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# The Role of Bacteriocins in Mediating Interactions of Bacterial Isolates from Cystic Fibrosis Patients

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**THE ROLE OF BACTERIOCINS IN MEDIATING INTERACTIONS OF  
BACTERIAL ISOLATES FROM CYSTIC FIBROSIS PATIENTS**

A Dissertation Presented

by

E. SUPHAN BAKKAL

Submitted to the Graduate School of the  
University of Massachusetts Amherst in partial fulfillment  
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

February 2011

Molecular and Cellular Biology Program

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BACTERIAL ISOLATES FROM CYSTIC FIBROSIS PATIENTS**

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E. Suphan Bakkal

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## **DEDICATION**

This thesis is dedicated to my beloved family, who has always been supportive in my life. No words can express how grateful I am to my mother (Filiz Bakkal), my father (Macit Bakkal), and my lovely sisters (Handan Bakkal-Caglayan and Nurdan Bakkal).

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## ABSTRACT

### THE ROLE OF BACTERIOCINS IN MEDIATING INTERACTIONS OF BACTERIAL ISOLATES FROM CYSTIC FIBROSIS PATIENTS

February 2011

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Cystic Fibrosis (CF) is a common autosomal genetic disorder in Caucasian populations. CF is caused by mutations in the *cfr* gene, which encodes the CF transmembrane conductance regulator (CFTR). CFTR regulates chloride and sodium ion transport across the epithelial cells lining the exocrine organs. Mutations in the *cfr* result in a failure to mediate chloride transport, which leads to dehydration of the mucus layer surrounding the epithelial cells. The mucus coating in the lung epithelia provides a favorable environment for invasion and growth of several opportunistic bacterial pathogens resulting in life threatening respiratory infections in CF patients.

*Pseudomonas aeruginosa* (Pa) and *Burkholderia cepacia* complex (Bcc) are associated with chronic lung infections and are responsible for much of the mortality in CF. Little is known about interactions between these two, often co-infecting, species. When in competition, it is not known whether Bcc replaces the resident Pa or if the two species co-exist in the CF lung.

Bacteriocins are potent toxins produced by bacteria. They have a quite narrow killing range in comparison to antibiotics and have been implicated in intra-specific and inter-specific bacterial competition brought on by limited nutrients or niche space. Both Pa and Bcc produce bacteriocins known as pyocins and cepaciacins, respectively. More than 90% of Pa strains examined to date produce one or more of three pyocin types: R, F, and S. A limited number of phenotypic surveys suggest that approximately 30% of Bcc also produce bacteriocins.

The goals of my thesis study were to determine if clinical strains of Pa and Bcc produce bacteriocins and to determine whether these toxins play a role in mediating intra- and inter-specific bacterial interactions in the CF lung. The final goal was to identify novel bacteriocins from clinical Pa and Bcc strains.

First, I designed a phenotypic bacteriocin survey to evaluate bacteriocin production in 66 clinical Pa (38) and Bcc (28) strains procured from CF patients. This study revealed that 97% of Pa strains and 68% of Bcc strains produce bacteriocin-like inhibitory activity. Further phenotypic and molecular based assays showed that the source of inhibition is different for Pa and Bcc. In Pa, much of the inhibitory activity is due to the well known S- and RF-type pyocins. S- and RF pyocins were the source of within species inhibitory activity while RF pyocins were primarily implicated in the between species inhibitory activity of Pa strains. In contrast, Bcc inhibition appeared to be due to novel inhibitory agents. Finally, I constructed genome libraries of *B. multivorans*, *B. dolosa*, and *B. cenocepacia* to screen for genes responsible for the inhibitory activity previously described in Bcc. ~10,000 clones/genome were screened, resulting in fifteen clones with the anticipated inhibition phenotype. Of these fifteen, only five clones had

stable inhibitory activity. These clones encoded proteins involved in various metabolic pathways including bacterial apoptosis, amino acid biosynthesis, sugar metabolism, and degradation of aromatic compounds. Surprisingly, none of Bcc clones possessed typical bacteriocin-like genes. These data suggest that, in contrast to all bacterial species examined in a similar fashion to date, Bcc may not produce bacteriocins. Instead, Bcc may be using novel molecular strategies to mediate intra- and inter-specific bacterial interactions.

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## CHAPTER 1

### BACKGROUND AND SIGNIFICANCE

*"Woe is the child who tastes salty from a kiss on the brow, for he is cursed, and soon must die."*

Northern European Folklore

#### Preface

Cystic Fibrosis (CF) was defined as a fibrocystic disease of secretory organs in the 1930s. In 1938, Dorothy Anderson, MD published a comprehensive study, which categorized 49 CF patients between 0-14.5 years old based on the age of death and clinical manifestations (Andersen, 1938). The major conclusion of this case study was that the disease creates diverse effects on multiple body systems, in particular the digestive and respiratory systems. Further, the majority of patients (44) died due to respiratory infections (Andersen, 1938). During the past 60 years, a vast amount of data has provided support to the notion that the disease was the causative agent of the resulting clinical outcomes; these clinical observations included salty-tasting skin, vitamin-A deficiency, poor growth, greasy, bulky stools, persistent coughing, wheezing, shortness of breath, malformation of pancreatic ducts, fertility problems, and lung infections (Darling *et al.*, 1953; Freedman *et al.*, 2000a; Freedman *et al.*, 2000b). One of the most important milestones in the history of CF was the discovery of the cystic fibrosis transmembrane regulatory receptor (*cftr*) gene, whose mutation is responsible for this disease (Kerem *et al.*, 1989; Riordan *et al.*, 1989; Rommens *et al.*, 1989).

Today, we define CF as an autosomal recessive genetic disorder, mainly affects Caucasian population with an average incidence of 1 in 3000 live births (Morrissey *et al.*, 2003). CF patients ultimately die in their mid-30s due to chronic lung infections that are

associated with high mortality (Govan & Deretic, 1996). *Staphylococcus aureus*, *Haemophilus influenzae*, *Stenotrophomonas maltophilia*, *Pseudomonas aeruginosa*, and *Burkholderia cepacia* complex are commonly isolated species from CF patients (Jones & Webb, 2003).

This thesis is focused on two bacterial species: *Pseudomonas aeruginosa* and *Burkholderia cepacia* complex. Both species are multi-drug resistant and became the major concern for CF patients in the past 30 years (Govan & Deretic, 1996). *Pseudomonas aeruginosa* is the most common infectious agent involved, which is normally acquired shortly after birth. Approximately 80% of adult CF patients have chronic *Pseudomonas* infections, which result in high levels of mortality (CFF2008; Govan & Deretic, 1996). In contrast, the *Burkholderia cepacia* complex affects a smaller portion of CF patients (~3%). However, colonization by *Burkholderia*, usually leads to rapid lung failure (Jones *et al.*, 2004; Kalish *et al.*, 2006; Tablan *et al.*, 1985).

There are numerous bacterial proteins thought to be associated with the pathogenicity of bacteria in CF lungs (Govan & Deretic, 1996; Mahenthiralingam *et al.*, 2005). In this thesis, I will specifically focus on bacteriocins produced by clinical *Pseudomonas aeruginosa* and *Burkholderia cepacia* complex. These bacteriocins are narrow spectrum toxins used by bacteria to kill strains of related species (Riley & Wertz, 2002a; Riley & Wertz, 2002b). I investigated (i) bacteriocin production of *Pseudomonas aeruginosa* and *Burkholderia cepacia* complex and their role in mediating both within and between species inhibitory activity; (ii) bacteriocin types produced by *Pseudomonas aeruginosa* and *Burkholderia cepacia* complex; (iii) putative bacteriocin genes of *Burkholderia cepacia* complex.

The following sections will provide general information about CF, CF symptoms, current treatment regimens, mechanism of the disease, bacteria colonizing CF lung (in particular *Pseudomonas aeruginosa* and *Burkholderia cepacia* complex), interaction between *Pseudomonas aeruginosa* and *Burkholderia cepacia* complex, bacteriocins of *Pseudomonas aeruginosa* and *Burkholderia cepacia* complex, and finally the significance of the project.

## **1.1 The Genetics of Cystic Fibrosis**

### **1.1.1 The *cftr* gene**

Cystic Fibrosis is an autosomal recessive genetic disorder, which is caused by mutations on the Cystic Fibrosis Transmembrane Regulator (*cftr*) gene (Morrissey *et al.*, 2003). The *cftr* gene is located on the long arm of chromosome 7 (7q.31.2). It spans a 215 kb region and consists of 27 exons and 26 introns (Figure 1-1a). The *cftr* gene encodes a transmembrane protein with two membrane-spanning domains (MSD), one regulatory domain (R), and two nucleotide-binding domains (NBD) (Figure 1-1b) (Riordan *et al.*, 1989).

## **1.2 The CFTR Protein**

The CFTR protein is an apical membrane Cl<sup>-</sup> ion channel on epithelial cells. The pore size of the channel is ~5.3 Å (Linsdell *et al.*, 1997). It is selectively permeable to Cl<sup>-</sup> ions. Water, urea, and ATP may also permeate through the CFTR channel (Hasegawa *et al.*, 1992).

Site directed mutagenesis studies have revealed the functions of each domain of the CFTR: The MSD domains contribute to ion selectivity and formation of Cl<sup>-</sup> channels;

The R domain connects two halves of the protein, and also has multiple sites for phosphorylation by protein kinase A and C (Figure 1-1b) (Riordan *et al.*, 1989).

The R domain controls channel gating activity by phosphorylation and dephosphorylation, and the NBD domains hydrolyze ATP to regulate channel gating (Sheppard & Welsh, 1999).

The two MSDs are composed of six transmembrane segments (M) (Figure 1-1b). There are six conserved residues K95 (M1), R134 (M2), R334 (M6), R335 (M6), R347 (M6), and R1030 (M10). A mutation in either arginine residue (R334 and R347) is associated with the onset of disease symptoms (Riordan *et al.*, 1989). These mutations lead to more than a 70% decrease in Cl<sup>-</sup> ion conductance (Tabcharani *et al.*, 1993). Positively charged arginine residues are required to interact with the negative charge on Cl<sup>-</sup> during Cl<sup>-</sup> conductance. Further, mutations in two basic lysine residues (K95 and K335) to acidic aspartate (D) and glutamate (E) changed ion selectivity of CFTR channel from Br<sup>-</sup>>Cl<sup>-</sup>>I<sup>-</sup>>F<sup>-</sup> to I<sup>-</sup>>Br<sup>-</sup>>Cl<sup>-</sup>>F<sup>-</sup> (Anderson *et al.*, 1991b). Finally, Cheung and Akabas used the substituted cysteine accessibility method to identify pore-lining residues in M1 and M6. They revealed 11 residues (I331, L333, R334, K335, F337, S341, I334, R347, T351, R352, and Q353) along M1 and M6, which contribute formation of Cl<sup>-</sup> channel (Akabas *et al.*, 1994; Cheung & Akabas, 1996).

The residues at intracellular and extracellular loops (ICL and ECL) of CFTR are important for membrane localization and functioning of the CFTR protein. Mutations in internal loops (ICL) altered gating behavior and channel regulation and no effect on pore formation. Mutations in ICL 1 and 2-increased mean closed time of the CFTR channel while ICL 3 and 4-decreased mean opened state of the channel (Seibert *et al.*, 1996).



Further, mutations in extracellular loops (ECL) contribute CFTR pore functioning (Sheppard & Welsh, 1999).

The NBDs of CFTR has three conserved domains (Walker A, Walker B, and LSGGQ) to interact with ATP (Walker *et al.*, 1982). These domains are associated with many CF-related mutations. The CFTR channel is tightly regulated by kinase and phosphatase activity, and also presence of cellular ATP levels (Anderson *et al.*, 1991a; Gadsby & Nairn, 1999). Activation of cAMP dependent protein kinase A (PKA) causes phosphorylation of serine residues on the R domain, which is followed by ATP hydrolysis at NBDs, opening the Cl<sup>-</sup> channel. Dephosphorylation of serine residues by protein phosphatases return the Cl<sup>-</sup> channel to a closed state (Gadsby & Nairn, 1999; Ostedgaard *et al.*, 2001).

There are two main models proposed for ATP-dependent gate opening of CFTR. In the first model (Figure 1-2a), there are three closed states (C1, C2, and C3) and two open states (O1 and O2). ATP binds NBDs in a closed state. At C1 state, both NBDs are empty. Then R domain phosphorylation results in ATP binding to the NBDs sequentially, first to NBD2 (C2 state) and then to NBD1 (C3 state). Hydrolysis of ATP at NBD1 opens the channel (O1 state). Then ATP at NBD2 hydrolyzed (O2 state) (Sheppard & Welsh, 1999). In the second model (Figure 1-2b), there are two closed (C1 and C2) and two open states (O1 and O2). The ion gate opens after R domain phosphorylation followed by ATP binding at NBD2 (O1 state). ATP hydrolysis at NBD2 (O2 state) followed by R domain phosphorylation and ATP binding at NBD1 leads to channel closing (Sheppard & Welsh, 1999).

### 1.3 Mutations Resulting in Disease

According to a 2004 World Health Organization (WHO) report, the incidence of CF in newborns in specific regions is as follows: US (1/3500), Europe (1/2-3,000), Africa (1/7056). We also see ranges of disease occurrence in Asia and the Middle East of (1/10-40,000) and (1/2-15,000), respectively (WHO 2004 CF Report) (Figure 1-3).

The most common CF mutations are missense mutations, point mutations of one nucleotide, which account for 48.7% of all CF mutations (Lommatzsch & Aris, 2009). There have been 1720 point mutations detected from CF patients, all of which are documented in the CF Genetic consortium database.

Mutations are classified into 5 classes (Class I-V) based on CFTR function (Figure 1-4). Class I mutations create premature stop codons due to frameshift or nonsense mutations, leading to truncated mRNA. Therefore, the CFTR protein is not expressed (Figure 1-4) (Gibson *et al.*, 2003). Class II mutations are the most common mutation and cause misfolded CFTR proteins. CFTR proteins does not fold into proper tertiary structures and therefore are degraded in the endoplasmic reticulum instead of being trafficked to the epithelial membrane (Figure 1-4) (Gibson *et al.*, 2003; Lommatzsch & Aris, 2009). The  $\Delta F508$  deletion (a 3 bp deletion in exon 10) is the most common mutation in class II mutations. Approximately 70% of CF patients have  $\Delta F508$  deletion (Bobadilla *et al.*, 2002).  $\Delta F508$  deletion affects the interaction between the N-terminus NBD1 and C-terminus of transmembrane segment-4, which is involved in channel gating (Lommatzsch & Aris, 2009). Class I and II mutations are the most frequently observed in CF patients. Their clinical manifestations are severe since

functional CFTR protein is not presented on the surface of the epithelial cell (Zielenski, 2000).

Class III mutations affect ATP binding to NBD1, NBD2, and therefore ATP hydrolysis, which is involved in channel activation. CFTR channels are not activated (Gibson *et al.*, 2003; Lommatzsch & Aris, 2009). Class IV mutations mostly occur in transmembrane domain 1, which is associated with pore forming. Therefore Class IV mutations affect chloride ion conductance (Akabas *et al.*, 1994). The CFTR is fully translated and trafficked to the membrane in Class III and IV mutations so the effect of these mutations are milder than those of Classes I and II (Zielenski, 2000).

Class V mutations affects the number of functional CFTRs on the membrane. Splice mutations are most common in this class. The disease phenotype may range from mild to severe since functional CFTR copying differs from patient to patient as well as in the epithelial cells of various organs (Nissim-Rafinia & Kerem, 2002; Nissim-Rafinia *et al.*, 2004).

## **1.4 Diagnosing CF**

### **1.4.1 Early warning signs**

CF patients are diagnosed with the disease in infancy and/or early childhood (Koch & Hoiby, 2000). The majority of patients are diagnosed in early childhood due to respiratory infections (50.5%), malnutrition (42.9%), or both (Rosenstein & Cutting, 1998). Nineteen percent of CF patients are diagnosed at birth due to *meconium ileus*, an intestinal obstruction leading to a twisting of the bowel and abdominal infection (Rosenstein & Cutting, 1998). Vomiting a few hours after birth is the first sign of

*meconium ileus* (Donnison *et al.*, 1966). Knowledge of family history accounts for 16.8% of CF diagnoses. Another symptom of the disease is steatorrhea (the presence of excess fat in stool), which occurs in 35% of CF patients (Rosenstein & Cutting, 1998).

## **1.4.2 Clinical Tests for CF**

Sweat tests, DNA mutation analysis, and nasal potential difference test are diagnostic tests used to detect CF in patients (Koch & Hoiby, 2000; Rosenstein & Cutting, 1998). In most cases, more than one test is used since diagnostic tests may be inconclusive due to various clinical manifestations depending on the type of mutation (Koch & Hoiby, 2000; Rosenstein & Cutting, 1998).

### **1.4.2.1 The Sweat Test**

Testing sodium and chloride concentration in sweat (Sweat test) is a noninvasive routine method used to diagnose CF (Koch & Hoiby, 2000). In this procedure, two electrodes are attached to the patient's arm. One of the straps has a disk with pilocarpine, which induces sweat glands. An electrical pulse stimulates pilocarpine diffusion through skin. Then a special sweat collection device is attached the skin where sweat glands are stimulated. A minimum of 15  $\mu$ l sweat must be collected during a 30-minute period (Naehrlich, 2007). A healthy person has 30-40 mM sodium and chloride concentration in their sweat while a CF patient has greater than 60-70 mM of sodium and chloride. This concentration difference is due to nonfunctional CFTR channels in the CF patient (Green *et al.*, 1985; Hodson *et al.*, 1983; Kirk & Westwood, 1989). However, this test is not 100% accurate. Some CF patients have normal salt levels in their sweat, which are dependent on the type of mutation in the *cftr* gene. This test also does not take into

account the fluctuation of sweat chloride levels with age (Lyczak *et al.*, 2002). Therefore, additional diagnostic tools are required to verify this test's findings.

#### **1.4.2.2 DNA Mutation Analysis**

DNA mutation analysis is a genetic test, which identifies the mutation in the *cftr* gene. DNA is isolated from either saliva or blood and is screened for the presence or absence of common CF mutations. These DNA screening kits (ex Tag-It™ Cystic Fibrosis Kit) are commercially available to physicians. The USA CF foundation recommends that all newborns be screened with this test since more than 10 million American are asymptomatic carriers of CF (CFF, 2008). The downside of this test is that it only detects the 70 most prevalent CF mutations, yet there are 1720 mutations listed in CF Genetic consortium database (CFF Consortium).

#### **1.4.2.3 Nasal Potential Difference Testing**

Nasal potential difference testing is used as a complementary diagnostic tool where sweat and genetic tests are inconclusive (Delmarco *et al.*, 1997). Sodium and chloride ion transport through CFTR on epithelial cells create transepithelial electrical potential (TEP). The nasal potential difference testing directly measures the transepithelial electrical potential through the ion channels. In this test, an electrode is placed in the nose of a patient and a series of solutions are applied to the nose. The solutions are a Ringer's saline solution (a salt solution used to obtain the baseline potential difference), amiloride solution that blocks sodium channels, a chloride-free solution, and finally isoproterenol solution that stimulates CFTR. A typical CF patient has a more negative baseline potential (-60 mV vs. -40 mV), a larger inhibition of TEP

after addition of amilorate, and a little or no change in TEP after addition of the chloride-free and isoproterenol solutions (Rosenstein & Cutting, 1998).

### **1.5 Pulmonary Symptoms of Cystic Fibrosis: A Vicious Cycle**

CF is a genetic disease affecting the epithelium surrounding secretory organs, in particular the lung epithelium (Davies & Bilton, 2009). While the other secretory organs are also affected, the predominantly life-threatening symptoms occur within the lungs (Gibson *et al.*, 2003). Defective and/or deficient CFTR causes thick, viscous mucous secretions in the airways, which are associated with an abnormal airway surface environment (Figure 1-5). Abnormal airway epithelia cause airway obstruction, which is followed by infection, inflammation, and finally bronchiectasis. The presence of infection, inflammation, and bronchiectasis leads to more obstruction in the bronchi, which leads to more infection and inflammation, which are the major driving forces in this vicious cycle.

### **1.6 Pulmonary Structure: Setting the Stage**

The lungs are organized as right and left lobes, connected through trachea. The trachea branches into two bronchi, which branch into tubular extensions- bronchioles. Finally, bronchioles branch into alveoli where oxygen is transported into capillaries through alveoli epithelia (Figure 1-6a).

The lung epithelium serves as a continuous lining around the lungs and provides a selective surface where ions and oxygen are transported into and out of the lung tissue (Matthay *et al.*, 2002). Airway portions of the lungs (bronchi and bronchioles) are covered with basal, ciliated and mucous secretory (Goblet) epithelia cells (Figure 1-6b).

Goblet cells are located distally and secrete mucin (MUC5AC, MUC5B) to form a mucus layer surrounding the epithelia (Figure 1-6b). Clara cells replace Goblet cells at the proximal portion of the airways. Finally, the alveoli are composed of type I and type II epithelial cells (Figure 1-6b and 1-6c) (Matthay *et al.*, 2002).

Type I cells are very large, thin, squamous epithelial cells with 50-100  $\mu\text{m}$  in diameter and constitute 95% of the alveolar surface (Figure 1-6c). They provide structural support to alveoli. Type II cells are cuboidal epithelial cells, approximately 10  $\mu\text{m}$  in diameter (Figure 1-6c). Type II cells function to secrete surfactant (e.g. phospholipids) to reduce surface tension in the lungs during pressure changes (Matthay *et al.*, 2002; Matthay *et al.*, 2005). Both types of cells are involved in active ion transport (or vectorial ion transport) at the proximal portion of the alveoli to maintain osmotic balance, and keep the lungs free of pathogens (Saumon & Basset, 1993).

Osmotic balance is required to keep the thickness of airway surface liquid (ASL) at optimum height for the efficient mucociliary clearance. ASL is a thin water surface, which lines the airway portion of the epithelial cells (Figure 1-7). It consists of a mucus layer (7 to 70  $\mu\text{m}$  thickness) and a periciliary liquid layer (PCL) (Gibson *et al.*, 2003) (Figure 1-7). The mucus layer traps foreign particles while the PCL layer separates the mucus layer from the epithelial cells. The osmotic balance is important to keep the thickness of the PCL at optimum height (approximately 7  $\mu\text{m}$ ) so cilia can outbeat and clear the foreign particles trapped in the mucus (mucociliary clearance) (Figure 1-7) (Boucher, 2004).

Osmotic balance in the lungs is maintained via sodium influx through apical amilorate-sensitive epithelial  $\text{Na}^+$  channels (ENaC) in response to an electrochemical

gradient generated by basolateral quabain-inhibitable  $\text{Na}^+/\text{K}^+/\text{ATPase}$  (Matthay *et al.*, 2002). Type II cells are easy to culture and they form monolayers with intact tight junctions in 3-4 days (Matthay *et al.*, 2005). These cells express apical amilorate-sensitive epithelial  $\text{Na}^+$  channels (ENaC), basolateral quabain-inhibitable  $\text{Na}^+/\text{K}^+/\text{ATPase}$  pump, apical  $\text{Ca}^{+2}$  activated  $\text{Cl}^-$  channel, CFTR, and basolateral  $\text{Na}^+/\text{K}^+-2\text{Cl}^-$  cotransporter to mediate  $\text{Na}^+$  absorption and to secrete  $\text{Cl}^-$ , respectively. Protein expression and immunocytochemical studies revealed the presence and distribution of two subunits of  $\text{Na}^+/\text{K}^+-\text{ATPase}$  ( $\alpha_1$  and  $\alpha_2$ ) in type I cell in rats (Ridge *et al.*, 2003). However, the role of type I cells in the vectorial ion transport is not certain due to inability to culture polarized type I alveolar cells and perform electrophysiological studies (Matalon & Davis, 2003).

### **1.7 Mucociliary Clearance and Bacterial Colonization of the Lung**

Most CF patients are born with a normal lung function, followed by bacterial colonization of the lung, leading to chronic infections in the lungs within just a few years of life (Boucher, 2004). More than 80% of CF patients die due to bacterial infections (Lyczak *et al.*, 2002). Defective mucociliary clearance (MC) is the major cause of bacterial colonization in the lungs. MC is an important part of the body's innate defense mechanism, which traps and clears inhaled pathogenic organisms from the airways in less than 6 h under normal conditions (Knowles & Boucher, 2002). This mechanism is arrested in CF patients, allowing bacteria to enter the lungs and begin the invasion.



### **1.7.1 Mucociliary Clearance (MC)**

MC utilizes three components to clear foreign bodies: cilia, a mucus layer, and a PCL (Figure 1-7). The height of the PCL is important to maintain osmotic balance in order to maintain effective MC. The optimum thickness of PCL is approximately 7  $\mu\text{m}$ . This height is required for efficient ciliary beating.

### **1.7.2 Inhibiting MC Clearance**

Both absorption of  $\text{Na}^+$  and  $\text{Cl}^-$  secretion is important to keep periciliary layer (PCL) height (or volume) constant. In CF patients, nonfunctional CFTR leads to impaired  $\text{Cl}^-$  secretion to the ASL, causing accelerated  $\text{Na}^+$  absorption via ENaC. Thus, water is transported into the epithelial cell due to reverse osmosis. Water diffusion leads to depletion of the PCL layer, which in turn causes the formation of mucus plaques since mucus secretion continues from goblet cells. The continuous supply of mucus thickens the mucus plaques to more than 100  $\mu\text{m}$  in thickness, where oxygen is limited. Thickened mucus plaques reduce the mucociliary transport of the foreign molecules. Further, oxygen is depleted into the deep surface of the epithelial cells leading to mucus hypoxia, which is a favored environment for certain bacterial species, in particular *Pseudomonas aeruginosa* (Worlitzsch *et al.*, 2002).

### **1.7.3 Resisting Bacterial Infection**

Thick mucus blocks the airways (airway obstruction), which results in infection and inflammation. The presence of pathogens in the airways induces the secretory apparatus (Goblet and Clara cells) to secrete antibacterials (proteins lysozyme, lactoferrin, secretory phospholipase A2, and secretory leukocyte protease inhibitor

(SLPI) to kill pathogens. Neutrophils are the first immune cells to arrive at the site of infection. They recognize chemicals (chemoattractants) produced by pathogens and/or damaged tissue. Cytokines (IL-1, IL-8, tumor necrosis factor alpha (TNF- $\alpha$ )), leukotrienes (LTB-4), anaphylatoxin C5a, and bacterial proteins (LPS, exotoxins) are the major chemoattractants for neutrophil to the infection site (Konstan & Berger, 1997).

Oxidants and proteases like elastase released by neutrophils degrade bacterial proteins as well as host lung tissue. This leads to a release of host DNA, which thickens the mucus further and is associated with pulmonary exacerbations, worsening due to infection and inflammation. Further, elastase induces generation of more chemoattractants, which induce attraction of more neutrophils to the area of infection, resulting in more tissue damage. Both inflammation and infection lead to structural damage of the lung, this is known as bronchiectasis (Boucher, 2004).

The common belief is that inflammation occurs after infection. A massive amount of neutrophil attraction to the site of infection is the signature of a CF lung. Some studies performed with infants revealed that the lungs are at the proinflammatory state before bacterial colonization (Khan *et al.*, 1995; Muhlebach *et al.*, 1999; Rosenfeld *et al.*, 2001). Studies show elevated levels of proinflammatory markers like adhesion molecules at which neutrophils recognize and bind (ICAM-1), chemoattractants (IL-6 and IL-8) and deficiency of IL-10, which inhibits production of chemoattractants-IL-6, IL-8, TNF (Elizur *et al.*, 2008). There is no direct link between the *cftr* gene mutation and the proinflammatory reaction observed in CF patients. However, other studies suggest inflammation might precede infection (Khan *et al.*, 1995; Muhlebach *et al.*, 1999; Rosenfeld *et al.*, 2001).

## 1.8 The Bacteria of Cystic Fibrosis

“Understanding the genetic defect underlying cystic fibrosis is only half the battle.

Identifying the specific bacterium infecting CF patients is just as important”.

John E. Herst and Karen E. Elliot

Bacterial infection is the main reason for high mortality in CF (Gibson *et al.*, 2003; Govan & Deretic, 1996). CF patients are born with a sterile lung, and then shortly after birth they start to develop bacterial infections (Boucher, 2004). It is believed that susceptibility of CF patients to certain bacterial species is age-related. CF patients become infected with *Staphylococcus aureus* and *Haemophilus influenzae* in early years and then *Pseudomonas aeruginosa* and *Burkholderia cepacia* complex replace these species during adolescence (Govan & Deretic, 1996). *Stenotrophomonas maltophilia* and *Achromobacter xylosoxidans* are the other species isolated from CF patients.

### *Staphylococcus aureus*:

*Staphylococcus aureus* (Sa) is a gram positive, non-motile, non-spore forming bacteria that is part of human upper respiratory tract flora (Williams, 1961). Forty percent of CF patients have Sa colonization during their first year of their life (CFF, 2008). In fact, it is usually the first pathogen to cause infection in CF patients (McCaffery *et al.*, 1999). Most of these initial infections result from endogenous colonization with patients' own organism (Govan, 2000).

In the pre-antibiotic era, most CF patients died due to Staph infections (Govan & Deretic, 1996). Today, CF patients are either under prophylactic (preventive) or intermittent treatment with antibiotics until the *Staphylococcus* is eradicated from their sputum cultures (McCaffery *et al.*, 1999). Penicillin, cephalosporins, macrolide, and

tetracycline antibiotics are among the commonly prescribed antibiotics (Beardsmore *et al.*, 1994; Szaff & Hoiby, 1982; Weaver *et al.*, 1994). It has been shown that prophylactic treatment of CF patients with floxacillin (penicillin-derivative) is beneficial and is associated with fewer hospital visits, reduced morbidity, and lower rates of infection, when compared to intermittent antibiotic treatment (Weaver *et al.*, 1994). However, another study revealed that prophylactic antibiotic therapy of patients under age 6 leads to significantly higher rates of *Pseudomonas aeruginosa* acquisition (approximately 50% more) when compared to patients with intermittent antibiotic treatment (Ratjen *et al.*, 2001). Further, aggressive antibiotic treatment is also associated with the emergence of resistant Staphylococcus, in particular methicillin resistant Sa (MRSA), one of the most vexing resistance challenges worldwide (Nordmann *et al.*, 2007). A recent survey of CF patient registry data from the US recorded between January 1996 and December 2008 has shown that detection of MRSA in CF patients is associated with more rapid lung failure and a significant decrease in life expectancy (Dasenbrook *et al.*, 2010).

*Haemophilus influenzae:*

*Haemophilus influenzae* (Ha) is among the most commonly isolated opportunistic pathogens from CF patients, in particular from newborns and children under the age of 10 years (CFF, 2008). The prevalence of Ha infection is approximately 30% among patients between 0-5 years old (Razvi *et al.*, 2009).

Ha infections are treated after detection of asymptomatic colonization or after the development of an infection. Amoxicillin, ciprofloxacin, and azithromycin are the most commonly prescribed antibiotics to treat Ha infections (Doring & Hoiby, 2004). Ciprofloxacin resistance has been observed in 40% of Ha strains isolated from CF

patients (Perez-Vazquez *et al.*, 2007). Further, vaccination of infants is used as a preventive approach. However, infections with uncapsulated and therefore non-typable Ha reduce the chance of success via vaccination (Bilton *et al.*, 1995).

The role of Ha infection in CF pathogenicity is unknown. It may form biofilms in lung epithelia and induce inflammatory reactions in the lower respiratory tract (Starner *et al.*, 2006). Inflammation due to Ha colonization at an early age causes lung damage in CF patients, which in turn provides an ideal environment for further colonization, particularly by *Pseudomonas* (Govan & Deretic, 1996; Lyczak *et al.*, 2002).

*Stenotrophomonas maltophilia*:

*Stenotrophomonas maltophilia* (Sa) is gram-negative, motile bacteria present in the natural environment (such as on plants and in soil) and the hospital environment. Sa is also present in approximately 13% of CF patients (CFF, 2008). Acquisition of Sa is mostly from the environment (Denton & Kerr, 1998). Doxycycline, ceftazidime, tobramycin, and ciprofloxacin are the most frequently used antibiotics to treat these infections. The exact role of Sa infections in CF pathogenicity is unknown. The common belief among clinicians is that these infections are more harmful to patients with already damaged lungs (Gladman *et al.*, 1992; Karpati *et al.*, 1994).

*Pseudomonas aeruginosa*:

*Pseudomonas aeruginosa* (Pa) is a gram-negative, multi-drug resistant versatile bacterium grows in soil, water, on plant, and animal tissues (Govan & Deretic, 1996). It is the most common infectious agent involved in the CF lung and it is often acquired shortly after birth (Li *et al.*, 2005). The prevalence of Pa infection is 30% of infants, 60% of adolescents, and 80% of adults with CF (CFF, 2008).

The initial source of Pa infection is unknown. Natural environment is one potential source of a CF patient's first acquisition (Barben *et al.*, 2005; Regnath *et al.*, 2004). Indeed, a recent genotype study of clinical Pa strains from newly infected CF patients and Pa strains isolated from various surfaces of the patients' houses revealed that 5.9% of the environmental isolates share the same genotypes with clinical isolates (Schelstraete *et al.*, 2008). Further, samples from hospital settings, including sinks, soap, tables, and tap water, are also contaminated with Pa (Doring *et al.*, 1996; Zimakoff *et al.*, 1983). The prevalence of Pa is 72% from patient's toilets, 44% on sinks, and 1.5% on other hospital surfaces (Festini *et al.*, 2007). The risk of potential Pa acquisition of a CF patient from clinical settings at each visit is 5.4% (Festini *et al.*, 2007).

Patient-to-patient contact is another route for Pa transmission. Speert *et al.*, 1987 showed that patients sharing the same hospital room are colonized with the same Pa strain (Speert & Campbell, 1987). Another study performed on patients attending the same CF holiday camp revealed that ¼ of the patients are infected with the same strain (Brimicombe *et al.*, 2008). Further, a genome fingerprinting study performed on siblings from 13 families showed that one to three Pa strains are identical in all siblings in 8 families (Grothues *et al.*, 1988).

During infancy, Pa is isolated intermittently from CF patients. Screening the presence of Pa in oropharyngeal (OP) and bronchoalveolar lavage (BAL) cultures are routine approaches. Patients are (i) intermittently infected if 50% or less of the preceding 12 months the patients are Pa culture positive, (ii) chronically infected if more than 50%

of the preceding 12 months they are culture positive, (iii) free if no Pa is detected (Lee *et al.*, 2003).

The first step in colonization is the attachment of Pa to the epithelial cell surface (Davies & Bilton, 2009). As described above, defective MC traps bacteria on the viscous mucus ASL where Pa is in close contact with the epithelial cell surface. Early Pa colonizers isolated from CF airways are motile (Gibson *et al.*, 2003). Pa flagellar mutants cause milder disease phenotypes in a murine model, suggesting that motility is important for early bacterial attachment and colonization (Feldman *et al.*, 1998). Further, recent studies suggested that Pa pili bind CFTR and epithelial cell receptor ganglioside asialo-GM1, which have been proposed as Pa receptors (de Bentzmann *et al.*, 1996; Pier *et al.*, 1996; Pier *et al.*, 1997). Interaction of Pa with ganglioside asialo-GM1 receptor induces NFK-B mediated expression of pro-inflammatory cytokines, which are major neutrophil attractants (Davies & Bilton, 2009). Therefore, colonization directly induces inflammation, which is associated with tissue destruction and additional infection cycles, as described above.

Attachment of Pa to the CFTR channel is followed by internalization into epithelial cells where the bacterium colonizes, forms a biofilm, and may secrete virulence factors to further invade lung tissue, all of which results in the development of chronic lung infections (Davies & Bilton, 2009). Pa employs a variety of exoproducts to invade lung tissue, such as elastase, protease, exotoxin A, siderophores, and antibiotic resistance proteins. Elastase and alkaline phosphatases are proteases, which cleave host immune proteins including immunoglobulins, cytokines (Heck *et al.*, 1990). Exotoxin A inhibits phagocytosis and siderophores (iron-binding proteins), like pyocyanin, slows down

ciliary beat frequency in the ASL and destroys tight junctions at epithelia, leading to lung tissue destruction (Haas *et al.*, 1991).

The most vexing feature of Pa lung invasion is the emergence of alginate forming mucoid variants over time (Hentzer *et al.*, 2001). Levy *et al.*, 2008 showed that nearly half of the patients in his study developed mucoidy Pa variants during the course of study.

These data revealed that gender, presence/absence of the  $\Delta F508$  deletion, Forced Expiratory Volume (FEV), and sputum type are all indicators of the potential establishment of mucoid Pa (Levy *et al.*, 2008).

Mucoid Pa form dense biofilms, which are virtually impossible to eradicate once established (Hentzer *et al.*, 2001). Biofilms are defined as polysaccharide matrix-enclosed, differentiated micro-colonies of bacteria growing on solid surfaces. Many bacterial pathogens adapt to their hosts by shifting from a free living planktonic mode of growth to biofilm formation when exposed to host defenses, nutrient deprivation, and oxidative or osmotic stresses (Fuqua *et al.*, 2001) (Jefferson, 2004). Biofilm formation in the lungs of CF patients creates a major health challenge. This is due in part to the fact that biofilms result in 10 to 1000-fold increase in antibiotic resistance and an increase in the ability to withstand host defense systems. Indeed, the formation of biofilms is the primary contributor to the mortality of CF patients (Giwercman *et al.*, 1991; Hoyle *et al.*, 1992; Miller & Bassler, 2001). The elevated levels of antibiotic resistance and virulence are due primarily to the phenotypic heterogeneity within the biofilm structure, to reduced growth rate of cells in biofilm, to the limited access of antibiotics into the interior regions of the biofilm structure, and to the impact of quorum signaling based-induction of the virulence genes (Lewis, 2001; Stoodley *et al.*, 2002). Quorum signaling (QS) is a cell-



density dependent communication between bacteria that occurs via secreted signaling molecules (acylated homoserine lactones (AHLs)). *Pa* has two hierarchical quorum signaling systems: LasI/LasR and RhlI/RhlR (Van Delden & Iglewski, 1998). A recent study has shown that QS mutants of *Pa* possess higher susceptibility to antibiotic treatment compared to wild type *Pa* (Shih & Huang, 2002).

Despite high levels of antibiotic resistance in CF lung biofilms, antibiotic treatment remains the major therapeutic approach to treatment. The most promising drugs for treating Pseudomonal infections are TOBI<sup>®</sup>, which is a solution of the anti-pseudomonal tobramycin designed for inhalation, and azithromycin, in either an oral suspension or capsules. These antibiotics differ in their modes of action. Tobramycin is an aminoglycoside, which inhibits protein synthesis and thus kills the target pathogen. Azithromycin is a macrolide, which improves the patients' lung function by reducing inflammation and alginate production, which is the main component of a biofilm's structure (Giamarellos-Bourboulis, 2008).

Long-term exposure of *Pa* to antibiotics selects for multi-drug resistant strains. In fact, nearly half of adult CF patients are chronically infected with multi-drug resistant *Pa*, which is associated with a more rapid decline in lung function (Lechtzin *et al.*, 2006). An antibiotic susceptibility study of *Pa* strains from sixty-seven CF centers revealed high levels of resistance, on average 75% of the strains were resistant to the 10 drugs examined, including tobramycin, gentamycin, ciprofloaxacin (Saiman *et al.*, 1996). In addition, *Pa* strains are intrinsically multi-drug resistant. Genome sequencing studies revealed that the genome of at least one *Pa* strain (PAO1) genome has five different drug efflux pumps (Lomovskaya & Watkins, 2001). Finally, a recent study revealed a novel

drug efflux mechanism in Pa, one that specifically mediates biofilm resistance to tobramycin, gentamycin, ciprofloxacin, which are among the most commonly prescribed antibiotics to treat CF lung infections (Zhang & Mah, 2008).

*Burkholderia cepacia complex:*

Members of the *Burkholderia cepacia* complex (Bcc) are gram-negative bacteria found in numerous environments including soil, water, as well as the roots of various plants (Mahenthiralingam *et al.*, 2005). The Bcc comprises a group of genetically diverse, but phenotypically similar bacteria, including nine genomovars: *Burkholderia ambifaria*, *Burkholderia anthina*, *Burkholderia cenocepacia*, *Burkholderia cepacia*, *Burkholderia dolosa*, *Burkholderia multivorans*, *Burkholderia pyrrocinia*, *Burkholderia stabilis*, and *Burkholderia vietnamiensis* (Mahenthiralingam *et al.*, 2000). In the 1930s, *Burkholderia* were classified as members of the *Pseudomonas* genus. Yabuuchi *et al.* 1992 proposed to re-classify these *Pseudomonas* species under the new genus *Burkholderia*, based on 16s rRNA sequences, phenotypic characteristics, and DNA-DNA hybridization (Yabuuchi *et al.*, 1992).

*B. cepacia* strains may be either pathogenic, beneficial or commensal, depending on the environment in which they are found. *B. cepacia* causes soft onion rot disease, which is a type of root tissue damage associated with yellow/brown coloring (Mahenthiralingam *et al.*, 2005). Further, a type IV toxin from *B. cenocepacia* and *B. vietnamiensis* causes a type of plant tissue destruction called plant-tissue water soaking (Engledow *et al.*, 2004). *B. cepacia* degrades chlorinated aromatic substrates found in pesticides and herbicides. For example *B. vietnamiensis* degrades toluene and decreases

the concentration of toluene in the water present in a sand aquifer (Mahenthiralingam *et al.*, 2005).

In the 1970s, members of the Bcc were first identified in the airways of CF patients (Govan & Deretic, 1996). Although these species remain a relatively rare source of infection in the CF lung (roughly 3% of CF patients are colonized with *B. cepacia*), the clinical manifestations can be severe (2008). Colonization may be asymptomatic or can result in progressive decline in lung function over a period of months. A smaller number of such infections (20%) result in cepacia syndrome, which is a fatal pneumonia that causes rapid death, within weeks to months (Jones *et al.*, 2004; Kalish *et al.*, 2006; Tablan *et al.*, 1985). *B. multivorans* and *B. cenocepacia* are the species from the Bcc most frequently isolated from CF patients with a prevalence of 38% and 50%, respectively (LiPuma *et al.*, 2001). However, all genomovars have been recovered from CF patients (Mahenthiralingam *et al.*, 2000; Mahenthiralingam *et al.*, 2005).

Various disease outcomes are explained by host and strain dependent factors. Although the process of Bcc colonization is unclear, these species encode numerous virulence factors (Mahenthiralingam *et al.*, 2005). A pathogenicity island is located in many of the Bcc genomes, which is absent in non-epidemic strains and rarely detected in environmental strains (Mahenthiralingam *et al.*, 1997). Surveys reveal that the presence of this islands is various between geographic regions. For example, the strains of *B. cepacia* infecting CF patients in Canada are more likely (80%) to encode the pathogenicity island than are strains from US patients (23%) (LiPuma *et al.*, 2001). The pathogenicity island contains quorum signaling factors, genes involved in fatty acid and amino acid metabolism, and several genes whose functions are unknown (Baldwin *et al.*,

2004). Baldwin *et al.*, 2004 also revealed that mutations in the amidase and quorum-sensing genes result in lower levels of strain persistence and reduced levels of inflammation in the lungs of a CF rat model. Additional pathogenicity is conferred by cell surface lipopolysaccharides (LPS), which provide resistance to cationic antibiotics (Shimomura *et al.*, 2003). Flagellar genes also contribute to strain virulence. Mutations in *fliG* gene, which encodes the ATPase subunit of the flagella, prevent attachment and invasion of the epithelial cells (Tomich *et al.*, 2002).

It is now becoming clear that both environment and clinical settings are potential reservoirs for Bcc infections. LiPuma *et al.*, 2002 used DNA sequence data to determine that a *B. cepacia* strain found in an epidemic, was also isolated from an unrelated soil sample (LiPuma *et al.*, 2002). However, frequently, patients are colonized with *B. cepacia* during a hospital visit or while at a CF camp (Gibson *et al.*, 2003). It is clear that *B. cepacia* strains can be transmitted from person to person (contagious) (LiPuma *et al.*, 1990). In response, National CF organizations prepared an infection control consensus document, which recommends segregation of CF patients infected with Bcc from those not infected. Further, they urge that CF patients remain at least three feet away from one another, even if neither is infected with Bcc (Mahenthiralingam *et al.*, 2005).

Treatment of Bcc pulmonary infections is problematic due to the intrinsic antibiotic resistance of most strains. The Bcc genomes possess multi-drug efflux pumps, LPS, and chromosomally encoded beta-lactamases, which provide resistance to various classes of antibiotics including aminoglycosides, chloramphenicol, quinolones, and cationic peptides (Aronoff, 1988; Burns *et al.*, 1989; Burns *et al.*, 1996; LiPuma, 1998a; LiPuma, 1998b). Combinatorial application of multiple antibiotics is the current method

of choice. These drug combinations usually include meropenem due to the low levels of meropenem resistance observed so far in Bcc (Aaron *et al.*, 2000; Lewin *et al.*, 1993). Minocyclin, amikacin, and ceftazidime are among the other antibiotics used in combination with meropenem (Gibson *et al.*, 2003).

### **1.9 Interaction of *Pseudomonas aeruginosa* (Pa) and *Burkholderia cepacia* complex (Bcc)**

Pa is the persistent species in CF lung infections. Bcc colonization is more rare, and, usually, follows an initial Pa colonization (Govan & Deretic, 1996). The outcome of these multiple rounds of colonization depends upon the patient and the bacterial species and strains involved. *B. cenocepacia* and *B. multivorans* are the most frequently isolated members of the Bcc (LiPuma *et al.*, 2001). It is not known why some Bcc species and strains are effective lung colonizers while others are not. Certainly there are interactions between the Pa and Bcc species in CF lungs.

The first evidence of communication between these two species comes from McKenney *et al.*, 1995. In this study, spent media, used to grow *B. cepacia* was supplemented with a cell free extract of Pa and the expression of siderophore, elastase, protease and lipase was monitored in *B. cepacia*. The levels of these virulence-related proteins were increased (McKenney *et al.*, 1995).

Weaver and Kolter (2004) investigated the effect of *B. cepacia* cell free extract on the expression of Pa genes. The genes upregulated in Burkholderia conditioned medium are the same as those induced under iron-limited conditions. These data reveal that Burkholderia conditioned medium has iron chelating activity (Weaver & Kolter, 2004).

Additionally Pa and Bcc strains interact through a process mediated by quorum signaling (QS). However, this communication is unidirectional, with Bcc strains capable of perceiving the QS molecules, acylated homoserine lactones (AHLs), released by Pa (Riedel *et al.*, 2001). Geisenberger *et al.* (2000) analyzed the AHL profiles of Pa strains in CF patients co-infected with both species and revealed that during co-infection a dramatic reduction of AHL production occurs in the Pa (Geisenberger *et al.*, 2000). Thus, the two species communicate, and the interaction results in an increased production of virulence factors. However, the final outcome, in terms of the patients' health, is also dependent upon strain- and patient-specific factors, which might explain why a mixed infection results in such dramatic differences in infectivity, pathogenicity and host response.

### **1.10 Bacteriocins**

Bacteriocins are narrow spectrum antibacterial proteins, which are produced by most bacterial species (Riley & Gordon, 1999; Riley & Wertz, 2002a; Riley & Wertz, 2002b). They are classified based upon the producer species; such as colicins produced by *E. coli*, pyocins produced by Pa (formerly named *pyocyanina*), and pesticins of *Yersinia pestis*. Colicins are one of the most thoroughly studied bacteriocin and are used as a model system to study bacteriocin structure, function, and evolution (Riley & Gordon, 1999; Riley & Wertz, 2002a; Riley & Wertz, 2002b).

Bacteriocins are high molecular weight proteins, produced by bacteria, usually under conditions of stress, such as when resources are limited (Riley & Gordon, 1999). They are encoded on both plasmids and chromosomes. The genes encoding bacteriocins are often found in clusters of genes, which include a toxin, immunity, and lysis genes.

The immunity gene encodes a protein, which confers immunity to that toxin, while the lysis gene encodes a protein that aids in the release of the toxin from the cell – often resulting in cell death. (Riley & Wertz, 2002a).

## **1. 10.1 Bacteriocins of *Pseudomonas aeruginosa* (Pa) and *Burkholderia cepacia* complex (Bcc)**

### **1.10.1.1 Pyocins: Bacteriocins of Pa**

Francois Jacob was the first scientist to describe the production of bacteriocins (known as pyocins) in Pa (formerly *P. pyocyanea*). He detected a UV induced, protease resistant compound, which causes lysis of a susceptible bacterium (Jacob, 1952). Pa is one of the most prolific bacteriocin producing species. More than 90% of Pa isolates produce pyocins (Govan & Deretic, 1996).

Pyocins fall into three types, known as R-, F-, and S pyocins. The R and F-type pyocins were the first pyocins described (Michel-Briand & Baysse, 2002). They share three key features: they can resist to proteases, resemble phage tails, and kill sensitive cells by depolarizing the cell membrane (Michel-Briand & Baysse, 2002). Two representative R and F-type pyocins, R2 and F2, are encoded in a large gene cluster, spans greater than 40 kb, located between the *trpE* and *trpF* genes in the genome of Pa strain PAO1 (Nakayama *et al.*, 2000). There are 44 open reading frames associated with the R2/F2 phenotypes, which include regulatory, lysis and toxin genes. The R2 and F2 pyocins show sequence similarity to the tail fiber genes of P2 and lambda phage, respectively (Nakayama *et al.*, 2000).

There are several R-type pyocins (R1, R2, R3, R4, and R5), which consist of extended sheets of 34 subunits, a base plate, tail fiber, and a core structure (Figure 1-8a).

A single bacterium is capable of producing 200 R-pyocins (Shinomiya, 1972). R-pyocin binds to membrane lipopolysaccharide (LPS) via the tail fiber (Ikeda & Egami, 1969). Contraction of tail fiber penetrates the cell membrane and produces a channel, or pore. The sensitive cell is then lysed due to the resulting depolarization of the cytoplasmic membrane (Uratani & Hoshino, 1984).

There are three F-type pyocins (F1, F2, and F3), which are flexible, non-contractile rods, 106 nm in length and 10 nm in width (Figure 1-8b). The rod structure consists of 23 annuli and a fiber structure consisting of both long and short filaments (Michel-Briand & Baysse, 2002).

The four known S-type pyocins (S1, S2, S3, and AP41) are protease sensitive bacteriocins, which resemble colicins in terms of their genetic structure and modes of action. They kill cells via DNase activity located at the C-terminal end of the toxin protein. Different S-pyocins may recognize different receptors; S2 and S3 recognize type I and II ferripyoverdine receptors, respectively (de Chial *et al.*, 2003).

The typical S-pyocin operon spans an approximately 2 kb region of DNA and includes two genes: the toxin gene which provides the killing activity and the immunity gene which provides immunity to that pyocin. There are four domains in the toxin protein: domain I produces receptor binding activity; the function of domain II is unknown; domain III enables translocation of the toxin through the plasma membrane; and domain IV provides killing activity (Michel-Briand & Baysse, 2002).

Two proteins regulate the synthesis of R, F and S pyocins: *ptrN* and *ptrR*, both of which are located upstream of the R2F2 gene cluster. *PtrN* is a transcriptional activator, which bind the regulatory sequence P-box located approximately 60-100 bp upstream of



the ribosome-binding site. The P-box consists of a 10-12 nt consensus sequence - ATTGnn(n)GT-nn(n). PtrR is a transcriptional repressor protein, which binds to PtrN, preventing its binding to the P-box. Under conditions of stress, such as DNA damage RecA cleaves PtrR, releasing PtrN. The binding of PtrN to the P box induces transcription of pyocin genes (Matsui *et al.*, 1993; Michel-Briand & Baysse, 2002; Nakayama *et al.*, 2000).

#### **1.10.1.2 Cepaciains: Bacteriocins of Bcc**

The bacteriocin produced by *B. cepacia* (known as *Pseudomonas cepacia* at that time) are called cepaciains (Govan & Harris, 1985). Govan *et al.*, 1985 has assayed a collection of *Pseudomonas cepacia*, as they were known then, using pyocin producer and sensitive strains as indicator strains to phenotypically identify bacteriocins produced by *B. cepacia*. This study revealed that 30% of *P. cepacia* strains possess bacteriocin-like killing activity. Further, the cepaciains looked like phage-tails when viewed under an electron microscope. Unfortunately, Govan's study is the only publication on cepaciains. No further molecular characterizations have been published.

### **1.11 CF Treatment Regimens**

#### **1.11.1 Physical therapy**

Physical therapy is applied to CF patients to clear mucus from their airways. Chest physical therapy, oscillating positive expiratory pressure, high frequency chest wall oscillation therapy, active cycle of breathing technique, autogenic drainage are all commonly employed approaches in treating CF patients (George *et al.*, 2009). These methods are noninvasive and aim to dislodge and clear mucus from lungs.

### **1.11.2 Anti-inflammatory therapy**

The goal of anti-inflammatory therapy is to suppress inflammation in the lungs, which causes tissue destruction and results in further infection. Ibuprofen and azithromycin are the most frequently used anti-inflammatory drugs (George *et al.*, 2009).

### **1.11.3 Mucus thinning drugs**

Mucus thinning therapy reduces the mucus, which clogs the airways of CF patients. This also results in a reduction of the bacterial load in the CF lungs. Pulmozyme is the most frequently prescribed mucus-thinning drug. Pulmozyme is a DNase, inhaled via a nebulizer, which degrades the patients own DNA that was released during inflammation (Frederiksen *et al.*, 2006).

### **1.11.4 Antibiotic therapy**

Antibiotics are used to treat pulmonary bacterial infections. Pa and Bcc infections are the most problematic to treat since both species are intrinsically multi-drug resistant. Antibiotics can be administrated orally, intravenously, and are inhaled. The most common therapeutic approach involves application of a cocktail of antibiotics, usually a combination of beta-lactamases and aminoglycosides to treat chronic Pa infections. A combination of meropenem and another class of antibiotic (tetracycline, aminoglycoside and/or cephalosporins) is used to treat Bcc, based on the sensitivity phenotype of the strains isolated from the lung (Gibson *et al.*, 2003).

### **1.11.5 Bronchodilator therapy**

A bronchodilator is a medicine that dilates bronchi and bronchioles. This therapy is administered during airway obstruction when mucus clogs the airways, and the patient having difficulty breathing (Eggleston *et al.*, 1991).

### **1.11.6 Lung transplantation**

Lung transplantation is the therapy applied to CF patients, which are at the end-stage of the disease. The lifespan of CF patients might increase up to 10 years. However, post transplantation infections in particular Pa infections are common and observed up to 90% of patients (Morton & Glanville, 2009).

## **1. 12 Research Questions**

The following research questions were the focus of my doctoral research:

1. Do clinical strains of Pa and Bcc isolated from CF lungs produce bacteriocins?
2. If so, what types of bacteriocins are produced and how specific or broad are their killing activity?
3. What roles do these bacteriocins serve in mediating intra- and inter-specific interactions of bacterial isolates from cystic fibrosis patients?

### **1.13 Problem Statement**

Over the past 60 years, a vast literature has focused on the bacteriocins produced by Pa and Bcc. Within the CF research community, most of this research has focused on phenotypic screens of pyocin and cepaciacin production of the two species (Edmonds *et al.*, 1972b; Farmer & Herman, 1969; Farmer & Herman, 1974; Fyfe *et al.*, 1984; Govan

& Harris, 1985; Jones *et al.*, 1973; Jones *et al.*, 1974a; Jones *et al.*, 1974b; Kageyama *et al.*, 1979; Moroz *et al.*, 1984; Pitt, 1988; Richardson *et al.*, 1991; Shriniwas, 1975; Zabransky & Day, 1969). However, none of this research has explored the potential role of bacteriocins in mediating within and between species killing of clinical Pa and Bcc strains. Given the high levels of bacteriocin production by both species, and the clear necessity that they interact during co-infections of the CF lung, this absence of research is striking.

#### **1.14 Purpose of this study**

The major goals of this study are to:

1. Determine if clinical strains of Pa and Bcc isolated from the CF lung produce bacteriocins.
2. Determine whether these toxins play a role in mediating intra- and inter-specific interactions of bacterial isolates from cystic fibrosis patients.
3. Identify bacteriocins produced by the two species.

#### **1.15 Methodology**

1. Employ phenotypic screens of bacteriocin production to determine which isolates from CF lungs produce bacteriocin-like inhibition phenotypes.
2. Employ phenotypic assays (protease digestion, freezing, and filtration) to assess whether the phenotypes observed are due to bacteriocins, phage or some unknown mechanism.
3. Employ multilocus pyocin sequence typing to determine the bacteriocin genes present in this strain collection.

4. Screen genomic libraries to identify bacteriocin genes that failed to amplify in the sequence typing characterization.

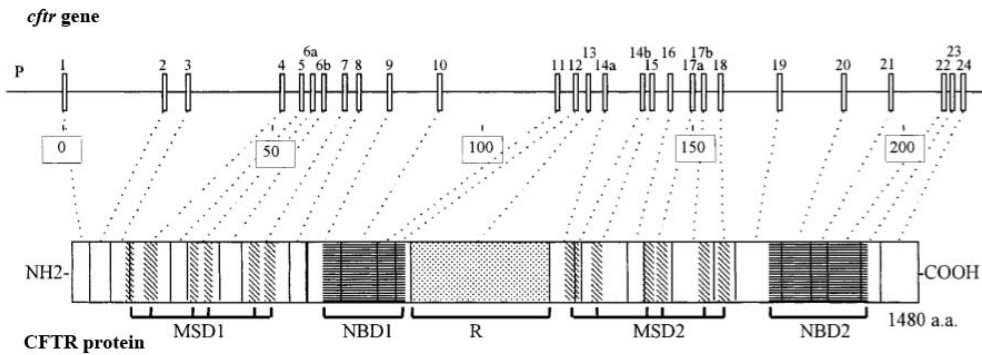
### **1.16 Significance**

Pa and Bcc infections are the cause of significant mortality in patients with cystic fibrosis. Pa is the primary, persistent species found in CF lung infections. This species is hard to treat due to its intrinsic resistance to many antibiotics. Bcc causes secondary lung infections, usually colonizing the CF lung after initial Pa colonization. Little is known about how these two species interact during the strain invasion and establishment process. This study seeks to contribute to our understanding of the role bacteriocins serve in mediating the bacterial strain and species interactions in the CF lung. First, an extensive strain by strain survey reveals the inhibition potential of both species. Second, the same survey provides insight into the role these toxins play in mediating intra- and inter-species interactions in the CF lung. Finally, the molecular characterization reveals which bacteriocins are produced by CF strains. The data produced in this study are the first step in understanding the role of bacteriocins in mediating bacterial strain interactions in the CF lung.

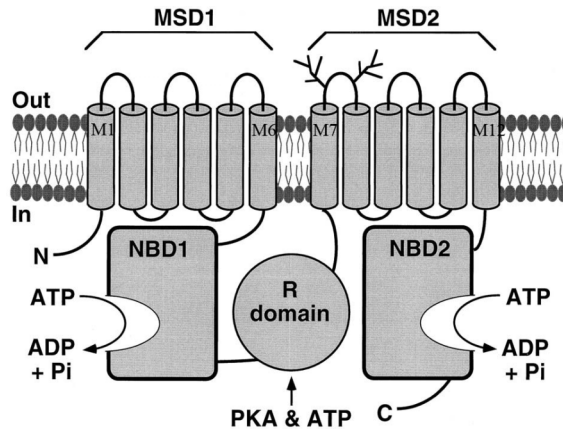
**Figure 1-1 Cystic Fibrosis Transmembrane Regulator (*cftr*) gene and its encoded protein presented on epithelial cells.**

a. The *cftr* gene spans 215 kb region and consists of 27 exons and 26 introns; b-The *cftr* gene encodes a transmembrane protein with two membrane spanning domains (MSD), regulatory domain (R), and two nucleotide binding domains (NBD) (Zielenski *et al.*, 2000; Sheperd *et al.*, 1999).

a. *cftr* gene and CFTR protein



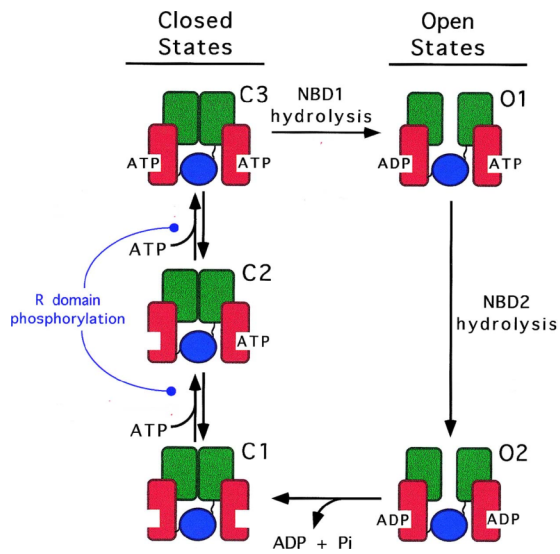
b. CFTR protein topology



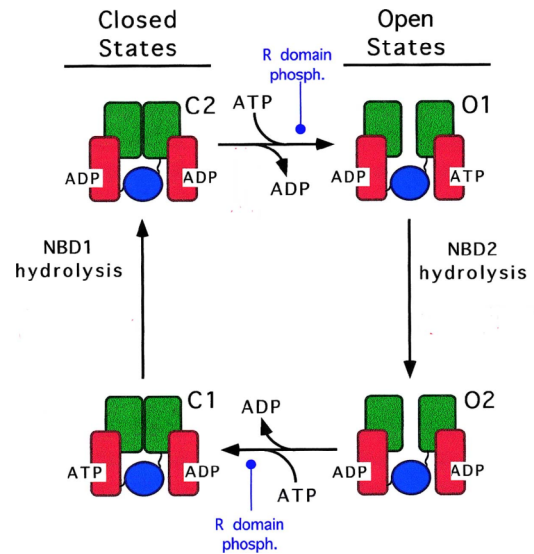
**Figure 1-2 Models of ATP dependent gating of CFTR channel**

a. Model 1 has three closed states (C1, C2, and C3), and two open states (O1 and O2). Sequential hydrolysis of ATP at NBDs opens the channels; b. Model 2 has two closed (C1 and C2) and two open states (O1 and O2). ATP hydrolysis at NBD2 (O2 state) followed by R domain phosphorylation, which leads to channel closing (Sheperd *et al.*, 1999).

a. Model 1



b. Model 2

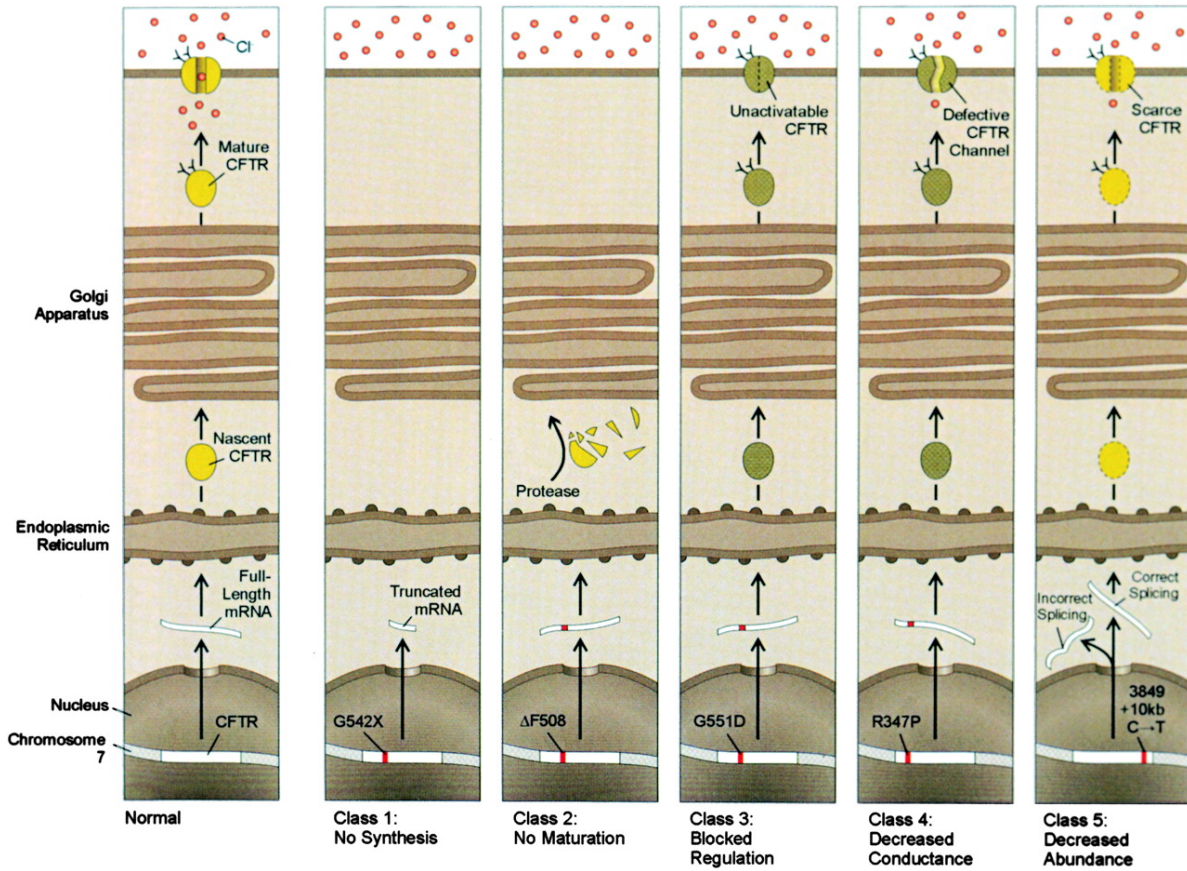






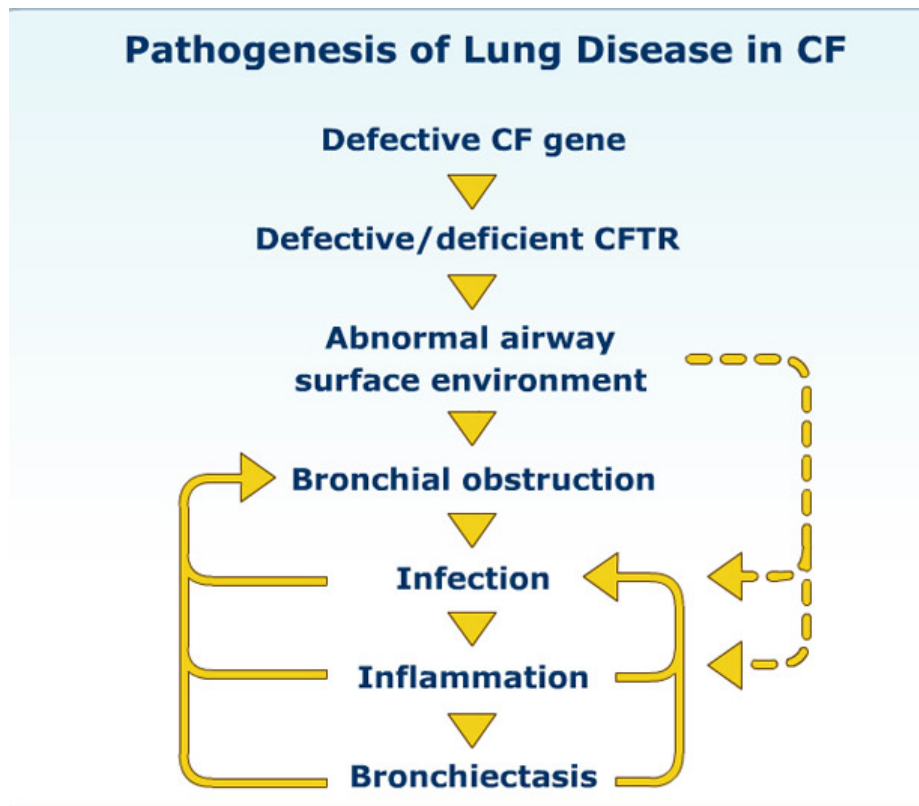
**Figure 1-4 Functional classification of CFTR mutations**

Mutations causing unfunctional or defective CFTR protein is classified into five classes (Class 1-5) (Gibson *et al.*, 2003).



**Figure 1-5 Cystic Fibrosis: the vicious cycle**

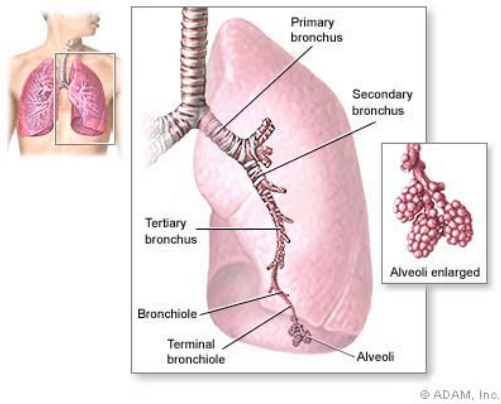
Pathogenesis of lung disease in CF is described as vicious cycle, in which abnormal airway surface cause airway obstruction, which is followed by infection, inflammation, and bronchiectasis (Konstan, 1997)



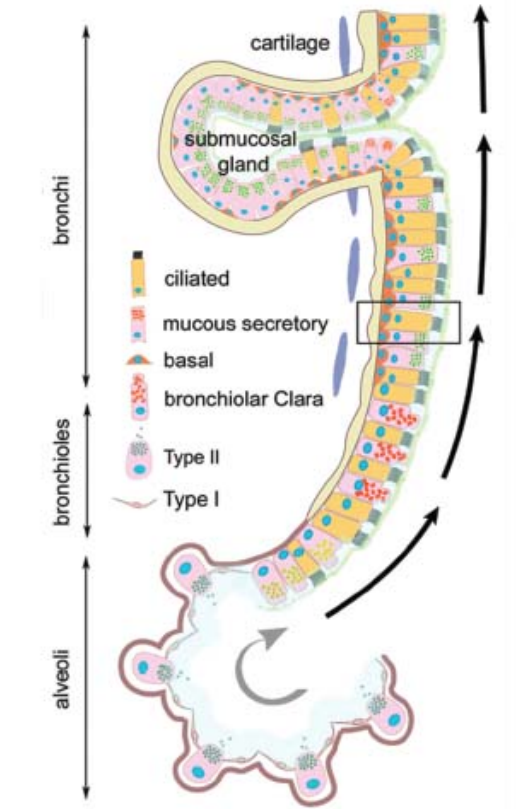
**Figure 1-6 Human lung epithelium**

a-Pulmonary structure of the lungs. Bronchus branches into bronchioles, which branch into alveoli; b- Pulmonary cells. Type I, type II epithelial cells, mucous secretory cells (Clara and Goblet) are epithelial cell types in lungs; c- Type I cells are large squamous epithelial cells and type II cells are cuboidal epithelial cells in lungs (Matthay *et al.*, 2002; Livraghi *et al.*, 2007)

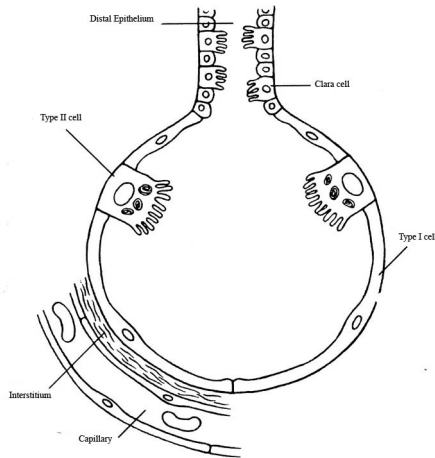
a.



b.

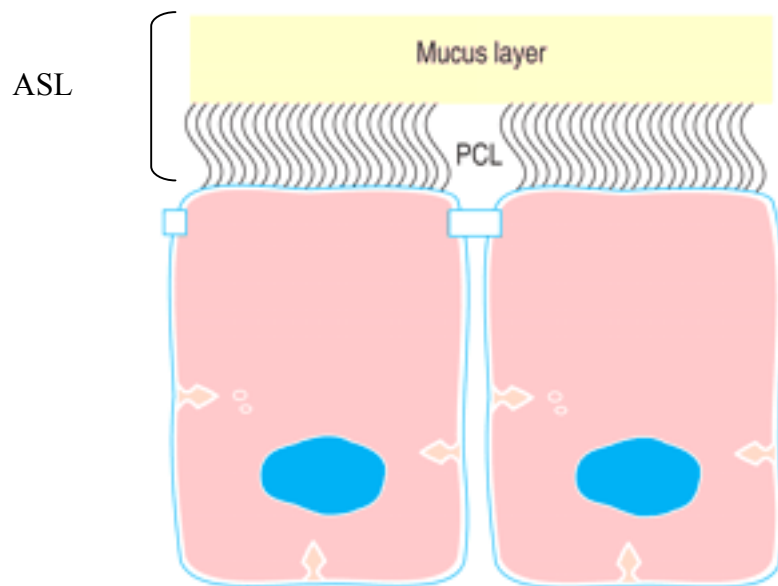


c.



**Figure 1-7 Airway surface liquid (ASL)**

ASL is a thin water surface, which lines the airway portion of the epithelial cells. ASL composed of mucous layer and periciliary liquid (PCL) (Boucher, 2004).

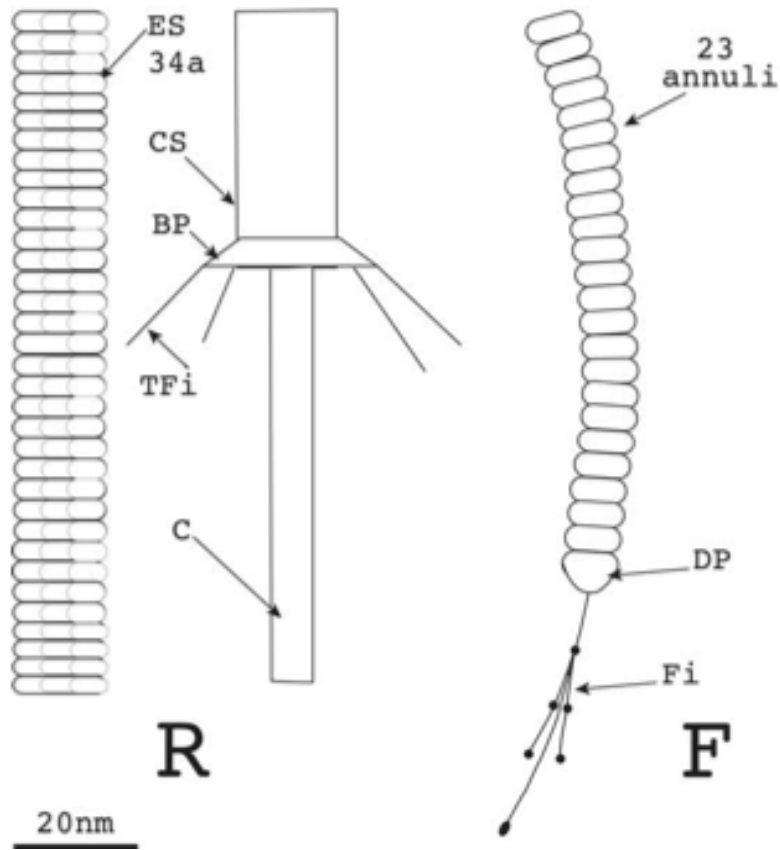


**Figure 1-8 Structures of R- and F- type pyocins**

a. R-type pyocin. R-type pyocins consist of extended sheets (ES), a base plate (BP), tail fiber (TF), and a core structure (CS); b-F-type pyocin. F-type pyocins consist of 23 annuli and a fiber (Fi) structure consists of both short and long filaments (Michael-Briand, 2002).

a. R-type pyocin

b. F-pyocin



## CHAPTER 2

### THE ROLE OF BACTERIOCINS IN MEDIATING INTERACTIONS OF BACTERIAL ISOLATES TAKEN FROM CYSTIC FIBROSIS PATIENTS

#### 2.1 Abstract

*Pseudomonas aeruginosa* (Pa) and *Burkholderia cepacia* complex (Bcc) lung infections are responsible for much of the mortality in cystic fibrosis (CF). However, little is known about the ecological interactions between these two, often co-infecting, species. This study provides the first report of the intra- and inter-species bacteriocin-like inhibition potential of Pa and Bcc strains recovered from CF patients. A total of 66 strains were screened and shown to possess bacteriocin-like inhibitory activity (Pa - 97% and Bcc - 68%), much of which acts across species boundaries. Further phenotypic and molecular based assays reveal that the source of this inhibition differs for the two species. In Pa, much of the inhibitory activity is due to the well known S and RF pyocins. In contrast, Bcc inhibition is due to unknown mechanisms, although RF-like toxins are implicated in some strains. These data suggest that bacteriocin-based inhibition may play a role in governing Pa and Bcc interactions in the CF lung and may, therefore, offer a novel approach to mediating these often-fatal infections.

## 2.2 Introduction

Individuals with cystic fibrosis (CF) face a lifelong battle with chronic bacterial lung infections. *Pseudomonas aeruginosa* (Pa) is the predominant infectious agent in the lungs of adult CF patients and most individuals are infected shortly after birth (CFF, 2008). Alginate-producing mucoid variants emerge over the course of several years and form dense bacterial biofilms in the CF lung. Once established, eradication of these infections is generally not possible (Govan & Deretic, 1996; Hentzer *et al.*, 2001).

In the 1970s, members of the *Burkholderia cepacia* complex (Bcc) of species were first identified in the airways of CF patients (Govan & Deretic, 1996). Although few patients colonized with Bcc (~3%), the clinical manifestations of these infections can be severe (CFF, 2008; Kalish *et al.*, 2006). Colonization by Bcc may be asymptomatic or result in progressive decline in lung function. A smaller number of infections result in “cepacia syndrome”, a fatal pneumonia that results in death (Jones *et al.*, 2004; Kalish *et al.*, 2006; Tablan *et al.*, 1985).

Given that CF patients are initially infected with Pa, Bcc strains must either compete with or act in synergy with established Pa biofilms (Al-Bakri *et al.*, 2004). How such interactions occur is unknown. In fact, little is known about the process of Bcc invasion and its outcomes. Do the strains from the two species peacefully co-exist? Do Bcc strains out-compete and displace pre-existing Pa strains? Few studies have sought to address how these two bacterial species interact in the CF lung (Al-Bakri *et al.*, 2004; McKenney *et al.*, 1995; Weaver & Kolter, 2004).

One factor known to mediate bacterial interactions is the production of potent toxins known as bacteriocins (Riley & Gordon, 1999). Unlike traditional antibiotics,

many bacteriocins have a relatively narrow killing range. They have been implicated in intra-specific competition brought on by limited nutrients (Riley & Wertz, 2002a). In some cases, however, bacteriocins are also able to kill more broadly and have been implicated as a primary mechanism for mediating microbial diversity (Kerr *et al.*, 2002).

Much is already known about pyocins, the bacteriocins produced by *Pseudomonas*. In fact, more than 90% of all *Pa* strains examined to date produce one or more of three pyocin types: S, R, and F (Fyfe *et al.*, 1984). S pyocins are high molecular weight proteins, which resemble the well-known colicins produced by *Escherichia coli*. They are protease sensitive and most kill by DNA degradation (Michel-Briand & Baysse, 2002). Four S-pyocins (S1, S2, S3, and AP41) have been studied extensively (Duport *et al.*, 1995; Sano & Kageyama, 1981; Sano *et al.*, 1993a; Sano *et al.*, 1993b; Sano, 1990) (Figure 2-1a). All S-pyocin operons share the presence of two genes: the larger (toxin gene) provides the killing activity, while the smaller (immunity gene) provides immunity against killing by that pyocin. The toxin gene is comprised of four domains: Domain I - produces receptor binding activity, which enables a pyocin to recognize a specific target, Domain II - has an unknown function, Domain III - enables translocation of the toxin through the plasma membrane, and Domain IV - produces the killing (DNAse) activity (Michel-Briand & Baysse, 2002).

The R and F pyocins, which resemble bacteriophage tails, are resistant to both nuclease and protease digestion and kill by depolarizing the cell membrane. The genes encoding these pyocins are found in a cluster (which is why they are often referred to as RF pyocins), and are located between *trpE* (anthranilate synthase component I) and *trpG* (anthranilate synthase component II) genes (Nakayama *et al.*, 2000; Shinomiya *et al.*,



1983). Figure 2.1(b) shows the genetic organization of pyocin R2F2 genes. The open reading frames (PRF3-PRF43) include regulatory, lysis, and R and F pyocin genes. The lysis genes (PRF9, PRF24, PRF25, and PRF26) are similar in sequence to the lysis cassettes of P2 and lambda phages (Nakayama *et al.*, 2000). There are 16 R and F pyocin genes (PRF10-23 and PRF28-43) (Nakayama *et al.*, 2000). The encoded proteins show high levels of sequence similarity to tail genes of P2 and lambda phage, respectively (Nakayama *et al.*, 2000). The function of PRF5-8 is unknown.

The S, R, and F pyocins are controlled by the same *ptrN* and *ptrR* regulatory genes (Figure 2-1b) (Michel-Briand & Baysse, 2002; Nakayama *et al.*, 2000). *PtrN* is a transcriptional activator, which is repressed by *PtrR* (Matsui *et al.*, 1993). Under DNA damage, *RecA* protein cleaves the *PtrR* repressor protein, which leads to expression of *ptrN*. *PtrN* binds to the regulatory sequence (P-box), thus inducing transcription of pyocin genes (Matsui *et al.*, 1993).

Stress conditions, such as might be encountered in a CF lung, may induce pyocin production. Microarray analysis of *Pa* genomes revealed that pyocin transcription is up-regulated by hydrogen peroxide and ciprofloxacin (Brazas & Hancock, 2005; Chang *et al.*, 2005). White & Curtis (2009) investigated the effect of pyocins in mixed-culture biofilms under aerobic and anaerobic conditions and revealed that pyocins sensitivity increased under anaerobic conditions. Further, Heo *et al.*, 2007 revealed that R pyocins provide a competitive advantage during growth in planktonic conditions.

Far less is known about cepaciocins, the bacteriocins produced by *Burkholderia*. Approximately 30% of *Burkholderia cepacia* (formerly *Pseudomonas cepacia*) strains produce cepaciocins (Govan & Harris, 1985). The few characterized cepaciocins

resemble R-type pyocins, as they are resistant to trypsin digestion and appear phage-tail-like when viewed under the electron microscope (Govan & Harris, 1985). Nothing further is known about the abundance, diversity or molecular biology of these toxins.

The goal of the present study is to determine if clinical strains of Pa and Bcc isolated from the CF lung produce bacteriocins and, if so, whether these play a role in mediating intra- and inter-specific bacterial interactions in the CF lung. To this end, a collection of clinical Pa and Bcc strains from CF patients was screened for bacteriocin production and sensitivity. This collection consists of strains isolated either as pairs (one strain of Pa and one of Bcc isolated from the same lung) or unpaired. Phenotypic screens for bacteriocin production coupled with typing methods to identify individual bacteriocins reveal a diversity of inhibitory mechanisms. Further, the patterns of within and between species inhibition suggests a role for these toxins in mediating bacterial interactions in the CF lung.

## **2.3 Materials and Methods**

### **2.3.1 Bacterial strains**

The bacterial strains used in this study include a total of 66 clinical strains of Pa (38) and Bcc (28) obtained from Children's Hospital, Boston MA. The study protocol was approved by the Committee on Human Research at Children's Hospital Boston and written informed consent was obtained. The clinical collection can be divided into two groups. The first is composed of 14 Pa and 7 Bcc paired isolates procured from 7 patients with CF. Each pair of Pa and Bcc strains was isolated from the same CF patient. The second group is composed of 24 Pa and 21 Bcc unpaired isolates, which were isolated

from 31 patients. The Bcc strains include 4 genomovars (1 *Burkholderia vietnamiensis*, 12 *Burkholderia multivorans*, 11 *Burkholderia dolosa*, and 4 *Burkholderia cenocepacia*). The reference strain collection includes bacteriocin producer strains which are Pa strains known to produce the following pyocins: S1, R4 (PML28), AP41 and F3 (PAF41), S2, R, F (NIH18), S2, R2, F2 (PAO1) (Ito, 1970; Kuroda & Kageyama, 1981; Nakayama *et al.*, 2000; Sano, 1990; Seo & Galloway, 1990). The reference collection also includes indicator strains sensitive to various combinations of pyocins: PML1516d (S1<sup>S</sup>S2<sup>S</sup>AP41<sup>S</sup>), NIH3 (S1<sup>S</sup>S2<sup>S</sup>AP41<sup>S</sup>), NIH3S1<sup>R</sup> (S1<sup>R</sup>S2<sup>S</sup>AP41<sup>S</sup>), NIH3S2<sup>R</sup> (S1<sup>S</sup>S2<sup>R</sup>AP41<sup>S</sup>), NIH3AP41<sup>R</sup> (S1<sup>S</sup>S2<sup>S</sup>AP41<sup>R</sup>), 3295 (AP41<sup>S</sup>), 3012 (AP41<sup>S</sup>F3<sup>S</sup>), 7NSK2 (S1<sup>S</sup>S3<sup>S</sup>), 7NSK2-*fpvA* (S1<sup>S</sup>S3<sup>R</sup>), PML14 (S1<sup>S</sup>R1<sup>S</sup>R2<sup>S</sup>R3<sup>S</sup>R4<sup>S</sup>R5<sup>S</sup>), 13s (S1<sup>S</sup>R1<sup>S</sup>R2<sup>S</sup>R3<sup>S</sup>R4<sup>S</sup>R5<sup>S</sup>), NIH5 (ATCC 25317) (F1<sup>S</sup>F2<sup>S</sup>F3<sup>S</sup>) (de Chial *et al.*, 2003; Kageyama *et al.*, 1979; Kuroda & Kageyama, 1981; Sano & Kageyama, 1981; Sano *et al.*, 1993a; Williams *et al.*, 2008). Additionally, four cloned S-pyocins (S1, S2, S3, and AP41) were employed to detect S-pyocin sensitivity of clinical Pa and Bcc strains (Duport *et al.*, 1995; Sano & Kageyama, 1993; Sano *et al.*, 1993b). There is no corresponding set of Bcc reference strains.

### **2.3.2 Bacteriocin production and sensitivity screen.**

The patch assay was used to identify bacteriocin-like inhibition, which involves overnight growth in 10 ml Luria Broth (LB) at 37 °C, shaken at 250 rpm. Six ml. of LB top agar (0.6%, w/v) mixed with 3 µl of Mitomycin C (0.5 µg ml<sup>-1</sup>; Sigma) and 100 µl of indicator cells (10<sup>8</sup> cells) were plated as a lawn on an LB plate (Pugsley & Oudega, 1987). The producer strain (~10<sup>6</sup> cells) was spotted on the indicator overlay by toothpick. Two spots per strain were applied. After overnight incubation at 37 °C the plates were

scored. If a strain produces an inhibition factor, such as a bacteriocin, active against the lawn, a zone of inhibition appears. The clinical collection was screened in an all-by-all assay, *i.e.* each strain was used as a potential inhibitor producer and indicator. The all-by-all patch assay was done in duplicate. All positive results were tested a third time.

The one-tailed Mann-Whitney U test was used for nonparametric comparison of inhibitory activities in order to identify significant differences in inhibition frequencies (Zar, 1999).

### **2.3.3 Phenotypic bacteriocin identification.**

The clinical collection was screened against the indicator strains using the patch assay. In addition, a cell-free extract of each putative bacteriocin-producing strain was subjected to trypsin digestion, filtration, and freezing to distinguish between protease sensitive and phage-like bacteriocins (Pugsley & Oudega, 1987; Riley *et al.*, 2003). Cell free extracts of putative producer strains were digested with trypsin ( $5\text{mg ml}^{-1}$ ) for 30 min at  $25^{\circ}\text{C}$ , which inactivates protease-sensitive bacteriocins, frozen at  $-70^{\circ}\text{C}$ , which tends to fracture phage tail type bacteriocins, and filtered in a 100 kDa Microcon YM-100 Centrifugal Filter Device (Millipore) using centrifugation at 14000 rpm for 12 min, which tends to retain phage tail-like bacteriocins. The treated cell free extracts were tested on the lawns of pyocin sensitive reference and clinical strains.

### **2.3.4 Molecular Screening**

Genomic DNA was isolated using the DNeasy Tissue kit (QIAGEN). The Gram-negative protocol was applied to  $2 \times 10^9$  cells from an overnight culture. A standard PCR protocol was used to screen genomes for the presence of all previously characterized S-

type pyocin genes (encoding S1, S2, S3, and AP41) and their corresponding immunity genes (Duport *et al.*, 1995; Sano & Kageyama, 1993; Sano *et al.*, 1993b). Multiple primer sets were designed based on existing pyocin sequences to amplify different regions of each S-pyocin and corresponding immunity genes (Appendix III). Further, primer sets specific to the regulatory region (P-box) of pyocins S1, S2, and AP41 were employed. Primers designed to amplify the RF-type pyocins targeted PRF-10, PRF-31, and PRF-38 genes were taken from the literature (Nakayama *et al.*, 2000). NCBI accession numbers of the sequenced pyocins, primer positions, primer sequences, amplicon sizes, and identities are given in Appendix III.

## **2.4 Results**

### **2.4.1 Inhibitory activity in Pa and Bcc strains**

A phenotypic screen for inhibitory activity and sensitivity was conducted on a collection of 66 clinical strains of Pa (38) and Bcc (28) isolated from CF patients. The collection consists of strains isolated as pairs, *i.e.* one Pa and one Bcc isolated from the same patient (taken from 7 patients, and resulting in 14 pairs; 14 Pa paired with 7 Bcc strains), and unpaired (24 Pa and 21 Bcc). Bacteriocin production and sensitivity was assayed in an all-by-all comparison, *i.e.* each strain was used as a putative producer and sensitive strain.

In this screen, Pa and Bcc strains showed different levels of inhibitory activity. The majority of the Pa strains (97%) were inhibitory (Table 2-1). Most (76%) inhibit both Pa and Bcc species, resulting in similar levels of intra- (92%) versus inter- (81%) specific inhibition. Only 3% of Pa strains show no inhibitory activity.

The Bcc strains showed lower levels of overall inhibitory activity (68 %) (Table 2-1). The one-tailed Mann-Whitney U test confirms significantly higher levels of total inhibition by Pa versus Bcc strains ( $P < 0.0005$ ). Similar to Pa, most Bcc strains (43%) inhibit both species, again resulting in similar levels of intra- (54%) versus inter- (57%) specific inhibition. A much larger fraction of Bcc strains (32%) display no inhibitory activity.

When the CF strain collection is divided into strains isolated as pairs or unpaired, the paired and unpaired Pa showed similar levels of total inhibition (Table 2-1). All paired and most unpaired (96%) Pa isolates possess inhibitory activity. Most of the paired Pa strains (86%; 12/14 strains) inhibit both species, while the remainder inhibit either Bcc (7%; 1/14 strains) or Pa (7%; 1/14 strains). Two of the paired Pa strains inhibit their Bcc pair mates. The inhibitory unpaired Pa show similar patterns of inhibition, 71% (17/24 strains) inhibit both species and the remainder inhibit either Bcc (4%; 1/24 strains) or Pa (21%; 5/24 strains).

The paired Bcc strains show significantly different inhibition patterns than their Pa pair mates (Table 2-1). Only 43% of paired Bcc isolates (3 strains) possess inhibitory activity, significantly lower levels than paired Pa ( $0.025 < P < 0.05$ ), and all of these strains inhibit both species, but not their pair mates (Table 2-1). The inhibition patterns of unpaired Bcc isolates are quite similar to those of unpaired Pa strains. Most of the unpaired Bcc are inhibitory (76%; 16/21 strains) and most of these (43%; 9/21 strains) inhibit both species. The remainder inhibit only Bcc (14%; 3/21 strains) or only Pa strains (19%; 4/21 strains) (Table 2-1).

Inhibition haplotypes (Pa and Bcc strains inhibited by a putative producer strain) were determined for each producer. The 37 Pa and 19 Bcc producers displayed 36 and 18 haplotypes, respectively (Figure 2-2). Further, all Pa isolates inhibit multiple Pa and Bcc; 12 Pa inhibit 1-10 strains (11 inhibition haplotypes), 8 inhibit 11-20 strains (8 inhibition haplotypes), 11 inhibit 21-30 strains (11 inhibition haplotypes), and 3 inhibit 31-40 strains (3 inhibition haplotypes). Three of the paired Pa isolates were able to inhibit most of the strains in the collection (41-50 strains). The Bcc isolates have a more limited inhibition range. The majority (17 strains) inhibit 1-10 strains (16 inhibition haplotypes). Two Bcc strains are more broadly inhibitory, acting against 13 or 24 strains.

#### **2.4.2 Bacteriocins of clinical Pa and Bcc strains are a source of inhibitory activity**

A series of phenotypic assays were employed to assess whether the observed inhibitory activities were due to bacteriocin or bacteriophage production. Cell free extracts of each producer were subjected to filtration (to retain phage-like bacteriocins), protease digestion (to digest protein moieties), and freezing (to fracture phage-like bacteriocins). These assays revealed that 13% of Pa produce substances that appear to be protease sensitive bacteriocins, 11% produce phage-like bacteriocins, and 63% produce both (Figure 2-3). Finally, eleven percent of the Pa could not be further characterized due to the loss of inhibitory activity in the cell-free extract.

Surprisingly, all of the Bcc producers lost inhibitory activity in their cell-free extracts. This result was further explored by concentrating the extracts 20 fold. One strain (4%) showed phage-like bacteriocin activity (Figure 2-3). None showed only protease-sensitive bacteriocin activity. Eleven percent showed both. One Bcc strain (4%) produced phage plaques indicating that the inhibitory activity is due to lytic activity of a

bacteriophage. Finally, fifty percent could not be characterized due to the complete loss of inhibitory activity even after 20-fold concentration of the extract (Figure 2-3).

### **2.4.3 Clinical strains have the potential to produce multiple bacteriocins**

A set of Pa reference strains enabled us to identify the source of some or all of the inhibition identified above. These reference strains are either producers of or are sensitive to specific S, R and F pyocins (de Chial *et al.*, 2003; Ito, 1970; Kageyama *et al.*, 1979; Kuroda & Kageyama, 1981; Nakayama *et al.*, 2000; Sano, 1990; Seo & Galloway, 1990; Williams *et al.*, 2008). Additionally, cloned pyocins (S1, S2, S3, and AP41) were also employed as references to identify S-pyocin sensitivity of clinical Pa and Bcc strains (Duport *et al.*, 1995; Sano & Kageyama, 1981; Sano *et al.*, 1993b). There are no corresponding reference strains for Bcc bacteriocins.

All strains were assayed for bacteriocin-like inhibitory activity against the Pa reference collection. The bacteriocin phenotypes predicted from these inhibition haplotypes are given in Table 2-2. Among the 37 Pa producer strains, 76% inhibit one or more of the reference strains. Strains predicted to possess the most bacteriocins (Pa I: S1, S2, S3, AP41, R2, R4, F2, F3 and Pa II: S1, S2, AP41, R2, R4, F2, F3) are the most common phenotypes encountered in the clinical Pa strains. These two phenotypes inhibit an average of 31 and 23 strains, respectively. Pa II strains possessing all but the S3 phenotype (PaII S3<sup>-</sup>) experience a 42% decrease in Bcc inhibition in comparison to Pa I. The Pa III (S1, R2, R4, F2) phenotype is found in only one strain, however it inhibits the greatest number of clinical strains, an average of 44 strains. Pa strains with Pa IV (S1, R2, R4, F2, F3) and Pa V (S1, S3, AP41, R2, R4, F2, F3) phenotypes inhibit the least number of clinical strains, an average of 6.5 and 9, respectively. Strains with the Pa VI



(S1, S2, AP41) phenotype kill an average of 17 clinical strains. Nine Pa producers (Pa VII) do not inhibit any of the reference strain and have the lowest level of inhibition, an average of 2.4 clinical strains inhibited. Among the 19 Bcc producer strains, only 16% inhibit any of the Pa reference strains. These three possess the Bcc I (S1, S2, AP41, F3), Bcc II (F3) and Bcc III (R2, F2) phenotypes (Table 2-2). Fifteen Bcc producers (Bcc IV) do not inhibit any of the reference strains and inhibit an average of 5.8 clinical strains.

#### **2.4.4 Clinical strains show sensitivity to multiple bacteriocins.**

The clinical strains were exposed to cloned S-pyocins (Duport *et al.*, 1995; Sano & Kageyama, 1981; Sano *et al.*, 1993b) and 89% were sensitive (Table 2-3). Sixty-two percent were sensitive to a single pyocins and 38% to multiple pyocins. Finally, none of the Bcc are sensitive to the cloned S-pyocins (Table 2-3).

#### **2.4.5 Source of the intra- and inter-specific inhibitory activity of clinical strains**

Based on inhibition haplotypes, 28 Pa are predicted to produce combinations of S and RF type pyocins, which kill within and between species (Figure 2-4). Two inhibit intra-specifically with S-pyocins, 24 with RF-type pyocins, and 25 with S and/or RF pyocins. Twenty-five act inter-specifically, all with RF-type pyocins. Finally, 14 produce novel bacteriocins that act intra-specifically (50%) or inter-specifically (50%).

In contrast, the majority of Bcc strains produce novel bacteriocin-like activity (Figure 2-4). Three produce RF-like factors and 12 produce novel bacteriocins, all of which act intra-specifically. Further, three produce RF-like, one produces S- and/or RF-like pyocins, and the remainder (13) produce novel inhibitors to inhibit inter-specifically (Figure 2-4).

#### **2.4.6 Molecular Screening: Clinical Pa strains possess multiple pyocin genes**

PCR primers specific to S-type (S1, S2, S3, and AP41) (Duport *et al.*, 1995; Sano & Kageyama, 1981; Sano *et al.*, 1993b) and RF-type (PRF-10, 31, and 38) pyocin genes were employed to screen for the presence of known pyocin-encoding genes (Appendix III). All Pa strains were amplification positive for one or more of the pyocin genes (Figure 2-5). Ninety-five percent possess one or more S-pyocin genes; 71% amplify the S1 gene, 71% S2 gene, 42% S3 gene, and 63% AP41 gene. A further 16% amplified from the pyocin AP41 immunity gene, but not the corresponding toxin gene. Ninety-five percent of the strains were amplification positive for one or more RF pyocin genes; 68% for PRF-10, 45% for PRF-31, and 24% for PRF38 (Figure 2-5). Finally, none of the Bcc strains were amplification positive for any of the pyocin genes (data not shown).

#### **2.5 Discussion**

Individuals with cystic fibrosis (CF) face a lifelong battle with chronic bacterial lung infections (Govan & Deretic, 1996). *Pseudomonas aeruginosa* is the most prevalent species in the CF lung and is associated with high mortality (Emerson *et al.*, 2002). CF patients are first colonized with nonmucoid Pa in childhood, which is replaced by mucoid variants in adulthood (Gibson *et al.*, 2003). Mucoid Pa form biofilms, which are virtually impossible to eradicate even with aggressive antibiotic therapy (Hentzer *et al.*, 2001). Secondary bacterial infections, such as with *Burkholderia cepacia*, although less common, are of particular concern because they are often associated with faster decline in lung function and may result in cepacia syndrome, which is a rapidly progressing and fatal pneumonia (Huang *et al.*, 2001; Lambiase *et al.*, 2006). What is remarkable about these secondary infections is that they require a strain to compete with, and perhaps even

displace, the established *Pseudomonas* strain (Lambiase *et al.*, 2006; Ledson *et al.*, 1998; Ledson *et al.*, 2002; McManus *et al.*, 2004). Further, the time interval between exposure and invasion may be as short as days to weeks (Ledson *et al.*, 1998; Tablan *et al.*, 1985; Whiteford *et al.*, 1995). This ability of Bcc strains to compete with, or even displace, the resident Pa strains stands in sharp contrast with our seeming inability to significantly impact long standing *Pseudomonas* lung infections with even the most aggressive use of broad spectrum antibiotics.

Bacteriocins, narrow spectrum antimicrobials, are recognized as one of the most common mechanisms by which bacteria mediate population and community level interactions (Kerr *et al.*, 2002; Riley & Gordon, 1999; Riley & Wertz, 2002a). Pa is a well-characterized and prolific bacteriocin producer. Indeed, studies indicate that nearly all clinical and only slightly fewer environmental (~70%) Pa strains produce pyocins (B.Bouhaddioui, 2002; Farmer & Herman, 1969; Jones *et al.*, 1974b; Zabransky & Day, 1969). Typing of a much smaller number of *Burkholderia cepacia* strains from clinical sources (previously known as *Pseudomonas cepacia*) revealed far fewer producers (30%) of putative bacteriocin-like toxins (labeled cepaciacins) (Govan & Harris, 1985). What little we know about cepaciacins suggests that some may be similar to the phage-tail like R-pyocins (Govan & Harris, 1985).

In an attempt to understand how Pa and Bcc strains interact in the CF lung, isolates of both species were surveyed for the production of bacteriocins. Not surprisingly, both species are prolific producers of bacteriocin-like toxins, with 97% of Pa and 68% of Bcc capable of inhibition (Table 2-1). For *Pseudomonas*, most of the inhibitory activity detected (76%) is due to S and RF pyocins (Table 2-2). In contrast, for

Burkholderia, most of the inhibition could not be characterized (Table 2-2). Only a small fraction (16%) was attributable to bacteriocin-like proteins (Table 2-2).

More surprisingly, the levels of between species inhibition is high, 81% of the Pa and 57% of the Bcc producers inhibit the other species (Table 2-1). Previous studies of Gram-negative bacteriocins have described bacteriocins as narrow spectrum toxins, referring to the observation that they are active against members of the same species and generally display restricted levels of inhibition outside of the producing species (Riley & Wertz, 2002a; Riley & Wertz, 2002b). A similar survey in closely related *Klebsiella* species revealed that only 20% of strains show inter-specific inhibitory activity (Riley *et al.*, 2003).

The high levels of inter-specific inhibition detected for Pa and Bcc may reflect the competitive interactions that occur between these species in the CF lung. Prior studies have suggested that these two species actively inhibit each other's growth. McKenney *et al.* (1995) revealed that the addition of cell free Pa exo-products to the growth medium used to cultivate Bcc enhances the production of siderophores, lipases and proteases. Further, Weaver & Kolter (2004) examined the impact of cell free extracts of Bcc on Pa gene expression and revealed that most of the up-regulated genes of Pa are normally induced under iron-limited conditions. The authors concluded that iron-limited conditions might be created in these pairwise growth conditions due to the iron chelator-ornibactin produced by Bcc. Indeed, iron-siderophores and S-pyocins (S2, S3) share the same receptors (type I and II ferripyoverdines, respectively) (Denayer *et al.*, 2007). Thus, S-pyocins are better absorbed by sensitive strains under iron-limited conditions (Ohkawa *et al.*, 1980). Given these facts, Bcc colonization in the CF lung may induce intra-specific

Pa inhibitory activity. Clearly, the effect of iron on inter-specific inhibitory activity of pyocins and cepaciocins bears further investigation. For these purposes, identification of cepaciocins and the corresponding cell surface receptors is required.

The majority of Pa inter-specific inhibitory activity is due to RF type pyocins (Figure 2-4). In fact, R pyocins inhibit a variety of Gram-negative bacteria (Blackwell *et al.*, 1979; Filiatrault *et al.*, 2001). This study provides the first substantial report of the potential role of RF pyocins in clinical Bcc inhibition. The remainder of the Pa inter-specific inhibitory activity is due to novel virulence factors. The majority of Bcc inter-specific inhibitory activity is due to novel factors, which require further investigation (Figure 2-4).

Another intriguing observation reported here is that most isolates of Pa from the CF lung produce multiple bacteriocins (Table 2-2 and Figure 2-5). In fact, the most frequently encountered bacteriocin types (Pa I-II) include essentially all characterized S and RF-pyocins. The observation of such high levels of Pa bacteriocin diversity is good news for those interested in the potential use of bacteriocins as narrow spectrum antimicrobials. Although the bacteriocin-like production patterns are complex, they offer a wide range of inhibition specificities.

Given the lack of effective antibiotic therapies for adult patients with CF, it is intriguing that these strains are inhibited by a diversity of Pa and Bcc bacteriocins (characterized and putative). Bacteriocins may be an effective alternative for mediating some of these long-standing infections in the CF lung. One requirement would be the production of bacteriocin sensitivity profiles to permit identification of the appropriate therapeutic bacteriocins. Of course, such treatment will select for bacteriocin resistance

(e.g. protease sensitivity, lipopolysaccharide (LPS) receptor modification) (Loutet *et al.*, 2006). However, given the plethora of potential bacteriocins, cocktails of toxins could be created, which would result in far lower rates of resistance evolution (Riley, personal communication). Bacteriocins are now frequently being considered for such therapeutic use (Gillor *et al.*, 2005). For example, they are employed as antibiotics for use in mastitis in dairy cows (Diez-Gonzalez, 2007), and to inhibit growth of pathogenic *Escherichia coli* in newborn piglets and foals (Gillor *et al.*, 2005).

The most striking difference between Pa and Bcc inhibition patterns involves their breadth of activity. Almost all Pa isolates are able to inhibit a wide range of Pa and Bcc strains (Figure 2-2). In contrast, Bcc strains, on average, inhibit a much more limited number of strains. Further, the Bcc inhibitory substances appear to be phenotypically unique. For example, standard bacteriocin assays are highly successful when applied to most species of Gram-negative bacteria (Pugsley & Oudega, 1987; Riley *et al.*, 2003). And yet, these methods are far less effective when applied to Bcc. Most intriguing was the complete loss of all inhibitory activity in Bcc cell free extracts when extracts are concentrated 20-fold concentrations. Inhibitory activity was not recovered despite its presence on plate-based screens. In fact, the most successful protocols for obtaining Bcc inhibition involved concomitant growth of both the producer and sensitive strains on a solid surface. Perhaps inhibition production requires severely restricted resources and/or high levels of competition. An alternative explanation could be the presence of unstable phage particles in lysates from producer strains (Summer *et al.*, 2004). We note that we were unable to propagate bacteriophage activity, only one Bcc strain was clearly shown to inhibit via phage (Figure 2-3). Recent studies on the bacteriophages of Bcc revealed

that bacteriophages are able to inhibit different Bcc genomovars. However, these phages had a relatively limited ability to inhibit Pa strains (Langley *et al.*, 2003; Seed & Dennis, 2005). Clearly, this intriguing loss of inhibitory activity for the majority of Bcc producers requires further investigation.

Pa strains are usually the persistent, resident bacterial species in the adult CF lung. A recent study revealed a clear example of how R-pyocins may play a role in mediating intra-specific competition and succession of Pa strains (Heo *et al.*, 2007). Further, pyocin production has been shown to impact intra-specific interactions in mixed biofilms (Waite & Curtis, 2009). It is rare for a Bcc strain to be able to invade these established populations. In the relatively infrequent cases of co-existence, (i.e. the paired strains in this study), the Pa and Bcc strains are usually unable to inhibit each other. Only two Pa (via RF pyocins) and none of the Bcc strains in this study were able to inhibit their pair-mates (Table 2-1 and Figure 2-4). This observation may simply reflect the outcome of prior competition, if one or the other was able to inhibit the invader or resident strain, they would not now coexist. Clearly most unpaired Pa and Bcc strains can inhibit each other (75% vs. 62%, respectively) even while the pair mates cannot (Table 2-1).

Future efforts will focus on two significant features of bacteriocin biology revealed by this study. First, it is surprising that no molecular investigations of cepaciacins exist. Given the importance of Bcc in human disease, the presence of these potent toxins should have generated prior interest. In contrast, numerous studies have focused on the characterization of pyocins. Preliminary studies designed to provide a molecular characterization of cepaciacin identified here caution us that this may not be a

simple exercise. To date, our efforts have not yet resulted in a single cloned cepaciacin, or even a bacteriocin-like inhibitory function from these Bcc strains. Second, the suggestion that bacteriocin-like inhibition may play a role in mediating strain dynamics in the CF lung serves as inspiration for future studies designed to characterize the potential of these narrow spectrum antimicrobials to serve in future CF therapeutics. Clearly, such toxins are able to mediate inter-specific interactions of Pa and Bcc. Whether we can employ these toxins for our own purposes in a clinical setting remains to be seen.

## **2.6 Acknowledgements**

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**Table 2-1. Inhibitory activity of Pa and Bcc from the CF lung**

	<u>Number of Pa isolates (%)</u>			<u>Number of Bcc isolates (%)</u>		
	PI <sup>*</sup> n=14	UPI <sup>‡</sup> n=24	Total N=38	PI <sup>*</sup> n=7	UPI <sup>‡</sup> n=21	Total N=28
No inhibition	0 (0)	1 (4)	1 (3)	4 (57)	5 (24)	9 (32)
Inhibits only Pa	1 (7)	5 (21)	6 (16)	0 (0)	4 (19)	4 (14)
Inhibits only Bcc	1 (7)	1 (4)	2 (5)	0 (0)	3 (14)	3 (11)
Inhibits both Pa and Bcc	12 (86)	17 (71)	29 (76)	3 (43)	9 (43)	12 (43)
Total inhibition	14 (100)	23 (96)	37 (97)	3 (43)	16 (76)	19 (68)
Paired inhibition	2 (14)	NA	2 (5)	0 (0)	NA	0 (0)

\*PI: Paired isolates; ‡UPI: Unpaired isolates. NA: Not applicable

**Table 2-2 Bacteriocin phenotypes of clinical Pa and Bcc strains**

Producer species*	Phenotype designation	Bacteriocin phenotype <sup>‡</sup>	Number of strains	Average number of Pa inhibited	Average number of Bcc inhibited	Total number of strains inhibited
Pa	Pa I	S1, S2, S3, AP41, R2, R4, F2, F3	10	19	12	31
	Pa II	S1, S2, AP41, R2, R4, F2, F3	12	16	7	23
	Pa III	S1, R2, R4, F2	1	18	26	44
	Pa IV	S1, R2, R4, F2, F3	2	5.5	1	6.5
	Pa V	S1, S3, AP41, R2, R4, F2, F3	1	6	3	9
	Pa VI	S1, S2, AP41	2	8.5	8.5	17
	Pa VII	-	9	1.8	0.6	2.4
Bcc	Bcc I	S1, S2, AP41, F3	1	6	4	10
	Bcc II	F3	1	4	2	6
	Bcc III	R2, F2	1	1	2	3
	Bcc IV	-	15	2.8	3	5.8

\* Pa: *Pseudomonas aeruginosa*; Bcc: *Burkholderia cepacia* complex

<sup>‡</sup> S type pyocins: S1, S2, S3, AP41; RF type pyocins: R2, R4, F2, F3

**Table 2.3 S-pyocin sensitivity of clinical Pa and Bcc strains**

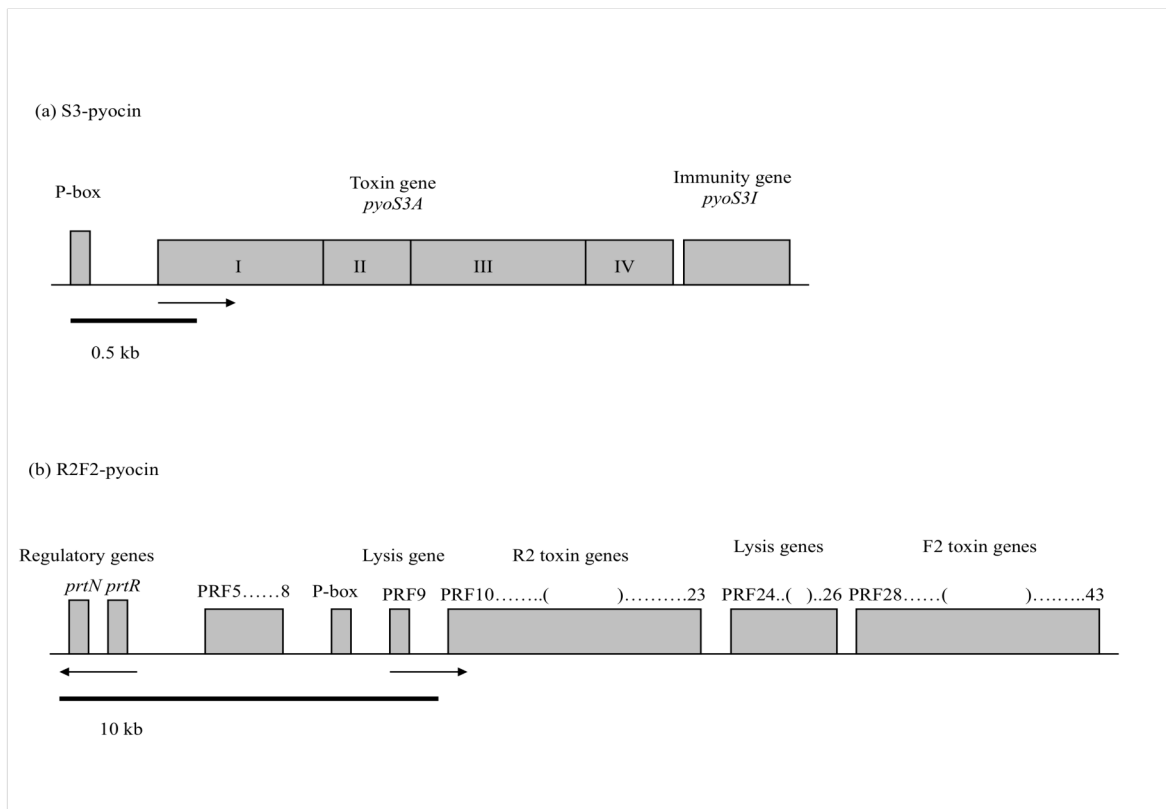
Source of pyocin	Pyocin sensitivity phenotype <sup>‡</sup>	Number of Pa strains (%) n=38	Number of Bcc strains (%) n=28	
Cloned S-pyocins	S1	3	0	
	S2	0	0	
	S3	13	0	
	AP41	5	0	
	S1, S2	6	0	
	S1, S3	0	0	
	S1, AP41	1	0	
	S2, S3	0	0	
	S2, AP41	0	0	
	S3, AP41	3	0	
	S1, S2, AP41	1	0	
	S1, S3, AP41	2	0	
	S2, S3, AP41	0	0	
	S1, S2, S3, AP41	0	0	
	Total sensitivity		34 (89)	0 (0)

<sup>‡</sup> Phenotypes are according to sensitivity to S-pyocin producing clones, e.g. S1: only S1 sensitivity; S1, S2: S1 and S2 sensitivity

## Figure 2-1 Genetic organization of exemplar pyocin genes

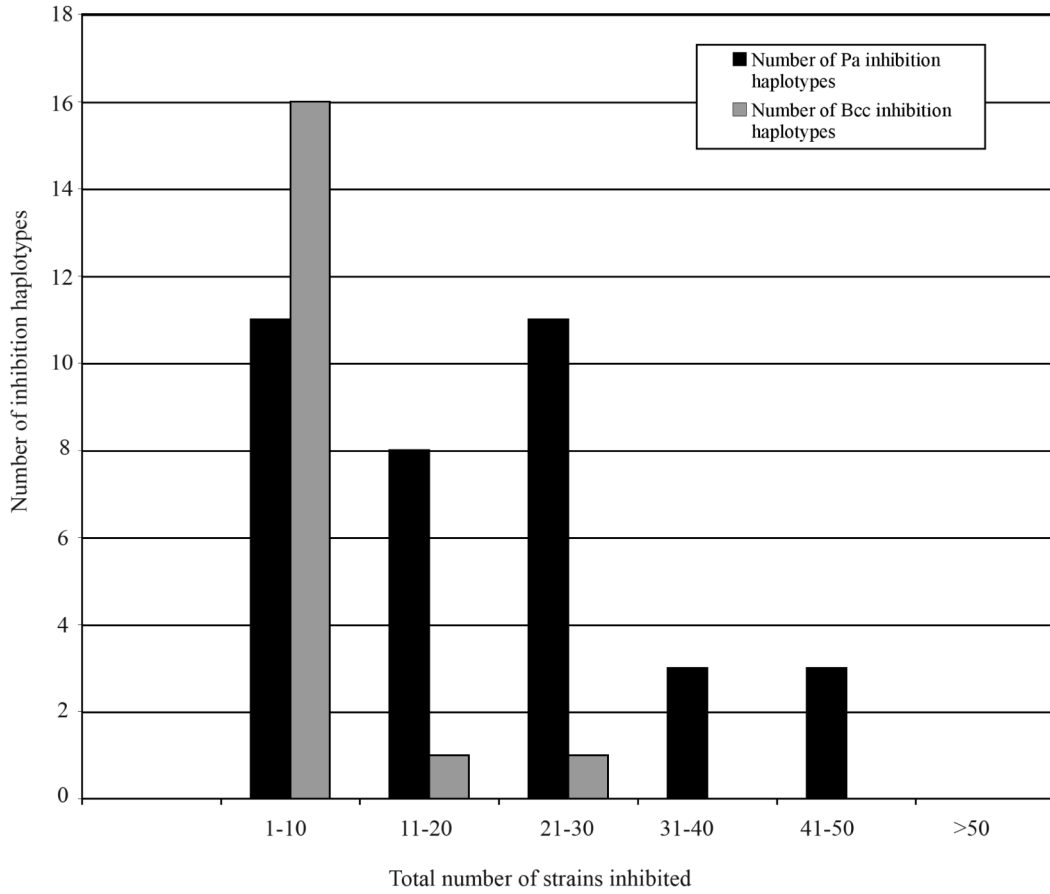
adapted from Michel-Briand & Baysse, 2002

- S3 pyocin: P-box refers to the binding site for PrtN (not shown); The toxin gene is divided into four domains, labeled I-IV.
- R2F2-pyocin. Arrows indicate the direction of transcription.



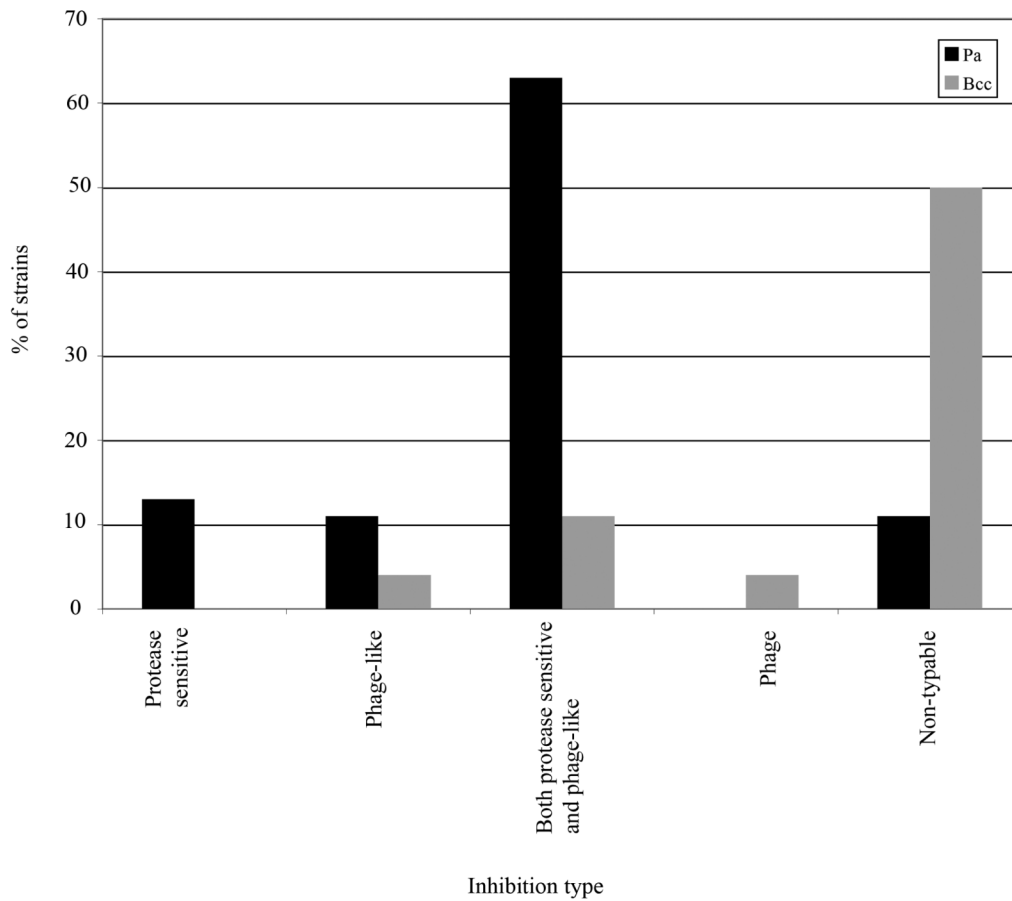
**Figure 2-2 Inhibition haplotypes of clinical Pa and Bcc strains**

Pa and Bcc strains inhibited multiple strains. For example, 12 Pa inhibit 1-10 strains. The Bcc isolates have a more limited inhibition range in comparison to Pa isolates.



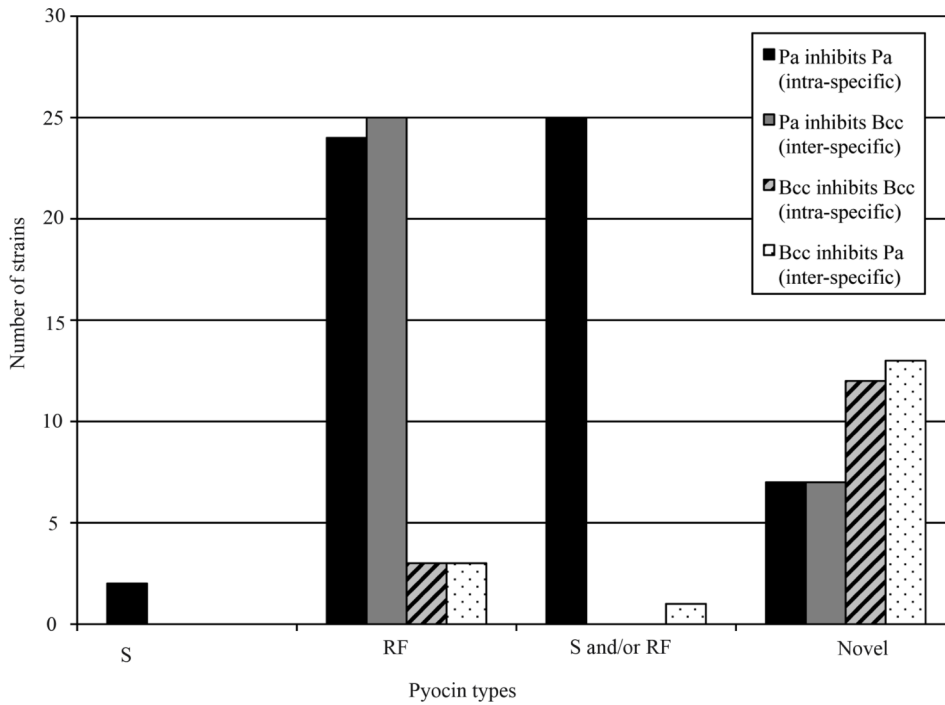
**Figure 2-3 Inhibition types produced by clinical Pa and Bcc strains**

Pa strains showed protease sensitive, phage-like, and/or both types of inhibition. Most of Bcc strains are nontypable. One Bcc strain showed phage activity.

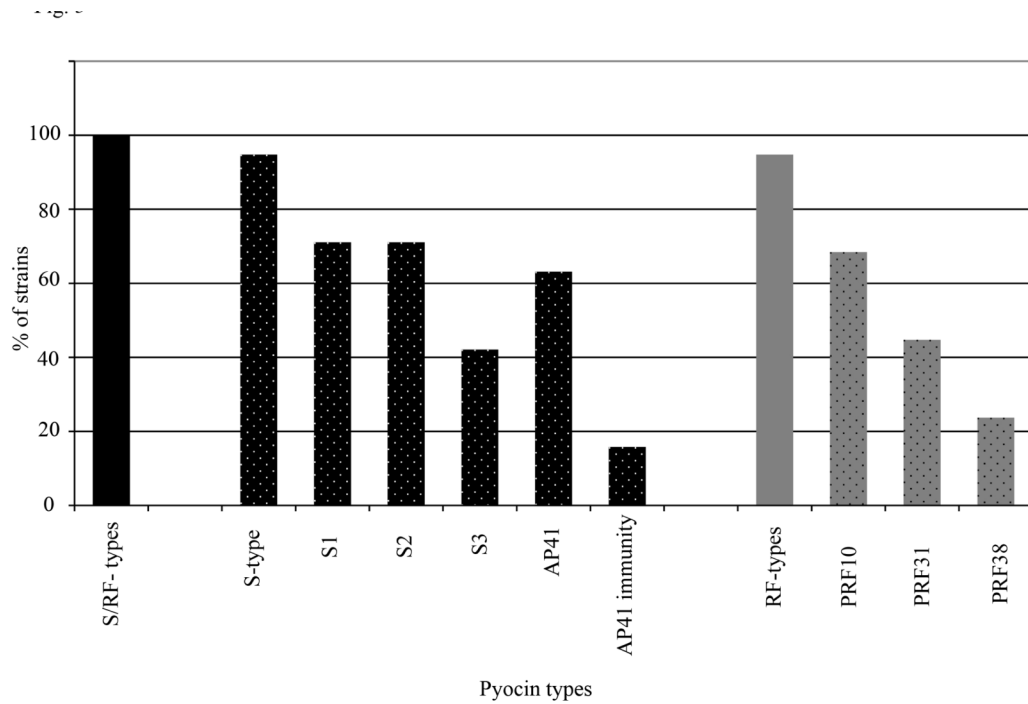


**Figure 2-4 Source of the intra- and inter-specific inhibitory activity of clinical Pa and Bcc strains**

Source of inhibitory activity of Pa and Bcc are different. In Pa, much of the intra- and inter-specific inhibitory activity was due to the activity of S- and RF-type pyocins, In Bcc, inhibitory activity is mostly novel or due to RF-like pyocins.



**Figure 2-5 Pyocin gene distribution in the genomes of clinical Pa and Bcc**  
 PCR primers were used to screen genomes of clinical Pa for the known S-pyocins (S1, S2, S3, AP41) as well as RF pyocins (PRF10-, PRF31, PRF38). All Pa bacteriocin producers (100%) possessed one or multiple S- and RF pyocin genes in their genome.





## CHAPTER 3

### CONSTRUCTION OF GENOMIC LIBRARIES TO IDENTIFY PUTATIVE GENES INVOLVED IN BACTERIOCCIN-LIKE INHIBITORY ACTIVITY OF B. CEPACIA COMPLEX

#### 3. 1 Abstract

A recent survey revealed a potential role for bacteriocins in mediating bacterial interaction in Cystic Fibrosis (CF) lungs. This study showed that 68% of *Burkholderia cepacia* complex (Bcc) strains isolated from lungs of CF patients possess potent inhibitory activity, which appears similar to the bacteriocin-like inhibition produced by many other bacterial species. Since most adult CF patients have pre-existing *Pseudomonas aeruginosa* (Pa) infections, Bcc strains must either compete with or act in synergy with the Pa already established in the lungs. Although this prior study made a compelling case for the potential role for these inhibitory substances in mediating bacterial interactions in the lungs, it could not identify the substances in question. In this study, genomic libraries of *B. multivorans*, *B. dolosa*, and *B. cenocepacia* were constructed to screen for genes responsible for the inhibitory activity previously described. ~10,000 clones/genome were screened, resulting in fifteen clones with the anticipated inhibitory activity. Of these fifteen, only five had stable inhibition activity, and were pursued further. These clones encoded proteins involved in various metabolic pathways including bacterial apoptosis, amino acid biosynthesis, sugar metabolism, and degradation of aromatic compounds. Surprisingly, none possessed typical bacteriocin-like

genes. These data suggest that Bcc may use novel inhibitory agents to mediate intra- and inter-specific interactions.

### **3.2 Introduction**

*B. cepacia* complex species, most of which are temperate soil saprophytes and plant pathogens, are associated with severe, often fatal, pulmonary infections in persons with cystic fibrosis.

Summer *et al.*, 2007

#### **3.2.1 *B. cepacia* complex (Bcc)**

In 1950, plant pathologist Walter Burkholder showed that *Pseudomonas cepacia* was different from other members of the *Pseudomonas* genus: they could grow on *m*-hydroxy benzoate or tryptamine as carbon sources (Mahenthalingam *et al.*, 2005). Further, they are able to catabolize more than 200 organic carbon sources; demonstrating enormous metabolic versatility (Lessie *et al.*, 1996). In 1992, *Pseudomonas cepacia*, *Pseudomonas solanacearum*, *Pseudomonas pickettii*, *Pseudomonas glandioli*, *Pseudomonas mallei*, *Pseudomonas pseudomallei*, and *Pseudomonas caryophylli* were incorporated into a new genus, *Burkholderia*, based on 16S rRNA sequences, DNA-DNA homology, lipid and fatty acid compositions, and phenotypic characteristics (Yabuuchi *et al.*, 1992).

In 1997, molecular and biochemical assays were performed on 128 tentatively classified *B. cepacia* strains. This study revealed five phenotypically similar, but genotypically distinct, *Burkholderia* species (genomovars), classified as the *B. cepacia* complex (Bcc). These species were named as *B. cepacia* (genomovar I), *B. multivorans*

(genomovar II), *B. cenocepacia* (genomovar III), *B. stabilis* (genomovar IV), and *B. vietnamiensis* (genomovar V) (Vandamme *et al.*, 1997). Later, *B. dolosa* (genomovar VI), *B. ambifaria* (genomovar VII), *B. anthina* (genomovar VIII), and *B. pyrrocinia* (genomovar IX) were added to the Bcc (Coenye *et al.*, 2001a; Coenye *et al.*, 2001b; Vandamme *et al.*, 2002).

### **3.2.2 *B. cepacia* complex: Friend or Foe?**

Burkholderia species are opportunistic pathogens (Mahenthiralingam *et al.*, 2005). *B. cepacia* was the first pathogen identified in the genus. It is the causative agent of soft rot in onions (Sotokawa & Takikawa, 2004). Subsequently, *B. glumae*, *B. plantarii*, *B. glandioli*, and *B. phytofirmans* were shown to cause diseases in such economically important crops such as rice, maize, potato, and tomatoes (Coenye & Vandamme, 2003; Sessitsch *et al.*, 2004). Members of the genus also engage in activities that are beneficial to humans, such as serving as bioremediation and biocontrol agents (Parke & Gurian-Sherman, 2001). Various species degrade aromatic compounds, crude oil, herbicides, and xenobiotics (Sousa *et al.*, 2010). Further, they produce antibiotics and iron scavenging siderophores that repress fungal growth (Chiarini *et al.*, 2006; Parke & Gurian-Sherman, 2001).

Members of the genus are also associated with human disease and are responsible for numerous nosocomial infections in immunocompromised patients, such as those with cancer or HIV (Mann *et al.*, 2010; Verghese *et al.*, 1994). The focus of this study, however, is their role in Pa-Bcc interaction in lungs of patients with cystic fibrosis (CF) (Mahenthiralingam *et al.*, 2005). Bcc infections in CF patients were first observed in the early 1980 (Tablan *et al.*, 1985), and remain relatively rare, only 3% of CF patients are

infected with a member of this species complex (CFF, 2008). However, clinical outcomes of such infections are both unpredictable and potentially severe, ranging from asymptomatic, to chronic, and even fatal infections (Mahenthiralingam *et al.*, 2002; Whiteford *et al.*, 1995). Nearly 20% of Bcc infected CF patients develop cepacia syndrome, a fatal, rapid onset pneumonia, occurring within days or weeks of their introduction into the lung (Govan & Deretic, 1996). The varied colonization outcomes may be due to strain, patient or treatment-based factors. Metabolic versatility, insertion sequences, genomic islands, bacteriophages, proteases, and exotoxins in Bcc are potential factors, which may serve to enhance their ability to invade and colonize the CF lung (Mahenthiralingam *et al.*, 2001; Mahenthiralingam *et al.*, 2005).

Bcc species have large genomes, up to 6-9 Mb, which are organized as multiple chromosomes, with 67% GC content (Holden *et al.*, 2009; Parke & Gurian-Sherman, 2001). The number of chromosomes and the total genome size varies among strains as well as within a genomovar (Table 3-1). Currently, the genomes of 38 Burkholderia strains have been sequenced, eleven of which are members of the Bcc species complex; including five strains of *B. multivorans*, two of *B. cenocepacia*, two *B. ambifaria*, one *B. cepacia*, and one *B. vietnamiensis* (NCBI, ; Pathema).

Bcc genomes are rich in insertion sequence (IS) elements, which are transposable DNA sequences ranging between 700-2500 bp in length. A recent study revealed one strain with 79 IS elements in its genome (Holden *et al.*, 2009). IS elements are capable of inserting themselves at multiple sites in the genome, leading to a variety of genetic rearrangements such as duplications, deletions, and inversions. Studies have revealed that several IS elements in *P. cepacia* can activate gene expression (Wood *et al.*, 1991) and

play a role in plasmid rearrangement (Byrne & Lessie, 1994). The presence of IS elements is proposed as one of the major driving forces of the versatility and adaptation of bacteria in different environmental niches (Mahillon & Chandler, 1998; Mira *et al.*, 2002).

Genomic islands (GI), which are clusters of genes in bacterial genomes also involved in adaptation of bacteria in different environments as well as involved in virulence, antibiotic resistance, and other metabolic processes (Hacker & Carniel, 2001). GIs range in size from 10-100 kb in length and are often associated with horizontal gene transfer events (Hacker & Carniel, 2001). These islands are often called pathogenicity islands (PI), if the genes are associated with increased pathogenicity.

Bcc genomes are rich in genomic islands. Fourteen GIs were identified in the recently published genome of *B. cenocepacia* J2315, distributed across all three chromosomes, some of which are unique to epidemic strain (Holden *et al.*, 2009). One GI, *cci*, encodes virulence and metabolism genes, including arsenic and antibiotic resistance genes, quorum sensing genes, amino acid and fatty acid metabolism genes, and transcriptional regulator genes (Baldwin *et al.*, 2004; Holden *et al.*, 2009). A second, BcenGI2 possesses genes that code for plasmid conjugation, while others, BcenGI9, 12, and 13, encode prophage genes (Baldwin *et al.*, 2004; Holden *et al.*, 2009).

The presence of bacteriophages in pathogen genomes is frequently associated with enhanced virulence (Wagner & Waldor, 2002). Bacteriophages can be lytic or lysogenic. Lytic phages can multiply in host cells using the host's transcription and translation machinery. The host cell is then lysed, disseminating phage progeny. Lysogenic phages integrate their DNA into the host chromosome; there is no subsequent

phage multiplication or host cell lysis. The phage DNA in this repressed state is called a prophage because it has the potential to produce phage progeny (Casjens, 2003; Synder & Champness, 2003). The host is not apparently adversely affected by the presence of prophage and the phage may persist in this state until induction by the presence of an inducing agent such as UV or oxidative stress (Barnhart *et al.*, 1976; Los *et al.*, 2010).

A number of bacteriophages have been isolated from members of Bcc strains. These include lysogenic (BcepMu, KS8, KS9, KS10, KS11, BcP15, DK4, CP1, CP75) and lytic (KS1, KS2, KS3, KS5, KS6) phages (Cihlar *et al.*, 1978; Hens *et al.*, 2005; Langley *et al.*, 2005; Matsumoto *et al.*, 1986; Summer *et al.*, 2004; Summer *et al.*, 2006). Seed *et al.*, 2005 performed a study to determine the host range of these bacteriophages by infecting 24 strains from the Bcc complex with them. The study revealed that the lytic bacteriophages (KS1, KS5, and KS6) have broader host ranges, including *B. cepacia*, *B. multivorans*, *B. cenocepacia*, *B. ambifaria*. The lysogenic phages are restricted to a more limited number of host species, including *B. cenocepacia* and *B. ambifaria* (Seed & Dennis, 2005).

Genomes of some bacteriophages in Bcc do encode putative proteins, which may be potential virulence factors involved in host cell lysis. Gene 53 from epidemic strain *B. cenocepacia* ET12 encodes acyltransferase, which modifies lipopolysaccharides (LPS), resulting in strain serotype conversion. The gp8 gene from the same strain resembles the ExeA protein of *Aeromonas hydrophilia*, which is involved in the secretion of toxins such as aerolysin (Summer *et al.*, 2007).

### 3.2.3 Burkholderia and Pseudomonas interaction

*P. aeruginosa* (Pa) is the predominant infectious agent in the lungs of adult CF patients (Govan & Deretic, 1996), found in approximately 30% of infants and 80% of adult CF patients (CFF). Chronic Pa infection is one of the primary health concerns for adult CF patients, ultimately resulting in death of the patient (Lyczak *et al.*, 2002).

Secondary Bcc infections have become a major concern over the past 30 years (Tablan *et al.*, 1985). Although all Bcc species can infect the lung, *B. multivorans* and *B. cenocepacia* are the species most frequently identified, with a prevalence of 38% and 50%, respectively (LiPuma *et al.*, 2001). In most cases, Bcc acquisition occurs in CF patients already colonized with Pa (Govan & Deretic, 1996). During this secondary infection it has been suggested that Bcc strains directly compete with the established Pa strains, although it is not known how such interactions occur. However, it is clear that Bcc-Pa co-infections are rare, suggesting that Bcc strains can successfully compete with and displace the resident Pa strains (Lambiase *et al.*, 2006; McManus *et al.*, 2004).

Unfortunately, few studies have focused on these multi-species bacterial interactions in the CF lung. What we do know from the limited studies available is that strain replacement is only observed among members of Bcc (Govan *et al.*, 1993; Ledson *et al.*, 1998; Mahenthiralingam *et al.*, 2001). A recent study revealed that in ~7% of CF patients examined, Bcc strains are replaced over time with other species of Bcc (Bernhardt *et al.*, 2003). Further, Yang *et al.*, 2006 revealed that co-infection with more than one Bcc strain may occur in the early phases, but is rare after the development of a chronic infection (Yang *et al.*, 2006). None of these studies focused on the factors that mediate such interactions.

Bacterial antagonism is common due to competition for limiting resources. Iron is one of the main limiting factors, which bacteria utilize during colonization and invasion. Weaver and Kolter have shown that members of the Bcc species complex compete more effectively for iron than strains of Pa (Weaver & Kolter, 2004). They investigated the effect of Bcc presence on Pa gene expression by incubating the Pa strain in the presence and absence of a Bcc cell free extract. Pa virulence gene expression is enhanced by the presence of Bcc in iron-limited environments. Further, their data showed that the Bcc iron scavenging siderophore (ornibactin) limits available iron to Pa, leading the induction of Pa virulence genes (Weaver & Kolter, 2004).

Bacteriocin production is another factor mediating bacterial antagonistic interactions. Aside from one recent study (Bakkal *et al.*, 2010), there are no additional publications that focus on the effect of bacteriocin production on bacterial interactions in the CF lungs. The Bakkal *et al.*, 2010 study revealed bacteriocin production in 97% of Pa and 68% of Bcc strains isolated from CF lungs (Bakkal *et al.*, 2010). In Pa, much of the inhibitory activity was shown to be due to S- and RF-type pyocins, bacteriocins of Pa. In Bcc, the source of inhibitory activity appears to be bacteriocin-like, but no bacteriocins of Bcc (cepaciaicins) have been described (Bakkal *et al.*, 2010).

The goal of the present study is to identify the sources of the observed Bcc inhibitory activity. Three genome libraries were created from *B. multivorans*, *B. dolosa*, and *B. cenocepacia* and screened for inhibition against sensitive Pa and Bcc strains. Five clones from the *B. multivorans* library had inhibitory activity against the Pa strain. DNA sequences of these clones were determined and their putative functions were identified.



They include bacterial apoptosis, amino acid biosynthesis, sugar metabolism, and degradation of aromatic compounds.

### **3.3 Materials and Methods**

#### **3.3.1 Bacterial strains**

Genomic libraries were prepared from *B. multivorans* (ATCC 17616), *B. dolosa* (AUO158), *B. cenocepacia* (02-228-1429). *Pseudomonas aeruginosa* (327-6-1422), *B. vietnamiensis* (03-260-0635), and *B. dolosa* (04-053-0423) were used as sensitive lawns to detect inhibitory activity produced during the library screen (Bakkal *et al.*, 2010). The genomes of *Pseudomonas aeruginosa* (PAO1), which encodes pyocin S2, and *Pseudomonas aeruginosa* (NIH3), which does not encode bacteriocins, served as positive and negative controls during the library screens (Bakkal *et al.*, 2010, ; Matsui *et al.*, 1993; Nakayama *et al.*, 2000) PAO1 produces a pyocin (S2). *Escherichia coli* XL1 Blue MRF' (Stratagene, Agilent Technologies) was used as the recipient strain for the genomic libraries.

#### **3.3.2 Construction of genomic library**

Genomic libraries were constructed according to the method described in Ausubel *et al.*, 2004 (Ausubel *et al.*, 2004). Genomic DNA was produced using the Gentra Puregene kit (QIAGEN), with the gram-negative DNA isolation protocol and  $5 \times 10^9$  cells from an overnight culture. *Bam* HI (10 U/ $\mu$ l; NEB) and/or *Bfu* CI (10 U/ $\mu$ l; NEB) were used to partially digest the genomic DNA at 37 °C for 30-50 minutes. The reaction was terminated by incubation at 65 °C and then run on a 0.6% SEAKEM<sup>®</sup> GTG agarose gel (FMC BioProducts) at 80 V for 5 hours. DNA fragments in the size range of 4-5 kb were

isolated from the gel using the QIAquick gel extraction kit (QIAGEN). These fragments were then ligated into a *Bam*HI digested and dephosphorylated pUC19 plasmid vector (NEB) (Figure 3-1). The ligated DNA was chemically transformed into *E. coli* XL1 Blue MRF' supercompetent cells (Stratagene, Agilent Technologies) (Figure 3-1). Blue/white colony screening was carried out on Luria Broth (LB) plates supplemented with 100 µg/µl ampicillin (Sigma), 40 mM IPTG (Sigma), and 0.08% X-Gal (Sigma). X-gal (bromo-chloro-indolyl-galactopyranoside) is a colorless substrate, which is metabolized by β-galactosidase to form an insoluble blue product (5-bromo-4 chloroindole). IPTG (Isopropyl β-D-1-thiogalactopyranoside) is used to induce transcription of the gene coding for β-galactosidase. If the ligation is successful, the insert DNA will interfere with the transcription of the β-galactosidase gene, resulting in white bacterial colonies; if the ligation is unsuccessful, the colonies will appear blue (Figure 3-1).

### **3.3.3 Phenotypic screening of genomic clones**

The patch assay was used to identify Pa and Bcc inhibitory activity among the genomic clones. Strains were grown overnight in 10 ml LB at 37 °C, shaken at 250 rpm. Six ml of LB top agar (0.6%, w/v) mixed with 100 µl of 10 mM IPTG, and 100 µl of sensitive cells ( $10^8$  cells) were plated as a lawn on an LB plate. All resulting white colonies from the genomic library were spotted on the sensitive Pa and/or Bcc lawn by toothpick. *B. multivorans* (ATCC17616) and XL1 Blue MRF' transformed with pUC19 plasmid were spotted on each lawn as positive and negative controls, respectively. After overnight incubation at 37 °C the plates were scored for the presence of inhibitory activity. More than 10,000 white colonies were screened per genomic library, resulting in 2-fold coverage of each genome. Each positive clone was screened a second time.

Plasmids were isolated from overnight cultures of genomic clones with putative inhibitory activity, and digested with *EcoRI* (10 U/μl; NEB) to identify the presence or absence of the DNA insert. Finally, inhibition phenotypes of the genomic clones were visualized and photographed using a Leica MZ 16 FA fluorescent dissecting scope.

### **3.3.4 Nucleotide sequencing**

Plasmid DNAs were isolated from overnight cultures of genomic clones using a plasmid isolation kit (QIAGEN). Plasmid DNAs were sequenced using Big Dye Terminator Cycle Sequencing mix with pUC19-forward (5'-AGC GGA TAA CAA TTT CAC ACA GGA-3') and pUC 19- reverse (5'-CGC CAG GGT TTT CCC AGT CAC GAC-3') primers (Applied Biosystems, Foster City, CA). The sequencing reaction was performed at 96 °C for 1 min, followed by 96 °C for 30 sec, 50 °C for 15 sec, and 60 °C for 4 min through 25 cycles. Homology searches were performed using the NCBI nucleotide–nucleotide Blast (blastn) algorithm on the combined GenBank/EMBL/DDBJ database. Further, gene annotations were performed via SEED, which is an annotation and analysis tool provided by the fellowship for interpretation of genomes (University of Chicago).

## **3.4 Results**

### **3.4.1 Genomic Library Construction**

The Bcc species chosen for this study represent the most frequently isolated species from CF patients. *B. multivorans* and *B. cenocepacia* are isolated from 38% and 50% of CF patients, respectively (LiPuma *et al.*, 2001). The incidence of *B. dolosa* from CF patients is only 2% (Coenye *et al.*, 2001a), but it is one of the most frequently isolated

species (39%) from our previous phenotypic bacteriocin screening study (Bakkal *et al.*, 2010).

Partially digested genomic DNAs of *B. multivorans* (ATCC17616), *B. dolosa* (AUO158), and *B. cenocepacia* (02-228-1429) were ligated to *Bam*HI digested and dephosphorylated pUC19 plasmids to construct genomic libraries of these strains. The genomes of *B. multivorans* (ATCC 17616) and *B. dolosa* (AUO158) have previously been sequenced and annotated in NCBI (NCBI, ; Pathema).

The Pa strain, PAO1, was used as a positive control in the genomic library construction. Pa PAO1 produces pyocin S2, an S-type bacteriocin (Seo & Galloway, 1990). It has been shown that PAO1 inhibits the growth of Pa NIH3 via the DNase activity of pyocin S2. Thus, Pa NIH3 was used as a lawn to detect the pyocin S2 activity of the corresponding genomic clones of Pa PAO1.

Approximately 10,000 white colonies/genome were screened on sensitive lawns of PAO1 and Bcc strains. A total of fifteen clones with inhibitory activity were identified, twelve from *B. multivorans* (ATCC 17616), two from *B. dolosa* (AUO158), and one from *B. cenocepacia* (02-228-1429). Additionally, three clones were identified from the genomic library of PAO1, the positive control for screening.

### **3.4.2 Phenotypic screening of genomic clones**

The phenotypic bacteriocin assay was repeated to verify inhibitory phenotypes of these fifteen genomic clones. Only six clones (Table 3-2) produced repeatable inhibitory activity (Figure 3-2a), five from *B. multivorans* (ATCC17616) and one from Pa (PAO1) (Figure 3-2a). *B. multivorans* (ATCC17616) showed an inhibition phenotype on a sensitive lawn of *P. aeruginosa* (327-6-1422), which consisted of the inhibition of growth

of the sensitive lawn under the producer Bcc colony (Figure 3-2b). Thus, the killing phenotype was defined as the loss of visible lawn growth under the putative producer clone. Genomic library clones (clones 2, 4, 6, 7, and 9) of *B. multivorans* (ATCC17616) possessed the described inhibitory phenotype (Figure 3-2a).

Pa (PAO1) showed an inhibition phenotype on the sensitive lawn Pa (NIH3) (Figure 3-2b). This phenotype consists of a thin inhibition zone around the producer PAO1 colony. The PAO1 genomic colony also possessed the same phenotype as the pyocin S2 producer Pa (PAO1) (Figure 3-2a).

### 3.4.3 Nucleotide sequencing

Forward and reverse sequencing primers of the pUC19 plasmid were used to sequence the clones. The resulting DNA sequences were employed in BLAST screens against existing genome sequences of *B. multivorans* (ATCC17616) and Pa (PAO1) (Table 3-2).

Genomic clone 2 had an insert of 714 nucleotides (nt), located on chromosome 1 of *B. multivorans* (ATCC17616). The NCBI blast showed that the DNA sequence corresponded to the DNA sequence of a hypothetical protein (Bm 1615) with an unknown function (Figure 3-3a). The flanking DNA sequences of Bm1615 encode another hypothetical protein (Bm1614) and a membrane protein (seven transmembrane helix protein) (Bm1616) (Figure 3-3a). The DNA sequence alignment of Bm1615 and clone 2 has shown that DNA sequence of the clone 2 covered majority of the hypothetical protein (453 nt) including its start codon (Figure 3-3b). Finally, the last 22 nt of Bm1616 are excluded in the clone 2.

Clone #4 had a DNA insert of 3,498 nt, containing genes encoding poly-beta-hydroxyalkanoate depolymerase, glucoamylase, and 3-carboxymuconate cyclase proteins (Figure 3-4a). These genes are also located on chromosome 1 of *B. multivorans* (ATCC17616).

Glucoamylase is an extracellular enzyme, common in fungus *Aspergillus*. This enzyme hydrolyzes starch into glucose via catalyzing hydrolysis of  $\alpha$ -1,4 and  $\alpha$ -1,6 glycosidic bond (Sauer *et al.*, 2000). Glucoamylase also induces biosynthesis of fungus toxin (aflatoxin), which causes infection in plant tissue (Mellon *et al.*, 2007). Genomic clone 4 contained 1929 nt of DNA sequence, encoding full length glucoamylase enzyme (Figure 3-4b)

The DNA insert of clone 4 also possessed partial DNA sequences, encoding poly-beta-hydroxyalkanoate depolymerase and 3-carboxymuconate cyclase (1222 nt and 210 nt, respectively)(Figure 3-4a and b). Both enzymes are involved degradation of aromatic hydrocarbons.

Poly-beta-hydroxyalkanoate depolymerase is an enzyme involved in degradation of polyhydroxyalkanoates (PHAs) (Madison & Huisman, 1999). PHAs are biopolyesters, which are synthesized by Archea, Gram-positive, and Gram-negative bacteria in response to nutrient limitations. Bacteria accumulate PHAs into inclusion bodies and store them in their cytoplasm. The PHAs are then degraded via enzymatic reaction and used as carbon and energy resources (Madison & Huisman, 1999).

3-carboxymuconate cyclase is also among the enzymes, responsible for degradation of aromatic hydrocarbons. It is one of the enzymes in the 3-oxodipate

pathway in fungi and bacteria. The 3-oxodipate pathway involves the conversion of hydroxybenzoate and catechol to succinate and acetyl-CoA (Cook & Cain, 1974).

Clone # 6 consisted of a 677 nt insert, which corresponded to a partial DNA sequence of amidohydrose II and 4-hydroxybenzoate transporter (285 nt and 423 nt, respectively) (Figure 3-5a and b). These genes are located on chromosome 3 of *B. multivorans* (ATCC17616).

Amidohydrolyase II belongs to the functional category of enzymes with unknown specificity (Pathema). The protein belongs to the amidohydrolyase superfamily consisting of hydrolase enzymes that catalyzes the cleavage of C-N, C-C, C-O, C-Cl, C-S, O-P bonds of organic substances. The group includes 771 enzymes, which are known to catalyse nonhydrolytic reactions including decarboxylation and hydration (Aimin *et al.*, 2007).

4-hydroxybenzoate transporter is also involved degradation of aromatic benzoate. This enzyme belongs to beta-ketoadipate pathway, which degrades 4-hydroxybenzoate to succinyl-CoA and Acetyl-CoA (Wright, 1993).

The blast result of clone 7 contained a 3,956 nt long DNA sequence, corresponding to the DNA sequence encoding 3-isopropyl malate dehydrogenase (1066 nt), 3-isopropyl malate dehydratase small subunit (651 nt), 3-isopropyl malate dehydratase large subunit (411 nt), aspartate semialdehyde dehydrogenase genes (1122 nt), and entericidin AB (132 nt) (Figure 3-6a and b). These genes are possessed by chromosome 2 of *B. multivorans* (ATCC17616).

3-isopropyl malate dehydrogenase, 3-isopropyl malate dehydratase small subunit, and 3-isopropylmalate dehydratase large subunit are among the enzymes responsible for

leucine biosynthesis. Leucine biosynthesis requires expression of four contiguous genes- *leuABCD* as a single operon in *E. coli*. The *leuA* and *leuB* genes encode 2-isopropylmalate synthetase and 3-isopropylmalate dehydrogenase, respectively. The *leuC* and *leuD* genes encode large and small subunits of isopropylmalate dehydratase (Tamakoshi *et al.*, 1998). In some organisms, organization of these genes could be different. For example, *leuB* is located separately from *leuACD* in *Leptospira interrogans* (Ding & Yelton, 1993).

3-Isopropylmalate dehydrogenase belongs to a family of metal dependent decarboxylating dehydrogenase enzymes. Thus, this enzyme requires divalent cations ( $Mg^{+2}$  or  $Mn^{+2}$ ) to become active. 3-Isopropylmalate dehydrogenase catalyzes the conversion of 3-isopropylmalate to 2-isopropyl-3-oxosuccinate (Hurley & Dean, 1994).

3-isopropylmalate dehydratase (or isopropylmalate isomerase) is composed of large (Leu C) and small (LeuD) subunits, which catalyzes isomerisation of 2-isopropylmalate and 3-isopropylmalate, via the formation of 2-isopropylmaleate. This enzyme performs the second step in the biosynthesis of leucine, and is present in most prokaryotes and many fungal species.

Aspartate semialdehyde dehydrogenase is one of the enzymes in lysine biosynthesis pathway. It catalyzes the conversion of beta-aspartyl phosphate to L-aspartate beta-semialdehyde, which is ultimately converted to L-lysine (Tunca *et al.*, 2004).

Finally, this clone encodes for entericidin AB, which is a toxin-antitoxin gene pair involved in bacterial apoptosis in *E. coli* (Bishop *et al.*, 1998). Entericidins are small, amphipathic alpha-helical cationic lipoproteins, which bind to membrane phospholipids. The genes are induced in stationary phase, when cells are starved for



energy. The producing cell is lysed due to the resulting disruption in membrane stability (Bishop *et al.*, 1998).

Blast result of clone # 9 corresponded to 37 nt long sequence of the GGDEF domain, which is 1479 nt long (Figure 3-7a and b). The GGDEF is a conserved domain with unknown function, located on chromosome 1 of *B. multivorans* (ATCC17616) (Pathema). This domain is found in a diversity of bacteria (Pei & Grishin, 2001). It has been suggested that the domain is observed in multi-domain proteins such as those involved in signaling pathways. For example, diguanylate cyclase (DGC) is an enzyme involved in the synthesis of c-di-GMP, which is a secondary messenger involved in various cellular processes including cell-to-cell signaling, motility, virulence, biofilm formation, and antibiotic production (Tamayo *et al.*, 2007).

Finally, the genomic clone of Pa (PAO1) had an insert of 4089 nt, which encodes an hypothetical protein, pyocin S2 toxin, and immunity proteins (Figure 3-8 a and b). Pyocin S2 is one of the S-type pyocins found in Pa (Michel-Briand & Baysse, 2002). The genomic clone of PAO1 has DNA sequence, which covers the pyocin S2 toxin gene (Pa1152) and the pyocinS2 immunity gene (Pa1153), which provides immunity to pyocin S2 (Figure 3-8b).

### **3.5 Discussion**

Patients with CF suffer from chronic lung infections that are associated with high mortality (Lyczak *et al.*, 2002). During infancy, the patient's airways and lungs are colonized intermittently with several species of bacteria, including *Haemophilus influenzae* (*H. influenzae*), *Staphylococcus aureus* (*S. aureus*), *Pseudomonas aeruginosa* (Pa), and *B. cepacia complex* (Bcc) (Hutchison & Govan, 1999). Infections may become

chronic, resulting in decreased lung capacity, continuous inflammation and ultimately result in the death of the patient (Govan & Deretic, 1996; Lyczak *et al.*, 2002).

CF patients' susceptibility to particular pathogen species may be age-related (Govan & Deretic, 1996). *H. influenzae* and *S. aureus* colonize the lungs of infants primarily, but can be found in the lungs through early childhood (Gibson *et al.*, 2003). *Pa* tends to colonize older patients, but can be found in up to 30% of young patients (2008). Early *Pa* colonizers are planktonic (free living) and these infections are relatively easily treated with broad-spectrum antibiotics, usually a combination of beta-lactamases and aminoglycosides (Hansen *et al.*, 2008). Over time, alginate-forming mucoid *Pa* variants emerge and dense biofilms develop in the lungs, resulting in chronic lung infections. These chronic, biofilm-based infections are difficult to eradicate since the biofilms enhance the innate antibiotic resistance of the strains (Hentzer *et al.*, 2001).

In the past thirty years, *Bcc* has emerged as a lung pathogen in CF patients (Govan & Deretic, 1996). Members of the species complex naturally reside in soil and on plants (Mahenthiralingam *et al.*, 2005). *Bcc* is known for its metabolic versatility, which may contribute to its ability to survive, and even thrive, in numerous environments, including the human lung. Although the prevalence of *Bcc* lung infections is low (~3% of CF patients), the clinical outcomes are unpredictable and can be quite severe (Kalish *et al.*, 2006). A small portion of those infected (20%) develop cepacia syndrome, which is a necrotizing pneumonia, leading to rapid lung failure and death (Jones *et al.*, 2004; Kalish *et al.*, 2006; Tablan *et al.*, 1985).

*Bcc* strains that invade the CF lung face numerous challenges, such as evading the host immune system, competing with the resident bacteria and colonizing the host tissue.

The focus of this study was an exploration of the virulence factors that play a role in one feature of Bcc invasion, the competition between Bcc and the bacteria already resident in the patient's lungs.

Bcc colonization usually involves competition with a resident strain of Pa (Govan & Deretic, 1996). In such a scenario, two kinds of species interactions may occur, synergism and antagonism, resulting in either co-infection or extinction of one species. The incidence of co-infection is quite low (Lambiase *et al.*, 2006; McManus *et al.*, 2004). In either case, the presence of Pa has been shown to have a detectable impact on Bcc, such as the promotion of virulence gene expression (Riedel *et al.*, 2001). Riedel *et al.* 2005 revealed that Bcc recognizes acylated homoserine lactones (AHLs) secreted by Pa, which are small molecules that induce expression of genes in Bcc involved in virulence and biofilm formation. The AHL recognition is unidirectional, Bcc recognizes AHL's produced by Pa, while no recognition of Bcc AHLs by Pa is detected (Riedel *et al.*, 2001).

Another factor known to impact bacterial interactions is the production of bacteriocins, which are potent toxins produced by bacteria to kill the strains of the same or related species (Riley & Wertz, 2002a; Riley & Wertz, 2002b). Studies in which bacteria are competed *in vitro* (on petri plates) and *in vivo* (in the mouse colon) reveal that bacteriocins are highly effective in mediating intra- and inter-specific competition, (Bakkal *et al.*, 2010; Michel-Briand & Baysse, 2002).

Pa is a prolific producer of bacteriocins, with more than 90% of the strains assayed identified as producers (Fyfe *et al.*, 1984). In contrast, far fewer strains of Bcc (~30%) produce bacteriocins (Govan & Harris, 1985). A survey of bacteriocin production

was undertaken with strains isolated from the lungs of CF patients. This study revealed that 97% of Pa and 68% of Bcc strains produce bacteriocin-like inhibitory activity (Bakkal *et al.*, 2010).

Detailed studies of Pa bacteriocin production have resulted in the identification of three pyocin types: S, R, and F (Michel-Briand & Baysse, 2002). S-pyocins are high molecular weight proteins, which possess DNase activity to inhibit (Sano & Kageyama, 1981; Sano & Kageyama, 1984; Sano *et al.*, 1993a; Sano *et al.*, 1993b). RF pyocins resemble phage tails, which depolarize the cell membrane (Nakayama *et al.*, 2000). The bacteriocins of Bcc have not been studied extensively. In fact, there is no molecularly characterized Bcc bacteriocin. One available phenotypic study revealed that Bcc bacteriocins resemble phage tails (Govan & Harris, 1985)

We employed genomic library screens of *B. multivorans*, *B. dolosa*, and *B. cenocepacia* to understand how Bcc mediates its interactions with Pa in the CF lung. The screening involved lawns of sensitive Pa and Bcc strains (Bakkal *et al.*, 2010). Fifteen clones, from three Bcc genomic libraries, possessed a putative inhibitory activity as revealed by inhibition zones on the sensitive lawns. However, the activity was lost in ten of the clones in subsequent replications of the screen. We also lost the inhibitory activity of two Pa PAO1 genomic clones (three clones identified in total).

The loss of inhibitory activity in ten Bcc and two Pa genomic clones can be explained simply by the absence of DNA sequence encoding immunity protein in genomic clones. In most bacteria, an immunity gene follows the bacteriocin gene (toxin), which are transcribed and translated together in bacteria. Immunity protein confers resistance to the toxin and thus, producer bacteria are immune to the produced bacteriocin

(Riley & Wertz, 2002a; Riley & Wertz, 2002b). Therefore, absence of immunity gene in genomic clones may result the lysis of *E. coli* recipient due to the toxicity of the cloned protein(s). Indeed, one Pa PAO1 genomic clone possessed DNA, encoding both pyocin S2 and corresponding immunity protein (Figure 3-8). In this clone, we observed clear inhibition of the sensitive lawn (Figure 3-2). Another reason of the loss of inhibitory activity might be the toxicity of Bcc and Pa bacteriocins (or novel toxins) to *E. coli* recipient. However, the bacteriocin producer Bcc and Pa strains were previously tested on *E. coli* XL1 Blue MRF' recipient. *E. coli* XL1 Blue MRF' recipient did not show sensitivity against these Pa and Bcc strains, which were used in genomic library construction.

The DNA isolated from the remaining five clones of *B. multivorans* was sequenced. The genes identified correspond to four major functional categories: amino acid biosynthesis, sugar metabolism, degradation of aromatic compounds, and bacterial apoptosis (Table 3-2).

### **3.5.1 Amino Acid and Sugar Metabolism**

An NCBI BLAST of the Bcc clones resulted in identification of genes for amino acid and sugar metabolisms. 3-isopropyl malate dehydrogenase, 3-isopropyl malate dehydratase small subunit, 3-isopropylmalate dehydratase large subunit, and aspartate semialdehyde dehydrogenase are involved in leucine and lysine biosynthesis (Black & Wright, 1954). The screen also revealed a sequence with homology to glucoamylase, a hydrolytic enzyme involved in sugar metabolism in fungus and bacteria, which converts starch into glucose (Sauer *et al.*, 2000). Further, this enzyme is involved the induction of a fungal toxin (aflatoxin). Thus, glucoamylase might have similar function in Bcc and

causes induction of toxin genes. Partial aflatoxin gene (Genbank accession number: AY864289.1) was blasted against 38 sequenced Bcc genomes to identify if these genomes possess aflatoxin-like proteins. However, none of these genomes have proteins, which share homology with aflatoxin of fungus *Aspergillus*.

### **3.5.2 Degradation of aromatic compounds**

The BLAST search of the genes (4-hydroxybenzoate transporters, poly-beta hydroxyalkonate) encoded in the Bcc genomic clones also revealed enzymes involved in degradation of aromatic compounds such as benzoate and polyhydroxyalkanoates (Nichols & Harwood, 1995). It is not clear how are these proteins can be involved in mediating Bcc competition in the CF lung? The primary reason of metabolizing toxic aromatic compounds is that they are used as energy sources where the nutrients are limited (Madison & Huisman, 1999). Thus, the presence of a competitor strain (Pa) in nature, as well as on solid LB agar medium, or even in a biofilm in the CF lung, may induce their expression, resulting in inhibition of Pa growth. It is also possible that the encoded proteins have secondary functions, which result in Pa inhibition.

### **3.5.3 Bacterial apoptosis**

The BLAST search revealed the presence of a bacterial apoptosis gene (entericidinAB) (Bishop *et al.*, 1998). Entericidins are produced at stationary phase, where nutrients are limited. These proteins bind to membrane phospholipids, and inhibit the cells via disruption of membrane stability (Bishop *et al.*, 1998). No further functional information is known about entericidin AB in Bcc.

The gene organization of the locus is similar to that of the bacteriocins of numerous Gram-negative bacteria, including *E. coli* (colicin) and *P. aeruginosa* (pyocin). An immunity gene follows the toxin gene, which are transcribed and translated together (Figure 3-9). Entericidin AB locus also has toxin/antitoxin gene pair. Entericidin A provides immunity to the toxin entericidin B. However, no sequence similarity was detected between entericidin AB and colicins/pyocins. Finally, there is no information about the ability of entericidin AB to lyse strains other than the producer. Our study is the first report suggesting that entericidin AB might be responsible for Bcc inhibitory activity.

#### **3.5.4 Proteins with unknown functions**

BLAST searches of the remaining sequences obtained from the genomic clones also revealed the presence of genes with unknown functions, amidohydrolyse II, GGDEF domain, and a hypothetical protein. Amidohydrolyse II catalyzes the cleavage of C-N, C-C, C-O, C-Cl, C-S, O-P bonds of organic substances (Aimin *et al.*, 2007). Thus, it may be involved in hydrolysis of vital proteins in Pa.

The GGDEF is a conserved domain, which is observed in multi-domain proteins such as those involved in signaling pathways including cell-to-cell signaling, motility, virulence, biofilm formation, and antibiotic production (Pei & Grishin, 2001; Tamayo *et al.*, 2007). Thus, GGDEF domain could be present in any protein involved in virulence signaling pathways, and in that manner, may play a role in Bcc inhibitory activity.

In summary, genomic screens for inhibitory activity in Bcc revealed a surprising absence of traditional bacteriocin genes. The five clones explored here possessed genes

involved in amino acid biosynthesis, energy metabolism, degradation of aromatic compounds, and bacterial apoptosis.

Why does Bcc, in contrast with all Gram-negative species examined to date, not have any identifiable bacteriocin genes? One simple explanation might be genomes of Bcc possess bacteriocins, which we were unable to identify in this study due to technical challenges. As it was mentioned, we lost the inhibitory activity in ten Bcc and two Pa genomic clones, which might be due to either toxicity of the cloned protein or lack of the corresponding immunity gene in the genomic clone. Additionally, Bcc bacteriocins might be relatively big proteins or phage tails, which might not be possible to identify with our approach since we cloned 4-5 kb DNA sequences. Thus, cloning larger DNA inserts of Bcc genome might allow us to identify bacteriocins of Bcc.

Another explanation is that Bcc has evolved a different solution to the challenge of bacterial competition. One solution may be found in the fact that Burkholderia has significantly more genes that encode proteins involved in such major metabolic pathways as energy metabolism, transport, and binding proteins are among these categories (Figure 3-10). Bcc genomes have ability to gain additional genes via horizontal gene transfer (Mahillon & Chandler, 1998; Tyler *et al.*, 1996; Wood *et al.*, 1991). For example, the glucoamylase gene identified in the Bcc clone 4 is originally detected in *Aspergillus*, which uses this enzyme to become pathogenic to plants (Mellon *et al.*, 2007). Perhaps these extra metabolic genes might provide a level of flexibility to Bcc, whose members has to adapt living in different environments (soil, plant, human tissue), where the level of bacterial competition is high.



This study revealed that members of Bcc might be using a different strategy to compete with Pa strains in our experimental conditions, where two species are in close contact. The genomic clones with inhibitory activity possess genes involved in major metabolic pathways, whose induction might provide Bcc strains a better growth advantage in the environment where competition is high and nutrients are limited. Revealing the exact roles of these genes is critical to our understanding the interaction between Pa and Bcc strains in CF lungs. Therefore, future efforts will focus on cloning of these genes identified in the genome library clones of *Burkholderia multivorans* (ATCC17616) to further characterize their potential roles in Bcc inhibitory activity.

### **3.6 Acknowledgements**

This work is supported by NIH Grants RO1GM068657-01 and RO1AI064588-01A2. We would like to thank Dr. Claudia Ordonez for providing us with clinical Pa and Bcc strains. We also would like to thank Dr. John LiPuma for providing *B. dolosa* AUO158. Finally, we would like to thank Dr. Sumiko Sano and Dr. Fred Ausebel for providing Pa NIH3 and Pa PAO1 strains, respectively. We thank Dr. Chris Vriezen and Chris Roy for helping phenotypic screening of Bcc genomic clones. Further, we would like to thank Sandra M. Robinson for intellectual support and editing of this manuscript.

**Table 3-1 Chromosomes and genome size of members of *B. cepacia* complex**

The number of chromosomes and the total genome size varies among strains as well as within the same genomovar.

Genomovar	Strain ID	Size of chromosomes (Mb)	Overall genome size (Mb)
Genomovar I ( <i>B. cepacia</i> )	ATCC 25416	3.5, 3.1, 1.1	7.7
	ATCC 17759	3.5, 3.3, 1.1	7.9
Genomovar II ( <i>B. multivorans</i> )	ATCC17616	3.4, 2.5, 0.9	6.8
	C5274	3.3, 2.4, 1.3	7.0
Genomovar III ( <i>B. cenocepacia</i> )	C4455	3.9, 3.0, 1.0	7.9
	CEP024	3.4, 2.7, 0.9	7.0
Genomovar IV ( <i>B. stabilis</i> )	LMG14294	3.7, 2.3, 1.3	8.2
	LMG7000	3.9, 3.3, 1.4	8.6
Genomovar V ( <i>B. vietnamiensis</i> )	ATCC53617	3.4, 3.2, 1.2, 1.1	8.9
	LMG18836	3.0, 1.8, 1.2	6.0
Genomovar VII ( <i>B. ambifaria</i> )	ATCC17760	3.4, 1.3	4.7
	BcF	3.5, 2.6, 1.3	7.4

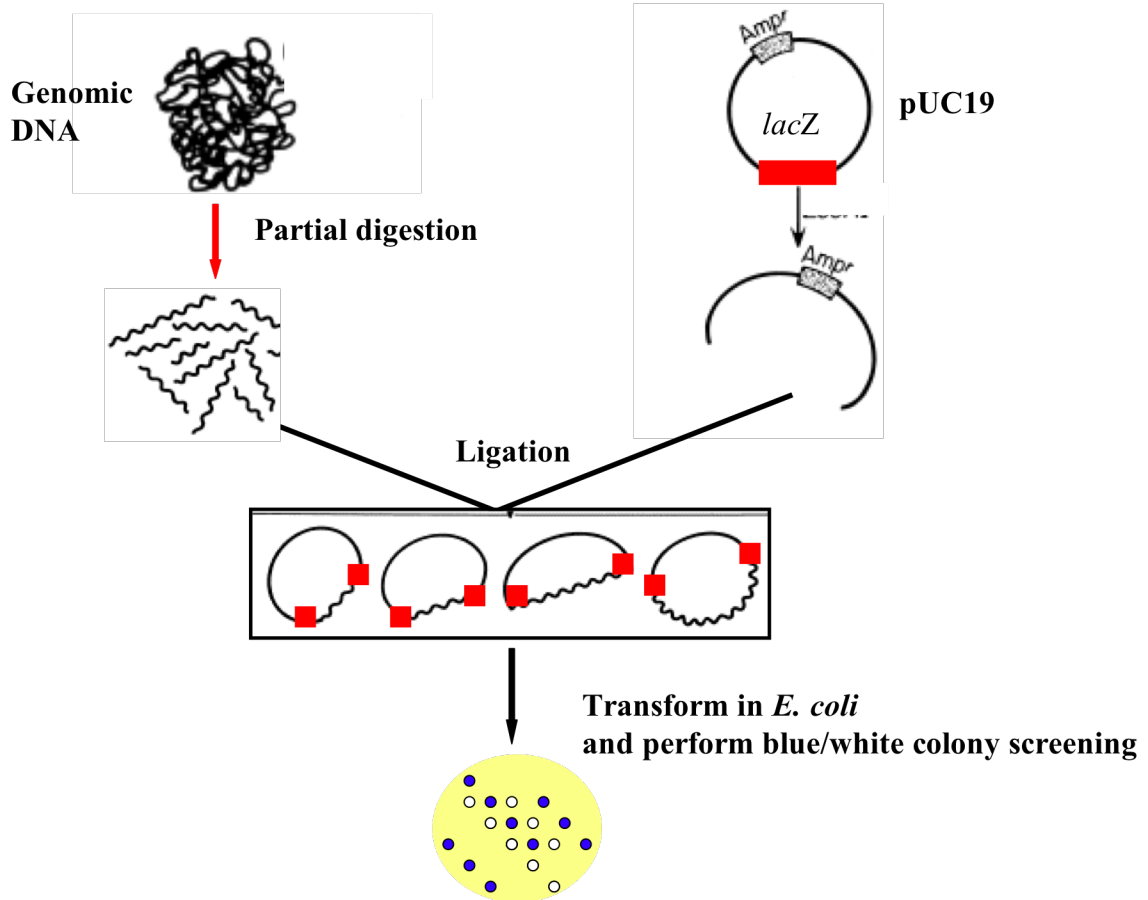
**Table 3-2 Genomic library clones of *B. multivorans* (ATCC17616) and *Pseudomonas aeruginosa* (PAO1)**

Inhibitory activity has detected in five genomic clones of *B. multivorans* (ATCC 17616) and one genomic clone of *P. aeruginosa* (PAO1). Insert DNAs were sequenced from these genomic clones, which are involved in various metabolic pathways including degradation of aromatic hydrocarbons, sugar metabolism, amino acid biosynthesis, and apoptosis in Bcc, and pyocin S2 synthesis in Pa.

Producer Strain	Clone Number	Length of the cloned DNA fragment (nt)	Gene ID	Corresponding genes in the cloned DNA fragment	Functional Category
<i>B. multivorans</i> ATCC17616	2	714	Bm1615	Hypothetical protein	Unknown function
	4	3498	Bm1065	Poly-beta-hydroxyalkanoate depolymerase	Degradation of aromatic compounds
	6	677	Bm1066	Glucoamylase	Sugar metabolism
			Bm1067	3-carboxymuconate cyclase	Sugar metabolism
			Bm6110	Amidohydrolase 2	Unknown function
			Bm6111	4-hydroxybenzoate transporter	Degradation of aromatic compounds
	7	3956	Bm4756	Aspartate-semialdehyde dehydrogenase	Amino acid biosynthesis
			Bm4757	3-isopropylmalate dehydrogenase	Amino acid biosynthesis
			Bm4758	3-isopropylmalate dehydratase small subunit	Amino acid biosynthesis
			Bm4759	Entericidin AB	Apoptosis
Bm4760			3-isopropylmalate dehydratase large subunit	Amino acid biosynthesis	
9	37	Bm4824	GGDEF domain	Unknown function	
<i>Pseudomonas aeruginosa</i> PAO1	1	4089	Pa1151	Hypothetical protein	Bacteriocin
			Pa1152	Pyocin S2	
			Pa1153	Pyocin immunity	

### Figure 3-1 Genomic library construction

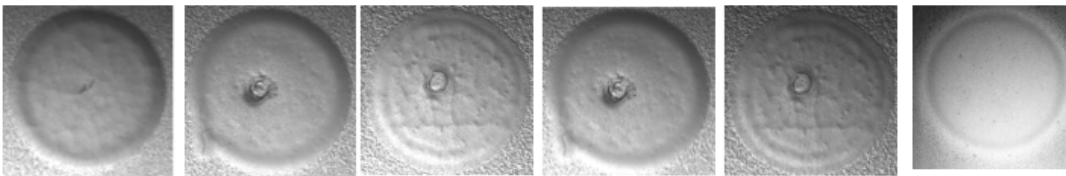
Genomic DNA of bacteriocin producer strain was partially digested. DNA inserts were ligated into *Bam* HI digested and dephosphorylated pUC19 vector. Blue/white colony screening was performed on *E. coli* recipients transformed with pUC19 clones.



**Figure 3-2 Inhibition phenotypes of genomic clones of *B. multivorans* (ATCC17616) and *Pseudomonas aeruginosa* (PAO1)**

a-Five *B. multivorans* (ATCC17616) clones (clone #2-9) and one *P. aeruginosa* (PAO1) clones (PAO1 clone) were detected as genomic clones with inhibitory activity. These clones showed inhibitory activity on the sensitive Pa strains; b-Bm ATCC 17616 and Pa PAO1 producer strains were tested on corresponding sensitive Pa strains as positive control. *E. coli* with puC19 was used as negative control.

a



clone #2

clone #4

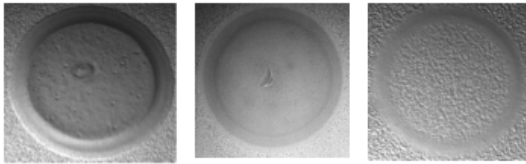
clone #6

clone #7

clone #9

PAO1 clone

b



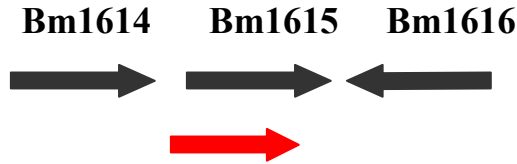
Bm ATCC17616  
Positive control

Pa PAO1  
Positive control

puC19 clone  
Negative control

**Figure 3-3 Blast result of the clone 2 of *B. multivorans* (ATCC17616) genome library**

a. Genomic organization of the clone 2 of *B. multivorans* (ATCC167616). Clone 2 encoded for a hypothetical protein (Bm1615).



**Clone2**

Bm1614: hypothetical protein  
 Bm1615: hypothetical protein  
 Bm1616: YbbM seven transmembrane helix protein

b. Alignment result of clone 2 and hypothetical protein of *B. multivorans* (ATCC17616)

```

Bm1615 -----
clone2      AGCGCTGAGCCGGCGCGTGGCCGGCATCGTCCGGTGGCGCGCAACGAGTGGGCGGCCTG 60

Bm1615 -----
clone2      CGTTCGTACGCGTGGCGCCAGGCCGTATCCGAGCCGGAGGCCATCGATACGAGCGCCAC 120

Bm1615 -----
clone2      GCGCGCGCGCTGCGGCTCGCTTCACGCCGTTTCGCGCCGGTTCGATCCGCTGCGCCGCT 180

Bm1615 -----
clone2      CCATGGTTCGCGCGCCGCGTGACGCGATGCCGATGTCGCGCTATGCTCGCCGCATCGTC 240

                                     Bm1615 ----->
Bm1615      -----ATGAAACATCGAATGCGCGTGCTGCTGTCGGCCGTCGC 38
clone2      CCTCTCCGCCAACGACCCTCCGATGAAACATCGAATGCGCGTGCTGCTGTCGGCCGTCGC 300
                *****

Bm1615      GTTCGTGCGGGCCATGCGCAGGCCGCCGACGACTGCAGCTTCGTGAAGAAAGTCGCGCT 98
clone2      GTTCGTGCGGGCCATGCGCAGGCCGCCGACGACTGCAGCTTCGTGAAGAAAGTCGCGCT 360
                *****

Bm1615      GCCGGCGCGGCAGCAGACGATCGTCTGTCGAGCGGCGCACTCGAGCCGTGCTCGACCGG 158
clone2      GCCGGCGCGGCAGCAGACGATCGTCTGTCGAGCGGCGCACTCGAGCCGTGCTCGACCGG 420
                *****

Bm1615      CAGCTATGCGGTGCGCGTCTATTCGACCGCGAACACGGCGCCCGGCTTCGATACCGACGA 218
clone2      CAGCTATGCGGTGCGCGTCTATTCGACCGCGAACACGGCGCCCGGCTTCGATACCGACGA 480
                *****

Bm1615      CTACGTGAGCGGCGCGCTGCATGCGCGGACGGCACCGTCACCGATGCGTTCGTGCGCGA 278
clone2      CTACGTGAGCGGCGCGCTGCATGCGCGGACGGCACCGTCACCGATGCGTTCGTGCGCGA 540
                *****

Bm1615      TCTCGGCGCGCGTGGCGCCGATGCGCTGATCGTGACGACGCGTTCGGCCGGGAGCGGCGG 338
clone2      TCTCGGCGCGCGTGGCGCCGATGCGCTGATCGTGACGACGCGTTCGGCCGGGAGCGGCGG 600
                *****
    
```

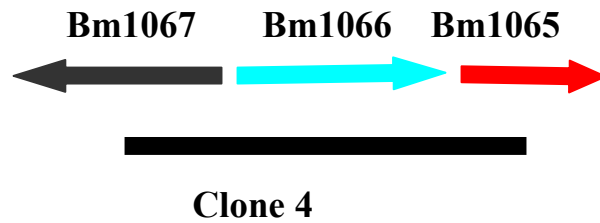
```

Bm1615      CTACGTCGGCGCCAGGCCTATCTGACGACGTCGCGCGCTGTGACGCTCGTCGCGTCGGT 398
clone2      CTACGTCGGCGCCAGGCCTATCTGACGACGTCGCGCGCTGTGACGCTCGTCGCGTCGGT 660
            *****
Bm1615      CGACGGGCTCGCGCCCGATGCCGATGTCGCGGGCGCGCTGCGTCGCGCGCTCGGGCGACG 458
clone2      CGACGGGCTCGCGCCCGATGCCGATGTCGCGGGCGCGCTGCGTCGCGCGCTCGG----- 714
            *****
Bm1615      TCGCGGCACGCACTGA 474
clone2      -----

```

**Figure 3-4 Blast result of the clone 4 of *B. multivorans* (ATCC17616) genome library**

a. Genomic organization of the clone 4 of *B. multivorans* (ATCC17616). Clone 4 encoded partially for 3-carboxymuconate cyclase and poly-beta-hydroxyalkolonate depolymerase proteins. This clone also encoded for glucoamylase protein (full protein).



Bm1065: Poly-beta-hydroxyalkolonate depolymerase  
 Bm1066: Glucoamylase  
 Bm1067: 3-carboxymuconate cyclase

b. Alignment result of clone 4 and Bm1065-67 of *B. multivorans* (ATCC17616)

```

          Bm1067 ←
Bm1065-67  TFACTGCACCGGTACGAGCTTGAAATCGACC GGCTTGCCGACCGCCACCTTGCGCGGATT 60
clone4     -----
Bm1065-67  CGCAGCGAGCTGCCCGCTGCCGACGTCGCGGCCGAACACGTAGAACGTGTCGCTGTCTCTG 120
clone4     -----
Bm1065-67  GTTGCCGACGATCAGCCACTTGCCGGTTCGGATCGATCAGGAATTCGCGCGGGCGTCCGGCC 180
clone4     -----
Bm1065-67  GAGGCTCGACTGGCGGCCGACGGTCTTCAGCCGGCCGTCGGCCTGGTTCACCGCGTAGAT 240
clone4     -----
Bm1065-67  CACGATCTCGTTTCGCGTCGCCGCGGTTGCTCACGTACAGGAAGCGGCCGTCCGGCGACAG 300
clone4     -----
Bm1065-67  GTGGATCGCGCCACCGCCGACCTTGCCCTTGAAGCCGGGCGCGATCATCGACACGGTCTC 360
clone4     -----

```

Bm1065-67 clone4 GATCGGCGTGAGCTTCCCGTCGTGATAGCCGAACACCTCGACCGACGCGTTCAGCTCGCT 420  
-----

Bm1065-67 clone4 CGTCACATACGCGAACCGGCCGTCGGCACTGAACACGAGATGGCGCGGCCCGCGCCGGC 480  
-----

Bm1065-67 clone4 CTTACCGGCGTATAACGCGTGTCCGGTCGGGCTGATCAGCCCGCGGCTGCCGTCGACCGT 540  
-----

Bm1065-67 clone4 GTAGCGATAGCCGTAGATCTTGTGTCGAGACCGAGATCCTGCACGAACAGATAGCGGCCGTC 600  
-----

Bm1065-67 clone4 GGGCGAGAACACCGTTCGAGTGGACGTGCGCGTTGTCTGTCGCCCTCTGACGGGCCCTT 660  
-----

Bm1065-67 clone4 CCCTTCGTGGTGCACGCTGAGCACGGCCGGTGCACGCCCGCTCGTCGCGGACCGGAAA 720  
-----

Bm1065-67 clone4 CAGCGCGAAGCTGCCGCCGGATCGGCCGCGACCGAATAGTTGGCCGTCACCAGATACTT 780  
-----

Bm1065-67 clone4 GCCGTCGGGCGACAGGCTCAAATAGCAAGGATCGTTCCCTCTGACGATACGCGATCGAT 840  
-----

Bm1065-67 clone4 GAAGGTGAGCGCGCCCGTCTTCGCATCGAAACGGAACGCGCTGATGCCGCCGCGCTGCGT 900  
-----

Bm1065-67 clone4 CGCGGGCCCGTTGTGCGCCGGCAGTTCGTTGACCGCATAACGACGCGACCGTTCGCGGCT 960  
-----

Bm1065-67 clone4 CGGCAACAGATACGACGGGTTACCGTCTTCGCCGACGATACCGGTGCGACACTACCGGT 1020  
-----CGGGTTCACCGTCTTCGCCGACGATACCGGTGCGACACTACCGGT 45  
\*\*\*\*\*

Bm1065-67 clone4 GCTCGTATCGAAGCGGTAAACGTAGATGCCGTCGCTGCCGGTGTTCGGTATAGGTGCCGAC 1080  
GCTCGTATCGAAGCGGTAAACGTAGATGCCGTCGCTGCCGGTGTTCGGTATAGGTGCCGAC 105  
\*\*\*\*\*

Bm1065-67 clone4 GAGCAGGTTGTAGACGCGTTCGCCGGTGCAGCGGATGCTGCGCGAATGCATGGGTGCG 1140  
GAGCAGGTTGTAGACGCGTTCGCCGGTGCAGCGGATGCTGCGCGAATGCATGGGTGCG 165  
\*\*\*\*\*

Bm1065-67 clone4 GGACAGGAAAGCACGAACGCGAAACCTTTCATCCAGTGAGCGAGCCTAAGCGGGAACCC 1200  
GGACAGGAAAGCACGAACGCGAAACCTTTCATCCAGTGAGCGAGCCTAAGCGGGAACCC 225  
\*\*\*\*\*

Bm1065-67 clone4 TCGTGTCTGAAGCGTGTGCGTACGCTCGCGTAAACGGTTGGGCATTGAATCCTCCTTGCA 1260  
TCGTGTCTGAAGCGTGTGCGTACGCTCGCGTAAACGGTTGGGCATTGAATCCTCCTTGCA 285  
\*\*\*\*\*

Bm1065-67 clone4 TCGAGTCGCTTCAAGTGTGAGATAGGACGAAACGCCGCGGTCATGCGCTCCGCTTCG 1320  
TCGAGTCGCTTCAAGTGTGAGATAGGACGAAACGCCGCGGTCATGCGCTCCGCTTCG 345  
\*\*\*\*\*

Bm1065-67 clone4 ATCGAGTATACGGGCGCGGCCAGCGCGTGAAGCTGTCCGTCCGCGGCCCGCATTTGTG 1380  
ATCGAGTATACGGGCGCGGCCAGCGCGTGAAGCTGTCCGTCCGCGGCCCGCATTTGTG 405  
\*\*\*\*\*

Bm1065-67 clone4 AACC CGAAACAAGGATTTGCCCGCCATGCCCGCCTTGATCGAAGACTATGCCCTCGTCG 1440  
AACC CGAAACAAGGATTTGCCCGCCATGCCCGCCTTGATCGAAGACTATGCCCTCGTCG 465  
\*\*\*\*\*

**Bm1066** →



Bm1065-67 clone4	<b>CGCAGCGCCACACGGCCGCGCTGATCGCAAAAGACGGCTCCGTCGACTGGCTGTGTGGC</b> GCGACGGCCACACGGCCGCGCTGATCGCAAAAGACGGCTCCGTCGACTGGCTGTGTGGC *****	1500 525
Bm1065-67 clone4	<b>CCCGGTTTCGACTCGGGCGCCTGCTTCGCGGCGCTCGTCGGCACACCCGAGCACGGTTCGCT</b> CCCGGTTTCGACTCGGGCGCCTGCTTCGCGGCGCTCGTCGGCACACCCGAGCACGGTTCGCT *****	1560 585
Bm1065-67 clone4	<b>GGCTGATCGCGCCGGCCGCGCAGCTCGCGATTACGCATACGACACGCCGCTATCGCGGGC</b> GGCTGATCGCGCCGGCCGCGCAGCTCGCGATTACGCATACGACACGCCGCTATCGCGGGC *****	1620 645
Bm1065-67 clone4	<b>ACACGCTGATTCTCGAAACCGATTACGAAAGTGCCGACGGCGCCGTGACCGTCGTCGACT</b> ACACGCTGATTCTCGAAACCGATTACGAAAGTGCCGACGGCGCCGTGACCGTCGTCGACT *****	1680 705
Bm1065-67 clone4	<b>TCATGCCGCCCGGCAACGGCTGGTCCGAGCTCGTGCGCATCGTCGTCGGCCGGCGCGCA</b> TCATGCCGCCCGGCAACGGCTGGTCCGAGCTCGTGCGCATCGTCGTCGGCCGGCGCGCA *****	1740 765
Bm1065-67 clone4	<b>CGATGAAGATGCGGATGGAACCTCGTGCTGCGCTTCGACTACGGCTTCTCAATCCCGTGGG</b> CGATGAAGATGCGGATGGAACCTCGTGCTGCGCTTCGACTACGGCTTCTCAATCCCGTGGG *****	1800 825
Bm1065-67 clone4	<b>TCACGCAGCTCACGCGGAAGACGGGATGAAGGCGATCGCCGGCCCCGACACGGTCTGTG</b> TCACGCAGCTCACGCGGAAGACGGGATGAAGGCGATCGCCGGCCCCGACACGGTCTGTG *****	1860 885
Bm1065-67 clone4	<b>TGCGCACGCGGTGCCGCTCACCGCAAGAACCTGCATACGCTCGCGGAATTCACGGTCA</b> TGCGCACGCGGTGCCGCTCACCGCAAGAACCTGCATACGCTCGCGGAATTCACGGTCA *****	1920 945
Bm1065-67 clone4	<b>GCGCCGACGAGCGCGTCCGTTCTCGTCGGCTATGCGCCCTCGCATCTGCGGCTGCCGC</b> GCGCCGACGAGCGCGTCCGTTCTCGTCGGCTATGCGCCCTCGCATCTGCGGCTGCCGC *****	1980 1005
Bm1065-67 clone4	<b>CCGCGCGCGATCCGCTGTGATGCTCGCGCGCACCGAGAATACTGGCTCGAATGGTCCG</b> CCGCGCGCGATCCGCTGTGATGCTCGCGCGCACCGAGAATACTGGCTCGAATGGTCCG *****	2040 1065
Bm1065-67 clone4	<b>GCCGCTGCCAGGTGCGCGGTGCTATGCGGCCGCGGTGCCGCGTTCGCTGATCACGCTGA</b> GCCGCTGCCAGGTGCGCGGTGCTATGCGGCCGCGGTGCCGCGTTCGCTGATCACGCTGA *****	2100 1125
Bm1065-67 clone4	<b>AGGCGCTCGCCTACGAGCCGACCGCGGGATCGTCGCGCGCCACCACGTCGCTGCCCG</b> AGGCGCTCGCCTACGAGCCGACCGCGGGATCGTCGCGCGCCACCACGTCGCTGCCCG *****	2160 1185
Bm1065-67 clone4	<b>AGAAGATCGGTGGCAACCGCAACTGGGACTACCGCTACTGCTGGCTGCGCGACGCGACGA</b> AGAAGATCGGTGGCAACCGCAACTGGGACTACCGCTACTGCTGGCTGCGCGACGCGACGA *****	2220 1245
Bm1065-67 clone4	<b>TCACGCTGCTCGCGTGTGCGCGCGGCTACTACGACGAAGCGCGCGCTGGCGCACGT</b> TCACGCTGCTCGCGTGTGCGCGCGGCTACTACGACGAAGCGCGCGCTGGCGCACGT *****	2280 1305
Bm1065-67 clone4	<b>GGCTCGGCCGCGTGTGCGCGGATCGCCGAGCAGATTCAGATCATGTACGGGATCGCGG</b> GGCTCGGCCGCGTGTGCGCGGATCGCCGAGCAGATTCAGATCATGTACGGGATCGCGG *****	2340 1365
Bm1065-67 clone4	<b>GCGAGCGCCGCTTGCCCGAGATGGAGCTCGACTGGCTGCCCGGCTATCAGGACTCGAAGC</b> GCGAGCGCCGCTTGCCCGAGATGGAGCTCGACTGGCTGCCCGGCTATCAGGACTCGAAGC *****	2400 1425
Bm1065-67 clone4	<b>CGGTGCGCGTTCGGCAACGGCGCGCGAACCAGCTGCAGCTCGACGTGTTTCGGCGAAGTGA</b> CGGTGCGCGTTCGGCAACGGCGCGCGAACCAGCTGCAGCTCGACGTGTTTCGGCGAAGTGA *****	2460 1485
Bm1065-67 clone4	<b>TGGCCGCGTGCATCTCGCGCGCTCGGTGGTCTGCAGGCCGACGATACGGTGTGGTCCG</b> TGGCCGCGTGCATCTCGCGCGCTCGGTGGTCTGCAGGCCGACGATACGGTGTGGTCCG *****	2520 1545
Bm1065-67	<b>TGCAGTGCGCGTGTCTCGATCACTCGAGAAGATCTGGCAGGAGCCCGACGAAGGGATCT</b>	2580

clone4	TGCAGTGC GCGCTGCTCGATCACCTCGAGAAGATCTGGCAGGAGCCCGACGAAGGGATCT *****	1605
Bm1065-67 clone4	GGGAAACGCGCGGGCGGCCGCCATTTCACGTTCTCGAAGGTGATGGCATGGGTCGCGT GGGAAACGCGCGGGCGGCCGCCATTTCACGTTCTCGAAGGTGATGGCATGGGTCGCGT *****	2640 1665
Bm1065-67 clone4	TCGACCGCGCGATCAAGTCCGCGGAGATGTTCCGCCCTGCCGGCTCGCTCGAGCGCTGGC TCGACCGCGCGATCAAGTCCGCGGAGATGTTCCGCCCTGCCGGCTCGCTCGAGCGCTGGC *****	2700 1725
Bm1065-67 clone4	GCGCGCTGCGCGACCGCATCCATGCCGACGTCTGCGAGAAGCGTGGCAGCAGCGCAAGC GCGCGCTGCGCGACCGCATCCATGCCGACGTCTGCGAGAAGCGTGGCAGCAGCGCAAGC *****	2760 1785
Bm1065-67 clone4	AGGCGTTCGCCCAAAGCTACGGCAGCGACGAGCTCGACGCGAGCGTGTGCTGATGCCGC AGGCGTTCGCCCAAAGCTACGGCAGCGACGAGCTCGACGCGAGCGTGTGCTGATGCCGC *****	2820 1845
Bm1065-67 clone4	TGCTCGGCTTCCTGCCGCCGAGGATCCGCGCATCGTCGGCAGGTGGAGGCGATCGAGC TGCTCGGCTTCCTGCCGCCGAGGATCCGCGCATCGTCGGCAGGTGGAGGCGATCGAGC *****	2880 1905
Bm1065-67 clone4	GGGAATGCTGCACGACGGGCTCGTGATGCGCTACCGCACGACCGAGTACGACGACGGCC GGGAATGCTGCACGACGGGCTCGTGATGCGCTACCGCACGACCGAGTACGACGACGGCC *****	2940 1965
Bm1065-67 clone4	TGCCGCCGGCGAAGGCACGTTTCTCGCGTGCAGTTTCTGGCTCGTCGACAACCTACGCGC TGCCGCCGGCGAAGGCACGTTTCTCGCGTGCAGTTTCTGGCTCGTCGACAACCTACGCGC *****	3000 2025
Bm1065-67 clone4	TGCTCGCCGGATCGACGACGCGCATCGGCTGTTTCAGTCGCCTGCTCGCGCTGTGCAACG TGCTCGCCGGATCGACGACGCGCATCGGCTGTTTCAGTCGCCTGCTCGCGCTGTGCAACG *****	3060 2085
Bm1065-67 clone4	ACCTCGGGCTGCTCGCGGAGGAATACGACCCGGTCGAGGGCGGGCTCGTCGGCAATTTC ACCTCGGGCTGCTCGCGGAGGAATACGACCCGGTCGAGGGCGGGCTCGTCGGCAATTTC *****	3120 2145
Bm1065-67 clone4	CGCAGGCGTTCGCGACGTGCGCTCGTGATACCGCGATGAACCTGATGCACCACGAAG CGCAGGCGTTCGCGACGTGCGCTCGTGATACCGCGATGAACCTGATGCACCACGAAG *****	3180 2205
Bm1065-67 clone4	AGGCGATGGCGCGCGGCCGGACAGCCGGCCGGCGGCCGCGACGGGCCGCTGACCGC AGGCGATGGCGCGCGGCCGGACAGCCGGCCGGCGGCCGCGACGGGCCGCTGACCGC *****	3240 2265
<b>Bm1065</b> →		
Bm1065-67 clone4	GGCCGGTGTATGGTAAGAGAAGCTCAAAATCGCAAAAATTTGATGAAAAATCCGGGAAAT GGCCGGTGTATGGTAAGAGAAGCTCAAAATCGCAAAAATTTGATGAAAAATCCGGGAAAT *****	3300 2325
Bm1065-67 clone4	GTTGCATGACACCAGGATCGTTGTCCGATATGATCGACACGACTGTCCGGCTGCACGTG GTTGCATGACACCAGGATCGTTGTCCGATATGATCGACACGACTGTCCGGCTGCACGTG *****	3360 2385
Bm1065-67 clone4	AACATTGCCGGACGATGACCCACAGGCACGCCGCGACGCCACGCGCCCTTGCGCAC AACATTGCCGGACGATGACCCACAGGCACGCCGCGACGCCACGCGCCCTTGCGCAC *****	3420 2445
Bm1065-67 clone4	CGTCCCCACGCGGGAGTAGTCTGCATGCTTTACCAACTGCACGAATTCAGCGGGCGAT CGTCCCCACGCGGGAGTAGTCTGCATGCTTTACCAACTGCACGAATTCAGCGGGCGAT *****	3480 2505
Bm1065-67 clone4	GCTGAGCCCGCTCACGGCCTGGGCCAGGCCGCTCGAAGTCTTCGCCAATCCGTCGAG GCTGAGCCCGCTCACGGCCTGGGCCAGGCCGCTCGAAGTCTTCGCCAATCCGTCGAG *****	3540 2565
Bm1065-67 clone4	CCCGTTTTCGCTGATCCCCGGCGCCGCGCATGGCGGCTGCGTACGAGCTGCTGTACCG CCCGTTTTCGCTGATCCCCGGCGCCGCGCATGGCGGCTGCGTACGAGCTGCTGTACCG *****	3600 2625

Bm1065-67 clone4	<b>GCTCGGCAAGGATTACGAGAAGCCGGAGTTCAACATTCATCAGATCGTCAAGGACGGCCA</b> GCTCGGCAAGGATTACGAGAAGCCGGAGTTCAACATTCATCAGATCGTCAAGGACGGCCA *****	3660 2685
Bm1065-67 clone4	<b>CAACATTCGGATCGTTCGAGCAGACGATCGTTCGAAAAGCCGTCTGCGGGTCTGCGCTT</b> CAACATTCGGATCGTTCGAGCAGACGATCGTTCGAAAAGCCGTCTGCGGGTCTGCGCTT *****	3720 2745
Bm1065-67 clone4	<b>CAAGCGCTATTCGGACGATGCCGATGCGGTACCGCAGCTGAAGGACGAACCGGTCGTGCT</b> CAAGCGCTATTCGGACGATGCCGATGCGGTACCGCAGCTGAAGGACGAACCGGTCGTGCT *****	3780 2805
Bm1065-67 clone4	<b>GGTCTGCGCGCCGCTGTTCGGGCCACCACTCGACGCTGCTGCGCGACACCGTGCACGCT</b> GGTCTGCGCGCCGCTGTTCGGGCCACCACTCGACGCTGCTGCGCGACACCGTGCACGCT *****	3840 2865
Bm1065-67 clone4	<b>GCTGCAGGATCACAAAGGTCTACATCACCGACTGGATCGACGCGCGGATGGTGCCGGTCTGA</b> GCTGCAGGATCACAAAGGTCTACATCACCGACTGGATCGACGCGCGGATGGTGCCGGTCTGA *****	3900 2925
Bm1065-67 clone4	<b>GGTGGCCCGTTCATCTGCACGACTACGTCGAGTACATCCAGGAATTCATCCGCCACAT</b> GGTGGCCCGTTCATCTGCACGACTACGTCGAGTACATCCAGGAATTCATCCGCCACAT *****	3960 2985
Bm1065-67 clone4	<b>CGGTGCGCGCAATCTGCACGTCGTATCGGTGTGTTCAGCCGACGGTGCCCGTCTCGCGGC</b> CGGTGCGCGCAATCTGCACGTCGTATCGGTGTGTTCAGCCGACGGTGCCCGTCTCGCGGC *****	4020 3045
Bm1065-67 clone4	<b>GATCTCGCTGATGGCGAGCCGCGGCGAGGACACGCGCTCACGATGACGATGATGGGCGG</b> GATCTCGCTGATGGCGAGCCGCGGCGAGGACACGCGCTCACGATGACGATGATGGGCGG *****	4080 3105
Bm1065-67 clone4	<b>CCCGATCGACGCGCGCCGACGCCGACTTCGGTGAATCGCTCGCGACGCAGCATTCGCT</b> CCCGATCGACGCGCGCCGACGCCGACTTCGGTGAATCGCTCGCGACGCAGCATTCGCT *****	4140 3165
Bm1065-67 clone4	<b>CGCGTGGTTCGAGAACAACGTGATCCACACGGTGCCGGCGAACTATCCGGGCGAAGGCCG</b> CGCGTGGTTCGAGAACAACGTGATCCACACGGTGCCGGCGAACTATCCGGGCGAAGGCCG *****	4200 3225
Bm1065-67 clone4	<b>TCAGGTGATCCGGGCTTCCTGCAGCACACGGGCTTCGTTCGGATGAATCCGGAGCGCCA</b> TCAGGTGATCCGGGCTTCCTGCAGCACACGGGCTTCGTTCGGATGAATCCGGAGCGCCA *****	4260 3285
Bm1065-67 clone4	<b>CGCGCAATCGCACTGGGACTTCTATCAGAGCCTGCTGCGGGCGACGAGGAAGACGCCGA</b> CGCGCAATCGCACTGGGACTTCTATCAGAGCCTGCTGCGGGCGACGAGGAAGACGCCGA *****	4320 3345
Bm1065-67 clone4	<b>AGCGCACCGCGCTTCTATGACGAATACAACGCGGTGCTCGACATGGCCGCCGAGTACTA</b> AGCGCACCGCGCTTCTATGACGAATACAACGCGGTGCTCGACATGGCCGCCGAGTACTA *****	4380 3405
Bm1065-67 clone4	<b>CCTCGAGACGATCCGCGTCTGTTCAGGAATTCGCCTCGCGGAAGGCACGTGGGACGT</b> CCTCGAGACGATCCGCGTCTGTTCAGGAATTCGCCTCGCGGAAGGCACGTGGGACGT *****	4440 3465
Bm1065-67 clone4	<b>CAACGGCGAACGCGTACGCCCGCAGGACATCAAGCGCACCGCACTGATGACGATCGAAGG</b> CAACGGCGAACGCGTACGCCCGCAGGACATCAAGCGCACCGCACTGATGACGATCGAAGG *****	4500 3498
Bm1065-67 clone4	<b>CGAGCTCGACGACATCTCGGGCAGCGGCCAGACGCACGTCGCGCACGAGCTGTGCACGGG</b> -----	4560
Bm1065-67 clone4	<b>CATTCGCGAGGATCAGCGCCGAGCTTGACCGCCGAGAAGTGCGGCCATTACGGGATCTT</b> -----	4620
Bm1065-67 clone4	<b>CTCCGGCCCGCTGGCGCACGATCATCTACCCGCAACTGCGCGACTTCATCCGCGAGCA</b> -----	4680

Bm1065-67 clone4 **TCGCGCCGAGCCGAAGAACGGCAGCGCGAAGGATCGCCCGGACGCAACGGCCGGCACGCT** 4740

Bm1065-67 clone4 **CGACACGCCCGGCCGATCGGGCCCGCGCTCGCGGCCGTCCCGGCCGGCCAGGCCAGCC** 4800

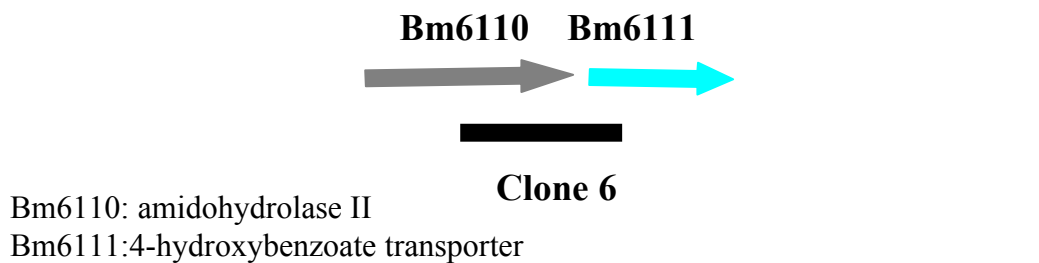
Bm1065-67 clone4 **CGAAACCGCGAAGGCCCGGCCACGCTCGCGAAACCGCGCGCGCGAAATCGTCGGGCAC** 4860

Bm1065-67 clone4 **GCTGTCCCGAAGGCCCGCACCCGGCCGCAAGCGCGGAGCGGCACACGCGCGAAGCCGGT** 4920

Bm1065-67 clone4 **TCGCGCACGCAAGGCCCGCTGA** 4942

**Figure 3-5 Blast result of clone 6 of *B. multivorans* (ATCC17616) genome library**

a. Genome organization of clone 6 of *B. multivorans* (ATCC17616). Clone 6 encoded for amidohydrolase II and 4-hydroxybenzoate transporter proteins. Both proteins were encoded partially.



b. Alignment result of clone 6 and Bm6110-11 of *B. multivorans* (ATCC17616)

**Bm6110** →

Bm6110-11 clone6 **ATGGCGAAGCGAAGCGGGTCCGCCAAGGAGACAGGAGAAACAGGATGGCAAGTGG** 60

Bm6110-11 clone6 **AAGACAATCGACATCAAGTCGTTCATCGACGACCGGCCGGTGTGCGCTACCAGTGGCTG** 120

Bm6110-11 clone6 **CTGGTTCGCGCTGTGCTGCCTCGTCGTGATCGCGGACGGGATGGACGTCGCGATCATGGG** 180

Bm6110-11 clone6 **TTCTGTCGACCGTCCGGTATTCCGCGACTGGGCGATCTCGCGCCGGAATTCGGGCTCGT** 240

Bm6110-11 clone6 **ATGAGCGCGGCCCGATCGGTCTCGTGATCGGTGCGCTCGTCGCGCCCGAGCGCGGAC** 300

Bm6110-11 clone6 **CGGTTCCGGGCGCAAGCGGTGCTGATCGCGTCCGGTGTGCTGTTCCGGCTCTTCACGATC** 360

Bm6110-11 clone6 GCGACCGCGTATGCGGGCTCGCCGGTTCAGATGGCCGTGCTGCGGCTGCTGACCGGCATC 420  
-----

Bm6110-11 clone6 GGGCTCGGCGCCGCGATGCCGAACACGACGACGCTGCTGTGCGGAGTATGCGCCGACGCG 480  
-----

Bm6110-11 clone6 ATGCGGTCGCTGATGATCAGATCATGTTACGGGCTTCAATCTCGGCTCGGCGCTGATC 540  
-----

Bm6110-11 clone6 GGCTTCGTCGCCGGCTGGCTGATTCGCTGCACGGCTGGCGCGGTTACTGCTGTTCGGC 600  
-----

Bm6110-11 clone6 GCGCGCTGCCGCTGCTGCTGATTCGCTGCAACTGTGGCTGCTGCCGAATCGGCGCG 660  
-----

Bm6110-11 clone6 CTGCTCGCCGTGCGCGGTGCGCCGTCGACGCGCATCGGCGCGTTGCTGAACCGCTCTGC 720  
-----

Bm6110-11 clone6 GCGCACGCTTCGACGGAACGAGACGTTCTGCTCGAACGAGCCGCGCTGCCGACGCGC 780  
-----

Bm6110-11 clone6 AAGCCGATCGGCGTGCTGTTCTCGCAAGGCTACGGCGTCGTCACGGTATCGCTGTGGATC 840  
-----

Bm6110-11 clone6 ACGTACTTCATGGGACTGCTCGTGATCTATCTGCTGACCGGCTGGCTGCCGACACTGATC 900  
-----

Bm6110-11 clone6 AAGGATGCGGGCCTGTGCGTACGACGCGCGAACGTGACGGCGATGTTCCAGATCGGC 960  
-----

Bm6110-11 clone6 GGGACGATCGGCGCGATCGGCGTCGGCTGGCTGATGGACAAGGTGCGGCCGACCCCGTG 1020  
-----

Bm6110-11 clone6 ATCGGCGCCGCTATCTCGGTGGCGCACTGTGCGTGGCCGTGCTCGCGTGGCCGGCGCG 1080  
-----

Bm6110-11 clone6 CTGTCGTCGTCGCTCGCGCTGCTCGTGTGTTGCGGCGGGCTTCTGCATGAGCGCGCGCAG 1140  
-----

Bm6110-11 clone6 ACGGGGCTGAACGCGTATGCGCCGGGCGCTACCCGACCGTTGCGCGCGCAACCGGCGTG 1200  
-----

Bm6110-11 clone6 AGCTGGATGCTCGGCATGGGCCGCTTCGGCAGCATTTCGGTTCGGCGATCGGCGGCGCG 1260  
-----GCATGGGCCGCTTCGGCAGCATTTCGGTTCGGCGATCGGCGGCGCG 47  
\*\*\*\*\*

Bm6110-11 clone6 CTGCTCGGCCTCGGCTGGAAGTTCGACGCGATCCTGTCGATGCTCGCGGTGCCCGCCACG 1320  
CTGCTCGGCCTCGGCTGGAAGTTCGACGCGATCCTGTCGATGCTCGCGGTGCCCGCCACG 107  
\*\*\*\*\*

Bm6110-11 clone6 CTGCGGCGATCGCGATCGTACGACGCGAGCGCGCGCCGACCTGAACCGAAGGAAATG 1380  
CTGCGGCGATCGCGATCGTACGACGCGAGCGCGCGCCGACCTGAACCGAAGGAAATG 167  
\*\*\*\*\*

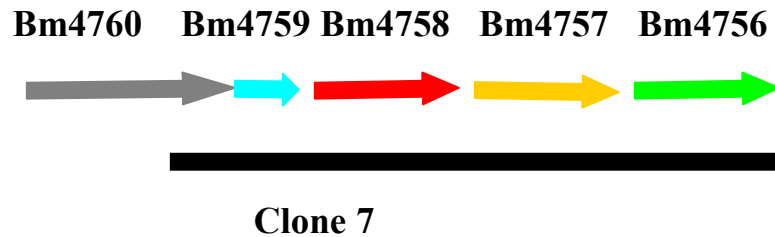
Bm6110-11 clone6 GAAAAACCGGCGCACTGACCGGCTCCGCACGATCGCGCGTCCCGGTCGCGGGCCGGCGC 1440  
GAAAAACCGGCGCACTGACCGGCTCCGCACGATCGCGCGTCCCGGTCGCGGGCCGGCGC 227  
\*\*\*\*\*

**Bm6111**

Bm6110-11 clone6	CGACACGCGGCATCCGGCCAGCACGTCATGCAACAGGAGTGTCAATGATCATCGATATTC CGACACGCGGCATCCGGCCAGCACGTCATGCAACAGGAGTGTCAATGATCATCGATATTC *****	1500 287
Bm6110-11 clone6	ACGGCCACTACACGACCGCGCCGAAGGCGCTCGAGACCTGGCGCAATCGCCAGATCGCGG ACGGCCACTACACGACCGCGCCGAAGGCGCTCGAGACCTGGCGCAATCGCCAGATCGCGG *****	1560 347
Bm6110-11 clone6	CGATTACAGCCCGTCCGAGCGGCCGAAGGTCGCGGAACGAACATCAGCGACGACGAGC CGATTACAGCCCGTCCGAGCGGCCGAAGGTCGCGGAACGAACATCAGCGACGACGAGC *****	1620 407
Bm6110-11 clone6	TGCGCGAGTCGCTCGAGCCCAACAGTTGCGGCTGATGCGCGAGCGCGGCCCTCGACCTGA TGCGCGAGTCGCTCGAGCCCAACAGTTGCGGCTGATGCGCGAGCGCGGCCCTCGACCTGA *****	1680 467
Bm6110-11 clone6	CGATCTTCAGCCCGCGCGGAGCTTCATGGCACACCACATCGGGACTTCGACGTGTCTGA CGATCTTCAGCCCGCGCGGAGCTTCATGGCACACCACATCGGGACTTCGACGTGTCTGA *****	1740 527
Bm6110-11 clone6	GCACCTGGGCCCGCGTCTGCAACGAGCTGTGCTACCGCGTGCACCGGCTGTACCCCGACC GCACCTGGGCCCGCGTCTGCAACGAGCTGTGCTACCGCGTGCACCGGCTGTACCCCGACC *****	1800 587
Bm6110-11 clone6	GCTTCGTGCCGCGGCGATGCTGCCGCAAAGCCCCGCGCTCGACGTGGCGACCTGCATTC GCTTCGTGCCGCGGCGATGCTGCCGCAAAGCCCCGCGCTCGACGTGGCGACCTGCATTC *****	1860 647
Bm6110-11 clone6	CGGAGCTCGTCCGGTTCGCTCGAGGCGTACGGCAACGTCGCGGTCAACCTGAATCCCGATC CGGAGCTCGTCCGGTTCGCTCGAGGCGTACG----- *****	1920 677
Bm6110-11 clone6	CGTCCGGCGGCCACTGGACGAGCCCGCGCTGTCGGACCGCTACTGGTATCCGCTGTACG -----	1980
Bm6110-11 clone6	AGAAGATGGTTCGAGTACGACATCCCCGCGATGATCCACGTGAGCACGAGCTGCAACGCGT -----	2040
Bm6110-11 clone6	GCTTTCACACGACCGGCGCGCACTACCTGAACCGGGACACGACCGCGTTCATGCAGTGCC -----	2100
Bm6110-11 clone6	TGACGTCCGATCTGTTCAAGGACTTTCGACGCTGCGCTTCGTGATTCGCGACGGCGGG -----	2160
Bm6110-11 clone6	GCGCGGTGCCGATCACTGGGGCGCTTTCGCGGCCCTCGCACAGGAACGAAGAAGCCGC -----	2220
Bm6110-11 clone6	TCCTGACCGAGCATCTACTCAACAACGTGTTCTTCGATACGTGCGTCTATCACCAGCCGG -----	2280
Bm6110-11 clone6	GCATCGATCTGCTGACCGCGTGATTCGGTTCGACAACATCTGTTTCGCGAGCGAGATGA -----	2340
Bm6110-11 clone6	TCGGCGCGGTTTCGCGGCATCGATCCGAGACCGGGCACTACTACGACGACACGAAGCGCT -----	2400
Bm6110-11 clone6	ACATCGAAGCGTCGGCACTCGACGCGCAGGCGGCTACAAGATTACGAAGCAATGCGC -----	2460
Bm6110-11 clone6	GCCGCGTGTATCCGCGCTGGATGCGCAACTGAAAGCGAAGGGGAAGTGA -----	2510

**Figure 3-6 Blast result of clone 7 of *B. multivorans* (ATCC17616) genome library**

a. Genomic organization of clone 7 of *B. multivorans* (ATCC17616). Clone 7 encoded for 3-isopropylmalate dehydratase large subunit, entericidinAB, 3-isopropylmalate dehydratase small subunit, 3-isopropylmalate dehydrogenase, and Aspartate-semialdehyde dehydrogenase proteins. 3-isopropylmalate dehydratase large subunit (Bm 4760) was partially encoded. Remainder genes were encoded as full length proteins.



Bm4756: Aspartate-semialdehyde dehydrogenase  
 Bm4757: 3-isopropylmalate dehydrogenase  
 Bm4758: 3-isopropylmalate dehydratase small subunit  
 Bm4759: entericidinAB  
 Bm4760: 3-isopropylmalate dehydratase large subunit

b. Alignment of genome 7 and Bm4756-60 of *B. multivorans* (ATCC17616)

	<b>Bm4760</b> →	
Bm4756-60 clone7	TTGATGTCACAACGACCCCGCATACCCATGGCACAGACTCTCTACGACAAACTGTGGAAT	60
Bm4756-60 clone7	TCCCACGTCGTCCACACCGAGGAAGACGGCACGGCATTGCTGTATATCGACCGTCAACTG	120
Bm4756-60 clone7	CTGCATGAAGTCACGAGCCCGCAGGCGTTCGAAGGGCTGAAGATCGCGCAGCGTCCGGTG	180
Bm4756-60 clone7	TGGCGCATCAGCGCGAACCTGGCCGTGTCGACCACAACGTGCCGACCACCGATCGCAGC	240
Bm4756-60 clone7	CACGGCATCGCCGATCCCGTCTCGAAGCTGCAGGTCGACACGCTCGACGCGAACTGCGAT	300
Bm4756-60 clone7	GCGTTCGGCATCACGCAGTTCAAGATGAACGACGTGCGTCAGGGCATCGTGACATCATC	360
Bm4756-60 clone7	GGCCCGAGCAGGGCGGACGCTGCCGGGCATGACGATCGTGTGCGGCGATTTCGCACACG	420

Bm4756-60 clone7 TCGACGCACGGCGCGTTTCGGCGCGCTCGCGCACGGCATCGGCACGTGCGAAGTCGAGCAC 480  
-----

Bm4756-60 clone7 GTGCTCGCGACGCAGACTTTGCTGCAGAAGAAGCAAGAACATGCTCGTGAAGGTCGAG 540  
-----

Bm4756-60 clone7 GCGCACTGCCGCGCGGCTGCACCCGAAGGACATCGTGCTCGGATCATCGGCAAGATC 600  
-----

Bm4756-60 clone7 GGCACGGCAGGCGGCACCGGCTATGCGATCGAATTCGGCGGCTCGACGATCCGCGCGCTG 660  
-----

Bm4756-60 clone7 TCGATGGAAGCCGGATGACGGTCTGCAACATGGCGATCGAAGCCGGCGCGCGCGGGC 720  
-----

Bm4756-60 clone7 ATGGTCCCGTCGACGACACGACGATCGACTACCTGAAGGGCCGTCGGTTTCGTGCCGACC 780  
-----

Bm4756-60 clone7 GCGCGGAATGGGATCAGGCCGTCGAATACTGGCGCCAGTTCAAGTCGGACGAAGCGCG 840  
-----

Bm4756-60 clone7 CATTTGATCGCGTCGTCGAGCTGAACCGGGCCGAGATCGTCCCGAGGTCACGTGGGGC 900  
-----

Bm4756-60 clone7 ACGTCGCCGAAATGGTACGTCGATCGACGGTCGCGTGCCCGATCCCGAGCGGAGAA 960  
-----

Bm4756-60 clone7 GATCCGGTCAAGCGCGACGCGATGGAGCGCGCTCGCCTACATGGCGCTCGAACCGAAC 1020  
-----

Bm4756-60 clone7 ACGCCGATCGAATCGATCAAGGTCGACAAGATCTTCATCGGCTCGTGACGAAACGCGCG 1080  
-----  
-----ATCGAATCGATCAAGGTCGACAAGATCTTCATCGGCTCGTGACGAAACGCGCGC 54  
\*\*\*\*\*

Bm4756-60 clone7 ATCGAGGACATCCGCGCGCCGCTACGTCGTGAAGAAGCTGAACCGTCGCGTCGCTCG 1140  
ATCGAGGACATCCGCGCGCCGCTACGTCGTGAAGAAGCTGAACCGTCGCGTCGCTCG 114  
\*\*\*\*\*

Bm4756-60 clone7 AACGTGCGGCTCGCGATGGTCTGCGGGCTCGGGCTCGTGAAGGCGCAGGCCGAGCGC 1200  
AACGTGCGGCTCGCGATGGTCTGCGGGCTCGGGCTCGTGAAGGCGCAGGCCGAGCGC 174  
\*\*\*\*\*

Bm4756-60 clone7 GAAGGCCTCGACAAGGTGTTTACCGATGCGGGCTTCGAATGGCGCGAGCCGGGCTGCTCG 1260  
GAAGGCCTCGACAAGGTGTTTACCGATGCGGGCTTCGAATGGCGCGAGCCGGGCTGCTCG 234  
\*\*\*\*\*

Bm4756-60 clone7 ATGTGTCTCGCGATGAACGCCGACCGCTCGAGCCGGCGAGCGCTGCGCCTCGACGTCG 1320  
ATGTGTCTCGCGATGAACGCCGACCGCTCGAGCCGGCGAGCGCTGCGCCTCGACGTCG 294  
\*\*\*\*\*

Bm4756-60 clone7 AACCGCAACTTCGAAGCCGGCAGGGCGGGCGGGCCGACGCACCTCGTCAGCCCCGCG 1380  
AACCGCAACTTCGAAGCCGGCAGGGCGGGCGGGCCGACGCACCTCGTCAGCCCCGCG 354  
\*\*\*\*\*

Bm4756-60 clone7 ATGGCGCGGGCCGCGCGATCGAAGGCCATTTTCGTGACATTCGCAAGCTGGGGTAAACGC 1440  
ATGGCGCGGGCCGCGCGATCGAAGGCCATTTTCGTGACATTCGCAAGCTGGGGTAAACGC 414  
\*\*\*\*\*

**Bm4759** →

Bm4756-60 clone7 GTGACGGCATCGAAATGGGTTGCGCGGCTCGCCGCGGCTGGCGCTCGCGGGGCTCGGC 1500  
GTGACGGCATCGAAATGGGTTGCGCGGCTCGCCGCGGCTGGCGCTCGCGGGGCTCGGC 474  
\*\*\*\*\*



Bm4756-60 clone7 **TTCCGGTCTCGCGGGCTGCAACACCGTCCGAGGCTTCGGCGAGGACGTCAATGCCGCCGGC** 1560  
**TTCCGGTCTCGCGGGCTGCAACACCGTCCGAGGCTTCGGCGAGGACGTCAATGCCGCCGGC** 534  
\*\*\*\*\*

Bm4756-60 clone7 **AGCGCACTGAAGCGCGCCGCGGAGTGA**CCCCGCCGAGAATTGTTCGATGTGCGCCGGCCG 1620  
**AGCGCACTGAAGCGCGCCGCGGAGTGA**CCCCGCCGAGAATTGTTCGATGTGCGCCGGCCG 594  
\*\*\*\*\*

**Bm4758** →

Bm4756-60 clone7 CCGGCCGGCCCTTCAACCGGATAGACGGATC**ATGGAAAAATTCACAGTGCATACCGGCGT** 1680  
**CGGCCGGCCCTTCAACCGGATAGACGGATC**ATGGAAAAATTCACAGTGCATACCGGCGT 654  
\*\*\*\*\*

Bm4756-60 clone7 **CGTGGCGCCGCTCGATCGCGAGAACGTTCGACACCGACGCGATCATTCCGAAGCAGTTCCCT** 1740  
**CGTGGCGCCGCTCGATCGCGAGAACGTTCGACACCGACGCGATCATTCCGAAGCAGTTCCCT** 714  
\*\*\*\*\*

Bm4756-60 clone7 **GAAATCGATCAAGCGCACGGGCTTCGGTCCGAACGCGTTCGACGAGTGGCGCTATCTTGA** 1800  
**GAAATCGATCAAGCGCACGGGCTTCGGTCCGAACGCGTTCGACGAGTGGCGCTATCTTGA** 774  
\*\*\*\*\*

Bm4756-60 clone7 **CCACGGCGAGCCGGGCCAGGACAACCTCGAAGCGTCCGCTGAACCCGGACTTCGTGCTGAA** 1860  
**CCACGGCGAGCCGGGCCAGGACAACCTCGAAGCGTCCGCTGAACCCGGACTTCGTGCTGAA** 834  
\*\*\*\*\*

Bm4756-60 clone7 **CCAGCCGCGCTACCAGGGCGCGTTCGATTTCTCGTCACGCGCAAGAACTTCGGTGC** 1920  
**CCAGCCGCGCTACCAGGGCGCGTTCGATTTCTCGTCACGCGCAAGAACTTCGGTGC** 894  
\*\*\*\*\*

Bm4756-60 clone7 **CTCGCGCGAGCACGCGCCGTGGCGCTGCAGCAGTACGGCTTCCGCGGATCATCGCGCC** 1980  
**CTCGCGCGAGCACGCGCCGTGGCGCTGCAGCAGTACGGCTTCCGCGGATCATCGCGCC** 954  
\*\*\*\*\*

Bm4756-60 clone7 **GAGCTTCGCGGACATCTTCTTCAACAACCTGCTTCAAGAACGGGCTGCTGCCGATCGTGCT** 2040  
**GAGCTTCGCGGACATCTTCTTCAACAACCTGCTTCAAGAACGGGCTGCTGCCGATCGTGCT** 1014  
\*\*\*\*\*

Bm4756-60 clone7 **GACCGAGCAGCAGGTCGATCATCTGATCAACGAGACGGTTCGCGTTCAACGGCTATCAGT** 2100  
**GACCGAGCAGCAGGTCGATCATCTGATCAACGAGACGGTTCGCGTTCAACGGCTATCAGT** 1074  
\*\*\*\*\*

Bm4756-60 clone7 **GACGATCGATCTCGAGGCGCAGGTCGTGCGCACGCCGACGCGCCGACTATCCGTTCTGA** 2160  
**GACGATCGATCTCGAGGCGCAGGTCGTGCGCACGCCGACGCGCCGACTATCCGTTCTGA** 1134  
\*\*\*\*\*

Bm4756-60 clone7 **GATCACCGCGTTCCGCAAGTACTGCCGTGTAACGGTTTCGACGACATCGGCCCTCACGCT** 2220  
**GATCACCGCGTTCCGCAAGTACTGCCGTGTAACGGTTTCGACGACATCGGCCCTCACGCT** 1194  
\*\*\*\*\*

Bm4756-60 clone7 **GCGTCACGCGGACAAGATCCGCCAGTTCGAAGCGGAGCGGCTCGCGAAGCAGCCGTGGCT** 2280  
**GCGTCACGCGGACAAGATCCGCCAGTTCGAAGCGGAGCGGCTCGCGAAGCAGCCGTGGCT** 1254  
\*\*\*\*\*

Bm4756-60 clone7 **CAACAACAAGCTGGTTCGGCTGA**AGTCGTCTTCGGGCGGGCGGCACGCCGCGGTTTCGC 2340  
**CAACAACAAGCTGGTTCGGCTGA**AGTCGTCTTCGGGCGGGCGGCACGCCGCGGTTTCGC 1314  
\*\*\*\*\*

**Bm4757** →

Bm4756-60 clone7 CCGCCAGCCATCACCCCTTATTCAGTCAGGAACAACGC**ATGAAGATTGCAGTGTGCGCGG** 2400  
**CCGCCAGCCATCACCCCTTATTCAGTCAGGAACAACGC**ATGAAGATTGCAGTGTGCGCGG 1374  
\*\*\*\*\*

Bm4756-60 clone7 **GCGACGGCATCGGTCCGAAATCGTCAACCGAAGCGGTGAAGGTGCTGAACGCACTCGACG** 2460  
**GCGACGGCATCGGTCCGAAATCGTCAACCGAAGCGGTGAAGGTGCTGAACGCACTCGACG** 1434  
\*\*\*\*\*

Bm4756-60 clone7 **AGAAGTTCGAACTCGAACAGGCGCCGGTTCGGCGGCGCGGGCTACGAGGCGAGGGGTATC** 2520  
**AGAAGTTCGAACTCGAACAGGCGCCGGTTCGGCGGCGCGGGCTACGAGGCGAGGGGTATC** 1494  
\*\*\*\*\*

Bm4756-60 clone7 **CGCTGCCGGACGCGACGCTGAAGCTCGCGAAGGAAGCCGACGCGATCCTGTTCCGGCGCGG** 2580  
**CGCTGCCGGACGCGACGCTGAAGCTCGCGAAGGAAGCCGACGCGATCCTGTTCCGGCGCGG** 1554

```

*****
Bm4756-60 clone7 TCGGCGACTGGAAGTACGACTCGTTCGAAACGCGCGCTGCGTCCCAGCAGGCGATTCTCG 2640
TCGGCGACTGGAAGTACGACTCGTTCGAAACGCGCGCTGCGTCCCAGCAGGCGATTCTCG 1614
*****

Bm4756-60 clone7 GCCTGCGCAAGCACCTCGAGCTGTTTCGCGAACTTCCGTCCGGCGATCTGCTATCCGCAGC 2700
GCCTGCGCAAGCACCTCGAGCTGTTTCGCGAACTTCCGTCCGGCGATCTGCTATCCGCAGC 1674
*****

Bm4756-60 clone7 TCGTTCGACGCGTTCGCCGCTGAAGCCCGAACTTGTTCGCGGGCCTCGACATCCTGATCGTGC 2760
TCGTTCGACGCGTTCGCCGCTGAAGCCCGAACTTGTTCGCGGGCCTCGACATCCTGATCGTGC 1734
*****

Bm4756-60 clone7 GCGAACTGAACGGCGACATCTATTTCGGCCAGCCGCGCGGCTGCGCGCGGACCCGAGC 2820
GCGAACTGAACGGCGACATCTATTTCGGCCAGCCGCGCGGCTGCGCGCGGACCCGAGC 1794
*****

Bm4756-60 clone7 GCCCGTTTCGCGGGCAGCGCGAAGGCTTCGACACGATGCGCTATTTCGGAGCCGGAAGTGC 2880
GCCCGTTTCGCGGGCAGCGCGAAGGCTTCGACACGATGCGCTATTTCGGAGCCGGAAGTGC 1854
*****

Bm4756-60 clone7 GCCGCATCGCGCACGTCGCGTTCAGGCCGCGCAAAAGCGCGCAAGAAGCTGCTGTTCGG 2940
GCCGCATCGCGCACGTCGCGTTCAGGCCGCGCAAAAGCGCGCAAGAAGCTGCTGTTCGG 1914
*****

Bm4756-60 clone7 TCGACAAGTCGAACGTCGTCGAGACGTCGCAGTTCGGCGGACATCATGATCGACGTGT 3000
TCGACAAGTCGAACGTCGTCGAGACGTCGCAGTTCGGCGGACATCATGATCGACGTGT 1974
*****

Bm4756-60 clone7 CGAAGGAATATGCGGACGTCGAGCTGTCGCACATGTACGTCGACAACGCGGCGATGCAGC 3060
CGAAGGAATATGCGGACGTCGAGCTGTCGCACATGTACGTCGACAACGCGGCGATGCAGC 2034
*****

Bm4756-60 clone7 TCGCGAAGGCGCGAAGCAGTTCGACGTGATCGTGACCGGCAACATGTTTCGGCGACATCC 3120
TCGCGAAGGCGCGAAGCAGTTCGACGTGATCGTGACCGGCAACATGTTTCGGCGACATCC 2094
*****

Bm4756-60 clone7 TGTCCGATGAAGCGTCGATGTCGACGGGCTCGATCGGCATGCTGCCGTCGGCGTCGCTCG 3180
TGTCCGATGAAGCGTCGATGTCGACGGGCTCGATCGGCATGCTGCCGTCGGCGTCGCTCG 2154
*****

Bm4756-60 clone7 ACAAGAACAACAAGGGTCTGTACGAGCCGTCGCACGGTTCGGCGCCGGACATCGCGGGCA 3240
ACAAGAACAACAAGGGTCTGTACGAGCCGTCGCACGGTTCGGCGCCGGACATCGCGGGCA 2214
*****

Bm4756-60 clone7 AGGGCATCGCGAATCCGCTCGCGACGATCCTGTCGGCCGCGATGCTGCTGCGCTATTTCGC 3300
AGGGCATCGCGAATCCGCTCGCGACGATCCTGTCGGCCGCGATGCTGCTGCGCTATTTCGC 2274
*****

Bm4756-60 clone7 TGAACCGCGCCGAGCAGGCCGACCGCATCGAGCGCGCGGTCAAACGGTGTTCGAAACAGG 3360
TGAACCGCGCCGAGCAGGCCGACCGCATCGAGCGCGCGGTCAAACGGTGTTCGAAACAGG 2334
*****

Bm4756-60 clone7 GCTACCGCACCGGCGACATCGCGACGCCGGGCTGCAAGCAGGTCGGCACGGCGGCGATGG 3420
GCTACCGCACCGGCGACATCGCGACGCCGGGCTGCAAGCAGGTCGGCACGGCGGCGATGG 2394
*****

Bm4756-60 clone7 GCGACGCGGTGGTTCGGGCCCTCTGAGCGCCGCAAGTCACGATGTAGAAAGGGCGCGA 3480
GCGACGCGGTGGTTCGGGCCCTCTGAGCGCCGCAAGTCACGATGTAGAAAGGGCGCGA 2454
*****

Bm4756-60 clone7 ACGGGCCGAAAAAATCGGTTTCGTTCCGCGACGGTTGGCCGTTGTGCGGGCAACCGGCTT 3540
ACGGGCCGAAAAAATCGGTTTCGTTCCGCGACGGTTGGCCGTTGTGCGGGCAACCGGCTT 2514
*****

Bm4756-60 clone7 TGTGTAGACTCGAACGATGGCGCTGATTTCCCTGATCTCCCGGTCCTGAAACTCCACGG 3600
TGTGTAGACTCGAACGATGGCGCTGATTTCCCTGATCTCCCGGTCCTGAAACTCCACGG 2574
*****

Bm4756-60 clone7 CAACACTGCCCGTGCGGGTGCGCGCGCCATCGTCATCACGAAAACCATACCACGAAAAC 3660
CAACACTGCCCGTGCGGGTGCGCGCGCCATCGTCATCACGAAAACCATACCACGAAAAC 2634
*****

```

Bm4756-60 clone7	GATCATTA AACGCTGATCGTCCGTGCTGCCCGCTGTTCCCGCGCGGTCACGCCGGG GATCATTA AACGCTGATCGTCCGTGCTGCCCGCTGTTCCCGCGCGGTCACGCCGGG *****	3720 2694
	<b>Bm4756</b> →	
Bm4756-60 clone7	GCGAAATGGCGGGAAGCTCCATTCAAAGGTTAGTC <b>ATGAACGTAGGTCCTCGTAGGTT</b> GCGAAATGGCGGGAAGCTCCATTCAAAGGTTAGTCATGAACGTAGGTCCTCGTAGGTT *****	3780 2754
Bm4756-60 clone7	<b>GGCGCGCATGGTCGGCAGCGTCTGATGCAGCGCATGCAGGAAGAGGGCGATTTCGACC</b> GGCGCGCATGGTCGGCAGCGTCTGATGCAGCGCATGCAGGAAGAGGGCGATTTCGACC *****	3840 2814
Bm4756-60 clone7	<b>TGATCGAACCGGTGTTTTCAGCACCAGCAACTCGGGCGGCAAGGCACCGTCTGTCGGA</b> TGATCGAACCGGTGTTTTCAGCACCAGCAACTCGGGCGGCAAGGCACCGTCTGTCGGA *****	3900 2874
Bm4756-60 clone7	<b>AAAACGAGACCACGCTGAAGGACGCGACCAACGTCGACGAACTGAAGAAGTGCACGTCA</b> AAAACGAGACCACGCTGAAGGACGCGACCAACGTCGACGAACTGAAGAAGTGCACGTCA *****	3960 2934
Bm4756-60 clone7	<b>TCATCAGTGCAGGGCGGCGACTACACGAATGACGTGTTCCCGAAGCTGCGCGCGGGC</b> TCATCAGTGCAGGGCGGCGACTACACGAATGACGTGTTCCCGAAGCTGCGCGCGGGC *****	4020 2994
Bm4756-60 clone7	<b>GCTGGAACGGCTACTGGATCGATGCGGCGTCTGCTGCGGATGAAGGACGACGCGGTCA</b> GCTGGAACGGCTACTGGATCGATGCGGCGTCTGCTGCGGATGAAGGACGACGCGGTCA *****	4080 3054
Bm4756-60 clone7	<b>TCATCCTCGATCCGGTGAACCTCGACGTCATCAAGGACGCGCTCGTCAAGGGCACGAAGA</b> TCATCCTCGATCCGGTGAACCTCGACGTCATCAAGGACGCGCTCGTCAAGGGCACGAAGA *****	4140 3114
Bm4756-60 clone7	<b>ACTTCATCGGCGGCAACTGCACGGTCAGCCTGATGCTGATGGCGCTCGGCGGCTGTTC</b> ACTTCATCGGCGGCAACTGCACGGTCAGCCTGATGCTGATGGCGCTCGGCGGCTGTTC *****	4200 3174
Bm4756-60 clone7	<b>GCGAGAACCTCGTCGATTGGATGACGGCCATGACGTATCAGGCCGCTCGGGCGCGGGC</b> GCGAGAACCTCGTCGATTGGATGACGGCCATGACGTATCAGGCCGCTCGGGCGCGGGC *****	4260 3234
Bm4756-60 clone7	<b>CGCAGAACATGCGTGAAGTGTGTCGAGATGGGCGCGCTGCACGGCGGTCAGCAGC</b> CGCAGAACATGCGTGAAGTGTGTCGAGATGGGCGCGCTGCACGGCGGTCAGCAGC *****	4320 3294
Bm4756-60 clone7	<b>AGCTCGCCGATCCGGCATCGGCGATCTCGACATCGATCGCCGCGTCTCGCCACGATGA</b> AGCTCGCCGATCCGGCATCGGCGATCTCGACATCGATCGCCGCGTCTCGCCACGATGA *****	4380 3354
Bm4756-60 clone7	<b>ACAGCGACGCGATGCCGACGAGCCACTTCGGCGTGCCTCGCGGTTTCGCTGATTCGGT</b> ACAGCGACGCGATGCCGACGAGCCACTTCGGCGTGCCTCGCGGTTTCGCTGATTCGGT *****	4440 3414
Bm4756-60 clone7	<b>GGATCGACAAGGATCTCGGCAACGGCATGTGCAAGGAAGAATGGAAGGGCGGCGCGGAA</b> GGATCGACAAGGATCTCGGCAACGGCATGTGCAAGGAAGAATGGAAGGGCGGCGCGGAA *****	4500 3474
Bm4756-60 clone7	<b>CGAACAAAGATCCTCGGCAAGCCGGCGATGGGCGAGCCGGGCTCGATTCCGGTCGACGGC</b> CGAACAAAGATCCTCGGCAAGCCGGCGATGGGCGAGCCGGGCTCGATTCCGGTCGACGGC *****	4560 3534
Bm4756-60 clone7	<b>TGTGCGTGGGATCGGCGCGATGCGCTGCCACTCGCAGGCGTGCATCAAGCTGAAGA</b> TGTGCGTGGGATCGGCGCGATGCGCTGCCACTCGCAGGCGTGCATCAAGCTGAAGA *****	4620 3594
Bm4756-60 clone7	<b>AGGACGTGCCGCTCGACGAGATCAACGGCATTCTCGCGTCCGCGAACGACTGGTGAAGG</b> AGGACGTGCCGCTCGACGAGATCAACGGCATTCTCGCGTCCGCGAACGACTGGTGAAGG *****	4680 3654
Bm4756-60 clone7	<b>TCGTGCCGAACGAGCGGCAAGCGTCGATGCACGATCTGTGCGCGGCAAGGTCACCGGCA</b> TCGTGCCGAACGAGCGGCAAGCGTCGATGCACGATCTGTGCGCGGCAAGGTCACCGGCA *****	4740 3714

```

*****
Bm4756-60  CGCTGACCGTGCCGGTCGGCCGCTGCGCAAGCTCGCGATGGGCGGCGAATACCTGTCGG 4800
clone7      CGCTGACCGTGCCGGTCGGCCGCTGCGCAAGCTCGCGATGGGCGGCGAATACCTGTCGG 3774
*****

Bm4756-60  CGTTCACGGTCGGCGATCAGTTGCTGTGGGGCGCGGCCGAACCGCTGCGCCGATGCTGC 4860
clone7      CGTTCACGGTCGGCGATCAGTTGCTGTGGGGCGCGGCCGAACCGCTGCGCCGATGCTGC 3834
*****

Bm4756-60  GCATCCTGCTCGACAAGTAAAGACGAGATCGTGCGCCGGCCGGCGCGCGATGCGACGCGCC 4920
clone7      GCATCCTGCTCGACAAGTAAAGACGAGATCGTGCGCCGGCCGGCGCGCGATGCGACGCGCC 3894
*****

Bm4756-60  TCGGTGCCTTCAACGGGCCGAGGCGCGTTTTTTATCGGCGGGCGCGTGCCTGCGGAAAATG 4980
clone7      TCGGTGCCTTCAACGGGCCGAGGCGCGTTTTTTATCGGCGGGCGCGTGCCTGCGGAAAATG 3954
*****

Bm4756-60  CGAAAGCCCCGAACCGCTCTCCCGCCGGGGCTTTCTTGCATCGATTCTTTGGCAGAG 5040
clone7      CG----- 3956
**

Bm4756-60  GAAGTGACGCGGTGCGCAGTATAAGGCACGCAGTTCGTGCGCATGTTGATAATGTTGTCG 5100
clone7      -----

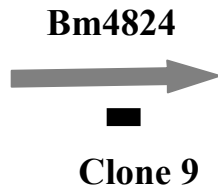
Bm4756-60  GGATTTACATCAATTGATGCTCGATTTTTGATAATGGCGTGCGGCGCGCAGTGGCGCATC 5160
clone7      -----

Bm4756-60  GGCCGCCTGTTCCCGTGTGCGCGCCGACGAATTCGGCCCCAACGCGATCGTGTGA 5217
clone7      -----

```

**Figure 3-7 Blast result of clone 9 of *B. multivorans* (ATCC17616) genome library**

- a. Genome organization of the clone 9 of *B. multivorans* (ATCC17616). Clone 9 encoded for GGDEF domain. This domain was partially encoded in clone 9.



Bm4824: GGDEF domain

- b. Alignment of the clone 9 and Bm4824 of *B. multivorans* (ATCC17616)

```

                Bm4824 ←-----
Bm4825  TCAGGCAGTCGGTTCGAATTGCGTGACCTTGTGCGTCCCCTCGCTTTCGCGTAATAAAG 60
clone9  -----

Bm4825  CGCTTCGTCGGCAGCCTTGATGATCGCGTCGGGTTGACGTCCTGATCGGGTTTCCAGCT 120
clone9  -----

Bm4825  CGCCAGGCCGATGCTCGCGGTGACGCGTCCGAACGCTGCGCACATGTTTCGAGGCCAG 180
clone9  -----

```

Bm4825 clone9 TTCGTTGATGGCGATTTCGAATCCGTTTCGGCAATCTGCGCGGGCCGTTCTCCGCCGTATC 240  
-----

Bm4825 clone9 GGGCAGCAGGACGACGAACTCTTCGCCCGCTAGCGGGCGGCGACGTCCCGGGCCGGCG 300  
-----

Bm4825 clone9 AATGTTCTCGCCGATGCAGCGCGGACTGCAGCGAGCGCATCGTCCGGCCTGGTGGCC 360  
-----

Bm4825 clone9 GTACGTGTCGTTGTAGGCCTTGAAGCGGTCGACGTCGACGAAGAGCAGCGAAAAGACCGA 420  
-----

Bm4825 clone9 TCGTACCCGGCGTGC GCGCGCCATTCGCGATCGAGCACCTCGCCGAAGCTGCGGCGGTT 480  
-----

Bm4825 clone9 GTTGAGACCCGTGAGCCCGTCGGTGC GCGCGGAGCAGGATCAGCTCCGATTCCGGCTCGCAT 540  
-----

Bm4825 clone9 CCTGCGCCGAGCTGCGCGCCGAGCAATACGGAAGCGCGATGAACGCAGCGCCGAACGT 600  
-----

Bm4825 clone9 CGCGACCAGCGCGCCGATCGTGAGCGCGGACGATGCCATGCCGCGTAAATGTCTCTGTT 660  
-----

Bm4825 clone9 GGCTTCGCGACCATCACGATCAGCGGAGGTTTCGGCAATGTCTTGAAGTAATACAGCGG 720  
-----

Bm4825 clone9 GCGCACCCGTCGATGGACGAGCGCTCGAGAAACGATCCTTCGGGTGCGCTTCGGAAGCG 780  
-----TCCTTCGGGTGCGCTTCGGAAGCG 24  
\*\*\*\*\*

Bm4825 clone9 GCGGAACGTGGCGCC TGGCTGATGTCCCGTCGATCGTATTCAGTTCGTAAGGCTGGCG 840  
GCGGAACGTGGCG----- 37  
\*\*\*\*\*

Bm4825 clone9 CATGACCATGATGCCGTCGGTCCGATTAGGGAAATCGATCCGTGCGGACCCAGCGCGAG 900  
-----

Bm4825 clone9 CCCGGCAAACAAC TTTGTGAAATACTCGAGGTTGATCGCGATCAATGCGACGCCGGCGAA 960  
-----

Bm4825 clone9 GGAGCCGTCCGGGTTTCGATACGCGACGGCTCAGCACGATGCTTGGTGTACC GCCGCGCAG 1020  
-----

Bm4825 clone9 CCGCGATGCGAATGGATCGCTGACGTAAAGCCCCACGTCCGGCGGGTCGCGGTGGACGGT 1080  
-----

Bm4825 clone9 GAAATATTTTCGATCCGAGAAGTTGCCGTGGCGGGCACGTCGTTCTGCGAGTTCGAGAAC 1140  
-----

Bm4825 clone9 GATATTGCCCTGCGCATCGAGGACCAGCATCGAACCGAGATATTGGGCCGTCATCGCGTG 1200  
-----

Bm4825 clone9 GTCGAACAGCACGCCGCGGCGAAGCGAGGCCGAAGCCGCATCACGTCGGGGCGCCGAAG 1260  
-----

```

Bm4825      CCCCTGGAGGACGGCCCTCGAGCGACAGATTGTAGAGCTCGACGTTGCGCGTGATGTCCGG  1320
clone9      -----

Bm4825      TTCGGCGACGAGCGCGAGATTGCGCGAAGTCTCGCGTGCCCTGTCCAGCGCATCCTGGCG  1380
clone9      -----

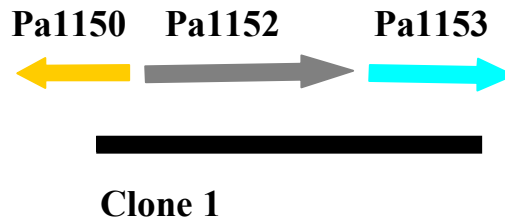
Bm4825      GCTCTGGAACAGCTGGAGTATGCAAAGCCCCATCAGCGCGAAGGCGATCAGCACGCCGAC  1440
clone9      -----

Bm4825      GCGGACGACATAGTGAGGCGCTGATGAGCGCGTTGCAT  1479
clone9      -----

```

**Figure 3-8 Blast result of clone 1 of Pa PAO1 genome library**

a. Genome organization of clone 1 of Pa PAO1. Clone 1 of Pa PAO1 encoded pyocin S2 (Pa 1152) and corresponding immunity protein (Pa1 153).



b. Alignment of clone 1 and Pa PAO1

```

Pa1151-53   GTCGGGCGAGCCGGCGGAGAGCGGGGCGTCTAGGGTGCGGGTTGCGGGGAGGCTGGC  60
clone1      GTCGGGCGAGCCGGCGGAGAGCGGGGCGTCTAGGGTGCGGGTTGCGGGGAGGCTGGC  60
*****

Pa1151-53   GGGGAGCGGAAGAGAGCTTTTATGGGATTTATGAACTGGATTGCAGAGTGGTGAATGTGC  120
clone1      GGGGAGCGGAAGAGAGCTTTTATGGGATTTATGAACTGGATTGCAGAGTGGTGAATGTGC  120
*****

Pa1151-53   GAACGGGAGGCTTGCCGATCGGAGGATACATGCAGGGCGTCCATCTCGGCCGTGGTGGTGG  180
clone1      GAACGGGAGGCTTGCCGATCGGAGGATACATGCAGGGCGTCCATCTCGGCCGTGGTGGTGG  180
*****

Pa1151-53   CAGCCCGCCTGTCAGTCAGGGCGCTGACAACCCAGGGTGACGACCAGTTCGCGGCCGCT  240
clone1      CAGCCCGCCTGTCAGTCAGGGCGCTGACAACCCAGGGTGACGACCAGTTCGCGGCCGCT  240
*****

Pa1151-53   CGCCAAGCCGGCGTCCGCCCCAGGGTCAGCGTGCGCGGCGTGTCTGGCGACTATCGAG  300
clone1      CGCCAAGCCGGCGTCCGCCCCAGGGTCAGCGTGCGCGGCGTGTCTGGCGACTATCGAG  300
*****

Pa1151-53   CAGCCCTCTTGAAGCACGAGGCTGTGCGGCGTGGCGCCCTGGCCAGCAGCCGATATCG  360
clone1      CAGCCCTCTTGAAGCACGAGGCTGTGCGGCGTGGCGCCCTGGCCAGCAGCCGATATCG  360
*****

Pa1151-53   CACCAGCGCCGAGGTACAGCCGATTCGCGGTTGAGCGCCGGGCTCAAGGGTGAGTGG  420
clone1      CACCAGCGCCGAGGTACAGCCGATTCGCGGTTGAGCGCCGGGCTCAAGGGTGAGTGG  420
*****

Pa1151-53   CAGGTGCAACTGGCCCTCCGCCGTGTGCGGTGACCCAGCCGGGCGGCGAGGCAGCGGA  480
clone1      CAGGTGCAACTGGCCCTCCGCCGTGTGCGGTGACCCAGCCGGGCGGCGAGGCAGCGGA  480

```

```

*****
Pa1151-53  GAGAGCATTGTCGAGCGTCGCCGCGTCGGCGAATGGGGCGGCCAGGCAGAGGCTGCCGAG 540
clone1      GAGAGCATTGTCGAGCGTCGCCGCGTCGGCGAATGGGGCGGCCAGGCAGAGGCTGCCGAG 540
*****

Pa1151-53  GAGCAGGGCTTTGGCGTCCATGTCGGGGTTCCGCTGGGGATGAAAGGGCATCATGGCTG 600
clone1      GAGCAGGGCTTTGGCGTCCATGTCGGGGTTCCGCTGGGGATGAAAGGGCATCATGGCTG 600
*****

Pa1151-53  AGGTGGCTTTATTCGTCGAACAATGGCTGGCCCTGCCGGTATATCGGCCAGGACTTCAAG 660
clone1      AGGTGGCTTTATTCGTCGAACAATGGCTGGCCCTGCCGGTATATCGGCCAGGACTTCAAG 660
*****

Pa1151-53  CGTTGCGGAAACGGCCTGACCAGTGCAGCGGCTTGCAATCAAAAACGCTGGGCTCGCAA 720
clone1      CGTTGCGGAAACGGCCTGACCAGTGCAGCGGCTTGCAATCAAAAACGCTGGGCTCGCAA 720
*****

Pa1151-53  TCAGCGTGGCTTTGTCCGGAAGCTCGCGAAGGGCGGCATGGATGAATTTTGAAAGTGGG 780
clone1      TCAGCGTGGCTTTGTCCGGAAGCTCGCGAAGGGCGGCATGGATGAATTTTGAAAGTGGG 780
*****

Pa1151-53  AGTTCGTTAATGAACCGAAGGTATGCGGCTTCCGTGACTGGCCGGGTAGATGAGGTGTG 840
clone1      AGTTCGTTAATGAACCGAAGGTATGCGGCTTCCGTGACTGGCCGGGTAGATGAGGTGTG 840
*****

Pa1151-53  CGTTAAGGAAATGACCGTTTGACGCGAGCGCGATTATAAGAGGCGAGAGAGCGCACCTTC 900
clone1      CGTTAAGGAAATGACCGTTTGACGCGAGCGCGATTATAAGAGGCGAGAGAGCGCACCTTC 900
*****

Pa1151-53  TGGATCATCTAAACACGGGATATTGAAGTTGATTGCAGTGTATTGCCGATGCATTGGGGC 960
clone1      TGGATCATCTAAACACGGGATATTGAAGTTGATTGCAGTGTATTGCCGATGCATTGGGGC 960
*****

Pa1151-53  TTATTGACTGGGAGAGGGTTCTCTAGGTGGGAGTCGCTCAATACATTACACTTTC A A A A T T 1020
clone1      TTATTGACTGGGAGAGGGTTCTCTAGGTGGGAGTCGCTCAATACATTACACTTTC A A A A T T 1020
*****

Pa1151-53  AATGTAGGGCGGATCTATGGCTGTCAATGATTACGAACCTGGTTCGATGGTTATTACACA 1080
clone1      AATGTAGGGCGGATCTATGGCTGTCAATGATTACGAACCTGGTTCGATGGTTATTACACA 1080
*****

Pa1151-53  TGTGCAGGGTGGTGGGCGTGACATAATCCAGTATATTCCTGCTCGATCAAGCTACGGTAC 1140
clone1      TGTGCAGGGTGGTGGGCGTGACATAATCCAGTATATTCCTGCTCGATCAAGCTACGGTAC 1140
*****

Pa1151-53  TCCACCATTGTCCACCAGGACCAAGTCCGTATGTCGGTACTGGAATGCAGGAGTACAG 1200
clone1      TCCACCATTGTCCACCAGGACCAAGTCCGTATGTCGGTACTGGAATGCAGGAGTACAG 1200
*****

Pa1151-53  GAAGCTAAGAAGTACGCTTGATAAGTCCCATTCAGAACTCAAGAAAAACCTGAAAAATGA 1260
clone1      GAAGCTAAGAAGTACGCTTGATAAGTCCCATTCAGAACTCAAGAAAAACCTGAAAAATGA 1260
*****

Pa1151-53  AACCTGAAGGAGGTTGATGAACTCAAGAGTGAAGCGGGGTTGCCAGGTAAGCGGTCAG 1320
clone1      AACCTGAAGGAGGTTGATGAACTCAAGAGTGAAGCGGGGTTGCCAGGTAAGCGGTCAG 1320
*****

Pa1151-53  TGCCAATGACATCCGCGATGAAAAGAGTATCGTTGATGCACTCATGGATGCCAAAGCAA 1380
clone1      TGCCAATGACATCCGCGATGAAAAGAGTATCGTTGATGCACTCATGGATGCCAAAGCAA 1380
*****

Pa1151-53  ATCGCTAAAGGCCATTGAGGATCGCCCGCCAATCTTTATACGGCTTCAGACTTTCCTCA 1440
clone1      ATCGCTAAAGGCCATTGAGGATCGCCCGCCAATCTTTATACGGCTTCAGACTTTCCTCA 1440
*****

Pa1151-53  GAAGTCAGAGTCGATGTACCAGAGTCAGTTGCTGGCCAGCCGAAAAATTCATGGAGAGTT 1500
clone1      GAAGTCAGAGTCGATGTACCAGAGTCAGTTGCTGGCCAGCCGAAAAATTCATGGAGAGTT 1500
*****

Pa1151-53  CCTGGATCGCCATATGAGTGAGCTGGCCAAAGCGTACAGCGCGGATATCTATAAGGCGCA 1560
clone1      CCTGGATCGCCATATGAGTGAGCTGGCCAAAGCGTACAGCGCGGATATCTATAAGGCGCA 1560

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**Pa1152**

```

*****
Pal151-53
clone1  AATCGCTATCTTGAACAAACGCTCAAGAGCTGGAGAATAAAGCCCGGTCATTGGAAGC 1620
        AATCGCTATCTTGAACAAACGCTCAAGAGCTGGAGAATAAAGCCCGGTCATTGGAAGC 1620
        *****

Pal151-53
clone1  AGAAGCCAGCGAGCCGCTGCTGAGGTGGAGGCGGACTACAAGGCCAGGAAGGCAAATGT 1680
        AGAAGCCAGCGAGCCGCTGCTGAGGTGGAGGCGGACTACAAGGCCAGGAAGGCAAATGT 1680
        *****

Pal151-53
clone1  CGAGAAAAAAGTGCAGTCCGAGCTTGACCAGGCTGGGAATGCTTTGCCTCAACTGACCAA 1740
        CGAGAAAAAAGTGCAGTCCGAGCTTGACCAGGCTGGGAATGCTTTGCCTCAACTGACCAA 1740
        *****

Pal151-53
clone1  TCCAACGCCAGAGCAGTGGCTTGAACGCGCTACTCAACTGGTTACGCAGGCATCGCCAA 1800
        TCCAACGCCAGAGCAGTGGCTTGAACGCGCTACTCAACTGGTTACGCAGGCATCGCCAA 1800
        *****

Pal151-53
clone1  TAAGAAGAAATTCAGACTGCAACAATGCCCTGATTGCCAAGGCACCCAATGCACTGGA 1860
        TAAGAAGAAATTCAGACTGCAACAATGCCCTGATTGCCAAGGCACCCAATGCACTGGA 1860
        *****

Pal151-53
clone1  GAAACAAAAGGCAACCTACAACGCCGATCTCCTAGTGGATGAAATCGCCAGCCTGCAAGC 1920
        GAAACAAAAGGCAACCTACAACGCCGATCTCCTAGTGGATGAAATCGCCAGCCTGCAAGC 1920
        *****

Pal151-53
clone1  ACGGCTGGACAAGCTGAACGCCGAAACGGCAAGGCGCAAGGAAATCGCTCGTCAAGCGGC 1980
        ACGGCTGGACAAGCTGAACGCCGAAACGGCAAGGCGCAAGGAAATCGCTCGTCAAGCGGC 1980
        *****

Pal151-53
clone1  GATCAGGGCTGCCAATACTTATGCCATGCCAGCCAATGGCAGCGTTGTGCCACCGCCGC 2040
        GATCAGGGCTGCCAATACTTATGCCATGCCAGCCAATGGCAGCGTTGTGCCACCGCCGC 2040
        *****

Pal151-53
clone1  AGGCCGGGGTCTGATCCAGGTCGCACAAGGCCGCCATCCCTTGCTCAAGCGATCTCCGA 2100
        AGGCCGGGGTCTGATCCAGGTCGCACAAGGCCGCCATCCCTTGCTCAAGCGATCTCCGA 2100
        *****

Pal151-53
clone1  TCGGATTGCCGCTCCTGGGCCGGGTCTGGCTTCAGCACCCCTCGGTGATGGCCGTGGGCTT 2160
        TCGGATTGCCGCTCCTGGGCCGGGTCTGGCTTCAGCACCCCTCGGTGATGGCCGTGGGCTT 2160
        *****

Pal151-53
clone1  TGCCAGTCTGACCTACTCCTCCCGACTGCCGAGCAATGGCAGGACCAAACGCCGATAG 2220
        TGCCAGTCTGACCTACTCCTCCCGACTGCCGAGCAATGGCAGGACCAAACGCCGATAG 2220
        *****

Pal151-53
clone1  CGTTCGTTACGCCCTGGGCATGGATGCCGCTAAATGGGGCTTCCCCAAGCGTAAACCT 2280
        CGTTCGTTACGCCCTGGGCATGGATGCCGCTAAATGGGGCTTCCCCAAGCGTAAACCT 2280
        *****

Pal151-53
clone1  GAACGCGGTTGCAAAGCCAGCGGTACCGTCGATCTGCCGATGCGCCTGACCAACGAGGC 2340
        GAACGCGGTTGCAAAGCCAGCGGTACCGTCGATCTGCCGATGCGCCTGACCAACGAGGC 2340
        *****

Pal151-53
clone1  ACGAGGCAACACGACGACCCCTTTCGGTGGTCAGCACCGATGGTGTGAGCGTTCCGAAAGC 2400
        ACGAGGCAACACGACGACCCCTTTCGGTGGTCAGCACCGATGGTGTGAGCGTTCCGAAAGC 2400
        *****

Pal151-53
clone1  CGTTCGGTCCGGATGGCGGCCACAAATGCCACGACAGGCCGTGACGAGGTTACGGTTCC 2460
        CGTTCGGTCCGGATGGCGGCCACAAATGCCACGACAGGCCGTGACGAGGTTACGGTTCC 2460
        *****

Pal151-53
clone1  CTCTACGACCGCAGAAGCGCCGCCACTGATCCTGACCTGGACGCGCGGAGTCTCCAGG 2520
        CTCTACGACCGCAGAAGCGCCGCCACTGATCCTGACCTGGACGCGCGGAGTCTCCAGG 2520
        *****

Pal151-53
clone1  AAACCAGAACCCTTCGAGTACCACTCCGGTCTGACCGAAGCCGGTGCCGGTATATGAGGG 2580
        AAACCAGAACCCTTCGAGTACCACTCCGGTCTGACCGAAGCCGGTGCCGGTATATGAGGG 2580
        *****

Pal151-53
clone1  AGCGACCCTTACACCGGTGAAGGCTACCCCGAAACCTATCCTGGGGTGATTACACTACC 2640
        AGCGACCCTTACACCGGTGAAGGCTACCCCGAAACCTATCCTGGGGTGATTACACTACC 2640
        *****

```



Pa1151-53 clone1	GGAAGACCTGATCATCGGCTTCCCGGCCGACTCGGGGATCAAGCCGATCTATGTGATGTT GGAAGACCTGATCATCGGCTTCCCGGCCGACTCGGGGATCAAGCCGATCTATGTGATGTT *****	2700 2700
Pa1151-53 clone1	CAGGGATCCGCGGGATGTACCTGGTGCTGCGACTGGCAAGGGACAGCCCGTCAGCGGTAA CAGGGATCCGCGGGATGTACCTGGTGCTGCGACTGGCAAGGGACAGCCCGTCAGCGGTAA *****	2760 2760
Pa1151-53 clone1	TTGGCTCGGCGCCCTCTCAAGGTGAGGGGGCTCCAATTCCAAGCCAGATTGCGGATAA TTGGCTCGGCGCCCTCTCAAGGTGAGGGGGCTCCAATTCCAAGCCAGATTGCGGATAA *****	2820 2820
Pa1151-53 clone1	ACTACGTGGTAAGACATTCAAAACTGGCGGGACTTTCGGGAACAATCTTGATAGTGT ACTACGTGGTAAGACATTCAAAACTGGCGGGACTTTCGGGAACAATCTTGATAGTGT *****	2880 2880
Pa1151-53 clone1	GGCTAATGATCCTGAGTTAAGTAAACAGTTTAACTCCTGGTAGTTTAGCTGTAATGAGAGA GGCTAATGATCCTGAGTTAAGTAAACAGTTTAACTCCTGGTAGTTTAGCTGTAATGAGAGA *****	2940 2940
Pa1151-53 clone1	TGGAGGGCTCCTTATGTCAGAGAGTCAGAACAGGCTGGCGGGAGAATAAAGATCGAAAT TGGAGGGCTCCTTATGTCAGAGAGTCAGAACAGGCTGGCGGGAGAATAAAGATCGAAAT *****	3000 3000
Pa1151-53 clone1	CCACCACAAGGTTTCAATAGCAGATGGAGGCGGCTTACAATATGGGGAACCTTGTGTC CCACCACAAGGTTTCAATAGCAGATGGAGGCGGCTTACAATATGGGGAACCTTGTGTC *****	3060 3060
	<b>Pa1153 →</b>	
Pa1151-53 clone1	AGTAACGCCAAAACGTCATATAGAAATCCACAAGGGAGGGAAGTGATATGAAGTCCAAGA AGTAACGCCAAAACGTCATATAGAAATCCACAAGGGAGGGAAGTGATATGAAGTCCAAGA *****	3120 3120
Pa1151-53 clone1	TTTCCGAATATACGGAAAAAGAGTTTCTTGAGTTTGTAAAGACATATACACAAACAATA TTTCCGAATATACGGAAAAAGAGTTTCTTGAGTTTGTAAAGACATATACACAAACAATA *****	3180 3180
Pa1151-53 clone1	AGAAAAAGTTCCCTACCGAGGAGTCTCATATTCAGCCGTGCTTGAATTTAAAAAATAA AGAAAAAGTTCCCTACCGAGGAGTCTCATATTCAGCCGTGCTTGAATTTAAAAAATAA *****	3240 3240
Pa1151-53 clone1	CGGAACACCCAAGCGGCTCAGACCTTCTTTACTACCCAACGAAAAATAGAGAAGATAGCC CGGAACACCCAAGCGGCTCAGACCTTCTTTACTACCCAACGAAAAATAGAGAAGATAGCC *****	3300 3300
Pa1151-53 clone1	CAGCTGGAGTTGTAAGGAAGTTAAAGAATGGCGTGCTTCCAAGGGCTTCTGGCTTTA CAGCTGGAGTTGTAAGGAAGTTAAAGAATGGCGTGCTTCCAAGGGCTTCTGGCTTTA *****	3360 3360
Pa1151-53 clone1	AGGCCGGTTAGTTGGCCATCAACAAGCCCGTTTCGACGGGGCTTGTTCATGTTGACGC AGGCCGGTTAGTTGGCCATCAACAAGCCCGTTTCGACGGGGCTTGTTCATGTTGACGC *****	3420 3420
Pa1151-53 clone1	TGCTCTATCTTGACAGTCTGTCTGAATATCTTGAAGACAATCACATTATGAGCCGCAA TGCTCTATCTTGACAGTCTGTCTGAATATCTTGAAGACAATCACATTATGAGCCGCAA *****	3480 3480
Pa1151-53 clone1	CCACCGCAAAACACCCATCGTCGGGCGCACGAGCTGCCGTAGCGAGCTCTCCACGTTGGC CCACCGCAAAACACCCATCGTCGGGCGCACGAGCTGCCGTAGCGAGCTCTCCACGTTGGC *****	3540 3540
Pa1151-53 clone1	CAGCAGCCACCTCTCCCTACTGGAAGTCAGGTCAATAACTGGTGCCACGTAAGGAT CAGCAGCCACCTCTCCCTACTGGAAGTCAGGTCAATAACTGGTGCCACGTAAGGAT *****	3600 3600
Pa1151-53 clone1	GGGCGTTCTACTGGCCCTCATAAGCCAGGCTGCTACGGCAGATCGTGTGCTAACCTC GGGCGTTCTACTGGCCCTCATAAGCCAGGCTGCTACGGCAGATCGTGTGCTAACCTC *****	3660 3660
Pa1151-53 clone1	AAGGGACACAACCCGCAAGAACCGCGCTCCCTGAAAAAGCGCCTGCTGCGCAAGTGATG AAGGGACACAACCCGCAAGAACCGCGCTCCCTGAAAAAGCGCCTGCTGCGCAAGTGATG *****	3720 3720

Pal151-53  
 clone1  
 AGCAAATGAGTACATCCAACATCCAGGCATTGGCCAGCACTGGGCACAGCTCGCCAAAGA 3780  
 AGCAAATGAGTACATCCAACATCCAGGCATTGGCCAGCACTGGGCACAGCTCGCCAAAGA 3780  
 \*\*\*\*\*

Pal151-53  
 clone1  
 AGCGGGATTCCGAGCCGACTATGACAGCACATCAACGCCAGAAACACATCTGGTCTGCGG 3840  
 AGCGGGATTCCGAGCCGACTATGACAGCACATCAACGCCAGAAACACATCTGGTCTGCGG 3840  
 \*\*\*\*\*

Pal151-53  
 clone1  
 AGTAACCCAAGAGTGGATTTCGAGAACACATCGTTGCCACCAACGATATGCGCCCGTTCGG 3900  
 AGTAACCCAAGAGTGGATTTCGAGAACACATCGTTGCCACCAACGATATGCGCCCGTTCGG 3900  
 \*\*\*\*\*

Pal151-53  
 clone1  
 CCTGCTGCATTTGCTGGGTTCAGGCATCGTTGCGCATGGAGCAAGCGTTGTGGCCGGAAGA 3960  
 CCTGCTGCATTTGCTGGGTTCAGGCATCGTTGCGCATGGAGCAAGCGTTGTGGCCGGAAGA 3960  
 \*\*\*\*\*

Pal151-53  
 clone1  
 TTACGAGCGGATGACTCGTAAGGTCAAGGGAGTCCAGCGGGAAGCTGAAGGCGACAACGC 4020  
 TTACGAGCGGATGACTCGTAAGGTCAAGGGAGTCCAGCGGGAAGCTGAAGGCGACAACGC 4020  
 \*\*\*\*\*

Pal151-53  
 clone1  
 CAAATCATACTTTATGAAGAAGTCATGCAGATAAGGCAGGAGCGCTTCGATAAAGCACG 4080  
 CAAATCATACTTTATGAAGAAGTCATGCAGATAAGGCAGGAGCGCTTCGATAAAGCACG 4080  
 \*\*\*\*\*

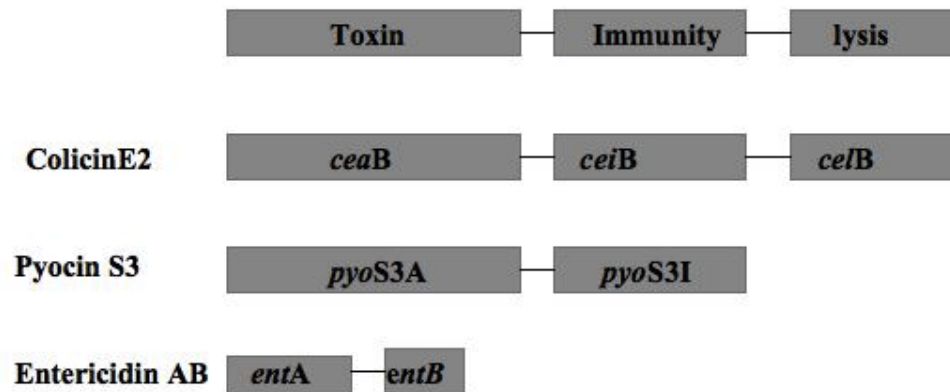
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 ACA----- 4083  
 \*\*\*

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 clone1  
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 -----

Pal151-53  
 clone1  
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 -----

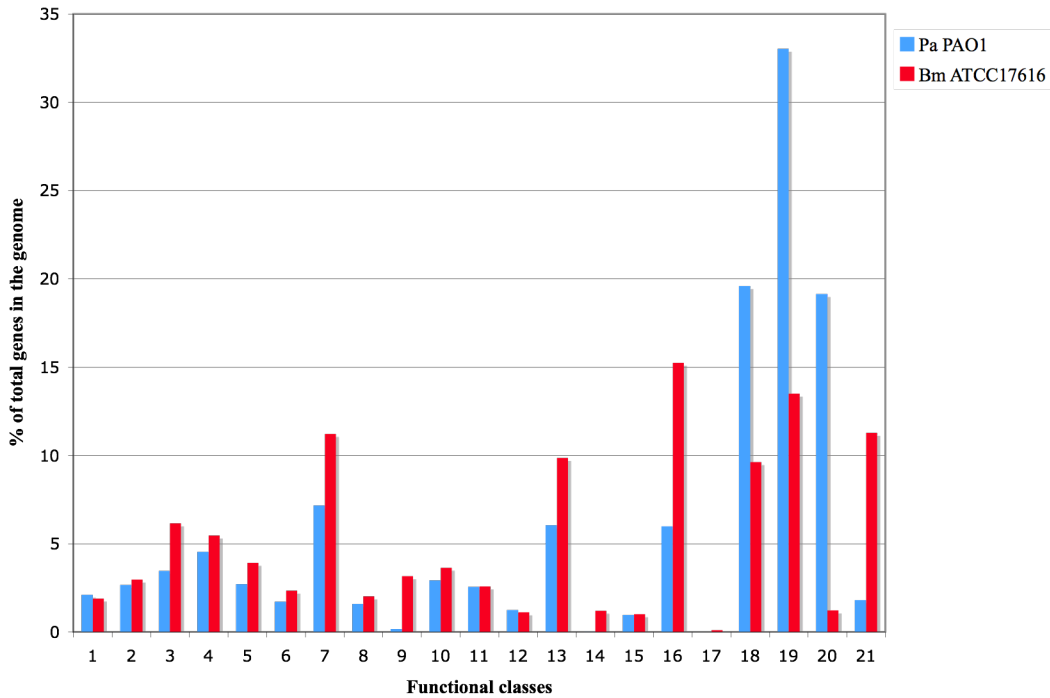
**Figure 3-9 Open reading frame organization of colicin E2, pyocin S3, and entericidinAB**

The genes encoding bacteriocins of enteric bacteria (i.e. colicin E2), Pa (i.e. pyocin S3), and entericidin AB are organized in a similar way. These proteins are toxin/antitoxin (immunity) complexes. An immunity gene (i.e. *ceiB*, *pyoS3A* *entA*) follows the toxin gene (i.e *ceaB*, *pyoS3I*, *entB*).



**Figure 3-10 Genome comparison of *B. multivorans* (Bm) ATCC17616 and *Pseudomonas aeruginosa* (Pa) PAO1**

Genes of Pa and Bcc were classified into 21 functional classes. Bm ATCC17616 genome possessed additional genes in majority of these functional classes in comparison to Pa PAO1 genome (Pathema).



- 1 Amino acid biosynthesis
- 2 Biosynthesis of cofactors, prosthetic groups, and carriers
- 3 Cell envelope
- 4 Cellular processes
- 5 Central intermediary metabolism
- 6 DNA metabolism
- 7 Energy metabolism
- 8 Fatty acid and phospholipids metabolism
- 9 Mobile and extrachromosomal element functions
- 10 Protein fate
- 11 Protein synthesis
- 12 Purines, pyrimidines, nucleosides, and nucleotides
- 13 Regulatory functions
- 14 Signal transduction
- 15 Transcription
- 16 Transport and binding proteins
- 17 Viral functions
- 18 Hypothetical proteins
- 19 Hypothetical proteins-conserved
- 20 Unclassified
- 21 Unknown function

## CHAPTER 4

### CONCLUSION

*“Two nebulisers in the morning with breathing exercises that take about half an hour. Then oral antibiotics, vitamins and occasionally steroids. I used to do chest physiotherapy but they gave me a PEP mask because (pause) well I just wanted to get on (with life) really rather than spend too long on treatment” by a CF patient*

*Kathryn Badlan, 2006*

#### **4.1 The Life of a CF patient**

Cystic Fibrosis (CF) is a rare genetic disorder that occurs in 1 in every 3,000 Americans, 1 in every 2-3,000 Europeans, and 1 in every 7,056 Africans (WHO, 2004). CF patients have a defective cystic fibrosis transmembrane regulator (CFTR) protein, which is a chloride ion channel on the apical surface of epithelial cells (Matthay *et al.*, 2005). The defective CFTR channel leads to decreased chloride secretion and increased sodium absorption across the cytoplasmic membrane. Imbalanced sodium and chloride transport leads to dehydration of the mucous across the epithelia, which results in failure of multiple organ systems including the digestive, reproductive, and respiratory systems (Gibson *et al.*, 2003).

CF patients suffer from vitamin-A deficiency, poor growth, persistent coughing, wheezing, shortness of breath, digestive complications, and fertility problems (Darling *et al.*, 1953; Freedman *et al.*, 2000a; Freedman *et al.*, 2000b). The most severe health

challenge, however, is the presence of chronic lung infections, which are associated with high mortality and shortened lifespans (George *et al.*, 2009).

The life of a CF patient is physically and emotionally challenging. Their daily routine includes repeated sessions of chest physiotherapy (to clear mucus from lungs), a high energy supplemented diet, digestive enzymes (taken with all meals to improve digestion), and finally oral and nebulized antibiotics (to treat lung infections) (Foster *et al.*, 2001). None of these treatments represent a cure, but significantly improve the quality of life, as well as increase the lifespan, of CF patients. The estimated median survival age of CF patients in the US was 37 years in 2008 . Even with aggressive therapies, CF patients ultimately die in their mid-30s (Gibson *et al.*, 2003; Govan & Deretic, 1996).

#### **4.2 The battle with bacterial infections: *Pseudomonas aeruginosa* and *Burkholderia cepacia* complex**

More than eighty percent of CF patients die due to respiratory failure (Lyczak *et al.*, 2002). Bacterial infections and the resulting lung tissue destruction are the major causes of respiratory failure (Gibson *et al.*, 2003; Govan & Deretic, 1996). Bacteria are trapped in the dehydrated mucus covering the epithelial cells of the airway, and thus escape from phagocytosis by the immune system, and are able to colonize in the airway and lungs (Gibson *et al.*, 2003). The presence of pathogens in the airway induces inflammation. Secretory cells release antibacterial proteins and neutrophils, which are attracted to the site of the infection (Konstan & Berger, 1997). However, rather than killing the invading bacteria, this process results in tissue destruction due to the degraded neutrophils releasing proteases which damage the lung tissue. The damaged tissue

provides food for the bacterial infection. Thus, inflammation leads to more bacterial colonization, which leads to more inflammation, resulting in a vicious cycle of host/pathogen interaction (Gibson *et al.*, 2003).

Most research on CF-based lung infections is focused on *Staphylococcus aureus*, *Haemophilus influenzae*, *Stenotrophomonas maltophilia*, *Pseudomonas aeruginosa* (Pa), and *Burkholderia cepacia* complex (Bcc) since these are the primary colonizers of the CF lung (Hutchison & Govan, 1999). It has been suggested that the prevalence of these species is age-related. *S. aureus* and *H. influenzae* are isolated in early years and are replaced with Pa and Bcc during adolescence (Govan & Deretic, 1996).

This thesis focused on the interaction between one pair of species, Pa and Bcc, which are associated with chronic lung infections in adult CF patients (Govan & Deretic, 1996). Once a chronic infection is established, eradication of Pa and Bcc is virtually impossible (Hentzer *et al.*, 2001) and the patients experience progressive lung failure and premature death (Jones *et al.*, 2004; Lyczak *et al.*, 2002; Tummler *et al.*, 1997).

Pa is the most persistent bacterial species in the CF lung. Approximately 30% of infants and 80% of adult CF patients are colonized with Pa. During infancy and childhood Pa is isolated only intermittently (Hansen *et al.*, 2008). At this stage, the Pa strains are usually in a planktonic form, which respond well to antibiotic treatment. As the disease progresses, alginate forming variants overtake the resident strain, resulting in mucoid production and biofilms formation (Govan & Deretic, 1996). Mucoid Pa form biofilms, which are associated with chronic lung infections and lead to progressive lung failure and death of CF patients (Hentzer *et al.*, 2001).

Bcc secondary infections have become a major concern in the last 30 years. Although the incidence of colonization is low (~3%), clinical outcomes are unpredictable and various - ranging from asymptomatic to severe chronic lung infections (Whiteford *et al.*, 1995). A small portion of CF patients succumbs to cepacia syndrome, which is fatal necrotizing pneumonia (Govan & Deretic, 1996). The prevalence of Bcc in infants is very low (less than one percent), most cases are reported after 5 years of age .

Combinatorial antibiotic therapy is the only treatment option for chronic lung infections in CF. Pa infections are usually treated with a combination of beta-lactamases and aminoglycosides (Aaron *et al.*, 2000; Hoiby *et al.*, 2001). However, the success of antibiotic treatments is limited by several factors.

The most critical factor is that both species are multi-drug resistant. One form of resistance is conferred by actively pumping the drugs through the cytoplasmic membrane. Five pump mechanisms have been described: resistance - nodulation - cell division (RND), major facilitator superfamily (MFS), small multi-drug resistance family (SMR), multidrug and toxic compound extrusion family (MATE), and ATP binding cassette family (ABC)(Paulsen, 2003). These pumps provide intrinsic antibiotic resistance to various classes of antibiotics including fluoroquinolones, beta-lactams, tetracycline, macrolides, chloramphenicol, trimethoprim, and sulfonamides (Poole & Srikumar, 2001). Genomes of Pa and Bcc encode all five mechanisms (Holden *et al.*, 2009; Stover *et al.*, 2000).

Biofilm formation is another factor contributing to antibiotic resistance. Bacteria in a biofilm are 10-1000 fold more resistant to antibiotic treatment than their planktonic counterparts (Mah & O'Toole, 2001). Both species form biofilms in CF lungs (Conway *et*



*al.*, 2002; Singh *et al.*, 2000), in fact, alginate-forming mucoid Pa variants are a signature of chronic Pa infections (Hentzer *et al.*, 2001). Antibiotic resistance in the biofilms may result from reduced growth rate, the anaerobic environment, and the presence of negatively charged alginate surrounding the biofilm structure, which traps the antibiotics (Hoiby *et al.*, 2001).

The situation is more complex with strains of Bcc. *In vitro* studies have shown that strains in both planktonic and biofilm form have similar levels of resistance to ceftazidime, ciprofloxacin, meropenem, minocycline, tobramycin, and trimethoprim/sulfamethoxazole (Peeters *et al.*, 2009). However, Caraher *et al.*, 2007 found elevated levels of resistance to meropenem and piperacillin-tazobactam in biofilm-based strains in comparison to planktonic forms, whereas the both showed similar levels of resistance to tobramycin and amikacin (Caraher *et al.*, 2007). These contradictory results may be due to differences in the alginates produced by the species. Alginate produced by Pa is an acetylated copolymer of beta-D-mannuronic acid and alpha-L-glucuronic acid, whereas Bcc produces a branched acetylated heptasaccharide composed of D-glucose, D-rhamnose, D-mannose, D-galactose, and D-glucuronic acid (Bylund *et al.*, 2006). Structural differences between these exopolysaccharides may explain the differential accessibility of various antibiotics in the biofilm structure.

The presence of hypermutable strains is another factor resulting in high levels of antibiotic resistance in CF strains. The emergence of hypermutable strains is thought to emerge due to the adaptation of bacteria to the continuously changing lung environment. Oliver *et al.*, 2000 compared the levels of antibiotic resistance of hypermutable (or mutator) and nonmutator Pa isolates from CF patients against ticarcillin, ceftazidime,

imipenem, gentamycin, tobramycin, amikacin, norfloxacin, and fosfomycin. The hypermutable Pa strains showed elevated resistance to all of the antibiotics (Oliver *et al.*, 2000).

In summary, CF patients experience long-term exposure to antibiotic treatment, which selects for multi-drug resistant strains. In addition, the bacterial strains in residence in their lungs encode several factors that ensure high levels of intrinsic resistance. Not surprising, nearly half of adult CF patients are chronically infected with multi-drug resistant Pa, which is associated with a more rapid decline in lung function (Lechtzin *et al.*, 2006).

New antibiotics are in the drug development pipeline, such as MP-376, a new formulation of levofloxacin; GS9310/11, an inhaled combination of the antibiotics fosfomycin and tobramycin; BAY Q3939, an inhaled version of ciprofloxacin; Arikace™, a liposomal formulation of amikacin (CF Foundation). We can anticipate that repeated exposure of these drugs will also result in the emergence of resistant strains. One goal of the present study is to better understand the nature of bacterial interactions in the CF lung, in order to identify new targets for therapeutic intervention.

Most adult CF patients are infected with Pa (Govan & Deretic, 1996). When a secondary Bcc infection occurs, the infecting strain must compete against the resident Pa strain(s). These competitive interactions are mediated by a variety of factors. A better understanding of these factors and the role they play in mediating Pa and Bcc interactions may provide novel insights into the development of alternative therapeutic approaches.

In my thesis, I specifically focused on one such factor, the bacteriocins produced by both species, and the role that these toxins play in mediating interactions between

these species. Bacteriocins are potent toxins produced by bacteria and are implicated in intra- and inter-specific competition of bacteria in various environments (Riley & Gordon, 1999; Riley *et al.*, 2003). Although the bacteriocins of Pa have been studied in great detail, far less is known about the corresponding toxins produced by Bcc species and even less is known about if or how they might contribute to bacterial interactions in the CF lung.

In my thesis, I focused on answering the following questions:

- Do clinical strains of Pa and Bcc isolated from CF lungs produce bacteriocins?
- If so, what types of bacteriocins are produced and how specific or broad are their killing activities?
- What roles do these bacteriocins serve in mediating intra- and inter-specific interactions of Pa and Bcc isolated from CF patients?

#### **4.3 Do clinical strains of Pa and Bcc isolated from CF lungs produce bacteriocins?**

To address this question, a bacteriocin assay was performed. The first step was to obtain a collection of isolates from CF lungs. This proved to be one of the most difficult challenges I encountered; it is extremely challenging to obtain access to such strain collections. However, I owe a great debt to Dr. Claudia Ordonez for providing me with a set of 66 clinical Pa (38) and Bcc (28) strains isolated from CF patients. The strain collection can be divided into two groups. The first is composed of 14 Pa and 7 Bcc paired isolates procured from 7 CF patients, each pair isolated from the same patient at the same time. The second group is composed of 24 Pa and 21 Bcc isolates procured

independently from 33 CF patients. The bacteriocin-screening assay employed each Pa and Bcc strain as a sensitive lawn and potential bacteriocin producer.

Previous studies of bacteriocin production in Pa and Bcc focused on bacteriocin typing and the use of such types to rapidly screen clinical isolates. These studies revealed that Pa is one of the most prolific Gram-negative producers of bacteriocins. Indeed, more than 90% of the strains tested produce bacteriocins, known as pyocins (B.Bouhaddioui, 2002; Bruun *et al.*, 1976; Edmonds *et al.*, 1972a; Farmer & Herman, 1969; Jones *et al.*, 1974b; Zabransky & Day, 1969). In contrast, prior typing of a much smaller number of Bcc strains from clinical sources revealed that only 30% produce bacteriocins, known as cepaciocins (Govan & Harris, 1985).

Our phenotypic assay revealed that 97% of Pa strains (37 strains) and 68% of Bcc strains (19 strains) possess bacteriocin-like inhibitory activity (Table 2-1). The frequency of production in Pa isolated from CF lungs is indistinguishable from strains isolated from other clinical sources. In contrast, the frequency of production in Bcc is far higher in strains isolated from CF lungs than from strains isolated from other clinical sources (Govan & Harris, 1985). The higher frequency of Bcc inhibitory activity in our study could be due to the fact that certain Bcc isolates (i.e. those which produce bacteriocins) are so successful in invading the CF lung, which requires interacting and/or competing with resident Pa strains. Further, the strains used to assay for inhibition activity were different in the prior study. The survey described here involved 28 strains of Bcc, representing 4 genomovars (*B. multivorans*, *B. dolosa*, *B. vietnamiensis*, and *B. cenocepacia*) and identified 19 bacteriocin-producing strains, of which ten are *B. multivorans*, six *B. dolosa*, two *B. cenocepacia*, and one *B. vietnamiensis*. *B. multivorans*

and *B. cenocepacia* are the most frequently isolated species from CF patients (LiPuma *et al.*, 2001). The previous study typed only *B. cepacia* (Govan & Harris, 1985).

#### **4.4 What types of bacteriocins are produced and how specific or broad are their killing activities?**

A set of reference strains was used in the bacteriocin screen identification of bacteriocin types. This collection included strains producing S and RF type pyocins and those sensitive to pyocins, as well as strains carrying plasmids with cloned S-type pyocins (pyocin S1, S2, S3, and AP41) (de Chial *et al.*, 2003; Duport *et al.*, 1995; Kageyama *et al.*, 1979; Kuroda & Kagiya, 1983; Sano & Kageyama, 1981; Sano *et al.*, 1993a; Sano *et al.*, 1993b; Williams *et al.*, 2008). Unfortunately, no cepaciocins have been fully characterized and there are no corresponding reference strains.

The Pa strains produced seven distinct bacteriocin phenotypes (based upon patterns of sensitivity to the reference collection), while the Bcc strains produced four phenotypes. Further, each Pa phenotype resulted from a combination of S and RF pyocins. In contrast to the bacteriocins of enteric bacteria, Pa is unique in producing numerous different pyocins per strain. However, there is no correlation between the number of bacteriocins produced and the number of strains inhibited. For example the Pa I bacteriocin phenotype produced at least eight pyocins (S1, S2, S3, AP41, R2, R4, F2, and F3) and inhibited 31 strains, of which 19 are Pa and 12 strains are Bcc (Table 2.2). Pa III pyocin phenotype III produces only 4 pyocins (S1, R2, R4, and F2), yet inhibited 44 strains of which, 18 were Pa and 26 Bcc (Table 2-2). The enhanced inhibition by strains with fewer pyocins could be due to the fact that particular combinations of pyocins may

be more inhibitory, or the fact that these strains may have also produced additional virulence factors, or novel bacteriocins.

Bacteriocin typing of Bcc strains was relatively challenging since there is no indicator strain collection including bacteriocin producer and sensitive Bcc strains. Burkholderia and Pseudomonas are closely related. They do co-exist in the same natural environment, and interact with each other. Further, their genome organization allows these bacteria to swap genes via horizontal gene transfer (Holden *et al.*, 2009; Stover *et al.*, 2000). In fact, Burkholderia species were formerly included within the genus Pseudomonas (for example, as *Pseudomonas cepacia*) (Yabuuchi *et al.*, 1992). Thus, I decided to use the same Pa reference strains to characterize bacteriocin phenotype of clinical Bcc strains. Indeed, this study revealed that 3 Bcc strains possessed bacteriocin phenotypes similar to those found in Pa. Bcc I phenotype produces 4 pyocins (S1, S2, AP41, F3), Bcc II phenotype produces F3 pyocin, and finally Bcc III phenotype produces R2 and F2 pyocins (Table 2-2). The bacteriocin phenotype of the remaining 15 Bcc strains could not be characterized, which suggests that they possessed novel toxins that provided inhibitory activity (Table 2-2).

#### **4.5 What roles do these bacteriocins serve in mediating intra- and inter-specific interactions of Pa and Bcc strains isolated from CF lung patients?**

The screening study revealed Pa and Bcc strains produced significantly different levels of intra- and inter-specific inhibition. The Pa strains showed similar levels of intra- and inter-specific inhibition (92 % and 81%, respectively) (Table 2-1). Bcc strains also showed similar levels of intra- and inter-specific inhibition, but at a much lower level (54% and 57%, respectively) (Table 2-1).

This study also revealed that the source(s) of the inhibitory activity is different for the two species. The intra-specific inhibitory activity for Pa strains was due primarily to S- and RF-pyocins, while inter-specific inhibition appeared to due to only RF pyocins (Figure 2-3). Not enough is known yet about cepaciacins to determine what types of toxins (i.e protease sensitive or phage-tail like) are acting.

#### **4.6 Further characterization of Bcc inhibitory activity**

Phenotypic and molecular studies revealed that the source of Bcc inhibitory activity is novel. Thus, genome libraries of three Bcc strains were constructed and screened against sensitive Pa and Bcc lawns to identify the source of the inhibition observed (Bakkal *et al.*, 2010). Fifteen genomic clones with putative inhibitory activity were identified from three Bcc genomes. Ten of the clones (*B. dolosa* and *B. cenocepacia*) did not have replicable inhibitory activity. The five remaining clones, which consistently maintained their inhibitory activity, were all from *B. multivorans*. The loss of inhibition in some of the clones is not surprising. The proteins produced could be toxic to the recipient *E. coli* due to the lack of cloned immunity gene, which confer resistance to the toxin.

The DNA inserts from the remaining five genomic clones were sequenced and compared to the genome sequence of *B. multivorans*. Instead, the Bcc genes identified are involved in amino acid biosynthesis, sugar metabolism, degradation of aromatic compounds, and bacterial apoptosis (Table 3-2). One explanation could be failure to detect Bcc bacteriocin genes due to technical challenges (Chapter 3.5). Another explanation could be Bcc strains use different strategies when it comes to bacterial competition. The genomes of Bcc strains possess numerous additional genes (Figure 3-

10), including the genes involved in primary metabolic pathways. Perhaps, instead of killing their competitors with highly costly bacteriocins, Bcc they used the advantage of their gene potential to grow and to adapt faster to any environment, where they are in competition with other species.

#### **4.7 The lessons learned from phenotypic bacteriocin screening and genome library of Pa and Bcc**

My thesis research has revealed that Bcc inhibitory agents appear to be phenotypically and genotypically different from that observed in the majority of Gram-negative bacteria. Most bacterial strains surveyed for an inhibition or killing activity are found, under further characterization, to encode one or more bacteriocin proteins. Riley (Riley & Gordon, 1999; Riley & Wertz, 2002a; Riley & Wertz, 2002b; Riley *et al.*, 2003) has gone so far as to label bacteriocins as the “weapon of choice” in bacteria. Even among the strains surveyed here, half of which were isolates of Pa, a plethora of bacteriocins were identified – all of which were encoded in the Pa genomes. In sharp contrast, none of the functions identified in the genome screens of Bcc bore any detectable similarity to the diversity of bacteriocins characterized in the literature. (Hardy, 1987; Riley *et al.*, 2003).

A further frustration was encountered when the standard bacteriocin assays were employed with Bcc. Inhibition phenotypes were constantly disappearing. A clear inhibition zone on a plate might disappear upon replication of that strain. Most of the inhibition zones observed in the standard plate assays also disappeared when subjected to the usual methods of concentration, such as is done with the crude lysate procedure (Bakkal *et al.*). Even with 20-fold concentrations during crude lysate preparation,



inhibition was lost in all of the Bcc strains. Further, only three of the 19 Bcc strains examined showed inhibition against the Pa indicator strain collection. These three Bcc strains were predicted to possess RF-like pyocins. Unfortunately, the PCR-based molecular screening methods employed here were unable to confirm the presence of any known RF-pyocin genes. Whether this absence is due to the fact that the pyocin-like genes are present but too divergent to amplify or they represent entirely novel bacteriocins remains unclear. BLAST-based examination of the 38 available Bcc genomes has also failed to detect the presence of any previously characterized bacteriocins.

The genome library of one of the Bcc strains surveyed was screened to identify the source of Bcc inhibitory activity. More than 10,000 genomic clones were screened on an appropriate sensitive lawn. As a positive control for the library construction and screening methods, a Pa strain, whose genome has been sequenced and is known to encode pyocin S2, was screened in an identical manner. It is surprising that no bacteriocin gene was identified from Bcc genome, while the first and the only gene detected from Pa PAO1 library was pyocin S2 as the source of inhibitory activity. Five inhibitory clones were identified from the Bcc strain. Sequence analysis determined that the Bcc clones are responsible for primary metabolic pathways including amino acid metabolism, carbohydrate metabolism, degradation of aromatic compounds, and bacterial apoptosis. Surprisingly, no bacteriocin-like genes were detected.

This library screen outcome was quite surprising. To our knowledge, no other Gram negative bacterial species examined at this level of detail has failed to reveal the presence of one or more recognizable bacteriocins. This result suggests that we should

not extrapolate from the existing surveys of bacteriocin-based inhibition. It is possible that some, or even much, of the inhibitory activities of Gram-negative bacteria are not bacteriocin-based. Until further molecular characterizations are carried out, it is premature to conclude the presence of bacteriocins, as was done even in the Bcc literature (Govan & Harris, 1985).

How then does Bcc produce inhibitory activity with the genes identified in the library screen? It is difficult to answer this question. First, we identified a number of primary metabolism genes, which are involved at specific steps of amino acid, carbohydrate metabolisms, and degradation of aromatic compounds. The remaining genes involved in these pathways are not isolated from our genomic clones. One explanation would be the proteins expressed in *E. coli* could be involved the very same metabolic pathways used in *E. coli*, and thus provided *E. coli* a growth advantage over the Pa lawn. Second, some of these genes are represented partially in *E. coli*, so the genomic clones do not express some of these genes (such as GGDEF domain, amidohydrolyse II) as functional proteins. Thus, the complete DNA sequence of these genes needs to be cloned to prove the Bcc inhibitory phenotype observed in genomic clones. Third, one apoptosis gene (Entericidin AB) was identified in clone 7. Like bacteriocins, entericidinAB is a toxin-antitoxin complex, whose open reading frame is organized similar to colicins of enteric bacteria and pyocins of Pa (Figure 3-11). A toxin gene and immunity gene are organized consecutively and transcribed together. Both pore former colicins and entericidin disrupt membrane stability and lyse the bacterial cells (Bishop *et al.*, 1998; Michel-Briand & Baysse, 2002; Riley & Wertz, 2002a). However, there is no sequence similarity between entericidin AB and pore former

colicins. Further, there is no literature suggesting that *E. coli* use entericin to inhibit bacterial growth. Instead, it has suggested that entericidins are produced by in a population, which is exposed to nutrient limitation (stationary phase). Thus, bacteria lyse themselves to provide nutrients to the remaining members of the population (Bishop *et al.*, 1998). Another interesting gene was glucoamylase from clone 4 of *B. multivorans* (ATCC17616). Glucoamylase is an extracellular enzyme, common in fungus *Aspergillus*. This enzyme hydrolyzes starch into glucose (Sauer *et al.*, 2000). More importantly, glucoamylase has a second function, which is induction of a fungal toxin (aflatoxin) biosynthesis (Mellon *et al.*, 2007). Bcc may be using this secondary function to induce similar toxin genes in its genome to inhibit other species. Finally, the inhibitory activity of the clones detected might be artifacts of the genomic library construction. Cloning and expression of identified Bcc genes is required to further verification and characterization of inhibitory activity in Bcc.

One goal of the present study is to better understand the nature of bacterial interactions in the CF lung in order to identify new targets for therapeutic intervention. In recent years, bacteriocins have been considered for therapeutic use in human health (Gillor *et al.*, 2005; Gillor & Ghazaryan, 2007). For example, bacteriocins from *Staphylococcus aureus* are used to treat skin infections. Bacteriocins from *Bacillus subtilis* are employed to treat vancomycin-resistant bacterial infections, while bacteriocin from *Lactococcus lactis* are effective in the treatment of peptic ulcers and bacteriocin from enteric bacteria are used to treat urinary tract infections (Gillor *et al.*, 2004; Gillor *et al.*, 2005). Future studies should focus on the efficacy of S and RF type pyocins on *in vitro* and *in vivo* single and mixed species biofilms of Pa and Bcc strains.

Finally, this study showed that Bcc strains might use primary metabolism to become more competent against Pa strains. We isolated five clones of *B. multivorans* (ATCC17616), which possessed inhibitory activity on a Pa lawn. Additional study needs to be done on these genomic clones to further prove this conclusion. First, genes possessed by these five clones should be cloned separately and the effect of protein expression on the Pa lawn should be investigated. Second approach could be creation of deletion mutants of Bcc for the genes detected in the genome library study. Finally, genome libraries of Bcc clones should be tested on Bcc lawns to observe the differences between within and between inhibitory activities.

One final study could be suggested on Pa inhibitory activity. It has been revealed that majority of Pa inhibitory activity is due to the presence of S- and RF pyocins. Indeed, we identified pyocin S2 in one genomic clone of Pa PAO1, which inhibits a Pa lawn. However, there are other pyocin-like genes annotated in Pa PAO1 genome. Interestingly, we only detected one S-pyocin (pyocin S2) in one Pa PAO1 genomic clone. This result may be due to sensitivity phenotype of the lawn. The lawn used to screen Pa PAO1 genomic library might be resistant to the other pyocin-like genes possessed in Pa PAO1 genome. Various lawns might be used to screen genomic clones to identify other pyocins in Pa PAO1 genome as well as in clinical Pa strains.

The treatment of Pa and Bcc chronic lung infections is a problem faced in the treatment of chronic CF lung infections. These species will survive and infect CF lungs as long as CF patients have defective CFTR on their epithelial cells. Antibiotics are the only available treatment option, but the multiple drug resistant nature of these species and emerging drug resistance to current antibiotics will make this option obsolete in the near

future. This study revealed sources of inhibitory agents, which are responsible for within and between species inhibitory activities of Pa and Bcc strains. Nature of bacterial interactions in the CF lung may reveal new targets for therapeutic intervention.

**APPENDIX A**

**CLINICAL PSEUDOMONAS AND BURKHOLDERIA STRAIN**

**COLLECTION**

Paired isolates <sup>1</sup>					
Study	Strain		Study	Strain	
ID	ID	Species	ID	ID	Species
1	03-195-0952	<i>B. cenocepacia</i>	19	181-0-63	<i>P. aeruginosa</i>
2	03-104-0962	<i>B. multivorans</i>	8	015-4-1076	<i>P. aeruginosa</i>
3	03-303-0655	<i>B. dolosa</i>	12	159-4-1077	<i>P. aeruginosa</i>
3	03-303-0655	<i>B. dolosa</i>	13	159-4-1078	<i>P. aeruginosa</i>
3	03-303-0655	<i>B. dolosa</i>	14	159-4-1178	<i>P. aeruginosa</i>
3	03-303-0655	<i>B. dolosa</i>	15	159-4-1606	<i>P. aeruginosa</i>
3	03-303-0655	<i>B. dolosa</i>	16	159-4-1607	<i>P. aeruginosay</i>
3	03-303-0655	<i>B. dolosa</i>	17	159-4-1674	<i>P. aeruginosa</i>
3	03-303-0655	<i>B. dolosa</i>	18	159-4-1675	<i>P. aeruginosa</i>
4	03-287-0857	<i>B. multivorans</i>	10	080-4-34	<i>P. aeruginosa</i>
4	03-287-0857	<i>B. multivorans</i>	11	080-4-1247	<i>P. aeruginosa</i>
5	03-350-1649	<i>B. dolosa</i>	20	203-1-1394	<i>P. aeruginosa</i>
6	03-318-1184	<i>B. dolosa</i>	9	030-7-33	<i>P. aeruginosa</i>
7	03-219-0968	<i>B. multivorans</i>	21	228-0-1396	<i>P. aeruginosa</i>

Unpaired isolates					
Study ID	Strain ID	Species	Study ID	Strain ID	Species
22	085-3-1211	<i>P. aeruginosa</i>	46	95-258-0446	<i>B. cenocepacia</i>
23	093-8-1498	<i>P. aeruginosa</i>	47	02-228-1429	<i>B. cenocepacia</i>
24	180-8-72	<i>P. aeruginosa</i>	48	03-342-1396	<i>B. cenocepacia</i>
25	180-8-75	<i>P. aeruginosa</i>	49	03-281-1317	<i>B. dolosa</i>
26	195-1-271	<i>P. aeruginosa</i>	50	03-277-0282	<i>B. dolosa</i>
27	195-1-272	<i>P. aeruginosa</i>	51	03-303-1206	<i>B. dolosa</i>
28	202-3-1393	<i>P. aeruginosa</i>	52	03-338-1539	<i>B. dolosa</i>
29	202-3-1504	<i>P. aeruginosa</i>	53	03-339-1377	<i>B. dolosa</i>
30	217-7-1505	<i>P. aeruginosa</i>	54	03-336-0575	<i>B. multivorans</i>
31	217-7-1506	<i>P. aeruginosa</i>	55	04-021-0261	<i>B. dolosa</i>
32	217-7-1507	<i>P. aeruginosa</i>	56	04-032-0605	<i>B. dolosa</i>
33	239-7-1215	<i>P. aeruginosa</i>	57	04-053-0423	<i>B. dolosa</i>
34	276-6-1714	<i>P. aeruginosa</i>	58	03-255-0518	<i>B. multivorans</i>
35	276-6-1715	<i>P. aeruginosa</i>	59	03-289-0554	<i>B. multivorans</i>
36	283-1-88	<i>P. aeruginosa</i>	60	03-338-0277	<i>B. multivorans</i>
37	283-1-89	<i>P. aeruginosa</i>	61	03-350-0955	<i>B. multivorans</i>
38	325-5-1421	<i>P. aeruginosa</i>	62	03-364-0829	<i>B. multivorans</i>
39	325-5-1428	<i>P. aeruginosa</i>	63	03-365-0241	<i>B. multivorans</i>
40	325-5-1533	<i>P. aeruginosa</i>	64	04-050-0866	<i>B. multivorans</i>
41	327-6-1422	<i>P. aeruginosa</i>	65	04-065-1420	<i>B. multivorans</i>

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42	327-6-1423	<i>P. aeruginosa</i>	66	03-260-0635	<i>B. vietnamiensis</i>
43	327-6-1424	<i>P. aeruginosa</i>			
44	327-6-1518	<i>P. aeruginosa</i>			
45	327-6-1519	<i>P. aeruginosa</i>			

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Paired isolate<sup>1</sup>: Seven Bcc strains are isolated as pairs with 14 Pa strains. Two Bcc strains (Study ID # 3 and #4) have multiple corresponding Pa isolates.



## APPENDIX B

### RESULT OF PHENOTYPIC BACTERIOCIN ASSAY OF CLINICAL PSEUDOMONAS AND BURKHOLDERIA STRAINS

Producer Strain #	Lawn # →															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
2	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
3	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
4	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
5	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
6	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
7	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	S
8	S	R	S	S	S	S	S	R	R	S	S	S	S	R	R	S
9	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
10	R	R	R	S	S	R	R	S	R	R	S	R	R	S	R	R
11	R	R	R	R	R	R	R	R	R	R	S	S	S	R	R	S
12	R	R	R	R	S	R	R	S	R	R	S	R	R	S	R	R
13	R	R	R	R	S	S	R	S	R	R	R	R	R	S	R	R
14	R	R	R	S	S	S	R	S	R	R	R	R	R	R	R	R
15	S	R	R	S	S	S	R	S	R	R	S	R	S	S	R	S
16	R	R	R	S	S	R	R	S	R	R	S	R	S	S	R	R
17	R	R	R	S	S	S	R	S	R	R	S	R	R	R	R	R
18	S	R	S	S	S	S	R	S	R	R	S	R	S	S	R	S
19	R	R	S	R	S	R	R	R	R	R	S	R	R	R	R	R
20	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
21	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S
22	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
23	R	R	R	R	R	S	R	S	R	R	S	R	S	S	R	R
24	R	R	R	R	R	R	S	S	R	R	S	R	R	S	R	R
25	R	S	R	S	R	R	R	S	R	R	S	R	R	S	R	R
26	R	R	R	R	R	R	R	S	R	R	S	R	R	S	R	R
27	R	R	R	R	R	R	R	R	R	R	R	S	S	R	R	S
28	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
29	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
30	R	R	R	S	S	R	R	S	R	R	S	R	R	S	R	R
31	R	R	R	S	R	R	R	S	R	R	S	R	R	S	R	R
32	R	R	R	R	R	R	R	R	R	R	R	S	S	R	R	S
33	R	R	R	R	R	R	R	S	R	R	R	R	R	S	S	R
34	R	R	S	S	S	R	R	S	R	R	S	R	R	R	R	R
35	R	R	S	R	S	S	R	S	R	R	S	R	R	S	R	R
36	R	S	S	S	S	S	S	R	R	S	R	R	S	S	R	R
37	R	R	R	R	R	R	R	S	R	R	S	R	R	S	R	R
38	R	S	R	R	R	R	R	S	R	R	S	R	R	S	R	R
39	R	R	R	R	R	R	R	S	R	R	S	R	R	S	R	R
40	R	R	R	R	R	R	R	S	R	R	S	R	R	S	R	R
41	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S
42	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
43	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
44	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
45	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	S
46	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R	S
47	S	S	S	R	S	S	R	R	R	R	R	R	R	S	R	R
48	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
49	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
50	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
51	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
52	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
53	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R
54	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
55	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
56	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
57	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R
58	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R	S
59	R	R	R	R	R	R	R	R	S	S	R	R	R	R	R	R
60	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
61	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
62	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S
63	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S
64	R	R	R	R	S	R	R	R	R	R	R	R	R	R	S	S
65	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R
66	R	R	R	R	S	S	R	R	S	R	R	R	R	R	R	S

Producer Strain #	Lawn # →															
	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
1	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
2	R	R	R	R	R	R	S	R	R	R	R	R	S	R	R	R
3	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
4	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R
5	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
6	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
7	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
8	S	R	R	S	S	S	S	R	R	R	S	R	R	R	R	S
9	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
10	R	S	R	S	S	S	S	R	R	R	S	S	R	R	R	S
11	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
12	R	R	R	S	S	S	R	R	R	R	S	S	R	R	R	S
13	R	R	R	S	S	S	R	R	R	R	R	S	R	R	R	R
14	R	R	R	R	S	S	R	R	R	R	S	R	R	R	R	R
15	R	R	R	S	R	S	R	S	R	S	S	S	S	S	S	R
16	R	R	R	S	S	S	R	R	R	R	S	S	R	R	R	S
17	R	R	R	R	S	S	R	R	R	S	S	S	R	R	R	S
18	R	R	R	S	R	S	R	S	S	S	S	S	R	S	S	R
19	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R	S
20	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
21	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
22	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
23	R	R	R	R	S	S	R	R	R	S	S	S	R	R	R	S
24	R	R	R	R	S	S	R	R	R	S	S	S	R	R	R	S
25	R	R	R	S	S	S	S	R	R	S	R	S	S	R	R	S
26	R	R	R	R	S	S	S	R	R	R	S	S	R	R	S	S
27	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
28	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
29	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
30	R	R	R	S	S	S	R	R	R	S	S	R	R	R	R	S
31	R	R	R	S	S	S	R	R	R	R	S	S	R	R	R	S
32	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
33	R	S	R	S	S	S	S	R	R	S	S	S	R	R	R	S
34	R	R	R	S	S	R	R	R	R	R	R	S	R	R	R	S
35	R	R	R	R	S	S	S	R	R	R	S	S	R	R	R	S
36	R	S	S	S	S	R	R	R	S	S	S	R	R	S	S	S
37	R	R	R	R	S	S	S	R	R	R	S	S	R	R	R	S
38	R	R	R	R	S	S	S	R	R	S	S	S	R	R	S	S
39	R	R	R	R	S	S	S	R	R	S	S	R	R	R	R	S
40	R	R	R	R	S	S	S	R	R	S	S	S	R	R	R	S
41	S	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R
42	S	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R
43	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R
44	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R
45	S	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R
46	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R	R
47	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R	R
48	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
49	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
50	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R
51	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R
52	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
53	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
54	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
55	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
56	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
57	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
58	R	R	R	S	R	R	R	S	R	R	R	R	S	R	R	R
59	S	R	R	R	S	R	R	R	R	R	S	R	R	R	R	R
60	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
61	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
62	R	S	R	R	R	R	R	R	R	R	R	S	R	R	R	R
63	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R
64	R	R	R	R	S	R	R	R	R	R	R	R	R	R	S	R
65	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R
66	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R

Producer Strain #	Lawn # →																
	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49
1	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
2	R	R	R	R	R	R	R	R	R	S	R	R	R	S	R	R	R
3	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
4	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R
5	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
6	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
7	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R
8	R	S	R	S	R	R	R	R	S	S	R	S	S	S	R	S	S
9	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S
10	R	R	R	S	R	R	R	R	S	S	S	S	S	R	R	S	S
11	R	S	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R
12	R	R	R	R	S	R	R	S	S	S	S	S	S	R	R	R	R
13	R	R	R	R	R	R	R	R	S	R	S	S	R	R	R	R	S
14	S	R	R	R	R	R	R	S	S	S	S	S	R	R	R	R	S
15	S	S	R	S	S	S	S	S	S	S	S	S	S	R	R	S	S
16	S	R	R	R	S	S	S	S	S	S	S	S	S	R	R	R	S
17	R	R	R	R	S	S	S	R	S	S	S	S	S	R	S	R	S
18	S	S	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S
19	R	R	R	R	R	R	R	S	S	S	S	R	R	R	R	R	R
20	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R
21	R	R	R	R	R	R	R	S	R	S	R	R	R	R	R	S	S
22	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
23	R	R	R	S	S	S	S	R	S	S	S	S	S	S	R	R	R
24	R	R	R	S	S	S	S	S	S	S	S	S	S	S	R	R	S
25	R	S	R	S	S	S	R	R	S	S	S	S	S	S	R	R	R
26	R	R	R	S	S	S	S	R	S	S	S	S	S	S	R	R	R
27	R	S	R	S	R	R	R	R	R	R	R	R	R	S	R	R	R
28	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
29	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
30	R	R	R	R	S	S	S	R	S	S	S	S	S	R	R	R	R
31	R	S	R	S	S	S	S	R	S	S	S	S	S	S	R	R	R
32	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R
33	R	R	R	S	S	S	S	R	R	R	S	S	S	S	R	R	R
34	R	R	R	S	R	R	S	R	S	S	S	S	S	R	R	S	R
35	R	R	R	S	R	R	R	R	S	S	S	S	S	R	R	R	R
36	R	S	R	R	S	R	S	R	S	R	S	S	S	S	R	R	R
37	R	R	R	R	R	R	R	R	S	S	S	S	R	R	R	R	R
38	R	S	R	S	R	R	R	R	S	S	S	S	S	R	R	R	R
39	R	R	R	R	S	R	R	R	S	S	S	S	S	R	R	R	R
40	R	R	R	R	S	R	R	R	S	S	S	S	S	R	R	R	R
41	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
42	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
43	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
44	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
45	R	S	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R
46	R	S	S	R	S	R	R	R	R	R	R	R	R	S	R	R	R
47	R	R	R	R	R	S	S	R	R	R	S	S	R	R	R	S	S
48	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
49	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
50	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
51	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
52	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
53	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S
54	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
55	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S
56	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
57	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R
58	R	R	R	R	R	S	R	R	R	R	R	R	R	S	R	S	R
59	R	R	S	R	R	S	R	R	R	R	R	R	R	R	S	R	R
60	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R
61	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
62	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R
63	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R
64	R	R	R	R	R	R	S	R	R	R	R	R	R	S	R	R	R
65	R	R	R	R	R	R	S	R	R	R	R	R	R	S	R	R	R
66	S	R	R	R	R	R	R	R	R	R	R	R	R	S	S	R	R

Producer Strain #	Lawn # →																
	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66
1	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
2	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
3	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
4	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
5	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
6	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
7	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
8	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S
9	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
10	S	S	S	S	S	S	S	S	S	R	S	S	S	S	R	R	S
11	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
12	S	S	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R
13	S	S	S	R	R	S	S	S	S	S	R	S	S	S	R	R	S
14	R	S	S	R	R	S	S	R	R	R	R	R	R	S	R	R	S
15	S	S	S	S	S	S	S	S	S	R	R	S	S	S	S	S	S
16	S	S	S	R	R	S	S	R	R	R	R	S	S	R	R	S	R
17	S	S	S	R	R	S	S	S	R	R	R	R	S	S	R	R	R
18	S	S	S	S	R	S	S	S	S	R	S	S	S	S	S	R	S
19	S	S	S	R	R	R	S	R	R	R	S	R	R	R	R	R	R
20	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R
21	S	S	S	R	R	S	S	S	R	R	R	S	S	R	R	R	S
22	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
23	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R
24	R	S	R	R	R	S	R	R	R	R	R	R	R	R	R	S	R
25	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R
26	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R
27	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R
28	R	R	S	R	R	S	R	R	R	R	R	R	R	R	R	R	R
29	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
30	R	S	S	R	R	S	S	S	R	R	R	R	R	R	R	R	R
31	R	S	R	R	R	S	S	S	R	R	R	R	R	S	R	R	R
32	R	R	S	R	R	R	R	S	R	R	R	R	R	R	R	R	R
33	R	R	R	R	R	S	R	R	R	S	R	R	R	R	R	R	R
34	S	S	S	R	R	S	S	S	R	R	S	S	S	S	S	R	S
35	R	S	S	R	S	S	S	S	R	R	S	R	S	S	R	R	S
36	R	S	S	R	R	S	S	S	S	R	S	R	S	S	S	S	S
37	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R
38	R	R	R	R	S	S	R	R	R	R	S	R	R	R	R	R	R
39	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R
40	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R
41	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
42	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
43	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
44	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
45	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R
46	R	R	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
47	R	R	S	S	R	R	S	S	S	R	S	S	S	R	R	S	R
48	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
49	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
50	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
51	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
52	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R
53	R	S	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R
54	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
55	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
56	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
57	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
58	S	S	R	S	R	S	R	R	S	R	R	R	R	R	R	R	R
59	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R
60	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
61	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
62	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
63	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
64	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
65	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
66	R	R	S	R	R	R	R	R	R	R	R	R	S	R	R	R	R

## APPENDIX C

### PRIMER PAIRS USED TO SCREEN PYOCIN GENES

Pyocin	Primer pair	Primer sequence (5' → 3')	Position*	Amplicon size (bp)	Amplicon identity <sup>‡</sup>	
Pyocin S1	1-F	GATATTGCAGTTTGATTGCTGTGTA	249	377	P, I	
	1-R	CATCTCGGAAGCTGAAGCTCGATCTC	625			
	2-F	ATGGCACGACCCATTGCTGACCTTA	370	256	I	
	1-R	CATCTCGGAAGCTGAAGCTCGATCTC	625			
	3-F	AGATCGGTGAACAAGCGGTGC	839			
Pyocin S2	3-R	ACAGCTATCCAGAATTGTTCCCG	2000	1162	I, III, IV	
	4-F	GATATTGAAGTTGATTGCAGT	236			
	4-R	TGAGGAAAGTCTGAAGCCGT	757	522	P, I	
	5-F	ATGGCTGTCAATGATTACGAAC	354			
	4-R	TGAGGAAAGTCTGAAGCCGT	757			
Pyocin S1/S2#	6-F	TTACACCGGTGAAGGCTAC	1709/1906 (S1/S2)	518/521	III (S1)	
	6-R	TCACTTCCCTCCCTTGTGGAT	2226/2426 (S1/S2)			(S1/S2)
	7-F	TAGCAGATGGAGGCGGCGTTAC	2138/2338 (S1/S2)	354	(S1/S2)	IV, imm (S1)
	7-R	CTAACCGGCCTTAAAGCCAGGAAG	2491/2691 (S1/S2)	(S1/S2)		IV, imm (S2)
	8-F	ATGAAGTCCAAGATTCCG	2228/2428 (S1/S2)	264	(S1/S2)	imm (S1)
	8-R	CTAACCGGCCTTAAAGCCA	2491/2691 (S1/S2)	(S1/S2)		imm (S2)
	Pyocin S3	9-F	ATGGCTGATGCACCACCG	146	854	I, II
		9-R	TCGTTGACGATCTGCTTGAC	999		
10-F		ATCTGATCGTGATCTTCCCG	1989	458	III, IV	
10-R		TCAGTACCACCCCTGTTCTTTG	2446			
11-F		ATGGAGAAGAAGCTGATCGTT	2446	462	imm	
11-R		CTATTTAGAACCAAGAAGAGC	2907			
Pyocin AP41	12-F	GATATTGTAGTTTGTGTCGIG	395	416	P, I	
	12-R	GGCAGTGTTTCGTTGGATTTTT	810			
	13-F	ATGAGCGACGTTTTTGACCTT	550	261	I	
	13-R	GGCAGTGTTTCGTTGGATTTTT	810			
	14-F	GAGGAGAAACTGGAGCAACG	1595	280	II, III	
	14-R	TTGAGAGTCCTGGAAACGCT	1874			
	14-F	GAGGAGAAACTGGAGCAACG	1595	1289	II, III, IV	
	15-R	TTATTTCTCCTTACGTTTA	2883			
	16-F	ATGGATATTAATAAACCTT	2886			
	16-R	TTAGCCAGCCTTGAAGCCA	3158			
Pyocin R	17-F	AGTTGATCGGCTTCTGGCCAGG	6245	307	PRF10	
	17-R	TGCGTCCACTCGACCAGCCAG	6551			
Pyocin F	18-F	AAGCCTGGACAGTTCGGCACTGA	21178	224	PRF31	
	18-R	TCAGTAGTGCTTCGTTGAGCTTGG	21401			
	19-F	AGGTTCGGTTTCCGTCACGCTG	2992	394	PRF38	
19-R	GTAACCTCAAGGCGTTGGCCGG	3385				

\* NCBI accession numbers: D12707 (pyocin S1); D12708 (pyocin S2); X77996 (pyocin S3); D12705 (pyocin AP41); AB030825 (R pyocin); AB046379 (F pyocin).

<sup>‡</sup> Amplicon identity: I-IV: Domains I-IV; P: P box; imm: immunity gene; PRF: Open reading frame numbers of pyocin R and F genes.

# Pyocin S1/S2: Primer sets-6F/R-8F/R are applicable for both S1 and S2 pyocins since S1 and S2 have similar sequences for region IV and immunity.

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