bacillaceae*; *Bacillus*	Similar to alkanal monooxygenase alpha chain	63%	5e-69
Most closely related functionally studied sequence			
Vibrio harveyi; Eubacteria; *Proteobacter*; gamma subdivision; *Vibrionaceae*; *Vibrio*	Alkanal monoxygenase alpha chain	69%	5e-06

similar to the alpha chain of alkanal monooxygenase. These were two different protein sequences. None of these genes have been studied functionally and were named due to their high similarity with alkanal monooxygenases from other species. The gene with the highest similarity to the ORF1 of *B. multivorans* that has been functionally studied was the alpha chain of alkanal monooxygenase encoded by the *lux*A gene of the *lux* operon found in *Vibrio harveyi*. The similarity between these two protein sequences gave an expect value of 5e-6 and similarity of 41% from amino acids 23 to 182 for ORF1 and 22 to 189 for LuxA, the N terminal half of the protein. The *lux* operon is responsible for production of luciferase in bioluminescent organism. The sequence alignment in figure 13 shows that the similarities listed in table 5 were across the entire protein sequences.

4.4.2 ORF 2

The nucleotide sequence and corresponding amino acid sequence of ORF2 are found in figure 11. The second *Sal* I site corresponding to the 5' end of the *Sal* I fragment, as labeled in figure 11, was found within the ORF2 coding region. ORF2 contained 1179 and the GC content for this sequence was 72%. The start codon is labeled in Figure 11 downstream of a putative RBS. A stop codon of this ORF was found 173 base pairs upstream of ORF3.

ORF2 encoded for a predicted protein of 392 amino acids, a 42.3-kDa protein with a predicted pI of 7.11. A gapped-BLASTP search of the NCBI nonredundant sequence database was performed using the amino acid sequence of ORF2 as the query sequence. The five proteins that gave the lowest expect values in the results of this BLASTP search are listed in Table 6. The lowest expect value of 2e-32 belonged to a putative ceramide glucosyltransferase of *Synechocystis sp*. The three other proteins in this table correspond to the ceramide glucosyltransferases of the mouse, rat and human. The last protein sequence listed in Table 6 is

```
Bmultiorf1     1  --------MIPFSVLDLAPIPAGADAAQAFRNTLDLAQHAERWGYRRYWLAEHHNMPGIAS
Ecolihyp       1  MTD---KTIAFSLLDLAPIPEGSSAREAFSHSLDLARLAEKRGYHRYWLAEHHNMTGIAS
Bsubalkan      1  MSNNQRKDTLLSVLNLSPVVQGGTIAESFRNSMDLARRAEEWGYHRYWLAEHHNIEGVAS
Bsubachain     1  -----MKKFDISVLSVAPLRQGETMKQCIDSAVSLAKAVDNMGYKRIWFAEHHNHDAYAS


Bmultiorf1    54  AATAVVIGHVAGGTKTIRVGSGGIMLPNHAPLVIAEQFGTLASLYPGRIDLGLGRAPGTD
Ecolihyp      58  AATSVLIGYLAANTTTLHLGSGGVMLPNHSPLVIAEQFGTLNTLYPGRIDLGLGRAPGSD
Bsubalkan     61  SATAVLIGHIAGGTKKIRVGSGGIMLPNHSSLVIAEQFGTLETLYPGRIDLGLGRAPGTD
Bsubachain    56  AATVSIVQHILANTKDIRVGSGGIMLPNHSPLIVAEQFGTLETLYPNRVDLALGRAPGTD


Bmultiorf1   114  QTTSRALRRDIIGSADSFDDVAELLRYFAEP-APGQRVRAVPGAGLDVPVWLLGSSLFS
Ecolihyp     118  QRTMMALRRHMSGDIDNFPRDVAELVDWFDAR-DPNPHVRPVPGYGEKIPVWLLGSSLYS
Bsubalkan    121  QLTARALRRNINSGEI-FEEQLEEIRNYHKPSGNVRNQVRAIPGEGIDVPIWLLGSSGFS
Bsubachain   116  QKTADVIRRSNHNGVFFFEREVNDILRFVGDK-SVQGEVRAYPGIGTHVPVFVLGSSTDS


Bmultiorf1   173  AQLAAMLGLPFAFASHEAPDYLMRALEVYRAQYRPSAAWPKPYAMVGVNVFAADTDDEAR
Ecolihyp     177  AQLAAQLGLPFAFASHEAPDMLFQAIHLYRSNFKPSARLEKPYAMVCINIIAADSNRDAE
Bsubalkan    180  ARLAGELGLPFAFAAHFSPANTVPALELYRNSFTPSDVLDEPYAMVGVTIIAADTNEKAQ
Bsubachain   175  AEIAAKLGLPYAFGAQFSPHSMEEALSIYRENFQPSSYLQEPYVIACINVIAAESIDEAS


Bmultiorf1   233  RLFTSLQQQFINLRRGTPGKLPPFVDVLEANEL--ELANVAHSLSEAAVGSRDTVRDKIR
Ecolihyp     237  FLFTSMQQAFVKLRRGETGQLPPFIQNMDQFWSPSEQYGVQQALSMSLVGDNAKVRHGLQ
Bsubalkan    240  HLATSHYQRFLDIVRGTPNQLKPPVEDMDQIWSPYHKAMVNEQLSSTIVGGPEEVKAKLE
Bsubachain   235  FISASHLQVYIDIYTNNLSKLIPPTENFLESLSQFELEIIHSRLGYTIMGDRETIRRELI


Bmultiorf1   291  DRIAQTGADELIITAQIYDHAARLRSFELAAQIRDELASERR---
Ecolihyp     297  SILREIDADEIMVNGQIFDHQARLHSFELAMDVKEEILG------
Bsubalkan    300  DFVKTIQADEIMVNSETFEHADRMRSFEIIADVWKNR--------
Bsubachain   295  DFQQMYHADELIVLSNIYELSKEIQSYEILKQVVDELFKKRMEQL
```

Figure 13. Amino acid sequence alignment for the ORF1 predicted protein and similar sequences identified in the gapped-BLASTP search using the ORF1 predicted protein sequence as the query sequence. Protein sequence alignment for *B. multivorans* ORF1 (Bmultiorf1) and the *E. coli* 37.1 kDa hypothetical protein found in the YhbW-Mtr intergenic region (Ecolihyp), *B. subtilis* similar to alkanal monooxygenases (Bsubalkan) and the *B. subtilis* similar to alkanal monooxygenase alpha chain sequence (Bsubαchain). Shading is black for identical amino acids and grey is for amino acids with similar properties. Similar properties refers to polarity, acidic or basic properties or similar charges at the same pH such that amino acid substitutions like these do not change the property of the protein.

Table 6. Genes and expect values from the results of a gapped-BLASTP search using *B. multivorans* putative ORF2 as the query sequence. The first four genes or hits from a gapped-BLASTP search using ORF2 as the query sequence are listed here with their respective expect values and percent similarity. Expect value is the number of hits one can "expect" to see by chance when doing a BLAST search on a database of a particular size (in this case it is the nonredundant database of NCBI). % Similarity is the % of amino acids residues that are identical or have similar properties to the residues in the query sequence. The unfinished fragment from *T. ferrooxidans* was identified in a TBLASTN search of the unfinished genomes.

Organism	Description	% similarity	Expect Value
Nonredundant database			
Synechocystis sp.; Bacteria; *Cyanobacteria*; *Chroococcales*; *Synechocystis*	ceramide glucosyltransferase	45%	2e-32
Mus musculus	ceramide glucosyltransferase	48%	4e-22
Rattus norvegicus	ceramide glucosyltransferase	49%	9e-22
Homo sapiens	ceramide glucosyltransferase	48%	1e-21
Incomplete genome databases			
Thiobacillus ferrooxidans; Bacteria; *Proteobacter*; gamma subdivision; *Thiobacillus*	unfinished fragment	61%	6e-54

from an unfinished fragment of *Thiobacillus ferrooxidans* which was identified in a TBLASTN search of the unfinished genomes database and showed 61% similarity to ORF2 of *B. multivorans*. Gapped-BLASTP searches of the ceramide glucosyltransferases listed in Table 6 gave the same results as seen in Table 6 suggesting that the sequence similarities in this table are real. The alignments for these genes are found in Figure 14. The human and rat protein sequences were not listed in the alignment because they are 96% similar to the mouse sequence. The sequence similarity for the protein sequences was observed throughout the entire protein. Many other proteins were identified in this BLASTP search, and, although the expect values were relatively high ranging from 5e-6 to 5.7, they all appeared to have a glucosyltransferase function (data not shown). One of these proteins was *P. aeruginosa* MigA (Expect value 0.66) known to encode a glucosyltransferase (Wang, J. et al., 1996).

Because of the similarity of *B. multivorans* ORF2 with the mammalian ceramide glucosyltransferases a phylogenetic tree was constructed using the neighbor joining distance matrix method of the PHYLIP package (Felsentein, J., 1993) in consultation with Dr. Fiona Brinkman, University of British Columbia and is shown in figure 15. The tree shows the relatedness of the different protein sequences as proportional to the length of the tree branches. The ceramide glucosyltransferase of human and mouse are the only functionally studied ceramide glucosyltransferase sequences available. The only complete genome sequence available from a multicellular organism (*Caenorhabditis elegans*), had three copies of genes described as putative ceramide glucosyltransferases. *Synechocystis sp.* is a cyanobacteria and the complete genome sequence was also available for this organism showed two copies of genes with sequence similarity to ceramide glucosyltransferase (http://www.kazusa.or.jp:8080/cyano). *Myxococcus xanthus,* a member of the delta subdivision of *Proteobacteria* and *Lactococcus*

Figure 14. Amino acid sequence alignment for the ORF2 predicted protein sequence and similar sequences identified in the gapped-BLASTP search using the ORF2 predicted protein sequence as the query sequence. Protein sequence alignment for *B. multivorans* putative ORF2 (Bmultiorf2) and the *Thiobacillus ferrooxidans* predicted amino acid sequence from the identified ORF (Tferrorf), Synechocystis *sp.* putative ceramide glucosyltransferase (synecere) and the mouse ceramide glucosyltransferase (mousecer). The rat and human sequences were not presented because they are 96% similar to the mouse sequence. Shading is black for identical amino acids and gray for amino acids with similar properties. Similar properties refers to polarity, acidic or basic properties or similar charges at the same pH such that amino acid substitutions like these do not change the property of the protein.

Figure 15. Phylogenetic tree of protein sequences similar to the ORF2 predicted protein sequence of *B. multivorans*. This tree was constructed using the neighbor joining distance matrix method (Felsentein, 1993). The length of the tree arms is proportional to the evolutionary distance of the protein sequences. ThioCer, *T. ferrooxidans* putative ORF; MouseCer, mouse ceramide glucosyltransferase; HumanCer, human ceramide glucosyltransferase; Celegans1, 2 and 3; *C. elegans* hypothetical ceramide glucosyltransferases copy 1, 2 and 3, respectively; Bmultiorf2, *B. multivorans* ORF2 predicted protein sequence; Mxanthus, *M. xanthus* hypothetical protein; Synecho1Cer and Synecho2Cer, *Synechocystis sp.* putative ceramide glucosyltransferase copies 1 and 2, respectively; Llactis, *L. lactis* hypothetical protein; RhizNodC, *R. legunimosarum* NodC protein. Bootstrap analysis for each branch is indicated as the number of times out of 100 that this branch occurred when this tree was constructed 100 times using a random input order of sequences. Appendix E shows the protein sequence alignments for the proteins presented in this tree and the results of the bootstrap analysis are also presented therein.

Llactis

Gram-positives

Nematodes

Mammals

Celegans2

Celegans1

MouseCer Humancer
99

100

Celegans3

99

72

Synecho2Cer

79

Bmultiorf2

37

Cyanobacter

73

58

33

beta
subdivision

Synech1Cer

55

ThioCer

Proteobacter

RhizNodC

alpha
subdivision

Mxanthus

delta
subdivision
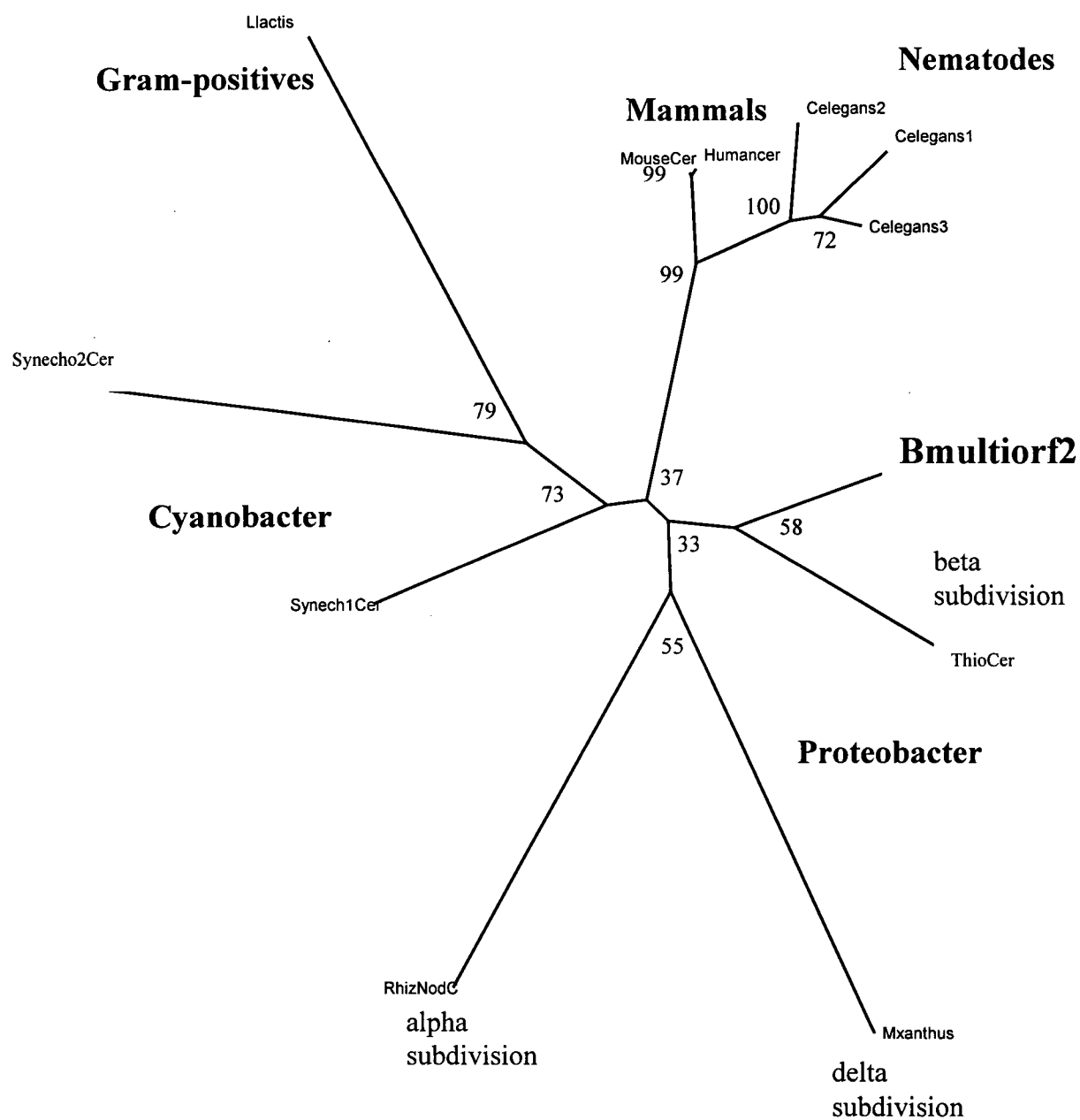
0.1

lactus, a Gram-positive organism, both had hypothetical proteins with similarity to ORF2 that were included in this tree. *Rhizobium leguminosarum* (a member of the alpha subdivision of *Proteobacter*) NodC protein was also included. The distance between the *B. multivorans* and human/mouse sequences was less than the distance between the *B. multivorans* and *C. elegans* sequences and these distances in this tree were therefore not consistent with recent horizontal gene transfer between mammals and *B. cepacia*. Appendix E shows the sequence alignments for all the proteins shown in the phylogenetic tree of figure 15.

4.4.3 ORF 3

The nucleotide sequence and predicted amino acid sequence of ORF3 are found in figure 11. ORF3 contained 1443 bases and the GC content for this gene was low for *B. multivorans* at 62%. ORF3 was found 173 basepairs downstream of ORF2 and the stop codon of this ORF3 was 3 basepairs upstream of the ORF4. The transposon, depicted in Figure 11 as a downward arrow, was inserted in the mutant genome at a location that corresponded to between bases 3759 and 3760 on this DNA fragment. The *Bam* HI site that corresponded to the 3' end of the *Bam* HI fragment is indicated in underlined bold characters.

ORF3 encoded for a predicted protein of 481 amino acids, a 54.0-kDa protein with a predicted pI of 7.59. PSORT program showed that the most likely location for *B. multivorans* ORF3 protein was cytoplasmic however, further studies would be required to confirm this. The predicted protein sequence of ORF3 was run in a gapped- BLASTP search and the protein sequences with the lowest expect values are listed in Table 7. The first three genes listed in table 7 are from *Pyrococcus horikoshii, Methanobacterium thermoautotrphicum* and *Thermotoga maritama* have not been studied functionally and are named as a result of similarity with other Mg-protophorphyrin IX monomethyl ester oxidative cyclases. Gapped-BLASTP searches of

Table 7. Genes and expect values from the results of a gapped-BLASTP search using the *B. multivorans* ORF3 predicted protein sequence as the query sequence. The first three proteins are hits from a gapped-BLASTP search and their respective expect values are listed. Expect value is the number of hits one can "expect" to see by chance when doing a BLAST search on a database of a particular size (in this case it is the non redundant database of NCBI). % similarity is the % of amino acid residues that are identical or have similar properties to the residues in the query sequence. The next two genes were also hits from same BLAST search but represent two genes that have been studied functionally. The last genes listed are unfinished fragments and the first two were found in a TBLASTN search of the unfinished genomes and the last fragment was identified from a TBLASTN search of the *T. ferrooxidans* genome.

Organism	Description	% Similarity	Expect Value
Nonredundant database			
Pyrococcus horikoshii Archaea; *Euryarchaeota; Thermococcales; Thermococcaceae*	498 amino acid long hypothetical methyl transferase	48%	3e-36
Methanobacterium thermoautotrophicum Archea; *Euryarchaeota; Methanobaeriales; Methanobacteriaceae*	Mg-protoporphyrin IX monomethyl ester oxidative cyclase	48%	2e-28
Thermotoga maritima Bacteria; *Thermotogales;* Thermotoga	Mg-protophorphyrin IX monomethyl ester oxidative cyclase	46%	5e-24
Functionally studied genes			
Streptomyces wedmorensis Eubacteria; Firmicutes; *Actinomycetes; Streptomycetes; Streptomyccetaceae; Streptomyces*	phosphonoacetaldehyde methylase FOM3	48%	1e-18
Streptomyces hygroscopicus Eubacteria; Firmicutes; *Actinomycetes; Streptomycetes; Streptomycetaceae; Streptomyces*	P-methyltransferase	46%	1e-10
Unfinished genomes database			
Thiobacillus ferrooxidans; Bacteria; *Proteobacter;* gamma subdivision; *Thiobacillus*	Two unfinished fragments identified	Fragment A 99% aa 8-43	7e-14
		Fragment B 72% aa 433-476	2e-08
		Fragment C 45% aa 212-367	0.003

these three genes showed the same results as the *B. multivorans* ORF3 suggesting that these similarities were real. The functionally studied genes that showed the highest similarity to the *B. multivorans* ORF3 were *Streptomyces wedmorensis* phosphoacetaldehyde methylase FOM3 and *Streptomyces hygroscopicus* P-methylase. Sequence alignments in figure 16 of all five protein sequences with the *B. multivorans* ORF3 showed protein sequence similarity throughout the proteins. When a TBLASTN search with the ORF3 protein sequence was performed on the unfinished genomes database two unfinished fragments from the *T. ferrooxidans* genome were identified. The first fragment showed 99% similarity with amino acid residues 8 to 43 of *B. multivorans* ORF3, the second fragment showed 72% similarity with amino acid residues 212 to 367. This second contig from *T. ferrooxidans* was the same contig that was identified as having similarity to part of *B. multivorans* ORF2. The stop codon of *T. ferrooxidans* ORF that had similarity to the *B. multivorans* ORF2, was found 24 base pairs upstream of the putative start site of the *T. ferrooxidans* ORF that had similarity to *B. multivorans* ORF3. This suggested that ORF2 and ORF3 like sequences existed in *T. ferrooxidans* and may be linked in this organism as well as in *B. multivorans*. The third fragment identified for *T. ferrooxidans* and listed in table 7 was found after a TBLASTN search of the *T. ferrooxidans* genome.

4.4.4 ORF 4

The nucleotide sequence and the corresponding amino acid sequence of the fourth open reading frame (ORF4) are found in Figure 11. This ORF contained 886 base pairs and the GC content for this gene was 74%. ORF4 was found 3 base pairs downstream of the ORF3.

ORF4 encoded for a predicted protein of 294 amino acids, 31.2-kDa protein with a predicted pI of 8.73. The predicted amino acid sequence of ORF4 was entered into a gapped-BLASTP search of the nonredundant database of NCBI and Table 8 lists the three genes, which

Figure 16. Amino acid sequence alignment for the ORF3 predicted protein sequence and similar sequences identified in the gapped-BLASTP similarity search. Protein sequence alignment for the *B. multivorans* putative ORF3 (BmultiORF3) and Pyromethyl, *Pyrococcus horikoshii* 498 amino acid long hypothetical methyl transferase; MethMgox, *Methanobacterium thermoautotrophicum* Mg-protoporphyrin IX monomethyl ester oxidative cyclase; ThermMgox, *Thermotoga maritima* Mg-protoporphyrin IX monomethyl ester oxidative cyclase; Swedmeth, *Streptococcus wedmorensis* phosphonoacetaldehyde methylase FOM3; Shygmeth, *Streptococcus hygroscopi* P-methyltransferase. Shading is black for identical amino acids and gray for amino acids with similar properties. Similar properties refers to polarity, acidic or basic properties or similar charges at the same pH such that amino acid substitutions like these do not change the property of the protein.

```
BmultiORF3   1 ------------------------MQATGAFMFTLFLQAPSYDGFDGGACSRYQ
Pyrmethyl    1 -----------------------------MKVMKILLVLEPTESAIKRVVCTTGI
MethMgox     1 -----------------------------DVVLINPEDRTAVENKLCFFLF
TherMgox     1 ----------------------------MRVLLINPYS-GGAYYYRLGAVYF
Swedmeth     1 -MTIGSLGSTEFALHGKPAIRWGDLPQRVGKPETRRYQKVLLLNPSA-TLFFHDLPRCTY
Shygmeth     1 MKHCIVVGYHETDMSGEELQLALEHGGDDLPAPIRSMLRTKITFGGSHYSYMDALSLGRN

BmultiORF3  24 AKREIRSFWYPTWIAQPAALVPGSRVVIAPADG-----------------LSVFIETLFI
Pyrmethyl   27 ---PLGIAYLIASMVFEEHDVI---IIDGLAED-----------------LTFSILIAFI
MethMgox    24 ---PLNIMILGASLEIASFSVI---IIDDDLRR-----------------MGVFIGIARL
TherMgox    23 ---PLGIMIISSSLKFKIHSVA---LIDMNVIK-----------------FDLFIRFN--
Swedmeth    59 ---IMLGIGYIAIVIEKYIYEVI---ILIVFAFGYYNAQPVDGDDQFLRYGLSDFIIVFV
Shygmeth    61 EGPGTDHAVTVSEIPSLGTLYLVNYLEEHGHPADFVN-----------SFSFFQLQLVK

BmultiORF3  66 --ANIYDLVIIHTSTP-SFPTDAMFAQDLFKMKFSMLVGMVGAKVM-------VDPHNSI
Pyrmethyl   62 IKKFIDPDIVGITATTS-AMYDAYTVAHIAFNINENVFVVMGGPHVT-------FTFELTM
MethMgox    60 VERINFFIVGITATTA-TIRTSLEYIFAIFDRLPNVLTVIGGPHPT-------FLFVDTL
TherMgox    57 --FRIYDVVGISADFV-RFPVVERTAKKAF--AQNVTVVMGGPHAT-------AYYFEII
Swedmeth   112 MKEFGPDVVGISSIFSNQADNVHHLLFLADLYTFEAVTAIGGAHAR-------VFFRACI
Shygmeth   109 LLADNFASVAITTTFYMMPAPVIELIIRFIRRHNANVPIIVGGELVDNQCREGRGDKLNRF

BmultiORF3 116 TASDAIIFVCRE-FFDYICKEIAF----GKPFAFIKGLSWFAK-DGSIEHNEA-------
Pyrmethyl  114 RECPCIFAVVRF-EGFLTFKELVFALSKFRELKGFILGLSYKF-NGKVPNEFP-------
MethMgox   112 RECRDLFVVVMG-EGEAFIVDIAFNHERFGSLDGVAGITYPF-GDRIRTNEP-------
TherMgox   105 QKG-LCFYVVLG-EGERAFSDLVFSIASNEKYPLIPGVAYMK--DGFVIALFS-------
Swedmeth   165 DDP-NLFAFFLG-EGEMTFLLWMFHLNGNVSDDAVHGIAWFLR-DGKVQIKFELPLISSM
Shygmeth   169 FIRVGAFYYFWESQGEAALAAVVGAIVNFDAPTFVPNFFLFSAATGEWFLTRK-------

BmultiORF3 163 RF-------------ILENMDELPFVAPVYK---PDIKIENYFIGYL-NYPYVSIYIGRG
Pyrmethyl  164 RF-------------LIQNVDEIPIPS-------YDI-LPMDKYKAD-GVPFGVVMTSRG
MethMgox   162 RF-------------LIEDLDEIPFPA-------PHI-VPFRDYETS-SQDAGGMITSRG
TherMgox   154 F--------------FLENLDDLPFPD-------FEK-VHLYRTKFA-GERATSLITSRG
Swedmeth   222 RFEGPETGKSSPMLSMAGELDHIPFPAWHHYNMEFYFEIKAYQSFGTVGSRVGQLYTSRG
Shygmeth   222 RF-------------FANDLNAFSV-------PWN--RFDPTVLG--P-TLSTRTAFS

BmultiORF3 206 GKSRGTFCLWPQTVSGHRYRTRSVENVLAFAKWIRDNMPEVKEIMFDDDTFTDDLPRAEA
Pyrmethyl  202 CPFNGVFCS-SFLQFGKPWPGHSVERVIEELSILH-YEYGIKEIFFLDDTFTLNKKRAIL
MethMgox   200 CVYPGRYCS-SFLIMFKKFFRSPENVVDEVEEIV-EVYGLHDIAFLDDTFMLHRPRARE
TherMgox   191 CPFNGEFCS-AFQFMFRFIFWRSIENVIDEIKIF--KKLGYGSVIFFDDNFTINPKFVVN
Swedmeth   282 CTAHFTFCT-TTHFWFQRLFRRFVQDVVDFVLRFR-DEYGIDFFHIQDDNIFNDMDHARE
Shygmeth   255 CAFSFAFCD-YPERAGALT-LADFSTVEREIFEEL--ADLGVKRVAFFDDTFNVPMRRFKD

BmultiORF3 266 TAIGLG--KLGITASCNAKANVPY---KTIKVFKENGLPLLLVGFESGDLQIL-VNIKKG
Pyrmethyl  260 ISLRIKQEGFDISWTASSFVNTFN--FKVAKAFKGGGCHTVVFGIESSASPFIL-FFGKG
MethMgox   258 ISEEIRFFNFDVSFVTFSSFVDMVQ--FSLFRDLRNAGMSTIYYGVESGCQRVL-DMMKKG
TherMgox   248 LCEEFMFKDFRFKFWAFSFADELLGRFDMVEAFSKAGCPMLFIGFESANDEVL-FEYGFN
Swedmeth   340 LFRAFK--EVFLPFATPQGTALWRMDFELFDLMAFSGAYQVTFAIESGVQRVLKFLIKFP
Shygmeth   311 LCRMLVFRLFFIEFFSYFFCGHAR-DPLVYDLMYFSGCRGVLLGIESGDDQVL-LNFDFR

BmultiORF3 320 VRTDFFRRFSADCFFLGIKIHGFFILGLF------GETKETIKKTIQYAKEINFHTIQVF
Pyrmethyl  317 ITPQQSIFAVFTAFFFLHALGSFIIGFF------DETRFEVEATIKFAKKLDIFYAFFT
MethMgox   315 IIVKQFEFAVFVTFFFVFVDFITSFILGYF------GEKPSEMDRTIDFSIKLDPIYSFY
TherMgox   307 LKSDIFFFVFFLLFFYKVDFFASFVIGAL------GFDTFKTIEKTVKFAKKLKASIVDFS
Swedmeth   398 LNLFRTSHLIFYARSLGMHVHGFFIIGMFPMCGNAGESIFFMQASYDYAEEAGFSSASFF
Shygmeth   369 AFTAHYQNGIFEQLFARGIFTHASFVVGFF------GESPGTVRNTIDFINSAGPDTFAVN

BmultiORF3 374 -----------LAAF----YPGT-TLYKQAIEFG--WMEENKTFNLVSKEGVQLAAFGYFF
Pyrmethyl  371 -----------IATF----YPGT-RIWEYAIAFN--LLLTMNWRKYTIID----PVMFLKF
MethMgox   369 -----------ILTF----FPGT-PIYAELKSQ---GLIEDDWENYIVIK----PVIFYEF
TherMgox   361 -----------ILTF----YPGT-AIFEKLKHL----IFEKDWRKFDGTH----LVFFHIN
Swedmeth   458 -----------AASF----IVGS-ELLRECIRGG--FVDPEESLYRMFYK---QGFFNVFG
Shygmeth   423 HWYYMQSTPIHVRAFQFDLTFGNGHTWSHRTMNSHQALEAADEIFDSVVNAAWMPVNGL

BmultiORF3 417 I--SRDEIYEHLEQFFREFYF--RFSKIWEIVR----EMLTSWDMMKRRLREGVEFFRFL
Pyrmethyl  410 F--TSFQISKLLRKAYISFYL--RPKVLIKDIFE--HHGFIIKRAIRGLLPVTYQA----
MethMgox   407 IGLFRFLFOKKFVKAYIKFYS--RPRYLLK-------HSYMIRVFFETLYPTYIEPKIPF
TherMgox   398 F--SPKFIRRLFIKAYYAVYT--SFPLIFR-------RGIPFLVRFLLFYREAFF------
Swedmeth   498 IW-DGFFIAEFAAFFNRDFNA--FRDRAYT----------PQFQWNANQY----------
Shygmeth   483 WGVPYLLGKGFSHAEIVRFLDLAKFLTVANVSRGSAEEFATAERRFFDFVTGLDLAPARY

BmultiORF3 469 FAHEA---
Pyrmethyl      --------
MethMgox   458 FKDR----
TherMgox       --------
Swedmeth       --------
Shygmeth   543 FTAGSEFR
```

Table 8. Genes and expect values from the results of a gapped-BLASTP search with *B. multivorans* ORF4 predicted protein sequence as the query sequence. The first four genes that were the result of a gapped-BLASTP search of the nonredundant database using the putative ORF 4 from *B. multivorans* with expect values less that 1.0 are listed. The fifth gene listed was identified in a gapped-BLAST search of the unfinished genomes. Expect value is the number of hits one can "expect" to see by chance when doing a BLAST search of a database of a particular size (in this case it is the nonredundant database of NCBI). % similarity is the % of amino acid residues that are identical or have similar properties to the residues in the query sequence.

Organism	Description	% Similarity	Expect Value
Bacillus stearothermophilus; Eubacteria; Firmicutes; Low GC content gram-positive bacteria; *Bacillaceae*; *Bacillus*	Cellobiose phosphotransferase system CelC	42%	5e-15
Bacillus cereus; Bacteria; Firmicutes; *Bacillus/Clostridium* group; *Bacillaceae*	YdjC-like protein	48%	8e-10
Escherichia coli; Eubacteria; *Proteobacteria*; gamma subdivision; *Enterobacteriaceae*; *Escherichia*	Hypothetical 27.8kDa protein, ORF found in *celF-katE* intergenic region	46%	4e-8
Thiobacillus ferrooxidans; Bacteria; *Proteobacter*; gamma subdivision; *Thiobacillus*	Unfinished fragment	62%	8e-54

gave the lowest, expect values. These were *Bacillus stearothermophilus cel*C, *Bacillus cereus ydj*C-like and *E. coli* hypothetical protein in the *cel*F-*kat*E region. Protein sequence alignments in figure 17 showed that the sequence similarity to those genes listed in Table 8 was throughout the amino acid sequence. The *celC* gene of *Bacillus stearothemophilus* has not been studied functionally. The *B. multivorans* predicted amino acid sequence from ORF4 showed similarity to a predicted protein from an ORF called *ydjC* found in the *cel*F-*kat*E region of *E. coli* at 46%. A similarity of 48% was exhibited with the *Bacillus cereus* YdjC-like protein, which was named as a result of its similarity with this same region in *E. coli*. A gapped-BLASTP search was performed on the unfinished genomes database and 61% similarity was found with a fragment from the *T. ferrooxidans* genome. The *T. ferrooxidans* contig identified did not overlap with previous two identified contigs with similarity to the ORF2 and ORF3 and it could not be concluded whether or not these contigs were adjacent in *T. ferrooxidans*.

4.4.5   ORF 5

The nucleotide sequence of the fifth open reading frame is found in figure 11 however further sequencing is required of this region. ORF5 was truncated at the *Sal* I site and a stop codon was not identified. A gapped-BLASTP search of the nonredundant database of NCBI of this incomplete amino acid sequence showed no sequence similarity with any known genes. A BLAST search of the unfinished genomes database showed 69% sequence similarity with a contig of *T. ferrooxidans* at the N-terminal. This suggested that these four genes may also be adjacent to each other and closely linked in *T. ferrooxidans* as was seen for *B. multivorans* however, no conclusions can be drawn until the genome sequencing project of *T. ferrooxidans* is finished.

```
BmultiORF4    1  VTTQRAAKALIFTADDFGLHERVNAAVERAHRDGVINAASLMVGAPAAQDAIARAPRLFS
Tferrorf      1  -------------------------------AHRDGEITIALMVAAPAAADAVARARALFG
BstecelC      1  -----MPHYCIVNADDFGYSKKVNYGILEAFQNGVVTSATLMANMPAAEHAARLAKDHFE
BcerydjC      1  ------MIKLIVNADDFGLTEGTNYGIIDGHINGLVNSTTMMMNMHGTEHAVRLAKEHNI
Ecolihyp      1  -----MERLLIVNADDFGLSKGQNYGILEACRNGIVTSTIALVNGQAIDHAVQLSRDEFS


BmultiORF4   61  LAVGLHLVIADKPATLPAREILALVGADGRFGGAMAKDKCRFFFLPHVRAQLRREIRAQF
Tferrorf     32  LGVGLHITIVDCTPLIPAEQVIDLVDEQGRFAADLWPRGVRYEFLPQVRKQLAAEIRAQI
BstecelC     56  LGVGIHFVLTCCRPLA---DVPSLVNENGEFP----RRGE-ALVGARRGD-IERELCAQL
BcerydjC     55  LGVGVHLVLTAGKPLIG--DVPSLVSSDGLFH----KQSV-VWEGKINPEEWEREWTAQI
Ecolihyp     56  LAICMHFVLTMKKPLT---AMPGLTR-DGVLG---KWIWQLAEEDALPLEEITQELVSQY


BmultiORF4  121  IAHAA-TGIPLDHVNAHKHEHLHPTVLSMIVEIGRDYGIRAVRIPYETT-----------
Tferrorf     92  AAHAA-TGIELDHANAHKHLHLHPTVLTQMLEVGKEEGIRAVRIPWEPLRAARCSGAKGV
BstecelC    107  KRIFS-FKITPTHIDSHHHVHEHPNVFPVVEQLAERYRIPILPWRTARPHRL--------
BcerydjC    108  KKLS-YGITPTHLDSHHHVHGLFILHDVLEKLAATYNVPIRKCEEFRAVR---------
Ecolihyp    109  LRHIELFGRKPTHLDSHHHVHMFPQIFPIVARFAAEQGIALRADRQMAFDLP--------


BmultiORF4  169  AP----VWLRFWIALVRAQLDRAGIAHNDYVVGIAQTGRMDEAVLLDALAALPP-GVGEI
Tferrorf    151  APTFWSIFLAPWLARMRRQLDRRRIMHNDQLYGLDATGHMDRERLLRMLRCLPATGVTEI
BstecelC    158  ----------ITVDVFFPDFYGDGLTKDRFISLIDRIG--DGQ-------------TAEV
BcerydjC    158  ----------FFSDVFYSDFYADGVTEDYFVKLKERVQ--GEQ-------------TVEI
Ecolihyp    161  ------VVN-LRTTQGFSSAFYGEEISESLFIQVLDDAGHRGDR------------SLFV


BmultiORF4  224  YCHPAEAGDGPITPIMADVRPADELDALLSPRVAAALARADVATKGHAAVFGTRDAQRGA
Tferrorf    211  YLHPAMVQTAALHALMPNHEPHAEWAALTSPRVKDYLRDQDHAKITFFDFVAR-------
BstecelC    193  MCHPAYIDVPLASGSSYCQQRVEEIAVLTDPTLVAEMAERGVQIITYREFYKL-------
BcerydjC    193  MVHPAXIDPELVKRSSYVMDRVKELRILTESELP----E-GIELVKF------------
Ecolihyp    201  MCHPAFIDN-TIRQSAYCEPRLTELDVLTSASLKGAIAQRGYRLGSYRDV----------


BmultiORF4  284  PAARRPGAQPS
Tferrorf         -----------
BstecelC         -----------
BcerydjC         -----------
Ecolihyp         -----------
```

Figure 17. Amino acid sequence alignments for the ORF4 predicted protein sequence and similar sequences identified in the gapped-BLASTP search using the ORF4 predicted protein sequence as the query sequence. Protein sequence alignment for the *B. multivorans* putative ORF4 and the putative ORF (BmultiORF4) from *Thiobacillus ferrooxidans* (Tferrorf), *Bacillus stearothermophilus* CelC (BstecelC), *Bacillus cereus* YdjC-like protein (BcerydjC) and the *E. coli* hypothetical 27.8-kDa protein in CelF-KatE intergenic region. Note that the *T. ferrooxidans* sequence is truncated at the start. Shading is black for identical amino acids and gray for amino acids with similar properties. Similar properties refers to polarity, acidic or basic properties or similar charges at the same pH such that amino acid substitutions like these do not change the property of the protein.

4.5 Subcloning of *Bam* HI, *Sal* I and *Pst* I fragments into pDN18

Since pBluescript does not replicate in *B. multivorans*, the *Bam* HI, *Sal* I and *Pst* I fragments were subcloned into the broad-host-range vector pDN18. pDN18 was transferred by triparental mating, using the helper plasmid pRK2013 into *B. multivorans* ATCC 17616. The plasmid pDN18 replicated in *B. multivorans* and was maintained at a low copy number. These fragments were subcloned in both directions into pDN18. The 8.0 Kb *Pst* I fragment, after ligation with *Pst* I digested pDN18, could not be electroporated or transformed into *E. coli* DH5α or XL1-Blue MR. Because ORF2, ORF3, ORF4, ORF5 and possibly more are all found on this 8.0 kb *Pst* I fragment this result suggested that expression of all these genes on a multi-copy number vector may be lethal to *E. coli*.

4.6 Complementation of Mutant 26D7

pDBS6, pDBS9, pDBS1, pDBS2 (detailed description of clones in table 1, Chapter 2) and pDN18 were conjugated by triparental matings into mutant 26D7. Figure 18A shows the *Bam* HI fragment cloned in opposite orientations in pDBS6 and pDBS9 and the *Sal* I fragment cloned in opposite orientations in pDBS1 and pDBS2. The arrow beneath the vector name in figure 18A indicates the direction of the *lac* promoter on the vector for each clone. Figure 18B shows ORF1 and ORF2 are found on the *Bam* HI fragment and ORF3, ORF4 and part of ORF5 are found on the *Sal* I fragment. To determine which clone complemented the mutation, MICs for cationic antimicrobials were assayed. These MIC experiments were executed in the absence of tetracycline selection (required for maintenance of the vector pDN18), because in the presence of tetracycline, all the clones, as well as the vector pDN18 appeared to complement the mutation. Under controlled conditions, where each well was inoculated with exactly 10^5 cells/ml, only the pDBS9 mutant clone appeared to show complementation. The MIC for the mutant carrying

Figure 18. Restriction map of the *Bam* HI and *Sal* I fragments and their respective orientations when cloned into pDN18. Panel A: arrows depict the orientation of ORF2 in clones pDBS6 and pDBS9 and the orientation of ORF3, 4 and 5 in clones pDBS1 and pDBS2 with respect to the *lac* promoter on the vector. Panel B: restriction enzyme map of the *Bam* HI and *Sal* I fragments. Clone pBS6 carried the *Bam* HI fragment of DNA that appeared to complement the knock out mutation in mutant 26D7. The *Bam* HI fragment contained two complete putative ORFs, ORF 1 and ORF 2. The *Sal* I fragment also contained two complete ORFs, ORF 3 and ORF 4 and the incomplete ORF5 and did not complement the mutation. The site of the transposon insertion in mutant 26D7 is indicated (▲).
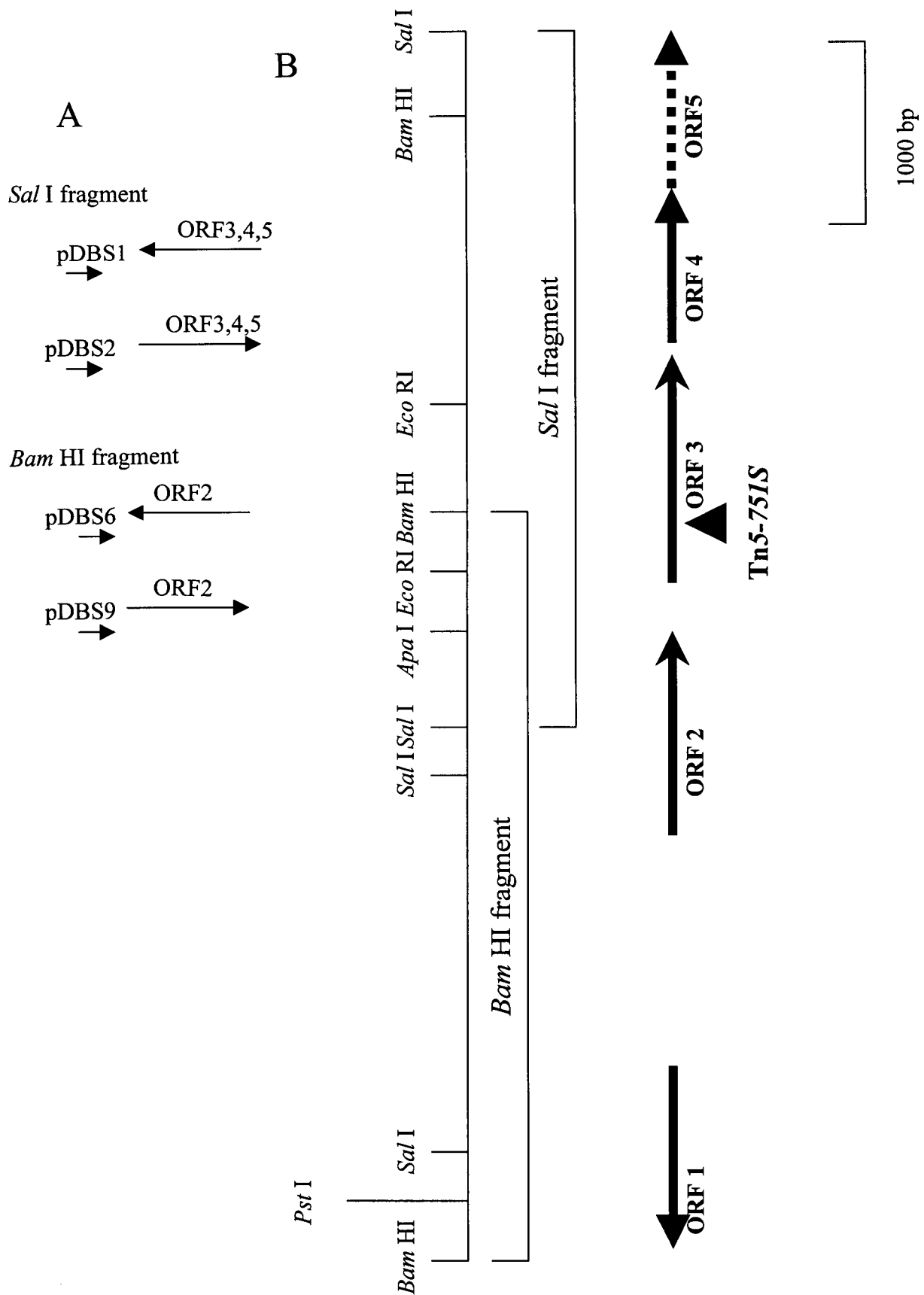
pDBS9 returned to the parent MIC of 64 µg/ml for both polymyxin B and colistin as shown in Table 9. pDBS6 did not complement the mutation in 26D7 and carried the same *Bam* HI fragment as pDBS9 in the opposing orientation (figure 18A). This suggested that the *lac* promoter might be driving gene expression. Mutant 26D7 carrying the complementing vector, pDBS9 showed reduced viability on LB plates and in both LB and Mueller-Hinton broth media with or without tetracycline for selection. In the MIC experiments this reduced viability of 26D7+pDBS9 was observed as reduced growth in all wells. The complemented mutant, 26D7+pDBS9 and the mutant 26D7 carrying the vector pDN18 both showed an increase in MIC of 31 µg/ml to 125 µg/ml to CP26 as shown in table 9. Thus, the presence of the vector cloned into mutant 26D7 caused an increase in MIC to CP26. Finally, all clones showed an MIC of 64 µg/ml for CEME. Therefore the complementing vector pDBS9 had no effect on the MIC for this peptide as shown in Table 9.

Table 9. MICs of cationic antimicrobial agents for the parent *B. multivorans* ATCC 17616, mutant 26D7 and 26D7+pDBS9. The parent strain (ATCC 17616), mutant 26D7 and 26D7 pDN18 and 26D7 pDBS9 (complementing mutant clone) were incubated for 24 hours at 37°C in the presence of increasing concentrations of the cationic antimicrobials: polymyxin B, colistin, a cecropin-melittin hybrid (CP-27) and a modified cecropin-melittin hybrid (CP26). The lowest concentration of antibiotic that inhibited growth of the strains tested was recorded. Results from one of three replicated experiments are shown.

Strain	polymyxin (μg/ml)	colistin (μg/ml)	CP26 (μg/ml)	CEME (μg/ml)
B. multivorans 17616	64	64	>500	64
26D7	8	8	31	64
26D7 pDN18	8	8	125	64
26D7 pDBS9	64	64	125	64
E. coli HB1005	0.5	0.5	15	15

4.7 Discussion

ORF1 predicted protein sequence showed 78% similarity to alkanal monooxygenases of *E. coli* and *Bacillus subtilis*. Since this ORF was transcribed in the opposite orientation, was located distantly upstream of ORF2, ORF3, ORF4 and ORF5 and the transposon was upstream from this ORF by 1,770 base pairs it was unlikely that this gene could have been affected by the transposon insertion. The sequence similarity searches showed a high degree of similarity for several genes that had not been studied functionally but were named based on their similarity to other alkanal monooxygenases. The gapped-BLASTP search of ORF1 showed similarity to these alkanal monooxygenases however the closest similarity to a functionally studied alkanal monooxygenase was relatively low and only across the N-terminal half of the protein. This alkanal monooxygenase gene (*lux*A) from *Vibrio harveyi* encodes for the alpha chain of luciferase (an alkanal monooxygenase) and includes one of the two active centers of luciferase at residues 100-115. Luciferase is an alpha-beta heterodimer monooxygenase that catalyzes the oxidation of a long chain aldehyde and releases energy in the form a visible light. From the results presented here it was not possible to conclude the exact function of ORF1 from *B. multivorans*.

ORF2 showed 45% protein similarity and 30% protein identity to a putative ceramide glucosyltransferase of *Synechocystis sp*. This enzyme has not been studied functionally in *Synechocystis* and was identified as a predicted protein upon sequencing of its entire genome. The mouse, rat, and human ceramide glucosyltransferases were all similar to the *B. multivorans* ORF2. The human ceramide glucosyltransferase has been shown to catalyze the first glycosylation step in the synthesis of glycosphingolipids (Ichikawa, S et al, 1998). Glycosphingolipids are not found in prokaryotes. These are a group of membrane components in eukaryotes that have the lipid portion embedded in the outer plasma membrane leaflet and the

carbohydrate chains extended to the outer environment. Other proteins that were identified in the gapped-BLASTP search with low level similarity were the MigA protein of *P. aeruginosa*, which has a glucosyltransferase function (Wang et al, 1996) and NodC protein of *Rhizobium sp.* NodC acts as an *N*-acetylglucosaminyltransferase, which is involved in the formation of the Nod factor backbone. Nod factors are lipooligosaccharides that contain a chitooligosaccharide chain with three to five *N*-acetylglucosamine residues. This similarity data suggested that ORF2 encoded for a protein product that is involved in sugar transfer. The similarity that ORF2 shared with ceramide glucosyltransferases from eukaryotes could be explained if the protein encoded by ORF2 catalyzes the transfer of a sugar molecule to a lipid molecule. Whether the substrate lipid molecule is lipid A of LPS or a phospholipid found in either the outer or inner membrane remains to be determined. Perhaps the addition of a sugar moiety to this membrane lipid by a phosphodiester link would reduce the negative charge of the lipid, reducing the ability of the cationic peptide to act on the membrane. The addition of a carbohydrate chain to a lipid may also hinder the ability of cationic peptides to access the negative charges found on bacterial lipids.

The phylogenetic tree for relatedness of the sequences identified as similar to *B. multivorans* ORF2 showed that the sequence from *B. multivorans* is relatively closely related to mammalian sequences. The fact that the *B. multivorans* ORF2 sequence seems to be more closely related to the mouse and human sequences than to the three *C. elegans* sequences suggested that this ORF may have had similar constraints on evolutionary divergence as mammalian sequences. However, it is difficult to draw any real conclusions because of the inadequate number of organisms in the tree with which to base conclusions.

The identification of a potential ceramide glucosyltransferase involved in cationic peptide resistance for *B. multivorans* could indicate the possibility that a lipid exists in the membrane of

this organism that is related to eukaryotic ceramide. One of the reasons that cationic peptides have no effect on eukaryotic membranes has to do with their lipid composition. Eukaryotic membranes consist of zwitterionic lipids like ceramide, not the anionic lipids that are seen in prokaryotes. It is interesting that *B. cepacia* has been shown to have a unique ornithine-containing lipid as well as a higher abundance of ornithine-containing lipids in general when compared with *P. aeruginosa* (Kawai et al. 1988). Ornithine is positively charged and as a result ornithine-containing lipids are zwitterionic with an overall positive charge.

ORF3 of *B. multivorans* showed the highest similarity (although only 48%) to the *Pyrococcus horikooshii* hypothetical methyltransferase. The putative Mg-protoporphyrin IX monomethyl ester oxidative cyclase enzymes of both *Methanobacterium thermoautotrophicum* and *Thermotoga maritima* were also identified. None of these have been studied functionally and all were identified as a result of genome sequencing projects and named because of their high sequence similarity with many methyltransferases and, in the cases of *M. thermoautotrophicum* and *T. mariima*, other Mg-protoporphyrin IX monomethyl ester oxidative cyclase enzymes. The more distantly related sequences from *S. wedmorensis* and *S. hygroscopicus* were also identified from this gapped-BLASTP search and these two methyltransferase have been studied functionally. Both of these enzymes have been shown to use methylcobalamin as the methyl donor and the positive charges on the methyl group allow electrophilic attack of the negative charges on the phosphate residue. The sequence similarity of these enzymes from *Streptomyces* with the Mg-protoporphyrin IX monomethyl ester oxidation may be due to the structural similarity of methylcobalamine and protoporphyrin (Hidaka et al., 1995). Thus the similarities are consistent with the suggestion that the *B. multivorans* ORF3 may function as a methyltransferase; however functional studies would be required to confirm that the predicted protein of ORF3 is truly a methyltransferase. It is possible that such a

methyltransferase might function to reduce the negative charge of phosphate residues found on the lipids or LPS of *B. multivorans.*

ORF 4 and ORF5 showed no significant sequence similarity with any known sequences in the gapped-BLASTP search of the NCBI nonredundant database.

The orientation and close proximity of ORF2, ORF3, ORF4 and ORF5 and the identification of the promoter sequence upstream of ORF2 suggested these genes may be members of a novel *B. multivorans* operon. This was supported by the identification of sequence similarity of all four genes with the sequences from *Thiobacillus ferrooxidans.* The contigs identified from *T. ferrooxidans* unfinished genome project, were aligned to determine whether or not they overlapped. The two contigs of the *T. ferrooxidans* genome sequence identified by ORF2 and ORF3 of *B. multivorans* did overlap. Unfortunately only the first 35 amino acids of the ORF3 of *T. ferrooxidans,* which showed 99% similarity with the *B. multivorans* ORF3 sequence, was used in these alignments because the contig was truncated at base 105. The distance between the ORF2 stop codon and ORF3 start codon of *T. ferrooxidans* was 24 base pairs. In this region a putative rho-independent terminator was identified which indicates that ORF2 and ORF3 may be transcribed as separate genes. It is possible that a promoter sequence may be found between these two ORFs but further studies would be required to determine this.

The fact that *T. ferrooxidans* and *B. multivorans* are both members of the beta subdivision of *Proteobacter* may be significant suggesting that grouping of these genes may be conserved within this subdivision. The *T. ferrooxidans* genome project is still in progress and most of the contigs identified were quite short indicating the project is still in its early stages. It will be interesting to determine whether or not these four genes are truly linked and whether or not a second copy of ORF2 exists in this organism as was seen in the complete genome sequences of both *Synechocystis sp.* and *C. elegans.* The *Synechocystis sp.* genome project is

finished and neither of the two putative ceramide glucosyltransferase enzymes identified were linked to a methyltransferase, although two methyltransferases with low sequence similarity to the ORF3 from *B. multivorans* were found in the genome of *Synechocystis sp.*

The *Bam* HI fragment in clone pDBS9 appeared to complement the insertion mutant 26D7 and carried intact ORF1 and ORF2 as well as only part of ORF3. This observation was surprising since ORF3 was the site of the transposon insertion in mutant 26D7. Because ORF1 was present on the complementing fragment, it was not possible to completely eliminate the involvement of ORF1. However, its genetic distance from the transposon insertion site and its orientation make it unlikely that ORF1 is involved in resistance to cationic peptides. ORF2, ORF3, ORF4 and ORF5 were all found in the same orientation and in close proximity of each other as shown in figure 18. Further sequencing downstream of the *Sal* I site is required to identify the stop codon for ORF5 and whether or not more ORFs exist downstream of ORF5. The intergenic region between ORF2 and ORF3 was cloned in pDBS1 and pDBS2 and contained a rho-independent terminator sequence directly upstream of the ORF3 start codon as shown in figure 11. This may explain why pDBS1 and pDBS2 could not complement the mutant phenotype in that this terminator probably inhibited expression of these genes off the *lac* promoter. The inability to clone a large fragment of this entire region into pDN18 (the *Pst* I fragment) may be due to expression of these genes on a multi-copy vector may have a lethal effect on the bacterial cell. The reduction in viability caused by the vector, pDBS9, when cloned into mutant 26D7 suggested that the fragment cloned in pDBS9 expressed ORF2 off the *lac* promoter caused a lethal effect on the bacteria as well. The results presented here suggest that expression of the genes may be tightly controlled and probably maintained at a low level.

The complementation studies suggested that ORF2 alone was capable of returning the mutant phenotype back to the parent phenotype. The ability of ORF2 to complement the mutant

phenotype by itself suggested that over-expression of ORF2 in the mutant may cause phenotypic suppression, overcompensating for the absence of ORF3 expression, ORF3 being the site of the transposon insertion. Complementation studies with ORF3 and ORF4 failed and it was not possible to determine whether ORF3 and ORF4 were expressed in the complementation experiment. However, sequence analysis showed the presence of a terminator upstream of ORF3, which most likely inhibited expression of ORF3 and ORF4 off the *lac* promoter in clone pDBS2. An increase in MIC for both the mutant carrying the vector pDN18 and the mutant carrying the clone pDBS9 was seen for CP26. In this case expression of the tetracycline pump (TetA) in the membrane of the cell may have allowed the mutant to become more resistant to CP26.

A novel group *B. multivorans* genes that may be involved in maintaining high levels of resistance to cationic peptides was identified. The possibility ORF2 may have a glucosyltransferase function was consistent with the identification of sugar transfer to LPS in *S. typhimurium* resulting in resistance to cationic peptides (Helander et al. 1994). The transfer of an Ara4N to a 4'-phosphate residue on lipid A reduces the negative charge of the LPS in *S. typhi* resulting in reduced binding of cationic peptides to the cell (Guo et al. 1994). However, the genes from these *S. typhi* studies are not the same as the ones found in this study. It is possible that the putative glucosyltransferase of ORF2 in *B. multivorans* could be involved in transferring a sugar residue to an inner or outer membrane lipid. ORF3, the site of the transposon insertion in mutant 26D7, was suggested to be a methyltransferase based on sequence similarity with other methyltransferase. The functionally studied methyltransferases that showed sequence similarity with ORF3 are all involved in transfer of a methyl group directly to a phosphate residue as intermediate steps in biosynthesis pathways (Hidaka et al. 1995, Kamigiri et al. 1992). ORF4 and ORF5 had no similarity to any studied proteins but their close proximity to each other and ORF3 suggests that these three genes may be co-expressed off a common promoter upstream of

ORF3. Knock out mutants directed in the four ORFs by allelic exchange would help identify which of the identified genes is important for resistance to cationic peptides or whether all of the genes found downstream of the transposon insertion are important for this phenotype. It would also be interesting to do PCR cloning of these ORFs, promoter regions and terminator to study where the promoter for these genes may be and whether the rho-independent terminator functions as a terminator between ORF2 and ORF3 such that they are expressed as separate genes or whether or not it functions as an attenuator and these genes are actually members of an operon.

Chapter 5 Phenotypic Characterization of Mutant 26D7

5.1 Basic Biochemistry and Microbiology

Gram stains of *B. multivorans* ATCC 17616, mutant 26D7 and randomly selected mutant 41E1 indicated a short Gram-negative rod (not shown). Biochemical tests were also done on *B. multivorans* ATCC 17616 and compared with mutant 26D7, the vector containing mutant, 26D7+ pDN18 and 26D7+pDBS9. Carbon assimilation tests were done. All 12 carbon-containing compounds listed in table 10 were assimilated by *B. multivorans*. Oxidation of glucose, maltose, lactose, adonitol and sucrose were tested and only sucrose could not be oxidized by *B. multivorans* ATCC 17616. Lack of sucrose oxidation is typical of genomovar II and genomovar IV strains of the *B. cepacia* complex. Lysine decarboxylase is found in all genomovars of the *B. cepacia* complex however, unlike the other genomovars, in general only half of the members of *B. multivorans* have this enzyme. *B. multivorans* ATCC 17616 strain was positive for lysine decarboxylase. Nitrate reduction to nitrites was observed and is typical for *B. multivorans* (Deborah Henry, University of B.C., personal communications). *B. multivorans* ATCC 17616 was negative for indole production, glucose acidification, urease production, β-galactosidase (esculin and PNPG reactions were negative) and protease (gelitine reaction was negative). No change in biochemistry was shown as a result of the transposon insertion or complementation with pDN18 as the vector as shown by comparison of the parent strain, mutant 26D7, 26D7+pDN18 and 26D7+pDBS9. All biochemical results were consistent with those for RAPD type 30 of *B. multivorans* or genomovar II of the *B. cepacia* complex (Mahenthiralingam, unpublished results). In particular genomovar II strains do not oxidize or ferment sucrose (Henry et al., 1997).

Table 10. Carbon assimilation and biochemical tests for *B. multivorans*.

	ATCC 17616	Mutant 26D7	26D7 pDN18	26D7 pDBS9
Assimilation of:				
Glucose	+	+	+	+
Arabinose	+	+	+	+
Mannose	+	+	+	+
Mannitol	+	+	+	+
N-acteyl-glucosamine	+	+	+	+
Maltose	+	+	+	+
Gluconate	+	+	+	+
Caprate	+	+	+	+
Adipate	+	+	+	+
Malate	+	+	+	+
Citrate	+	+	+	+
Phenyl-acetate	+	+	+	+
Oxidation of:				
Glucose	+	+	+	+
Maltose	+	+	+	+
Lactose	+	+	+	+
Adonitol	+	+	+	+
Sucrose	-	-	-	-
Lysine decarboxylation	+	+	+	+
Nitrate reduction	+	+	+	+
Indole production	-	-	-	-
Glucose acidification	-	-	-	-
Arginine dihydrolase	-	-	-	-
Urea	-	-	-	-
Esculin	-	-	-	-
Gelatine	-	-	-	-
PNPG*	+	+	+	+
oxidase	+slow	+slow	+slow	+slow

*p-nitrophenyl-β-D-galactopyranoside

5.2 Neutrophil Killing Assay

The *in vitro* chronic granulomatous disease (CGD) neutrophil killing assay was performed for both the mutant and parent strain in the presence of R5 serum (complement component 5 deficient). CGD neutrophils were incubated at a ratio of 1:1 with bacteria. CGD neutrophils were able to kill *P. aeruginosa* M2 by reducing the log colony forming units by one log (6.29 to 5.25) in two hours. The positive control *B. multivorans* JTC, the parent strain *B. multivorans* ATCC 17616 and the mutant 26D7 were able to grow in the presence of CGD neutrophils as shown in figure 19. Thus mutant 26D7 remained resistant to killing by CGD neutrophils. All strains of bacteria were killed equally well by normal neutrophils.

5.3 Outer Membrane Permeability

NPN is a hydrophobic compound that fluoresces weakly in a hydrophilic environment but strongly in a hydrophobic environment. The outer membrane of Gram-negative bacteria acts as a barrier to uptake of such hydrophobic compounds as NPN. Polymyxin B and other polycationic compounds can interact with the divalent cation LPS binding sites of the outer membrane resulting in weakening of this barrier, allowing uptake of NPN into the bacterial cell membrane which is measured by an increase in NPN fluorescence. This has been demonstrated for both *P. aeruginosa* and *E. coli* (Hancock, 1984, Loh, 1984). *B. cepacia* is resistant to membrane permeabilization by polymyxin B and other polycations (Moore, 1986). Since mutant 26D7 had an 8-fold reduction in MIC to polymyxin compared to the wild-type parental strain it was important to determine whether the outer membrane permeability of mutant 26D7 had changed. The mutant and the parent strain were exposed to increasing concentrations of polymyxin in the presence of NPN. The results in figure 20 show that the parent strain was resistant to permeabilization by NPN even in the presence of high concentrations of polymyxin

CGD Neutrophil Killing Assay
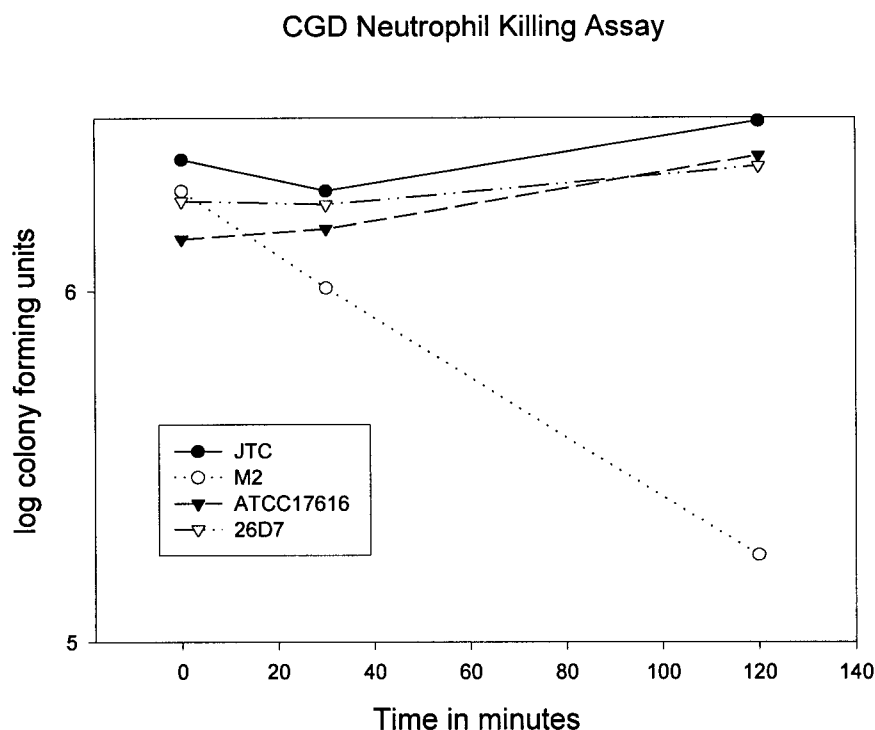
Figure 19. CGD neutrophil killing assay for *B. multivorans* ATCC 17616 and mutant 26D7. CGD neutrophils were incubated at 37°C 1:1 with bacteria and samples were taken at 0, 30 and 120 minutes. Viable colony counts were determined for each time point and log to the base ten recorded. JTC, *B. multivorans* strain JTC; M2, *P. aeruginosa* strain M2; ATCC 17616, *B. multivorans* parent strain; 26D7, *B. multivorans* transposon mutant. JTC is resistant to killing by CGD neutrophils and was used as the positive control. M2 is sensitive to killing by CGD neutrophils and was used as a negative control. No difference in killing by CGD neutrophils was observed for the parent and the mutant strains.
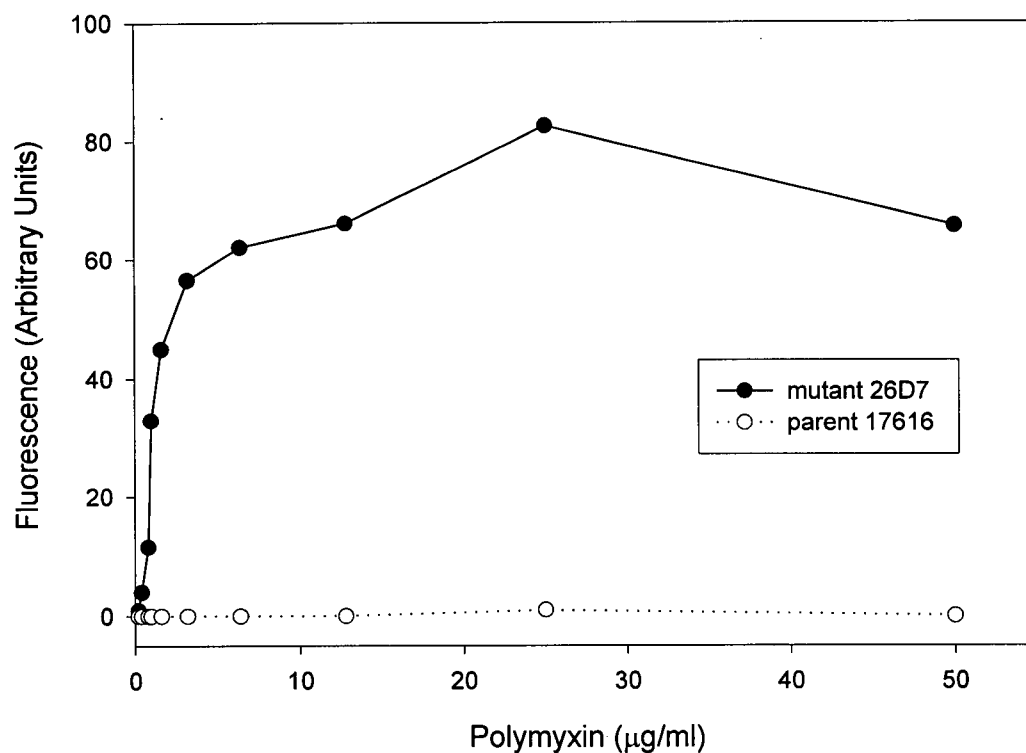
Figure 20. The effect of increasing concentrations of polymyxin B on the outer membrane permeability of *B. multivorans* ATCC 17616 and mutant 26D7. Polymyxin B was titrated into a cuvette containing 1.0 ml of intact bacterial cells OD_{600} 0.5 in HEPES buffer (pH 7.2) containing 5 μM CCCP and 10 μM NPN. NPN fluoresces in a hydrophobic environment. An increase in change in fluorescence corresponds directly to an increase in the uptake of NPN into the outer membrane. Mutant 26D7 showed an increased permeability to NPN in the presence of 1.6 μg/ml polymyxin while the parental strain remained impermeable at 50 μg/ml (one of three replicate experiments shown).

since no increase in fluorescence was observed. In contrast, the mutant was permeable to NPN in the presence of 1.6 μg/ml of polymyxin as shown by the large increase in fluorescence at concentrations of 1.6 μg/ml and higher. The transposon insertion in mutant 26D7, therefore, had changed the outer membrane permeability such that in the presence of polymyxin, NPN was able to penetrate the bacterial cell.

5.4 Dansyl-polymyxin binding to the Outer Membrane

Interaction of dansylated polymyxin (DPX) with LPS has been shown to enhance fluorescence of the fluorescent antibiotic. DPX has been shown to bind purified LPS but not whole cells of *B. cepacia* (Moore et al., 1986). Since mutant 26D7 is more susceptible to lysis by polymyxin B and more permeable in an NPN assay, it was important to determine whether the mutant was better at binding DPX than the wild-type parent strain. As expected, there was no increase in fluorescence when whole cells of the parent strain ATCC 17616 were exposed to DPX when compared with buffer and DPX alone as shown in figure 21. The mutant 26D7, like the parent strain also showed no increase in fluorescence when exposed to DPX. This suggested that intact cells of the polymyxin susceptible mutant, 26D7 remained incapable of binding DPX. Since DPX is an LPS-binding probe, it could be tentatively concluded that no obvious alteration in LPS had occurred.

5.5 Membrane Proteins

Membrane proteins were isolated as described in section 2.17 and separation into inner and outer membrane fractions was attempted on a sucrose gradient. Separation of inner and outer membrane proteins was crude and a clear separation was not seen for the parent and mutant strain. The parent strain ATCC 17616 and mutant 26D7 had a total of five membrane fractions loaded in lanes 2 to 7 and 10, 11 on the gel in figure 22. Samples were alternated for easy

Dansyl-polymyxin Binding Assay
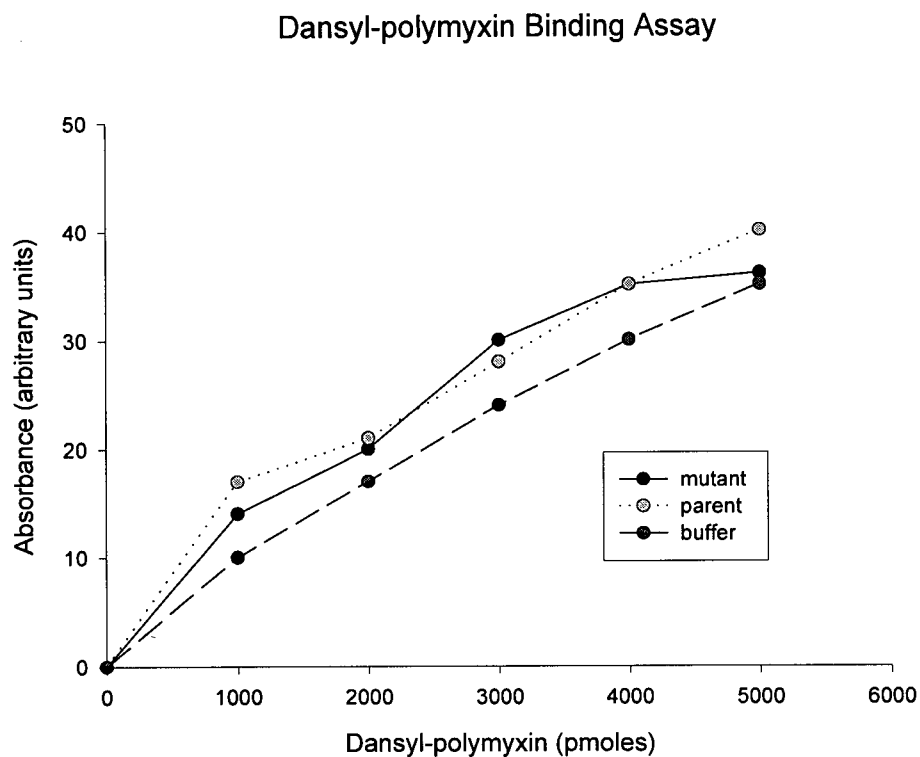
Figure 21. Binding of dansyl-polymyxin (DPX) to intact parent and mutant cells. DPX was titrated into a cuvette containing 1.0 ml of 5 mM HEPES buffer (pH 7.35), 5.0 mM CCCP and cells with an OD_{600} of 0.250. The whole cells of both the mutant and parent *B. multivorans* did not bind DPX as indicated by the absence of increase in fluorescence by DPX upon addition to cells when compared with the cuvette containing buffer alone.

Figure 22. Outer and inner membrane protein profiles of the parent strain, mutant 26D7, 26D7pDBS9 and 26D7pDN18. Outer and inner membrane proteins were purified as described in section 2.17. The major OMPs of *B. multivorans* are marked on the right. Lanes alternate between the parent and mutant strains for direct comparison. Samples were not equally loaded and the gel was coomassie blue stained. 26D7pDN18 and 26D7 pDBS9 are run side by side to for comparison between complemented mutant and mutant with vector. Lane 1 corresponds to the biorad broad range markers described in section 2.19. Lane 2, 4, 6, 10 are the parent ATCC 17616; Lane 3, 5, 7 are mutant 26D7; Lane 8, 12 are 26D7pDN18, Lane 9, 13 are 26D7pDBS9. Arrows point to the region in the gel where differences are observed.
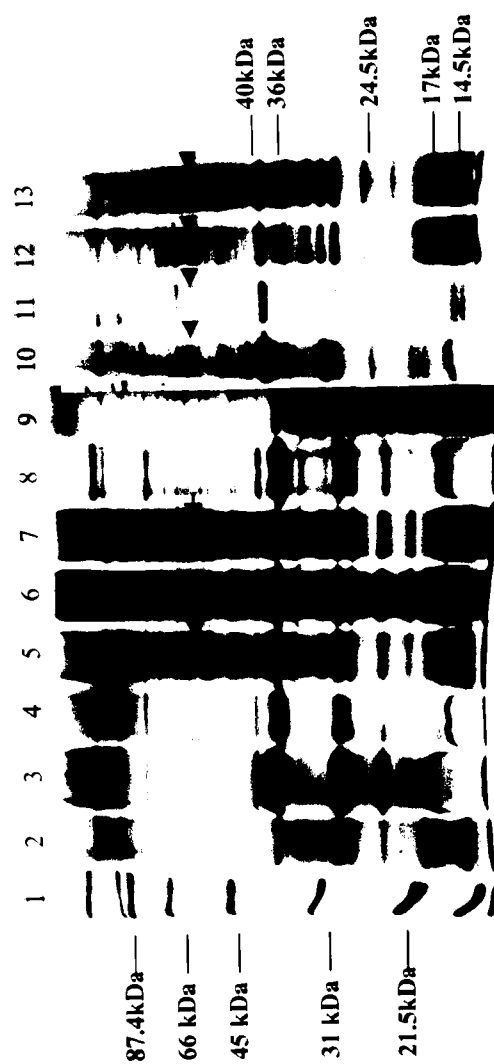
comparison between mutant and parent strains and between the complemented mutant (26D7pDBS9) and the mutant that carried the vector alone (26D7pDN18). Careful inspection suggested that there was a minor membrane protein of approximately 55-kDa missing in mutant 26D7 (lanes 3, 5, 7 and 11) and present in the parent (Lanes 2, 4, 6 and 10) indicated by an arrow in figure 22. This 55-kDa protein was absent in the membrane fractions of 26D7pDBS9 (Lane 9 and 13) and 26D7pDN18 (Lane 8 and 12). This minor membrane protein was the right size for the putative gene product of the putative ORF3, predicted to be 54.7-kDa however, further studies would be required to confirm whether this protein actually corresponds to the ORF3 predicted protein product. These findings also contradict the PSORT and ProteinPredict results that suggested that ORF3 encoded for a cytoplasmic protein. The complementing fragment in pDBS9 did not carry ORF3 explaining why both the mutant carrying the vector pDN18 and the mutant carrying the complementing clone, pDBS9 both lacked the 54.7kDa protein band in figure 22. Below the 54.7-kDa band there appeared to be a more significantly expressed protein found only in lanes 3, 5, and 7 corresponding to the mutant and not found in lanes 2, 4 and 6 corresponding to the parent strain. This was in the correct size range for flagellin protein, which is 50-kDa in size. Electron micrographs of the mutant and the parent strain shown in figure 23 indicated that the mutant did express flagella but that the parent strain did not. This correlated well with the observations in the protein gel in figure 22.

5.6 Lipopolysaccharide Ladders

LPS profiles of the parent strain, mutant 26D7, 26D7+pDN18, 26D7+pDBS9, random mutant 41E1, ATCC 17616+pTGL166 showed that the profiles for all strains were the same (figure 24). The parent strain *B. multivorans* ATCC 17616 is susceptible to human serum and does not have O-antigen present on its LPS so a serum resistant *P. aeruginosa* PAK was included in lane 7 and showed a complete LPS ladder. The core region of the *B. multivorans* strains shows a doublet

A B
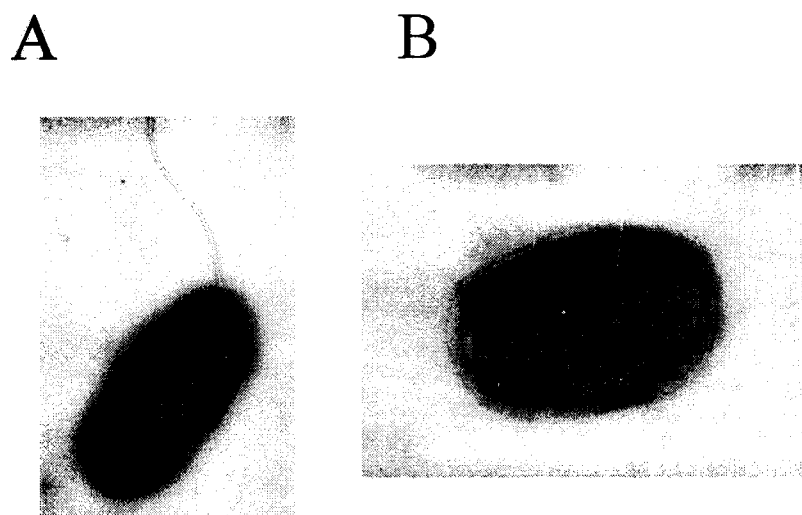
Figure 23. Electron microscopy of *B. multivorans* parent strain and mutant 26D7. Panel A is mutant 26D7 showing the presence of a flagellum as seen on the mutant. One half of the mutant bacteria did carry a flagellum. Panel B is the parent strain showing no flagellum, and none of the parent strain seen had flagella.
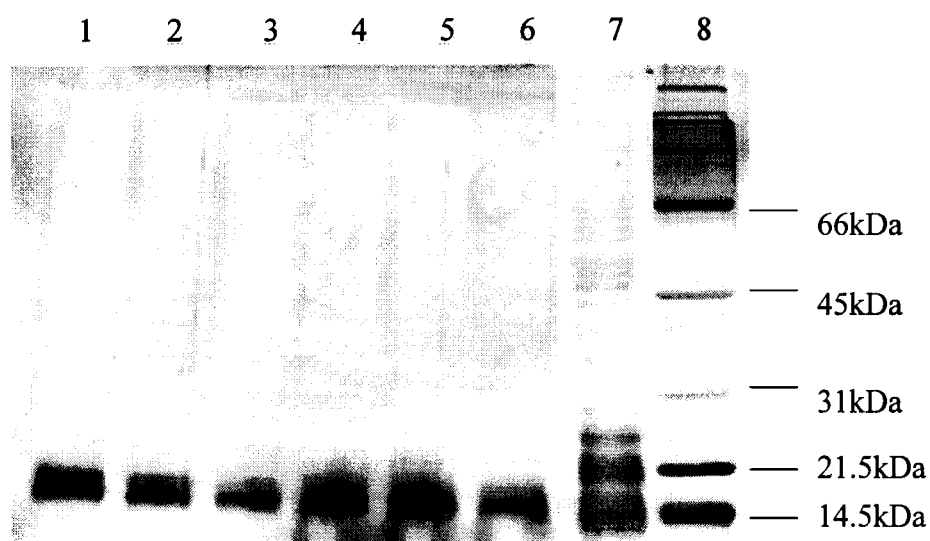
Figure 24. Lipopolysaccharide profiles for parent strain *B. multivorans* ATCC 17616 and mutant strains. Whole cell lysates were prepared as described in section 2.18 and silver stained specifically for polysaccharides as described in section 2.19. 1.0 µl of the whole cell lysate for each sample was loaded on a 12.5% polyacrylamide gel except for the *P. aeruginosa* sample for which 5.0 µl of the whole cell lysate was loaded. Lane1, 26D7pDBS9; Lane 2, 26D7pDN18; Lane 3, 26D7; Lane 4, 41E1, Lane 5, ATCC 17616pTGL166; Lane 6, ATCC 17616; Lane 7, *P. aeruginosa* PAK; Lane 8, broad range protein standards (BioRad).

band as shown in figure 24, quite different from that of *P. aeruginosa* PAK which showed the typical three banding pattern (lane 7 in figure 24).

5.7 Thin Layer Chromatography

Extractable lipids were purified from *B. multivorans* ATCC 17616 and mutants 26D7 and 41E1 as described in section 2.21. Two-dimensional TLC was performed on 10 µg of extracted lipids as described in section 2.22. Mutant 41E1, a random mutant selected from the transposon insertion library, did not have reduced resistance to polymyxin. Panel C were the control lipids, cardiolipin (CL), phosphatidyl glycerol (PG) and phosphatidyl ethanolamine (PE). Mutant 41E1 and the parent strain showed identical 2D TLC patterns confirming that the transposon insertion alone did not effect the extractable lipid composition of *B. multivorans* (panels B and D, figure 25). Mutant 26D7 appeared to have a possible different or changed lipid beneath PE-1 (marked as A in figure 25). Spot A was consistently present in repeated 2D TLCs of the mutant as shown in panel A. Spot A would occasionally appear in the parent 2D TLC as shown in panel D, however, it was always much less abundant when compared to the mutant 2D TLC. It was also interesting that the PE-1 spot in the mutant always appeared to be less than that for the parent strain (compare panel A and B) even though the plates were always loaded equally with 10 µg of total lipid. Also, marked B in the same figure, mutant 26D7 lacked an unknown glycolipid. All the lipids in these chromatograms were identified by comparison with the control lipids (panel C) as well as comparison with published 2D chromatograms (Yabuuchi et al., 1992 and Kawai et al., 1988).
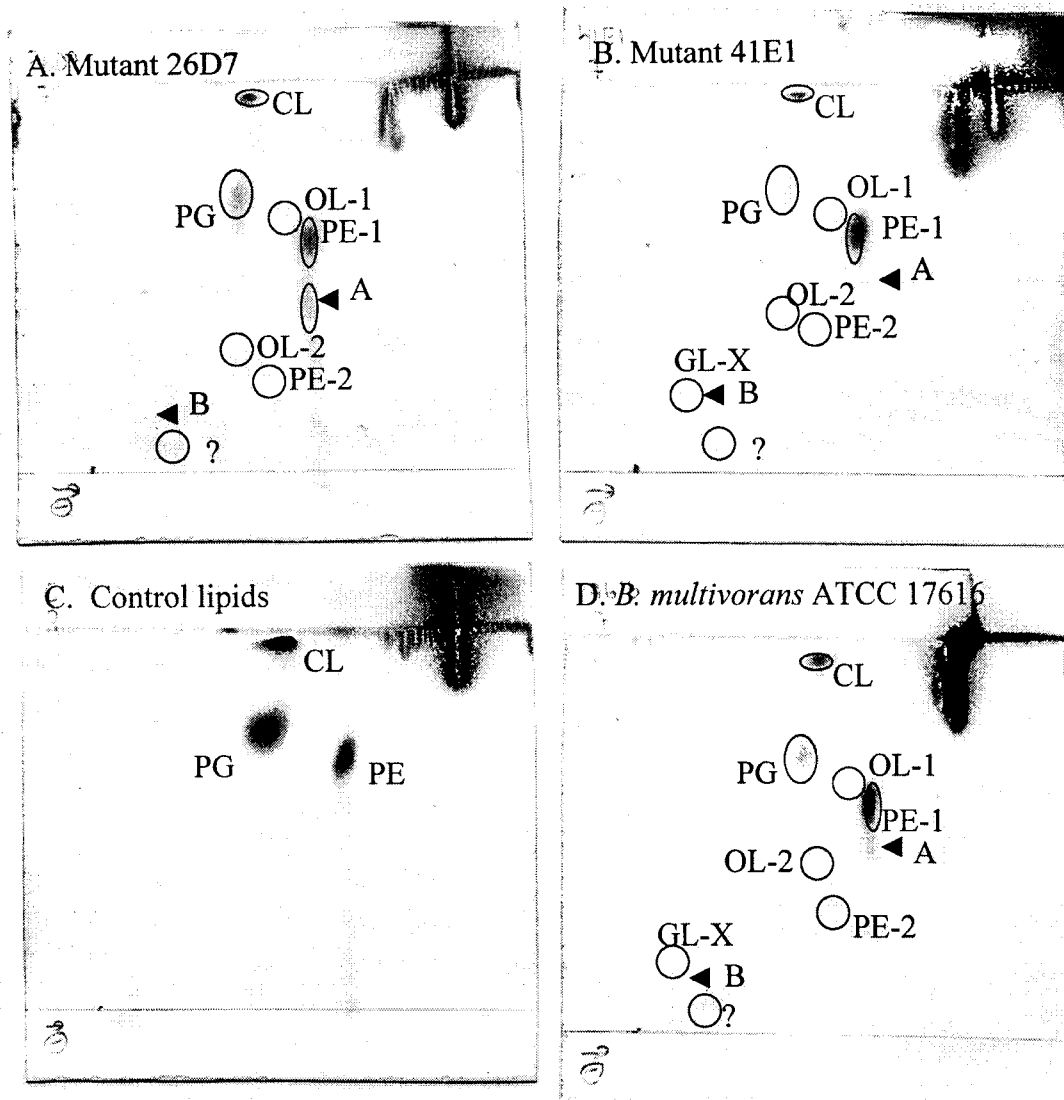
Figure 25. Two-dimensional thin-layer chromatograms of total extractable lipids. Arrow A points to the new lipid or shifted lipid in mutant 26D7 but not the parent or mutant 41E1. Arrow B points to the glycolipid that is absent in mutant 26D7. CL, cardiolipin (diphosphotidylglycerol); PG, phosphotidylglycerol; OL-1 and -2, ornithine containing lipids 1 and 2, PE-1 and -2, phosphotidylethanolamine; GL-X, unknown glycolipid; ? Unknown lipid. The solvent in the horizontal direction was chloroform/methanol/water (65:25:4) and in the vertical direction chloroform/methanol/acetic acid (65:25:10).

5.8 Discussion

Chronic granulomatous disease (CGD) neutrophils are dependant on nonoxidative killing mechanisms and resistance of *B. multivorans* and other members of the *B. cepacia* complex to CGD neutrophils was considered a possible mechanism of pathogenesis. The mutant identified here with enhanced susceptibility to cationic peptides was tested in a neutrophil killing assay to determine whether CGD neutrophils were capable of killing this relatively susceptible mutant 26D7. This mutant was found to be equally resistant to killing by CGD neutrophils as the parent strain and the control *B. multivorans* JTC strain. This supported that the MIC of 8 μg/ml to polymyxin B for this mutant was still resistant in comparison with other bacterial strains such as *P. aeruginosa*. This can be compared to the 12 strains of *P. aeruginosa* tested by Speert et al, 1994, for which all strains were susceptible to killing by CGD neutrophils.

Studies of the effect of polymyxin B on the permeability of the outer membrane of *B. multivorans* and *B. cepacia* show that these strains of bacteria were resistant to the effects of polymyxin B on NPN uptake. Even at extremely high concentrations of polymyxin B, these bacterial cells (Moore et al. 1984) never took up NPN. When the mutant strain was tested in this same assay for outer membrane permeability only low concentrations of polymyxin B were required for the mutant cells to become permeabilized by NPN. This permeability change was not accompanied by an increased ability of dansylated polymyxin B to bind intact mutant cells as is seen in other organisms such as *P. aeruginosa* (Loh et al., 1984). Neither the parent nor the mutant strain was able to bind dansylated polymyxin B. This suggested that the phenotypic increase in permeability of the mutant was not accompanied by a change in LPS that might affect binding to polymyxin B. LPS ladders were identical for the mutant and parent strain. A minor LPS modification such as a single sugar modification might not be easily observed on this kind of a gel nor would I expect to see the effects of a change in LPS charge.

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Membrane proteins were compared for the mutant and parent strains as well as the complemented mutant. A minor membrane protein that was missing in the mutant strain corresponded to the predicted size of the putative protein product from ORF3 of 54.7-kDa. This contradicted the PSORT data and ProteinPredict data that suggested ORF3 is a cytoplasmic protein. The transposon had inserted into the coding region of this ORF supporting its absence in the mutant strain. The complemented mutant was missing this same protein which corresponds with the fact that this ORF was not found on the DNA cloned in pDBS9. Further studies are required to determine whether or not ORF3 corresponds to a membrane protein and whether or not it exists as an inner membrane protein or outer membrane protein. Similar studies would be interesting for the ORF2 gene product since this was predicted to be an inner membrane protein. Good separation of inner and outer membrane protein fractions of *B. multivorans* would be required. This could be done by sucrose-gradient isopycnic centrifugation, followed by collection of several fractions from that gradient. To determine which sucrose gradient fraction contains inner or outer membrane proteins, the following experiments could be done. Each fraction isolated can be tested for NADH oxidase activity, which is an inner membrane marker. Separation of each fraction by SDS-PAGE followed by transfer to a membrane for immunobloting with an antibody against any one of the five OMPs identified for *B. mutivorans* (Anwar et al. 1993) could be done. This and KDO assays (specific for the fractions containing LPS) could act as outer membrane markers. Determination of which marker is associated with which fraction and then identification of which fractions the ORF2 and ORF3 proteins are associated would help identify the cellular localization of ORF2 and ORF3.

The expression of flagella in the mutant but not the parent strain was probably due to the presence of the transposon and the mutagenesis methods because a randomly chosen mutant also expressed flagella. Regulation of promoter transcription of flagella biosynthesis is complex and

is organized into a transcriptional hierarchy of three classes of genes and several sigma factors are involved. Each later class of genes requires al the genes of the previous class to be expressed. Class 1 genes are regulated in response to numerous environmental stimuli such as cAMP levels and temperature. These gene products interact to activate class 2 promoters, which encode the proteins needed for assembly of the basal body-hook plus another regulatory gene *fliA*. This gene product encodes for an alternative σ factor required for transcription from Class 3 promoters. These are the genes required for the final stages of flagellar assembly and the chemotactic signal transduction pathway (Hughes et al. 1998). The most likely explanation for flagellin expression in mutant 26D7 and the randomly selected mutant, is that these mutants underwent a genetic modification at elevated temperatures since during the mutagenesis procedure this strain was incubated at 47°C for 60 hours.

It appeared that when the 2D TLCs of the parent and mutant strain were compared, there might be a shift in PE-1 in the mutant or possibly a different lipid such that it does not travel as far in the second solvent. If a sugar could not be transferred onto either PE-1 or a lipid that travels to the same spot, the mobility of this lipid would change. As well, there was an unknown glycolipid missing in the mutant TLC when compared with the parent TLC. This lipid was identified as an unknown glycolipid by Yabuuchi et al, 1992 using 2D TLC and was not seen in *B. mallei, B. gladioli* or *B. solanacearum*. In another study, an unknown glycolipid was also seen in 2D TLC unique to *B. cepacia* when compared with *P. aeruginosa, P. Fluorescens* and *P. stutzeri* (Kawai et al, 1988). These observations suggest that a unique lipid may exist in the membrane of *B. cepacia* that is glycosylated and may be absent in the mutant identified in this study. Other studies have shown that *B. cepacia* membranes contain approximately 4 times less phosphatidyl glycerol than *P. aeruginosa* and that *B. cepacia* contains an 'ornithine containing' lipid and a glycolipid not found in *P. aeruginosa* (Kawai et al. 1991). Mass spectrophotometry

of all the spots on the TLC plates would give a more definitive answer as to whether these lipid differences in the mutant are significant and how they relate to the phenotype of reduced resistance to cationic peptides.

Mutant 26D7 has a reduced MIC to polymyxin B but was still resistant in the CGD neutrophil assay, showed no change in LPS ladder and no change in LPS binding of DPX. The mutant did show a change in membrane permeability and a change in lipid profile on a TLC plate. This suggested that the permeability change observed in the NPN assay and the reduced MIC to polymyxin B may be due to a lipid change on the inner or outer membrane, perhaps independent of LPS.

The proposed mechanism of action of cationic peptides on Gram-negative bacteria has been proposed to involve, in order, LPS binding, outer membrane permeabilization and then cytoplasmic membrane permeabilization. The observations here are consistent with the conclusion that the mutation in mutant 26D7 has affected the membrane permeabilization step. A second mutation in the LPS of *B. multivorans* to increase the binding ability of cationic peptides may be required for full susceptibility. Perhaps the ability of CEME to enter the bacterial cell is more dependent on its enhanced ability to bind to the LPS of intact cells than CP26. Perhaps, a change in binding to LPS would have been required to see a change in MIC for CEME and CP26, rather than only CP26.

Chapter 6 General Discussion

Transposon insertional mutagenesis was employed here to construct a mutant of *Burkholderia multivorans* rendered susceptible to killing by cationic peptides. Only three transposon insertion systems had been shown to be successful in producing mutant libraries of *B. cepacia* complex organisms at that time (Jayaswal et al., 1991, Pidcock et al., 1988, Cheng, et al., 1994). All of the *B. cepacia* strains that were tested as candidates for transposon mutagenesis showed high levels of resistance to aminoglycosides. Both Tn5 (Pidcock et al., 1988) and Tn5-259 (Jayaswal et al., 1991), carry the kanamycin resistance cassette as the selectable marker and were therefore not useful for the strains in this study. The method of Cheng et al, 1994 was employed here because the vector carried a tetracycline cassette and the transposon carried a trimethoprim cassette both antibiotics of which ATCC 17616, the strain chosen in this study, was susceptible to. Other transposon mutagenesis systems tried were the mini-tn5 transposon derivatives of de Lorenzo et al., 1990. After several different attempts at mutagenesis one library was constructed in *B. multivorans* strain ATCC 17616. Two other clinical strains also had susceptibility to trimethoprim and tetracycline but transposon insertion never worked. Transposon mutagenesis is known to be extremely difficult in *B. cepacia* complex organisms and successful attempts have only been documented twice since the start of this project (Abe et al., 1996 and Lewenza et al., 1999).

Screening of 6,000 mutants from the *B. multivorans* ATCC 17616 insertion library resulted in the identification of 40 putative polymyxin susceptible mutant. Mutants identified in the screen were tested in a standard MIC assay. Only 8 showed a significant reduction in MIC to polymyxin (greater than 2-fold) when compared to the parent strain and only one mutant (26D7) was selected for further characterization. It would be interesting to sequence the DNA at the

transposon insertion site in those other mutants identified in Figure 7. This would confirm whether those mutants that appeared to have the transposon inserted into the same site as 26D7 by Southern analysis were siblings of 26D7, novel insertions in the same region of DNA or novel insertions in completely different sites on the chromosome of *B. multivorans*. At the time of insertional mutagenesis, the cells were grown for 60 hours at 47°C in order to see loss of the plasmid pTGL166. This allowed for the formation of siblings to be a likely event. Since the rate of spontaneous mutagenesis in *B. cepacia* complex organisms is 2 in 1×10^6 cells grown in the presence of selection pressure it is not likely that the mutants in this study are a result of spontaneous mutagenesis (van Waasbergen et al. 1998). The mutants in this study were selected by screening and never saw selection pressure. One mutant out of 6,000 was selected for study and it is unlikely that this mutant is a result of a spontaneous mutation at another site in the genome.

In order to try and link the phenotypic change in mutant 26D7 to the transposon insertion, complementation of the mutant phenotype was attempted. Since electroporation experiments failed with strain ATCC 17616 a mobilizable vector was required. Vector pDN18 was chosen because it carried a tetracycline cassette, the only antibiotic to which 26D7 was sensitive. Two separate fragments were cloned into pDN18, one carrying ORF1 and ORF2 and the other fragment carried part of ORF2, ORF3, ORF4 and part of ORF5. ORF1 and ORF2 appeared to complement the mutant in an MIC assay with polymyxin however the transposon insertion was within ORF3 and the fragment containing this ORF did not complement the mutation in 26D7. Since it was not possible to make a direct conclusion from these complementation experiments other methods will have to be employed to try and confirm that the transposon insertion caused the phenotypic change in mutant 26D7. Allelic exchange is known to be difficult in *B. cepacia* complex organisms and has only just been reported in the literature for the first time (Lewenza,

S. et al. 1999) using the suicide vector pEX18Tc. With availability of this vector, it may be possible to try allelic exchange in 17616 to knock out the putative ceramide glucosyltransferase, putative methyltransferase or any of the putative genes downstream of the transposon insertion. Knock out of these different genes would aid in identifying exactly which of the ORFs identified near the transposon insertion was required for cationic peptide resistance, NPN permeability and changes in TLC patterns.

The transposon had inserted itself into ORF3, which was upstream of several closely linked genes with the putative ribosome binding sites for these ORFs overlapping with the upstream coding regions. Further studies to determine whether or not a promoter region is upstream of ORF3 would be required. These concerns could be addressed by cloning potential promoter regions next to a reporter gene, such as *lacZ*, however these experiments work best with constitutively active promoters which may not be the case here. It would also be interesting to do Northern blot analysis to determine the length of the mRNA transcript that the ORF3 gene is on and which of the genes (using a different probe specific for each gene) near the transposon insertion are found on this transcript. To determine if ORF2 is co-expressed with ORF3 and the downstream genes, one could delete or knock out by allelic exchange the promoter region upstream of ORF2 and look for changes in ORF3 expression on a protein gel. Since cloning of the genes in this region proved to be extremely difficult and the results here suggested that these genes expressed off a vector may be lethal, these cloning experiments could be difficult to perform.

The GC content of ORF3 at 62% was considerably lower than that expected for *B. multivorans*, which has an average GC content of 72%. This suggests possible gene transfer of this methyltransferase from another organism with a lower GC content, however the codon usage in this ORF was found to be very similar to that expected *B. cepacia*. IS element sequences are

usually associated with recently transferred genes on the outside ends of the gene sequence. It was observed that a methyltransferase is found downstream of a ceramide glucosyltransferase in *T. ferroxidans* but none of the other organisms in the phylogenetic tree (Fig. 15). With the completion of more genome sequencing projects it will be interesting to see whether identified ceramide glucosyltransferases are found in close proximity to methyl transferases.

At this point the most compelling evidence to support that the transposon insertion is the cause of the phenotypic change in mutant 26D7 is that the identified genes encode for proteins that probably play a role in lipid modification. ORF2 encoded for a predicted protein with sequence similarity to ceramide glucosyltransferase, which is known to be important for the transfer of a glucose residue to a ceramide, a lipid found only in eukaryotes. ORF3, a putative phosphomethyltransferase could potentially be involved in transferring a methyl group to phosphate residue on a lipid. The modification of lipids, specifically LPS, has already been shown to be important for resistance to cationic peptides. The transfer of a sugar residue to a phosphate residue in LPS causes resistance to cationic peptides in *S. typhimurium* (Guo et al. 1998). Addition of a palmitate to LPS in both *S. typhimurium* (Guo et al. 1997) and *P. aeruginosa* (Ernst et la. 1999) resulting in a hepta-acylated form of LPS, increases the resistance of both these organisms to cationic peptides. In two closely related organisms, knock out of LPS structural genes has been associated with loss of resistance to cationic peptides. In *R. solanocearum* a heptosyl transferase (*rfa*A) was identified as being involved in resistance to cationic peptides from plants. The *rfa*A gene is involved in LPS core biosynthesis. In *B. pseudomallei* it has also been shown that genes involved in LPS core oligosaccharide biosynthesis are important for resistance to cationic peptides (Burtnick et al., 1999). This group also identified a second group of genes that affect the *lytB* gene, the product of which is known to affect phospholipid and peptidoglycan synthesis. The data collected here suggests that the

architecture of the *B. multivorans* cell prevented cationic peptides from interacting with target groups of either LPS or phospholipids. This was seen by the change in outer membrane permeability of the mutant as seen in the NPN assay and the changes seen in the lipid profiles of the mutant when compare with the parent by 2D TLC. Further characterization of these lipid changes will be required to determine the exact lipid change in the mutant. Mass spectrophotometry of the spots on the TLC plates will aid in identifying the exact change that has occurred in the mutant.

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APPENDIX A: MEDIA

Luria Britani Broth and Agar

NaCl	10.0g
Bacto-yeast extract	5.0g
Bacto-tryptone	10.0g

make up to 1.0 Litre with distilled water
add 15.0 g granulated agar for solid media
sterilize by autoclaving

Mueller-Hinton Broth

soluble starch	1.5g
casamino acids	17.5g
Beef Infusion	300.0g

make up to 1.0 Litre with distilled water
sterilize by autoclaving

SOB

Bacto-tryptone	20.0g
Bacto-yeast extract	5.0g
NaCl	0.5g

make up to 1.0 Litre with distilled water
sterilize by autoclaving

Minimal Salts (10X)

dipotassium orthophosphate (K_2HPO_4)	105.0g
potassium phosphate (KH_2PO_4)	45.0g
ammonium suphate	10.0g
sodium citrate	5.0g

make up to 1.0 Litre with distilled water
sterilize by autoclaving

Supplemented Minimal Salts Media

Minimal Salts	100.0ml
Casamino Acids (2.0%)	10.0ml
Mannitol (5.0%)	100.0ml
Distilled water	780.0ml
Agar	15.0g

mix agar and distilled water
sterilize by autoclaving
cool to 56°C
add sterile minimal salts, casamino acids and mannitol warmed to 56°C to the sterilized water-agar.

APPENDIX B: TABLE OF PRIMERS

Table 11. Primer sequences.

PRIMER	SEQUENCE
Primers specific for sequencing off the end of Tn*5-751*	
Tn5-1	5'-GAG-GTC-ACA-TGG-AAG-TCA-G-3'
DS-1	5'-GTC-ACA-TGG-AAG-TCA-GAT-C-3'
Neomycin (kanamycin) cassette primers	
N727-3	5'-ATC-GAC-AAG-ACC-GGC-TTC-CAT-CCG-A-3'
N725-5	5'-TCA-GCG-CAG-GGG-CGC-CCG-GTT-CTT-T-3'
Tetracycline cassette primers	
Tet-1	5'-CAC-CAA-CTT-ATC-AGT-GAT-AAA-GA-3'
Tet-2	5'-AGT-TGC-ATG-ATG-AAG-AAG-AC-3'
Sequencing primers	
Seq1R	5'-GCG-CTT-CTT-ATG-CTC-GAT-3'
Seq1L	5'-ACT-AGC-TCG-CCG-ACG-ATT-3'
Seq2L	5'-GTT-CGG-TAC-GCA-GGT-CGT-CT-3'
Seq2R	5'-GCC-TGC-ATA-GTT-CGA-TTC-GT-3'
R1	5'-ATC-GAA-CTA-TGC-AGG-CTA-CC-3'
R2	5'-GCG-TCC-GAT-CCT-CGA-GAA-CA-3'
R3	5'-AGA-CGC-TGA-AGG-TGA-TGA-AG-3'
R4	5'-ATC-ACC-ATC-TCG-AGC-AGT-TC-3'
R5	5'-GTT-GCG-ATC-CAC-CAC-AGA-3'
Bam-1	5'-AAG-TAG-CGC-AGC-AGT-TCG-3'
R6	5'-AAC-GCC-GCG-AGC-CTG-ATG-3'
R7	5'-ACA-GCA-GCG-CGT-CGA-GTT-3'
LR-1	5'-ATC-CGC-ACA-ACT-CGC-TGA-CG-3'
LR-2	5'-ACC-GTG-CTG-TCG-ATG-ATC-GT-3'
LR-3	5'-GAA-CTG-CTC-GAG-ATG-GTG-AT-3'
LR-4	5'-GAT-GAA-GGT-GCC-GTG-GAT-CT-3'
RL-1	5'-TTC-GAA-CTG-CGC-ACG-GAT-CT-3'
RL-2	5'-ATT-CAC-ACG-CTC-GTG-CAG-3'
RL-5	5'-CAG-CTC-GGC-GAG-CCA-GAA-3'
Bam-2	5'-GCG-TGG-TTC-GGC-AGC-ATG-AT-3'
RL-6	5'-CTT-GAG-CAC-GCT-GAC-CGG-TT-3'
RL-7	5'-AAG-TCG-TCC-GCG-GTG-AAG-TA-3'
B2	5'-TGT-TGT-GGT-GTT-CGG-CGA-GC-3'
LR-5	5'-CTG-CTC-GAC-TGG-CTG-CTG-AT-3'
LR-6	5'-ACG-GCA-CGA-ATC-TGA-AGG-TC-3'

APPENDIX C: PARTIAL DIGESTS OF CHROMOSOMAL DNA

Purified chromosomal DNA was partially digested with *Sau*3A. In order to obtain large

Chromosomal fragments of between 35 and 40 kb, five reactions were set up as follows:

On ice, 50 μg of chromosomal DNA (quantitated by Absorbance readings at OD_{260}) was mixed

with the amount of reaction buffer and BSA required for a final volume of 500 μl. 5.0 μl was

removed and added to 1.0 μl of 6x DNA loading buffer with EDTA. In a 2.5 μl volume, 0.075

Units of *Sau*3A were added to the reaction mix. The reaction mix was then aliquoted into 5

tubes. These tubes were incubated at 37°C for 5, 10, 15, 20 and 25 minutes. At each time point

5 μl was removed from the sample, added to DNA loading buffer with EDTA and placed on ice.

The remaining 95 μl was phenol:chloroform extracted, ethanol precipitated, resuspended in

sterile distilled water and frozen at –20°C. All the 5.0 μl samples mixed with the DNA loading

buffer were resolved on a 0.8% agarose gel. The 5 minute sample was chosen as the sample to

be used for construction of the cosmid library (see figure 11).
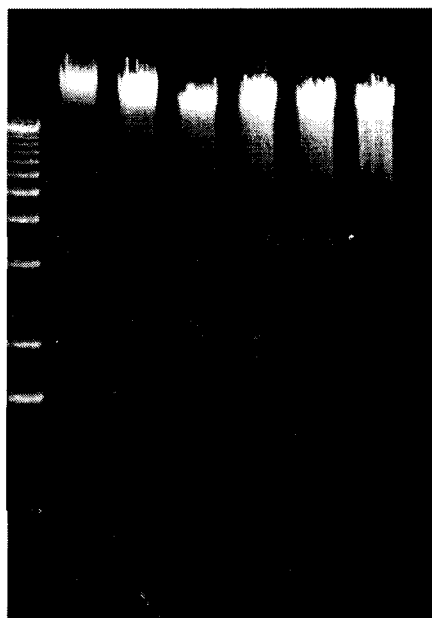
Figure 26. *B. multivorans* strain ATCC 17616 DNA partially digested with *Sau* 3A. B. *multivorans* strain ATCC 17616 DNA was partially digested with the restriction enzyme Sau3A. Lane 1 is the one-kilobase ladder. Lanes 2 to 7 represent the samples that were digested for 0, 5, 10, 15, 20 and 25 minutes respectively in the presence 0.015 Units of *Sau* 3A. Restricted DNA was resolved on a 0.8% agarose gel and stained with EtBr. DNA from the 5 minute digest was used for construction of the cosmid library.

APPENDIX D: QUALITY OF *B. multivorans* ATCC 17616 COSMID BANK

After packaging of the ligated insert and cosmid into λ phage as per the manufacturer's instructions (Stratagene catalogue #251301), transfection of *E. coli* XL1 Blue MR was performed. The transfection titre was low at 4.46×10^5 cfu/ml of undiluted packaged library. 19 colonies were randomly selected from this library and restriction digested with *Bam* HI (see Figure 28).

1 2 3   4   5   6   7   8 9 10 11 12 13 14 15 16 17 18
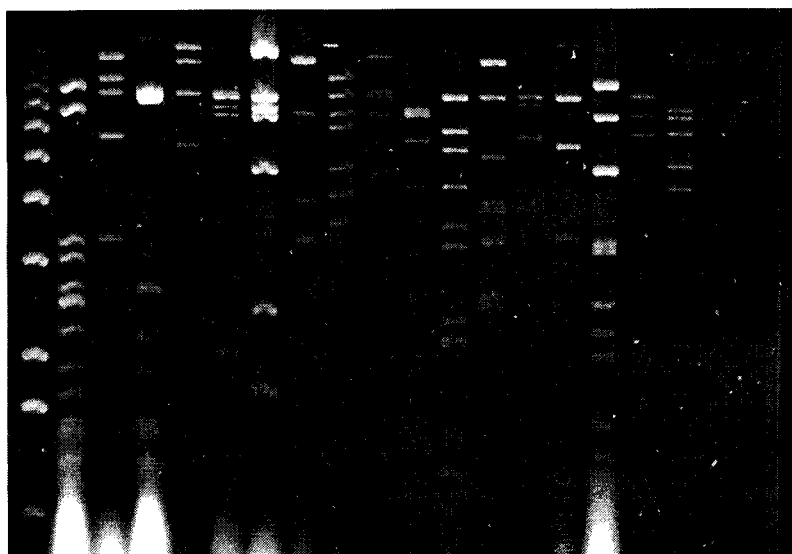
Figure 27 Restriction digests of random clones from the *B. multivorans* ATCC 17616 cosmid bank. Random cosmid clones were selected from the SuperCos 1 library and restriction digested with *Bam* HI. The resulting DNA fragments were resolved on a 1.0% agarose gel and stained with EtBr. Each clone tested exhibited a different pattern suggesting that each cosmid contains a different random chromosomal DNA insert. Insert sizes ranged from approximately 26.6 kb to 42.6 kb and the average size was 29.0 kb. Lane 1 is the 1.0 kb ladder and lanes 2-18 represent randomly selected clones.

APPENDIX E: SEQUENCE ALIGNMENT FOR THE PHYLOGENETIC TREE

```
Bceporf2      1  --------------------------------------------------------------
ThioCer       1  --------------------------------------------------------------
Synech1Cer    1  --------------------------------------------------------------
MouseCer      1  --------------------------------------------------------------
Humancer      1  --------------------------------------------------------------
Celegans1     1  MCKYGKSNLAAVASSTSSIIGAAAAAVAEAQPSASPSTSSSFLLLEVPFRHLLRLQPPPY
Celegans3     1  -----------MGDKITGLEVEIIPEISEVQPESLQEQNASSLYYPLFS------QYIPL
Celegans2     1  --------MEAANEVVNLFASQATTPSSLDAVTTLETVSTPTFIFPEVSDSQILQLMPAT
Mxanthus      1  --------------------------------------------------------------
RhizNodC      1  --------------------------------------------------------------
synecho2Cer   1  -----------------MPQFPWKDNDAELSPLEAFLAEWDDPEAEEEDFRNDFFRGS
Llactis       1  -------------------------------------------------------------M


Bceporf2      1  ---------MTTPAVSL-LDWLLIAEAVAAAAYALLAAFAPRPRVPPTAA---------RD
ThioCer       1  ---------MCWWIGGPAALLSLAAVVYLLLALRAIARWHPVLPERDAAVSG----DILC
Synech1Cer    1  ---------MNEGQWGEIMSWLCLLPISGGIVYNLITVFTTSLETARSLP-------KQD
MouseCer      1  --------MALLDLAQEGMALFGFVLFVLWLMHEMSIILTRLHINKKATDK----QPYS
Humancer      1  --------MALLDLALEGMAVFGFVLFLVLWLMHFMAIIYTRLHINKKATDK----QPYS
Celegans1    61  FIAGTRRMAAQLDVTIL-IAIVGFVFVFCLYLIHIIALSYSKYRLHEKVK-------EDS
Celegans3    44  LLTGTKKILEDIDYCTC-FAVFGVVFVSALYFLHIVGLCYGKYRLHRPTK-------PNP
Celegans2    53  LYSGMNWLRDHLDGFSL-LALSHCIFVSVLYLVHIIAFFYSIYRLHEKVE-------PDP
Mxanthus      1  --------------MSALVLASLAWCAVATGFSAVACVRLSRARAGAAP-------QPG
RhizNodC      1  ---------MTLLATIS--IAAISLYAMLSTVYKSAQVFHARRTTISTTPAKD---IETN
synecho2Cer  42  EGRRKKAAVMLMAIWTVVITLHYWWGSWLVWALTGALSLQALRIMKATPEEA---PPLL
Llactis       2  TIYIVWRILYTLPHGSAISVLLLSLLIAEIGSFVLSITEYFLFWKKNTKEDKELVELHP


Bceporf2     43  GAEPVSVLKP---LCGAEPH----LYENLATFCEQHHFR--YEVIEGVAS-AGDPAIAVV
ThioCer      48  DGPGVSVLKP---LHGDEGD----LYAALRSFCVQDYPA--FE1VFGVQR-PDDPAVTVV
Synech1Cer   45  FQPGVSVLKP---VRGLEKN----LEANLRTIAQQNYPA--YEVIYCVQL-PQDPALPIV
MouseCer     49  KLPGVSILKP---LKGVDPN----LINNLETHFELDYPK--YEVILCVQL-HDDPAIDVC
Humancer     49  KLPGVSILKP---LKGVDPN----LINNLETHFELDYPK--YEVILCVQL-HDDPAIDVC
Celegans1   113  SLPGVSILKP---IVGKDNN----LYENIESFFTTQYHK--YELLFCFNS-SDDEAVEVV
Celegans3    96  SLPGVSILKP---IIGADAN----LYTNLETHFTTQYHK--FELLFCFDR-SDDPAVKVV
Celegans2   105  TLPGVSVLKP---IVGTDKN----LYQNLSFFTSQYHS--FELLFCFHS-EEDEAIEVV
Mxanthus     39  AVIPVLLLKP---VDAPTPR----ELENLAQSIDYAGEL--EQVVVSPYR-PR--LAPGV
RhizNodC     47  PVISDVIVL---CFNELPIV---LSECLASLAEQDYAG--KLRIYVVDL-GS-KNRDAV
synecho2Cer  99  TGDASTVPYEQVCLMVAAKNEEAVIGKIVQQLCSLDYFGD-RHEWVIVDLNSTLRTPAIL
Llactis      62  NYLTVDLFIA---TYNESSS---ILKRTIIASKKVRYFDLRKVELYVLDLGDREEIALIA


Bceporf2     93  ERLRAAYFDRLIALVVDARVHGTNLKVSNLINLADRAKYLRLVIADSDIAVAPDYLERVT
ThioCer      98  QRLQAEFFALALRWVCTEARIGSNPKVNNLAGILALCRYITLVISDADISVGPHYLRQIC
Synech1Cer   95  KKLQAEFGPEKIIVAVHQIEQGANGKVNNLLGGLKHAKYLILVISDSDTNLRPDYLATMV
MouseCer     99  KKLLGKYPNVIARLFIGGKKVGLNPKLNNLMPAYEVAKYDLIWICDSGLRVIPDTLTDMV
Humancer     99  KKILGKYPNVIARLFIGGKKVGLNPKLNNLMPGYEVAKYDLIWICDSGLRVIPDTLTDMV
Celegans1   163  KCLMKKYLKVIAKLFFGGETVGLNPKINNMMPAYRSALYPLILVSDSGIFMRSLGVLDMA
Celegans3   146  ESIVKKYISVISTMFFGGEKIGLNPKINNMMPAYRIAKYQLIMISDSGIFMKSLAVLDMA
Celegans2   155  RSLIKKHLNIBAKILFEGEPVGMNPKVNNMMPAYRAARYPLVLISDSAIFMRPLGILDMA
Mxanthus     87  RWLPSEP-------LTPNRKVGHLLYALDVLFVGDR----VVLSVDADVAVTGALVEGLA
RhizNodC     97  VAQRAALAD-LERFNFTILPKNVG-KRKAIAAITQSSG-DLIINVDSDTTIAPLVVSKLA
synecho2Cer 158  DQLRQQYLQLKVVRRGASASGGKSGALNEVLAQTQG---DIVGVFDADANVPKDLLRRVV
Llactis     116  KELNVKYIRRL---NNENAKAG-N--LNNALKETKG---DLVVTLDADMVPRVDFLEKTV


Bceporf2    153  APLAD-ASVGVVTCLYHARSVGG---------------------FWTRIGAQFVD----
ThioCer     158  ASLQN-RDVGVVTCLYRARPVAT---------------------FWSRVLAG---
Synech1Cer  155  SPLGD-RLVGCVTTPFKLTQAQT---------------------WYEGLELL---
MouseCer    159  NQMT--EKVGIVHGLPYVADRQG---------------------LAATLEQ----
Humancer    159  NQMT--EKVGIVHGLPYVADRQG---------------------LAATLEQ----
Celegans1   223  TTVMSHEKMALVTQTPYCKDREG---------------------LDAAFEQ----
Celegans3   206  STVMSHETMALVTQTPYCKDRKGKSKNCAPIQILKRLTLIFIVVNVLACFNIALSISVWA
Celegans2   215  TTVMSHEKMASVTQIPYCKDRQG---------------------LHAAFEQ----
Mxanthus    136  VPVA---AGPPEHRGLPVGPRDG-----------------------ASRAVAG----
RhizNodC    154  HKYRD-PAVGAAMGQMKASNQADT--------------------NLTRLID----
synecho2Cer 215  PYFAS-PTFGALQVRKAIANEAVN--------------------FWTRGQG----
Llactis     167  GYFED-SKVGFLQAPQTFFNNDP--------------------YQFNFFSEKNLN---
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Bceporf2     186  --AWFAPTVRITHLGC---SSRFGFGATLALTRDTLERIGG------LRVLRDELADDFW
ThioCer      188  --QVNGLFLPSVLAARLGPNIFCGGATMALRRPTLAAIGG------LPRLANQLADDYW
Synech1Cer   185  --SINADFMPSVLFAEVTGASKACLGPSIAIRRSTLTEIGG------LESLADYIVEDFE
MouseCer     187  --VYFGTSHPRSYTSANVTGFKCVTGMSCLMRKDVLDQAGG------LIAFAQYIAEDYF
Humancer     187  --VYFGTSHPRYYTSANVTGFKCVTGMSCLMRKDVLDQAGG------LIAFAQYIAEDYF
Celegans1    253  --MYFGTSHGRTYLAGNCMDFVGSTGMSSMMKKEALDECGG------ISNFGGYLAEDYF
Celegans3    266  TLIYFGTSHARIYLAGNCLQFNCPTGMSSMMKKEALDECGG------FAAFSGYLAEDYF
Celegans2    245  --IFFGTSHARLYIVGNFLGVVGSSGMSSMMKKSALDECGG------MEKFGEYLAEDYF
Mxanthus     163  --LLRYTHHSFRALDAMSAGAKAVCGKALGLSPVAVESLR--------R-LADHIGEDLE
RhizNodC     184  -METWLACNEERAAQARFGAVMGCCGPCAMYRRSAMLSILLDQYETQLYRGKPSDFGEDRH
synecho2Cer  245  --AEMALDAYFQQQRIVTGGIGELRGNGQFVARQALDAVGG--------WNEQTITDDLD
Llactis      201  --NDQDFFMRRIENQKDIYNSVMYIGSNAVFRRAALESIGG--------FSTGVITEDLA


Bceporf2     235  LAELPRRLGRRTVLSEVEVATDVIEASFGPLWHRETRWLRTIRS---------------L
ThioCer      240  LGAYSRQLGQATLLADYVVDTEVREANFRAFYQHALRWSRTTRS---------------V
Synech1Cer   237  LGQRVWTGGLKMVLLPYVLDAGVNLDNWTNWWSHQLYWDQNTYL---------------A
MouseCer     239  MAKAIADRGWRFSMSTQVAMQNSGSYSISQFQSRMIRWTKLRIN---------------M
Humancer     239  MAKAIADRGWRFAMSTQVAMQNSGSYSISQFQSRMIRWTKLRIN---------------M
Celegans1    305  FGRELANRGYLSALGSHPALQNSSSVSVSSFLDRICRWVKLRIA---------------M
Celegans3    320  FGKKLASRGYKSGLSTHPALQNSAAVTMTSFTDRVCRWVKLRMA---------------M
Celegans2    297  FAKALTSRGCKAALSTHPALQNSASVTVLSFFNRIGRWIKLRIA---------------M
Mxanthus     212  LAKRLHAQGLVLSSAPAVVPLD-AEVLWAAPLSRFTRWMQVLAS---------------H
RhizNodC     243  ITILMLSAGFRTEYVPSAIAATVVPDTMGVYLRQQLRWARSTFR---------------D
synecho2Cer  295  LTIRLHLFQWKVGILVNPPVEEEGVTIAIALWHQRNRWAEGGYQRY-----LDYWRWICT
Llactis      251  TGMFIQAKGWKTRFVNKNLASGLAPENFSDLIKQRDRWSRGNIQVARKWLPLKIKGLNKV


Bceporf2     280  NEA----------------GFAFLFITF----TAPWLAIAAALALKLDGT-----------
ThioCer      285  QPL----------------GHTFSFLTY----PLPLVLLLAPWYGLWGG-----------
Synech1Cer   282  RPL----------------PFLATILIR------AVPFAFLFTFCHWSEP-----------
MouseCer     284  LPA----------------TIICEPISE----CFVASLIIGWAAHHVFR-----------
Humancer     284  LPA----------------TIICEPISE----CFVASLIIGWAAHHVFR-----------
Celegans1    350  LPH----------------ILIVEPLQD----CFPSGLIMAFSINHLVG-----------
Celegans3    365  MPQ----------------IIFVEPLQD----CFPSALIISFSLNYIAN-----------
Celegans2    342  MPH----------------LMVVEPLQNGKFNFTPFLEVFIWLFREFTSP-----------
Mxanthus     256  RPA----------------LYPTVPLLFTP--TLPLAVIAAALGSPVLAG-----------
RhizNodC     288  TLL--------------ALPVLEGLDRYLTLDAIGQNVGLLILALSVL-----------
synecho2Cer  350  QLMGWKK--------KLDLFSFLLMQY----LLPTAAVPDLLMALWQRR-----------
Llactis      311  QKLLYMDGIHYWFSGIYKMIFMLAPLWFVLFGFYSLNARFSGILTFWLPSFIASQLAFNR


Bceporf2     310  VAGTVAGVAAAAGT--GGRLVLHAR------GEDG------------------WRAYWRD
ThioCer      314  VPLGVVLLLRLVYH--RQIMHKLSA------DGSF------------------GVALLG-
Synech1Cer   310  WAWLILFITLATRY--GTAAIVAQE------LKDG------------------ETIRYLP
MouseCer     313  WDIMVFFMCHCLAW--FIFDYIQLR------GVQG------------------GTLCFSK
Humancer     313  WDIMVFFMCHCLAW--FIFDYIQLR------GVQG------------------GTLCFSK
Celegans1    379  LNIMPILILHTTYW--FSMDYSLMN------SMQN------------------GKLSFSP
Celegans3    394  IDMLTTIMLHVVFW--ITMDCMVMC------KMQV------------------------
Celegans2    376  FIFIKAVLAPTIVW--RNNKEKLSW------GGRI------------------RTSKNS-
Mxanthus     288  SVVGLVAVRTLLAL--RLAGLHAPV------ADAG------------------RAYAVTD
RhizNodC     322  TGIGQFALTATIPW--WTILVIGSMTLVRCSVAAY------------------RARELRF
synecho2Cer  387  FPLITPLSYLAIGESCWGMYYGLKR-----LTPSE------------------GESPWQQ
Llactis      371  VSQGTQSILLTNIYETVMAPFISYSVISDAVLKSKKGFTVTNKGYNTNKKYYNWRLSLPL


Bceporf2     344  LPLV-A--VRDTLLALEQLVAAFGT--------QVVWRG------ARMTVVG--GERAAA
ThioCer      347  ---------EFLGIWIWFHALFAR--------HVAWRG------SQEAIG---ADGRMD
Synech1Cer   344  LLLL-----RDCFGLVEFALSFSQR--------QVLWRG------ITYRLTK--GGKMIP
MouseCer     347  LDYAVAWFTRESMTTYIFLSALWDP--------TISWRT------GRYRLR---CGGTAE
Humancer     347  LDYAVAWFTRESMTTYIFLSALWDP--------TISWRT------GRYRLR---CGGTAE
Celegans1    413  LQFMLIWLIRDSPLLSSLSKLFYNQ--------QFNGET------MFFIWHG--VDRYFH
Celegans3         ------------------------------------------------------------
Celegans2    409  ---------QKVPEAVSLSKGAV------------------------------------
Mxanthus     322  WLLG------EALLIAAFLRSLWRQG-------TVTWRG------QTYVLRP--GGRMIR
RhizNodC     362  LGFALHTLVNIFLLIPLKAYALCTLS-------NSDWLSR-----GSVALAPTVGQQGAT
synecho2Cer  424  MPALLARTIGGTIYMFHWLIINPAVTA------RMAERP------KRLKWVKTVHGAATE
Llactis      431  LILLFFSIIALCKSIFVIFNILPFESGKDAIYINAFWLLYNVFILIFAVLVPFERPRFRK
```

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```
Bceporf2      385 AVEGGDGR---------------------------------------------------
ThioCer       380 GHDGAKR----------------------------------------------------
Synech1Cer    383 ASPIPKS----------------------------------------------------
MouseCer      390 EILDV------------------------------------------------------
Humancer      390 EILDV------------------------------------------------------
Celegans1     457 QNVDCFIYSFYFQISLTPNSSFLSVILFLNIIVNPLTTSSESLKSGLLSLHQLHC---
Celegans3         ----------------------------------------------------------
Celegans2         ----------------------------------------------------------
Mxanthus      361 VAPELSGGPG-------------------------------------------------
RhizNodC      410 KMPGRATSEIAYSGE--------------------------------------------
synecho2Cer   472 DALELKQS---------------------------------------------------
Llactis       491 SERFLSSKEAQLLDKESHLIIDCKVMDWNELGAGITIECNNKIELKEEQKIILSVNGY
```

Majority-rule and strict consensus tree program, version 3.572c

Species in order:

    Synech1Cer
    Synech2Cer
    Llactis
    MouseCer
    Humancer
    Celecgans1
    Celegans2
    Celegans3
    RhizNodC
    Mxanthus
    Bceporf2
    Thiocerglu

Sets included in the consensus tree

Set (species in order)   How many times out of 100.00

```
......***.. ..                100.00
...*****.. ..                  99.00
...**..... ..                  99.00
.**....... ..                  79.00
......*.*.. ..                 72.00
.......... **                  58.00
.........** ..                 55.00
...******* **                  37.00
.........** **                 33.00
```

Sets NOT included in consensus tree:

Set (species in order)   How many times out of 100.00

```
......**... ..                 28.00
.**......*. ..                 23.00
.........** *.                 18.00
..........* *.                 17.00
..........* **                 16.00
.*******. ..                   15.00
.******.. ..                   14.00
...*****.* **                  13.00
.********* ..                  11.00
...******* *.                  10.00
.********* *.                  10.00
.*.******* **                   9.00
...*****.* ..                    8.00
..*......*. ..                   6.00
.**......** ..                   6.00
...*******.  ..                  6.00
...*****.* *.                    5.00
.*******. **                     4.00
...******.  ..                   4.00
.*......** **                    4.00
..*.....** ..                    3.00
.*......... **                   3.00
.**.....** **                    3.00
```
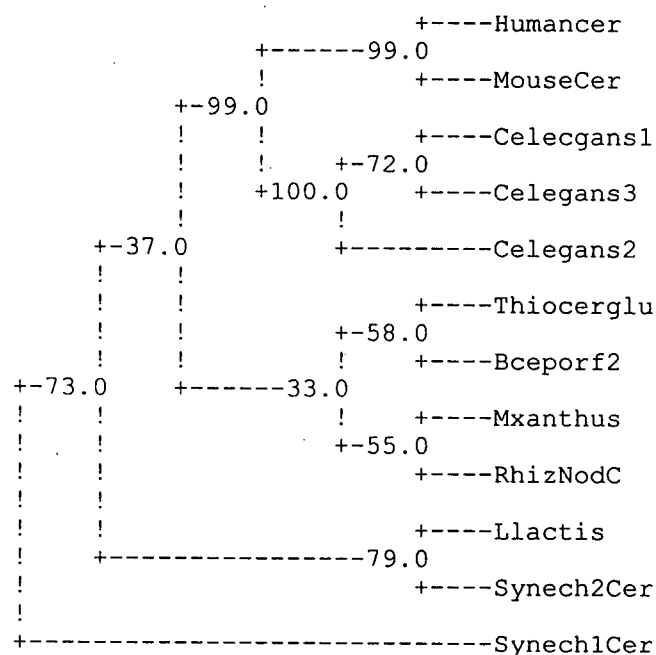
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.*******..  **              2.00
.........*.  *.             2.00
..******..  ..             2.00
.*.*****..  **             2.00
.........*  .*             2.00
..********  **             2.00
.*.......*.  **            1.00
.********.  *.             1.00
.**.....*.  **             1.00
..*......*  ..             1.00
.**..****.  ..             1.00
.*.*****.*  **             1.00
.**......*  ..             1.00
.........*.  **            1.00
....****..  ..             1.00
...*****..  *.             1.00
..*.....**  **             1.00
.*......*.  ..             1.00
.*.*******  *.             1.00
.*........  .*             1.00
.********.  .*             1.00
...*****..  **             1.00
.*.*****..  *.             1.00
.**.......  .*             1.00
.*.......*  **             1.00
..********  ..             1.00
```

CONSENSUS TREE:
the numbers at the forks indicate the number
of times the group consisting of the species
which are to the right of that fork occurred
among the trees, out of 100.00 trees

```
                              +----Humancer
                   +------99.0
                   !          +----MouseCer
           +-99.0
           !    !             +----Celecgans1
           !    !.    +-72.0
           !    +100.0    +----Celegans3
           !         !
     +-37.0          +--------Celegans2
     !    !
     !    !             +----Thiocerglu
     !    !      +-58.0
     !    !      !      +----Bceporf2
+-73.0    +------33.0
  !  !             !    +----Mxanthus
  !  !           +-55.0
  !  !             +----RhizNodC
  !  !
  !  !             +----Llactis
  !  +-----------------79.0
  !                +----Synech2Cer
  !
  +---------------------------Synech1Cer
```

remember: this is an unrooted tree!