UNIVERSITY OF CALGARY

Quorum-sensing in *Burkholderia cenocepacia* and *Burkholderia vietnamiensis*

by

Rebecca J. Malott

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF MICROBIOLOGY AND INFECTIOUS DISEASES

CALGARY, ALBERTA

JANUARY, 2007

Your file Votre référence
ISBN: 978-0-494-26244-3
Our file Notre référence
ISBN: 978-0-494-26244-3

Abstract

The *Burkholderia cepacia* complex (Bcc) is a group of closely related bacterial species that are found throughout the environment and are known for their biotechnological applications. The Bcc is also recognized as emerging opportunistic pathogens. The Bcc utilizes *N*-acyl-homoserine lactone (AHL)-mediated quorum-sensing systems for regulation of diverse physiological processes, including many that are involved in virulence. The *cepIR* system is distributed throughout the Bcc. In addition to *cepIR, B. cenocepacia* strains that possess the *cenocepaica* island contain *cciIR* and *B. vietnamiensis* strains contain *bviIR*. The main objective of this thesis was to define a role for these additional systems and determine their relationship to *cepIR*.

The *cciIR* genes were determined to comprise a functional quorum-sensing system. AHL production profiles indicated that the major product of CciI is *N*-hexanoyl-L-homoserine lactone (HHL). The *cciI* and *cciR* genes were found to be co-transcribed. CciR negatively regulates the expression of *cciIR* and *cepI*. Transcriptional analysis and AHL production profiles of quorum-sensing mutants established that *cepIR* and *cciIR* are arranged in a hierarchy, with CepR being essential for expression of *cciIR*. The *cciIR* system was shown to be involved in the regulation of protease production, biofilm formation and swarming motility. An additional LuxR homologue, BCAM0188, was identified and determined to be specific to *B. cenocepacia* strains. BCAM0188 is potentially a part of the *B. cenocepacia* quorum-sensing network.

In the environmental *B. vietnamiensis* strain G4 it was determined that BviR positively regulates the expression of *bviI*. Unlike the *cepIR* genes in other Bcc species, *cepIR* is not auto-regulated in G4. The *cepIR* and *bviIR* systems are arranged in a

hierarchy, where CepR is required for expression of *bviI*. The regulatory network in the clinical strain PC259 is similar to G4, except CepR positively regulates *cepI* and negatively regulates *cepR*. AHL production and *bviI* expression in *B. vietnamiensis* isolates are variable. Three of seven strains did not express *bviI*. Molecular investigations indicated that an additional regulatory element may be involved in the regulation of *bviIR* in certain *B. vietnamiensis* strains. These studies provide further understanding of the intricate role quorum-sensing plays in Bcc gene regulation.

Acknowledgements

This chapter of my scientific career would not have been a success without the support of many collegues, friends and family members. Foremost, I would like to express my appreciation to my supervisor, Dr. P. Sokol for the opportunity study in her laboratory. I am most grateful for her mentorship. I would like thank, for their time and knowledgeable input to my studies, the members of my supervisory committee; Dr. M.G. Surette, Dr. D.G. Storey and Dr. G. Chaconas. I would like to acknowledge Dr. R. Devinney, Dr. R.J. Turner Dr. M.R. Parsek for serving as external examiners at my candidacy and/or final defence. I am appreciative of project funding support from the Canadian Cystic Fibrosis Foundation and studentship funding from the Alberta Heritage Foundation for Medical Research.

I have been very fortunate to work alongside an excellent group of scientists in the Sokol laboratory and the Bacterial Patogenesis Research Group. I would like to specifically thank M.B. Visser and C. Kooi for their guidance and invaluable technical assistance. K.L. Tomlin, G. Rammage, D.G. Storey and H. Ceri are acknowledged for their efforts towards our productive collaborative study on quorum-sensing in *B. cenocepacia* biofilms. I would like to thank A. Baldwin and E. Mahenthiralingam for discovering the *cciIR* system and inspiring the major part of my thesis. I would like to acknowledge the members of the International *Burkholderia cepacia* working group for their stimulating exchange of scientific ideas.

Finally, I am deeply grateful to my family and friends who have been an incredible support during my academic career. I'd like to thank my mother Janet, my father Kenith, my brother Andy and his partner Tracey for all of their love and support.

To Carla, who helped me find my inner strength; my dear friend Edie, who showed me that the beaker is always half full; and my partner Mark who stood beside me for every success and challenge, I am forever grateful.

Dedication

In memory of my Grandfather; the person who showed me the joy of tinkering, and what marvels could be accomplished at a workbench.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

3-hydroxy-DHL	*N*-(3-hydroxydecanoyl)-L-homoserine lactone
3-hydroxy-OHL	*N*-(3-hydroxyoctanoyl)-L-homoserine lactone
3-oxo-deDHL	*N*-(3-oxotetradecanoyl)-L-homoserine lactone
3-oxo-DHL	*N*-(3oxodecanoyl)-L-homoserine lactone
3-oxo-doDHL	*N*- (3-oxododecanoyl)-L-homoserine lactone
3-oxo-HHL	*N*-(3oxohexanoyl)-L-homoserine lactone
AHL	*N*-acyl-homoserine lactone
Ap	ampicillin
Bcc	*Burkholderia cepacia* complex
BCESM	*B. cepacia* epidemic strain marker
BHL	*N*- (butyryl)-L-homoserine lactone
bp	base pair
CAS	chrome azurol S
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane regulator
CGD	chronic granulomatous disease
Cm	chloramphenicol
cm	centimeter
CPM	counts per minute
CPS	counts per second
CTAB	hexadecyltrimethyl ammonium bromide
Da	dalton
ddH_20	double distilled water
DHL	*N*-decanoyl-L-homoserine lactone
DNA	Deoxyribonucleic acid
doDHL	*N*-dodecanoyl-L-homoserine lactone
DPD	4,5-dihydroxy-2,3-pentonedione
EDTA	ethylenediamine tetraacetic acid
ET12	electrophoretic-type 12
g	gram
Gm	gentamicin
h	hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HHL	*N*-hexanoyl-L-homoserine lactone
IL-8	interleukin-8
kb	kilobase
kDa	kilodalton
Km	kanamycin
kV	kilovolt
l	liter
LB	Luria-Bertani
LC-MS	Liquid Chromatography-Mass Spectrometry
LPS	lipopolysaccharide
M	molar

Mb	megabase
MLRT	multilocus restriction typing
mM	millimolar
mRNA	messenger ribonucleic acid
NCBI	National Center for Biotechnology Information
nM	nanomolar
nm	nanometer
no.	number
^{o}C	degrees centigrade
OD	optical density
OHL	*N*-octanoyl-L-homoserine lactone
ONPG	*o*-nitrophenol-β-D-galactosidase
PCR	polymerase chain reaction
PDHC	Philadelphia – District of Columbia
PIA	*Pseudomonas* isolation agar
PTSB	peptone trypticase soy broth
RAPD	Random amplified polymorphic DNA
RNA	ribonucleic acid
RT-PCR	reverse –transcriptase PCR
SDS	sodium dodecyl sulphate
Sp	spectinomycin
Tc	tetracycline
TCE	trichloroethylene
Tp	trimethoprim
Tris	trishydroxymethylaminomethane
TSB	trypticase soy broth
TSB-DC	trypticase soy broth-dialysed and chelated
UV	ultraviolet
v/v	volume per volume
x g	times gravity
X-gal	5-bromo-4-chloro-3-indoly-β-galactopyranoside
Ω	ohm
µF	microfarad
µg	microgram
µl	microlitre
µm	micrometer
µm	micomolar

Chapter One: Introduction

1.1 The *Burkholderia cepacia* complex

The *B. cepacia* complex (Bcc) is a group of nine closely related bacterial species with extreme genetic diversity and metabolic versatility. Bcc bacteria are Gram-negative, non-spore forming bacilli that are found in soil, water, and the rhizosphere of plants. The multifarious Bcc exist in both beneficial and detrimental associations with the environment and humans (127, 156). Over the past twenty-five years, the Bcc has emerged as a group of opportunistic pathogens of particular importance in people with cystic fibrosis (CF) and chronic granulomatous disease (CGD) (88, 149). Bcc pneumonia and septicemia are life threatening for CGD patients (97). For CF patients infected with Bcc, high morbidity and mortality rates have been documented (128). Clinical risks of Bcc infection include the innate multi-resistance to available antibiotics and patient to patient spread of transmissible strains (72, 73, 123). The beneficial biocontrol, bioremediation and plant growth promoting capabilities of Bcc bacteria could make a large ecological and economic impact by providing alternatives to current chemical based strategies, however the risk of Bcc in CF patients has severely restricted their use (22, 30, 156). Genotypically identical Bcc strains have been isolated from both CF patients and environmental sources, suggesting that the acquisition of Bcc bacteria adapted for infection in humans occurs directly form the natural environment (117).

1.1.1 Taxonomy and nomenclature

B. cepacia, previously know as *Pseudomonas cepacia*, was originally described in 1950 by W.H. Burkholder as a phytopathogen that caused soft rot of onions (15). Bacteria that were initially identified as *B. cepacia* were more recently distinguished

from the type species using a polyphasic approach (31, 123) and subsequent identification of species-specific features allowed formal bionomial designation of the nine species that currently comprise the Bcc. The Bcc includes: *B. cepacia* (214), *B. multivorans* (214), *B. cenocepacia* (213), *B. stabilis* (215), *B. vietnamiensis* (214), *B. dolosa* (217), *B. ambifaria* (29), *B. anthina* (212), and *B. pyrrocinia* (212). Phylogenetic analysis of the *recA* gene has indicated that *B. cenocepacia* can be further classified into at least two distinct strain lineages, A and B (124). Each of the species comprising the Bcc has been isolated from environmental sources and CF patients (30).

1.1.2 Genome

The extreme metabolic capacity of Bcc strains is attributed to a large genetic make up (107). The total genome size of Bcc species is extremely variable, ranging from 4 to 9 Mb on two to four large chromosomes and one or more plasmids (156). The Wellcome Trust Sanger Institute completed the genome sequence of *B. cenocepacia* J2315 and determined that it has a genome size of 8.065 Mb divided among three chromosomes (3.87 Mb, 3.217 Mb and 92.7 kb) (http://www.sanger.ac.uk/Projects/B_cepacia/). The Joint Genome Institute began a draft genome sequence of *B. vietnamiensis* G4 and predicts the genome to be 8.5 Mb (http://genome.jgi-psf.org/draft_microbes/bur08/bur08.home.html).

In addition to the large nucleic acid potential, Bcc species are also littered with an extensive array of insertion sequences that may be involved in genomic rearrangements, contributing to the evolution and adaptability of the Bcc (107). The bacteriophage, BcepMu, has been identified in the *B. cenocepacia* J2315 genome that contains potential virulence factors (196). A survey of 33 Bcc species determined that BcepMu is only

present in *B. cenocepacia* strains belonging to the ET12 transmissible lineage, with the exception of strain K56-2 (196). The J2315 genome also contains multiple genomic islands that were acquired by horizontal gene transfer. These acquired islands comprise ten percent of the total genome (127). The *cenocepacia* island, was the first island to be extensively characterized. It is a 31.7 kb low GC content genomic island flanked by 13 bp of imperfect direct repeats and contains thirty-five predicted open reading frames involved in both virulence and metabolism (8).

1.2 Bcc in the environment

1.2.1 Interactions with plants

Bcc species are able to promote plant growth through the production of the plant growth hormone indoleacetic acid, and by fixing atmospheric nitrogen (22). *B. vietnamiensis* strains are able to fix atmospheric nitrogen and enhance crop yields presumably by increasing the available nitrogen in the rhizosphere (63). Inoculation of rice with *B. vietnamiensis* significantly increases grain yield and is potentially an economic alternative to nitrogen based fertilizers (204).

The agricultural damage caused by Bcc on plants is minimal compared to other *Burkholderia* phytopathogens such as *B. glumae* that has recently emerged as the most important bacterial pathogen of rice in Japan, Korea and Taiwan (30). The commercial crop that is negatively affected by Bcc bacteria are onions, in which Bcc bacteria cause soft rot or 'sour skin' disease (15). A plasmid-encoded pectate hydrolase, PehA, has been identified as the principal secreted virulence factor in these onion infections (68). This enzyme cleaves the major cell wall component, polygalacturonate, leading to maceration of the onion tissue.

1.2.2 Bioremediation and biocontrol applications

Individual Bcc strains complete metabolic processes that make the strains employable for biocontrol and bioremediation purposes. *B. cepacia, B. cenocepacia, B. ambifaria* and *B. pyrrocinia* produce several antimicrobial agents that protect commercially valuable crop plants from root rot and "damping off" of seedlings (22, 156). *B. ambifaria* strain BC-F suppresses the growth of soil-borne fungal pathogens that cause cucumber, pepper, tomato and soybean diseases (113, 133). Bcc biocontrol strains have been proven effective during *in vitro* and field tests and have the potential to replace chemical control measures (22, 30, 156).

The most notable bioremediation strain of the Bcc is *B. vietnamiensis* G4. When induced by phenol or tryptophan, strain G4 produces *ortho*-monooxygenase that co-degrades the groundwater contaminants toluene (179) and trichloroethylene (TCE) (152). Once a universal solvent, TCE is now a widespread environmental toxin (14). A constitutive *ortho*-monooxygenase mutant has been shown to efficiently eliminate TCE in groundwater (96).

1.3 Bcc as opportunistic human pathogens

The initial report of a Bcc infection in humans was in US military troops on a 19 day swamp training exercise in northern Florida who contracted jungle or swamp rot. The Bcc infection presented with macerated hyperkeratonic lesions on the soldier's toe webs (200). Bcc species are being recognised with increasing frequency as nosocomial pathogens (116). Hospital acquired Bcc pneumonia and bacteremia predominantly occur as a result of acute, point-source outbreaks (229). An outbreak of *B. cenocepacia* bacteremia in 53 patients resulted in a 47 percent mortality rate, illustrating the

devastating capacity of Bcc nosocomial infections (229). Bcc species are able to survive in disinfectants (148), hospital water (151), and ultrasound gel (86), and are thought to be widely distributed in the hospital setting.

The most prominent cases of Bcc infections are with CF and CGD patients. CGD is a genetic disorder that affects leukocyte function (97). The phagocytic cells of CGD patients are unable to generate bactericidal reactive oxygen radicals and must depend on non-oxidative means, such as cationic peptides, to kill invading bacteria. Bcc bacteria are resistant to non-oxidative killing, thus allowing for effective colonization of the lung, leading to aggressive pulmonary and bacteremic infections in CGD patients (192).

CF is a genetic disorder that results in defective chloride channel function of epithelial cells. Mutations and sequence variations within the CF transmembrane conductance regulator (CFTR) gene result in the disease (17). The most common mutation in the CFTR that results in disease is a single codon deletion of a phenylalanine at position 508, however, there have been over 1000 naturally occurring mutations described that result in CF (122). CF is currently the most common lethal autosomal recessive genetic disorder among Caucasians, with an occurrence of approximately 1 in 2500 births (17). Those who are homozygous for mutant alleles of the CF gene have severe defects in chloride ion transport. The imbalance in ion transport causes the epithelial cells to build-up sticky, dehydrated mucus in the airways of the lungs, the ducts of the pancreas and the male sex ducts. In the lungs, thickened airway secretions impair mucociliary clearance, increasing epithelial adherence of inspired microorganisms, resulting in persistent microbial colonization (72). Eighty to ninety-five percent of CF patients succumb to respiratory failure initiated by a chronic bacterial infection and

concomitant airway inflammation (122). The microbial pathogens most commonly isolated from CF patients are *Pseudomonas aeruginosa* and *Staphylococcus aureus* (37). The incidence of Bcc infection in CF patients is considerably lower. In the United States, 3 percent of CF patients are infected with Bcc species where 57 percent are infected with *P. aeruginosa* (37). Bcc bacteria, however, are the most problematic for persons with CF. Bcc infection is considered to be a significant independent risk factor for the morbidity and mortality in CF patients and is known to impact survival (115). It has also been reported that CF patients infected with Bcc bacteria experience a more rapid deterioration of lung function than those infected with *P. aeruginosa* (36).

1.3.1 Bcc epidemiology in CF patients

A broad spectrum of disease states has been reported for Bcc infections, including a chronic asymptomatic carriage, and accelerated decline in pulmonary function to a rapid, and often fatal, deterioration in lung function (88). The bacterial mechanisms contributing to Bcc infection are yet to be defined. There are also likely yet to be defined host factors that play a critical role in the outcome of Bcc infection in CF patients (115).

The Bcc species vary greatly in their pathogenic potential, frequency of colonization, transmissibility and geographic distribution. Epidemiological studies from Canada, the United States and Italy all report that *B. cenocepacia* is the most prevalent Bcc species recovered from CF patients (1, 118, 193). Eighty percent of Bcc isolates recovered from CF patients in Canada are *B. cenocepacia* (193). *B. multivorans* is the second most prevalent species, representing between 5 percent in Canada and 37 percent in the United States of Bcc infections isolated from CF patients (1, 118, 193). The remaining of the Bcc species constitute approximately 5 percent (1, 118, 193). There is a

low incidence reported for *B. vietnamiensis* infections in CF patients, comprising 1.6 percent of Canadian and 5.1 percent of United States CF patient Bcc isolates (118, 194). *B. vietnamiensis* was not isolated from CF patients in the Italian study (1). Present focus for *B. vietnamiensis* research is for its bioremediation and crop growth enhancement properties. However, an eleven patient epidemiological study in Brazil reported an equal incidence of *B. cenocepacia* and *B. vietnamiensis* isolates from CF patients, illustrating the potential of *B. vietnamiensis* as a CF pathogen (46).

In addition to *B. cenocepacia* being the dominant CF pathogen, the majority of Bcc strains identified as transmissible are *B. cenocepacia* (118, 123). The highly transmissible *B. cenocepacia* clones, including the electrophoretic-type 12 (ET12), Midwest and Philadelphia-DC (PHDC) transmissible lineages were identified in outbreaks in Europe and North America (20, 126).

Markers that are associated with the transmissibility of the *B. cenocepacia* ET12 lineage include a hybrid of insertion sequences IS*402* and IS*1356*, a cable pili which has been demonstrated to mediate adherence of the bacteria to respiratory mucins, and the *B. cepacia* epidemic strain marker (BCESM) (126, 171, 197, 205). The BCESM is a 1.4 kb DNA fragment that was identified during random amplified polymorphic DNA (RAPD) typing of Bcc isolates that had exhibited patient-to-patient spread (126). This marker is widely applied in infection control as a clinical risk marker (123, 128, 194). The BCESM contains a single coding sequence for a putative negative transcriptional regulator, *esmR* (126). Baldwin et al. (8), determined that the BCESM is part of the *cenocepacia* island. These three factors are not fully indicative of transmissibility for all lineages considering

the PHDC clone lacks the *cblA* gene and the BCESM (20), suggesting that the factors for patient to patient spread are lineage specific.

1.3.2 *B. cenocepacia* virulence factors

B. cenocepacia is the most adapted opportunistic pathogen of the Bcc, therefore understanding the virulence of *B. cenocepacia* has been the focus of many research efforts. The following is a brief description of the best characterized *B. cenocepacia* virulence factors.

B. cenocepacia is able to acquire iron from the environment via four siderophores: salicylic acid, ornibactin, pyochelin and cepabactin (143, 144, 186, 188). Salicylic acid and ornibactin are the most prevalent siderophores produced by the Bcc (39). Mutants in *orbA,* the gene encoding the outer membrane receptor for ferric-ornibactin, and *pvdA*, the gene encoding for L-ornithine *N*(5)-oxygenase, an enzyme involved in ornibactin biosynthesis, were determined to have decreased virulence in a chronic model of respiratory infection (187, 219).

B. cenocepacia produce at least two extracellular metaloproteases that contribute to virulence (35, 94). The *zmpA* gene encodes for a zinc metalloprotease that is capable of cleaving biologically relevant substrates including: α-1 proteinase inhibitor, α_2-macroglobulin, type IV collagen, human fibronectin and gamma interferon (93). The *zmpB* gene encodes for a zinc metalloprotease with a more broad specificity. ZmpB is also able to cleave: α-1 proteinase inhibitor, α_2-macroglobulin, type IV collagen and human fibronectin, as well as lactoferrin, transferrin, and human immunoglobulins (94).

The *B. cenocepacia* lipopolysaccharide (LPS) contributes to pathogenicity by inducing a strong immune response that can contribute to host cell damage and by

playing a role in antibiotic resistance (218). LPS is suggested to be at least partly responsible for the difference in the pathophysiology of Bcc bacteria compared to *P. aeruginosa,* since the activity of Bcc LPS is four to five times more endotoxic, and induces increased neutrophil burst activity and increased induction of interleukin-8 (IL-8) from epithelial cells (127). A recent study by Loutet et al. (119), identified the LPS core oligosaccharide biosynthesis genes *hldA* and *hldD,* and determined that the core LPS oligosaccharide is involved in the resistance of *B. cenocepacia* to antimicrobial peptides and the ability of the bacteria to colonize the host.

Two secretion systems that contribute to the virulence of *B. cenocepacia* have been identified (53, 201). A type IV secretion system is responsible for the secretion of a plant cytotoxic protein that contributes to the plant water soaking phenotype of onion infections (53). A type III secretion system is involved in virulence, since a mutant in *bscN* that encodes for an ATP-binding protein potentially involved in generating the energy required for virulence protein secretion is less virulent than wild-type *B. cenocepacia* (201).

B. cenocepacia is able to invade and survive in respiratory epithelial cells (16), macrophages (138), and in the amoebae, *Acanthamoeba polyphaga* (98, 137). The ability of bacteria to live within a host allows for the evasion of host defences, tissue destruction and systemic spread of the pathogen (108). Investigation by Lamothe et al. (98), determined that *B. cenocepacia* survives within the amoebae cell with minimal or no replication in an acidic vacuole that evades lysosomal fusion. This vacuole has been designated as the *B. cepacia* containing vacuole. It has been suggested that amoebae could act as a reservoir for *B. cenocepacia* in the soil (98, 137). A Bcc species has yet to

be recovered from an environmental amoeba isolate, however, the close relative to the Bcc, *Ralstonia* (previously *Burkholderia*) *pikettii* has been documented to live within amoebae in the environment (145).

Bcc strains are also capable of forming biofilms on inert surfaces as well as on well differentiated epithelial cells (34, 84, 175, 202). Biofilms are extensive, matrix enclosed microbial communities of significant clinical importance because they are resistant to antimicrobial agents and are often associated with persistent and chronic infections (89). Although a direct role for biofilm formation in *B. cenocepacia* infection has not been established, the biofilm mode of growth increases the resistance of *B. cenocepacia* to antibiotics *in vitro* (203). Bacterial motility has been implicated as an important factor for the initial stages of biofilm formation (153). *B. cenocepacia* isolates have demonstrated a swarming motility phenotype and it is believed that the biofilm formation and swarming motility phenotypes may contribute to the pathogenesis of the organism.

Recently, genomic approaches have been used to identify *B. cenocepacia* virulence factors. A modified signature-tagged mutagenesis screen identified 102 transposon mutants that were attenuated for survival in a chronic lung infection model (85). Genes identified were grouped into functional categories including: genes involved in cellular metabolism, regulation, DNA replication and repair, cell surface proteins and polysaccharide production. A suppression-subtractive hybridization approach was pursued to identify genes that were unique to *B. cenocepacia* that may account for the increased virulence of this Bcc species (12). Several *B. cenocepacia*-specific genes were identified and most of these genes were unique to the ET12 transmissible lineage.

The means by which virulence genes are regulated in *B. cenocepacia* is not yet well understood. However, it is known that the Bcc *cepIR* quorum-sensing system is involved in regulating virulence factors and contributes to the full virulence of *B. cenocepacia* (52, 130, 216). The role of the *cepIR* system in regulating *B. cenocepacia* virulence traits will be reviewed in detail later in this chapter (Section 1.5)

1.4 Quorum-sensing

Quorum-sensing is a form of genetic regulation typically mediated by the accumulation of one or more self-produced signal compounds in the environment. At low cell density, basal levels of the signal molecules are produced and diffuse away from the cell. The concentration of signal in the environment increases as a function of increasing cell density. Once a minimal threshold concentration of signal is reached, the bacteria respond by altering gene expression (147). There are different mechanisms for quorum-sensing in bacteria. Gram-positive bacteria generally employ a modified oligopeptide-mediated system, and Gram-negative bacteria typically utilize an acylated homoserine lactone (AHL)-mediated system (147). The LuxS interspecies communication system is used in numerous bacteria, both Gram-positive and Gram-negative. The signalling molecules produced by LuxS are distinct but related molecules derived from the highly reactive 4,5-dihydroxy-2,3-pentonedione (DPD) (223). The archetypical *Vibrio harveyi* LuxS system uses a furanosyl borate diester as a signalling molecule (21).

Quorum-sensing systems enable bacteria to synchronize particular functions on a population scale. The traits that tend to be controlled by quorum-sensing systems are often functions that are most useful when a sufficient community of bacteria is present.

These include luminescence, conjugation, biofilm formation, antibiotic production and virulence (75).

1.4.1 AHL mediated quorum-sensing

The archetypal AHL-mediated quorum-sensing system is the *luxIR* system of *V. fischeri* (Fig. 1.1). This system is comprised of two principal regulatory proteins: LuxI and LuxR. LuxI functions to catalyze the synthesis of the AHL signalling molecule, *N*-(3oxohexanoyl)-L-homoserine lactone (3-oxo-HHL). The AHLs bind to and activate the sensor/transcriptional regulator, LuxR. Active LuxR recognizes and binds to a conserved 20 bp inverted repeat sequence proximal to the target luciferase operon, termed the *lux* box, to mediate transcription, resulting in the production of luminescence by *V. fischeri* (58).

1.4.1.1 AHL biosynthesis

LuxI-type AHL synthases direct amide bond formation between the acyl portion, derived from a fatty-acid precursor conjugated to an acyl-carrier protein (ACP) and the homoserine lactone moiety that is derived from *S*-adenosylmethionine (SAM) (58, 59). Both substrates are common components of cellular metabolism. AHLs have a common homoserine lactone ring moiety. The acyl chain can vary between 4 and 18 carbons in length, the C-3 position can be a fully oxidized carbonyl, carry a hydroxyl group or be fully reduced and can vary in the degree of unsaturation (58, 136). These amphipathic signalling molecules are freely diffusible across the cell membrane. However, AHLs with long acyl side chains are thought to require active transport (161).

LuxI-type proteins typically contain ten conserved amino acids. The carboxy terminus is less conserved and is thought to be responsible for the acyl side chain

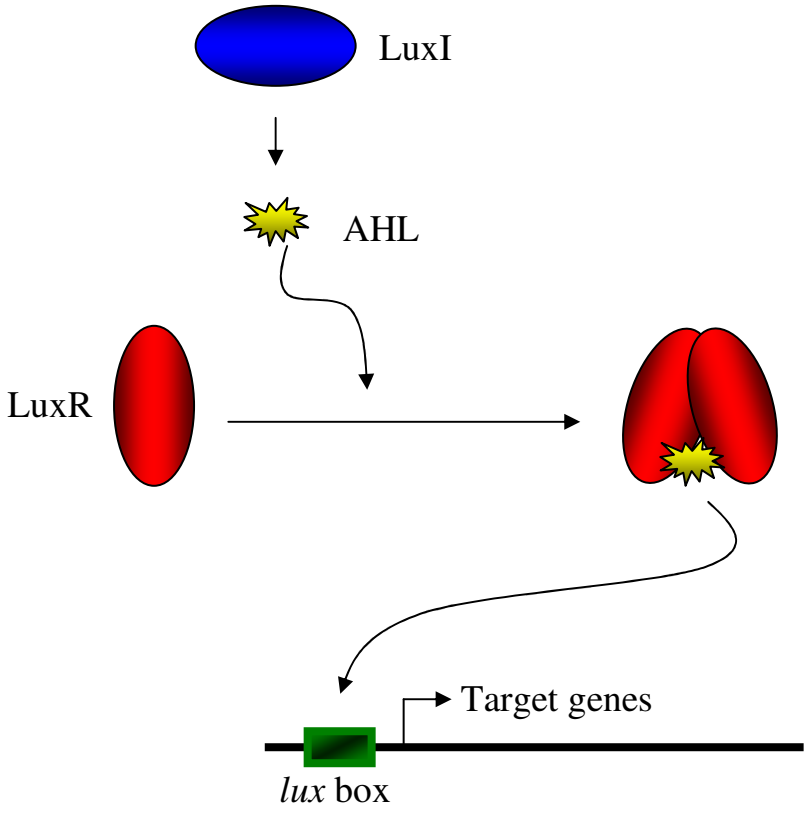


Figure 1.1. Model of AHL-mediated quorum-sensing. The AHL synthase, LuxI, catalyzes the synthesis of the AHL signaling molecule. The AHL binds to and induces LuxR. The active AHL-bound LuxR multimer recognizes and binds to the conserved *lux* box sequence within the target gene promoter, mediating transcription (58).

specificity of AHLs (58, 59). AHL synthesis reactions are precise since LuxI proteins recognize only the ACP containing a specific acyl chain moiety (199). The crystal structures of the LuxI homologues LasI, from *P. aeruginosa* (71), and EsaI, from *Pantoea stewartii* (224), have been solved and revealed a common binding site for the acyl moiety of the acyl-ACP. These studies also identified a threonine in the acyl-chain binding site that is important for hydrogen bonding to the 3-oxo position of acyl-ACP, leading to the preference of 3-oxo-AHL synthesis by EsaI and LasI. Mutations of this key binding pocket residue lead to loss of enzyme specificity (70). The acyl moiety binding pocket of LasI is larger than that of EsaI, which is consistent with LasI producing an AHL with a longer side chain. These studies indicate that the length and derivatization of the acyl side chains of AHLs are determined by differences in the AHL synthase binding cavities (199).

1.4.1.2 LuxR structure and function

Site-directed mutation studies of LuxR-type transcriptional regulators and the crystal structure of the *Agrobacterium tumefaciens* LuxR homologue, TraR complexed with its cognate AHL and target DNA have lead to the conclusion that LuxR-type proteins are comprised of an amino terminal AHL interaction domain and a carboxy terminal helix-turn-helix DNA binding domain (92). LuxR-type regulators share seven conserved residues (61). Several studies have demonstrated that only compounds that are closely related to the system's cognate AHL are capable of inducing gene expression, indicating that the transcriptional regulators are sensitive to alterations in AHLs (147).

A weight of evidence indicates that LuxR-type proteins reside in the cytoplasm or are loosely associated with the inner cytoplasmic membrane. Interaction with the

diffusible AHL triggers a conformational change that affects multimerization of the protein (58, 59). AHL-transcriptional regulator complexes bind to a specific DNA target sequence, referred to as the *lux* box. Typical *lux* boxes range from 18 to 22 bp, have dyad symmetry and are located proximal to the – 35 promoter element (58, 59). LuxR-type proteins can act as transcriptional activators and/or repressors (58).

1.4.1.3 Bacteria with multiple AHL-mediated quorum-sensing systems

Quorum-sensing in many bacteria is comprised of multiple systems to allow bacteria to integrate sensory information, providing flexibility to the genetic network (199). In general, multiple quorum-sensing systems can be set up in hierarchies, either in series or in parallel. Having systems arranged in series allows for the regulation of genes in a temporally defined manner where as systems arranged in parallel allows for the regulation of discrete groups of genes or converging to regulate and identical set of genes (199). Quorum-sensing networks can also be competitive, function with specific on/off switches and/or be responsive to host cues (223).

One of the more extensively studied bacteria with multiple AHL-mediated quorum-sensing systems is *P. aeruginosa*. There are two *luxIR* homologues in *P. aeruginosa* termed *lasIR* and *rhlIR*, that utilize *N*- (3-oxododecanoyl)-L-homoserine lactone (3-oxo-doDHL) and *N*- (butyryl)-L-homoserine lactone (BHL), respectively (159, 160). These systems are arranged in a hierarchical manner, with *lasIR* and *rhlIR* acting in series. Together, these two systems are responsible for the regulation of a large number of genes including many that play a role in virulence (41, 158, 159). There are complex interactions of additional regulators in the quorum-sensing system of *P. aeruginosa* including: QscR, Vfr, GacAS, RelA, RsaL, PQS, VqsR and RpoS (173). Other bacterial

species including *Yersinia pseudotuberculosis, A. tumefaciens, Rhizobium leguminosarum* and *Ralstonia solancearum* also have multiple regulatory systems that are organized in a hierarchical relationship to control the expression of various genes (6, 56, 163, 228).

1.5 Bcc quorum-sensing

1.5.1 The *cepIR* system

B. cenocepacia was determined to have an AHL-mediated quorum-sensing system by Lewenza et al. (109), who discovered the *luxIR* homologues *cepIR* while screening transposon insertion mutants that hyperproduce siderophores. A model of the Bcc *cepIR* quorum-sensing system is in Fig. 1.2. The *cepI* and *cepR* genes are divergently transcribed with a 727 bp intergenic region. CepI contains each of the 10 amino acids that are conserved among all LuxI family members. CepI catalyses the synthesis of two AHLs, *N*-hexanoyl-homoserine lactone (HHL) and *N*-octanoyl-homoserine lactone (OHL) (Fig. 1.3) (109, 110). CepR contains six of the seven amino acids that are identical in many of the LuxR-like proteins, and has a conserved AHL binding domain and a DNA binding domain (109).

Quorum-sensing systems that are highly homologous to *B. cenocepacia cepIR* have been identified in all Bcc species investigated thus far and the major product of CepI, OHL, is produced by all Bcc species (69, 121, 233). Lutter et al. (121), cloned and

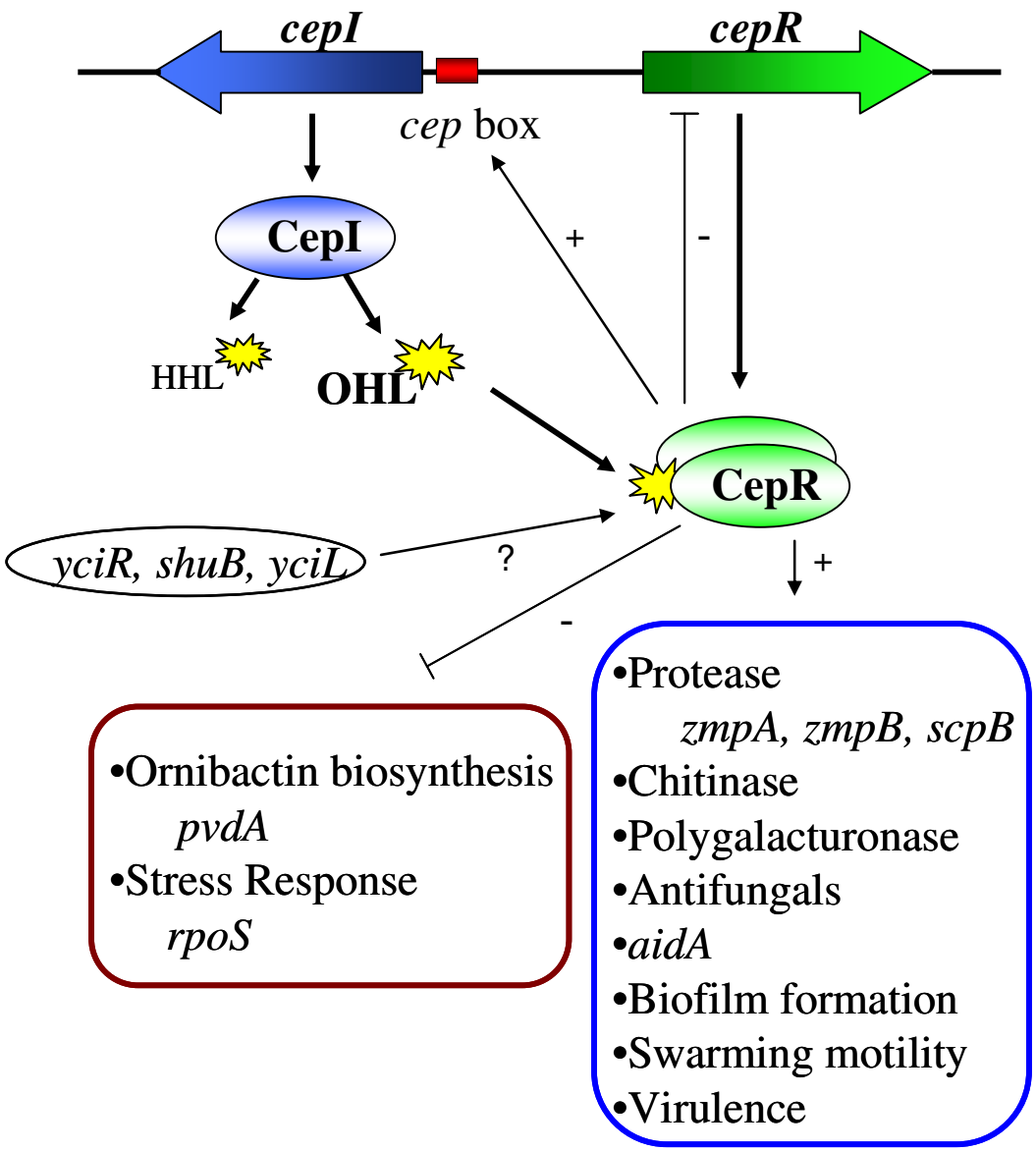


Figure 1.2. Model of the *cepIR* quorum-sensing system of the Bcc. The AHL synthase, CepI produces OHL and minor amounts of HHL. The signalling molecules bind to and activate the transcriptional regulator, CepR. In this active, presumably multimeric form, CepR-AHL is able to mediate the transcription of its target genes. CepR-OHL binds to the *cep* box to positively regulate *cepI*. CepR is involved in the negative regulation of *cepR,* but the mechanism has yet to be determined. Three genes, *yciR, suhB* and *yciL* are believed to encode higher-level regulators of the *cepIR* system (52, 130, 216, 225).

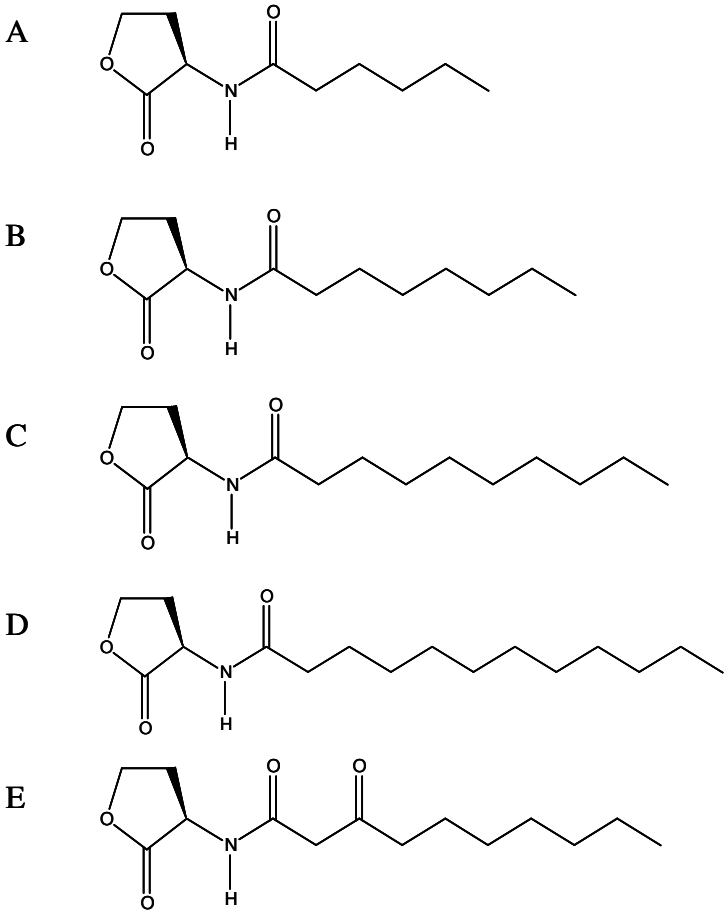


Figure 1.3. Chemical structures of the AHLs produced by Bcc bacteria. A. *N*-hexanoyl-L-homoserine lactone (HHL), B. *N*-octanoyl-L-homoserine lactone (OHL), C. *N*-decanoyl-L-homoserine lactone (DHL), D. *N*-dodecanoyl-L-homoserine lactone (doDHL) and E. *N*-(3oxodecanoyl)-L-homoserine lactone (3-oxo-DHL).

sequenced *cepIR* homologues from representative strains of *B. cepacia, B. multivorans, B. stabilis, B. vietnamiensis* and *B. dolosa* and determined their percent identity compared to the CepIR homologues of a *B. cenocepacia* strain. The CepR proteins were highly conserved with a percent identity ranging from 92 to 99 percent. The CepI proteins of *B. cepacia*, *B. stabilis* and *B. vietnamiensis* exhibited slightly lower sequence identity to CepI of *B. cenocepacia*, ranging from 90 to 96 percent. Lutter et al. (121), were not able to PCR amplify *cepI* from *B. multivorans* or *B. dolosa.* Gotschlich et al. (69), successfully cloned and sequenced *cepI* homologues from *B. multivorans* and *B. dolosa* strains and concluded that these proteins are less conserved than CepR homologues in the Bcc. Due to the sequence variability among the *cepI* homologues, *cepI* is being considered as part of a group of variable genes to be used in multilocus restriction typing (MLRT) of the Bcc (27).

The quantity and type of AHLs produced by the Bcc species was found to vary dramatically (34, 69, 231). Some *B. vietnamiensis* strains produce AHLs in addition to HHL and OHL, including *N*-decanoyl-L-homoserine lactone (DHL), *N*-dodecanoyl-L-homoserine lactone (doDHL) and *N*-(3oxodecanoyl)-L-homoserine lactone (oxoDHL) (Fig. 1.3) (33, 155). DHL production has also been detected for *B. ambifaria* strain AMMD^{T} (231). The amount of OHL produced by CepI is at least ten fold greater than HHL, as determined through analysis by Liquid Chromatography-Mass Spectrometry (LC-MS) techniques (34, 69, 110). However, the molar ratio of HHL:OHL is highly strain dependent (34, 69, 231).

Studies into the intra-regulation of the *cepIR* system revealed that *cepIR* is auto-regulated in that CepR positively regulates *cepI* and negatively regulates itself (110).

CepR controls the expression of *cepI* by binding to a *lux* box-like sequence that is centered approximately 44 bp upstream of the *cepI* transcriptional start site (225). The CepR binding site or *cep* box is a 16 bp sequence with imperfect dyad symmetry. CepR requires OHL for proper folding and it has been suggested that CepR functions as a dimer (225). OHL is the most active AHL for the *cepIR* system, but other unsubstituted, long chains AHLs are also able to induce CepR dependent gene expression (2, 225).

Recent proteomic and molecular studies have identified many CepR target genes and suggest that the *cepIR* quorum-sensing system is a global regulatory system that can activate and repress gene expression in the Bcc (3, 166, 225). Transcriptional analysis of a *B. cepacia* promoter library identified 28 genes that are positively regulated by CepR (3). Ridel et al. (166), determined that five percent of the *B. cenocepacia* proteome was downregulated and one percent was upregulated in a H111 *cepI* mutant. A transposon mutagenesis of a *B. cenocepacia cepI* mutant identified seven genes that are induced by OHL (225). A recent promoter library study in a *B. cenocepacia cepI* mutant has determined the largest list of OHL responsive genes to date (Subsin, Chambers, Visser and Sokol, in press). The responsive genes were grouped into the following functional categories; adaptation/resistance, secretion, metabolism, membrane/surface structure, regulation, DNA repair/replication/translation and transport/binding proteins.

One gene, a homologue of *aidA* (autoinducer dependent) of *R. solanacearum,* was identified by the majority of the above studies as being CepIR regulated, (3, 166, 225). A specific function for AidA has yet to be established. However, it is known that in *B. cenocepacia,* AidA is an intercellular protein that is essential for slow killing of the nematode *Caenorhabditis elegans* (82). Disrupting *aidA* has little effect on fast killing of

C. elegans, suggesting that the protein plays a role in the accumulation of the strain in the nematode gut and that AidA is required for establishing an infection-like process rather than acting as a toxin (82).

Characterization of *cepI* and *cepR* mutants in *B. cepacia, B. cenocepacia* and *B. ambifaria* has revealed numerous regulatory roles for this quorum-sensing system. The *B. cepacia cepIR* system negatively regulates the stationary phase sigma factor *rpoS*, positively regulates protease production and contributes to onion maceration by positively regulating polygalacturonase production (2). The *cepIR* homologous quorum-sensing system in *B. ambifaria* strain BC-F positively regulates antifungal production. Mutants constructed in *B. ambifaria cepI* and *cepR* were not able to suppress damping-off of cucumber and failed to inhibit the growth of *Saccharomyces cerevisiae* (233).

The *cepIR* quorum-sensing system in *B. cenocepacia* is involved in the positive regulation of swarming motility, mature biofilm development, chitinase production, extracellular protease production and the negative regulation of siderophore biosynthesis (83, 109-111). The *cepR* mutant, *B. cenocepacia* K56-R2, produced more siderophores than the parent, as determined by chrome azurol S (CAS) assays (109). This phenotype was characterized further by identifying that ornibactin is specifically regulated by the *cepIR* system since ornibactin is the only one of the four siderophores produced in greater amounts in the *cepR* mutant compared to the parent. Ornibactin biosynthesis was confirmed to be negatively controlled by the *cepIR* system with transcriptional analysis of the ornibactin biosynthesis gene, *pvdA* (110).

Mutants in *B. cenocepacia cepR* and *cepI* are unable to produce protease(s), as demonstrated by an absence of clearing around growth on skim-milk agar (109). Further

transcriptional analysis determined that the *zmpA* and *zmpB* zinc metalloprotease genes are positively regulated by *cepIR* (65, 94). A random transposon mutagenesis experiment designed to find CepR regulated genes also identified a serine protease positively regulated the *cepIR* quorum-sensing system (120). By sequence analysis, this protease has been determined to be a member of the serine-carboxyl protease family with 63% identity to *Pseudomonas* species 101 sedolisin (154). This protease has been termed ScpB and preliminary characterization indicates that it is a periplasmic protease that does not cleave casein (64).

The swimming behaviour of *cepI* and *cepR* mutants in *B. cenocepacia* H111 were completely indistinguishable from the parent, indicating that the swimming phenotype of *B. cenocepacia* is not regulated by the *cepIR* system; however, there was no swarming motility observed in the *cepIR* mutant strains (83). The swarming motility phenotype of the *cepI* and *cepR* mutants were restored by complementing the strains with exogenous OHL, or the surfactants serrawettin or surfactin, suggesting that quorum-sensing controls the production of a biosurfactant that is required for swarming motility in *B. cenocepacia* (83).

The involvement of quorum-sensing in *B. cenocepacia* biofilm formation was first identified by screening transposon mutants for a decreased ability to produce biofilms (83). The *cepIR* system has been found to be essential for the differentiation of microcolonies in *B. cenocepacia* H111 (83). The *cepI* and *cepR* mutant biofilms differed from the parent *B. cenocepacia* H111 in substratum coverage and thickness. Tomlin et al. (203), also observed structural and temporal impairments of biofilms that were formed by *cepI* and *cepR* mutants in *B. cenocepacia* K56-2 when compared to the parent.

Molecular investigation into biofilm deficient mutants of *B. cenocepacia* H111 has led to the identification of three putative regulatory factors of the *cepIR* quorum-sensing system, encoded by the genes *yciR*, *shuB* and *yciL* (84). Each of these genes are located upstream of the *cepIR* system in the H111 quorum-sensing network and are thought to encode higher-level regulators that act on genes outside the *cepIR* regulon to affect AHL synthesis and biofilm formation.

1.5.2 CepIR quorum-sensing and virulence

Since the *cepIR* quorum-sensing system is known to be involved in the regulation of specific virulence factors, the role of this system in pathogenesis has been investigated in various infection models including plant, invertebrate and animal (2, 95, 190). The *cepIR* locus contributes to onion pathogenicity in the onion maceration model as both *cepI* and *cepR* mutants in *B. cepacia* have attenuated maceration compared to the parent (2). A functional *cepIR* system is also required for efficient killing of *C. elegans* by *B. cenocepacia* (95). It is believed that the *cepIR* system is required for the bacteria to enter the intestinal lumen of the nematode. Two animal models have been used to determine that the *cepIR* quorum-sensing system contributes to the pathogenesis of *B. cenocepacia* K56-2 (190). Employing the chronic agar bead infection model in rats, Sokol et al. (190), determined that *cepIR* contributes to the maximum virulence of *B. cenocepacia* and hypothesized that this is due to the regulation of the production of extracellular virulence factors, resulting in increased lung injury. Investigations with wild type and $Cftr^{(-/-)}$ mice in the short-term intranasal colonization mouse model determined that a *cepI* mutant was less virulent in both types of mice and was unable to invade in the $Cftr^{(-/-)}$ mice (190). These studies indicate that the *cepIR* system contributes to the virulence of *B.*

cenocepacia in both acute and chronic pulmonary infections. AHLs have been detected in sputum (146) and mucopurulent respiratory secretions (18) from CF patients infected with Bcc bacteria, providing indirect evidence that quorum-sensing is functioning to regulate virulence factors during pulmonary infections in CF patients.

1.5.3 AHL-mediated interactions with *P. aeruginosa*

P. aeruginosa and *B. cenocepacia* clinical isolates can produce mixed-biofilms *in vitro*, suggesting that *B. cenocepacia* has the ability to interact with other microorganisms in the CF lung (167, 202). *P. aeruginosa* PA01 exoproducts have been shown to increase the production of virulence factors including: siderophore, lipase and protease in *B. cenocepacia* (139).

Due to the level of AHL similarity between *P. aeruginosa* and the Bcc, quorum-sensing may be involved in interspecies gene regulation during CF pulmonary infections. *B. cenocepacia* has been reported to detect and respond to *P. aeruginosa* AHLs in mixed biofilms and co-infected mouse lungs (167). This study showed that *B. cenocepacia* CepR could detect and respond to the AHLs produced by *P. aeruginosa*, but *P. aeruginosa* LasR was unable to detect and respond to the AHLs produced by *B. cenocepacia*. Conversely, Lewenza et al. (111), demonstrated that the *P. aeruginosa* RhlIR system was able to respond to *B. cenocepacia* AHLs when present in sufficient concentrations for activation. *B. cenocepacia* produces levels of AHL in the nanomolar range *in vitro*, whereas *P. aeruginosa* produces AHL in the micromolar range. Successful cross-feeding of a *P. aeruginosa rhlI* mutant occurred when the cross-feeding strain contained a high copy plasmid with *cepI* that produced greater amounts of AHL than the parent. Lewenza et al. (111), also determined that the ability of the respective

quorum-sensing transcriptional regulators to respond to heterologous AHLs and regulate transcription was variable between target genes. Protease expression was regulated by heterologous AHL but, other phenotypes including twitching motility, swarming motility and biosurfactant production are not be regulated by heterologous AHL. Further investigation is needed to determine a role of AHLs in interspecies interactions during mixed *B. cenocepacia* and *P. aeruginosa* infections.

1.5.4 Bcc *cepIR* quorum-sensing as target for antimicrobial therapy

Bcc infections are difficult to treat due to the inherent resistance of the organism to most antibiotics therefore, new alternative therapies are required. Traditional antibiotic therapy aims to treat chronic bacterial infections based on compounds that kill the organism or inhibit growth. A modern drug design, termed antipathogenic therapy, targets the regulatory systems that govern the expression of virulence factors (78). The rationale behind antipathogenic drugs is that the virulence of the organism is attenuated, resulting in decreased effectiveness of colonization, thus allowing the bacteria to be cleared by the host's immune system.

Quorum-sensing is an attractive antipathogenic therapy target because of the role that the system plays in the regulation of multiple virulence factors in many bacteria (185). There has been a significant amount of progress in determining effective ways to interfere with quorum-sensing systems. The major current strategies involve blocking the response regulator with AHL analogs, destroying the signal via AHL degrading enzymes or halting the synthesis of the AHL by blocking the biosynthesis pathway (185).

AHL analogues are capable of blocking quorum-sensing signal transduction by blocking or competitively interfering with the binding of native AHL signal to the

receptor LuxR-type protein. There have been many reports describing the application of AHL analogs to inhibit the quorum-sensing systems of various bacteria (78). Initial analogue studies took advantage of the natural halogenated furanone AHL analogs secreted by *Delisea pulchra* (132). Synthetic derivatives of these analogs have proven effective in antagonizing the quorum-sensing systems of *P. aeruginosa* (182, 183). Treatment with a synthetic AHL analogue inhibited the *P. aeruginosa* quorum-sensing mechanism in a mouse model of pulmonary infection, leading to an increased clearance of the organism from the mouse lung (79).

An inhibitor of *B. cenocepacia* quorum-sensing was identified with an *in silico* based approach (168). The characterized end-point inhibitory compound, compound 3, is surprisingly not related to AHL signal molecules or furanone compounds, despite evolving from a structural homologue of 3-oxo-doDHL. Compound 3 was determined to inhibit the quorum-sensing system of *B. cenocepacia* with high specificity, since it strongly inhibited the Bcc quorum-sensing related phenotypes of protease production, swarming motility and biofilm formation. The expression of *aidA* was also decreased in the presence of compound 3. Compound 3 successfully decreased the rate of *C. elegans* killing without affecting the growth of the bacteria or the development of the nematode.

A strategy to reduce AHL activity has also been pursued for inhibition of the Bcc quorum-sensing systems. AHLs can be enzymatically degraded. The *Bacillus aiiA* gene encodes for an AHL lactonase (49) and *Ralstonia* species contain the AHL acylase, AiiD (114), both of which degrade AHLs. The potential ability of these enzymes to interfere with the quorum-sensing systems of pathogenic bacteria in attempts to attenuate virulence is referred to as quorum-quenching (48). Wooperer et al. (231), determined

that heterologous expression of *aiiA* in all nine Bcc species eliminated or significantly reduced AHL accumulation. The presence of *aiiA* reduced protease production, swarming motility, biofilm formation and virulence in *C. elegans* in the majority of the strains tested. The quorum-quenching approach would be most applicable for *Burkholderia* anti-phytopathogenics since it is acceptable to perform transgenic procedures to produce food crops that express *aiiA*. Employing the AHL degrading enzyme approach for human therapeutic strategies is not yet feasible since it would be difficult to deliver physiologically active quorum quenching enzymes to the site of infection (185).

1.5.5 Multiple quorum-sensing systems in *B. cenocepacia* and *B. vietnamiensis*

B. cenocepacia and *B. vietnamiensis* contain AHL-mediated quorum-sensing systems additional to *cepIR.* The *cciIR* system was identified in the *B. cenocepacia* epidemic strain J2315 during characterization of the *cenocepacia* island (8). An additional *luxI* homologue was identified in *B. vietnamiensis* DBO1 using random Tn*Mod* mutagenesis (43) and was later determined to be a part of a system distinct from *cepIR,* designated as *bviIR* by Lutter et al. (121). The regulatory role of these additional quorum-sensing systems and their relationship with the *cepIR* system has yet to be determined. Further understanding of the role of quorum-sensing in the regulation of Bcc genes may lead to possible therapeutic strategies and potential mechanisms for safely administering Bcc strains for bioremediation and biological control applications.

1.6 Hypothesis

Quorum-sensing systems contribute to complex regulatory networks that control dynamic expression of the bacterial genome. It is hypothesized that the quorum-sensing systems of *B. cenocepacia, cepIR* and *cciIR,* as well as the quorum sensing systems of *B. vietnamiensis, cepIR* and *bviIR,* are involved in the co-ordinate regulation of genes, including those involved in virulence.

1.7 Objectives

1. To determine the role of CciI and CciR in the pathogenesis of *B. cenocepacia* K56-2

2. To investigate the regulatory properties of CciR in *B. cenocepacia* K56-2

3. To identify and perform preliminary characterization of additional LuxR homologues in *B. cenocepacia* K56-2

4. To investigate the role of the *cepIR* and *bviIR* quorum-sensing systems in *B. vietnamiensis*

Chapter Two: Materials and Methods

2.1 Strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 2.1. Cultures were routinely grown at 37°C, in Miller's Luria broth (LB) (Invitrogen, Burlington, ON) with shaking (200 rpm) or on 1.5% Lennox LB agar plates with the exception of *Agrobacterium tumefaciens* which was grown at 30°C. When appropriate, the following concentrations of antibiotics were used: 100 μg/ml of trimethoprim (Tp) and 200 μg/ml of tetracycline (Tc) for *Burkholderia. cenocepacia;* 100 μg/ml of Tp, 30 μg/ml of Tc, 20 μg/ml of gentamycin (Gm) and 80 μg/ml of chloramphenicol (Cm) for *B. vietnamiensis* G4; 100 mg/μl of Tp, 250 μg/ml of Tc and 400 μg/ml of kanamycin (Km) for *B. vietnamiensis* PC259; 100 μg/ml of Tp for all other *B. vietnamiensis* strains; 1.5 mg/ml Tp, 15 μg/ml of Tc, 25 μg/ml Gm, 35 μg/ml of Cm and 50 μg/ml of Km for *Escherichia coli;* and 4.5 μg/ml Tc and 50 μg/ml of spectinomycin (Sp) for *A. tumefaciens.* Antibiotics were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON). When blue/white screening was used during routine cloning procedures 40 μg/ml of 5-bromo-4-chloro-3-indoly-β-galactopyranoside (X-gal) was added to the medium. For genetic manipulations including RNA extraction from *B. cenocepacia* strains as well as luminescence assays and alfalfa model of infection cultures were grown in LB. For protease, β-galactosidase, and luminescence assays cultures were grown in 0.25% trypticase soy broth (TSB) (Difco, Franklin Lakes, NJ) with 5% Bacto-peptone (Difco) (PTSB) at 37°C. For chrome azurol S (CAS) assays cultures were grown in succinate medium supplemented with ornithine (10mM) (142). For examination of swarming

Table 2.1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Description	Reference
Strains		
A. tumefaciens		
A136	Ti plasmidless host	C. Fuqua
E. coli		
DH5α	F⁻ ϕ80*lacZ*ΔM15 Δ(*lac*ZYA-*arg*F) *rec*A1 *end*A *gyr*A96 *thi-1 hsd*R17 *sup*E44 *rel*Al *deo*R U169	Invitrogen
TOP10	F⁻ *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80lacZΔM15 ΔlacX74 *deo*R *rec*A1 *ara*D139 Δ*(ara-leu)*7697 *gal*U *gal*K *rps*L *(St^R)* endA1 *nupG*	Invitrogen
HB101	*supE44 hsdS20* (rBmB) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	(172)
B. cepacia complex		
B. cepacia		
ATTC25416^T	Onion isolate	(125)
B. multivorans		
LMG13010^T	CF isolate	(125)
C5393	CF isolate	(125)
B. cenocepacia		
J2315	CF isolate, BCESM +, *cblA* +	(125)
BC7	CF isolate, BCESM +, *cblA* +	(125)
C5424	CF isolate, BCESM +, *cblA* +	(125)
C6433	CF isolate, BCESM +, *cblA* -	(125)
C1394	CF isolate BCESM +, *cblA* -	(125)
PC184	CF isolate, BCESM +, *cblA* -	(125)
CEP511	CF isolate, BCESM +, *cblA* -	(125)
J415	CF isolate, BCESM -, *cblA* -	(125)
ATTC 17765	UTI isolate, BCESM+, *cblA* -	(125)
PC715j	CF isolate, BCESM-, *cblA*-	(140)
H111	CF isolate, BCESM-	(169)
K56-2	CF isolate, BCESM +, *cblA* +	(125)
K56-R2	*cepR::*Tn*5*-OT182 derivative of K56-2, Tc^R	(109)
K56-I2	*cepI:: dhfRII* derivative of K56-2, Tp^R	(109)
K56-dI2	Δ*cepI* derivative of K56-2	(129)
K56-2*cciI*	*cciI:: dhfRII* derivative of K56-2, Tp^R	(8)

K56-2*cciR*	*cciR:: dhfRII* derivative of K56-2, Tp^R	This study
K56-2*cciIR*	Δ*cciIR* derivative of K56-2	This study
K56-2*cepI,cciIa*	Δ*cepI, cciI:: dhfRII* derivative of K56-2 with a spontaneous mutation affecting ornibactin biosynthesis, Tp^R	This study
K56-2*cepI,cciIb*	Δ*cepI, cciI:: dhfRII* derivative of K56-2, Tp^R	This study
K56-*2cepR,cciIR*	*cepR::dhfRII,* Δ*cciIR* derivative of K56-2, Tp^R	This study
K56-2M188a	BCAM0188:: *dhfRII* derivative of K56-2, Tp^R	This study
K56-2M188b	BCAM0188:: *dhfRII* derivative of K56-2, Tp^R	This study
B. stabilis		
LMG 14294	CF isolate	(125)
LMG 14086	Respiratory isolate	(125)
B. vietnamiensis		
PC259	CF isolate	(99)
FC466	CF isolate	(214)
FC441	CGD isolate	(125)
C2822	CF isolate	E. Mahenthiralingam
$FC369^T$	Rice isolate	(63)
DBO1	Environmental isolate	(222)
G4	water treatment facility isolate	(152)
IMT0-61	*bviI::aacCI* derivative of G4, Gm^R	(33)
RMT-14	*bviR:: aacCI* derivative of G4, Gm^R	(33)
G4*cepI*	*cepI::cat* derivative of G4, Cm^R	This study
G4*cepR*	*cepR::cat* derivative of G4, Cm^R	This study
G4*cepI*GSV	*cepI::*pRMSVI derivative of G4, Gm^R	This study
G4*cepR*GSV	*cepR*::pRMSVR derivative of G4, Gm^R	This study
PC259*cepR*	*cepR::dhfRII* deriviative of PC259, Tp^R	This study
B. dolosa		
$LMG19468^T$	CF isolate	J. LiPuma
LMG18943	CF isolate	(28)
B. ambifaria		
CEP0996	CF isolate	(29)
LMG 17828	Soil isolate	(29)
B. athina		
$LMG\ 20980^T$	Soil isolate	(32)
LMG 20982	Hospital environmental isolate	J. R. W. Govan
B. pyrrocinia		
LMG 21822	Soil isolate	(32)

P. aeruginosa		
PAO1	CF respiratory isolate	(81)
Plasmids		
pEX18Tc	Suicide vector, *sacB,* Tc^R	(80)
pUCP26	Broad-host-range vector, Tc^R	(226)
pUCP28T	Broad-host-range vector, Tp^R	(176)
pCR®2.1Topo	Cloning vector for PCR products, Ap^R, Km^R	Invitrogen
pRK2013	ColEl Tra $(RK2)^+$, Km^R	(55)
pRK415	Broad-host-range vector, Tc^R	(90)
p34E-Tp	Source of Tp resistance cassette, Tp^R	(45)
p34S-Cm	Source of the Cm resistance cassette, Cm^R	(43)
pCF218	IncP plasmid expressing TraR, Tc^R	(234)
pCF372	pUCD2 with a *traI-lacZ* fusion, Sp^R	(60)
pSLA3.2	pUCP28Twith 3.2 kp SphI fragment from K56-2 containing *cepIR* genes, Tp^R	(109)
pSLS225	pUCP26 with 1.5 kb *Sph*I-*Kpn*I fragment containing *cepI*, Tc^R	(111)
pEXCEPI	pEX18Tc containing the *cepI* gene disrupted by the Tp resistance cassette, Tp^R, Tc^R	(109)
pEXCEPR	pEX18Tc containing the *cepR* gene disrupted by the Tp resistance cassette, Tp^R, Tc^R	(110)
pRM186-Tp	pEX18Tc containing the *cciR* gene disrupted by the Tp resistance cassette at the MluI site, Tp^R, Tc^R	This study
pRM4.3	pCR®2.1TOPO with the 4.3 kb XhoI fragment containing *cciIR*, Km^R, Ap^R	This study
pRM1T4	pCR®2.1TOPO with the 850 bp PCR amplified *cciI* fragment, Ap^R, Km^R	This study
pRM164	pUCP26 with the EcoRI fragment from pRM14T containing *cciI* in the same orientation as the *lacZ* promoter, Tc^R	This study
pRM164R	pUCP26 with the EcoRI fragment from pRM14T containing *cciI* in the opposite orientation as the *lacZ* promoter, Tc^R	This study
pRM165	pUCP26 with a 1.1 kb NcoI/ApaLI fragment from pRM4.3 containing *cciR*, Tc^R	This study
pRM166	pUCP26 with a 1.6 kb SphI/KpnI fragment from pSLA3.2, containing *cepR*, Tc^R	This study
pRM187-KO	pEX18Tc with 4.3 kb EcoRI/XbaI fragment from pRM4.3 containing *cciIR* with a 637 bp MluI/NcoI deletion removing the *cciIR* promoter region and 5' end of *cciR*, Tp^R, Tc^R	This study

pCciI-Tp	pEGM105-Tc suicide vector containing the *cciI* gene disrupted by the Tp resisitance cassette, $Tp^{R,}$ Tc^{R}	(8)
pMS402	*luxCDABE*-based promoter reporter vector, Km^{R}, Tp^{R}	(50)
pRM432	pMS402 containing the 1.8 kp PCR amplified *cepR* promoter fragment from K56-2, Km^{R}, Tp^{R}	This study
pCP300	pMS402 containing the 300 bp PCR amplified *cepI* promoter fragment from K56-2, Km^{R}, Tp^{R}	(129)
pRM445	pMS402 containing the 813 bp ClaI *cciIR* promoter fragment, Km^{R}, Tp^{R}	This study
pRM446	pMS402 containing the 391 bp SacII/SalI predicted *cciI* promoter fragment, Km^{R}, Tp^{R}	This study
pSG206	pUCP26 with a *zmpA-lacZ* fusion, Tc^{R}	(190)
pSG208	pUCP28T with a *zmpA-lacZ* fusion, Tp^{R}	(190)
pRM1T8	pCR®2.1TOPO containing the 472 bp internal BCAM0188 fragment, Ap^{R}, Km^{R}	This study
pRM1T6	pCR®2.1TOPO containing the 1.6 Kb BCAM0188 fragment, Ap^{R}, Km^{R}	This study
pRM613	pUCP26 containing a 1.3 kb SstI/SmaI fragment from pRM1T6 including BCAM0188, Tc^{R}	This study
pRM516	pRK415 containing the 1.6 kb BCAM0188 fragment from pRM1T6, Tc^{R}	This study
pRMTX6	pCR®2.1TOPO containing the 1.6 Kb BCAM0188 fragment with a 33 bp SacII deletion and incorporation of the Tp resistance cassette, Ap^{R}, Km^{R}	This study
pRM1X6	pEX18Tc containing the disrupted BCAM0188 fragment from pRMTX6, Tp^{R}, Tc^{R}	This study
pRM418	pMS402 containing the 480 bp BCAM0188 promoter fragment, Km^{R}, Tp^{R}	This study
pRM6.8	pUCP26 with a 6.8 kb BanHI fragment containing the *cepIR* locus from PC259	This study
pRM282	pUCP28T with a 1.5 kb PCR amplified *cepR* fragment from G4, Tp^{R}	This study
pRM283	pUCP28T with a 2.3 kb PstI fragment from pRM6.8 containing *cepI,* Tp^{R}	This study
pRM284	pUCP28T with a 1.1kb PCR amplified *bviR* fragment from G4, Tp^{R}	This study
pRM285	pUCP28T with a 997 bp PCR amplified *bviI* fragment from G4, Tp^{R}	This study

pRM2X1	pEX18Tc with a 2.4 kb PCR amplified *cepR* fragment from G4, Tc^R	This study
pRM2X1-Cm	PRM2X2 with the *cepR* gene disrupted by a Cm cassette at the PstI site, Tc^R, Cm^R	This study
pRM2T2	pCR2.1Topo with a 2.2 kb PstI fragment from pRM6.8 containing *cepI,* Ap^R, Km^R	This study
pRM2T2-Cm	pRM2T2 with the *cepI* gene disrupted by a Cm cassette at the MluI site, Km^R, Ap^R, Cm^R	This study
pRM2X2-Cm	pEX18Tc with the disrupted *cepI* fragment from pRM2T2-Cm, Tc^R, Cm^R	This study
pRM452	pMS402 containing the 1.8 kb PC259 *cepR* promoter fragment, Km^R, Tp^R	This study
pGSV3	Mobilizable suicide vector, Gm^R	(44)
pRM2T5	pCR2.1Topo containing the 426 bp fragment internal to *cepI* from G4, Ap^R, Km^R	This study
pRMSVI	pGSV3 containing the EcoRI fragment from pRM2T5 from G4, Gm^R	This study
pRM2T4	pCR2.1Topo containing the 653 bp fragment internal to *cepR* from G4, Ap^R, Km^R	This study
pRMSVR	pGSV3 containing the EcoRI fragment from pRM2T4, Gm^R	This study
pRM453	pMS402 containing the 769 bp PC259 *cepI* promoter fragment, Km^R, Tp^R	This study
pRM455	pMS402 containing the 983 bp PC259 *bviI* promoter fragment, Km^R, Tp^R	This study
pRM462	pMS402 containing the 266 bp G4 *cepR* promoter fragment, Km^R, Tp^R	This study
pRM463	pMS402 containing the 266 bp G4 *cepI* promoter fragment, Km^R, Tp^R	This study
pRM464	pMS402 containing the 695 bp G4 *bviR* promoter fragment, Km^R, Tp^R	This study
pRM465	pMS402 containing the 983 bp G4 *bviI* promoter fragment, Km^R, Tp^R	This study
pRM475	pMS402 containing the 983 bp FC466 *bviI* promoter fragment, Km^R, Tp^R	This study
pRM485	pMS402 containing the 983 bp FC441 *bviI* promoter fragment, Km^R, Tp^R	This study

CF, cystic fibrosis; CGD, chronic granulomatous disease; BCESM, *B. cepacia* epidemic strain marker; *cblA,* cable pilus gene; Ap, ampicillin; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Sp, spectinomycin; Tp, trimethoprim; Tc, tetracycline; *aacCI*, gentamicin resistance gene; *cat,* chloramphenicol resistance gene; *dhfRII,* trimethoprim resistance gene

motility cultures were grown in nutrient broth (Difco) supplemented with 0.5% glucose. For AHL extractions, *B. vietnamiensis* RNA extraction, luminescence assays, alfalfa model of infection and biofilm assays cultures were grown in TSB. For luminescence assays cultures were also grown in TSB-dialysed and chelated (TSB-DC), low salt LB (1.5 mg/ml), and ¼ LB. AT media supplemented with 0.5 % glucose (87) was used to grow *A. tumefaciens* (pCF218)(pCF372) for AHL bioassays. Chemicals for media and buffers were purchased from Sigma or Invitrogen unless otherwise stated. Media, solutions and buffers were made in double distilled water (ddH_20) unless otherwise stated.

2.2 DNA manipulations

2.2.1 Recombinant DNA manipulations

DNA manipulations were performed using standard techniques as described by Sambrook et al. (172). Agarose, 1 kb plus DNA ladder, restriction endonucleases and T4 DNA polymerase were purchased from Invitrogen. T4 DNA ligase was purchased from New England Biolabs (Mississauga, ON) or Invitrogen. Shrimp alkaline phosphatase was purchased from Roche (Mannheim, Germany). DNA fragments used in cloning procedures were purified with a QIAquick gel extraction kit (QIAGEN) or the QIAquick PCR purification kit (QIAGEN) as per the manufacturer's protocol. DNA was routinely quantified using the the Warburg-Christian method and a Beckman DU 640 spectrophotometer (Beckman, Coulter, Mississauga, ON). DNA fragments were routinely separated on 0.7 to 1.0 percent agarose gels prepared with 1X Tris-actetate-EDTA buffer (for 1 l of 50X; 242 g trishydroxymethylaminomethane (tris), 100 ml 0.5 M ethylenediaminetetraacetic acid (EDTA), 57.1 ml glacial acetic acid) at between 50 and

100 volts depending on the size of fragment of interest. The DNA fragments were visualized by staining with 2 µg/ml ethidium bromide solution. Gel photographs were taken with a Kodak digital camera with Kodak 1D analysis software (Eastman Kodak Company, Rochester, NY)

2.2.2 Genomic DNA isolation

Large scale genomic DNA isolation was performed as described by Goldberg and Ohman (67). Forty ml overnight cultures were grown in LB and the cells were collected by centrifugation at 4354 x g for 10 minutes. The cells were washed twice with ET buffer (10 mM Tris, 10 mM EDTA, pH 8.0) and resuspended in 10 ml of cold TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) with 1 mg of lysozyme and incubated at 37^{o}C for 15 minutes. To lyse the cells 1.2 ml of sarkosyl-pronase (10 % sarkosyl, 5 mg pronase/ ml of TE) was added and the cells were incubated at 37^{o}C for one hour. DNA was extracted from the aqueous phase four times with 10 ml of phenol saturated with TES (10 mM Tris, 1 mM EDTA, 50 mM NaCl) and three times with 10 ml of chloroform: isoamyl alcohol (24:1). The DNA was precipitated from the final aqueous phase by the addition of 1.2 ml of 3.0 M $CH_3CO_2NH_4$ and 20 ml of isopropanol. The precipitated DNA was spooled onto a glass rod, re-suspended in 2-3 ml of TE buffer and stored at 4^{o}C.

Small scale genomic DNA isolation was adapted from Ausubel et al. (7). A 5 ml overnight culture was grown in LB and 1.5 ml of culture was pelleted by centrifugation at 12000 to 16000 x g for 10 minutes. The cell pellet was resuspended in 567 µl of TE buffer, 30 µl of 10% Sodium dodecyl sulfate (SDS) and 3 µl of (20 mg/ml) proteinase K and incubated at 37^{o}C for 1 hour. One hundred microlitres of 5 M NaCl and 80 µl of hexadecyltrimethyl ammonium bromide (CTAB)/NaCl (10 % CTAB, 4.1% NaCl) were

added and the suspension was incubated at 65°C for 10 minutes. DNA was extracted from the suspension three times with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and two times with chloroform/isoamyl alcohol (24:1). The DNA was precipitated from the aqueous phase with 0.6 volumes of isopropanol and collected by centrifugation for 5 minutes at 12000 to 16000 x g. The pellet was washed with 70 percent ethanol, dried in a speed vacuum microfuge (Labconco, Kansas City, MO), resuspended in 100 µl of TE and stored at 4°C.

Genomic DNA for PCR was isolated based on the protocol described by Walsh et al. (221). One loop full of cells was suspended in five percent Chelex (Bio-Rad, Richmond, CA) in TE and boiled for 15 minutes. The cellular debris was removed by centrifugation at 5000 to 6000 x g for 10 minutes. The top 50 µl of supernatant was transferred to a clean microfuge tube. The DNA concentration was normalized to 50 ng/µl and stored at -20°C.

2.2.3 Plasmid DNA isolation

Plasmid DNA from *B. cenocepacia, B. vietnamiensis* and *E. coli* was isolated using a modified alkaline lysis protocol. Overnight cultures were grown in 5 ml of LB and the cells were collected by centrifugation for 10 minutes at 3024 x g. The cells were resuspended in 220 µl of GET buffer (50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA). To lyse the cells, 330 µl of solution II (1M NaOH, 1 % SDS) was added, mixed by inversion and incubated on ice for 5 minutes. The cellular debris was removed by adding 330 µl of solution III (5M CH_3CO_2K) and centrifugation at 12000 to 16000 x g. Ribonuclease A (RNase A) was added to a final concentration of 20 µg/ml and the

mixture incubated at 37°C for a minimum of 20 minutes. Plasmid DNA was extracted from the suspension twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and once with an equal volume of chloroform/isoamyl/alcohol (24:1). The plasmid DNA was precipitated by the addition of 0.6 volumes of isopropanol and incubation at -20°C for 10 minutes. The precipitated plasmid DNA was collected by centrifugation at 12000 to 16000 x g for 15 minutes at 4°C, washed with 70 percent ethanol and dried in the speed vacuum centrifuge. The plasmid DNA was resuspended in 50 µl of sterile ddH_2O and stored at -20°C. Plasmid DNA isolation from *E. coli* was also preformed using the QIAprep mini prep kit (QIAGEN, Mississauga, ON) and the GenElute™ plasmid mini prep kit (Sigma) according to the protocol outlined by the manufacturer.

2.2.4 Polymerase chain reaction (PCR)

Oligonucleotide primers (Table 2.2) were synthesized at Invitrogen or at the University of Calgary Core DNA and Protein Services (Calgary, AB). Routine PCR reactions were performed using Platinum *Taq* polymerase (Invitrogen) in a standard thermal cycler such as the Perkin Elmer 480 or Perkin Elmer 2400 thermal cyclers (Perkin Elmer Life Sciences Inc., Woodbridge, ON). Reactions mixtures contained 1x PCR enhancer solution (Invitrogen) or Q solution (QIAGEN), 1X PCR Buffer, 0.2 mM dNTPs, 4 mM $MgCl_2$, 3.2 pmol of each primer, 50 to 200 ng of template DNA, and 2.5 U of polymerase in a 50 µl total volume. Denaturation was performed for 5 minutes at 95°C followed by 30 cycles as follows; denaturation at 95°C for 1 minute, annealing at 52°C to 60°C based on the Tm of the primer (Table 2.2) for 1 minute and elongation at 72°C for 1 minute per kb of amplicon. Colony PCR was typically used to screen

Table 2.2. Oligonucleotide primers used in this study.

Name	Sequence (5' - 3')	Tm (°C)[f]	Restriction Site[g]	Reference
M13F	GTAAAACGACGGCCAGT	52		Invitrogen
M13R	CAGGAAACAGCTATGAC	50		Invitrogen
pZE.05	CCAGCTGGCAATTCCGA	54		(50)
pZE.06	AATCATCACTTTCGGGAA	50		(50)
cciF	GCCTCATTGTGCACTCGTG[a]	60		This study
cciR	GGTGGCACTGACATCGAAAG[a]	62		This study
ccRBam	CATCGCGGATCCCGCCAT[a]	60	BamHI	This study
ccRHin	ATGTGCTAAGCTTGATCGACC[a]	62	HindIII	This study
INcciIF	CATCTTCGCTGGCAGTTTCG[a]	62		This study
INcciIR	AACGCTGGTAAAGCCGTGC[a]	60		This study
INcciRF	TTACGCGCAACGAGACTACG[a]	62		This study
INcciRR	AAAATCTTCATCGCTTCGGCG[a]	62		This study
cciRscrF	ACGGACTGGCGAGATTCACC[a]	64		This study
cciRscrR	GTCCAATCGATGAGGCTGCC[a]	64		This study
CPRxho	GTTCCGGCTCGAGCGGCG[a]	64	XhoI	This study
CPRbam	CATGAAGCGGATCCTCAGCG[a]	64	BamHI	This study
cciIRTF	TCGCGGTCGTACGATTCA[a]	56		This study
cciIRTR	TTGCACCGATCAGGTAGGC[a]	56		This study
cciRRTF	ACGCGAGGCACTCTTGTTG[a]	60		This study
cciRRTR	GCCGACATCAGAGGCTTGAA[a]	62		This study
irBRRTF	ATTGATCCCATTCGGTCAGG[a]	60		This study
irBRRTR	CGCCTCCATTGTTGGCATA[a]	58		This study
InM188F	TTCGGCTACGTCACCATCG[a]	60		This study
InM188R	CCGCATGCGTCTTGTTCA[a]	56		This study
M188F	GCCGAGATAGAGCGAGCAGA[a]	64		This study
M188R	TCAGCCATGAGTTGACCGAG[a]	62		This study
M188Xho1B	CCGGACTCGAGATCGC[a]	54	XhoI	This study
M188Xho2B	GTAGTGCCTCGAGACAGTCG[a]	64	XhoI	This study
M188scF	CGACTGTTTCGACGCACTACA[a]	64		This study
M188scR	GATTTCGTAGGCCGTCTTGC[a]	62		This study
G4cepRKOF	CGGATCGGTACCTTGGGATG[b]	64	KpnI	This study
G4cepRKOR	CCGCAAGCTTCCCGTTTCAC[b]	64	HindIII	This study
G4cepRHind	CATTTCAAGCTTGAGCTGGACC[b]	66	HindIII	This study
G4cepIF	TCAATCCCGCCGATCAAG[c]	56		This study
G4cepIR	GCGCGAAAGACCTGAGACTG[c]	64		This study
bviIKOF	GTCCGAGGATCCAGAGCG[d]	60	BamHI	This study
bviIKOR	GCACGCGAAGCTTCACGG[d]	60	HindIII	This study
bviRKOF	CACGGTCGTCTAGACGAGG[d]	62	XbaI	This study
bviRKOR	GGCCGAAGCTTGATGAATCG[d]	62	HindIII	This study

cepIF	CAGGCGGCGATAGCTTG[e]	56		This study
cepIR	CACAGATCCGAGGACATCCA[e]	62		This study
UnicepIF	GACCTTCGTTCACGAGGAAG[c]	62		This study
UnicepIR	CGTCACGCCGATCAGCTGC[c]	64		This study
cepRF	GAGAAAGAATGGAACTGCGC[e]	60		This study
cepRR	TTGTTCACGTGGAAGTTGAC[e]	58		This study
R2Cla3RE	GAACGAAGGTCTGCATGGATG[e]	64		(112)
ExcepI	GCCTGCAGGGCACAACGACGCC TATCATGC[e]	98		(110)
cepRProF	GGCCGCTCGCGACATGGT[b]	62		This study
cepRProR	CCGCGGCGCTGAATTGTTGG[b]	66		This study
bviRUF	GACCAGCTCGAGGTAGCCG[d]	64	XhoI	This study
bviRUR	CAGTGGTCGGATCCGAGCG[d]	64	BamHI	This study
bviIproX	AATAGCCCTCGAGTGGCC[d]	58	XhoI	This study
bviIproB	CGAATTGGATCCATTATCGG[d]	58	BamHI	This study
INcepI	GCGGATCCACCAGACGCCCATC TACCTGCTTCG[e]	108		(110)
RTbviIF	CACGGAGAACGCAATGAGG[d]	60		This study
RTbviIR	CACGCGGATACCCTTTACGTC[d]	66		This study
RTbviRF	CAGACGTGGGTCGAACGCTA[d]	64		This study
RTbviRR	ATAGTTGGCCGTGTTGGCG[d]	60		This study
RTsigAF	AATGACCGAGGCGAACCTG[b]	60		This study
RTsigAR	TCTTGTCTTCCGGCATCTCC[b]	62		This study

[a] Primer designed based on the *B. cenocepacia* J2315 sequence (http://www.sanger.ac.uk/Projects/B_cenocepacia/private/)

[b] Primer designed based on the incomplete *B. vietnamiensis* G4 sequencing project (http://genome.jgi-psf.org/draft_microbes/bur08/bur08.home.html)

[c] Primer designed based on the *B. vietnamiensis* PC259 *cepIR* sequence (accession no. AF337814) (121)

[d] Primer designed based on the *B. vietnamiensis* DBO1 *bviIR* sequence (accession no. AF296284) (121)

[e] Primer designed based on the *B. cenocepacia* K56-2 *cepIR* sequence (accession no. AF019654) (109)

[f] Melting temperature (Tm) of the primers was determined with the formula (4 x G or C) + (2 x A or T).

[g] Restriction endonuclease sites incorporated into primers are underlined on sequence

transformants with one bacterial colony as template and an extended initial denaturation time of 10 minutes.

Phusion High-Fidelity DNA Polymerase (Finnzymes, Espoo, Finland) was used when high-fidelity PCR amplification was required for cloning genes or promoter regions. Reactions contained 1x Phusion HF buffer, 0.2 mM dNTPs, 500 nM of each primer, 3 percent DMSO, 50-100 ng of template DNA and 1 U of polymerase in a 50 µl total volume. Following an initial denaturation 30 seconds at 98^{o}C the reaction cycled as follows; denaturation of 15 seconds at 98^{o}C, annealing at a temperature based on the Tm of the primers (Table 2.2) for 30 seconds, elongation at 72^{o}C for 30 seconds per kb of amplicon, for 30 cycles, followed by a 10 minute final elongation at 72^{o}C.

PCR amplification of the BCAM0188 region was problematic, therefore the FailSafe^{TM} PCR System (Epicentre Biotechnologies, Madison, WI) was used to amplify the 470 bp internal BCAM0188 fragment with InM188F and InM188R primers and the 1.6 kb fragment containing BCAM0188 with M188F and M188R primers according to the manufacturer's instructions. For amplification of BCAM0188 from *B. cenocepacia* strains, Platinum *Taq* polymerase was used as above with 5 mM of $MgCl_2$ and a touch down reaction cycle as follows; an initial denaturation at 95^{o}C for 5 minutes followed by denaturation at 95^{o}C for 1 minute, annealing for 3 cycles each at 70^{o}C, 67^{o}C, 65^{o}C, 63^{o}C, 61^{o}C, 59^{o}C, 57^{o}C, 55^{o}C, 53^{o}C, 50^{o}C, elongation at 72^{o}C for 1 minute per kb of amplicon.

2.2.5 Labelling DNA probes

DNA probes for Southern hybridization were randomly labelled with [α^{32}P-dCTP] (GE Healthcare Bio-Sciences, Piscataway, NJ) using the Rediprime^{TM} II Random Prime Labelling System (GE Healthcare) according to the protocol provided by the

manufacturer. Unincorporated radioactive material was removed by applying the probe to a handmade column consisting of glass wool and 1 ml of Sephadex G-50 (GE Healthcare) slurry in ddH_2O and centrifuged at 3000 x g for three minutes. The radio-labelled probes were stored at -20^oC.

2.2.6 Southern hybridizations

The downward alkaline transfer of DNA from agarose gel to membrane was performed based on the protocol of Meng (141). DNA was digested with appropriate restriction endonucleases and electrophoresed with a 0.7 percent agarose gel. The gel was depurinated in solution I (0.25 N HCl) for 25 minutes, denatured in solution II (1.5 M NaCl, O.5 M NaOH) for 30 minutes and either washed in solution III (1.0 M Tris, 2.0 M NaCl, pH 5.0) for five minutes for transfer via a vacuum blotting apparatus with 20X SSC (3 M NaCl, 0.3 M Trisodium citrate) as the transfer buffer or washed in 0.5X solution II for 25 minutes prior to conventional transfer. DNA was transferred to Gene Screen Plus membranes (Perkin Elmer) for 1 hour via the vacuum blotting apparatus or for 4 hours via conventional transfer. Transferred membranes were pre-hybridized at 65^oC for 30 minutes to 1 hour in hybridization buffer (50 % dextran sulphate, 10 % SDS). The radio-labelled probe was denatured by boiling for 5 minutes and 1 x 10^7 counts per second CPS of the denatured probe was added to the hybridization buffer along with 100 µl of salmon sperm DNA (Invitrogen). Hybridization was performed overnight at 65^oC in a VWR 5420 hybridization oven (VWR International, Mississauga, ON) with constant rotation. The membrane was then washed twice in solution A (2X SSC) for 5 minutes at room temperature, twice in solution B (2X SSC, 1% SDS) at 65^oC for 30 minutes and

twice in solution C (0.1X SSC) at room temperature for 30 minutes. The membrane was dried under a heat lamp and exposed to Kodak X-Omat AR film at -70°C.

2.2.7 Colony hybridizations

The DNA from bacterial colonies was transferred to nylon membranes using the protocol described by Woods (230). Colonies of interest were picked and patched onto agar plates and grown overnight. The colonies were transferred to sterile Colony/Plaque Screen Hybridization (Perkin Elmer) transfer membranes. The colonies were lysed from under the membrane for 15 minutes by placing the membrane on blotting paper dampened with lysis buffer (0.5 M NaOH, 1.5 M NaCl). The membranes were blotted on fresh blotting paper to remove excess lysis buffer and neutralized on blotting paper dampened with neutralization buffer (1 M Tris, 1.5 M NaCl, pH 7.0) for 5 minutes. To remove the cellular debris the membranes were washed with 3X SSC for between 15 and 20 minutes at room temperature, then at 65°C overnight in 3X SSC with 0.1 % SDS in a shaking water bath. The membranes were dried with a heat lamp and hybridized in 2.2.6.

2.2.8 Sucrose density centrifugation

A ten to fourty percent sucrose gradient was prepared using a gradient maker with 5.2 ml each of 10 percent and 40 percent sucrose solutions (20 mM Tris, 10 mM EDTA, 50 mM NaCl, 10 or 40 % sucrose) in a polyallomer centrifuge tube (Senton Scientific, Los Gatos, CA). Approximately 75 µg of digested genomic DNA was loaded onto the gradient and centrifuged at 90709 x g for 18 hours at 25°C. DNA fractions were collected by puncturing the bottom of the centrifuge tube with an 18 gauge needle and collecting 10-15 drops (500 to 700 µl) per fraction.

2.2.9 DNA sequencing and analysis

Nucleotide sequencing was performed by University of Calgary Core DNA and Protein Services or Macrogen Inc. (Seoul, Korea). The M13F and M13R primers were used for routine sequencing of DNA fragments cloned into pCR®2.1Topo (Invitrogen), pEX18Tc (80), pUCP28T (176), pUCP26 (226) and pRK415 (90). The pZE.05 and pZE.06 (50) primers were used for sequencing promoter fragments cloned into pMS402 (50). The nucleotide sequences for the *cciIR* locus and BCAM0188 were obtained from the *B. cenocepacia* J2315 sequencing project (http://www.sanger.ac.uk/Projects/B_cenocepacia/private/). The nucleotide sequences for portions of the *cepIR* and *bviIR* loci as well as the *sigA* gene were obtained from the *B. vietnamiensis* G4 sequencing project (http://genome.jgi-psf.org/draft_microbes/bur08/bur08.home.html). Sequence analysis was performed with Artemis (170), DNAMAN (Lynnon Biosoft, Vandreuil, PQ) the gapped BLASTX (5) and BLASTP (5) sequence analysis software. The Conserved Domain Database at The National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) was used to predict conserved domains present in CciR and BCAM0188 with conserved domains search software (135) and the conserved domains database (134).

2.3 Bacterial transformations and conjugation

2.3.1 Electroporation

Plasmids or ligation mixtures were introduced into *E. coli, B. cenocepacia* strains and *B. vietnamiensis* G4 by electroporation using a Gene Pulser (Bio-Rad). *E. coli* electrocompetent cells were prepared as follows; an overnight culture was sub-cultured

(1/100) into 40 ml of fresh LB and grown to mid-late log phase (absorbance of 0.5 to 1.0 at 500 nm). The cells were incubated on ice for 30 minutes and collected by centrifugation at 3024 x g for 10 minutes at 4°C. The cells were washed twice in 20 ml of cold 1 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). The cells were washed in 2 ml of 15 percent glycerol, resuspended in 1 ml of 15 percent glycerol and stored at -70°C. DNA was added to 100 µl of electrocompetent cells and incubated on ice for 5 minutes. The suspension was transferred to a 0.2 cm electroporation cuvette (Bio/Can Scientific, Mississauga, ON) and cells were pulsed with the voltage at 2.5 kV (12.5kV/cm), with the discharge capacitor at 25 µF and the resistance at 200 Ω. The cells were recovered in 300 µl of SOC (2 % tryptone, 0.5 % yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) at 37°C for 1 hour with shaking at 200 rpm. *B. cenocepacia* and *B. vietnamiensis* G4 electrocompotent cells were prepared in the same manner, replacing the HEPES buffer with MEB (1 mM $MgCl_2$, 1 mM HEPES) (42) and incubating at room temperature.

2.3.2 Chemical transformation of *E. coli*

Chemically competent *E. coli* DH5α cells were prepared based on the protocol of Ausubel et al., (7). A sub-culture (1/40) of an overnight culture was grown in 20 ml of LB until mid-log phase of growth (approximate absorbance of 0.5 at 600 nm). The cells were incubated on ice for 30 minutes and collected by centrifugation at 4354 x g for 10 minutes at 4°C. The cells were resuspended in 10 ml of cold 50 mM $CaCl_2$ and incubated on ice for 30 minutes. The collected cells were resuspended in 2 ml of $CaCl_2$ with 15 percent glycerol for storage at – 70 °C. Plasmid DNA or ligation mixture was added to 100 µl of cells and incubated on ice for 15 minutes. The cells were heat shocked at 42°C

for 45 seconds and immediately returned to ice. The cells were recovered in 300 µl of SOC for 1 hour at 37°C. Cells were plated on media with appropriate antibiotic selection.

Plasmids and ligation mixtures were also introduced into *E. coli* TOP10 (Invitrogen) chemically competent cells according to the manufacturer's instructions.

2.3.3 Bacterial Conjugation

Plasmids were mobilized into *B. cenocepacia* and *B. vietnamiensis* strains by tri-parental mating employing *E. coli* HB101 (pRK2013) (55) as the mobilization strain. Overnight cultures were grown in 5 ml of LB, sub-cultured 1/100 for the *E. coli* strains and 1/10 for *B. cenocepacia* and *B. vietnamiensis* strains into 10 ml of LB and grown for 5 hours. The turbidity of the cultures was quantified at an optical density of 600 nm. Mating strains were mixed at a ratio of 1:1:4 or 1:1:6 (recipient:mobilizer:donor). The cells were centrifuged at 5000 to 6000 x g for 10 minutes, resuspended in 100 µl of SOC and spotted on SOB (2 % tryptone, 0.5 % yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 1.5% agar) plates. Matings were incubated at 37°C for 24 hours. The mating was scraped off of the SOC medium with a sterile cotton swab and resuspended in 1 to 5 ml of LB. The transconjugants were plated onto PIA (Difco) containing appropriate antibiotic selection.

2.4 Cloning of quorum-sensing genes

2.4.1 Cloning of *cciI, cciR, cepR* and BCAM0188 from *B. cenocepacia* K56-2

The *cciI* gene was PCR amplified from K56-2 using the cciF and cciR primers and the resulting 850 bp PCR fragment was cloned into pCR®2.1Topo (Invitrogen). The nucleotide sequence was determined to confirm the absence of PCR errors. The resulting *cciI* clone was designated pRM1T4. An EcoRI fragment of pRM1T4 containing *cciI* was

sub-cloned into pUCP26 (226) in both orientations, resulting in pRM164 and pRM164R. A 4.3 kb DNA fragment containing *cciIR* was shot-gun cloned from XhoI fragments separated by sucrose gradient fractionation. The fraction containing the *cciIR* fragment was identified by Southern hybridization with a 449 bp *cciR* probe and a 450 bp *cciI* probe and cloned into the XhoI site of pCR®2.1Topo (Invitrogen), resulting in pRM4.3. The *cciI* and *cciR* probes were PCR amplified using the primers INcciIF with INcciIR and INcciRF with INcciRR respectively. Positive clones were identified using colony hybridization assays (230). A 1.1 kb NcoI/ApalI fragment containing *cciR* was cloned from pRM4.3 into pUCP26 (226) and the resulting plasmid was designated pRM165. A 1.6 kb SphI/KpnI fragment containing *cepR* was cloned from pSLA3.2 (109) into pUCP26 (226) resulting in pRM166. A 472 bp fragment internal to BCAM0188 was PCR amplified using the primers InM188F and InM188R and cloned into pCR®2.1Topo (Invitrogen) for maintenance, resulting in pRM1T8. The region containing the entire BCAM0188 open reading frame was cloned by PCR amplifying a 1.6 kb fragment with the M188F and M188R primers and cloning the fragment into pCR2.1Topo, resulting in pRM1T6. The nucleotide sequence was determined to confirm the absence of PCR errors. For complementation of the BCAM0188 mutants, a 1.3 Kb SstI/SmaI fragment from pRM1T6 was subsequently cloned into pUCP26 (226) resulting in pRM613 and a 1.6 Kb HindIII/XbaI fragment from pRM1T6 was subsequently cloned into pRK415 (90) resulting in pRM516.

2.4.2 Cloning of *cepI, cepR, bviI* and *bviR* from *B. vietnamiensis*

The *cepR, bviI* and *bviR* genes were PCR amplified from *B. vietnamiensis* G4 and cloned into the broad host range vector pUCP28T (176) as follows. The *cepR* clone,

pRM282, was constructed by amplifying a 1.5 kb fragment with the G4cepRKOF and G4cepRHind primers. The *bviI* clone, pRM285, was constructed by amplifying a 997 bp fragment with the bviIKOF and bviIKOR primers. The *bviR* clone, pRM284, was constructed by amplifying a 1.1 kb fragment with the bviRKOF and bviRKOR primers. The nucleotide sequence of all positive clones was determined to confirm the absence of PCR errors**.** A 6.8 kb BamHI fragment containing *cepIR* was shotgun cloned from BamHI fragments from *B. vietnamiensis* PC269 separated by sucrose gradient fractionation. The fragment containing *cepIR* was identified by Southern hybridization with a 627 *cepI* probe and cloned into pUCP26 (226). The *cepI* probe was amplified using the cepIF and cepIR primers. A positive clone was identified using colony hybridization assays (230) with the same *cepI* probe and designated as pRM6.8. A 2.2 kb PstI fragment containing *cepI* was sub-cloned from pRM6.8 into pUCP28T (176) resulting in pRM283.

2.5 Construction of quorum-sensing mutants

2.5.1 Construction of the *cciR, cciIR, cepI,cciI* and *cepR,cciIR* mutants in *B. cenocepacia* K56-2

The 2.0 kb fragment containing the *cciR* gene and flanking region was amplified from K56-2 using the primers ccRBam and ccRHin and cloned into the BamHI/HindIII sites of pEX18Tc (80). The *cciR* gene was disrupted by incorporation of the Tp resistance cassette from p34E-Tp (45) into the MluI site of *cciR* by blunt end ligation, resulting in pRM186-Tp (Fig. 2.1a). The vector was transferred into K56-2 using pRK2013 as the mobilizing plasmid (55). Transconjugants were plated onto

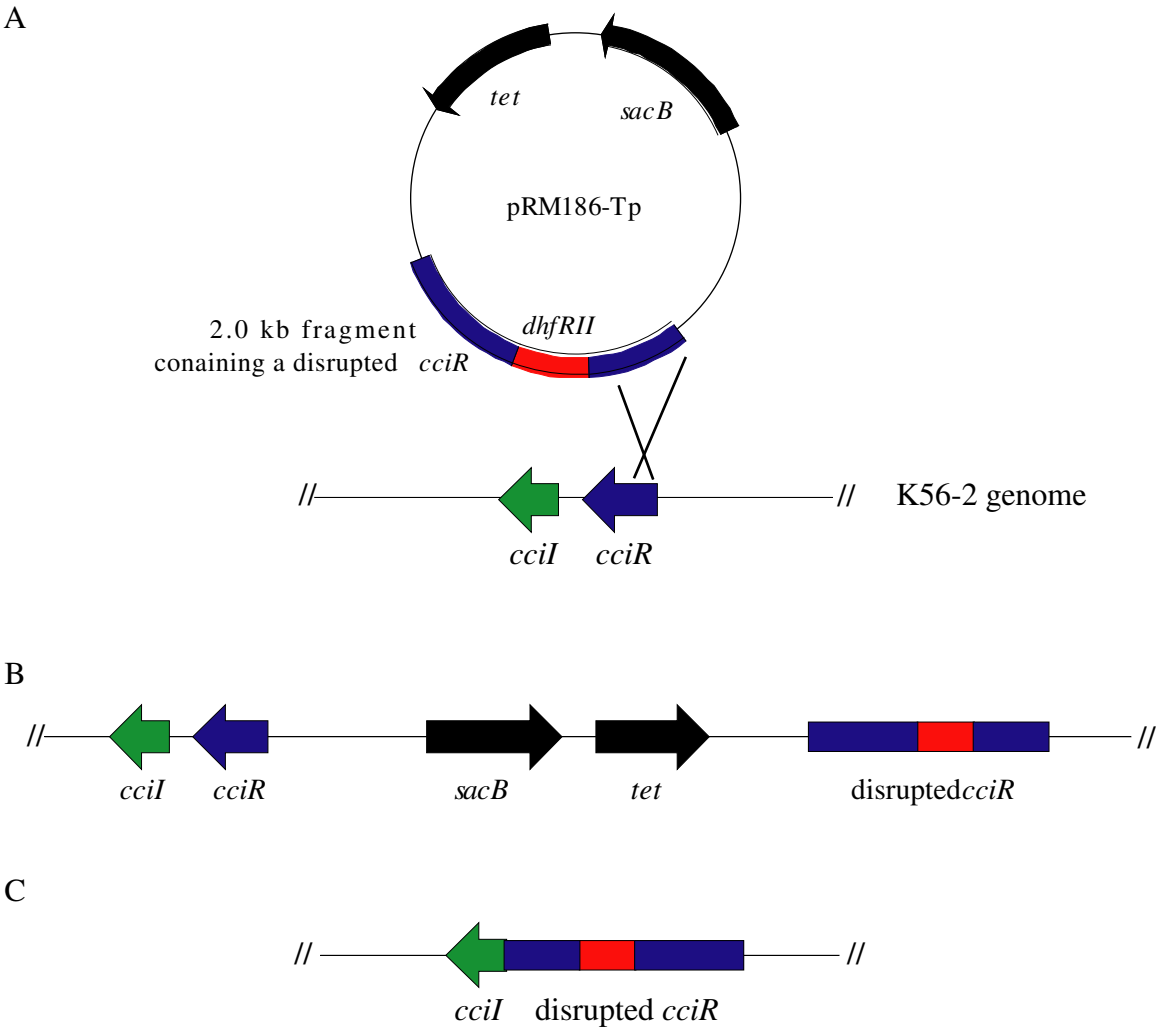


Figure 2.1. Schematic representation of quorum-sensing mutant construction using K56-2*cciR* as an example.

(A) The *cciR* open reading frame and flanking DNA was cloned into pEX18Tc (80). The *cciR* gene was disrupted by incorporation of the Tp resistance cassette containing the *dhfRII* gene. The allelic exchange vector was incorporated into K56-2 by conjugation.

(B) After a singer crossover, the pRM186-Tp vector will integrate into the K56-2 genome resulting in a merodiploid.

(C) With a second crossover, the disrupted *cciR* allele will replace the parent allele resulting in the haploid *cciR::dhfRII* mutant, K56-2*cciR*.

Pseudomonas isolation agar (PIA) (Difco) containing Tp to select for single cross-over events resulting in merodiploids (Fig. 2.1b). The haploid *cciR::dhfRII* mutant (K56-2*cciR*) (Fig. 2.1c) was identified by screening for Tc sensitivity. Allelic exchange was confirmed in by PCR with the IncciRF and IncciRR primers and by Southern hybridization with a 449 bp probe that was PCR amplified with IncciRF and IncciRR primers.

To construct an unmarked Δ*cciIR* mutant, the 4.3 kb *cciIR* fragment from pRM4.3 was cloned into pEX18Tc and *cciR* was disrupted by deleting a 637 bp NcoI to MluI fragment resulting in pRM187-KO. This deletion removed the 5' end of *cciR* and the *cciIR* promoter region. Allelic exchange was conducted as described above, resulting in a Δ*cciI,cciR* double mutant (K56-2*cciIR*). Allelic exchange was confirmed by PCR using the cciRscrF and cciRscrR primers and by Southern hybridization using a 449 bp probe amplified with IncciRF and IncciRR primers. A *cepR::dhfRII,*Δ*cciIR* mutant (K56-2*cepR,cciIR*) was constructed in the same manner with K56-2*cciIR* and the *cepR* allelic exchange vector, pEXCEPR (110). Allelic exchange was confirmed by PCR with the cepRF and cepRR primers and by Southern hybridization using a 650 bp probe amplified with cepRF and cepRR primers. A Δ*cepI,cciI::dhfRII* mutant (K56-2*cepI,cciI*) was constructed in K56-dI2 (129) using the allelic exchange vector pCciI-Tp (8). Two independent Δ*cepI,cciI::dhfRII* mutants, designated K56-2*cepI,cciIa* and K56-2*cepI,cciIb*, were isolated. K56-2*cepI,cciIa* appeared to have a spontaneous mutation that affected ornibactin biosynthesis. Allelic exchange was confirmed by PCR with the IncciIF and IncciIR primers. To construct the BCAM0188::*dhfRII* mutant a SacII digest

of pRM1T6 removed 33 bp of BCAM0188 and the Tp resistance cassette from a SmaI digest of p34E-Tp (45) was inserted into the BCAM0188 open reading frame by blunt end ligation resulting in pRMTX6. The disrupted BCAM0188 fragment was sub-cloned into pEX18Tc with HindIII and XbaI resulting in pRM1X6. Allelic exchange was performed as described above. Two individual clones of the BCAM0188 mutant were characterized and are designated as K56-2M188a and K56-2M188b. Allelic exchange was confirmed by PCR with M188scF and M188scR primers and by Southern hybridization with a 472 bp probe PCR amplified with InM188F and InM188R primers.

2.5.2 Construction of *cepI* and *cepR* mutants in *B. vietnamiensis*

To construct a *cepI::cat* mutant in *B. vietnameiensis* G4, a 2.2 kb PstI fragment from pRM6.8 containing *cepI* was blunt ended and cloned into the EcoRV site of pCR2.1Topo (Invitrogen) resulting in pRM2T2. The *cepI* open reading frame was disrupted by incorporation a SmaI fragment from p35S-Cm (43) harbouring the Cm resistance cassette into the MluI site of *cepI* yielding pRM2T2-Cm. The disrupted fragment was sub-cloned into pEX18Tc (80) with BamHI and XbaI resulting in pRM2X2-Cm, which was transferred into G4 by conjugation. Transconjugants were plated onto PIA (Difco) with Cm to select for single-crossover events. Attempts to identify a *cepI::cat* mutant by screening Cm resistant colonies for Tc sensitivity, and loss of the plasmid were unsuccessful. Attempts were made to construct the *cepI* mutant with pEXCEPI (109) and other vectors with varying amounts of flanking DNA and different resistance cassettes. None were successful; therefore, G4*cepI* is a merodiploid. The mutant was confirmed by PCR with the internal *cepI* primers UnicepIF and UnicepIR.

To construct a *cepR::cat* mutant in G4 a 2.4 kb fragment containing *cepR* was PCR amplified using the G4cepRKOF and G4cepRKOR primers and cloned into pEX18Tc (80) resulting in pRM2X1. The *cepR* open reading frame was disrupted at the PstI site by the Cm resistance cassette from p34S-Cm (43) resulting in pRM2X1-Cm. Attempts to construct a *cepR::cat* mutant were carried out as outlined above, again resulting in the merodiploid, G4*cepR*. The mutation was confirmed by PCR with the primers cepRF and cepRR. The *cepR* mutant in PC259 (PC259*cepR*) was constructed using the allelic exchange vector pEXCEPR (110). The mutation was confirmed by PCR with the cepRF and cepRR primers and Southern hybridization using a 650 bp *cepR* probe PCR amplified with the same primers.

2.6 Construction of *cepI* and *cepR* insertion mutants in G4

The *cepI* and *cepR* genes in *B. vietnamiensis* G4 were inactivated by insertion of the pGSV3 (44) suicide vector into the open reading frame of the gene. A 426 bp internal *cepI* fragment was PCR amplified using UnicepIF and UnicepRR primers and the resulting fragment was cloned into pCR®2.1Topo (Invitrogen) resulting in pRM2T5. The insertional activation vector, pRMSVI, was constructed by excising the *cepI* fragment from pRM2T5 with EcoRI and cloning it into pGSV3 (44). The *cepR* insertional activation vector, pRMSVR, was constructed in a similar manner by PCR amplification of a 647 bp fragment internal to *cepR* with cepRF and cepRR primers and cloning the fragment into pCR®2.1Topo (Invitrogen) yielding pRM2T4. To construct pRMSVR the EcoRI internal *cepR* fragment was sub-cloned from pRM2T4 into pGSV3 (44). The insertional activation vectors were mobilized into G4 by conjugation using pRK2013 (55) as the mobilization vector. Transconjugants were plated onto PIA (Difco) containing Gm

to select for insertion of the vector into the open reading frame of either *cepI* or *cepR* and the insertion was confirmed by PCR with the G4cepIF and G4cepIR primers for the *cepI* mutant (G4*cepR*SV) and with the G4cepRKOF and G4cepRKOR primers for the *cepR* mutant (G4*cepR*SV).

2.7 Analysis of BCAM0188 in the Bcc

The presence of BCAM0188 in seventeen strains representing the nine Bcc species was determined by Southern hybridization. The genomic DNA of the strains was digested with BamHI, electrophoresed on a 0.7 percent agarose gel and transferred to a membrane as described in 2.2.6. A 488 bp EcoRI fragment from pRM1T8 was used as the BCAM0188 probe fragment. The probe was radio-labeled with ^{32}P using as outlined above. The presence of BCAM0188 in 12 *B. cenocepacia* strains was determined by PCR with the M188scF and M188scR primers. The amplified products were electrophoresed on a 1.0 percent agarose gel and visualized by ethidium bromide staining and exposure to UV.

2.8 Analysis of the *bviI* and *bviR* genes in *B. vietnamiensis* strains

The presence of the *bviIR* genes in seven *B. vietnamiensis* strains was determined by PCR with primers RTbviIF and RTbviIR for *bviI* and RTbviRF and RTbviRR for *bviR*. The amplified products were electrophoresed on a 1.0 percent agarose gel and visualized by ethidium bromide staining and exposure to UV.

The *bviIR* loci from FC466, FC441, C2822 and G4 were cloned by PCR amplification of a 2.6 Kb fragment from genomic DNA using the bviIKOR and bviRKOR primers. Sequencing of the loci was performed on both strands with the M13F, M13R, bviIKOR, bviRKOR, RTbviIF, RTbviIR, RTbviRF and RTbviRR primers.

Sequence analysis was preformed with DNAMAN Sequence Analysis Software. The nucleotide sequence for the G4 *bviIR* locus was confirmed with the incomplete *B. vietnamiensis* G4 sequencing project (http://genome.jgi-psf.org/draft_microbes/bur08/bur08.home.html). Only 1.2 kb of the G4 *bviIR* locus was available through the sequencing project. The G4, FC466, FC441, and C2822 *bviIR* sequences were deposited into the NCBI database with the respective accession numbers EF032807, EF032808, EF032809 and EF032810.

2.9 Construction of *luxCDABE* transcriptional fusions

A *cepR-luxCDABE* transcriptional fusion, pRM432, was constructed by amplifying the *cepR* promoter region as described by Lewenza et al. (110) by PCR with the CPRxho and CPRbam primers. The 1.8 kb *cepR* promoter fragment was cloned upstream of the promoterless *luxCDABE* operon in pMS402 (50). The *cepI-luxCDABE* transcriptional fusion, pCP300, contains a 300 bp fragment containing the *cepI* promoter region (129). The promoter regions for *cciI* and *cciR* were predicted *in silico* using SoftBerry BPROM (http://www.softberry.com). For construction of the *cciI* and *cciR* promoter fusions a 391 bp SalI/SacII fragment and a 813 bp ClaI fragment from pRM4.3, containing the respective predicted promoter regions, were cloned into the EcoRV site of pCR®2.1Topo (Invitrogen). A XhoI/BamHI fragment containing the *cciI* or *cciR* promoter regions were subsequently cloned into pMS402 (50) and designated pRM446 and pRM445, respectively.

The remainder of the quorum-sensing gene transcriptional fusions were constructed by first predicting the promoter *in silico* using SoftBerry BPROM (http://www.softberry.com). Predicted promoter fragments were amplified by PCR and

cloned upstream of the *luxCDABE* operon in the promoter cloning site of pMS402 (50). When primers were used that did not have incorporated restriction sites (Table 2.2) the promoter fragment was initially cloned into the EcoRV site of of pCR®2.1Topo (Invitrogen) and subsequently cloned into the XhoI/BamHI site of pMS402. The sequence and orientation of the transcriptional fusions was confirmed by sequence analysis.

The primers used to amplify the promter fragments and the resulting transcriptional fusions are as follows. The BCAM0188-*luxCDABE* transcriptional fusion, pRM418, was constructed by amplifying a 645 bp product with the M188Xho2a and M188Xho2b primers. The *cepI*$_{G4}$-*luxCDABE* transcriptional fusion, pRM463, was constructed by amplifying a 266 bp promoter fragment using the primers R2Cla3RE and ExcepI (110). The *cepR*$_{G4}$-*luxCDABE* transcriptional fusion, pRM462, was constructed by amplifying a 227 bp *cepR* promoter fragment with the cepRProF and cepRProR primers. The *bviR*$_{G4}$-*luxCDABE* transcriptional fusion, pRM464, was constructed by amplifying a 695 bp promoter fragment with the bviRUF and bviRUR primers. The *bviI*$_{G4}$-*luxCDABE* transcriptional fusion, pRM465, was constructed by amplifying a 983 bp fragment with the bviIproX and bviIproB primers. The *bviI-luxCDABE* transcriptional fusions; pRM455, pRM475, pRM485, pRM495 and pRM415, were constructed using the respective genomic DNA (PC259, FC466, FC441, FC369^{T} and DBO1) as a template. The *cepI*$_{PC259}$-*luxCDABE* transcriptional fusion, pRM453, was constructed by amplifying a 769 bp promoter fragment from PC259 DNA with INcepI and ExcepI primers (110). The *cepR*$_{PC259}$-*luxCDABE* transcriptional fusion, pRM452, was constructed as described for pRM432 using PC259 genomic DNA as the template.

2.10 Luminescence assays

Overnight cultures were sub-cultured to an initial absorbance at 600 nm of 0.02 in 20 ml medium. Assays were performed with triplicate cultures. At selected times 100 µl aliquots were removed and the luminescence in counts per second (CPS) and turbidity at an absorbance of 600 nm or 620 nm were measured using a Wallac Victor^2 Multi-label counter (Perkin Elmer) or a MicroBeta TriLux microplate scintillation and luminescence counter (Perkin Elmer). The samples were read in black, clear bottom, 96 well microtitre plates (Corning Inc., Corning, NY). The level of promoter expression is reported as the ratio of luminescence to turbidity or relative luminescence. Luminescence assays for screening for gene expression in a 96 well plate format were performed as above with overnight cultures being sub-cultured (1/100) into 150 µl of medium. For luminescence assays supplemented with *P. aeruginosa* PA01 conditioned media, an overnight culture of PA01 was sub-cultured (1/100) into 50 ml of TSB and grown to an optical density of 1.5 at 600 nm. The supernatant was collected by centrifugation at 4354 x g for 10 minutes and the pH was adjusted to 7.0. The conditioned media was sterilised through a 0.22 µM filter (Millipore, Billerica, MA) and was added at 2, 5 or 10 percent of the total assay culture volume.

2.11 β-galactosidase assays

Overnight cultures were sub-cultured to an initial absorbance at 600 nm of 0.02 in 20 ml of PTSB. Assays were performed with triplicate cultures. Throughout the time course one ml aliquots were removed and β-Galactosidase activity was measured as previously described (164). Cells were lysed by mixing with 60 µl of chloroform, 30 ml of 0.1 percent SDS and placed on ice. The lysate was added to a total volume of 1 ml

Buffer Z (1.16 g $NaH_2PO_4 \cdot H_2O$, 1.90 g Na_2PO_4, 200 µl of 0.1 M $MgCl_2$, 400 ml of 1 M $MgSO_4$ to a final volume of 200 ml sterile ddH_2O; with the addition of 135 µl of β-mercaptoethanol to 50 ml of Buffer Z prior to assay) in a plastic 2 ml cuvette (DiaMed Lab Supplies Inc., Mississauga, ON). To initiate the reaction 200 µl of 4 mg/ml *o*-nitrophenol-β-D-galactosidase (ONPG) was added to the cuvette until the solution turned yellow and the reaction was terminated with the addition of 500 µl of STOP buffer (1 M Na_2CO_3). The time for the colour change to occour was timed in minutes. The absorbance at 420 nm and 550 nm of the reaction and at 600 nm for the assay culture were determined. The enzyme activity, expressed as Miller Units, was calculated with the following formula:

$$1000 \text{ X } \frac{[OD_{420} - (1.75 \text{ X } OD_{550})]}{t \text{ X } v \text{ X } OD_{600}}$$

where t is the length of time in minutes for the colour change and v is the volume of lysate used in the assay in millilitres.

2.12 RNA manipulations

2.12.1 Isolation of total mRNA

For isolation of *B. cenocepacia* K56-2 total mRNA, overnight cultures were sub-cultured (1/100) into 20 ml of LB at an initial turbidity of 0.02 at 600 nm and grown for 10 h. Total RNA was isolated from approximately 1 x 10^9 cells with a QIAGEN RNeasy mini kit (QIAGEN) according to the manufactures instructions. RNA was treated with amplification-grade DNaseI (Invitrogen) before use. For isolation of *B. vietnamiensis* total mRNA, overnight cultures were sub-cultured (1/100) into 20 ml of TSB and grown for 24 h. Total RNA was isolated from approximately 1 x 10^9 cells with the RiboPure™-

Bacteria RNA isolation kit (Ambion, St. Austin, TX) according to the manufacturer's instructions with the following modification; two times the crude aqueous RNA sample volume was loaded onto the purification column. For quantification, the RNA sample was diluted 1/50 in TE and the absorbance at 260 nm and 280 nm were determined and the total RNA concentration was calculated with the assumption that an absorbance of 1.0 at 260 nm is equal to 40 µg/ml of RNA. The ratio of absorbance at 260 nm to 280 nm was also calculated to determine the purity of the sample, with ratios between 1.8 and 2.1 considered acceptable. RNA was treated with amplification-grade DNaseI (New England Biolabs) before use.

2.12.2 Reverse-transcriptase (RT)-PCR

RT-PCR was performed using a Titan One-tube RT-PCR kit (Roche) according to the manufacturer's instructions. For each 50 µl reaction 0.2 mM of dNTPs, 5 mM dithiothreitol (DTT), 1.5 mM $MgCl_2$, 5 U of RNase inhibitor, 0.4 µM of each primer, 1X RT-PCR buffer, 1 reaction of Titan^2 enzyme mix and 50 or 75 ng of RNA was used. cDNA was synthesized by reverse transcription at 50^{o}C for 40min. Denaturation was performed for 2 min at 96^{o}C followed by 35 cycles of PCR as suggested by the manufacturer followed by a final elongation step at 68^{o}C for 7 min. To ensure that there was no DNA contamination in the RNA samples, Platinum *Taq* polymerase (Invitrogen) was used instead of the reverse transcriptase enzyme mixture with the same reaction conditions.

Primer sets were designed to amplify internal to *cciI,* internal to *cciR* and the *cciIR* intergenic region to yield 380 bp, 280 bp and 532 bp products respectively. The primers and annealing temperatures are as follows; *cciI* (64^{o}C): cciIRTF and cciIRTR;

cciR (58°C): cciRRTF and cciRRTR; *cciIR* intergenic region (58°C): irBRRTF and irBRRTR. Primer sets were designed to internally amplify *bviI, bviR* and *sigA* to yield 297 bp, 297 bp, and 347 bp products respectively. The *sigA* gene encodes for the principal sigma factor (19) and was used as a control gene. A homologue of *B. cepacia sigA* was identified in the *B. vietnamiensis* G4 sequencing project (http://genome.jgi-psf.org/draft_microbes/bur08/bur08.home.html). *B. vietnamiensis* G4 SigA is 89.93% identical to SigA of *B. cepacia* (accession no. AAD03549) (19). The primers and annealing temperatures used are as follows; *bviI* (58°C): RTbviIF and RTbviIR; *bviR* (64°C): RTbviRF and RTbviRR; *sigA* (62°C): RTsigAF and RTsigAR. RTbviRF and RTbviRR primers yielded an approximately 745 bp non-specific contaminating band (data not shown). This band was sequenced and a BLAST (5) search indicated that the fragment has homology to *B. vietnamiensis* LMG10929T 23s rRNA and is not related to *bviR.*

2.13 Phenotypic assays

2.13.1 AHL production

AHL production was assessed by AHL-TLC bioassays as described by Lewenza et al, (110) (Fig. 2.2). For crude AHL extractions from *B. cenocepacia* and *E. coli* strains overnight cultures were sub-cultured into 40 ml of TSB with an initial turbidity of 0.05 at 600 nm and grown for 12 hours with constant shaking (200 rpm) at 37°C. Clarified culture fluid was collected by centrifugation at 3024 x g for 10 minutes. The AHLs were

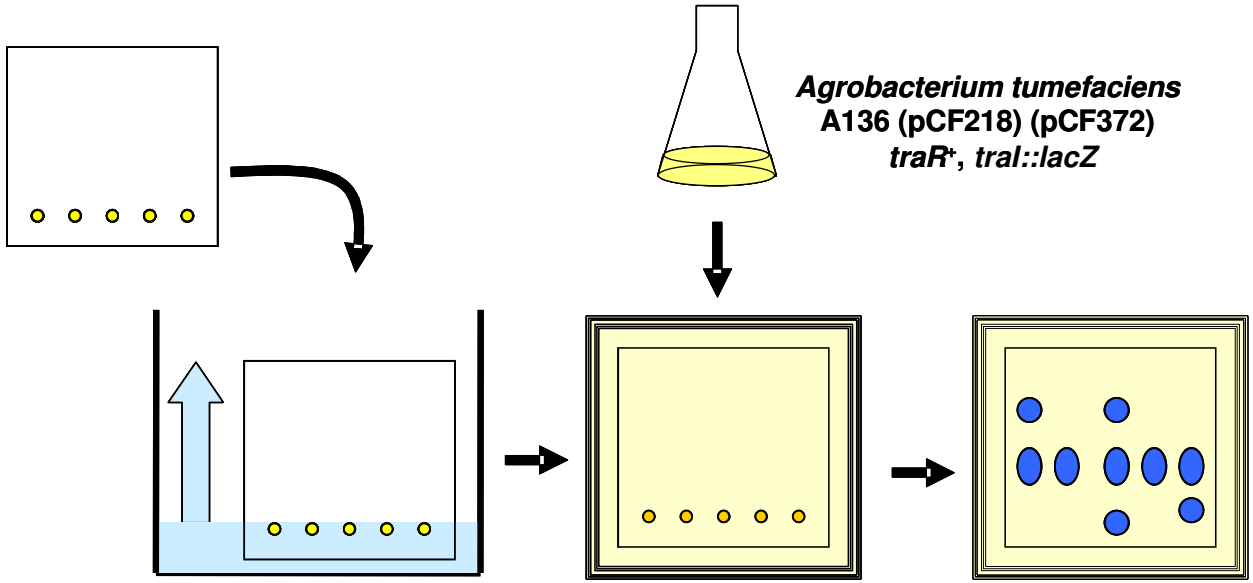


Figure 2.2. Schematic representation of the AHL-TLC bioassay. Crude AHL extracts were spotted onto a C_{18} reverse phase TLC plate and resolved in 70 percent methanol. The TLC plate was air-dried and placed in an assay tray. The plate was overlaid with molten AT agar containing the *A. tumefaciens* (pCF218)(pCF372) reporter strain and 60 µg/ml X-gal. The plate was incubated at 30°C for 24-48 hours for the AHL spots to be detected.

extracted twice with equal volumes of acidified ethyl acetate (0.1 ml of glacial acetic acid per litre). Extracts were concentrated using a rotary evaporator (BUCHI RE11 Rotoevaporator) at 40-45^{o}C. The AHLs were re-suspended in 2 ml of acidified ethyl acetate and dried using a speed vacuum microfuge. Dried AHL samples were stored at -20^{o}C until assayed. Thawed AHL samples were re-suspended in 100 µl of acidified ethyl acetate for analysis. Crude AHL extracts from *B. vietnamiensis* strains were performed as above with the following modifications; overnight cultures were sub-cultured (1/100) into 40 ml of TSB and grown for 24 hours at 30^{o}C, the AHLs were extracted once from the clarified culture fluid with equal volumes of acidified ethyl acetate and thawed AHL samples were re-suspended in 60 µL of acidified ethyl acetate for analysis.

Samples were spotted onto C_{18} reversed-phase TLC plate (Whatman, Florham Park, NJ) and developed in methanol-water (70:30, vol/vol). The resolved plate was allowed to dry. An agar overlay containing the *A. tumefaciens* A136 (pCF218)(pCF372) AHL reporter strain that is able to identify AHLs with 3-oxo-, 3-hydroxy-, and 3-unsubsituted side chains ranging from 6 to 16 carbons in length (178) was prepared by sub-culturing (1/100) an overnight culture into 30 ml of LB and growing the culture to an optical density of 0.3 at 600 nm. Cells were collected by centrifugation at 3024 x g for 10 minutes, resuspended in 20 ml of AT medium and incubated at 30^{o}C for 30 minutes. The culture was added to 150 ml of molten AT agar supplemented with 60 µg/ml of X-gal and 0.5 percent glucose. The overlaid TLC plates were incubated at 30^{o}C for 24 or 48 hours. Synthetic AHL standards (Sigma) were used as reference standards in the following quantities; 2.5 µmol of HHL, 0.5 µmol of OHL, 5.0 µmol of DHL.

2.13.2 Protease activity

Protease activity was determined using skim milk as a substrate (189). Cultures were grown overnight and a sub-cultured (1/100) into 10 ml PTSB. At mid log phase of growth the cultures were normalized to an optical density of 0.3 at 600 nm and spotted (2 µL) in triplicate onto dialysed 1.5 percent brain heart infusion agar containing 10 percent skim milk. The plates were incubated at 37°C for 24 or 48 hours and the zones of clearing around the growth were measured. For AHL add-back assays, 2.5 nM and 10.0 nM concentrations of synthetic HHL and 2.5 nM of synthetic OHL dissolved in 20 percent acetonitrile were spread on the agar prior to spot inoculation.

2.13.3 Swarming motility

Overnight cultures were normalized and 1 µl was spot inoculated onto semi-solid nutrient agar plates (0.5% agar) supplemented with 0.5% glucose (111). The plates were incubated at 37°C for 24 to 48 hours and the diameter of the swarm zone was measured.

2.13.4 CAS activity

Ornibaction production was measured by Chrome Azurol S (CAS) assays (177). Siderophores present in the culture supernatant are able to remove the iron from the CAS dye complex, resulting in a blue to orange colour change that can be quantitatively measured at an absorbance of 630 nm. All glassware was acid-washed with 3 M HCl prior to use to remove trace amounts of iron. Overnight cultures were grown in succinate medium with 10 mM ornithine, sub-cultured (1/100) and normalized. The cultures were grown for 40h at 32°C. CAS assays were preformed with 100 µl of supernatant fluid mixed with 400 µl of sterile ddH_20 and 500 µl of CAS dye (177) in a 2 ml plastic cuvette. The assay was incubated at room temperature, in the dark, overnight and the

absorbance at 630 nm was determined in a Philips PYE UNICAM PU8800 dual beam spectrophotometer (Markham, ON). To normalize for cell density the absorbance at 630 nm was divided by the turbidity of the culture at an absorbance of 600 nm and this ratio was reported as CAS activity.

2.13.5 Biofilm formation

The following procedure was adapted for use with polysterene pegs from methods originally developed by Christiansen (23, 24). Biofilms were formed on the MBEC™ System or on NUNC 96 pegged transfer lids (Nalge Nunc International, Rochester, NY) by placing the pegs in a 96-well microtiter plate. Inoculation suspensions of each strain were prepared in TSB, normalized to an optical density of 0.4 at 600 nm and inoculated 1/30 into 150 µl of TSB. The device was incubated for 24 hours on a rocking table at 37°C, after which the lid was removed and rinsed briefly to remove loose biomass in a trough containing double distilled water. The lid was air dried for 10 minutes, stained with 1.0 percent crystal violet in a trough for 1 minute, and rinsed 3 times in separate troughs containing double distilled water. The stained pegs were decolourised with 175 µl of methanol in a microtiter plate for 1 minute. The quantity of crystal violet was measured using a Wallac Victor^2 Multi-label counter (Perkin Elmer) set to measure absorbance at 540 nm.

2.13.6 Growth on nitrogen-free media

The nitrogen-free medium, Baz (54) was used to assess the ability of *B. vietnamiensis* to fix atmospheric nitrogen. Strains were streaked onto the medium and grown in aerobic and anaerobic conditions. *B. vietnamiensis* FC369^T and *E. coli* DH5α were used as positive and negative controls respectively.

2.13.7 Biochemical tests with API 20 NE

Biochemical characteristics of the *B. vietnamiensis* quorum-sensing mutants were determined with API 20 NE tests (bioMerieux, St. Laurent, PQ) according to the protocol outlined by the manufacturer.

2.13.8 Virulence in the alfalfa model of infection

The alfalfa infection model was performed as previously described by Bernier et al., (11). *Medicago sativa* (alfalfa) variety 57Q77 seeds (Pioneer Hi-Bred International, Inc, Johnston, IA) were immersed in concentrated sulphuric acid (approximately 10 ml for 300 seeds) for 20 min to disinfect and accelerate germination and then washed with 500 ml of sterile ddH_20 four times. The seeds were suspended in 60 ml of sterile ddH_2O in and incubated at 32°C with shaking for 6 to 8 h to encourage uniform imbibition and germination. Ten seedlings were placed per plate of water agar (1% Difco Bacto Agar, 1% Difco Noble agar). One leaf of each seedling was wounded by piercing the leaf with a 21 gauge needle. Immediately after the leaves were wounded, the seedlings were surface inoculated with 10 µl containing approximately 1.5 x 10^9 cells in 0.85 percent NaCl or with 10 µl of 0.85 percent NaCl as a control. The plates of infected seedlings were sealed with parafilm and incubated in at 30°C or 37°C under a desk lamp at an intensity of 640 lx. The initial inoculum was quantified by serial dilution in 0.85 percent NaCl and spread plating onto LB agar plates. After seven days the seedlings were visually monitored for disease symptoms including; yellow leaves, stunted roots, and brown necrotic regions.

2.14 Statistical analysis

Statistical analysis including unpaired *t*-test and analysis of variance (ANOVA) was performed with INSTAT software (GraphPad Software, San Diego, Calif.). A *P* value of < 0.05 was considered significant. The Welch correction was applied when comparing tow populations with unequal standard deviations. Post tests were applied when multiple analysis were made; Bonferroni for multiple comparisons, Tukey-Kramer or Student- Newman-Keuls when the Bonferroni correction was considered too stringent, and Dunnett when comparing multiple values to a control.

Chapter Three: Characterization of the *cciIR* quorum-sensing system in an epidemic strain of *Burkholderia cenocepacia*

3.1 Introduction

The epidemic *B. cenocepacia* strain, K56-2, produces the AHL signalling molecules OHL and HHL. A K56-2 *cepI* mutant, K56-I2, does not produce detectable levels of OHL, but produces small amounts of HHL (110). A *cepI* mutant in *B. cenocepacia* strain H111 is devoid of AHL production (83), suggesting that there is an additional AHL synthase present in K56-2 that is not present in H111. A novel set of *luxIR* homologues was identified during characterization of the *cenocepacia* island (8). BCAM0239a and BCAM0240 lie within the ***c****eno****c****epacia* **i**sland, approximately 14 kb upstream of the BCESM open reading frame *esmR,* and were named *cciI* and *cciR* respectively. This was the first account of a quorum-sensing system present on a genomic island (8). The *cciI* gene was found to be always associated with an intact *cenocepacia* island and was present in all *B. cenocepacia* strains possessing the original BCESM (8). Epidemiological studies have demonstrated that the BCESM is present in the majority of *B. cenocepacia* group A strains isolated from CF patients but is absent in the most group B CF strains (118, 194). Unlike strain K56-2, strain H111 does not contain the BCESM or the *cciI* gene (E. Mahenthiralingam, personal communication). The absence of *cciI* in H111 is a probable explanation for the difference in AHL production between the *cepI* mutants in K56-2 and H111. In CF patient populations where BCESM positive *B. cenocepacia* strains are prevalent, the infections are associated with transmissibility, virulence, and mortality (128, 193). A *cciI* mutant constructed in K56-2 (K56-2*cciI*) was shown to have attenuated virulence in a rat chronic respiratory

infection model (8). The inflammation in animals infected with K56-2*cciI* was approximately 50% less than that observed in animals infected with K56-2, despite similar numbers of bacteria being present in the lungs. This data indicates that the CciI AHL synthase contributes significantly to the virulence of *B. cenocepacia* strains containing the *cenocepacia* island.

The objectives of this study were to determine if the *cciIR* genes encode for a functional quorum-sensing system responsible for the production of HHL in the K56-2 *cepI* mutant, determine if the *cciIR* system is involved in the regulation of virulence associated phenotypes, and if a regulatory relationship exists between the *cepIR* and *cciIR* quorum-sensing systems in the *B. cenocepacia* K56-2.

3.2 Results

3.2.1 The *cciIR* genes encode for an AHL mediated quorum-sensing system

The *cciI* and *cciR* genes are 623 and 788 bp respectively and are arranged in tandem with a 254 bp intergenic region. The *cciI* gene product shares 36 percent identity with SolI of *R. solancearum* (accession no. AAC45948) (56), 34 percent identity with CepI of *B. cenocepacia* (accession no. AAD12727) (109) and 23 percent identity with LuxI of *V. fischeri* (accession no. 1403259B) (47) (Fig. 3.1a). CciI contains nine of the ten residues that are conserved among LuxI AHL synthases (157). A predicted *lux* box–type sequence has been identified and is centered 90 bp upstream of the predicted *cciI* transcription start (Fig. 3.1b).

The *cciR* gene product is 24 percent identical to CepR of *B. cenocepacia* (accession no AAD12726) (109), 22 percent identical to SolR of *R. solancearum*

A

```
                                  *   *     *           *  *
CciI    .VPSIFAGSFDDMPTTMHRRLGVFRYDVFVGRLGWQLPGADATSLTEWDQ     49
SolI    ..MRTFVHGGGRLPEGIDAALAHYRHQVFVGRLGWQLPMADGT..FERDQ     46
CepI    ..MQTFVHEEGRLPHELAADLGRYRRRVFVEQLGWALPSANES..FERDQ     46
LuxI    MTIMIKKSDFLAIPSEEYKGILSLRYQVFKQRLEWDLVVENNL...ESDE     47

         *                    *               *
CciI    FDRGRTIHVVSVDQAQHICGCARLIPTTQPYLLQTLCAPSAALHLPR..A     97
SolI    YDRDDTVYVVARDEGGTICGCARLLPTTRPYLLKDVFASLLMHGMPPPES     96
CepI    FDRDDTVYVFARNADGDMCGCARLLPTTRPYLLKSLFADLVAEDMPLPQS     96
LuxI    YDNSNAEYIYACDDTENVSGCWRLLPTTGDYMLKSVFPELLGQQSAPK.D     96

           *  *
CciI    PTVWELSRFAARCGANPT...MRASTGMQLFPAILAIAASLGATCVIGAM    144
SolI    PEVWELSRFAARSGAPCPRSGRADWAVRPMLASVVQCAAQRGARRLIGAT    146
CepI    AAVWELSRFAATDDEGGP..GNAEWAVRPMLAAVVECAAQLGARQLIGVT    144
LuxI    PNIVELSRFAVGKNSSKIN.NSASEITMKLFEAIYKHAVSQGITEYVTVT    145

CciI    TRAVARLYQRCGLALQLLDTA...ETADRPAYLIGAIEITRSTFTNLGCD    191
SolI    FVSMVRLFRRIGVRAHRAGPV...RCIGGRPVVACWIDIDASTCAALGIP    193
CepI    FASMERLFRRIGIHAHRAGPP...KQVDGRLVVACWIDIDPQTFAALGIE    191
LuxI    STAIERFLKRIKVPCHRIGDKEIHVLGDTKSVVLSMPINEQFKKAVLN..    193

CciI    AHELLAAVTWLGAHAT                                      207
SolI    SASAAPGPVLQ.....                                      204
CepI    PGQAARQAIAA.....                                      202
LuxI    ................                                      193
```

B

```
        cciI    TCCTGTCAACTTTATCGGCG      20
        cepI    ..CTGTAAGAGTTACCAG..      16
        luxI    ACCTGTAGGATCGTACAGGT      20
```

Figure 3.1. Multiple alignments of amino acid sequences from LuxI homologues and *lux* box promoter elements.

(A) Amino acid alignment of *B. cenocepacia* CciI with the LuxI homologues; SolI of *R. solancearum* (accession no. AAC45948) (56), CepI of *B. cenocepacia* (accession no. AAD12727) (109) and *V. fischeri* LuxI (accession no. 1403259B) (47). The alignment was generated using DNAMAN sequence analysis software. The shaded regions highlight amino acids conserved in at least three of the four proteins. The ten invariant amino acids characteristic of LuxI homologues are denoted by an asterisk above the sequence (157).

(B) Comparison of the lux box sequences in the promoter regions of *B. cenocepacia cciI* with *B. cenocepacia cepI* (225), and *V. fischeri luxI* (47).

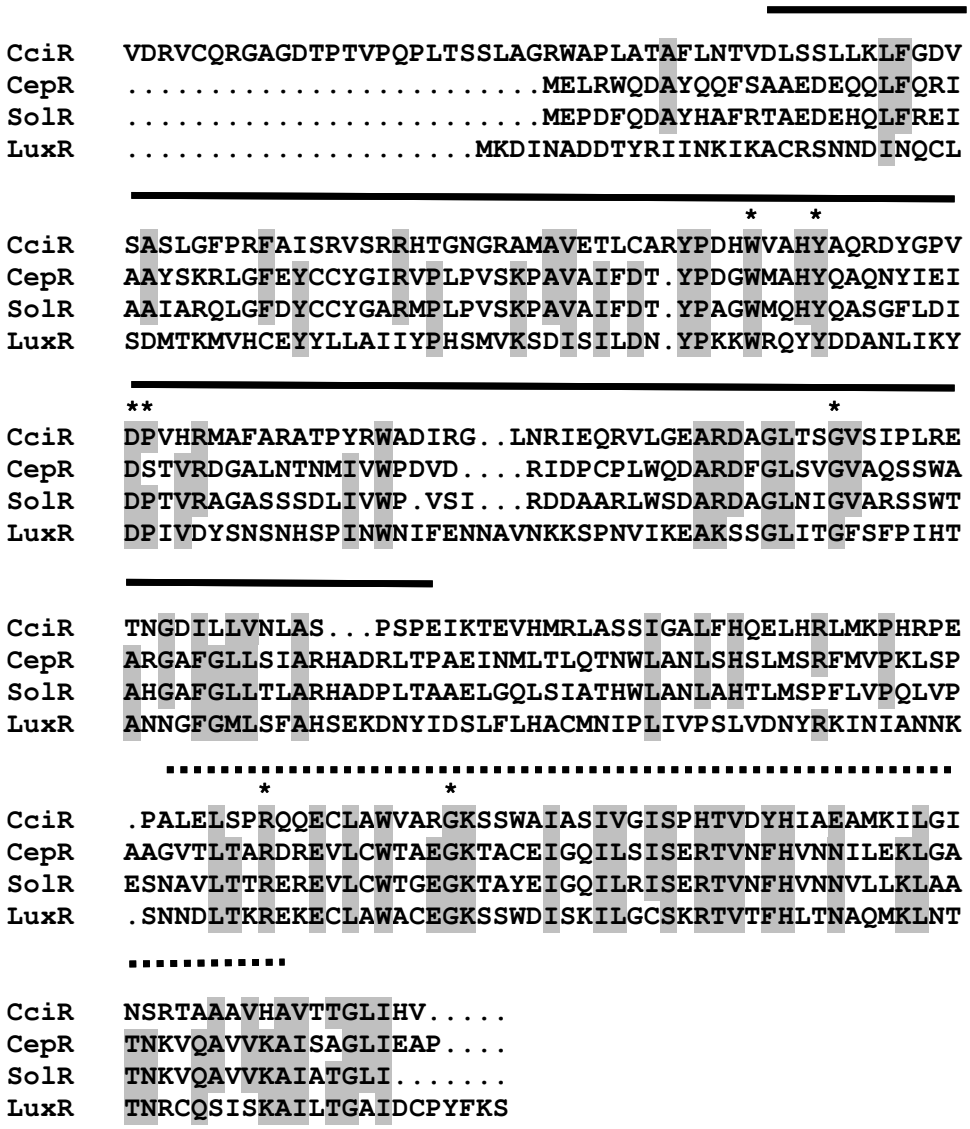


Figure 3.2. Multiple alignments of amino acid sequences from LuxR homologues. CciR of *B. cenocepacia* is aligned with CepR of *B. cenocepacia* (accession no AAD12726) (109), SolR of *R. solancearum* (accession no. AAC45947) (56) LuxR of *V. fischeri* (accession no. 1403259A) (47). The alignment was generated using DNAMAN sequence analysis software. The shaded regions highlight amino acids conserved in at least three of the four proteins. The seven residues that are conserved among LuxR transcriptional regulators (61) are denoted by an asterisk above the sequence. The AHL binding domain from residues (39 to 164) (134, 135) is indicated by a solid line above the sequence and the helix-turn-helix DNA binding motif from residues 197 to 254 is indicated by a dashed line above the sequence.

(accession no. AAC45947) (56) and 18 percent identical to LuxR of *V. fischeri* (accession no. 1403259A) (47) (Fig. 3.2). CciR contains all seven residues that are conserved among LuxR transcriptional regulators (61). A motif search indicated that CciR contains both an AHL binding and a helix-turn-helix DNA binding domain (135) (Fig. 3.2)

To determine if *cciI* encodes for a functional AHL synthase, the *cciI* gene was cloned and expressed in *E. coli* DH5α. The AHLs present in the culture supernatant of *E. coli* DH5α (pRM1T4), which contains *cciI,* were analyzed using the *A. tumefaciens* TLC-AHL bioassay. *E. coli* DH5α containing *cciI* produced AHLs that co-migrated with synthetic HHL (Fig. 3.3a, lane 4). An AHL that co-migrated with synthetic OHL was weakly detectable only after the plate was allowed to develop for greater than 72 hours. *E. coli* DH5α (pSLS225), which contains *cepI* produced AHLs that co-migrated on the TLC plate with both HHL and OHL (Fig. 3.3a, lane 2)

To confirm that *cciI* directs the synthesis of HHL and minor amounts of OHL, extracts of K56-2*cciI* and K56-2*cepI,cciIa* were analyzed using the *A. tumefaciens* TLC-AHL bioassay. The *cepI,cciI* mutant was constructed by allelic exchange and confirmed by PCR (Fig. 3.4a). K56-2*cciI* produced slightly less HHL compared to the parent strain (Fig. 3.3b, compare lanes 1 and 2). HHL production was restored to parental levels when *cciI* was present *in trans* (Fig. 3.3b, compare lanes 1 and 3). There were no detectable AHLs in extracts from K56-2*cepI,cciIa* indicating that *cepI* and *cciI* are collectively responsible for AHL synthesis in K56-2 (Fig. 3.3c, lane 1). HHL or OHL

Figure 3.3. TLC-AHL bioassays of the K56-2 quorum-sensing mutants. Ethyl acetate extracts were chromatographed on C_{18} reverse-phase TLC plates developed with methanol-water (70:30 vol/vol). The spots were visualized using the *A. tumefaciens* reporter strain.

(A) Heterologous expression of *cciI* in *E. coli.* Lane 1, *E. coli* DH5α (pUCP26); Lane 2, *E. coli* DH5α (pSLS225); Lane 3: *E. coli* TOP10 (pCR®2.1TOPO); Lane 4, *E. coli* TOP10 (pRM1T4); Lane 5, synthetic AHL standards.

(B) AHL production profile of K56-2*cciI* and the complemented mutant. Lane 1, K56-2 (pUCP26); Lane 2 K56-2*cciI* (pUCP26); Lane 3, K56-2*cciI* (pRM164); Lane 4, synthetic AHL standards.

(C) AHL production profile of K56-2*cepI,cciIa* and the complemented mutant. Lane 1, K56-2*cepI,cciIa* (pUCP26); Lane 2, K56-2*cepI,cciIa* (pRM164); Lane 3, K56-2*cepI,cciIa* (pSLS225); Lane 5, synthetic standards.

(D) AHL production profiles of the transcriptional regulator mutants. Lane1, K56-2; Lane 2, K56-R2 (*cepR*); Lane 3, K56-2*cciR;* Lane 4, K56-2*cciI,cciR*; Lane 5, K56-2*cepR,cciIR*; Lane 6,synthetic standards.

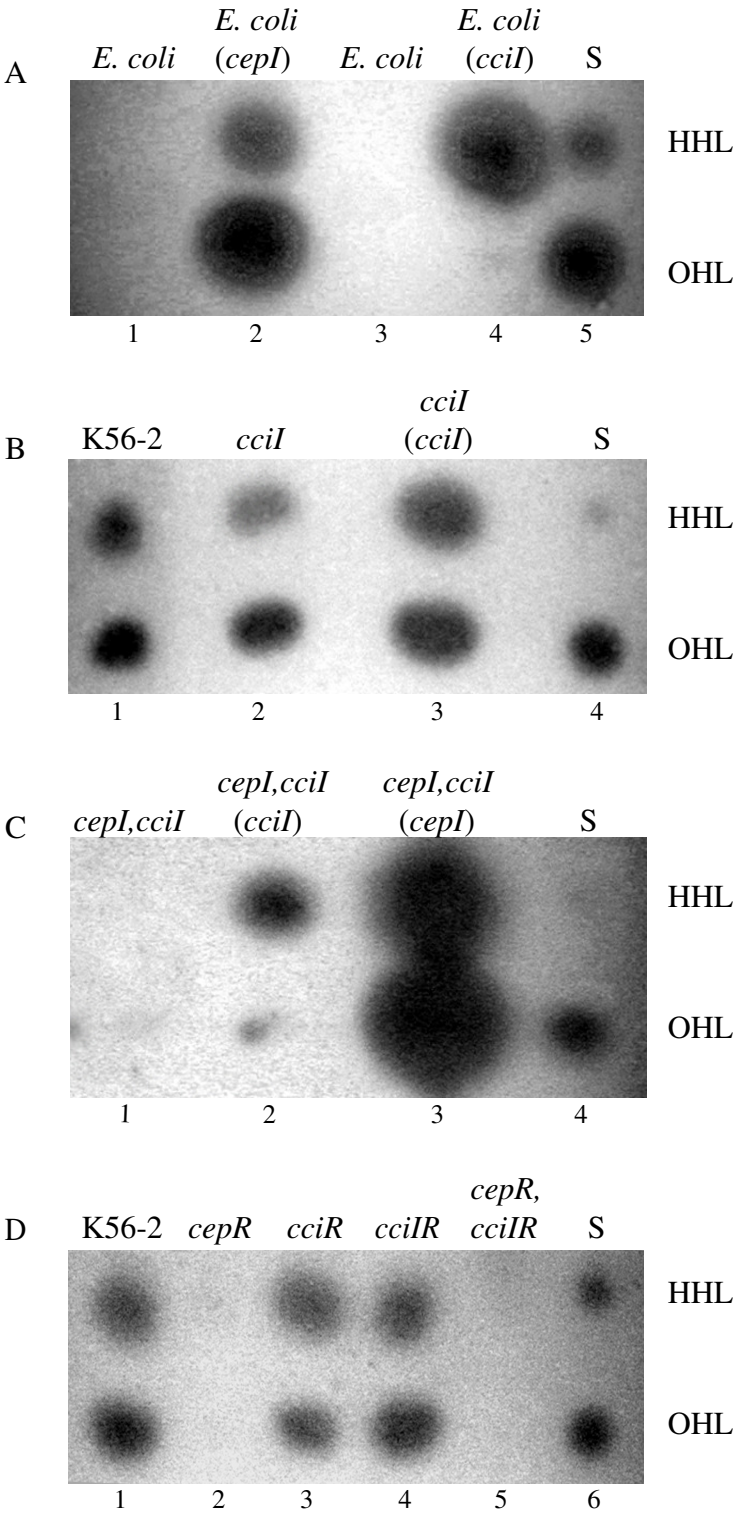

A
E. coli
E. coli (cepI)
E. coli
E. coli (cciI)
S
HHL
OHL
1 2 3 4 5
B
K56-2
cciI
cciI (cciI)
S
HHL
OHL
1 2 3 4
C
cepI,cciI
cepI,cciI (cciI)
cepI,cciI (cepI)
S
HHL
OHL
1 2 3 4
D
K56-2
cepR
cciR
cciIR
cepR, cciIR
S
HHL
OHL
1 2 3 4 5 6

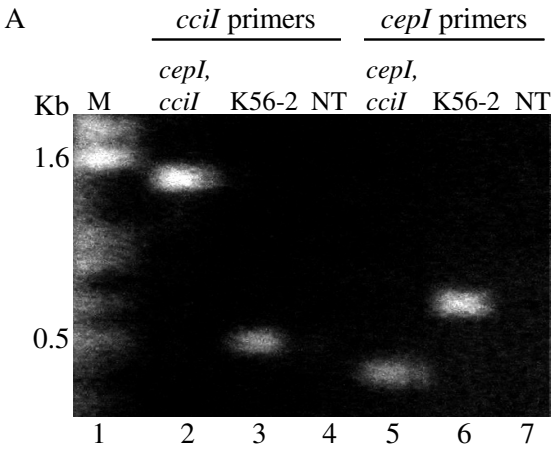
Figure 3.4. Confirmation of quorum-sensing mutant construction.

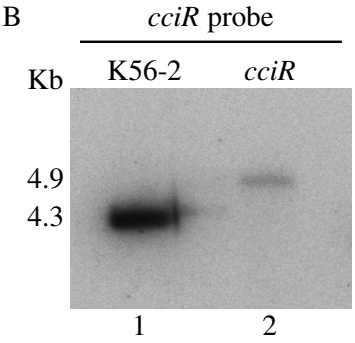
(A) PCR analysis of the K56-2*cepI,cciIb* mutant. An identical PCR analysis was performed for the K56-2*cepI,cciIa* mutant (data not shown). Products were electrophoresed on a 1.0% agarose gel. Lane 1; 1 Kb plus ladder (Invitrogen), lane 2; *cciI* product amplified from K56-2*cepI,cciIb* genomic DNA with the InccIIF and InccIR primers, lane 3, *cciI* product amplified from K56-2 genomic DNA with the InccIIF and InccIR primers, lane 4, no template control with the InccIIF and InccIR primers, lane 5; *cepI* product amplified from K56-2*cepI,cciIb* genomic DNA with the cepIF and cepIR primers, lane 6; *cepI* product amplified from K56-2 genomic DNA with the cepIF and cepIR primers, lane 7; no template control with the cepIF and cepIR primers. An increase of 1 kb in the amplified *cciI* fragment is due to the incorporation of the Tp resistance cassette. A 0.3 kb decrease in the amplified *cepI* fragment is due to the deletion of an NruI fragment internal to *cepI*. M; DNA ladder, NT; no template control.

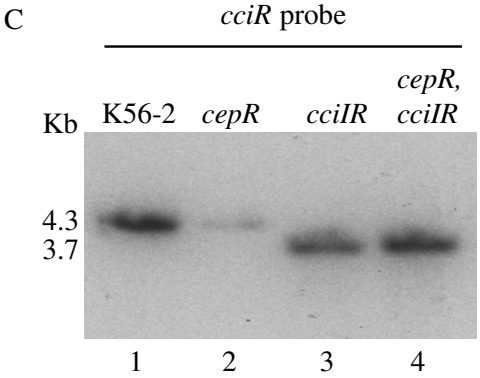
(B) Southern blot of K56-2*cciR* genomic DNA hybridized with a *cciR* probe. Genomic DNA was digested with XhoI and hybridized with a PCR amplified 450 bp *cciR* fragment labelled with ^{32}P. Lane 1; K56-2, Lane 2; K56-2*cciR*. An increase of 0.6 kb in the labelled fragment is due to the incorporation of the Tp resistance cassette.

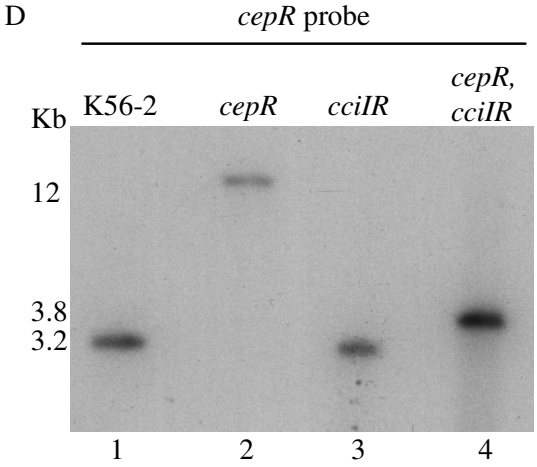
(C) Southern blot of K56-2*cciIR* and K56-2*cepR* genomic DNA hybridized with a *cciR* probe. Genomic DNA was digested with XhoI and hybridized with a PCR amplified 450 bp *cciR* fragment labelled with ^{32}P. Lane 1; K56-2, lane 2; K56-R2, lane 3; K56-2*cciIR*, lane4; K56-*2cepR,cciIR*. A 0.65 kb decrease in the hybridized fragment is due to the deletion mutation.

(D) Southern blot of K56-2*cepR,cciIR* genomic DNA hybridized with a *cepR* probe. Genomic DNA was digested with SphI and hybridized with a PCR amplified 650 bp *cepR* fragment labelled with ^{32}P. Lane 1; K56-2, lane 2; K56-R2, lane 3; K56-2*cciIR*, lane4; K56-*2cepR,cciIR*. The 10 kb increase in size of the hybridized fragment in K56-R2 is due to the incorporation of Tn5-OT182 and the 0.6 kb increase in the hybridized fragment in K56-2*cepR,cciIR* is due to the incorporation of the Tp resistance cassette.


A
cciI primers
cepI primers
cepI, cciI
cepI, cciI
Kb
M
K56-2
NT
K56-2
NT
1.6
0.5
1
2
3
4
5
6
7


B
cciR probe
Kb
K56-2
cciR
4.9
4.3
1
2


C
cciR probe
Kb
K56-2
cepR
cciIR
cepR, cciIR
4.3
3.7
1
2
3
4


D
cepR probe
Kb
K56-2
cepR
cciIR
cepR, cciIR
12
3.8
3.2
1
2
3
4

production was restored in K56-2*cepI,cciIa* by complementation with *cepI* or *cciI* (Fig. 3.3c,lanes 2 and 3), confirming the results obtained by heterologous expression in *E. coli* (Fig. 3.3a). K56-R2 (*cepR*) has previously been shown to produce small amounts of HHL (110). Mutants in *cciR, cciIR* and *cepR,cciIR* were constructed and confirmed by Southern hybridization (Fig. 3.4c-d). The K56-2*cciR* and the K56-2*cciIR* mutant AHL production profiles were similar to K56-2 (Fig. 3.3d, comparing lanes 1, 3 and 4) indicating that CciR is not required for AHL production. K56-2*cepR,cciIR* produced very small amounts of HHL and OHL that were weakly detectable only when the plate was overdeveloped (Fig. 3.3d, lane 5) confirming that CepR is required for optimum production of both HHL and OHL.

3.2.2 Characterization and expression of the *cciIR* operon

Transcriptional analysis of *cciIR* was performed to determine if these genes regulate their own transcription. The promoter regions of *cciI* and *cciR* were predicted *in silico* and *cciI-luxCDABE* and *cciR-luxCDABE* transcriptional fusions were constructed based on these predictions (Fig. 3.5b). The expression of *cciI-luxCDABE* and *cciR-luxCDABE* was compared in K56-2 and expression was only observed for *cciR-luxCDABE.* Additional *cciI-luxCDABE* transcriptional fusion constructs were made by cloning varying lengths of the region upstream of *cciI* in front of the promoterless *luxCDABE* operon. None of these constructs resulted in the expression of the *luxCDABE* operon.

RT-PCR was used to determine if *cciI* and *cciR* are co-transcribed. Three primer sets were designed to internally amplify *cciI, cciR*, and the intergenic region between *cciR* and *cciI* (Fig. 3.5c). A product was amplified for each gene as well as the intergenic

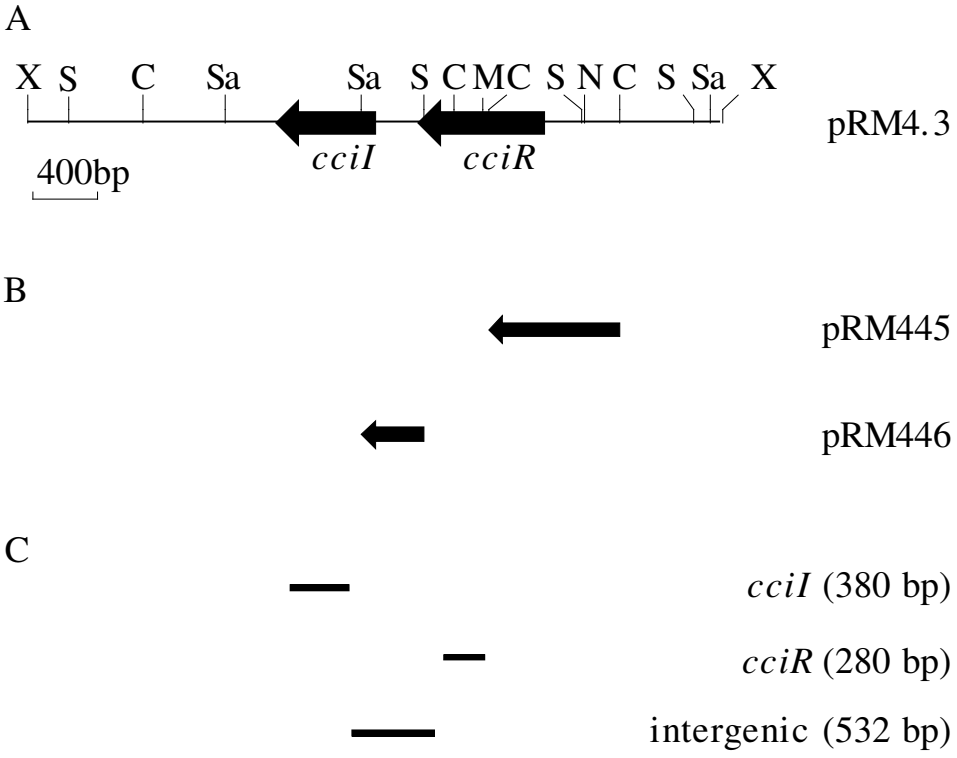


Figure 3.5. Physical and genetic map of the *cciIR* locus of *B. cenocepacia* based on the J2315 sequence.

(A) Map of the 4.3 kb XhoI insert in pRM4.3 containing *cciIR*. The arrows indicate the location and orientation of the genes. The Tp cassette was incorporated into the MluI site of *cciR* to construct K56-2*cciR*. A MluI-NcoI fragment was deleted to construct K56-2*cciI,cciR.* Abbreviations: C, ClaI; N, NcoI: M, MluI; Sa, SalI; S, SacII; X, XhoI.

(B) The *cciI* and *cciR* promoter regions used in the *lacZ* fusion constructs pRM446 and pRM445.

(C) Locations of the RT-PCR products for *cciI, cciR* and the *cciIR* intergenic region.

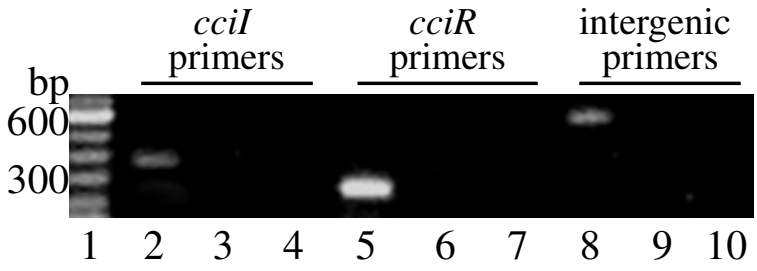


Figure 3.6. RT-PCR analysis of the *cciIR* transcript. Products were electrophoresed on a 1.0 % agarose gel. Lane 1; 100 bp ladder (Invitrogen), Lane 2; *cciI* RT-PCR product with cciIRTF and cciIRTR primers, Lane 3; no template control with cciIRTF and cciIRTR primers, Lane 4; Taq polymerase control with cciIRTF and cciIRTR primers. Lane 5; *cciR* RT-PCR product with cciRRTF and cciRRTR primers, Lane 6; no template control with cciRRTF and cciRRTR primers, Lane 7; Taq polymerase control with cciRRTF and cciRRTR primers, Lane 8; *cciIR* intergenic RT-PCR product with irBRRTF and irBRRTR primers, Lane 9; no template control with irBRRTF and irBRRTR primers; Lane 10; Taq polymerase control with irBRRTF and irBRRTR primers.

region, indicating that *cciI* and *cciR* are on the same transcript (Fig. 3.6, lanes 2, 5 and 8) therefore, *cciI* is transcribed from a promoter located upstream of *cciR*. To further confirm the absence of a promoter region proximal to *cciI*, heterologous expression of *cciI* with and without the control of the *lacZ* promoter was performed. The *cciI* gene and predicted promoter region was cloned into pUCP26 (226) in both orientations, one in the same orientation as the *lacZ* promoter present on the vector and one in the reverse orientation. There was a minimal amount of HHL produced by *E. coli* DH5α (pRM164R) with the *cciI* gene cloned in the opposite orientation to *lacZ* (Fig. 3.7, lane 3) compared to substantial HHL production by *E. coli* DH5α (pRM164), containing *cciI* gene the same orientation as the *lacZ* promoter. The quantity of HHL production by the product of *cciI* expressed with the aid of the *lacZ* promoter is greater than the amount produced by CepI (pSLS225) (Fig. 3.7. comparing lanes 2 and 4) as observed above (Fig. 3.3a).

To determine if *cciIR* is auto-regulated, the expression of *cciIR-luxCDABE* was compared in K56-2 and the K56-2 *cciIR* mutant. The expression of *cciIR-luxCDABE* was significantly greater in K56-2*cciIR* than in K56-2 from 12 to 16 hours ($P < 0.05$ ANOVA, Bonferroni). The two fold increase in expression indicates that the *cciIR* system negatively regulates its own expression (Fig. 3.8).

3.2.3 Regulatory role of CciR in *B. cenocepacia* K56-2

To determine if there is a regulatory relationship between the *cepIR* and the *cciIR* genes, expression of the *cepI-luxCDABE* and *cepR-luxCDABE* transcriptional fusions were compared in K56-2 and K56-2*cciIR* There was a significant increase in *cepI-luxCDABE* expression in K56-2*cciIR* compared to K56-2 ($P < 0.05$; unpaired *t* test)

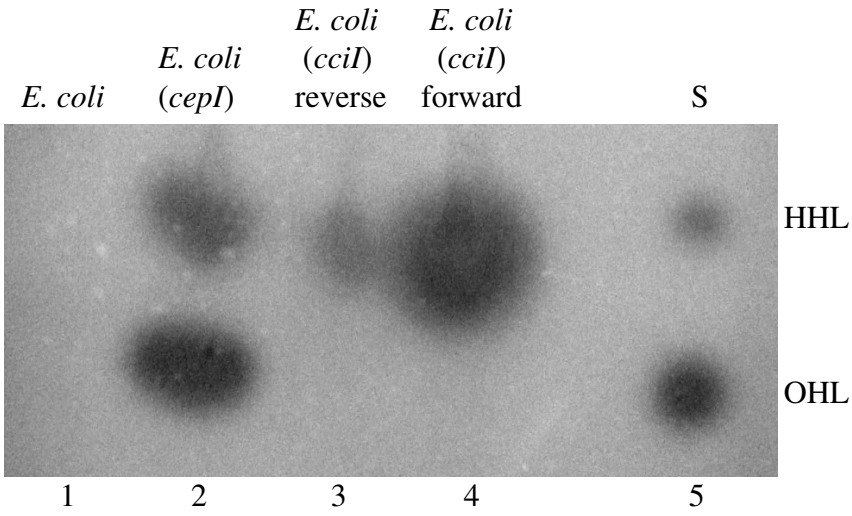


Figure 3.7. TLC-AHL bioassay of heterologous expression of *cciI* in *E. coli*. Ethyl acetate extracts were chromatographed on C18 reverse-phase TLC plates developed with methanol-water (70:30 vol/vol). The spots were visualized using the *A. tumefaciens* reporter strain. Lane 1, *E. coli* DH5α (pUCP26); Lane 2, *E. coli* DH5α (pSLS225) containing the *cepI* gene from K56-2; Lane 3: *E. coli* DH5α (pRM164R) containing the *cciI* gene in reverse orientation to the *lacZ* promoter; Lane 4, *E. coli* DH5α (pRM164) containing the *cciI* gene from K56-2 in the same orientation as the *lacZ* promoter; Lane 5, synthetic AHL standards.

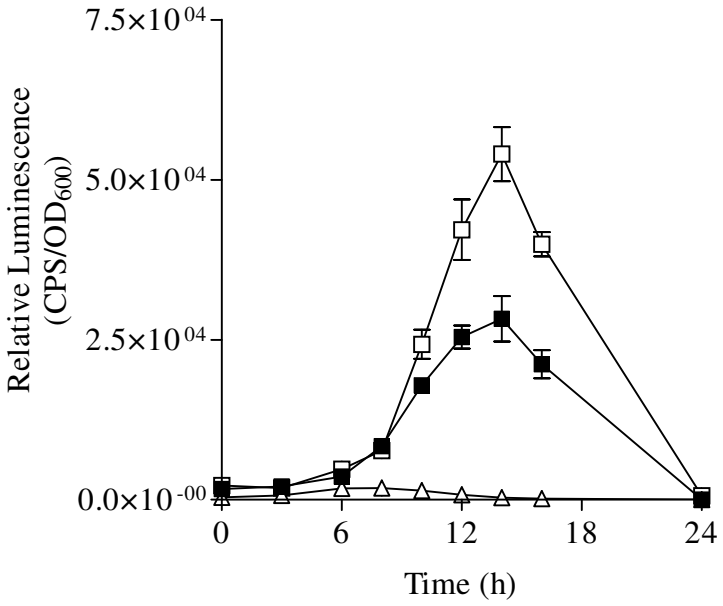


Figure 3.8. Effect of CepR and CciR on *cciIR* expression. Expression was monitored throughout growth in PTSB plus 100 μg/ml of Tp. All values are the means ± SD of triplicate cultures and are representative of two individual experiments: ■, K56-2; □, K56-2*cciIR*; Δ, K56-R2 (*cepR*). The expression of *cciIR-luxCDABE* (pRM445) in K56-2 is significantly less than the expression in K56-2*cepR,cciIR* from 12 to 16h and significantly greater than the expression in K56-R2 (*cepR*) from 6 to 16h during the time course ($P < 0.05$, ANOVA, Bonferroni).

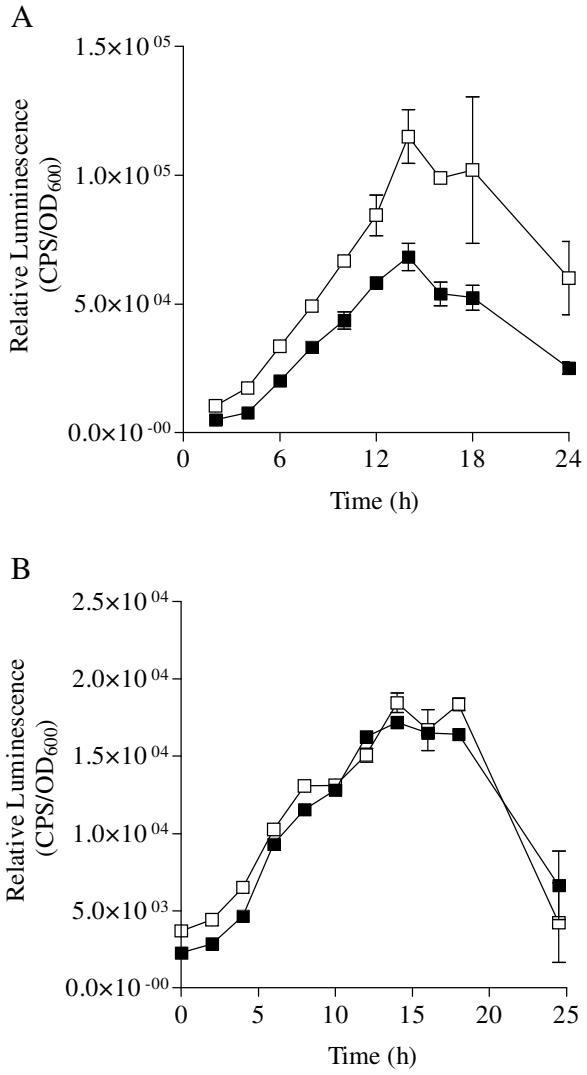


Figure 3.9. Effect of CciIR on *cepI* and *cepR* expression. Expression was monitored throughout growth in PTSB plus 100 μg/ml of Tp. All values are the means ± SD of triplicate cultures and are representative of two individual experiments: ■, K56-2; □, K56-2*cciIR*. (A) The effect of *cciR* on *cepI* expression. The expression of *cepI::luxCDABE* (pCP300) in K56-2*cciIR* is significantly greater than the expression in K56-2 at all of the time points ($P < 0.02$; *t*-test). (B) The effect of *cciR* on *cepR* expression. The expression of *cepR::luxCDABE* (pRM432) in K56-2 is not significantly different than the expression observed in K56-2*cepR,cciIR*.

indicating that *cciIR* is involved in the negative regulation of *cepI* (Fig. 3.9a). The expression of *cepR-luxCDABE* in K56-2*cciIR* was the same as K56-2 demonstrating that *cciIR* does not regulate *cepR* expression (Fig. 3.9b). Expression of *cciIR-luxCDABE* was compared in K56-2 and the K56-R2 (*cepR*), (Fig. 3.8). The expression of *cciIR-luxCDABE* was reduced to almost background levels in K56-R2 (*cepR*) indicating that *cepR* is required for *cciIR* expression.

The *cepIR* system was previously shown to positively regulate the expression of the zinc metalloprotease gene, *zmpA* (190). To determine if *zmpA* is also regulated by the *cciIR* system the expression of a *zmpA-lacZ* transcriptional fusion was examined in K56-2, K56-I2 (*cepI)*, K56-2*cciI* and K56-2*cciR* (Fig. 3.10). The expression of *zmpA-lacZ* in K56-2*cciR*, was significantly less than in the parent strain in stationary phase at 24 and 32 h and the expression of *zmpA-lacZ* in K56-2*cciI* was significantly less than in the parent strain during the entire growth curve ($P < 0.01$ ANOVA, Bonferonni). The respective two fold and 5 fold differences in *zmpA* expression indicate that the *cciIR* system is involved in the positive regulation of *zmpA*.

The *cepIR* system positively regulates the "autoinducer dependent' gene, *aidA,* in *B. cepacia* and *B. cenocepacia* (3, 82). To determine if the *cciIR* system also regulates *aidA,* the expression of *aidA-luxCDABE* was examined in K56-2 and K56-2*cciIR* (Fig. 3.11). The expression of *aidA-luxCDABE* was significantly greater in the K56-2 *cciIR* mutant from hours twelve to sixteen along the time course ($P < 0.05$, unpaired *t*-test, Welch correction) indicating that CciIR is involved in the negative regulation of *aidA*.

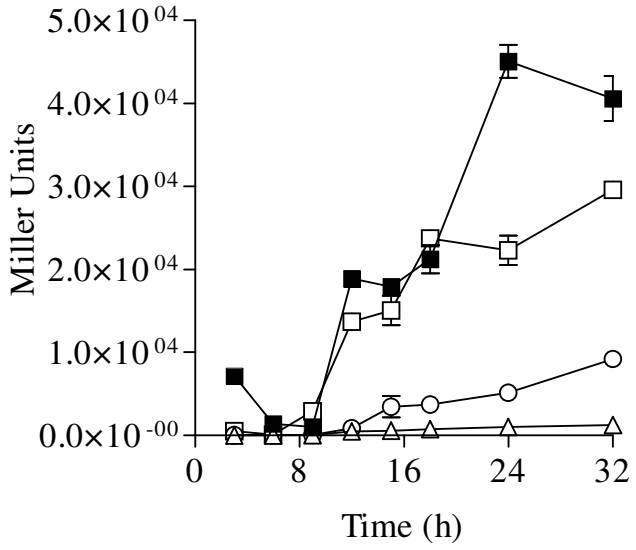


Figure 3.10. Effect of CepI, CciI and CciR on *zmpA* expression. β-Galactosidase activity was monitored throughout growth in PTSB and reported in Miller units. ■, K56-2 (pSG208); □, K56-2*cciR* (pSG206);○, K56-2*cciI* (pSG206);Δ, K56-I2 (*cepI*) (pSG206). Values shown are the mean ± SD of triplicate cultures and are representative of two individual experiments. The difference in *zmpA* expression between K56-2 and K56-2*cciR* is significantly different at time points 3h to 12h and 24h to 32h, the difference between K56-2 and K56-2*cciI* or K56-I2 (*cepI*) was significantly different at all time points between 3h and 32h and the difference between K56-2*cciI* and K56-I2 (*cepI*) was significantly different at 32h ($P< 0.01$ ANOVA, Bonferonni).

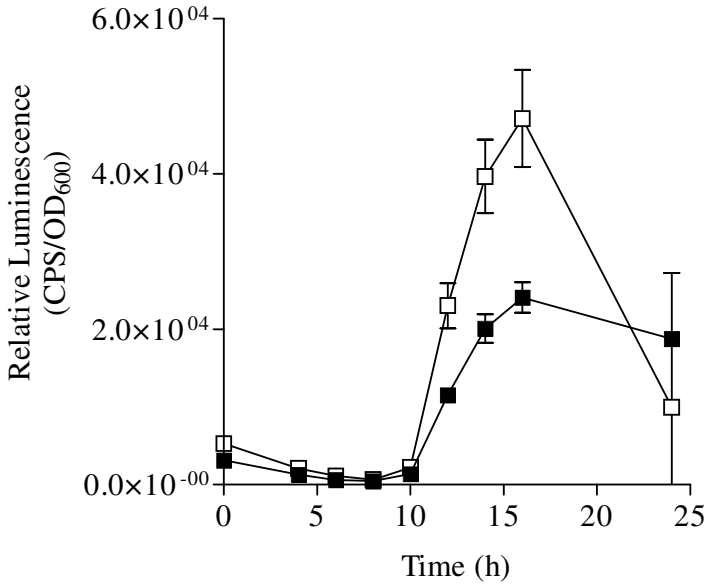


Figure 3.11. Effect of CciIR on *aidA* expression. Expression was monitored throughout growth in PTSB plus 100 μg/ml of Tp. All values are the means ± SD of triplicate cultures and are representative of three individual trials: ■, K56-2; □, K56-2*cciIR*. The expression of *aidA-luxCDABE* (pAid301) is significantly greater in K56-2*cciIR* than K56-2 from 12 to 16 hours along the time course ($P < 0.05$, unpaired *t*-test, Welch).

3.2.4 Characterization of the *B. cenocepacia* K56-2 quorum-sensing mutants

Previously, *cepI* and *cepR* mutants were shown to be protease deficient (109). To evaluate the effect of mutations in the *cciI* and *cciR* quorum-sensing genes on the protease activity of K56-2, skim milk assays were performed (Fig. 3.12). K56-2*cciI* produced significantly greater protease activity than the parent strain ($P < 0.01$, ANOVA, Dunnett) (Table 3.1). This phenotype is opposite to that observed for K56-I2 (*cepI)*, and K56-2*cepI,cciIa*, which do not produce detectable protease activity. K56-*cciR* produces less protease activity than the parent, but more protease activity than K56-R2 (*cepR*) or K56-2*cepR,cciIR.*

The ability of the *cciI* gene to complement a mutation in *cepI* and the *cepI* gene to complement a mutation in *cciI* was investigated with respect to protease activity. Plasmids containing *cciI* or *cepI* were introduced into the heterologous AHL synthase mutant and the protease activity of each strain was determined on skim milk agar (Table 3.2). Introduction of *cciI* into K56-I2 (*cepI)* or *cepI* into K56-*2cciI* did not restore protease activity to parental levels. Plasmids containing either *cciI* or *cepI* were introduced into K56-2*cepI,cciIa.* The protease activity of the mutant was restored when a plasmid containing *cepI* was introduced but not upon the introduction of a plasmid containing *cciI.* These experiments indicate that *cepI* is unable to compensate for the mutation in *cciI,* and *cciI* is unable to compensate for the mutation in *cepI.* Complementation of K56-I2 (*cepI*) and K56-2*cciI* with *cepI* or *cciI in trans*, respectively, restored protease activity to the parental phenotype.

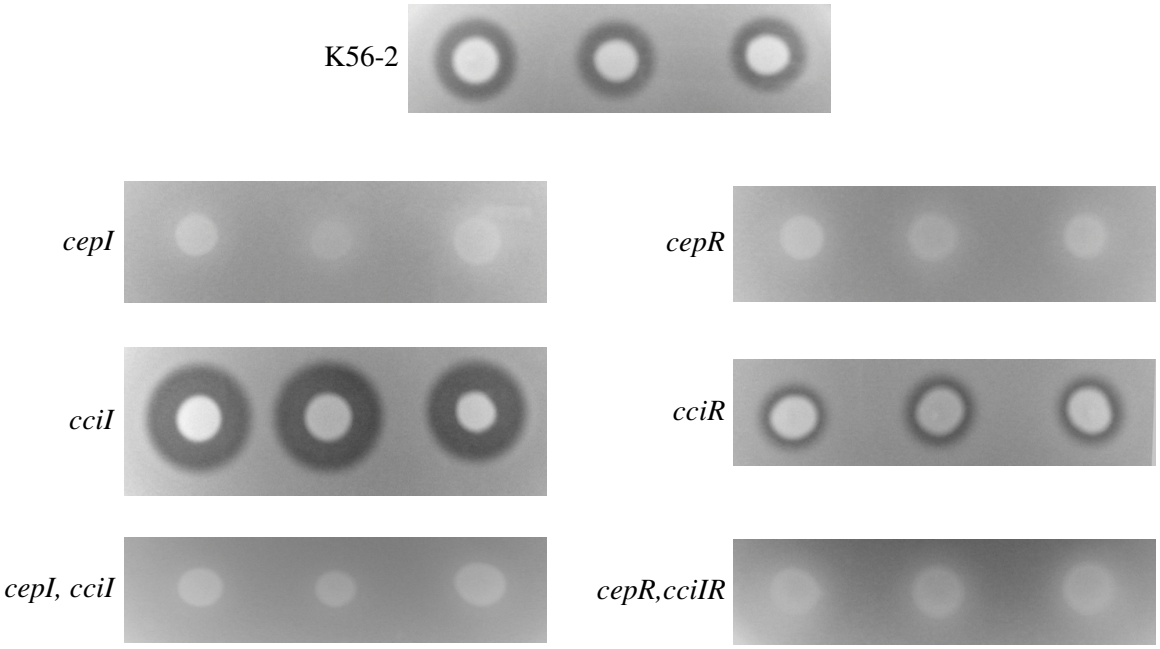


Figure 3.12. Protease activity of the quorum-sensing mutants. Overnight cultures were sub-cultured (1/100) and grown to an optical density at 600 nm of 0.3 and spotted (2µL) onto dialyzed brain heart infusion agar containing 10 percent skim milk. Triplicate zones of clearing of parent K56-2 compared to the quorum-sensing mutants after 24h incubation at 37°C. *cepI;* K56-I2, *cepR;* K56-R2, *cciI;* K56-2*cciI, cciR;* K56-2*cciR, cepI,cciI;* K56-2*cepI,cciIa, cepR,cciIR;* K56-2*cepR,cciIR.*

Table 3.1. Extracellular protease activity and swarming motility of *B. cenocepacia* quorum-sensing mutants.

Strain	Zone of clearing [a] (mm)	Zone of swarming [b] (cm)
K56-2	2.23 ± 0.35	4.50 ± 0.20
K56-I2	0.00 ± 0.00	2.67 ± 0.15
K56-R2	0.00 ± 0.00	1.13 ± 0.12
K56-2*cciI*	3.88 ± 0.28	2.33 ± 0.15
K56-2*cciR*	1.25 ± 0.27	4.16 ± 0.76
K56-2*cepI,cciIa*	0.00 ± 0.00	1.23 ± 0.06
K56-2*cepR,cciIR*	0.00 ± 0.00	1.50 ± 0.26

[a] Values are the average zone of clearing ± SD for six replicates. The data shown are representative of two independent experiments. All of the mutants had significantly different protease activity than K56-2 ($P < 0.01$, ANOVA, Dunnett)

[b] Values are the average swarming zone ± SD at 24 h of three replicates and are representative of three independent experiments. All of the mutants, except for K56-2*cciR*, had significantly smaller swarming zones than the parent strain, K56-2 ($P < 0.01$, ANOVA, Dunnett).

Table 3.2: Complementation of the extracellular protease activity and swarming motility of the AHL synthase mutants with *cepI* or *cciI in trans*.

Strain	Gene present *in trans*	Zone of clearing [a] (mm)	Zone of swarming [b] (cm)
K56-2 (pUCP26)	None	3.13 ± 0.28	2.67 ± 0.06
K56-I2 (pUCP26)	None	0.50 ± 0.52	1.77 ± 0.15
K56-I2 (pRM164)	*cciI*	0.00 ± 0.00	1.90 ± 0.00
K56-I2 (pSLS225)	*cepI*	4.67 ± 0.52[c]	2.30 ± 0.10[c]
K56-2*cciI* (pUCP26)	None	4.46 ± 0.40	1.90 ± 0.1
K56-2*cciI* (pRM164)	*cciI*	2.33 ± 0.41[c]	3.57 ± 0.57[c]
K56-2*cciI* (pSLS225)	*cepI*	4.42 ± 0.38	3.30 ± 0.35[c]
K56-2*cepI,cciIa* (pUCP26)	None	0.00 ± 0.00	0.48 ± 0.03
K56-2*cepI,cciIa* (pRM164)	*cciI*	0.00 ± 0.00	0.97 ± 0.57[c]
K56-2*cepI,cciIa* (pSLS225)	*cepI*	4.17 ±0.26[c]	2.90 ± 0.20[c]

[a] Values are the average zone of clearing ± SD for a minimum of six replicates and are representative of three independent experiments.

[b] Values are the average swarming zone ± SD after 24 hours of three replicates and are representative of three independent experiments.

[c] Significantly different from the mutant with the vector alone ($P < 0.01$, ANOVA, Bonferonni).

The effect of exogenous HHL and OHL concentrations on the protease activity of K56-I2 (*cepI)*, K56-2*cciI* and K56-2*cepI,cciIa* was examined by adding 2.5 to 10.0 nM synthetic HHL or 2.5 nM synthetic OHL to the skim milk agar prior to inoculation (Table 3.3). The protease zones of K56-I2 (*cepI*) and K56-2*cepI,cciIa* were restored to the size of the parent when 2.5 nM OHL was added to the agar. Restoration of the protease zones in K56-I2 (*cepI*) was also observed when 2.5 nM HHL was added to the agar; however, K56-2*cepI,cciIa* required more HHL to restore the protease zones to paerntal levels (Table 3.3). The protease zone size of K56-2*cciI* was reduced to parental levels when 2.5 nM HHL was added to the agar but was not affected by either 10 nM HHL or 2.5 nM OHL (Table 3.3).

Previously, *cepI* and *cepR* mutants were shown to have severely impaired swarming motility (83). The effect of mutations in *cciI* and *cciR* on swarming motility was evaluated (Fig. 3.13). K56-*2cciI,* K56-I2 (*cepI*), K56-2*cepI,cciIa* had significantly less swarming motility than the parent strain ($P < 0.01$ ANOVA, Dunnett) (Table 3.1), indicating that AHL production is essential for maximal swarming motility in K56-2. The zones of swarming motility for K56-I2 (*cepI*) and K56-2*cciI* were increased to parental levels when their respective mutations were complemented with the genes *in trans.* When the *cciI* gene was introduced into K56-I2 (*cepI*) the swarming motility phenotype was not restored; however, when *cepI* was introduced into K56-2*cciI* the zone of swarming increased to parental levels (Table 3.2). The zone of swarming motility for K56-2*cepI,cciIa* was only fully complemented when *cepI* was present *in trans* (Table 3.2).

Table 3.3. Effect of exogenous AHL on extracellular protease activity.

Strain	Zone of clearing (mm) [a]				
	No addition	Acetonitrile	2.5 nM HHL	10.0 nM HHL	2.5 nM OHL
K56-2	2.25 ± 0.42	2.33 ± 0.41	1.83 ± 0.75	2.17 ± 0.52	4.00 ± 0.00[b]
K56-I2	0.00 ± 0.00[c]	0.00 ± 0.00[c]	3.83 ± 0.29[b,c]	4.16 ± 0.29[c]	4.83 ± 0.29[b]
K56-2*cciI*	3.91 ± 0.14[c]	3.83 ± 0.29[c]	2.33 ± 0.57[b]	3.33 ± 0.29	4.33 ± 0.29
K56-2*cepI,cciIa*	0.00 ± 0.00[c]	0.00 ± 0.00[c]	0.83 ± 0.76	3.17 ± 0.29[b]	4.00 ± 0.00[b]

[a] Values are the average zone of clearing ± SD for three replicates and are representative of two independent experiments.

[b] Significantly different from the acetonitrile control for the same strain ($P < 0.01$, ANOVA, Bonferroni).

[c] Significantly different from K56-2 on the same medium.

\

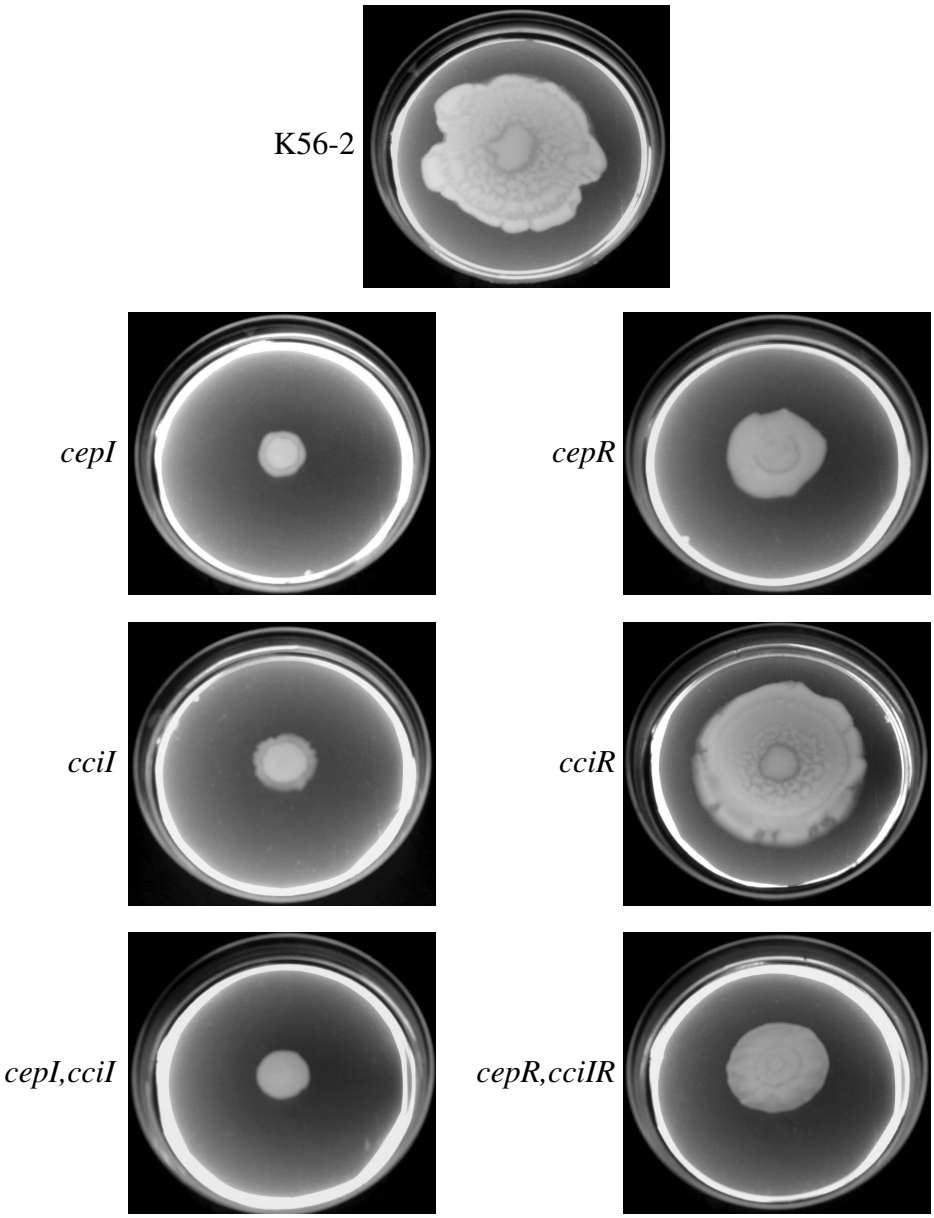


Figure 3.13. Swarming motility of the quorum-sensing mutants. Overnight cultures were normalized and spot inoculated (1µl) onto nutrient broth agar (0.5%) plates with 0.5% glucose and incubated at 37°C for 48h. *cepI;* K56-I2, *cepR;* K56-R2, *cciI;* K56-2*cciI, cciR;* K56-2*cciR, cepI,cciI;* K56-2*cepI,cciIa, cepR,cciIR;* K56-2*cepR,cciIR*

The *cepR* mutant, K56-R2, and K56-2*cepR,cciIR* also exhibited significantly less swarming motility than the parent strain ($P < 0.01$ ANOVA, Dunnett) (Table3.1); however, the ability of K56-2*cciR* (Table 3.1) and K56-2*cciIR* (data not shown) to swarm was not altered. These data confirm that AHL production is essential for maximal swarming motility as K56-R2 (*cepR*) and K56-2*cepR,cciIR* do not produce significant amounts of AHLs whereas K56-2*cciR* produces parental AHL levels (Fig. 3.3d).

The role of *cciIR* in the regulation of ornibactin production was monitored using a quantitative CAS assay (Fig. 3.14). There was little difference in the amount of CAS activity present in culture supernatants with the exception of K56-I2 (*cepI*), which has previously been shown to produce more ornibactin (109) and K56-2*cepI,cciIa,* which had no CAS activity. Attempts to complement CAS activity in K56-2*cepI,cciIa* with either *cciI* or *cepI in trans* were unsuccessful (data not shown). Therefore a second *cepI,cciI* mutant was constructed (designated K56-2*cepI,cciIb*). This mutant had parental levels of CAS activity (Fig. 3.14) suggesting that K56-2*cepI,cciIa* has a secondary mutation that affects ornibactin biosynthesis. K56-2*cepI,cciIb* and *cepI,cciIa* have the same protease and swarming phenotypes (data not shown). These data, together with the complementation data in Table 3.2, confirm that both *cepI* and *cciI* influence protease production and swarming motility, but only *cepI* influences ornibactin biosynthesis.

To determine if the *cciIR* system is involved in biofilm formation in K56-2, the quorum-sensing mutants were grown on the MBEC™ assay system and stained with crystal violet as a semi-quantitative measure of cell attachment. The crystal violet bound by both K56-I2 and K56-R2 biofilms was significantly less than that of the parent strain K56-2 ($P<0.05$, ANOVA, Bonferroni) (Fig. 3.15). The attachment deficiency

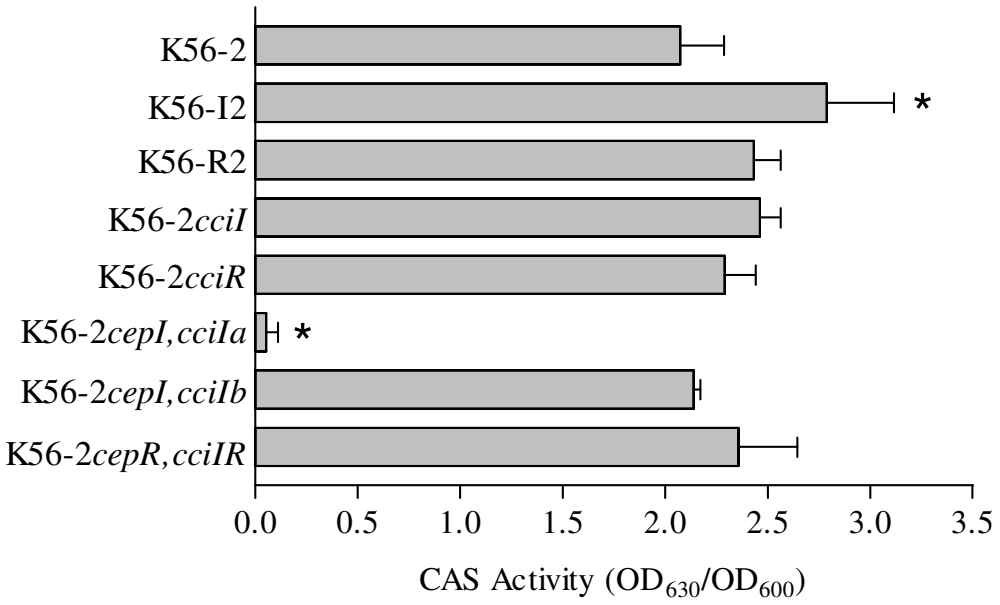


Figure 3.14. Effect of *cepIR* and *cciIR* mutations on ornibactin biosynthesis. Cultures were grown in succinate medium supplemented with 10 mM ornithine for 40h. Spent supernatants were added to the CAS dye complex and the activity was evaluated by absorbance at 630 nm. Absorbance was normalized to culture turbidity (absorbance at 600 nm). The values shown are the average ± SD for three replicate cultures and are representative of three independent experiments. The asterisks indicate that K56-I2 *cepI* and K56-2*cepI,cciIa* had significantly different CAS activity than the parent K56-2 ($P<$ 0.01 ANOVA, Dunnett).

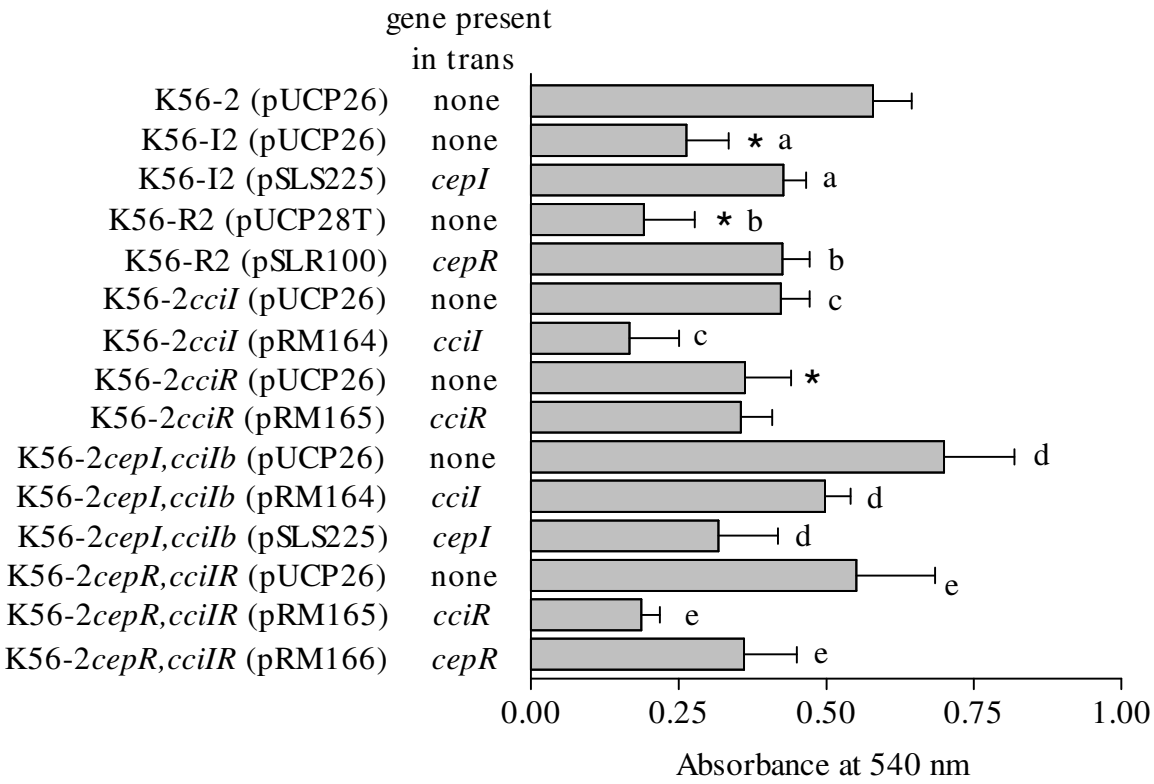


Figure 3.15. Effect of *cepIR* and *cciIR* mutations on biofilm formation. Quantification of cellular matter is represented by absorbance at 540 nm of crystal violet stain bound to 24 hour biofilms cultured on the MBEC™ assay system. The values shown are the mean ± SD of at least 5 replicate biofilms and are representative of three independent experiments. The asterisks indicate strains that produced significantly less biomass than K56-2 ($P < 0.05$ ANOVA, Bonferroni). Letters a through e indicate complemented strains that produce significantly different biomass than the mutant containing the vector control ($P < 0.05$ ANOVA, Bonferroni).

could be partially restored upon providing the mutants with wild type copies of the genes (Fig. 3.15). There was less biofilm formed by K56-2*cciI* and K56-2*cciR* however, the deficit could not be complemented by providing the respective genes *in trans.* The addition of *cciI* on a multi-copy plasmid to K56-2*cciI* actually resulted in a significant decrease in biomass formation ($P<0.05$, ANOVA, Bonferroni). The amount of stained biomass formed by the and K56-2*cepI,cciIb* and K56-*2cepR,cciIR* mutants were indistinguishable from the K56-2 parent strain (Fig. 3.15) however, the double AHL synthase mutant appears to produce more pellicle on the side of glass flasks at the air-liquid interface when grown in routine planktonic cultures. A significant decrease in adherence was noted upon providing K56-2*cepI,cciIb* with the functional *cepI* or *cciI* gene and by providing K56-2*cepR,cciIR* with functional *cepR* or *cciR* on multi-copy vectors ($P < 0.05$, ANOVA, Bonferroni) (Fig. 3.15).

Virulence of the K56-2 quorum-sensing mutants was assessed in the alfalfa model of infection (Table 3.4). In trial one, the infected alfalfa seedlings were incubated at 37^{o}C for seven days. All of the infected sprouts showed signs of disease including yellowed leaves, stunted root growth and brown necrotic lesions. In trial two, the infected alfalfa seedlings were incubated at 28^{o}C for seven days (Table 3.4). The decrease in incubation temperature resulted in a decrease in sprouts with disease symptoms similar for the parent and the quorum-sensing mutants, indicating that virulence in alfalfa is not regulated by quorum-sensing in K56-2.

3.3 Discussion

These studies have demonstrated that the *cciIR* quorum-sensing system associated with epidemic strains of *B. cenocepacia* is a functional part of the *cepIR* regulatory

Table 3.4. Virulence of the quorum-sensing mutants in an alfalfa infection model.

Strain	Percent of seedlings with symptoms Trial 1[a]	Percent of seedlings with symptoms Trial 2[b]
K56-2	100	85
K56-I2	100	95
K56-R2	100	95
K56-2*cciI*	100	85
K56-2*cciR*	100	85
K56-2*cciIR*	100	95
K56-2*cepI,cciIa*	100	90
K56-2*cepR,cciIR*	100	90
Saline control	0[c]	0[c]

[a] Trial 1 was performed with 20 sprouts at 37°C
[b] Trial 2 was performed with 20 sprouts at 28°C
[c] Saline control was tested on 10 sprouts

network and contributes to the regulation of known virulence factors. Phylogenetic analysis demonstrates that the *cepIR* and *cciIR* systems are highly distinct (129). The CepIR system clusters with other Bcc and *B. pseudomallei* quorum-sensing systems indicating that this system is ancestral to the *Burkholderia* genus whereas the CciIR system was likely acquired by horizontal gene transfer when the *cenocepacia* island was incorporated into the *B. cenocepacia* genome (129). The presence of an ancestral and a more recently acquired AHL–dependent quorum-sensing system has been observed in other species. The *P. aeruginosa lasIR* system is considered to be the ancestral system and the *rhlIR* system considered to be more recently acquired (106). The *rhlIR* system has been incorporated into the regulatory pathway of the *lasIR* system (101, 162). Both of these pathways are involved in the virulence of the organism in a variety of infection models (184) and it is believed that the acquisition of the *rhlIR* system has aided *P. aeruginosa* in honing gene regulation during evolution (106).

Transcriptional analysis of *cciIR* in the presence and absence of functional CepR and phenotypic characterization of double AHL synthase and response regulator mutants demonstrated that the *B. cenocepacia cciIR* system has been incorporated in series into the *cepIR* regulatory network. This arrangement allows for the regulation of genes in a temporally defined manner (199). The predicted *cciIR* promoter region does not contain a CepR binding box (225). The delayed initiation of *cciIR* expression compared to the expression observed for *cepI* and *cepR* in the parent (comparing Fig. 3.8 and Fig. 3.9b) is consistent with the regulatory arrangement of the *cepIR* and *cciIR* systems. Since both systems have been shown to be involved in the virulence of *B. cenocepacia* (8, 190) and *B. cenocepacia* stains that possess the cci are often associated with transmissibility and

increased severity of clinical outcome (8), the evolution of the *cepIR* and *cciIR* quorum-sensing regulatory network may contribute to the invasiveness and transmissibility observed with *B. cenocepacia*.

Pathogenicity islands tend to contain genes encoding for regulatory elements (76). These regulatory elements can be specific for genes encoded on the pathogenicity island and may also regulate genes in the rest of the genome. As well, regulatory systems encoded on pathogenicity islands may be regulated by systems encoded outside of the genomic region. Each of these regulatory arrangements is observed with the *cciIR* quorum-sensing system. The *cciIR* system was shown to be involved in negative auto-regulation as well as positive regulation of the *zmpA* and negative regulation of *aidA* genes which are not located within the cci. The *cepIR* quorum-sensing system is involved in the positive regulation of the *cciIR* quorum-sensing system. There are most likely additional regulatory genes involved in the *cepIR* regulatory cascade in epidemic strains of *B. cenocepacia.* The three identified regulators of the H111 *cepIR* quorum-sensing system (*yciR, suhB* and *yciL*) are postulated to influence *cepIR* by post-transcriptional control of *cepR* expression or by affecting the activity status of the CepR (84). It is yet to be determined if these regulatory elements are also involved in the quorum-sensing cascade of K56-2.

The tandem organization of the *cciIR* genes is different than the other two quorum-sensing systems in the Bcc as the genetic organization of *cepIR* and *bviIR* systems is divergent. The *luxIR* homologous systems are organized in different genetic arrangements including, divergent, convergent, tandem and unlinked without an apparent correlation to species or genus (51). A *lux* box sequence was identified upstream of *cciI,*

therefore it was unexpected that *cciI* and *cciR* are co-transcribed. The predicted *lux* box may be a vestigial promoter sequence utilized prior to the cci being transferred into *B. cenocepacia.*

The effect of mutation of the K56-2 quorum-sensing genes on AHL production, protease production, ornibactin production, swarming motility, biofilm formation and alfalfa virulence are summarized in Table 3.5. The *cciIR* system regulates protease production and swarming motility. The hyper-production of protease by K56-2*cciI* was surprising since *cciI* positively influences *zmpA* expression. Corbett et al. (35), showed that a *zmpA* mutant of K56-2 produce very little protease activity in the skim milk agar assay. The decrease in protease production observed for K56-2*cciR*, is most likely due to the decrease in *zmpA* expression in this mutant. The predicted result for the absence of functional CciI would also be a decrease in protease production. The most plausible explanation for the observed increase in protease production is that *cciI* is involved in the negative regulation of an additional protease. During the course of this study, a second metalloprotease, ZmpB, was identified in *B. cenocepacia* K56-2 (94). The expression of *zmpB* was significantly less in all of the quorum-sensing mutants except K56-2*cciI* (94). The expression of *zmpB* in K56-2*cciI* was significantly greater than in the parent accounting for the increase of protease production in this mutant. A K56-2 *zmpB, cciI* mutant has been constructed and this mutant does not exhibit protease activity (B. Subsin, personal communication), further illustrating that the increase in protease production of K56-2*cciI* is due to the increased expression of *zmpB* in this mutant.

Both CciI and CepI are responsible for the production of the same AHLs; however, the ratio of HHL to OHL production by the two synthases is different. The

Table 3.5. Summary of the quorum-sensing mutant phenotypes.

Strain	HHL production	OHL production	Protease production	Swarming motility	Ornibactin production	Biofilm formation	Virulence in alfalfa
K56-I2	D	-	-	-	I	-	+
K56-R2	D	-	-	-	+	-	+
K56-2*cciI*	D	+	I	-	+	+	+
K66-2*cciR*	+	+	D	+	+	D	+
K56-2*cepI,cciIb*	-	-	-	-	+	+	+
K56-2*cepR,cciIR*	-	-	-	-	+	+	+

+; parental phenotype, D; decrease in phenotype compared to parent, I; increase in phenotype compared to parent, -; phenotype negative

quantity of each AHL present appears to be critical as K56-2*cepI,cciIa* required more HHL to restore protease production to parental levels than K56-I2 (*cepI*), which produces minor amounts of HHL. Protease activity in the K56-2*cciI* mutant was only reduced by the addition of HHL to the medium and not OHL. The AHL synthases *cepI* and *cciI* are not redundant, since *cciI* does not restore protease activity or the swarming phenotype in the *cepI* mutant, and both *cepI* and *cciI* mutants are reduced in virulence (8, 190).

The *cciIR* system is involved in the positive regulation of swarming motility through the production of AHLs by CciI. The regulation of swarming motility by quorum-sensing systems is believed to allow optimal dissemination of the bacteria when the population is saturating a particular niche and may allow bacteria to progress from local infection sites to other organs (198, 227).

Mutations in the *cepIR* system have a significant effect on the establishment of biofilms, however the mutants in the *cciIR* system only resulted in a minor decrease in biofilm formation. Both the *cciI* and *cciR* mutants produce considerable amounts of HHL and OHL indicative of a functional *cepIR* system that could be compensating for the mutations in *cciIR*. The results from the complementation with wild-type genes *in trans* on high copy vectors did not fully restore the biofilm phenotypes and resulted in unexpected increases and decreases in biofilm formation of the strains. As outlined above, the quantity of AHLs present is critical; suggesting that complementing the mutants with multiple copies of either AHL synthase or transcriptional regulator genes would set the regulatory network off balance, leading to unpredictable biofilm formation. Surprisingly, the double AHL synthase and transcriptional regulator mutants did not have a decrease in biofilm attachment. Biofilm formation is the only phenotype tested where

the phenotype of the double mutants did not match the single *cep* mutants (Table 3.5). A dryer colony morphology and an increase in pellicle formation has been observed for K56-2*cepI,cciIb*. The *cepI,cciI* mutant biofilm is also more sensitive to ciprofloxacin treatment (203) indicating that the architecture of the K56-*2cepI,cciIb* biofilm may be structurally different from the parent. The major role of quorum-sensing in biofilm development is in the transition of the microcolony to the mature biofilm (77), thus the MBEC^{TM} assay that assesses the ability of a bacterium to attach and form biofilms may not have been a sensitive enough assay system to discern the effects of the mutations. Mutants in *cepI* and *cepR* in strain H111 and K56-2 are deficient in mature biofilm development and have a decrease in substratum coverage compared to the parent when grown in flow cells (83, 203). Artificial flow cells allow for biofilm development to be followed in real time, monitored by confocal epifluorescence microscopy (83, 203). Future studies with the *cciI* and *cciR* single mutants as well as the *cepI,cciI* and *cepR,cciR* mutants using this technology may further elucidate the role of quorum-sensing in mature biofilm development of K56-2.

The high level of virulence for the *B. cenocepacia* quorum-sensing mutants in the alfalfa model of infection was unexpected considering that mutants in *B. cepacia cepI* and *cepR* resulted in a decrease in onion pathogenesis (2). *B. plantarii cepI* and *cepR* homologue mutants do not cause rice seedling blight (191) and a *Pectobacterium* sp. strain A2JM AHL synthase mutant was shown to be less destructive to mung bean sprouts (165), illustrating that quorum-sensing is involved in phytopathogenesis of seedlings by other bacteria. Higher plants species including pea, soybean, rice, tomato, crown vetch and the model legume *Medicago truncatula* have all been shown to secrete

substances that stimulate or repress bacterial quorum-sensing systems (9), therefore it is possible that the alfalfa seedlings are producing a substance that is interfering with the K56-2 quorum-sensing systems. Another possibility is that, since alfalfa is not a natural host for *B. cenocepacia,* this virulence assay circumvents the quorum-sensing dependent step of infection. However, the most likely expleanation is that quorum-sensing is not involved in the colonization of alfalfa seedlings by K56-2.

The phenotypes of the *cciI* and *cciR* mutants differed with respect to AHL production, protease activity, and swarming motility, although transcriptional analysis of *zmpA* suggests that the *cciIR* system operates as a unit to positively regulate the expression of *zmpA*. Presumably, both CepR and CciR respond to HHL and OHL as their cognate AHL synthases produce these signalling molecules. The difference in phenotypes of K56-2*cciR* and K56-2*cciI* could be due to the parental levels of AHLs produced by K56-2*cciR* compared to K56-2*cciI,* which produces less HHL. The expected decrease in HHL production in K56-2*cciIR,* where the *cciIR* promoter is deleted, is likely compensated by the absence of negative regulation of *cepI* by CciR.

Not all of the phenotypes regulated by the *cepIR* system were regulated by the *cciIR* system (Table 3.5) indicating that CciR has a distinct regulon. A proteomic study is currently underway in the Sokol laboratory comparing the proteomic profiles of K56-2, K56-2*cciIR,* K56-R2 (*cepR*) and K56-2*cepR,cciIR* to begin defining the CepR and CciR proteomes. Defining a CciR proteome will aid in understanding the contribution of the *cciIR* system to the virulence of *B. cenocepacia* strains containing the *cenocepacia* island.

Chapter Four: Identification and preliminary characterization of an additional *luxR* homologue in *Burkholderia cenocepacia*

4.1 Introduction

The *B. cenocepacia* genome sequencing project (http://www.sanger.ac.uk/Projects/B_cenocepacia/) provides the means to conveniently identify additional components of the quorum-sensing regulatory network. The *B. pseudomallei* genome sequence (http://www.sanger.ac.uk/Projects/B_pseudomallei/) aided in the identification of three sets of *luxIR* homologues, *pmlIR, bpmIR2* and *bpmIR3* and two additional *luxR* homologues, *bpmR4* and *bpmR5* (206, 209). Ulrich et al. (206), demonstrated that each gene of the *B. pseudomallei* quorum-sensing network is involved in pathogenicity in showing that Syrian golden hamsters infected with the *B. pseudomallei* quorum-sensing mutants had an increase in the LD_{50} compared to the parent. It would be unexpected to identify an additional AHL synthase gene in the *B. cenocepacia* genome since the K56-2*cepI,cciI* mutant was devoid of detectable AHLs (Fig. 3.3c). However, the possibility of additional LuxR homologues present in the genome remains.

Genome sequence analyses of many bacteria indicate that the number of LuxR homologues present in a genome is often in excess of the number of LuxI homologues (57). The "orphaned" LuxR homologues that are without an associated AHL synthase are predicted to respond to endogenously synthesized AHLs. The enteric bacteria *E. coli* and *Salmonella enterica* serovar Typhimurium do not produce AHL signalling molecules, but contain an orphaned LuxR homologue, SdiA, that responds to signals generated only by other microbial species (4).

The *P. aeruginosa* quorum-sensing network contains the orphan regulator QscR (*q*uorum-*s*ensing *c*ontrol *r*epressor) that negatively modulates the activity of LasR and RhlR (25). It is believed that the mechanism of QscR activity is partially independent of transcriptional control, through the formation of QscR-LasR and QscR-RhlR heterodimers, or through competition for AHLs or competition for DNA binding sites (25, 102). QscR contributes to the regulation of virulence of *P. aeruginosa* since a *qscR* mutant is hyper-virulent in the *Drosophila melanogaster* model of infection (25). A recent microarray study determined that QscR is capable of regulating *P. aeruginosa* genes and that LasR, RhlR and QscR have overlapping but distinct regulons (105). QscR requires 3-oxo-DHL to actively bind DNA, but exhibits a relaxed AHL specificity compared to LasR that may enable QscR to also respond to exogenous AHLs in mixed bacterial populations (103).

The objectives of this study were to identify additional LuxR homologues in the *B. cenocepacia* genome, to characterize the distribution of the identified LuxR homologues within the Bcc and to determine if the identified genes played a role in the regulation of quorum-sensing associated phenotypes.

4.2 Results

4.2.1 Identification of BCAM0188 as a LuxR homologue

Additional LuxR homologues were identified *in silico* by BLAST search (5) of the *B. cenocepacia* J2315 genome sequence (http://www.sanger.ac.uk/Projects/B_cenocepacia) with CepR (accession no. AAD12726 (109). Multiple open reading frames with predicted helix-turn-helix DNA binding motifs typical of LuxR response regulators were identified distributed throughout the genome.

Only one of these open reading frames, BCAM0188, also contained an AHL binding domain (Fig. 4.1). BCAM0188 contains all seven residues that are conserved among LuxR transcriptional regulators (61) (Fig. 4.2). BCAM0188 is located on chromosome 2 and is without a proximal *luxI* homologue.

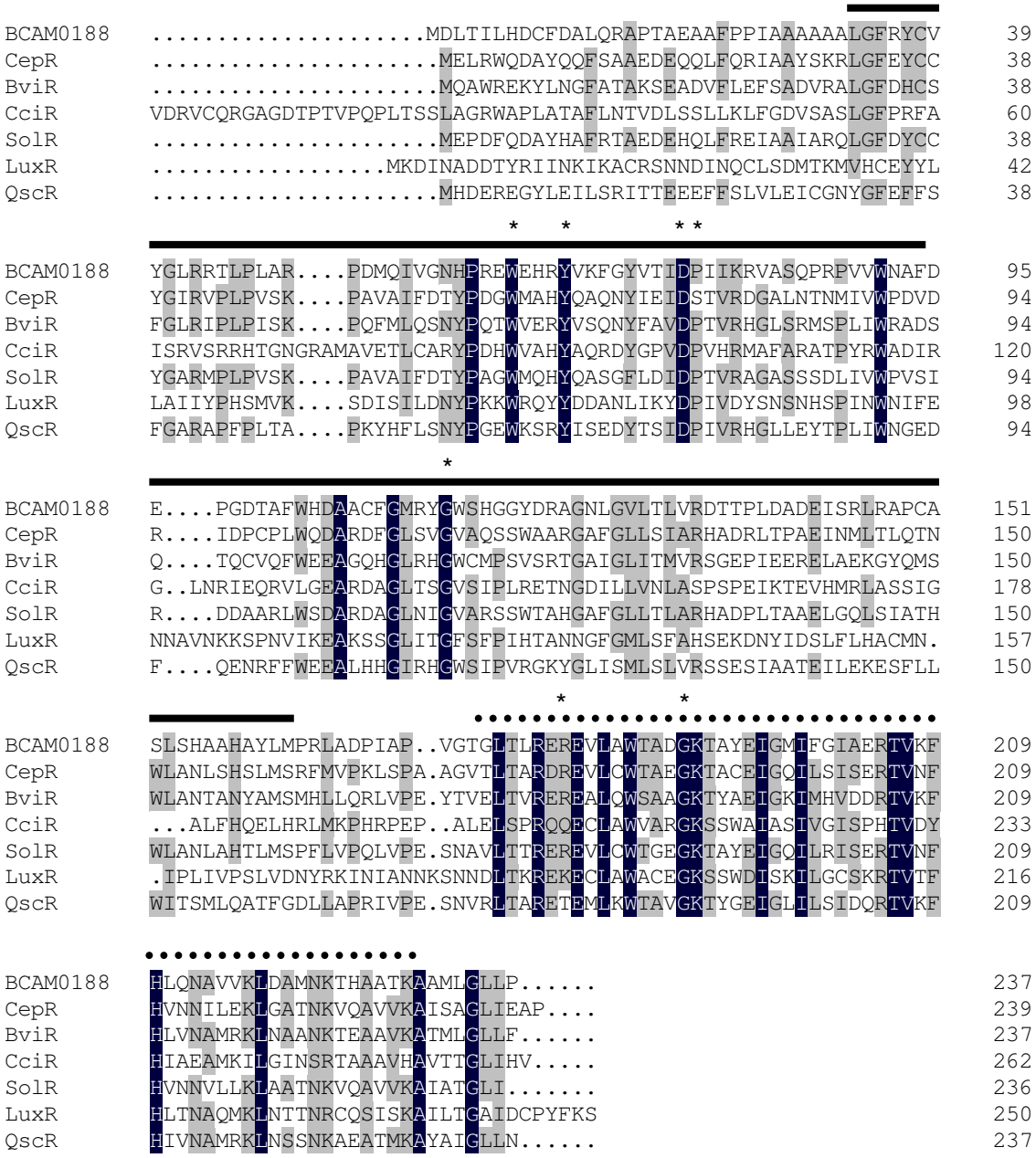
BCAM0188 encodes for a 237 amino acid product with a predicted molecular mass of 26046 Da. The product of BCAM0188 is 38 percent identical to SolR of *R. solanacearum* (accession no. AAC4597) (56), 23 percent identical to LuxR of *V. fischeri* (accession no. 1403259A) (47) and 18 percent identical to QscR of *P. aeruginosa* (accession no. AAG05287) (195) (Fig. 4.2). The BCAM0188 product shares the following homologies with Bcc LuxR homologues; 36 percent identity with BviR of *B. vietnamiensis* (accession no. AAD12726) (121), 36 percent identity with CepR of *B. cenocepacia* (accession no. AAD12726) (109) and 21 percent identity with *B. cenocepcacia* CciR (http://www.sanger.ac.uk/Projects/B_cenocepacia/private/) indicating that BCAM0188 is a distinct Bcc LuxR homologue (Fig. 4.2).

4.2.2 Distribution of BCAM0188

CepR is distributed throughout the Bcc where as CciR is only present in *B. cenocepacia* strains that contain the *cenocepacia* island (8, 69, 121). To determine the distribution of BCAM0188 in the Bcc, Southern hybridization with a probe internal to BCAM0188 was performed with seventeen strains representing the nine Bcc species (Fig. 4.3). The BCAM0188 probe only hybridized with the *B. cenocepacia* strains K56-2, J2315 and Pc715j. To determine if the presence of BCAM0188 is common among *B. cenocepacia* strains, primers M0188scR and M0188scR were used to amplify a fragment internal to BCAM0188 from each *B. cenocepacia* strain in the Bcc strain panel (125) as

Figure 4.1. Conserved domain prediction for BCAM0188. The conserved AHL binding (Autoind_bind) and helix-turn-helix DNA binding (HTH_LUXR) domains of BCAM0188 were predicted using conserved domains search software (135) and the conserved domains database (134).

Figure 4.2. Multiple alignment of BCAM0188 with other LuxR homologues. BCAM0188 of *B. cenocepacia* (http://www.sanger.ac.uk/Projects/B_cenocepacia/private/) is aligned with CepR of *B. cenocepacia* (accession no AAD12726 (109), BviR of *B. vietnamiensis* (accession no. AAk35156) (121), CciR of *B. cenocepacia* (http://www.sanger.ac.uk/Projects/B_cenocepacia/private/), SolR of *R. solancearum* (accession no. AAC45947) (56), LuxR of *V. fischeri* (accession no. 1403259A) (47) and QscR of *P. aeruginosa* (accession no. AAG05287) (195). The alignment was generated using DNAMAN sequence analysis software. The conserved residues are shaded black and the similar residues are shaded in grey. The seven residues that are conserved among LuxR transcriptional regulators (61) are denoted by an asterisk above the sequence. The AHL binding domain from residues (33 to 163) is indicated by a solid line above the sequence and the helix-turn-helix DNA binding motif from residues 175 to 230 is indicated by a dashed line above the sequence.


BCAM0188 .....MDLTILHDCFDALQRAPTAEAAFPPIAAAAAALGFRYCV 39
CepR .....MELRWQDAYQQFSAAEDEQQLFQRIAAYSKRLGFEYCC 38
BviR .....MQAWREKYLNGFATAKSEADVFLEFSADVRALGFDHCS 38
CciR VDRVCQRGAGDTPTVPQPLTSSLAGRWAPLATAFLNTVDLSSLLKLFGDVSASLGFPRFA 60
SolR .....MEPDFQDAYHAFRTAEDEHQLFREIAAIARQLGFDYCC 38
LuxR .....MKDINADDTYRIINKIKACRSNNDINQCLSDMTKMVHCEYYL 42
QscR .....MHDEREGYLEILSRITTEEEFFSLVLEICGNYGFEFFS 38
BCAM0188 YGLRRTLPLAR....PDMQIVGNHPREWEHRYVKFGYVTIDPIIKRVASQPRPVVWNAFD 95
CepR YGIRVPLPVSK....PAVAIFDTYPDGWMAHYQAQNYIEIDSTVRDGALNTNMIVWPDVD 94
BviR FGLRIPLPISK....PQFMLQSNYPQTWVERYVSQNYFAVDPTVRHGLSRMSPLIWRADS 94
CciR ISRVSRRHTGNGRAMAVETLCARYPDHWVAHYAQRDYGPVDPVHRMAFARATPYRWADIR 120
SolR YGARMPLPVSK....PAVAIFDTYPAGWMQHYQASGFLDIDPTVRAGASSSDLIVWPVSI 94
LuxR LAIIYPHSMVK....SDISILDNYPKKWRQYYDDANLIKYDPIVDYSNSNHSPINWNIFE 98
QscR FGARAPFPLTA....PKYHFLSNYPGEWKSRYISEDYTSIDPIVRHGLLEYTPLIWNGED 94
BCAM0188 E....PGDTAFWHDAACFGMRYGWSHGGYDRAGNLGVLTLVRDTTPLDADEISRLRAPCA 151
CepR R....IDPCPLWQDARDFGLSVGVAQSSWAARGAFGLLSIARHADRLTPAEINMLTLQTN 150
BviR Q....TQCVQFWEEAGQHGLRHGWCMPSVSRTGAIGLITMVRSGEPIEERELAEKGYQMS 150
CciR G..LNRIEQRVLGEARDAGLTSGVSIPLRETNGDILLVNLASPSPEIKTEVHMRLASSIG 178
SolR R....DDAARLWSDARDAGLNIGVARSSWTAHGAFGLLTLARHADPLTAAELGQLSIATH 150
LuxR NNAVNKKSPNVIKEAKSSGLITGFSFPIHTANNGFGMLSFAHSEKDNYIDSLFLHACMN. 157
QscR F....QENRFFWEEALHHGIRHGWSIPVRGKYGLISMLSLVRSSESIAATEILEKESFLL 150
BCAM0188 SLSHAAHAYLMPRLADPIAP..VGTGLTLRREREVLAWTADGKTAYEIGMIFGIAERTVKF 209
CepR WLANLSHSLMSRFMVPKLSPA.AGVTLTARDREVLCWTAEGKTACEIGQILSISERTVNF 209
BviR WLANTANYAMSMHLLQRLVPE.YTVELTVRERERALQWSAAGKTYAEIGKIMHVDDRTVKF 209
CciR ...ALFHQELHRLMKPHRPEP..ALELSPRQQECLAWVARGKSSWAIASIVGISPHTVDY 233
SolR WLANLAHTLMSPFLVPQLVPE.SNAVLTTRERERVLCWTGEGKTAYEIGQILRISERTVNF 209
LuxR .IPLIVPSLVDNYRKINIANNKSNNDLTKREKECLAWACEGKSSWDISKILGCSKRTVTF 216
QscR WITSMLQATFGDLLAPRIVPE.SNVRLTARETEMLKWTAVGKTYGEIGLILSIDQRTVKF 209
BCAM0188 HLQNAVVKLDAMNKTHAATKAAMLGLLP..... 237
CepR HVNNILEKLGATNKVQAVVKAISAGLIEAP.... 239
BviR HLVNAMRKLNAANKTEAAVKATMLGLLF..... 237
CciR HIAEAMKILGINSRTAAAVHAVTTGLIHV..... 262
SolR HVNNVLLKLAATNKVQAVVKAIATGLI..... 236
LuxR HLTNAQMKLNTTNRCQSISKAILTGAIDCPYFKS 250
QscR HIVNAMRKLNSSNKAEATMKAYAIGLLN..... 237

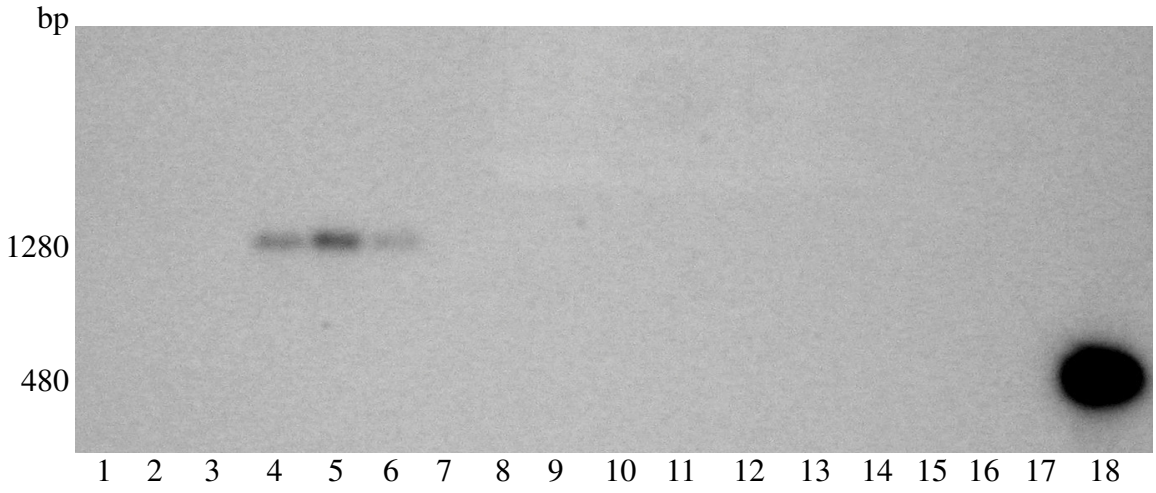


Figure 4.3. Detection of BCAM0188 in Bcc strains by Southern hybridization. Genomic DNA was digested with BamHI and hybridized with a 470 bp EcoRI fragment from pRM1T8 internal to BCAM0188 labelled with ^{32}P. Lane 1, *B. cepacia* ATTC25416^{T}; lane 2, *B. multivorans* LMG13010^{T}; lane 3, *B. multivorans* C5393; lane 4, *B. cenocepacia* K56-2; lane 5, *B. cenocepacia* J2315; lane 6, *B. cenocepacia* Pc715j; lane 7, *B. stabilis* LMG14294; lane 8, *B. stabilis* LMG14086; lane 9, *B. vietnamiensis* PC259; lane 10, *B. vietnamiensis* G4; lane 11, *B. dolosa* LMG18943; lane 12, *B. dolosa* LMG19468^{T}*;* lane 13, *B. ambifaria* Cep0996; lane 14, *B. ambifaria* LMG17828; lane 15, *B. athina* LMG20980; lane 16, *B. athina* LMG20982; lane 17, *B. pyrrocinia* LMG21822; lane 18, BCAM0188 probe fragment.

well as strains H111 and PC715j (Fig. 4.4). All *B. cenocepacia* strains tested contained BCAM0188.

4.2.3 Preliminary characterization of a BCAM0188 mutant

To determine if BCAM0188 is involved in the regulation of known *B. cenocepacia* quorum-sensing regulated phenotypes, a BCAM0188 mutant was constructed in K56-2 (Fig. 4.5) and was designated K56-2M188a. The AHL production profile of K56-2M188a was determined by AHL-TLC bioassay with the *A. tuemefaciens* A136 (pCF218) (pCF372) reporter strain (Fig. 4.6). The AHL production profile of K56-2M188a was indistinguishable from K56-2 (Fig. 4.6, compare lanes 1 and 2).

Protease production of the BCAM0188 mutant was assessed by skim milk assays. K56-2M1088a produced greater protease activity than K56-2 (Fig. 4.7a). However the protease activity of the mutant was not returned to parental levels upon addition of BCAM0188 *in trans* (Fig. 4.7b). To ensure that the hyper-protease production phenotype of BCAM0188a was not due to a secondary mutation in K56-2, a second BCAM0188 mutant designated as K56-2M188b was characterized. K56-2M188b also produced greater protease activity than K56-2 ($P < 0.001$, ANOVA, Student-Newman-Keuls) (Table 4.1). Incorporation of pUCP26 or pRM613 containing BCAM0188 into K56-2M188b resulted in a significant decrease in protease production compared to the mutant alone ($P< 0.001$ ANOVA, Student-Newman- Keuls) (Table 4.1). There was no difference observed between the protease production of K56-2 (pUCP26), K56-2M188b (pUCP26) and K56-2M188b (pRM613) (Table 4.1). The vector pUCP26 used for complementation is a high-copy cloning vector (226). To determine if the vector effects in the complementation experiment were due to the

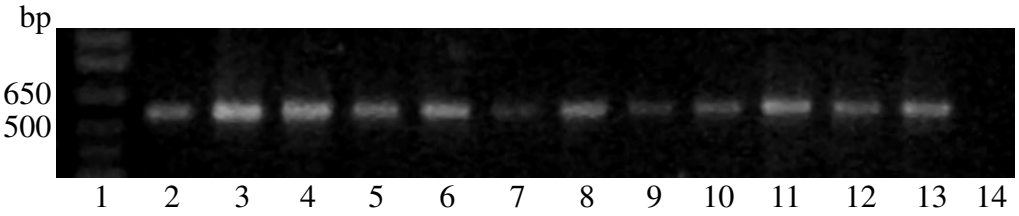


Figure 4.4. Detection of BCAM0188 in *B. cenocepacia* strains by PCR. Products were amplified with the M188scF and M188scR primers and electrophoresed on a 1.0 percent agarose gel. Lane 1, 1 Kb plus ladder (Invitrogen); lane 2, C5424 (ET12 lineage); lane 3, BC7 (ET12 lineage); lane 4, K56-2 (ET12 lineage); lane 5, C6433 ; lane 6, C1394 ; lane 7, PC184 (midwest lineage) ; lane 8, Cep511 ; lane 9 ; J415 ; lane 10, ATCC17765 ; lane 11, Pc715J; lane 12, J2315; lane 13, H111; lane 14, no template control.

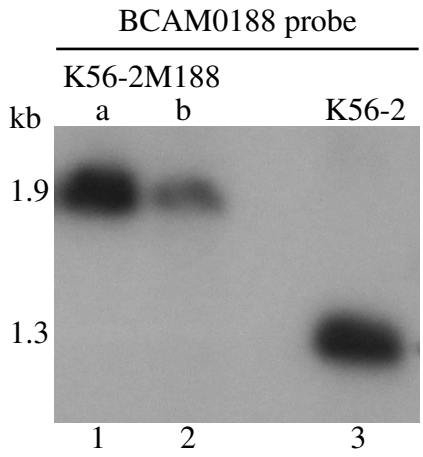


Figure 4.5. Confirmation of BCAM0188 mutant construction by Southern hybridization. Genomic DNA was digested with BamHI and hybridized with a 470 bp EcoRI fragment from pRM1T8 internal to BCAM0188 labelled with ^{32}P. Lane 1, K56-2M188a; lane 2 K56-2M188b; lane 3, BCAM0188 probe fragment. The 0.6 kb increase in the hybridized fragment is due to the incorporation of the Tp resistance cassette.

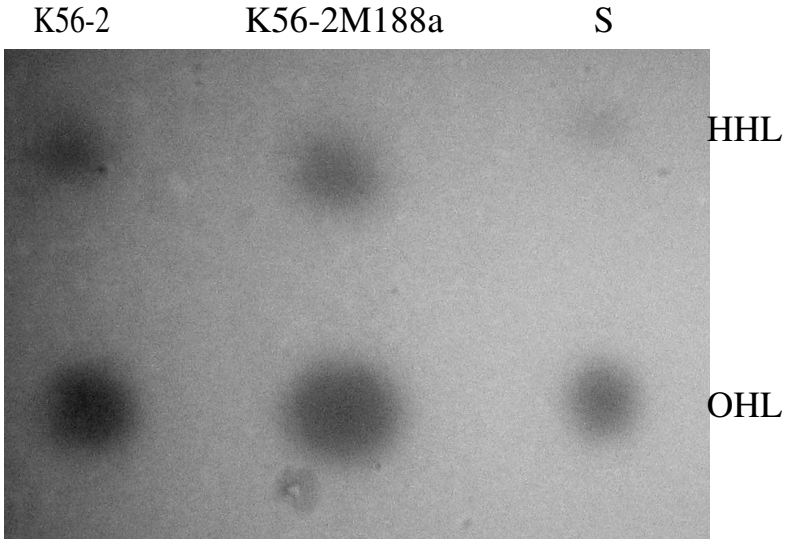


Figure 4.6. AHL production profile of the BCAM0188 mutant. Ethyl acetate extracts were chromatographed on C_{18} reverse-phase TLC plates developed with methanol-water (70:30 vol/vol). The AHLs were visualized using the *A. tumefaciens* (pCF218) (pCF372) reporter strain. S, synthetic standards.

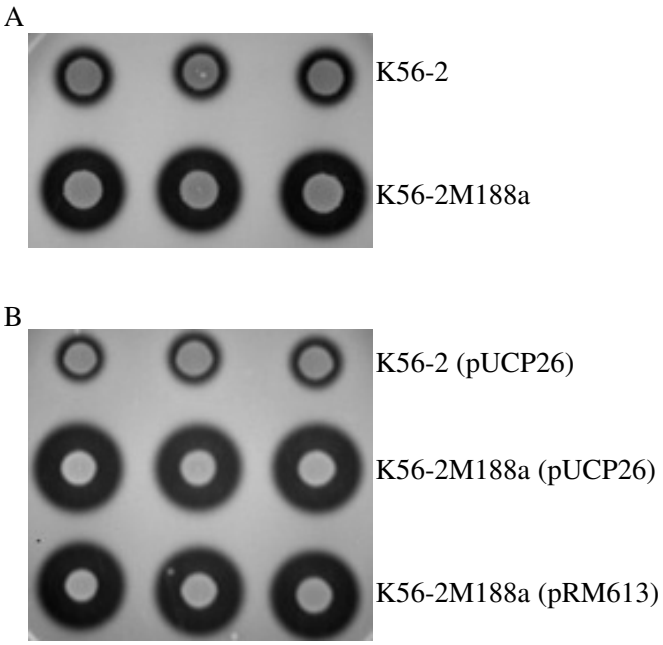


Figure 4.7. Protease activity of the BCAM0188a mutant. Overnight cultures were sub-cultured (1/100) and grown to an optical density at 600 nm of 0.3 and spotted (2µl) onto dialyzed brain heart infusion agar containing 10 percent skim milk. Triplicate zones of clearing of each strain are shown after 24h incubation at 37^{o}C.

(A) Comparison of the parent protease activity compared to the BCAM0188a mutant.

(B) Complementation of the BCAM1088a mutant with BCAM0188 *in trans* on the cloning vector pUCP26.

Table 4.1. Extracellular protease activity and swarming motility of the BCAM0188 mutants.

Strain	Gene present *in trans*	Zone of clearing[a] (mm)	Swarm zone diameter[b] (mm)
K56-2	None	1.9 ± 0.2	50.8 ± 6.0
K56-2M188b	None	3.0 ± 0.0[d]	32.8 ± 6.2[f]
K56-2(pUCP26)	None	2.2 ± 0.3	55.8 ± 6.2
K56-2M188b (pUCP26)	None	2.2 ± 0.3[e]	46.7 ± 3.0
K56-2M188b (pRM613)	BCAM0188	2.0 ± 0.0[e]	51.3 ± 7.6

[a] Values are the average zone of clearing ± SD for six replicates.
[b] Values are the average swarming zone ± SD for three replicates.
[c] Significantly less than K56-2 (P<0.001, unpaired t-test)
[d] Significatly less than K56-2 ($P < 0.001$, ANOVA, Student-Newman-Keuls)
[e] Significantly different from K56-2M188b ($P < 0.001$, ANOVA, Student-Newman-Keuls)
[f] Significantly less than K56-2 ($P < 0.05$, ANOVA, Student-Newman-Keuls).

presence of the high copy vectors, the lower copy cloning vector pRK415 (90) and pRM516, containing BCAM0188 were introduced into K56-2M188b. A preliminary skim milk assay indicated that introduction of pRK415 partially reduced the protease production of K56-2M188b compared to K56-2M188b alone and that the incorporation of pRM516 containing BCAM0188 had no effect on the protease production of the BCAM0188 mutant.

The *cepIR* and *cciIR* quorum-sensing systems were found to be involved in the regulation of swarming motility in *B. cenocepacia* [(83), Table 3.1]. The BCAM0188a mutant exhibited less swarming motility than K56-2 (Fig. 4.8). This decrease in swarming motility was also observed for the BCAM0188b mutant ($P < 0.05$, ANOVA, Student, Newman-Keuls) (Table 4.1). The swarming motility of K56-2M188b (pUCP26) was slightly lower than K56-2 (pUCP26) and was slightly increased when BCAM0188 is added *in trans* (Table 4.1).

The effect of BCAM0188 on the regulation of ornibactin biosynthesis was assessed by quantifying CAS activity. The BCAM0188a and BCAM0188b mutants have a significant decrease in CAS activity ($P = 0.02$, unpaired *t*-test and $P < 0.001$, ANOVA, Student-Newman-Keuls) (Fig. 4.9) and incorporation of either pUCP26 or pRM613 containing BCAM0188 into K56-2M188b restored the CAS activity to parental levels (Fig. 4.9b). Incorporation of pRK415 alone also restored the CAS activity of K56-2M188b to parental levels (Fig. 4.10a). The initial cultures of the BCAM0188 mutants were grown in the presence of 100 μg/ml of trimethoprim prior to subculture for the CAS assay and the strains containing pUCP26, pRK415, pRM613 or pRM513 were

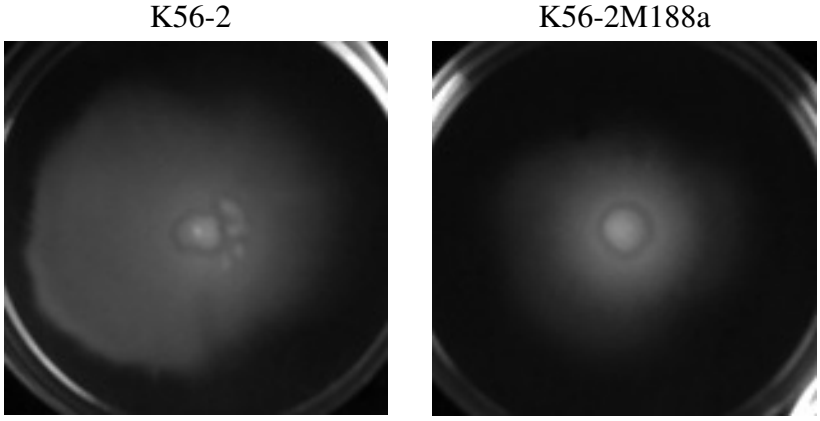


Figure 4.8. Swarming motility of the BCAM0188a mutant. Overnight cultures were normalized and spot inoculated (1µl) onto nutrient broth agar (0.5%) plates with 0.5% glucose and incubated at 37^{o}C for 48h.

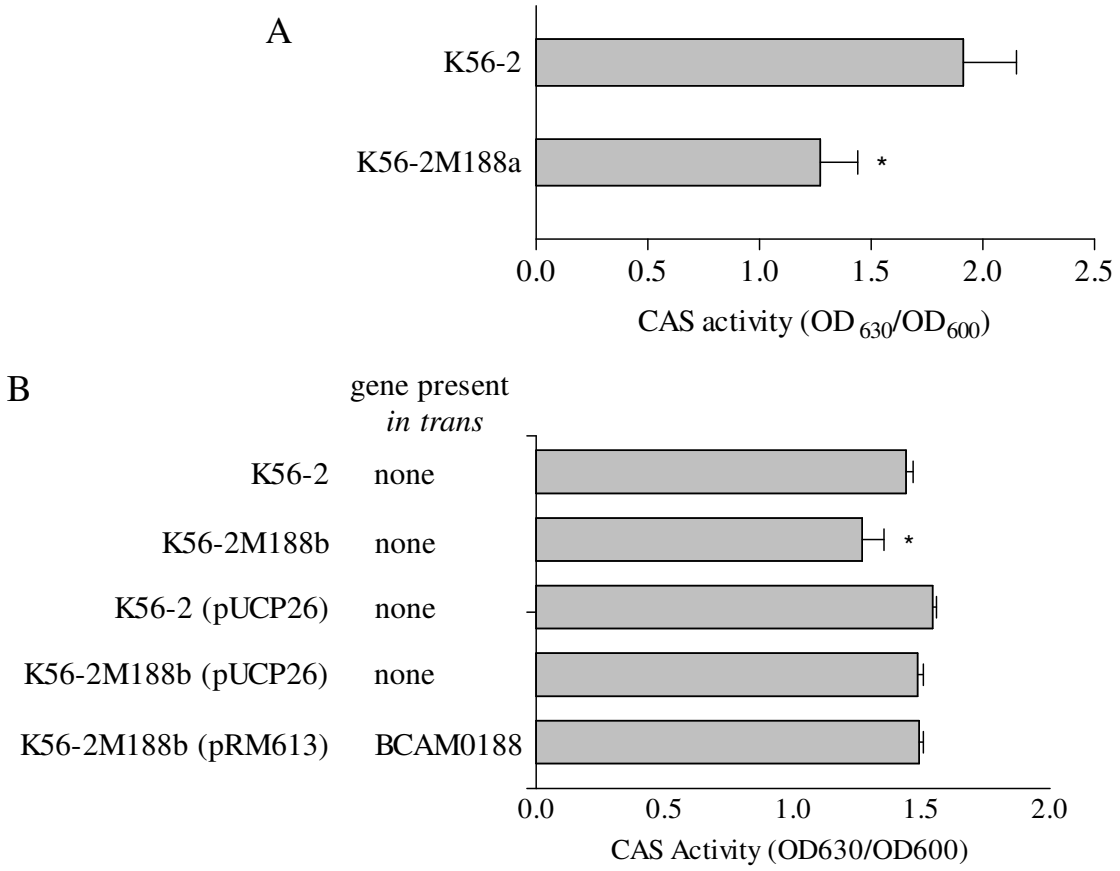
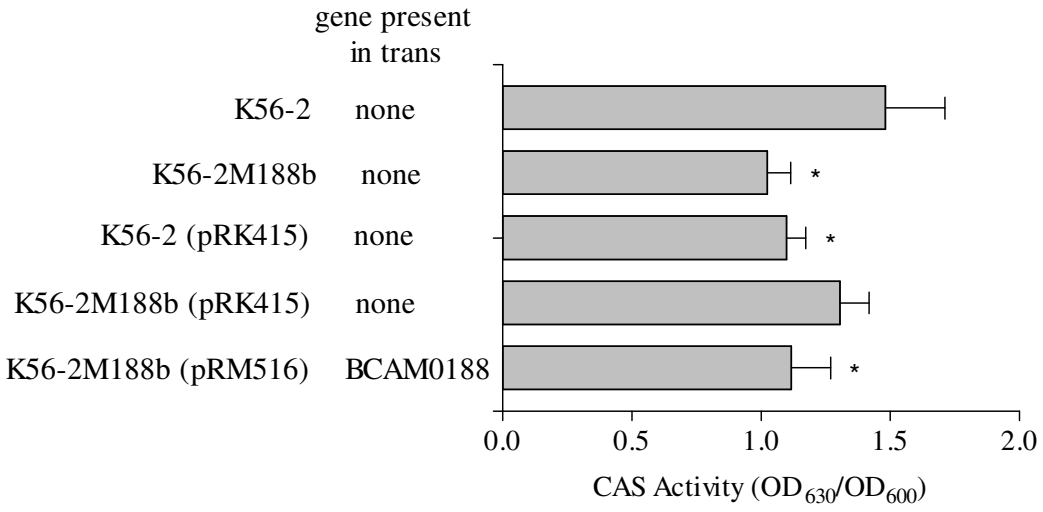


Figure 4.9. Effect of BCAM0188 on ornibactin biosynthesis. Cultures were grown in succinate medium supplemented with 10 mM ornithine for 40h. Spent supernatants were added to the CAS dye complex, and the activity was determined by absorbance at 630 nm. The readings were normalized by culture turbidity (absorbance at 600 nm). The values are the mean ± SD for three replicate cultures.

(A) Ornibactin biosynthesis of K56-2M188a. The CAS activity of K56-2M188a is significantly less than the parent K56-2 ($P = 0.02$, unpaired *t*-test).

(B) Ornibactin biosynthesis of K56-2M188b. The CAS activity of K56-2M188b is significantly less than the parent K56-2 ($P < 0.001$, ANOVA, Student-Newman-Keuls).

A



B

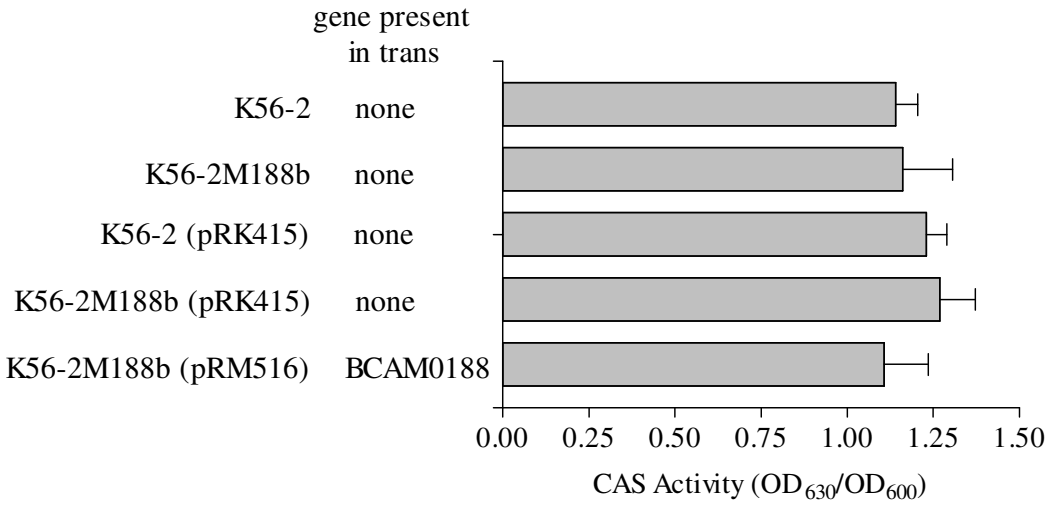


Figure 4.10. Effect of antibiotics on ornibactin biosynthsis of the BCAM0188 mutant. Cultures were grown in succinate medium supplemented with 10 mM ornithine for 40h. Spent supernatants were added to the CAS dye complex, and the activity was determined by absorbance at 630 nm. The readings were normalized by culture turbidity (absorbance at 600 nm). The values are the mean ± SD for three replicate cultures.

(A) Ornibactin biosynthesis of K56-2M188b with 100 μg/ml Tp in the initial culture. The asterisks denote strains that produce significantly less than CAS activity than the parent K56-2 ($P < 0.5$, ANOVA, Student-Newman-Keuls).

(B) Ornibactin biosynthesis of K56-2M188b without 100 μg/ml Tp in the initial culture.

initially grown in the presence of 200 μg/ml of tetracycline for maintenance of the plasmid. To determine if the antibiotics present in the initial cultures were affecting CAS activity, the CAS assay was performed without antibiotics in the initial culture or subculture. The CAS activity of K56-2M188b was indistinguishable from the parent, and there was no difference in the CAS activity of K56-2 (pRK415), K56-2M188b (pRK415) and K56-2M188b (pRM513) (Fig. 4.10b), indicating that the decrease in CAS activity initially observed for K56-2M188b (Fig. 4.7b and Fig. 4.8a) may be due to the antibiotics present in the subculture.

The effect of BCAM0188 on biofilm formation was determined by quantifying the amount of cellular matter formed by K56-2M188b on polystyrene pegs. The amount of biomass formed by the mutant strain was similar to the parent K56-2 (Fig. 4.11), indicating that BCAM0188 is not involved in biofilm formation.

4.2.4 Transcriptional analysis of BCAM0188

To determine if BCAM0188 is a part of the *B. cenocepacia* quorum-sensing regulatory network, a BCAM0188-*luxCDABE* transcriptional fusion was constructed based on an *in silico* promoter prediction. Expression above background levels was not detectable for BCAM0188-*luxCDABE* in K56-2 when grown in the following media conditions; TSB, PTSB, TSB-DC, LB, ¼ LB and low salt LB at 37^{o}C. To ascertain if BCAM0188 was expressed at different temperatures, K56-2 (BCAM0188-*luxCDABE,* pRM418) was grown in TSB and LB at room temperature (22^{o}C) and 42^{o}C. There was no expression detected at either temperature tested. Since BCAM0188 was predicted to be involved in density dependent gene regulation, expression of K56-2 (BCAM-

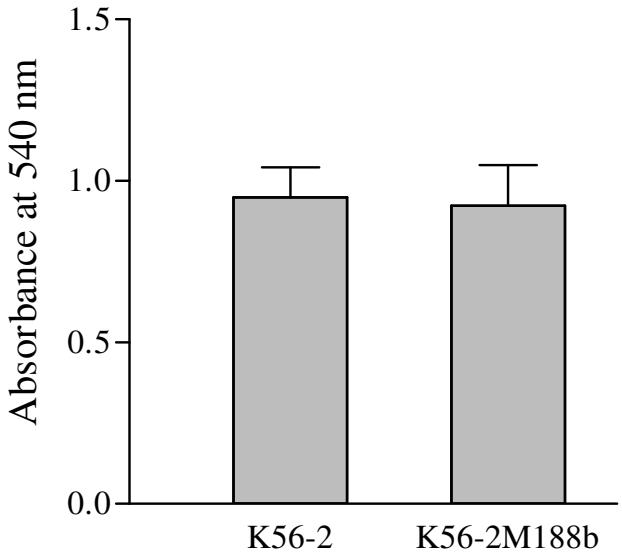


Figure 4.11. Effect of BCAM0188 on biofilm formation. Biofilms were formed on polysterene pegs grown in TSB for 24 hours and the cellular matter was quantified by absorbance at 540 nm of crystal violet bound to the biofilm. The values are the mean ± SD of at least nine pegs per strain.

luxCDABE, pRM418) was monitored for up to 50 hours of growth in TSB to determine if expression was initiated in late stationary phase. Expression above background levels was not detected at any measured time points. It has been previously demonstrated that the *cepIR* system is induced by *P. aeruginosa* AHLs (111, 167). To determine if exogenous AHLs produced by *P. aeruginosa* could induce BCAM0188 expression, K56-2 (BCAM0188-*luxCDABE,* pRM418) was grown in the presence of two, five and 10 percent *P. aeruginosa* PAO1 spent culture supernatant yet, no effect was detected. The possibility of BCAM0188 being negatively regulated by CepR or CciR was determined by incorporating BCAM0188-*luxCDABE* into K56-R2 (*cepR*) and K56-*2cciIR.* Low levels of BCAM0188-*luxCDABE* expression were detected in K56-R2 (*cepR*) (Fig. 4.12), indicating that that CepR may be involved in negative regulation of BCAM0188. Expression studies with certain pMS402 based *luxCDABE* transcriptional fusions in K56-R2 are not reproducable (C. Chambers, B. Subsin, M. Visser, unpublished observations). To confirm the expression of BCAM0188-*luxCDABE* in K56-R2 (*cepR*), ten additional transformants of K56-R2 (BCAM0188-*luxCDABE,* pRM418) were assessed for expression. BCAM0188-*luxCDABE* expression was not detected in any of the additional transformants.

4.3 Discussion

This study identified an additional LuxR homologue that is specific to *B. cenocepacia.* Due to its predominance as a CF pathogen, current research efforts are aimed at understanding what factors set *B. cenocepacia* apart from the rest of the Bcc with regards to virulence. Bernier and Sokol (12) used a suppression-subtractive hybridization

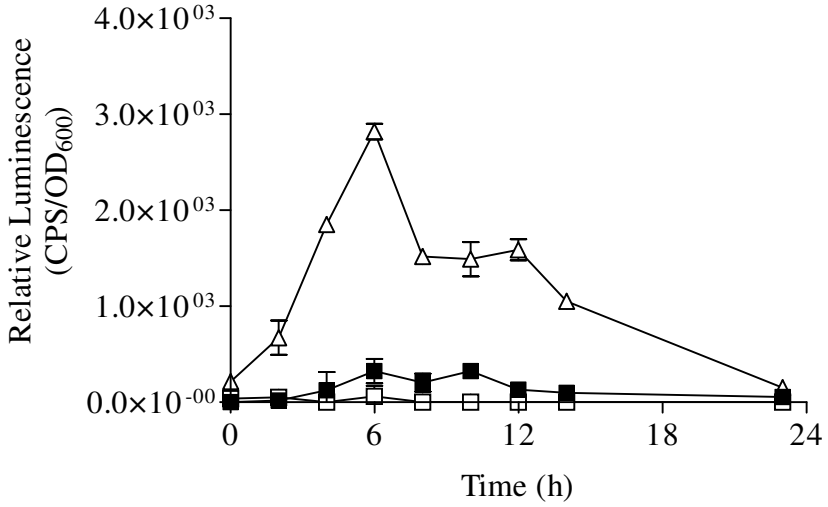


Figure 4.12. Effect of CepR and CciIR on BCAM0188 expression. Expression was monitored throughout growth in TSB plus 100 µg/ml Tp. All values are the means ± SD of triplicate cultures. ■, K56-2 (BCAM0188-*luxCDABE,* pRM418); Δ, K56-R2 (*cepR*) (BCAM0188-*luxCDABE*, pRM418); □, K56-2*cciIR* (BCAM0188-*luxCDABE*, pRM418).

approach to identify genes that are unique to *B. cenocepacia.* Although this study did not identify BCAM0188, it identified numerous *B. cenocepacia* specific genes and found that many of these genes were only present in strains of the ET12 lineage. The BCAM0188 open reading frame was identified in all *B. cenocepacia* species tested, including representative strains from the ET12, Midwest transmissible lineages, as well both *B. cenocepacia* type A and B lineages, suggesting that BCAM0188 is not specifically associated with epidemic strains. BCAM0188 could potentially regualte virulence traits that would lead to increased morbidity and mortality in those infected with *B. cenocepacia* strains.

An increase in protease production as well as decreased swarming motility and ornibactin biosynthesis were observed for the BCAM0188 mutant. However, these phenotypes could not be restored when BCAM0188 was provided *in trans.* Both vector and antibiotic effects were observed during the complementation studies. The incorporation of pUCP26 alone appeared to alter the phenotype of the BCAM0188 mutant. This cloning vector has been used previously for complementing quorum-sensing mutants without indication of an effect on the phenotypes assessed (Table 3.2). The CAS activity of the BCAM0188 mutant was significantly less than the parent only when the initial culture of the mutant was grown in the presence of trimethoprim. This observation, taken with the lack of mutant complementation and the lack of BCAM0188-*luxCDABE* expression *in vitro* suggests that the altered phenotypes observed for the BCAM0188 mutant were due to effects of growth in trimethoprim and not due to the effect of the mutation in BCAM0188.

There were no defects in growth of the mutant when grown in the presence of trimethoprim, suggesting that the antibiotic may be affecting gene expression. The amount of trimethoprim carried over from the original cultures to the assay subcultures would have been minimal. Micro-array studies with *P. aeruginosa* grown in the presence of inhibitory or sub-inhibitory levels of ciprofloxacin indicated the addition of antibiotics at both levels dramatically altered the transcriptome of *P. aeruginosa* (13). The hypothesis that trimethoprim affects the expression of BCAM0188 is currently difficult to prove experimentally since the *luxCDABE* expression vector, pMS402 (50), strictly requires the presence of trimethoprim for maintenance of the vector in *B. cenocepacia* and BCAM0188 expression could not be detected using a BCAM0188-*luxCDABE* transcriptional fusion under *in vitro* conditions. An RT-PCR strategy for examining BCAM0188 expression could be pursued to resolve this issue. The expression of the *luxI* homologue, *bviI,* in *B. vietnamiensis* strain PC259 was detected by RT-PCR but was not detectable using a $bviI_{PC259}$-*luxCDABE* transcriptional fusion (Table 5.2).

Numerous *in vitro* conditions were assessed in attempt to detect expression of BCAM0188-*luxCDABE.* The possibility remains that BCAM0188 requires cues from the host or environment for expression. In the phytopathogen, *A. tumefaciens,* the *luxR* homologue *traR* is only expressed in the presence of host-produced opines that are released from the crown gal tumour (62). With respect to human opportunistic infections, the *rhlIR* quorum-sensing system of *P. aeruginosa* has recently been shown to be enhanced by the cytokine interferon gamma (IFN-γ) (232). Another possibility is that BCAM0188 is negatively regulated by an as yet unidentified component of the *B. cenocepacia* quorum-sensing network. Expression of BCAM0188-*luxCDABE* was not

detected in the *cciIR* mutant and was detected for only one biological replicate in the *cepR* mutant. A *cep* box (225) could not be identified upstream of BCAM0188.

The lack of detection of gene expression using a *luxCDABE* transcription fusion has been reported for another *B. cenocepacia* specific gene. The open reading frame BCAL1122 is homologous to *E. coli* hypothetical protein YjfI and is only found in *B. cenocepacia* strains belonging to the ET12 lineage (12). A *yjfI* mutant constructed in K56-2 did not have altered biofilm, swarming or swimming phenotypes (12) and *in vitro* expression of *yjfi-luxCDABE* could not be detected (S. Bernier, unpublished observation). The *yjfI* mutant however, had a significant decrease in lung pathology in a rat agar bead model of infection (12) and was shown to be important for survival of *B. cenocepacia* in the rat (85). The current working hypothesis is that there may be a role for BCAM0188 in transcriptional regulation as a part of the quorum-sensing network in *B. cenocepacia* strains, expressed only in the presence of specific host or environmental factors.

Chapter Five: Characterization of the *cepIR* and *bviIR* quorum-sensing systems in clinical and environmental strains of *B. vietnamiensis*

5.1 Introduction

In addition to *cepIR, B. vietnamiensis* strains contain the *bviIR* quorum-sensing system (121). PCR amplification and Southern hybridization analysis revealed that *B. vietnamiensis* is the only Bcc species that contains the *bviIR* system (33, 121). A *B. vietnamiensis* strain G4 *bviI* mutant was reported to not produce detectable levels of any AHL except minor amounts of OHL (33) indicating that BviI is responsible for the synthesis of all AHLs produced by G4 including; HHL, OHL, DHL, oxoDHL and doDHL. Despite all tested *B. vietnamiensis* strains containing the genes for two quorum-sensing systems, AHL production has been shown to be strain dependent with regards to type and quantity of AHL produced (34, 69, 231).

Few phenotypes are known to be regulated by the *bviIR* system. One study suggested that an undefined *B. vietnamiensis* G4 transposon mutant with decreased AHL production showed reduced antibiotic production, although this antibiotic has yet to be characterized (155). In the same study, it was determined that the degradation of toluene and thus the expression of *ortho*-monooxygenase is not regulated by quorum-sensing (155). There is also evidence that the *bviIR* system is not involved in siderophore production (33).

The objectives of this study were to further characterize the *bviIR* and *cepIR* quorum-sensing systems of *B. vietnamiensis* G4 including the regulatory relationship between the two quorum-sensing systems. In this study we also investigate the basis for

variability of AHL production in clinical and environmental *B. vietnamiensis* strains at a molecular level.

5.2 Results

5.2.1 Transcriptional analysis of *bviIR* and *cepIR*

To determine if BviR is involved in the regulation of the *bviIR* system, a comparative expression analysis of $bviI_{G4}$ and $bviR_{G4}$-*luxCDABE* transcriptional fusions in G4 and a G4 *bviR* mutant was performed. The expression of *bviI* was reduced to almost background levels in the *bviR* mutant, indicating that BviR is required for the expression of *bviI* (Fig. 5.1a). The expression of *bviR* was similar in G4 and the G4 *bviR* mutant, demonstrating that BviR is not involved in auto-regulation (Fig. 5.1b).

To determine if CepR is involved in the regulation of *cepIR* in *B. vietnamiensis*, transcriptional analysis of $cepI_{G4}$ and $cepR_{G4}$-*luxCDABE* fusions in G4 and a G4 *cepR* mutant was performed. A merodiploid *cepR* mutant was constructed and confirmed by PCR analysis (Fig. 5.2a). Unexpectedly, the expression of *cepI* and *cepR* was similar in both the parent and the *cepR* mutant (Fig. 5.3) indicating that CepR does not regulate either *cepI* or *cepR* in *B. vietnamiensis* G4.

To determine if the lack of CepR auto-regulation was common in *B. vietnamiensis* strains, similar comparative expression studies were performed in strain PC259 and a PC259 *cepR* mutant constructed by allelic exchange (Fig. 5.2b). The expression of *cepR* was significantly greater in the PC259 *cepR* mutant than in the parent strain during the growth curve from hour three to twelve ($P < 0.05$, unpaired *t*-test, Welch) (Fig. 5.4a) indicating that CepR is involved in negative auto-regulation. The expression of *cepI* was

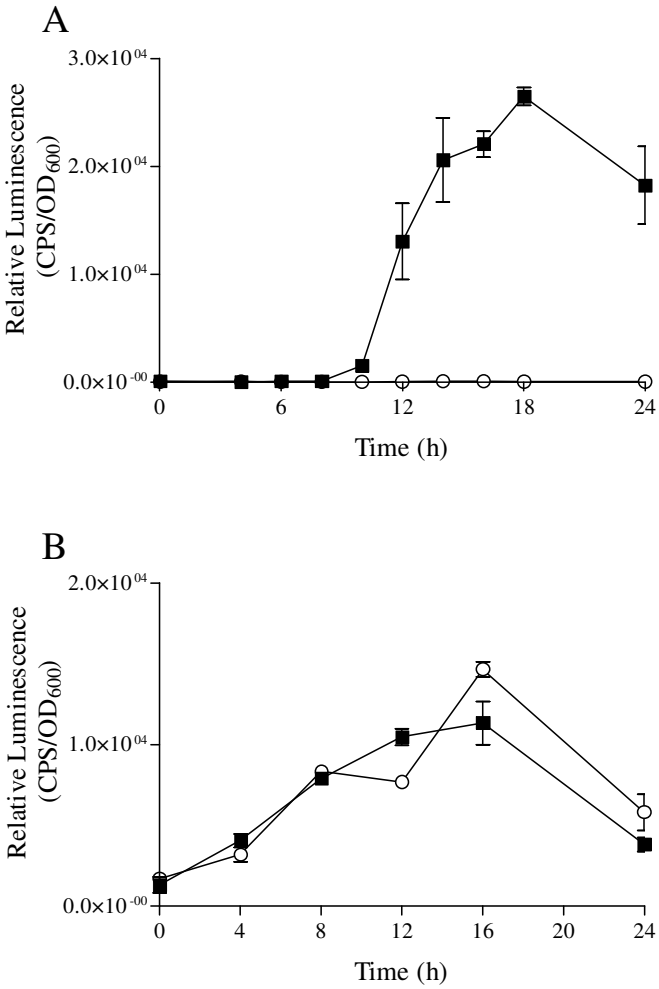


Figure 5.1. Effect of BviR on *bviI* and *bviR* expression in G4. Expression was monitored throughout growth in TSB plus 100 µg/ml Tp. All values are the means ± SD of triplicate cultures and are representative of two individual experiments. ■, G4; ○, G4 *bviR* mutant (RMT-14).
(A) Effect of BviR on *bviI* expression (*bviI*$_{G4}$-*luxCDABE,* pRM465).

(B) Effect of BviR on *bviR* expression (*bviR*$_{G4}$-*luxCDABE,* pRM464).

Figure 5.2. Confirmation of *B. vietnamiensis* quorum-sensing mutant construction by PCR. Products were electrophoresed on a 1.0% agarose gel. L, DNA ladder; NT, no template control.

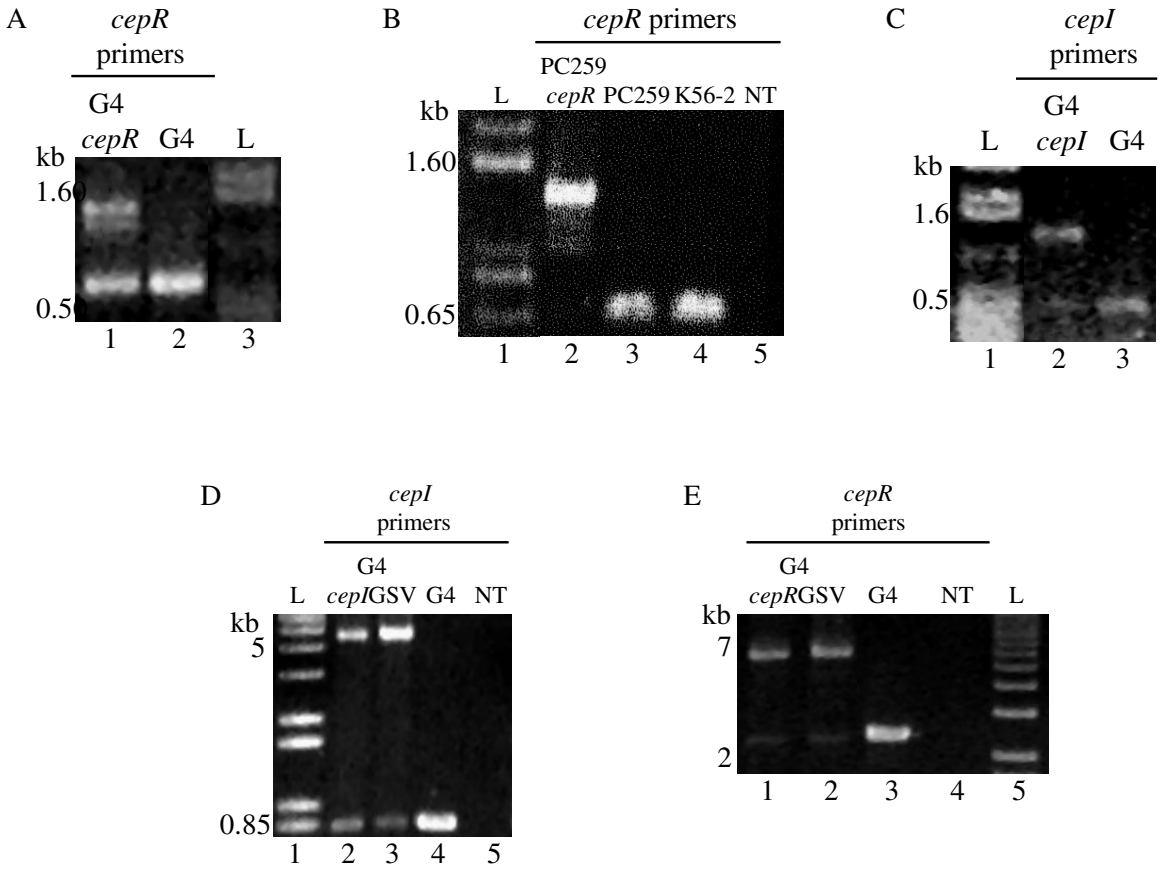
(A) Confirmation of G4*cepR* by PCR with the cepRF and cepRR primers. Lane 1, G4*cepR;* lane 2, G4; lane 3, 1 kb plus ladder. The mutant is a merodiploid and contains a wild type and mutant copy of *cepR.* The 0.9 kb increase in size of the mutant product is due to the incorporation of the chloramphenicol resistance cassette.

(B) Confirmation of PC259*cepR* by PCR with the cepRF and cepRR primers. Lane 1, I kb plus ladder (Invitriogen); lane 2, PC259*cepR*; lane 3, PC259; lane 4, pSLA3.2 (containing *cepIR* from K56-2); lane 5, no template control. The 0.6 kb increase in the size of the mutant product is due to the incorporation of the trimethoprim resistance cassette.

(C) Confirmation of G4*cepI* by PCR with the unicepIF and unicepIR primers. Lane 1, 1 kb plus ladder (Invitrogen); lane 2 G4*cepI*; lane 3, G4. The mutant is a merodiploid and contains a wild type and mutant copy of *cepI*. The 0.9 kb increase in size of the mutant product is due to the incorporation of the chloramphenicol resistance cassette.

(D) Confirmation of G4*cepI*GSV by PCR with G4cepIF and G4cepIR primers. Lane 1, 1 kb plus ladder (Invitrogen); lane 2, G4*cepI*GSVa; lane 3, G4*cepI*GSVb; lane 4, G4; lane 5, no template control. The mutant is a merodiploid and contains a wild type and mutant copy of *cepI.* The 4.0 kb increase in size of the mutant product is due to the incorporation of pRMSVI.

(E) Confirmation of the G4*cepR*GSV mutant by PCR with the cepRKOF and cepRKOR primers. Lane 1 G4*cepI*GSVa; lane 2, G4*cepI*GSVb; lane 3, no template control; Lane 5, 1 kb plus ladder (Invitrogen). The mutant is a merodiploid and contains a wild type and mutant copy of *cepR.* The 3.8 kb increase in the size of the mutant product is due to the incorporation of pRMSVR.


A
cepR primers
G4
cepR G4 L
kb
1.60
0.5
1 2 3
B
cepR primers
PC259
L cepR PC259 K56-2 NT
kb
1.60
0.65
1 2 3 4 5
C
cepI primers
G4
L cepI G4
kb
1.6
0.5
1 2 3
D
cepI primers
G4
L cepIGSV G4 NT
kb
5
0.85
1 2 3 4 5
E
cepR primers
G4
cepRGSV G4 NT L
kb
7
2
1 2 3 4 5

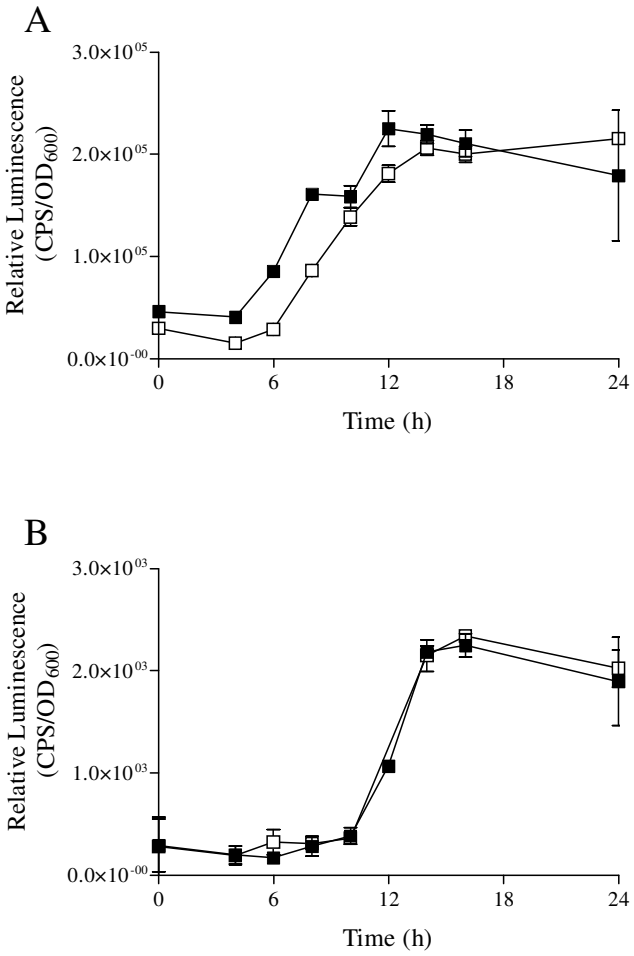


Figure 5.3. Effect of CepR on *cepI* and *cepR* expression in G4. Expression was monitored throughout growth in TSB plus 100 µg/ml Tp. All values are the means ± SD of triplicate cultures and are representative of two individual expreiments. ■, G4; □, G4*cepR*.

(A) Effect of CepR on *cepI* expression (*cepI*$_{G4}$-*luxCDABE,* pRM453).

(B) Effect of CepR on *cepR* expression (*cepR*$_{G4}$-*luxCDABE,* pRM4462).

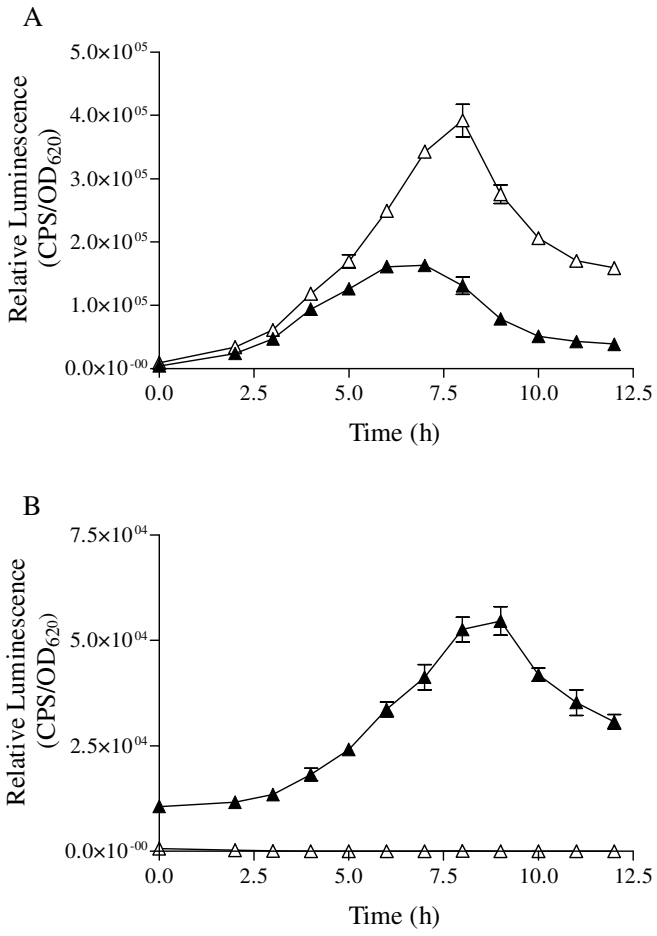


Figure 5.4. Effect of CepR on *cepI* and *cepR* expression in PC259. Expression was monitored throughout growth in TSB plus 100 µg/ml Tp. All values are the means ± SD of triplicate cultures and are representative of two individual experiments. ▲, PC259; Δ, PC259*cepR*.

(A) Effect of CepR on *cepR* expression (*cepR*$_{PC259}$-*luxCDABE*, pRM452). The expression of pRM452 was significantly greater in PC259*cepR* from three to twelve hours ($P < 0.05$, unpaired *t*-test, Welch).

(B) Effect of CepR on *cepI* expression (*cepI*$_{PC259}$-*luxCDABE*, pRM453).

reduced to almost background levels in the PC259 *cepR* mutant (Fig. 5.4b), indicating that CepR is required for *cepI* expression in PC259.

To determine if there is a regulatory relationship between the *cepIR* and *bviIR* systems, transcriptional analysis of *cepI*$_{G4}$-*luxCDABE* and *cepR*$_{G4}$-*luxCDABE* transcriptional fusions were compared in G4 and the G4 *bviR* mutant, and the expression of *bviI*$_{G4}$-*luxCDABE* and *bviR*$_{G4}$-*luxCDABE* were compared in G4 and G4*cepR*. The expression of *cepR* and *cepI* were similar in G4 and the G4 *bviR* mutant (Fig. 5.5), indicating that BviR is not involved in the regulation of the *cepIR* system. The expression of *bviR* was also similar between the parent and *cepR* mutant (Fig. 5.6a), however the expression of *bviI* in the G4 *cepR* mutant was reduced to almost background levels (Fig. 5.6b) indicating that CepR positively regulates *bviI* in G4.

Assessment of the regulatory relationship between *cepIR* and *bviIR* in PC259 was attempted, however expression of the *bviI*$_{PC259}$-*luxCDABE* fusion, pRM455 was not detected in PC259 (Fig. 5.7). To determine if CepR positively regulates *bviI* in PC259, reverse transcriptase (RT)-PCR was performed to amplify *bviI* and *bviR* from PC259 and the PC259 *cepR* mutant total RNA. There was less *bviR* product (Fig. 5.8, compare lanes 3a and 3b) and no detectable *bviI* product (Fig. 5.8, lane 2b) for the PC259 *cepR* mutant indicating that CepR positively regulates the *bviIR* system in PC259. The *sigA* gene was used as a control and there was no observable difference in expression between PC259 and the PC259 *cepR* mutant (Fig. 5.8, compare lanes 4a and 4b).

5.2.2 Phenotypic characterization of *B. vietnamiensis* quorum-sensing mutants

AHL-TLC bioassays of the G4 *bviI* and *bviR* mutants were performed (Fig. 5.9a). Ethyl acetate extracts of spent culture supernatants were chromatographed and AHLs

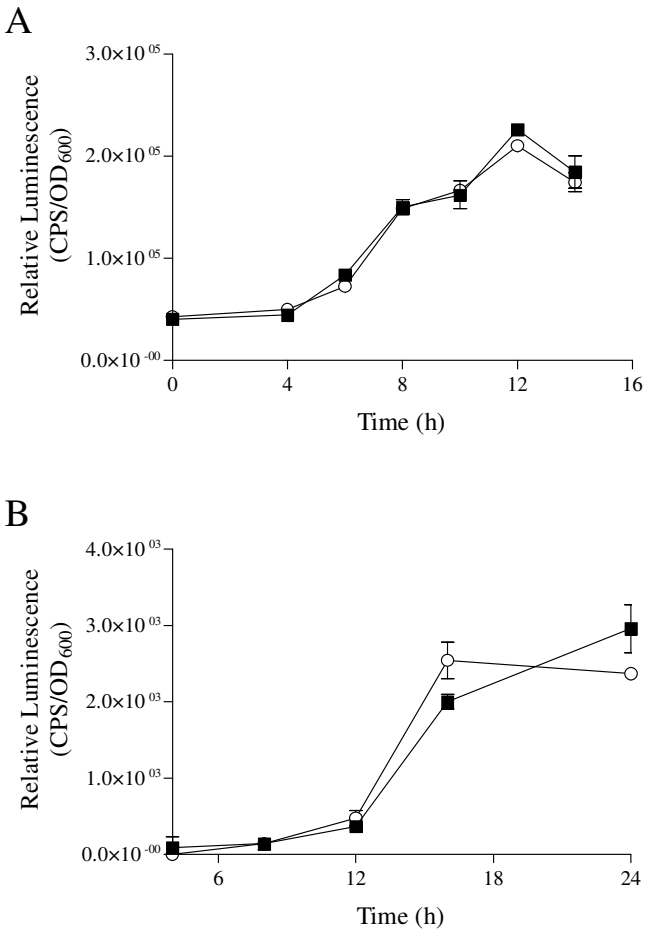


Figure 5.5. Effect of BviR on *cepI* and *cepR* expression in G4. Expression was monitored throughout growth in TSB plus 100 µg/ml Tp. All values are the means ± SD of triplicate cultures and are representative of two individual experiments. ■, G4; ○, G4*bviR* (RMT-14).
(A) Effect of BviR on *cepI* expression (*cepI*$_{G4}$-*luxCDABE,* pRM463).

(B) Effect of BviR on *cepR* expression (*cepR*$_{G4}$-*luxCDABE,* pRM462).

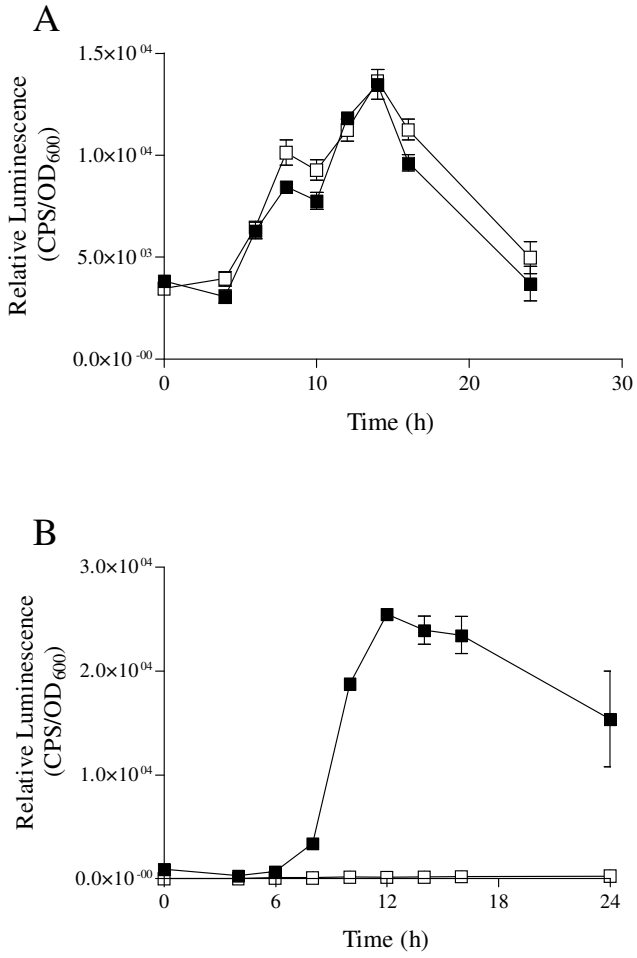


Figure 5.6. Effect of CepR on *bviI* and *bviR* expression in G4. Expression was monitored throughout growth in TSB plus 100 µg/ml Tp. All values are the means ± SD of triplicate cultures and are representative of two individual experiments. ■, G4; □, G4*cepR*.

(A) Effect of CepR on *bviR* expression ($bviR_{G4}$-*luxCDABE,* pRM464).

(B) Effect of CepR on *bviI* expression ($bviI_{G4}$-*luxCDABE,* pRM465).

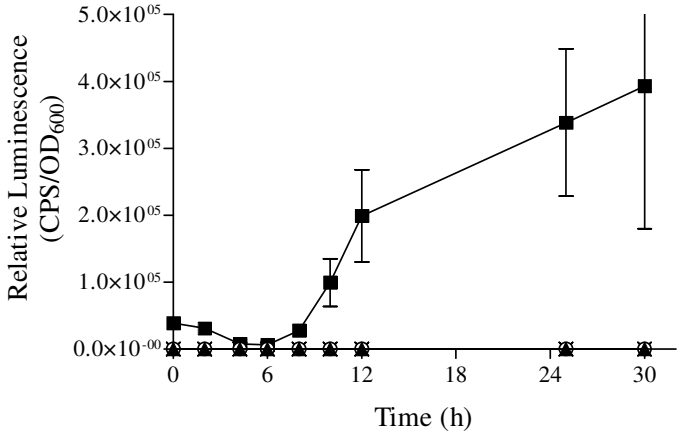


Figure 5.7. Expression of *bviI-luxCDABE* in *B. vietnamiensis* strains. Expression was monitored throughout growth in LB plus 100 µg/ml Tp. All values are the means ± SD of triplicate cultures and are representative of two individual experiments. ■, G4 (*bviI*$_{G4}$-*luxCDABE,* pRM465); ▲, PC259 (*bviI*$_{PC259}$-*luxCDABE,* pRM455); ○, FC466 (*bviI*$_{FC466}$-*luxCDABE*, pRM475); ×, FC441 (*bviI*$_{FC441}$-*luxCDABE,* pRM485).

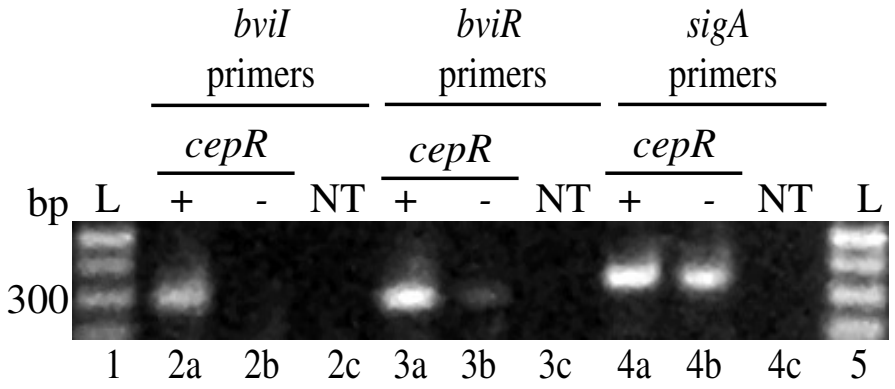


Figure 5.8. Effect of CepR on *bviI and bviR* expression in PC259 by RT-PCR. Products were electrophoresed on a 1.0% gel. L, DNA ladder; NT, no template control.. Lane 1, 1 kb plus ladder (Invitrogen); lanes 2a-c, product amplified by RTbviIF and RTbviIR; lanes 3a-c, product amplified by RTbviRF and RTbviRR; lanes 4a-c, product amplified by RTsigAF and RTsigAR; lane 5, 1 kb plus ladder (Invitrogen). a, PC259 RNA; b, PC259*cepR* RNA; c, no template control.

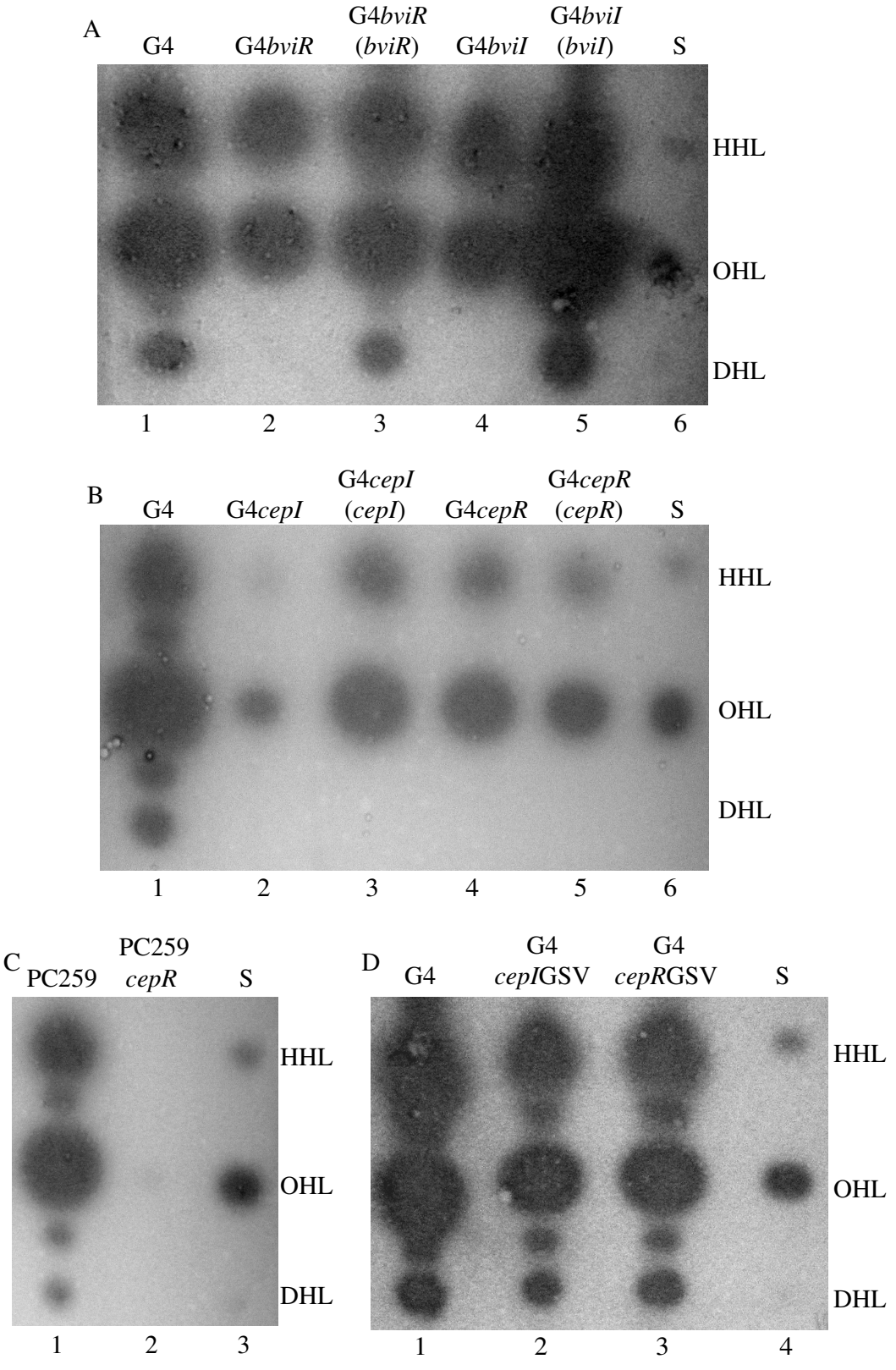
Figure 5.9. AHL production profiles of the *B. vietnamiensis* quorum-sensing mutants. Ethyl acetate extracts were chromatographed on C_{18} reverse-phase TLC plates developed with methanol-water (70:30 vol/vol). The AHLs were visualized using the *A. tumefaciens* (pCF218) (pCF372) reporter strain. S, synthetic standards.

(A) AHL production profile of the G4 *bviI* and *bviR* mutants. Lane 1, G4 (pUCP28T); lane 2, G4*bviR* (pUCP28T); lane 3, G4*bviR* (pRM612); lane 4, G4*bviI* (pUCP28T); lane 5, G4*bviI* (pRM611); lane 6, synthetic standards.

(B) AHL production profiles of the G4 *cepI* and *cepR* mutants. Lane 1, G4 (pUCP28T); lane 2, G4*cepI* (pUCP28T)*;* lane 3, G4*cepI* (pRM283)*;* lane 4, G4*cepR* (pUCP28T); lane 5, G4*cepI* (pRM282)*;* lane 6, synthetic standards.

(C) AHL production profile of the PC259 *cepR* mutant. Lane 1, PC259; lane 2, PC259*cepR*; lane 3, synthetic standards.

(D) AHL production profiles of the G4 *cepI* and *cepR* mutants constructed with pGSV3. Lane 1, G4; lane 2, G4*cepI*GSV; lane 3, G4*cepR*GSV.


A
G4
G4*bviR*
G4*bviR* (*bviR*)
G4*bviI*
G4*bviI* (*bviI*)
S
HHL
OHL
DHL
1
2
3
4
5
6
B
G4
G4*cepI*
G4*cepI* (*cepI*)
G4*cepR*
G4*cepR* (*cepR*)
S
HHL
OHL
DHL
1
2
3
4
5
6
C
PC259
PC259 *cepR*
S
HHL
OHL
DHL
1
2
3
D
G4
G4 *cepI*GSV
G4 *cepR*GSV
S
HHL
OHL
DHL
1
2
3
4

were visualized using an *A. tumefaciens* A136 (pCF218) (pCF372) AHL reporter strain agar overlay. G4 produced detectable amounts of HHL, OHL and DHL (Fig. 5.9a, lane 1). DHL production was not detected for the G4 *bviI* or *bviR* mutant (Fig. 5.9a, lanes 2 and 4). The production of DHL was restored when *bviI* and *bviR* were present *in trans* (Fig. 5.9a, lanes 3 and 5), thus corroborating the role of BviR in the positive regulation of *bviI*. Considerable amounts of HHL and OHL remained in the AHL production profiles of the *bviIR* mutants (Fig. 5.9a, lanes 2 and 4), thereby confirming that the *bviIR* system is not required for the expression of *cepIR*.

The G4 *cepR* mutant produces marginally less HHL and OHL than the parent but no detectable amounts of DHL (Fig. 5.9b, compare lanes 1 and 4), confirming the role of CepR in the positive regulation of *bviI* and in the negative regulation of *cepI*. Unexpectedly, AHL production was not restored in G4*cepR* when a wild type copy of *cepR* was added *in trans*. A merodiploid G4 *cepI* mutant was constructed and confimed by PCR analysis (Fig. 5.2c). The AHL production profile of the G4 *cepI* mutant has considerably less HHL and OHL than the parent (Fig. 5.9b, compare lanes 1and 2) and no detectable DHL. HHL and OHL, but not DHL production was increased when the mutant was complemented with a wild type copy of *cepI* (Fig. 5.9b, lane 3), confirming that *cepI* is expressed and encodes for a functional AHL synthase in G4. PC259 produces detectable amounts of HHL, OHL and DHL (Fig. 5.9c, lane 1). The AHL production profile for the PC259 *cepR* mutant was devoid of detectable AHL (Fig. 5.9c, lane 2), confirming that CepR is involved in the positive regulation of the AHL synthase genes *cepI* and *bviI* in this strain.

The discrepancies between the G4 *cepR* merodiploid and the PC259 *cepR* haploid mutant, with regards to regulation of *cepIR* and AHL production, as well as the lack of full complementation of the G4 *cepI* and *cepR* mutants prompted a second mutant construction strategy for G4 aimed at eliminating the wild type copy. Mutant construction has proven successful in *B. mallei* and *B. pseudomallei* using the pGSV3 vector (150). This strategy incorporates pGSV3 (44) into the open reading frame of interest. G4 *cepI* and *cepR* mutants were constructed with this approach and were designated G4*cepI*GSV and G4*cepR*GSV respectively. The mutant cultures contained cells with wild type *cepI* and *cepR* genes (Fig. 5.2d and e). The presence of the wild type cells decreased with increasing Gm concentrations (data not shown), indicating that there may be revertants in the population. The AHL production profiles of G4*cepI*GSV and G4*cepR*GSV were unaffected (Fig. 5.9d) therefore; these mutants were not studied further.

To determine if the *bviIR* quorum-sensing system is involved in phenotypes known to be quorum-sensing regulated in *B. cenocepacia*, siderophore biosynthesis, biofilm formation, and swarming motility assays were performed (83, 109-111). Extracellular protease production assays were not performed as *B. vietnamiensis* does not produce proteases (65, 69). The effect of *bviIR* mutations on nitrogen fixation was also determined since this phenotype has been shown to be quorum-sensing regulated in other nitrogen-fixing species (38). G4 did not exhibit swarming motility or the ability to grow on nitrogen free media. The levels of siderophore biosynthesis in the G4 *bviI* and *bviR* mutants were indistinguishable from the parent G4 (Fig. 5.10). The *bviI* and *bviR* mutants exhibited marginally less biofilm formation; however, this decrease was not

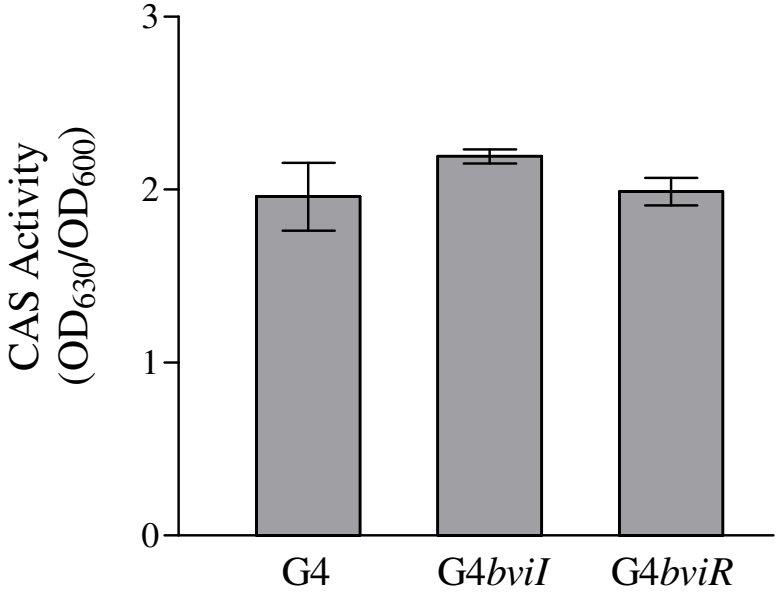


Figure 5.10. Effect of BviI and BviR on ornibactin biosynthesis in G4. Cultures were grown in succinate medium supplemented with 10 mM ornithine for 40h. Spent supernatants were added to the CAS dye complex, and the activity was determined by absorbance at 630 nm. The readings were normalized by culture turbidity (absorbance at 600 nm). The values are the mean ± SD for three replicate cultures.

restored when *bviI* and *bviR* were added *in trans* (Fig. 5.11). Although G4 did not cause infections in alfalfa relative to the positive control, *B. cenocepacia* K56-2, fewer seedlings showed signs of disease when inoculated with G4 *bviI* and *bviR* mutants than when infected with the parent (Table 5.1). Further investigations of phenotypes that may be regulated by the *bviIR* system were analyzed by API 20 NE strips. The *bviI* and *bviR* mutants did not exhibit a phenotype distinguishable from the parent in any of the API 20 NE biochemical tests (data not shown).

5.2.3 Analysis of AHL production in clinical and environmental strains of *B. vietnamiensis*

AHL production in the Bcc is strain dependent with respect to quantity and type of AHL produced (34, 69, 231). Since the regulation of the quorum-sensing systems appears to be strain dependent, further investigation into the differences in AHL production by *B. vietnamiensis* strains was pursued. The AHL production profiles of seven *B. vietnamiensis* strains were determined by AHL-TLC bioassays (Fig. 5.12). AHL production by *B. vietnamiensis* strains is variable with FC466 producing low amounts of AHLs; to FC441 and C2622 producing HHL and OHL; to PC259, FC369^{T}, DBO1 and G4 producing considerable quantities of multiple AHLs (Fig. 5.12). Only one of four clinical strains, but all three environmental strains produced DHL, suggesting that the *bviIR* genes may be expressed less in clinical strains than environmental strains, since OHL and HHL could be produced by CepI in these strains.

The presence of the *bviI* and *bviR* genes in the seven *B. vietnamiensis* strains was confirmed by PCR analysis with primers that amplified DNA fragments internal to *bviI* and *bviR* (Fig. 5.13). To determine if these genes are expressed in the clinical strains that

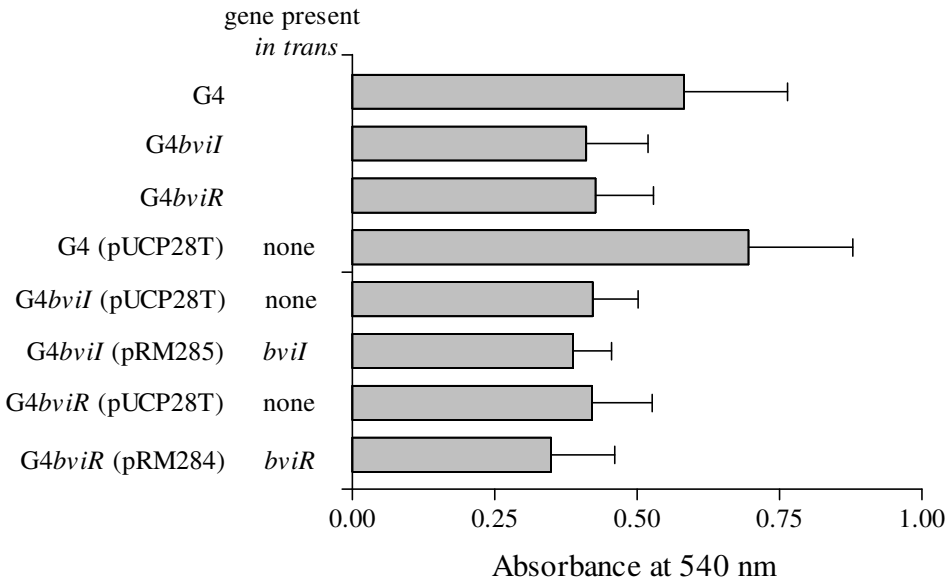


Figure 5.11. Effect of BviI and BviR on biofilm formation in G4. Biofilms were formed on polystyrene pegs grown in TSB for 48h and the cellular matter was quantified by absorbance at 540 nm of crystal violet bound to the biofilm. The values are the mean ± SD of at least nine pegs per strain and represent two individual experiments. Plasmids pRM284 and pRM285 contain *bviR* and *bviI* respectively.

Table 5.1: Effect of BviI and BviR on virulence in an alfalfa model of infection

Strain	Percent of seedlings with symptoms Trial 1	Percent of seedlings with symptoms Trial 2
K56-2	85 [a]	80 [b]
G4	20 [a]	12.5 [b]
G4*bviI*	5 [a]	5 [b]
G4*bviR*	0 [a]	5 [b]
Saline	0 [c]	20 [c]

[a] twenty seedlings infected
[b] fourty seedlings infected
[c] ten seedlings infected.

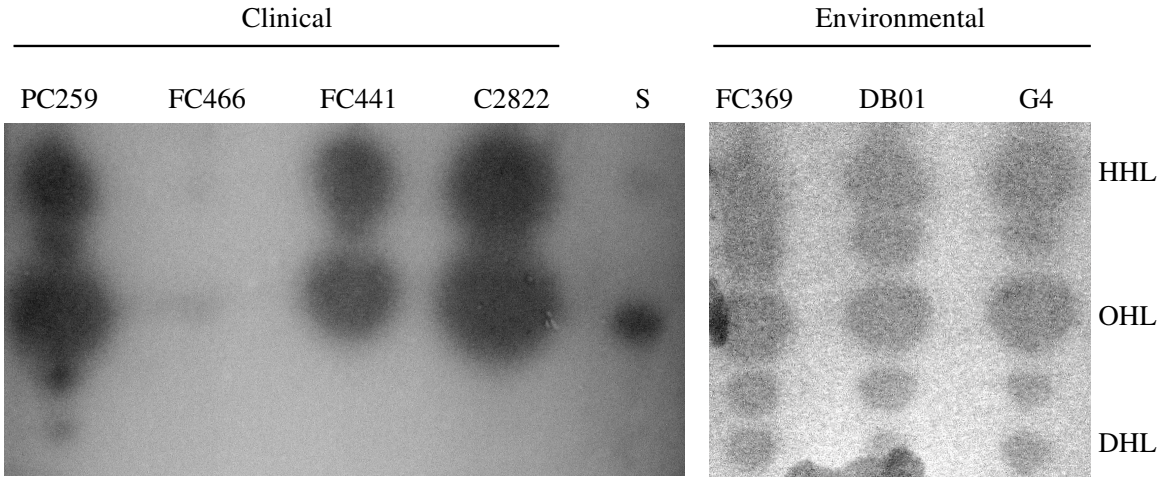


Figure 5.12. AHL production profiles *B. vietnamiensis* strains. Ethyl acetate extracts were chromatographed on C_{18} reverse-phase TLC plates developed with methanol-water (70:30 vol/vol). The AHLs were visualized using the *A. tumefaciens* (pCF218) (pCF372) reporter strain. S, synthetic standards.

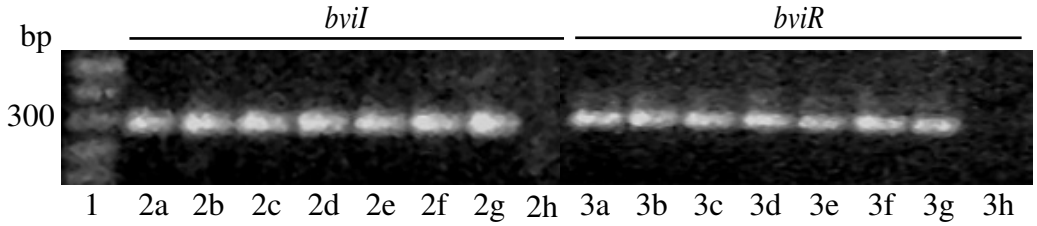


Figure 5.13. Amplification of the *bviI* and *bviR* genes in *B. vietnamiensis* strains by PCR. Products were electrophoresed on a 1.0 percent agarose gel. Lane 1, 1 kb plus ladder (Invitrogen); lanes 2 a-h, products amplified with RTbviIF and RTbviIR primers; lane 3 a-h, products amplified with RTbviRF and RTbviRR primers. a, PC259; b, FC466; c, FC441; d, C2822; e, FC369T; f, DBO1; g, G4; h, no template control.

do not produce DHL, RT-PCR was performed (Fig. 5.14). There was a decrease in amplification of the *bviR* RT-PCR products from the non-DHL producing strains; FC466, FC441 and C2822 compared to the DHL producing strains; PC259 and G4 (Fig. 5.14a, compare lanes 3a-e). There were no detectable *bviI* RT-PCR products from FC466, FC441 and C2822 (Fig. 5.14a, lanes 2b-d). There were no distinguishable differences between strains in the amplification of the control gene, *sigA* (Fig. 5.14a, lanes 4a-e). These data indicate that the *bviI* and *bviR* genes are poorly expressed in the three non-DHL producing strains

To ensure that the variance in *bviI* and *bviR* gene expression between the five strains was not due to differences in primer efficiency, RT-PCR was performed with increasing cycle numbers. RT-PCR with 20 cycles did not yield visible products (Fig. 5.14b). At 25 cycles, there were similar *sigA* products amplified for each strain and *bviI* products amplified only for PC259 and G4 (Fig. 5.14b). At 30 cycles, the results are as initially reported (Fig. 5.14a) except, the *bviR* products for FC466 and FC441 are not visible (Fig. 5.14b) indicating that the RTbviRF and RTbviRR primers may be less efficient in these strains.

The *bviI* RT-PCR data was confirmed for strains PC259, FC466 and FC441 by transcriptional analysis with *bviI-luxCDABE* transcriptional fusions of each *bviI* promoter in their respective strain background (Fig. 5.7). The only discrepancy was that the expression of *bviI-luxCDABE* in PC259 was at background levels (Fig. 5.7) where as a *bviI* RT-PCR product was obviously amplified (Fig. 5.14a, lane 2a).

Given that BviR is implicated in the positive regulation of *bviI* and that the expression of *bviR* was lower in the three non-DHL producing clinical strains,

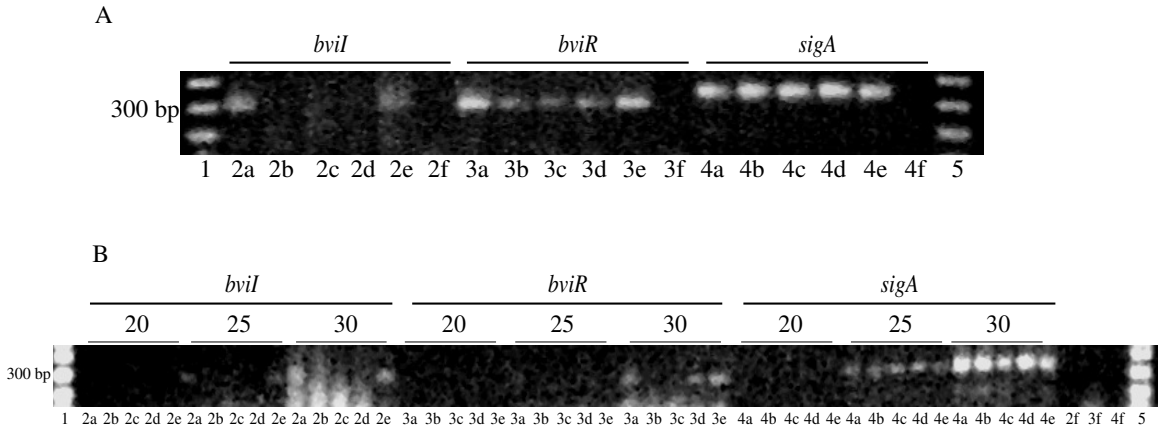


Figure 5.14. Transcriptional analysis of *bviI* and *bviR* in non-DHL producing strains by RT-PCR. Products were electrophoresed on a 1.0% agarose gel. Lanes 1 and 5, 1 kb plus DNA ladder (Invitogen); lanes 2 a-f, *bviI* products amplified with the RTbviIF and RTbviIR primers; lanes 3 a-f, *bviR* products amplified with the RTbviRF and RTbviRR primers; lanes 4 a-f, *sigA* products amplified with the RTsigAF and RTsigAR primers. a, PC259; b, FC466; c, FC441; d, C2822; e, G4; f, no template control.
(A) RT-PCR analysis of *bviI* and *bviR* under optimized amplification conditions.

(B) Analysis of primer efficiency for RT-PCR by performing the amplification reactions with 20, 25 or 30 cycles. The cycle number is indicated above the gel.

heterologous expression of *bviR*$_{G4}$ was performed to determine if the absence of *bviI* expression in these strains was due to a lack of induction by BviR (Fig. 5.15). The presence of *bviR*$_{G4}$ *in trans* restored DHL production in each of the strains (Fig. 5.15, lanes 1, 3 and 5), suggesting that the absence of *bviI* expression in the non-DHL producing strains is due to a hindered induction of *bviI* by BviR.

The *bviIR* loci of the three non-DHL producing strains and G4 were sequenced to determine if there were any sequence differences in the non-DHL producing strains that could lead to a lack of DHL synthesis. The sequence of *bviIR* from DBO1 (accession no. AF296284) (121) was also compared. The translated *bviI* sequence was identical in all six strains (Fig. 5.16) demonstrating that each strain encodes a functional AHL synthase. The translated *bviR* sequence was identical at all but residue 106 (Fig. 5.17). This difference was not related to the ability to produce DHL since C2822 and G4 contained a glycine and FC466 and FC411 contained an aspartate at this position. Therefore *bviR* encodes for a functional transcriptional regulator in all strains. There are nucleotide differences in the intergenic region proximal to the *bviI* start codon (Fig. 5.18a). The predicted -10 and -35 promoter elements did not contain sequence differences that correlate with *bviI* expression levels. A *lux*-box-type consensus sequence, identical in all five strains, was predicted to be centered 56 bp upstream of the *bviI* start codon (Fig. 5.18a) and designated as the *bviI*$_1$ box (Fig. 5.18b). A *cep* box consensus sequence, based on the *B. cenocepacia* CepR binding site (225) (Fig. 5.18b) was predicted to be centered 337 bp upstream of the *bviI* start codon and is designated as the *bviI*$_2$ box This predicted CepR binding site was identical in all strains except for G4 (Fig. 5.18a).

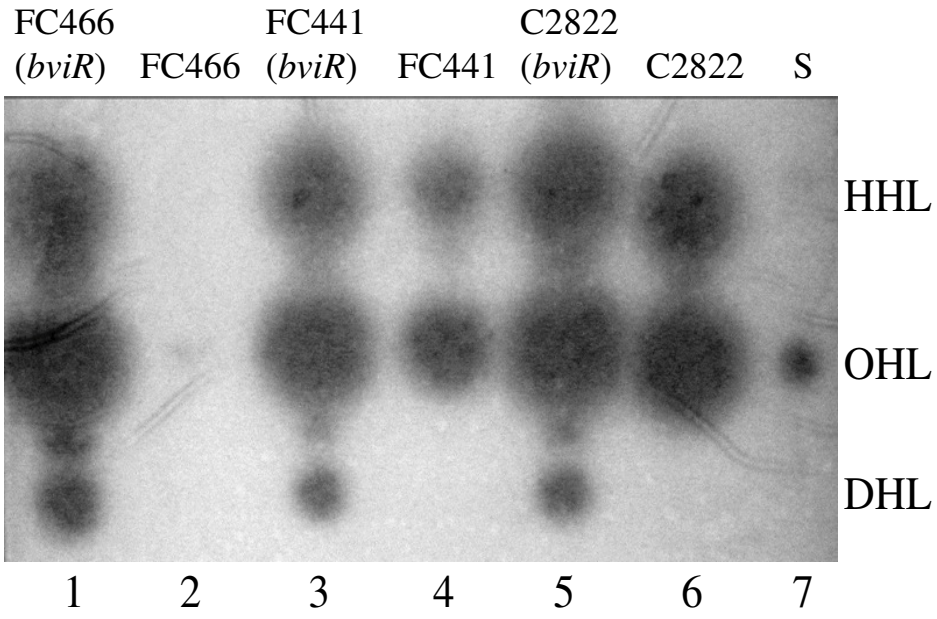


Figure 5.15. Heterologous expression of *bviR*$_{G4}$ in non-DHL producing clinical strains. Ethyl acetate extracts were chromatographed on C_{18} reverse-phase TLC plates developed with methanol-water (70:30 vol/vol). The AHLs were visualized using the *A. tumefaciens* (pCF218) (pCF372) reporter strain. S, synthetic standards. Lane 1, FC466 (pRM284); lane 2, FC466 (pUCP28T); lane 3, FC411 (pRM284); lane 4, FC441 (pUCP28T); lane 5, C2822 (pRM284); lane 6, C2822 (pUCP28T); lane 7, synthetic standards.

```
FC466    MLTLLSGRSADLNRETMYQLAKYRHKVFIQELGWTLPTDNGIEFDNFDHADTLYVIARDR     60
FC411    MLTLLSGRSADLNRETMYQLAKYRHKVFIQELGWTLPTDNGIEFDNFDHADTLYVIARDR     60
C2822    MLTLLSGRSADLNRETMYQLAKYRHKVFIQELGWTLPTDNGIEFDNFDHADTLYVIARDR     60
G4       MLTLLSGRSADLNRETMYQLAKYRHKVFIQELGWTLPTDNGIEFDNFDHADTLYVIARDR     60
DBO1     MLTLLSGRSADLNRETMYQLAKYRHKVFIQELGWTLPTDNGIEFDNFDHADTLYVIARDR     60

FC466    NGEIVGCGRLLPTDEPYLLGDVFPTLMGDAALPHAPDVWELSRFAMSMPRGESLTAEESW    120
FC411    NGEIVGCGRLLPTDEPYLLGDVFPTLMGDAALPHAPDVWELSRFAMSMPRGESLTAEESW    120
C2822    NGEIVGCGRLLPTDEPYLLGDVFPTLMGDAALPHAPDVWELSRFAMSMPRGESLTAEESW    120
G4       NGEIVGCGRLLPTDEPYLLGDVFPTLMGDAALPHAPDVWELSRFAMSMPRGESLTAEESW    120
DBO1     NGEIVGCGRLLPTDEPYLLGDVFPTLMGDAALPHAPDVWELSRFAMSMPRGESLTAEESW    120

FC466    QNTRAMMSEIVRVAHAHGANRLIAFSVLGNERLLKRMGVNVHRAAPPQMIEGKPTLPFWI    180
FC411    QNTRAMMSEIVRVAHAHGANRLIAFSVLGNERLLKRMGVNVHRAAPPQMIEGKPTLPFWI    180
C2822    QNTRAMMSEIVRVAHAHGANRLIAFSVLGNERLLKRMGVNVHRAAPPQMIEGKPTLPFWI    180
G4       QNTRAMMSEIVRVAHAHGANRLIAFSVLGNERLLKRMGVNVHRAAPPQMIEGKPTLPFWI    180
DBO1     QNTRAMMSEIVRVAHAHGANRLIAFSVLGNERLLKRMGVNVHRAAPPQMIEGKPTLPFWI    180

FC466    EIDEQTRAALNLDGLERVGGVPPKTLRRPDASRALEQSV                         219
FC411    EIDEQTRAALNLDGLERVGGVPPKTLRRPDASRALEQSV                         219
C2822    EIDEQTRAALNLDGLERVGGVPPKTLRRPDASRALEQSV                         219
G4       EIDEQTRAALNLDGLERVGGVPPKTLRRPDASRALEQSV                         219
DBO1     EIDEQTRAALNLDGLERVGGVPPKTLRRPDASRALEQSV                         219
```

Figure 5.16. Multiple alignment of *B. vietnamiensis* BviI sequences. The alignment was generated using DNAMAN sequence analysis software. The shading indicates conserved amino acids at all residues.

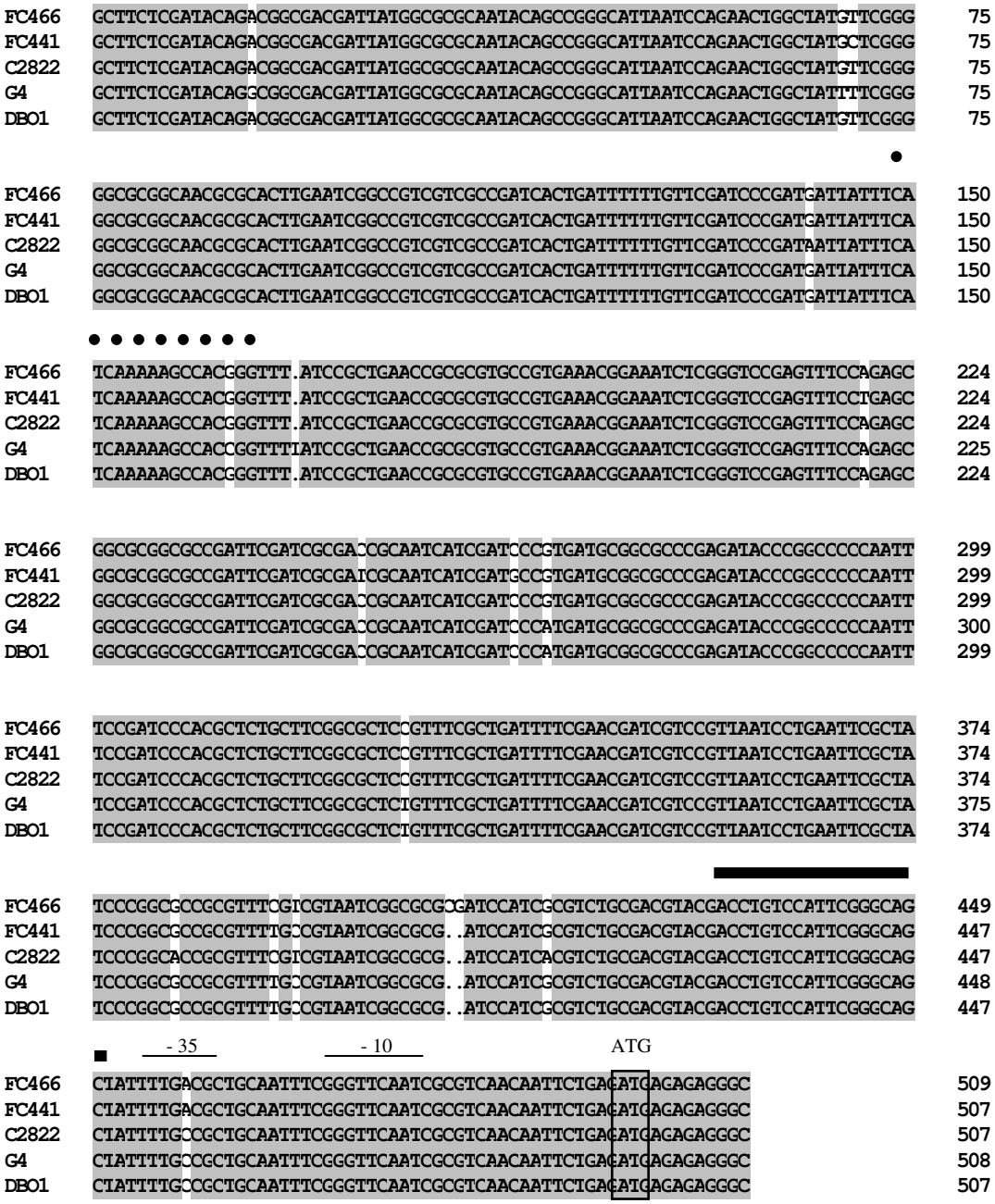
```
FC466    MQAWREKYLNGFATAKSEADVFLEFSADVRALGFDHCSFGLRIPLPISKPQFMLQSNYPQ     60
FC411    MQAWREKYLNGFATAKSEADVFLEFSADVRALGFDHCSFGLRIPLPISKPQFMLQSNYPQ     60
C2822    MQAWREKYLNGFATAKSEADVFLEFSADVRALGFDHCSFGLRIPLPISKPQFMLQSNYPQ     60
G4       MQAWREKYLNGFATAKSEADVFLEFSADVRALGFDHCSFGLRIPLPISKPQFMLQSNYPQ     60
DBO1     MQAWREKYLNGFATAKSEADVFLEFSADVRALGFDHCSFGLRIPLPISKPQFMLQSNYPQ     60

FC466    TWVERYVSQNYFAVDPTVRHGLSRMSPLIWRADSQTQCVQFWEEADQHGLRHGWCMPSVS    120
FC411    TWVERYVSQNYFAVDPTVRHGLSRMSPLIWRADSQTQCVQFWEEADQHGLRHGWCMPSVS    120
C2822    TWVERYVSQNYFAVDPTVRHGLSRMSPLIWRADSQTQCVQFWEEAGQHGLRHGWCMPSVS    120
G4       TWVERYVSQNYFAVDPTVRHGLSRMSPLIWRADSQTQCVQFWEEAGQHGLRHGWCMPSVS    120
DBO1     TWVERYVSQNYFAVDPTVRHGLSRMSPLIWRADSQTQCVQFWEEAGQHGLRHGWCMPSVS    120

FC466    RTGAIGLITMVRSGEPIEERELAEKGYQMSWLANTANYAMSMHLLQRLVPEYTVELTVRE    180
FC411    RTGAIGLITMVRSGEPIEERELAEKGYQMSWLANTANYAMSMHLLQRLVPEYTVELTVRE    180
C2822    RTGAIGLITMVRSGEPIEERELAEKGYQMSWLANTANYAMSMHLLQRLVPEYTVELTVRE    180
G4       RTGAIGLITMVRSGEPIEERELAEKGYQMSWLANTANYAMSMHLLQRLVPEYTVELTVRE    180
DBO1     RTGAIGLITMVRSGEPIEERELAEKGYQMSWLANTANYAMSMHLLQRLVPEYTVELTVRE    180

FC466    REALQWSAAGKTYAEIGKIMHVDDRTVKFHLVNAMRKLNAANKTEAAVKATMLGLLF       237
FC411    REALQWSAAGKTYAEIGKIMHVDDRTVKFHLVNAMRKLNAANKTEAAVKATMLGLLF       237
C2822    REALQWSAAGKTYAEIGKIMHVDDRTVKFHLVNAMRKLNAANKTEAAVKATMLGLLF       237
G4       REALQWSAAGKTYAEIGKIMHVDDRTVKFHLVNAMRKLNAANKTEAAVKATMLGLLF       237
DBO1     REALQWSAAGKTYAEIGKIMHVDDRTVKFHLVNAMRKLNAANKTEAAVKATMLGLLF       237
```

Figure 5.17. Multiple alignment of *B. vietnamiensis* BviR sequences. The alignment was generated using DNAMAN sequence analysis software. The shading indicates conserved amino acids at all residues. Residue 106 is the only differing residue.

Figure 5.18. Multiple alignments of the nucleotide sequences proximal to *bviI* and *lux* box promoter elements of *B. vietnamiensis* species. The alignments were generated using DNAMAN sequence analysis software. Shaded regions highlight nucleotides conserved in at least three of the sequences.
(A) Comparison of the 500 bp upstream of *bviI* in *B. vietnamiensis* strains. The ATG start codon, -10 and -35 promoter elements are indicated above the sequence. The two *lux* box regions are indicated above the sequence; solid line; $bviI_1$ box and dotted line; $bviI_2$ box.

(B) Comparison of the *lux* box sequences in the promoter regions of *bviI* with *B. cenocepacia cepI* (225) and *V. fischeri luxI* (47).

A



B

bviI$_1$	ACCTGTCCATTCGGGCAGCT	20
bviI$_2$	..ATCAAAAAGCCTCGGG..	16
cepI	..CTGTAAGAGTTACCAG..	16
luxI	ACCTGTAGGATCGTACAGGT	20

To determine if the absence of *bviI* expression in the non-DHL producing strains is due to sequence differences upstream of *bviI*, expression of *bviI*$_{G4}$-*luxCDABE* in PC259, FC466, FC441 and C2822 (Fig. 5.19a) and expression of *bviI*$_{PC259}$-*luxCDABE* in FC369^{T}, DBO1 and G4 (Fig. 5.19b) was examined. There was expression *bviI*$_{PC259}$-*luxCDABE* in FC369^{T}, DBO1 and G4 but there was no detectable *bviI*$_{G4}$-*luxCDABE* expression in FC466, FC441 and C2822 indicating that the lack of *bviI* expression in these three clinical strains is not due to mutations in the *bviI* promoter region.

It is possible that the lack of DHL production in these three clinical strains may be due to CepR not inducing *bviI* since CepR positively regulates *bviI* in both G4 and PC259. Heterologous expression of *cepR*$_{G4}$ in FC466, FC441 and C2822 was performed to determine if CepR is able to induce *bviI* (Fig. 5.20). Addition of *cepR*$_{G4}$ increased the production of HHL and OHL in FC466 (Fig. 5.20, compare lanes 1 and 2), but it did not restore DHL production in FC466, FC441 or C2822 (Fig. 5.20, lanes 1, 3 and 5), indicating that lack of DHL is not due to lack of CepR functioning as a positive regulator of *bviI* in these strains.

5.3 Discussion

The *B. vietnamiensis cepIR* and *bviIR* systems are a part of a hierarchical regulatory network. The *cepIR* and *bviIR* regulatory relationship was established by transcriptional analysis, assessment of the AHL production profiles of quorum-sensing mutants and by identifying a potential CepR binding site (225) upstream of the *bviI* start codon. The initiation of *cepI* expression at least six hours before *bviI* expression is consistent with the idea that the CepIR system is required for the expression of *bviI*. Phylogenetic analysis established the *cepIR* system is the ancestral quorum sensing

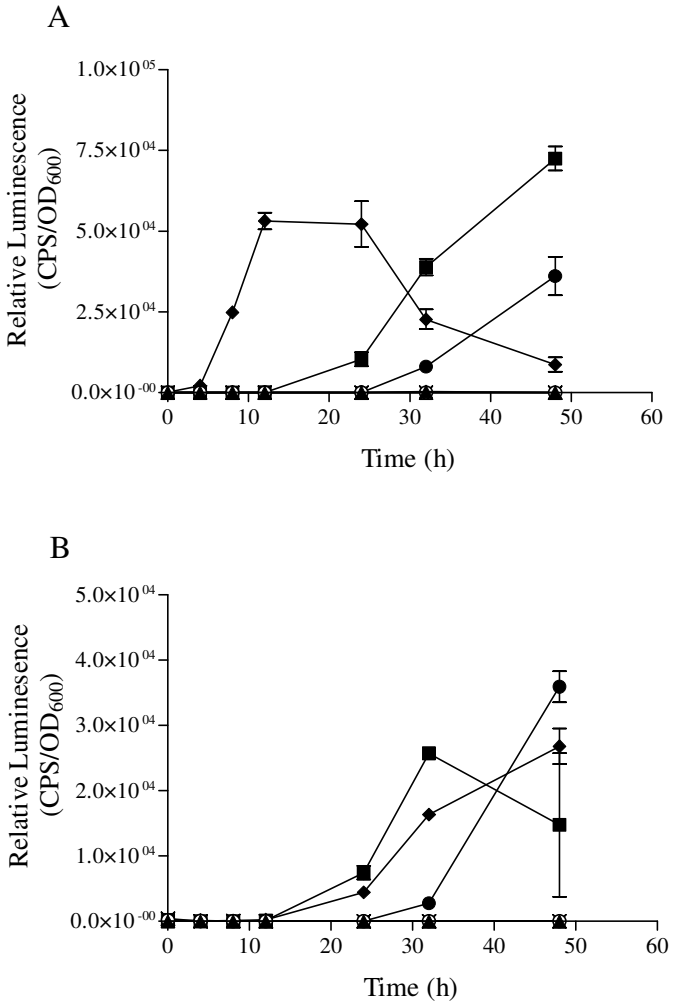


Figure 5.19. Expression of *bviI*$_{G4}$ and *bviI*$_{PC259}$ in *B. vietnamiensis* strains. Expression was monitored throughout growth in TSB plus 100 µg/ml Tp. All values are the means ± SD of triplicate cultures and are representative of two individual experiments. ■, G4; ▲, PC259; ○, FC466; ×, FC441; Δ, C2822; ●, FC369^{T}; ♦, DBO1.
(A) Expression of *bviI*$_{G4}$ (*bviI*$_{G4}$-*luxCDABE,* pRM465).

(B) Expression of *bviI*$_{PC259}$ (*bviI*$_{PC259}$-*luxCDABE*, pRM455).

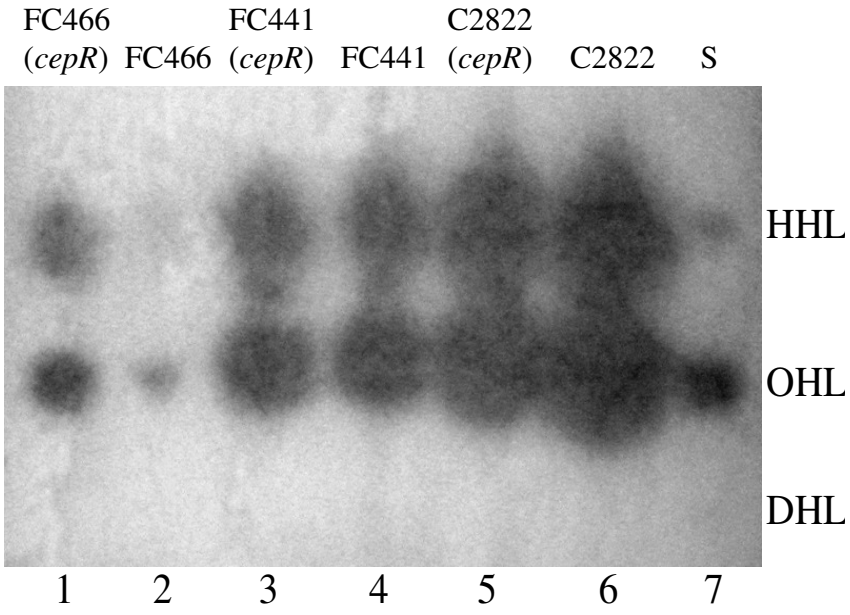


Figure 5.20. Heterologous expression of *cepR*$_{G4}$ in non-DHL producing strains. Ethyl acetate extracts were chromatographed on C_{18} reverse-phase TLC plates developed with methanol-water (70:30 vol/vol). The AHLs were visualized using the *A. tumefaciens* (pCF218) (pCF372) reporter strain. S, synthetic standards. Lane 1, FC466 (pRM282); lane 2, FC466 (pUCP28T); lane 3, FC411 (pRM282); lane 4, FC441 (pUCP28T); lane 5, C2822 (pRM282); lane 6, C2822 (pUCP28T); lane 7 synthetic standards.

system for the Bcc and that the *bviIR* system is distinct (129), therefore the *bviIR* system is presumed to been acquired independently and subsequently incorporated into the CepIR regulatory network. A principal role for the *cepIR* system in *B. vietnamiensis* quorum-sensing is contrary to the previous hypotheses that indicated a role for *bviIR* as the dominant system based on the AHL production profiles of the G4 *bviI* and *bviR* mutants (33). The difference in results and interpretation may be due to the difference in AHL reporters used to characterize the AHL production profile of the G4 *bviI* and *bviR* mutants in the two studies. Conway and Greenberg (33) used an AHL radiotracer assay and an *E. coli* based AHL bioassay to examine the AHL production profiles of the *bviI* and *bviR* mutants. The radiotracer assay quantifies the amount of radiolabelled methionine incorporated into AHLs during synthesis and distinguishes the AHLs present by HPLC (181) and the *E. coli* biosensor (pHV200I^{-}) used is based on *V. fischeri luxIR* system and is most sensitive to 3-oxo-HHL (159). The *A. tumefaciens* reporter used in the current study is most sensitive to 3-oxoacyl-homoserine lactones with side chains of eight to twelve carbons and is able to detect unsubstituted-homoserine lactones with side chains ranging from six to twelve carbons (178). If the assay employed by Conway and Greenberg (33) was as sensitive to HHL and OHL as the *A. tumefaciens* based assay, they would likely have detected greater AHL production by the *bviIR* mutants and a greater role for CepI in AHL production.

The Conway and Greenberg study (33) and the current study are in agreement regarding the role of *bviR* in the positive regulation of *bviI*. Auto-regulation is common in AHL–mediated quorum sensing systems to achieve exponential activation of the system (223). Feedback was observed for the *cepIR* system in PC259 since CepR

positively regulated *cepI* and negatively regulated itself. The G4 *cepIR* system, however, did not exhibit auto-regulation. There was a marked difference in the AHL production profiles for the *cepR* mutants in PC259 and G4 that is most likely due to the differences in the role of CepR in auto-regulation. In the partial G4 genome sequence (http://genome.jgi-psf.org/draft_microbes/bur08/bur08.home.html), a CepR binding site identical to that published for *B. cenocepacia cepI* (225) was identified upstream of *cepI* illustrating potential for *cepIR* auto-regulation. G4*cepR* is a single cross over mutant and therefore, could still be producing functional copies of CepR that are hindering observation of the effect of CepR during comparative transcriptional analysis.

The AHL production profiles of the quorum-sensing mutants confirmed the transcriptional analysis experiments. The G4 *cepI* mutant AHL production profile contains less HHL and OHL than the parent, presumably due to the lack of synthesis of these AHLs by CepI, however, the G4 *cepI* mutant did not produce detectable levels of DHL. With no detectable DHL in the AHL production profile of the G4 *bviI* mutant, it is unlikely that CepI synthesizes DHL in G4. With less AHL present in the environment of the *cepI* mutant, there would be less of the active AHL bound form of CepR, leading to a decrease in *bviI* expression and the synthesis of DHL. The failed restoration of DHL production in the G4 *cepI* and *cepR* mutants when a wild type copy of the respective gene was added *in trans* was unexpected, especially considering that complementation of the G4 *bviI* and *bviR* mutants resulted in AHL production greater than or equal to parental levels. The construct pRM283, containing the *cepI* gene and pRM282, containing the *cepR* gene, expressed *cepI* and *cepR* since there was an increase in HHL and OHL production in the *cepI* mutant harbouring pRM283 and in FC466 harbouring pRM282.

CepR is not the cognate regulator of *bviI,* therefore it is possible that complementing the mutants with *cepI* or *cepR in trans* would only lead to partial restoration and insufficient levels of DHL for detection. The *A. tumefaciens* reporter strain is one hundred times less sensitive to DHL than OHL (18). Employing an AHL reporter strain more sensitive to long chain AHLs such as *P. putida* F117 (pKR-C12) (231) may have detected DHL in the G4 *cepI* and *cepR* mutants complemented with *cepI* and *cepR*, respectively.

It is curious that all of the environmental isolates tested produced DHL where as all of the clinical isolates examined either did not produce DHL or had less *bviI* expression than G4 (Table 5.2). The ability of Bcc species to produce AHLs has been extensively surveyed and a clear correlation between quantity or type of AHL produced and origin of the strain has not developed (34, 69, 231). These three studies employed different methodologies, but agree with the current study on the AHLs produced by all strains except for FC441 where one study reported DHL production by this strain (231) (Table 5.3). All three environmental isolates were reported to produce DHL in four independent studies, whereas four out of the ten clinical isolates are reported not to produce DHL or not to produce DHL as their predominant AHL (34, 69, 231) (Table 5.2), suggesting less DHL production in *B. vietnamiensis* clinical strains.

The current study has taken the analysis of AHL variability further by investigating AHL production at the molecular level. It has been suggested that differences between non-pathogenic strains and those which cause infections may occur at the level of gene regulation, rather than presence or absence of genes (156). Heterologous expression of $bviR_{G4}$ in the clinical non-DHL producing strains restored

Table 5.2. Summary of AHL production, *bviIR* gene presence and *bviIR* expression in clinical and environmental *B. vietnamiensis* strains.

Strain	Source of isolate	HHL production[a]	OHL production[a]	DHL production[a]	Presence of *bviIR* genes[b]	Expression of *bviI*	Expression of *bviR*[g]
PC259	Clinical	+	+	+	+	+[c]/-[d,e]	+
FC466	Clinical	(+)	(+)	-	+	-[c,d,e]	(+)
FC441	Clinical	+	+	-	+	-[c,d,e]	(+)
C2822	Clinical	+	+	-	+	-[c,e]	(+)
FC369^T	Environmental	+	+	+	+	+[c,f]	n/d
DBO1	Environmental	+	+	+	+	+[f]	n/d
G4	Environmental	+	+	+	+	+[c,d,,f]	+

HHL; *N*-hexanoyl-homoserine lactone, OHL; *N*-octanoyl-homoserine lactone, DHL; *N*-decanoyl-homserine lactone, +; positive, (+); weak positive, -; negative, n/d; not determined

[a] Production of AHLs was determined by AHL-TLC bioassays using *A. tumefaciens* A136 (pCF218) (pCF372) as the reporter strain

[b] Presence of the *bviIR* genes was determined by PCR amplification of *bviI* and *bviR* with primers internal to the genes

[c] The expression of *bviI* was assessed by reverse transcriptase-PCR with primers internal to *bviI*

[d] The expression of *bviI* was analyzed by transcriptional analysis of *bviI-luxCDABE* fusions (pRM455, pRM465, pRM475, pRM485) in their respective host backgrounds

[e] The expression of $bviI_{G4}$ was analyzed by transcriptional analysis of $bviI_{G4}$-*luxCDABE* fusion (pRM465)

[f] The expression of $bviI_{PC259}$ was analyzed by transcriptional analysis of $bviI_{PC259}$-*luxCDABE* fusion (pRM465)

[g] The expression of *bviR* was assessed by reverse transcriptase-PCR with primers internal to *bviR*

Table 5.3. Summary of AHLs produced by *B. vietnamiensis* strains from four independent studies.

Strain	Source	Presence of DHL (this study) [a]	Presence of DHL (69) [b]	Presence of DHL (231) [c]	Most predominant AHL (34) [d]
PC259	CF patient	(+)	-	n/d	OHL
FC466	CF patient	-	-	n/d	OHL
FC441	CGD patient	-	-	+	OHL
C2822	CF patient	-	n/d	n/d	OHL
LMG 6998	Blood	n/d	+	-	n/d
LMG 6999	Neck Absess	n/d	+	+	n/d
R-128	CF patient	n/d	+	n/d	n/d
R-723	CF patient	n/d	-	n/d	n/d
R-921	CF patient	n/d	+	+	n/d
C9178	CF patient	n/d	n/d	n/d	OHL
FC369^T	Rice rhizosphere	+	+	+	DHL
DBO1	Rice	+	n/d	n/d	DHL
G4	Water treatment facility	+	n/d	+	n/d

DHL; *N*-decanoyl-homserine lactone, +; positive, (+); weak positive, -; negative, n/d; not determined

[a] Determined by TLC-AHL bioassay with the *A. tumefaciens* A136 (pCF218) (pCF372) reporter strain that detects the 3-oxo-AHLs with side chains ranging from C_4 to C_{12} and 3-unsubstituted-AHLs with side chains of 6 to 12 carbons (178).

[b] Determined by TLC-AHL bioassay with the *E. coli* MT102 (pSB403) reporter that is most sensitive to 3-oxo-HHL, but also senses longer chain AHLs including 3-oxo-DHL and 3-oxo-doDHL and the *E. coli* (pSB1075) reporter that is most sensitive to long chain AHLs and most sensitive to 3-oxo-DHL and 3-oxo-doDHL (69).

[c] Determined by cross-streaking the strain with the *P. putida* F117 (pKR-C12) reporter strain that is highly sensitive to 3-oxo-doDHL, 3-oxo-DHL and long chain AHLs (231).

[d] Determined by a radiotracer assay where cultures were labled with L[1-^{14}C]methionine during growth and ethyl acetate extractions of culture supernatants were fractionated by HPLC and fractions containing AHLs were quantified with a scintillation counter (34).

DHL production implicating inefficient induction of *bviI* by BviR as the reason for the absence of DHL production in these three strains. Transcriptional analysis of the $bviI_{G4}$-*luxCDABE* in the non-DHL producing strains and of $bviI_{PC259}$-*luxCDABE* in the DHL producing strains determined that the lack of *bviI* expression is not due to mutations in the *bviI* promoter region, suggesting that an unknown upstream regulatory element is influencing the expression of *bviI.* Heterologous expression of $cepR_{G4}$ in the clinical non-DHL producing strains did not restore DHL production, therefore CepR is not the affected upstream regulatory element. There is a possibility that *B. vietnamiensis* species possess additional quorum-sensing systems that are influencing the expression of the *bviIR* and *cepIR* systems. *B. pseudomallei* and *B. thailandensis* possess three complete AHL-mediated quorum sensing systems and two additional *luxR* homologues (206, 208) and *B. mallei* contains two *luxI* and four *luxR* homologues (207). The regulatory network of the systems has yet to be characterized. Searching the incomplete G4 genome sequence (http://genome.jgi-psf.org/draft_microbes/bur08/bur08.home.html) did not reveal additional *luxI* or *luxR* homologues but at the time of investigation this sequencing project was in the early stages of development and did not even contain the complete *cepI* open reading frame. The possibility also exists that the clinical non-DHL producing strains could have an additional system to the ones that are present in G4 as only *B. cenocepacia* strains that contain the *cenocepacia* island, contain the *cciIR* system (8).

This study has resulted in further characterization of the *B. vietnamiensis* quorum-sensing regulatory network yet, little is known about what is regulated by these systems. The G4 *bviI* and *bviR* mutants appeared less virulent in alfalfa. It is difficult to interpret if this decrease in virulence is biologically significant considering the parent is not as

virulent as *B. cenocepacia* K56-2. The G4 *bviI* and *bviR* mutants also appeared to produce less biomass than the parent. The mutant phenotypes could not be restored when functional copies of the genes were provided *in trans.* Complete restoration of biofilm formation was also not achieved with the *B. cenocepacia* quorum-sensing mutants (Fig. 3.13), suggesting that the quantity of AHLs present is also critical for biofilm formation in *B. vietnamiensis.*

Since the G4 *bviIR* mutants retain the ability to produce HHL and OHL, it is assumed that the *cepIR* system is still functional in these mutants and compensates for mutations in *bviIR* making it difficult to detect phenotypic differences in the mutants. It might be possible to detect quorum-sensing regulated phenotypes by constructing *cepI,bviI* double mutants, however construction of such mutants in *B. vietnamiensis* strains proves difficult as it is less amenable to genetic manipulation as other Bcc species. There is a possibility of analyzing the effect of the absence of AHLs in *B. vietnamiensis* strains by employing a quorum-quenching approach with heterologous expression of *aiiA*, encoding for lactonase (231). With this approach Wooperer et al. (231), determined that *aidA,* a *cepIR* regulated gene of unknown function that is involved in nematode virulence (82), is also regulated by quorum-sensing in *B. vietnamiensis.* The quorum-quenching approach, however, does not make it possible to distinguish which quorum-sensing system is involved in the regulation of *aidA* in *B. vietnamiensis.*

Quorum-sensing systems in *B. cepacia* and *B. cenocepacia* regulate many physiological processes (52, 216). Proteomic (166) and molecular based analysis (Subsin, Chambers, Visser, and Sokol, in press), (225) of quorum-sensing mutants in *B. cenocepacia* have been successful in identifying numerous quorum-sensing regulated

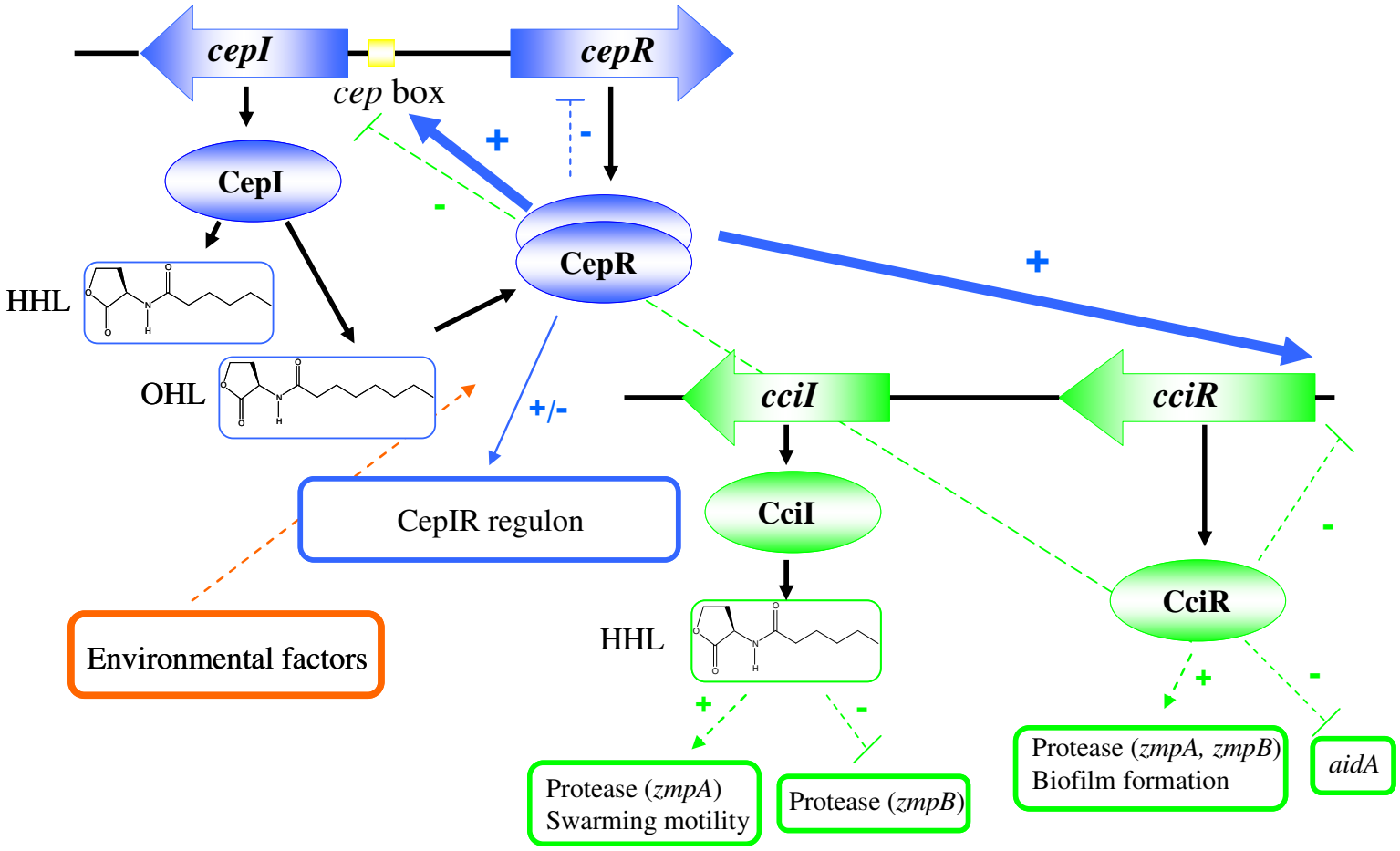
genes. With further development of molecular tools for *B. vietnamiensis* including a completed genome sequence, the role of quorum-sensing in regulating both environmentally beneficial phenotypes and pathogenic traits may be elucidated.

Chapter Six: Discussion and future directions

Bcc bacteria are a microbial challenge that connects agricultural and medical microbiologists. This fascinating organism can reduce an onion to a macerated pulp, protects other crops from disease, and devastates the health and social life of CF patients (74). Much effort has gone into understanding both the beneficial and virulence traits of the Bcc, but little is known about the regulation of the diverse and dynamic phenotypes of these organisms. It is probable that the adaptability of Bcc bacteria to infect humans is not only due to the presence of certain genes, but to gene regulation (156). Quorum-sensing is a form of density dependent gene regulation. The AHL-mediated *cepIR* system that is distributed throughout the Bcc (69, 121) has been widely characterized and reviewed (52, 130, 216). This thesis focused on two Bcc species that contain AHL-mediated quorum-sensing systems in addition to *cepIR.* These studies determined that both the *cepIR* and *cciIR* systems of *B. cenocepacia* strains that contain the *cenocepacia* island and the *cepIR* and *bviIR* systems of *B. vietnamiensis* are functional and that they are arranged in a hierarchical manner with the ancestral *cepIR* system being essential for expression of *cciIR* and *bviI.* This thesis also identified an additional LuxR homologue, BCAM0188, exclusive to *B. cenocepacia* that is potentially a part of the quorum-sensing network in this Bcc species. The current models for quorum-sensing in *B. cenocepacia* and *B. vietnamiensis,* developed during this thesis are presented in Figures 6.1 and 6.2.

The *cciIR* system is involved in the regulation of known quorum-controlled phenotypes. A *cciR* mutant strain produces less proteases and forms less biofilm and the *cciI* mutant does not swarm and hyper-produces protease compared to the parent.

Figure 6.1. Quorum-sensing in *B. cenocepacia* K56-2. The *cepIR* system is composed of the AHL synthase CepI that synthesizes OHL and HHL. The signalling molecule, OHL, binds to and activates CepR. Active CepR positively regulates the expression of *cepI* and negatively regulates the expression of *cepR*. OHL-CepR controls an extensive regulon including *cciIR*. The major signalling molecule synthesized by CciI is HHL. The *cciI* and *cciR* genes are co-transcribed. CciR negatively regulates the *cciIR* operon and *cepI*. The genes and phenotypes that are regulated by CciIR are listed in the green boxes. The orphan LuxR homologue, BCAM0188 was identified in the *B. cenocepacia* genome. A role for BCAM0188 has yet to be elucidated. Environmental factors may also integrate with the quorum-sensing systems. The weight of the blue and green arrows indicates the magnitude of gene expression induced by the quorum-sensing regulators. The heavy solid arrows indicate where a regulator is required for the expression of a particular gene and dashed arrows indicate a lesser, but significant effect on gene expression.


cepI
cepR
cep box
CepI
CepR
HHL
OHL
+
-
+/-
cciI
cciR
CepIR regulon
CciI
CciR
HHL
Environmental factors
Protease (zmpA)
Swarming motility
Protease (zmpB)
Protease (zmpA, zmpB)
Biofilm formation
aidA

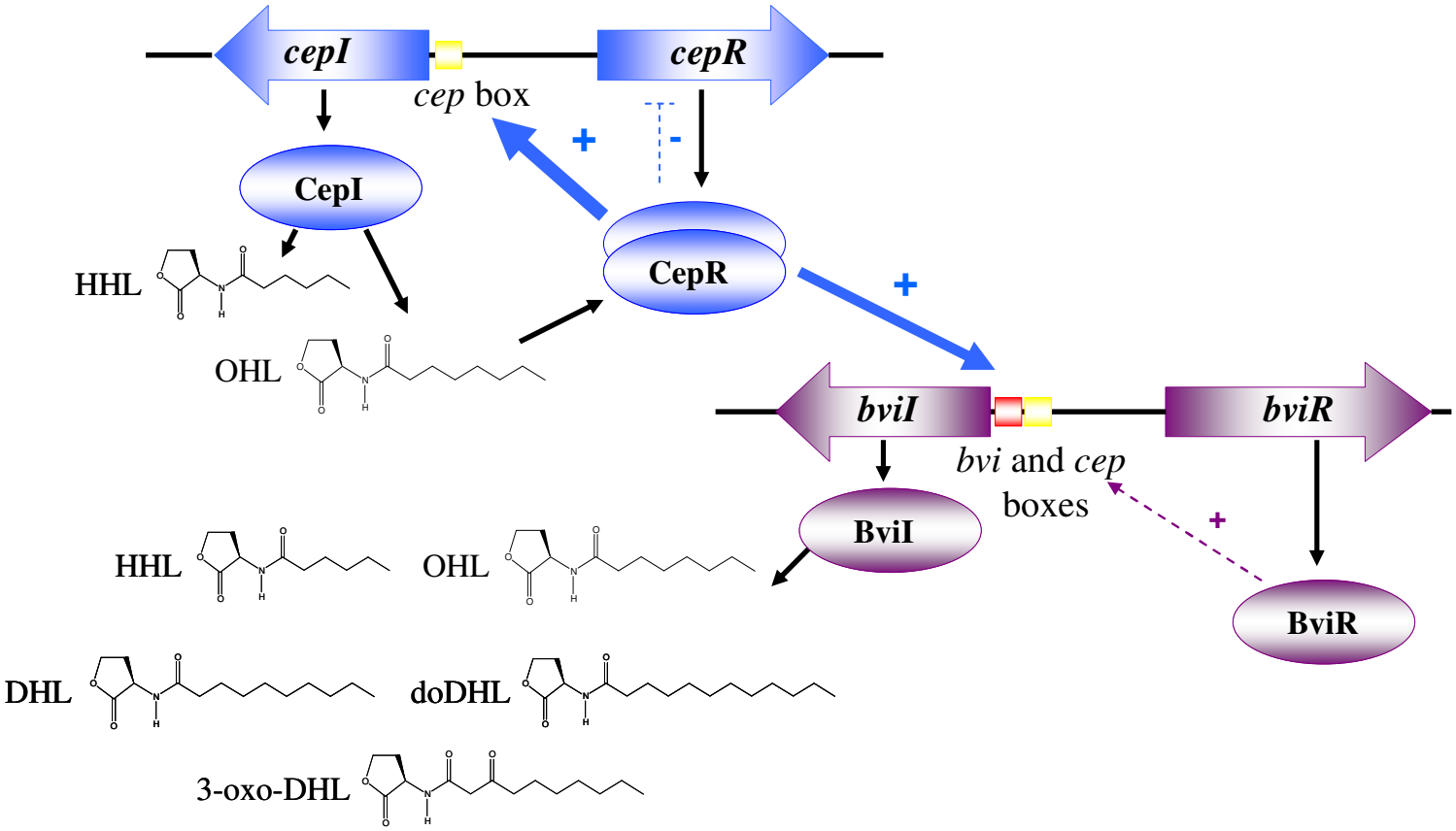


Figure 6.2. Quorum-sensing in *B. vietnamiensis*. *B. vietnamiensis* strains contain the *cepIR* and *bviIR* quorum-sensing systems. The AHL synthase CepI produces OHL and minor amounts of HHL and BviI produces HHL, OHL, DHL, doDHL and 3-oxo-DHL. The inter-regulation of the *cepIR* system has been shown to be strain dependent. CepR is required for the expression of *bviI* in both PC259 and G4. In PC259, CepR positively regulates *cepI* and exhibits negative auto-regulation. The weight of the blue and purple arrows indicates the magnitude of gene expression induced by the quorum-sensing regulators. The heavy solid arrows indicate where a regulator is required for the expression of a particular gene where as the dashed arrows indicate a lesser, but significant effect on gene expression.

Protease production contributes to *B. cenocepacia* virulence (35, 94). The regulation of protease by the *cepIR* system is thought be in part responsible for the decrease in virulence of the *cepI* and *cepR* mutants in two animal infection models (190). The *cciI* mutant has been previously shown to be involved in the pathogenesis of K56-2 using the rat agar bead model of chronic pulmonary infection (8). Since the *cciI* mutant produces greater protease activity than the parent, the role of *cciI* in regulating *B. cenocepacia* virulence must be through regulating a different virulence factor or factors.

To further understand the role of the *cciIR* quorum-sensing system in *B. cenocepacia* virulence, the *cciR, cepI,cciI* and *cepR,cciIR* mutants could be tested in a variety of infection models. During the course of this thesis, preliminary virulence studies of the K56-2 *cciR, cepI,cciI* and *cepR,cciIR* mutants in the rat agar bead model of chronic pulmonary infection were performed. Interestingly, the *cepI,cciI* mutant was cleared from the lungs fourteen days post infection of 4/5 rats in an initial study and 2/5 of the rats in a subsequent study (R. Malott, C. Kooi and J. De Buck, unpublished observations). These data suggest that together, the AHL synthases of K56-2 contribute to the ability of K56-2 to establish pulmonary infections.

B. cenocepacia has previously demonstrated splenic invasion in a mouse agar bead model of chronic pulmonary infection (26). CepI has been shown to be involved in invasion during acute intranasal infection studies performed with $Cftr^{(-/-)}$ mice (190). A modified gentamicin protection assay using A549 pulmonary epithelial cells with gentamicin and ceftazidime was shown to correlate well with spenic invasion in a mouse model (26). Therefore, the role of the *cciIR* system in invasion could be tested with the quorum-sensing mutants in cell culture or mouse model experiments.

Quorum-sensing does not appear to be involved in the regulation of plant virulence in *B. cenocepacia.* The *B. cenocepacia* quorum-sensing mutants are indistinguishable in their ability to cause infections in alfalfa and the *cepI* and *cepR* mutants do not differ from the parent in onion pathogenesis (112). However, the *cepI* and *cepR* mutants in H111 are significantly less efficient at killing *C. elegans* under fast-toxin mediated and chronic colonization conditions (95). The *C. elegans* model offers a simple and economical alternative to mammalian host models (180) to test the virulence of the K56-2 quorum-sensing mutants.

This thesis identified *B. cenocepacia* BCAM0188 as a potential orphan LuxR homologue, but, *in vitro* expression of BCAM0188 was not demonstrated. The current hypothesis is that BCAM0188 requires environmental or host factors for expression, as outlined in chapter 4. The *C. elegans* infection model could be used to determine if BCAM0188 is expressed in the presence of the nematode host. Kothe et al., (95) used a green fluorescent protein (GFP) tag to observe the ability of H111 to accumulate in the *C. elegans* intestine by fluorescence microscopy. A BCAM0188-*gfp* transcriptional fusion could be constructed with the predicted BCAM0188 promoter region. The transcriptional fusion could be transferred into K56-2 and fed to *C. elegans.* The expression of BCAM0188 may be observed by fluorescence microscopy over a time course.

Proteomic and molecular based studies have demonstrated that the *B. cenocepacia cepIR* regulon includes numerous genes, not just those characterized as virulence factors (166, 225), Subsin, Chambers, Visser, and Sokol, in press). In *P. aeruginosa,* micro-arrays have been used to further define the *lasIR* and *rhlIR* quorum-sensing network (79, 174, 220). A *B. cenocepacia* J2315 whole genome micro-array has recently been

developed in a project supported by the Cystic Fibrosis Foundation and will soon be available for the CF research community. The array contains 11000 features including probes for all of the genes annotated within the J2315 genome (http://www.sanger.ac.uk/Projects/B_cenocepacia/private/) as well as probes against unique genes annotated within the *B. cenocepacia* HI2424 (http://genome.jgi-psf.org/draft_microbes/burce/burce.home.html) or AU1054 (http://genome.jgi-psf.org/draft_microbes/burca/burca.home.html) genomes (104). This thesis has contributed to the progress of the micro-array project in that the *B. cenocepacia* homologue of *sigA,* the gene identified for use as a control gene during RT-PCR analysis, has been selected as a candidate control gene for the micro-array. Preliminary micro-array experiments indicate that *B. cenocepacia sigA* (BCAM0918) steadily expresses regardless of the experiment conditions (P. Drevinek, E. Mahenthiralingam, personal communication).

The *B. cenocepacia* micro-array could be used to determine distinct *cepIR* and *cciIR* regulons by comparing the expression profiles of the *cepR, cciR* and *cepR,cciR* mutants with the parent. If conditions for BCAM0188 expression are elucidated, trancriptome analysis of the BCAM0188 mutant compared to the parent could determine the role for this orphan LuxR homologue in the *B. cenocepacia* quorum-sensing network. Further understanding of the AHL responsive regulon could be determined by transcriptome analysis of the *cepI,cciI* mutant in response to exogenous AHLs. This approach was used by three independent research groups to define the *P. aeruginosa* quorum-sensing regulon (79, 174, 220). Schuster et al. (174), compared the information obtained from the *lasI,rhlI* mutant with expression profiling data from a *lasR,rhlR* mutant

to define the quorum-controlled regulon in *P. aeruginosa* (174). The information obtained from the *B. cenocepacia* regulator mutants and the AHL responsive studies could be compared to define the role for quorum-sensing in gene regulation in *B. cenocepacia.*

From studies with *P. aeruginosa* it is becoming apparent that AHL-mediated quorum-sensing systems are a part of a larger picture of genome regulation (173). In *P. aeruginosa,* the *lasIR* and *rhlIR* systems are imbedded in a global regulatory network (173). This could also be the case for *cepIR* and *cciIR* in *B. cenocepacia.* The *B. cenocepacia* CepR regulon contains many down stream regulators and regulatory systems, including *cciIR* (225), (Subsin, Chambers, Visser, and Sokol, in press). The following is a brief description of two example down-stream regulators. A LuxR homologue with domains associated with the FixJ-NarL family, but no AHL binding domain was identified during two independent screens for OHL-responsive genes (225), (Subsin, Chambers, Visser, and Sokol, in press). This homologue was not pursued further during the course of this thesis because of it did not have an AHL binding domain. However, in *P. aeruginosa* VsqR is a transcriptional regulator belonging to the FixJ-NarL family that does not contain an AHL binding domain but is essential for AHL production (173). Immediately downstream of the potential LuxR homologue, Weingart et al., (225) identified an OHL responsive gene with a protein product containing GGDEF/EAL motifs. These motifs are usually found in multi-domain proteins linked to signal sensing and signal transduction domains (100). The motifs represent a signal-transduction network that is able to convert sensory information into the production of a secondary messenger cyclic di-GMP. Most GGDEF/EAL proteins are associated with

exopolysaccharide production in biofilms (100). It has recently been hypothesized that GGDEF/EAL domains play a role in the shiny colony morphology of *B. cenocepacia* (10). Shiny *B. cenocepacia* are deficient in biofilm formation and are less virulent than their rough counterparts (10). Studies are currently underway in the Sokol laboratory to determine the potential role of this quorum-controlled GGDEF/EAL regulator in the shiny phenotype.

Higher-level regulators could also influence the expression of *cepIR*. Studies with *P. aeruginosa* quorum-sensing responsive gene fusions showed a delayed response in the presence of exogenous AHLs until the stationary phase of growth, suggesting that the activation of quorum-controlled genes requires factors additional to accumulation of AHLs (173). In *B. cenocepacia,* the induction of the CepR-regulated gene *zmpA* is not temporally different in the presence or absence of exogenous OHL (190), supporting the view that additional factors are involved in regulating CepR. Three putative higher-level of CepR have been identified in *B. cenocepacia* H111; *yciR, suhB* and *yciL* (84). The distribution of these genes in the Bcc and their specific roles in regulation has not been defined. This thesis identified the orphan LuxR homologue, BCAM0188 that could be involved in the quorum-sensing regulatory network of *B. cenocepacia.* It is unlikely that BCAM0188 regulates the CepIR system since AHL production is unaffected in the BCAM0188 mutant. Environmental factors could also be integrated into the quorum-sensing regulatory network. The effect of various growth conditions on the expression of a *cepI-lacZ* transcriptional fusion in *B. cenocepacia* K56-2 has indicated that *cepI* expression varies depending on the media conditions used for growth and that *cepI* induction is effected by factors other than cell density (131).

In contrast to the *B. cenocepacia* quorum-sensing network, there is little known about the *B. vietnamiensis cepIR* and *bviIR* quorum-sensing regulons. This thesis determined that the regulation of these systems is variable between strains. In G4 there is a potential role for quorum-sensing in the regulation of bioflim formation and alfalfa virulence since G4 *bviI* and *bviR* mutants produced less biofilms and were less virulent than the parent. The virulence study results were difficult to interpret considering the parent is not significantly virulent in alfalfa. *B. vietnamiensis* G4 is considered to be virulent in *C. elegans* (231). When the AHLs produced by G4 were quenched by the presence of the lactonase AiiA, the strain was not pathogenic. Therefore, it may be worthwhile investigating the virulence of the G4 quorum-sensing mutants in *C. elegans* to determine a clearer role for quorum-sensing in regulating virulence in *B. vietnamiensis.*

A two-dimensional (2-D) gel electrophoresis approach could be used to begin identifying *B. vietnamiensis* quorum-controlled proteins. This approach was used to identify 55 quorum-controlled proteins, making up 5.6 percent of detected proteins from *B. cenocepacia* H111 (166). The proteomic profiles of the G4 quorum-sensing mutants as well as the PC259 *cepR* mutant could be compared to the parent and each other. Comparing mutants in both strains may reveal further differences in quorum-sensing between *B. vietnamiensis* strains.

This thesis has demonstrated that there is overlap in the AHLs produced by the *B. cenocepacia* AHL synthases CepI and CciI and the *B. vietnamiensis* AHL syntheses CepI and BviI. This is in contrast with the *P. aeruginosa* AHL synthases, LasI and RhlI that produce distinct AHLs (159, 160). Other *Burkholderia* species with multiple AHL synthases produce common AHLs. *B. thailandensis*, *B. mallei* and *B. pseudomallei* also

contain multiple quorum-sensing systems (121, 206-208). The regulatory relationships, if any, between these systems has yet to be described. *B. pseudomallei* PmlI, BpmI2 and BpmI3 have been shown to direct the synthesis of OHL, DHL, *N*-(3-hydroxyoctanoyl)-L-homoserine lactone (3-hydroxy-OHL) and *N*-(3-hydroxydecanoyl)-L-homoserine lactone (3-hydroxy-DHL) (206, 209). *B. pseudomallei* BpmI2 and BpmI3 also direct the synthesis of *N*-(3-oxotetradecanoyl)-L-homoserine lactone (3-oxo-deDHL) (206). *B. thailandensis* BtaI1, BtaI2 and BtaI3 are responsible for the production of OHL, DHL and HHL respectively (208). *B. mallei* BmaI1 and BmaI3 produce OHL, DHL and 3-hydroxy-OHL and OHL, DHL and 3-hydroxy-DHL, respectively (207). The specific role of these AHL signalling molecules in *Burkholderia* quorum-sensing has yet to be determined.

The benefit to *B. cenocepacia* of containing two AHL synthases that produce HHL was initially unclear. This thesis demonstrated that CepI and CciI are not redundant and that the ratios of AHLs play a role in gene regulation. Recently, CepR has been shown to be inhibited by high quantities of HHL (225). It is believed that HHL is able to bind to CepR without activating the regulator and that the inhibition occurs because of HHL out-competing OHL for the AHL binding site (225). These data suggest that the addition of *cciI* on the *cenocepacia* island has allowed for negative control of the *B. cenocepacia* quorum-sensing regulon. This hypothesis is further supported by the data from this thesis demonstrating an increase in *cepI* expression in the *cciR* mutant. The negative effect of CciIR on the *cepIR* system could also explain the increase in *aidA* expression in the *cciR* mutant since *aidA* is positively regulated by CepR (225).

In *B. cepacia* and *B. cenocepacia*, CepR is known to be most induced by OHL (2, 225). CepR is optimized to detect and respond to AHLs with acyl chains that are unsubstituted, fully reduced and are 8 to 12 carbons in length (225). CepR is activated by DHL and doDHL (225), suggesting that the products of BviI could be inducing the CepIR system, increasing the complexity of the *cepIR/bviIR* quorum-sensing hierarchy. The AHLs that best induce BviR have yet to be determined. This thesis showed that the expression of *bviI* requires functional BviR. The BviR-AHL response could be investigated by heterologous expression of a *bviI* transcriptional fusion in *E. coli* in the presence of BviR as outlined by Aguilar et al. (2) for the investigation of the induction of CepR by various AHLs in *B. cepacia*.

Evidence from this thesis suggests that there is less *bviI* expression and thus less DHL production in clinical *B. vietnamiensis* strains. From an evolutionary standpoint, there would be a selective advantage for strains to exhibit decreased expression of *bviI* since there is a large metabolic cost involved in AHL production (91). Expressing only the *cepI* AHL synthase may be less costly to the organism. The lack of *bviI* expression could also be explained as a pathoadaptive advantage. *B. mallei* is lacking a set of *luxIR* homologues (*bpmIR2*) that are present in both *B. pseudomallei* and *B. thailandensis* (207). *B. mallei* is a pathoadaptive obligate animal pathogen that is proposed to be a clone of *B. pseudomallei* (66, 150). It has been suggested that *B. mallei* has undergone genomic modifications that have resulted in the loss of *bpmIR2* and that these alleles are required for the regulation of traits not specific to the *in vivo* pathogenesis of the organism (207). Although quorum-sensing is involved in Bcc virulence (2, 8, 95, 190), there has yet to be a correlation between the quantity and type of AHL produced by a Bcc

strain and its virulence. Lack of DHL production by a particular strain is not linked to biofilm formation (34) or nematode virulence (231).

The initial efforts towards an antipathogenic therapeutic strategy for Bcc opportunistic infections are promising (168, 231). A complete quorum-sensing regulon for Bcc bacteria needs to be defined before the impact of these strategies on the bacteria will be fully understood. Both positive and negative regulation of Bcc virulence factors by quorum-sensing systems has been demonstrated (110). Interfering with the operation of its quorum-sensing system could potentially lead to an increase in the virulence of an organism. Genomic and proteomic technology will aid in further characterization of the quorum-sensing regulons in the Bcc. Investigation into the response of the quorum-controlled genes to the host environment must also be pursued to obtain a complete picture of the effect of disabling the regulatory network.

There must be knowledge of all quorum sensing systems present in particular bacteria before perusing antipathogenic strategies directed to these systems. *P. aeruginosa lasIR* mutants gave rise to spontaneous mutations under selective conditions that restored production of certain *lasIR* regulated virulence factors (211). However, these mutations did not occur when both the *lasIR* and *rhlIR* systems were inactivated. These data stress the importance of targeting all quorum sensing systems of the bacteria to prevent the possibility of the untargeted regulatory system evolving to compensate for the system disabled by the therapeutic agent (210).

The identification and further characterization of additional components of the *B. cenocepacia* and *B. vietnamiensis* quorum-sensing, could possibly lead to additional antipathogenic therapeutic targets. As observed with *P. aeruginosa,* global regulator

factors, including GacA, Vfr and RelA (40) are involved in the positive regulation of the *lasIR* quorum-sensing system. It has been proposed that these regulatory factors would be ideal targets for potential disruption of the quorum-sensing system of *P. aeruginosa* (185).

In closing, *B. cenocepacia* strains that harbour the *cenocepacia* island and *B. vietnamiensis* strains contain multiple quorum-sensing systems. These systems are arranged in a hierarchical manner with the ancestral *cepIR* system being required for the expression of either *cciIR* in *B. cenocepacia* or *bviI* in *B. vietnamiensis.* The *cciIR* system is involved in the regulation of known quorum-controlled phenotypes. The orphan LuxR regulator, BCAM0188 has been identified and determined to be specific to *B. cenocepacia.* Quorum-sensing appears to be variable in the Bcc with regards to the genes that are present, their expression, the quantity, and type of AHL signalling molecules produced. It is presumed that the variability in quorum-sensing contributes to the complexity and variability of this group of bacteria. This thesis has determined how distinct AHL-mediated quorum-sensing systems operate in the Bcc and how these systems interact with each other to regulate gene expression. These findings may eventually lead to strategies to manipulate these multifarious organisms so they can be used for their benefit with minimal human risk.

References

1. **Agodi, A., E. Mahenthiralingam, M. Barchitta, V. Giannino, A. Sciacca, and S. Stefani.** 2001. *Burkholderia cepacia* complex infection in Italian patients with cystic fibrosis: prevalence, epidemiology, and genomovar status. J Clin Microbiol **39:**2891-2896.

2. **Aguilar, C., I. Bertani, and V. Venturi.** 2003. Quorum-sensing system and stationary-phase sigma factor (*rpoS*) of the onion pathogen *Burkholderia cepacia* genomovar I type strain, ATCC 25416. Appl. Environ. Microbiol. **69:**1739-1747.

3. **Aguilar, C., A. Friscina, G. Devescovi, M. Kojic, and V. Venturi.** 2003. Identification of quorum-sensing-regulated genes of *Burkholderia cepacia*. J. Bacteriol. **185:**6456-6462.

4. **Ahmer, B. M.** 2004. Cell-to-cell signalling in *Escherichia coli* and *Salmonella enterica*. Mol Microbiol **52:**933-945.

5. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J Mol Biol **215:**403-410.

6. **Atkinson, S., J. P. Throup, G. S. Stewart, and P. Williams.** 1999. A hierarchical quorum-sensing system in *Yersinia pseudotuberculosis* is involved in the regulation of motility and clumping. Mol. Microbiol. **33:**1267-1277.

7. **Ausubel, F., R. Brent, R. Kingston, D. Moore, J. Seidman, J. Smith, and K. Struhl.** 1989. Current protocols in molecular biology, vol. 1. John Wiley & Sons, Inc., New York.

8. **Baldwin, A., P. A. Sokol, J. Parkhill, and E. Mahenthiralingam.** 2004. The *Burkholderia cepacia* epidemic strain marker is part of a novel genomic island encoding both virulence and metabolism-associated genes in *Burkholderia cenocepacia*. Infect. Immun. **72:**1537-1547.

9. **Bauer, W. D., and J. B. Robinson.** 2002. Disruption of bacterial quorum sensing by other organisms. Curr Opin Biotechnol **13:**234-237.

10. **Bernier, S. P.** 2006. Virulence studies in the *Burkholderia cepacia* complex. University of Calgary, Calgary.

11. **Bernier, S. P., L. Silo-Suh, D. E. Woods, D. E. Ohman, and P. A. Sokol.** 2003. Comparative analysis of plant and animal models for characterization of *Burkholderia cepacia* virulence. Infect Immun **71:**5306-5313.

12. **Bernier, S. P., and P. A. Sokol.** 2005. Use of suppression-subtractive hybridization to identify genes in the *Burkholderia cepacia* complex that are unique to *Burkholderia cenocepacia*. J Bacteriol **187:**5278-5291.

13. **Brazas, M. D., and R. E. Hancock.** 2005. Ciprofloxacin induction of a susceptibility determinant in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother **49:**3222-3227.

14. **Bruckner, J. V., B. D. Davis, and J. N. Blancato.** 1989. Metabolism, toxicity, and carcinogenicity of trichloroethylene. Crit Rev Toxicol **20:**31-50.

15. **Burkholder, W.** 1950. Sour skin, a bacterial rot of onion bulbs. Phytopathology. **40:**115-118.

16. **Burns, J. L., M. Jonas, E. Y. Chi, D. K. Clark, A. Berger, and A. Griffith.** 1996. Invasion of respiratory epithelial cells by *Burkholderia* (*Pseudomonas*) *cepacia*. Infect Immun **64:**4054-4059.

17. **Bye, M. R., J. M. Ewig, and L. M. Quittell.** 1994. Cystic fibrosis. Lung. **172:**251-270.

18. **Chambers, C. E., M. B. Visser, U. Schwab, and P. A. Sokol.** 2005. Identification of N-acylhomoserine lactones in mucopurulent respiratory secretions from cystic fibrosis patients. FEMS Microbiol Lett **244:**297-304.

19. **Chang, H. K., and G. J. Zylstra.** 1998. Novel organization of the genes for phthalate degradation from *Burkholderia cepacia* DBO1. J Bacteriol **180:**6529-6537.

20. **Chen, J. S., K. A. Witzmann, T. Spilker, R. J. Fink, and J. J. LiPuma.** 2001. Endemicity and inter-city spread of *Burkholderia cepacia* genomovar III in cystic fibrosis. J Pediatr **139:**643-649.

21. **Chen, X., S. Schauder, N. Potier, A. Van Dorsselaer, I. Pelczer, B. L. Bassler, and F. M. Hughson.** 2002. Structural identification of a bacterial quorum-sensing signal containing boron. Nature **415:**545-549.

22. **Chiarini, L., A. Bevivino, C. Dalmastri, S. Tabacchioni, and P. Visca.** 2006. *Burkholderia cepacia* complex species: health hazards and biotechnological potential. Trends Microbiol **14:**277-286.

23. **Christensen, G. D., W. A. Simpson, A. L. Bisno, and E. H. Beachey.** 1982. Adherence of slime-producing strains of *Staphylococcus epidermidis* to smooth surfaces. Infect. Immun. **37:**318-326.

24. **Christensen, G. D., W. A. Simpson, J. J. Younger, L. M. Baddour, F. F. Barrett, D. M. Melton, and E. H. Beachey.** 1985. Adherence of coagulase-

negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. J. Clin. Microbiol. **22:**996-1006.

25. **Chugani, S. A., M. Whiteley, K. M. Lee, D. D'Argenio, C. Manoil, and E. P. Greenberg.** 2001. QscR, a modulator of quorum-sensing signal synthesis and virulence in *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. U S A **98:**2752-2757.

26. **Cieri, M. V., N. Mayer-Hamblett, A. Griffith, and J. L. Burns.** 2002. Correlation between an in vitro invasion assay and a murine model of *Burkholderia cepacia* lung infection. Infect. Immun. **70:**1081-1086.

27. **Coenye, T., and J. J. LiPuma.** 2002. Multilocus restriction typing: a novel tool for studying global epidemiology of *Burkholderia cepacia* complex infection in cystic fibrosis. J. Infect. Dis. **185:**1454-1462.

28. **Coenye, T., J. J. LiPuma, D. Henry, B. Hoste, K. Vandemeulebroecke, M. Gillis, D. P. Speert, and P. Vandamme.** 2001. *Burkholderia cepacia* genomovar VI, a new member of the *Burkholderia cepacia* complex isolated from cystic fibrosis patients. Int. J. Syst. Evol. Microbiol. **51:**271-279.

29. **Coenye, T., E. Mahenthiralingam, D. Henry, J. J. LiPuma, S. Laevens, M. Gillis, D. P. Speert, and P. Vandamme.** 2001. *Burkholderia ambifaria* sp. nov., a novel member of the *Burkholderia cepacia* complex including biocontrol and cystic fibrosis-related isolates. Int. J. Syst. Evol. Microbiol. **51:**1481-1490.

30. **Coenye, T., and P. Vandamme.** 2003. Diversity and significance of *Burkholderia* species occupying diverse ecological niches. Environ Microbiol **5:**719-729.

31. **Coenye, T., P. Vandamme, J. R. Govan, and J. J. LiPuma.** 2001. Taxonomy and identification of the *Burkholderia cepacia* complex. J. Clin. Microbiol. **39:**3427-3436.

32. **Coenye, T., P. Vandamme, J. J. LiPuma, J. R. Govan, and E. Mahenthiralingam.** 2003. Updated version of the *Burkholderia cepacia* complex experimental strain panel. J. Clin. Microbiol. **41:**2797-2798.

33. **Conway, B. A., and E. P. Greenberg.** 2002. Quorum-sensing signals and quorum-sensing genes in *Burkholderia vietnamiensis*. J. Bacteriol. **184:**1187-1191.

34. **Conway, B. A., V. Venu, and D. P. Speert.** 2002. Biofilm Formation and Acyl Homoserine Lactone Production in the *Burkholderia cepacia* Complex. J. Bacteriol. **184:**5678-5685.

35. **Corbett, C. R., M. N. Burtnick, C. Kooi, D. E. Woods, and P. A. Sokol.** 2003. An extracellular zinc metalloprotease gene of *Burkholderia cepacia*. Microbiology **149:**2263-2271.

36. **Corey, M., and V. Farewell.** 1996. Determinants of mortality from cystic fibrosis in Canada, 1970-1989. Am J Epidemiol **143:**1007-1017.

37. **Cystic Fibrosis Foundation.** 2005. Patient Registry 2004, Annual Report. Cystic Fibrosis Foundation.

38. **Daniels, R., D. E. De Vos, J. Desair, G. Raedschelders, E. Luyten, V. Rosemeyer, C. Verreth, E. Schoeters, J. Vanderleyden, and J. Michiels.** 2002. The *cin* quorum sensing locus of *Rhizobium etli* CNPAF512 affects growth and symbiotic nitrogen fixation. J Biol Chem **277:**462-468.

39. **Darling, P., M. Chan, A. D. Cox, and P. A. Sokol.** 1998. Siderophore production by cystic fibrosis isolates of *Burkholderia cepacia*. Infect. Immun. **66:**874-877.

40. **de Kievit, T. R., and B. H. Iglewski.** 2000. Bacterial quorum sensing in pathogenic relationships. Infect. Immun. **68:**4839-4849.

41. **de Kievit, T. R., Y. Kakai, J. K. Register, E. C. Pesci, and B. H. Iglewski.** 2002. Role of the *Pseudomonas aeruginosa las* and *rhl* quorum-sensing systems in *rhlI* regulation. FEMS Microbiol. Lett. **212:**101-106.

42. **Dennis, J. J., and P. A. Sokol.** 1995. Electrotransformation of *Pseudomonas*. Methods Mol. Biol. **47:**125-133.

43. **Dennis, J. J., and G. J. Zylstra.** 1998. Plasposons: modular self-cloning minitransposon derivatives for rapid genetic analysis of gram-negative bacterial genomes. Appl. Environ. Microbiol. **64:**2710-2715.

44. **DeShazer, D., D. M. Waag, D. L. Fritz, and D. E. Woods.** 2001. Identification of a *Burkholderia mallei* polysaccharide gene cluster by subtractive hybridization and demonstration that the encoded capsule is an essential virulence determinant. Microb Pathog **30:**253-269.

45. **DeShazer, D., and D. E. Woods.** 1996. Broad-host-range cloning and cassette vectors based on the R388 trimethoprim resistance gene. Biotechniques. **20:**762-764.

46. **Detsika, M. G., J. E. Corkill, M. Magalhaes, K. J. Glendinning, C. A. Hart, and C. Winstanley.** 2003. Molecular typing of, and distribution of genetic markers among, *Burkholderia cepacia* complex isolates from Brazil. J. Clin. Microbiol. **41:**4148-4153.

47. **Devine, J. H., C. Countryman, and T. Baldwin.** 1988. Nucleotide sequence of the *luxI* and *luxR* genes and structure of the primary regulatory region of the *lux* regulon of *Vibrio fischeri* ATCC 7744. Biochemistry **27:**837-842.

48. **Dong, Y. H., L. H. Wang, J. L. Xu, H. B. Zhang, X. F. Zhang, and L. H. Zhang.** 2001. Quenching quorum-sensing-dependent bacterial infection by an N-acyl homoserine lactonase. Nature **411:**813-817.

49. **Dong, Y. H., J. L. Xu, X. Z. Li, and L. H. Zhang.** 2000. AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of *Erwinia carotovora*. Proc Natl Acad Sci U S A **97:**3526-3531.

50. **Duan, K., C. Dammel, J. Stein, H. Rabin, and M. G. Surette.** 2003. Modulation of *Pseudomonas aeruginosa* gene expression by host microflora through interspecies communication. Mol. Microbiol. **50:**1477-1491.

51. **Eberl, L.** 1999. N-acyl homoserinelactone-mediated gene regulation in gram-negative bacteria. Syst Appl Microbiol **22:**493-506.

52. **Eberl, L.** 2006. Quorum sensing in the genus *Burkholderia*. Int J Med Microbiol **296:**103-110.

53. **Engledow, A. S., E. G. Medrano, E. Mahenthiralingam, J. J. LiPuma, and C. F. Gonzalez.** 2004. Involvement of a plasmid-encoded type IV secretion system in the plant tissue watersoaking phenotype of *Burkholderia cenocepacia*. J Bacteriol **186:**6015-6024.

54. **Estrada-De Los Santos, P., R. Bustillos-Cristales, and J. Caballero-Mellado.** 2001. *Burkholderia*, a genus rich in plant-associated nitrogen fixers with wide environmental and geographic distribution. Appl Environ Microbiol **67:**2790-2798.

55. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. Proc. Natl. Acad. Sci. U S A **76:**1648-1652.

56. **Flavier, A. B., L. M. Ganova-Raeva, M. A. Schell, and T. P. Denny.** 1997. Hierarchical autoinduction in *Ralstonia solanacearum*: control of acyl-homoserine lactone production by a novel autoregulatory system responsive to 3-hydroxypalmitic acid methyl ester. J. Bacteriol. **179:**7089-7097.

57. **Fuqua, C.** 2006. The QscR quorum-sensing regulon of *Pseudomonas aeruginosa*: an orphan claims its identity. J Bacteriol **188:**3169-3171.

58. **Fuqua, C., and E. P. Greenberg.** 2002. Listening in on bacteria: acyl-homoserine lactone signalling. Nat. Rev. Mol. Cell Biol. **3:**685-695.

59. **Fuqua, C., M. R. Parsek, and E. P. Greenberg.** 2001. Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. Annu. Rev. Genet. **35:**439-468.

60. **Fuqua, C., and S. C. Winans.** 1996. Conserved cis-acting promoter elements are required for density-dependent transcription of *Agrobacterium tumefaciens* conjugal transfer genes. J. Bacteriol. **178:**435-440.

61. **Fuqua, C., S. C. Winans, and E. P. Greenberg.** 1996. Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcriptional regulators. Annu. Rev. Microbiol. **50:**727-751.

62. **Fuqua, W. C., and S. C. Winans.** 1994. A LuxR-LuxI type regulatory system activates *Agrobacterium* Ti plasmid conjugal transfer in the presence of a plant tumor metabolite. J Bacteriol **176:**2796-2806.

63. **Gillis, M., T. Van, R. Bardin, M. Goor, P. Hebbar, A. Willems, P. Segers, K. Kersters, T. Heulin, and M. Fernandez.** 1995. Polyphasic taxonomy in the genus *Burkholderia* leading to an emended description of the genus and proposition of *Burkholderia vietnamiensis* sp. nov. for N_2-fixing isolates from rice in Vietnam. Int J Syst Bacteriol **45:**274-289.

64. **Gingues, S.** 2004. Proteases of the *Burkholderia cepacia* complex. University of Calgary, Calgary.

65. **Gingues, S., C. Kooi, M. B. Visser, B. Subsin, and P. A. Sokol.** 2005. Distribution and expression of the ZmpA metalloprotease in the *Burkholderia cepacia* complex. J Bacteriol **187:**8247-8255.

66. **Godoy, D., G. Randle, A. J. Simpson, D. M. Aanensen, T. L. Pitt, R. Kinoshita, and B. G. Spratt.** 2003. Multilocus sequence typing and evolutionary relationships among the causative agents of melioidosis and glanders, *Burkholderia pseudomallei* and *Burkholderia mallei*. J Clin Microbiol **41:**2068-2079.

67. **Goldberg, J. B., and D. E. Ohman.** 1984. Cloning and expression in *Pseudomonas aeruginosa* of a gene involved in the production of alginate. J Bacteriol **158:**1115-1121.

68. **Gonzalez, C. F., E. A. Pettit, V. A. Valadez, and E. M. Provin.** 1997. Mobilization, cloning, and sequence determination of a plasmid-encoded polygalacturonase from a phytopathogenic *Burkholderia* (*Pseudomoas*) *cepacia*. Mol Plant Microbe Interact **10:**810-851.

69. **Gotschlich, A., B. Huber, O. Geisenberger, A. Togl, A. Steidle, K. Riedel, P. Hill, B. Tummler, P. Vandamme, B. Middleton, M. Camara, P. Williams, A.**

Hardman, and L. Eberl. 2001. Synthesis of multiple *N*-acylhomoserine lactones is wide-spread among the members of the *Burkholderia cepacia* complex. Syst. Appl. Microbiol. **24:**1-14.

70. **Gould, T. A., J. Herman, J. Krank, R. C. Murphy, and M. E. Churchill.** 2006. Specificity of acyl-homoserine lactone synthases examined by mass spectrometry. J Bacteriol **188:**773-783.

71. **Gould, T. A., H. P. Schweizer, and M. E. Churchill.** 2004. Structure of the *Pseudomonas aeruginosa* acyl-homoserinelactone synthase LasI. Mol Microbiol **53:**1135-1146.

72. **Govan, J. R., and V. Deretic.** 1996. Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. Microbiol. Rev. **60:**539-574.

73. **Govan, J. R., J. E. Hughes, and P. Vandamme.** 1996. *Burkholderia cepacia*: medical, taxonomic and ecological issues. J. Med. Microbiol. **45:**395-407.

74. **Govan, J. R., and P. Vandamme.** 1998. Agricultural and medical microbiology: a time for bridging gaps. Microbiology **144 (Pt 9):**2373-2375.

75. **Gray, K. M.** 1997. Intercellular communication and group behavior in bacteria. Trends Microbiol. **5:**184-188.

76. **Hacker, J., and J. B. Kaper.** 2000. Pathogenicity islands and the evolution of microbes. Annu. Rev. Microbiol. **54:**641-679.

77. **Harrison, J. J., R. J. Turner, L. R. Marques, and H. Ceri.** 2005. Biofilms: A new understanding of these microbial communities is driving a revolution that may transform the science of microbiology. American Scientist **93:**508-515.

78. **Hentzer, M., and M. Givskov.** 2003. Pharmacological inhibition of quorum sensing for the treatment of chronic bacterial infections. J Clin Invest **112:**1300-1307.

79. **Hentzer, M., H. Wu, J. B. Andersen, K. Riedel, T. B. Rasmussen, N. Bagge, N. Kumar, M. A. Schembri, Z. Song, P. Kristoffersen, M. Manefield, J. W. Costerton, S. Molin, L. Eberl, P. Steinberg, S. Kjelleberg, N. Hoiby, and M. Givskov.** 2003. Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. Embo J **22:**3803-3815.

80. **Hoang, T. T., R. R. Karkhoff-Schweizer, A. J. Kutchma, and H. P. Schweizer.** 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene **212:**77-86.

81. **Holloway, B. W., U. Romling, and B. Tummler.** 1994. Genomic mapping of *Pseudomonas aeruginosa* PAO. Microbiology **140 (Pt 11):**2907-2929.

82. **Huber, B., F. Feldmann, M. Kothe, P. Vandamme, J. Wopperer, K. Riedel, and L. Eberl.** 2004. Identification of a novel virulence factor in *Burkholderia cenocepacia* H111 required for efficient slow killing of *Caenorhabditis elegans*. Infect Immun **72:**7220-7230.

83. **Huber, B., K. Riedel, M. Hentzer, A. Heydorn, A. Gotschlich, M. Givskov, S. Molin, and L. Eberl.** 2001. The *cep* quorum-sensing system of *Burkholderia cepacia* H111 controls biofilm formation and swarming motility. Microbiology **147:**2517-2528.

84. **Huber, B., K. Riedel, M. Kothe, M. Givskov, S. Molin, and L. Eberl.** 2002. Genetic analysis of functions involved in the late stages of biofilm development in *Burkholderia cepacia* H111. Mol. Microbiol. **46:**411-426.

85. **Hunt, T. A., C. Kooi, P. A. Sokol, and M. A. Valvano.** 2004. Identification of *Burkholderia cenocepacia* genes required for bacterial survival in vivo. Infect Immun **72:**4010-4022.

86. **Hutchinson, J., W. Runge, M. Mulvey, G. Norris, M. Yetman, N. Valkova, R. Villemur, and F. Lepine.** 2004. *Burkholderia cepacia* infections associated with intrinsically contaminated ultrasound gel: the role of microbial degradation of parabens. Infect Control Hosp Epidemiol **25:**291-296.

87. **Ishizuka, T., T. Oyama, M. Sato, T. Hisada, H. Takagi, T. Hamada, T. Kimura, K. Kashiwabara, and M. Mori.** 2003. Fatal pneumonia caused by *Burkholderia cepacia* 9 months after resection of aspergilloma. Respirology. **8:**401-403.

88. **Isles, A., I. Maclusky, M. Corey, R. Gold, C. Prober, P. Fleming, and H. Levison.** 1984. *Pseudomonas cepacia* infection in cystic fibrosis: an emerging problem. J. Pediatr. **104:**206-210.

89. **Jackson, K., R. Keyser, and D. J. Wozniak.** 2003. The role of biofilms in airway disease. Semin Respir Crit Care Med **24:**663-670.

90. **Keen, N. T., S. Tamaki, D. Kobayashi, and D. Trollinger.** 1988. Improved broad-host-range plasmids for DNA cloning in gram-negative bacteria. Gene **70:**191-197.

91. **Keller, L., and M. G. Surette.** 2006. Communication in bacteria: an ecological and evolutionary perspective. Nat Rev Microbiol **4:**249-258.

92. **Koch, B., T. Liljefors, T. Persson, J. Nielsen, S. Kjelleberg, and M. Givskov.** 2005. The LuxR receptor: the sites of interaction with quorum-sensing signals and inhibitors. Microbiology **151:**3589-3602.

93. **Kooi, C., C. R. Corbett, and P. A. Sokol.** 2005. Functional analysis of the *Burkholderia cenocepacia* ZmpA metalloprotease. J Bacteriol **187:**4421-4429.

94. **Kooi, C., B. Subsin, R. Chen, B. Pohorelic, and P. A. Sokol.** 2006. *Burkholderia cenocepacia* ZmpB is a broad-specificity zinc metalloprotease involved in virulence. Infect Immun **74:**4083-4093.

95. **Kothe, M., M. Antl, B. Huber, K. Stoecker, D. Ebrecht, I. Steinmetz, and L. Eberl.** 2003. Killing of *Caenorhabditis elegans* by *Burkholderia cepacia* is controlled by the *cep* quorum-sensing system. Cell Microbiol. **5:**343-351.

96. **Krumme, M. L., K. N. Timmis, and D. F. Dwyer.** 1993. Degradation of trichloroethylene by *Pseudomonas cepacia* G4 and the constitutive mutant strain G4 5223 PR1 in aquifer microcosms. Appl Environ Microbiol **59:**2746-2749.

97. **Lacy, D. E., D. A. Spencer, A. Goldstein, P. H. Weller, and P. Darbyshire.** 1993. Chronic granulomatous disease presenting in childhood with *Pseudomonas cepacia* septicaemia. J. Infect. **27:**301-304.

98. **Lamothe, J., S. Thyssen, and M. A. Valvano.** 2004. *Burkholderia cepacia* complex isolates survive intracellularly without replication within acidic vacuoles of *Acanthamoeba polyphaga*. Cell Microbiol **6:**1127-1138.

99. **Larsen, G. Y., T. L. Stull, and J. L. Burns.** 1993. Marked phenotypic variability in *Pseudomonas cepacia* isolated from a patient with cystic fibrosis. J. Clin. Microbiol. **31:**788-792.

100. **Lasa, I.** 2006. Towards the identification of the common features of bacterial biofilm development. Int Microbiol **9:**21-28.

101. **Latifi, A., M. Foglino, K. Tanaka, P. Williams, and A. Lazdunski.** 1996. A hierarchical quorum-sensing cascade in *Pseudomonas aeruginosa* links the transcriptional activators LasR and RhIR (VsmR) to expression of the stationary-phase sigma factor RpoS. Mol. Microbiol. **21:**1137-1146.

102. **Ledgham, F., I. Ventre, C. Soscia, M. Foglino, J. N. Sturgis, and A. Lazdunski.** 2003. Interactions of the quorum sensing regulator QscR: interaction with itself and the other regulators of *Pseudomonas aeruginosa* LasR and RhlR. Mol Microbiol **48:**199-210.

103. **Lee, J. H., Y. Lequette, and E. P. Greenberg.** 2006. Activity of purified QscR, a *Pseudomonas aeruginosa* orphan quorum-sensing transcription factor. Mol Microbiol **59:**602-609.

104. **Leiske, D., A. Karimpour-Fard, and R. T. Gill.** 2006. A whole-genome microarray for *B. cenocepacia*. Presented at the International *Burkholderia cepacia* Working Group Meeting, Gent, Belgium.

105. **Lequette, Y., J. H. Lee, F. Ledgham, A. Lazdunski, and E. P. Greenberg.** 2006. A distinct QscR regulon in the *Pseudomonas aeruginosa* quorum-sensing circuit. J Bacteriol **188:**3365-3370.

106. **Lerat, E., and N. A. Moran.** 2004. The evolutionary history of quorum-sensing systems in bacteria. Mol. Biol. Evol. **21:**903-913.

107. **Lessie, T. G., W. Hendrickson, B. D. Manning, and R. Devereux.** 1996. Genomic complexity and plasticity of *Burkholderia cepacia*. FEMS Microbiol. Lett. **144:**117-128.

108. **Levy, A., B. J. Chang, L. K. Abbott, J. Kuo, G. Harnett, and T. J. Inglis.** 2003. Invasion of spores of the arbuscular mycorrhizal fungus *Gigaspora decipiens* by *Burkholderia* spp. Appl Environ Microbiol **69:**6250-6256.

109. **Lewenza, S., B. Conway, E. P. Greenberg, and P. A. Sokol.** 1999. Quorum sensing in *Burkholderia cepacia*: identification of the LuxRI homologs CepRI. J. Bacteriol. **181:**748-756.

110. **Lewenza, S., and P. A. Sokol.** 2001. Regulation of ornibactin biosynthesis and *N*-acyl-L-homoserine lactone production by CepR in *Burkholderia cepacia*. J. Bacteriol. **183:**2212-2218.

111. **Lewenza, S., M. B. Visser, and P. A. Sokol.** 2002. Interspecies communication between *Burkholderia cepacia* and *Pseudomonas aeruginosa*. Can. J. Microbiol. **48:**707-716.

112. **Lewenza, W. S.** 2000. Quorum Sensing in *Burkholderia cepacia*. University of Calgary, Calgary, AB.

113. **Li, W., D. P. Roberts, P. D. Dery, S. Meyer, S. Lohrke, R. D. Lumsden, and K. P. Hebbar.** 2002. Broad spectrum anti-biotic activity and disease suppression by the potential biocontrol agent *Burkholderia ambifaria* BC-7. Crop Prot **21:**129-135.

114. **Lin, Y. H., J. L. Xu, J. Hu, L. H. Wang, S. L. Ong, J. R. Leadbetter, and L. H. Zhang.** 2003. Acyl-homoserine lactone acylase from *Ralstonia* strain XJ12B

represents a novel and potent class of quorum-quenching enzymes. Mol Microbiol **47:**849-860.

115. **LiPuma, J. J.** 2003. *Burkholderia* and emerging pathogens in cystic fibrosis. Semin Respir Crit Care Med **24:**681-692.

116. **Lipuma, J. J.** 2005. Update on the *Burkholderia cepacia* complex. Curr Opin Pulm Med **11:**528-533.

117. **LiPuma, J. J., T. Spilker, T. Coenye, and C. F. Gonzalez.** 2002. An epidemic *Burkholderia cepacia* complex strain identified in soil. Lancet **359:**2002-2003.

118. **LiPuma, J. J., T. Spilker, L. H. Gill, P. W. Campbell, 3rd, L. Liu, and E. Mahenthiralingam.** 2001. Disproportionate distribution of *Burkholderia cepacia* complex species and transmissibility markers in cystic fibrosis. Am. J. Respir. Crit. Care. Med. **164:**92-96.

119. **Loutet, S. A., R. S. Flannagan, C. Kooi, P. A. Sokol, and M. A. Valvano.** 2006. A complete lipopolysaccharide inner core oligosaccharide is required for resistance of *Burkholderia cenocepacia* to antimicrobial peptides and bacterial survival *in vivo*. J Bacteriol **188:**2073-2080.

120. **Lutter, E.** 2002. Identification of Quorum Sensing Regulated Genes in *Burkholderia cepacia*. University of Calgary, Calgary.

121. **Lutter, E., S. Lewenza, J. J. Dennis, M. B. Visser, and P. A. Sokol.** 2001. Distribution of quorum-sensing genes in the *Burkholderia cepacia* complex. Infect. Immun. **69:**4661-4666.

122. **Lyczak, J. B., C. L. Cannon, and G. B. Pier.** 2002. Lung infections associated with cystic fibrosis. Clin. Microbiol. Rev. **15:**194-222.

123. **Mahenthiralingam, E., A. Baldwin, and P. Vandamme.** 2002. *Burkholderia cepacia* complex infection in patients with cystic fibrosis. J. Med. Microbiol. **51:**533-538.

124. **Mahenthiralingam, E., J. Bischof, S. K. Byrne, C. Radomski, J. E. Davies, Y. Av-Gay, and P. Vandamme.** 2000. DNA-Based diagnostic approaches for identification of *Burkholderia cepacia* complex, *Burkholderia vietnamiensis, Burkholderia multivorans, Burkholderia stabilis,* and *Burkholderia cepacia* genomovars I and III. J. Clin. Microbiol. **38:**3165-3173.

125. **Mahenthiralingam, E., T. Coenye, J. W. Chung, D. P. Speert, J. R. Govan, P. Taylor, and P. Vandamme.** 2000. Diagnostically and experimentally useful panel of strains from the *Burkholderia cepacia* complex. J. Clin. Microbiol. **38:**910-913.

126. **Mahenthiralingam, E., D. A. Simpson, and D. P. Speert.** 1997. Identification and characterization of a novel DNA marker associated with epidemic *Burkholderia cepacia* strains recovered from patients with cystic fibrosis. J. Clin. Microbiol. **35:**808-816.

127. **Mahenthiralingam, E., T. A. Urban, and J. B. Goldberg.** 2005. The multifarious, multireplicon *Burkholderia cepacia* complex. Nat Rev Microbiol **3:**144-156.

128. **Mahenthiralingam, E., P. Vandamme, M. E. Campbell, D. A. Henry, A. M. Gravelle, L. T. Wong, A. G. Davidson, P. G. Wilcox, B. Nakielna, and D. P. Speert.** 2001. Infection with *Burkholderia cepacia* complex genomovars in patients with cystic fibrosis: virulent transmissible strains of genomovar III can replace *Burkholderia multivorans*. Clin. Infect. Dis. **33:**1469-1475.

129. **Malott, R. J., A. Baldwin, E. Mahenthiralingam, and P. A. Sokol.** 2005. Characterization of the *cciIR* Quorum-Sensing System in *Burkholderia cenocepacia*. Infect Immun **73:**4982-4992.

130. **Malott, R. J., and P. Sokol.** 2003. Cell-cell signaling mechanisms of the *Burkholderia cepacia* complex, p. 277-293. *In* S. G. Pandalai (ed.), Recent Research Developments in Infection and Immunity, vol. 1. Transworld Research Network, Trivandrum, India.

131. **Malott, R. J., and P. A. Sokol.** 2002. Environmental conditions that influence *cepI* expression in *Burkholderia cepacia* K56-2. Presented at the Canadian Society of Microbiologists, Saskatoon, SK.

132. **Manefield, M., R. de Nys, N. Kumar, R. Read, M. Givskov, P. Steinberg, and S. Kjelleberg.** 1999. Evidence that halogenated furanones from *Delisea pulchra* inhibit acylated homoserine lactone (AHL)-mediated gene expression by displacing the AHL signal from its receptor protein. Microbiology **145 (Pt 2):**283-291.

133. **Mao, W., J. A. Lewis, R. D. Lumsden, and K. P. Hebbar.** 1998. Biocontrol of selected soilborne diseases of tomato and pepper plants. Crop Prot **17:**535-542.

134. **Marchler-Bauer, A., J. B. Anderson, P. F. Cherukuri, C. DeWeese-Scott, L. Y. Geer, M. Gwadz, S. He, D. I. Hurwitz, J. D. Jackson, Z. Ke, C. J. Lanczycki, C. A. Liebert, C. Liu, F. Lu, G. H. Marchler, M. Mullokandov, B. A. Shoemaker, V. Simonyan, J. S. Song, P. A. Thiessen, R. A. Yamashita, J. J. Yin, D. Zhang, and S. H. Bryant.** 2005. CDD: a Conserved Domain Database for protein classification. Nucleic Acids Res **33:**D192-196.

135. **Marchler-Bauer, A., and S. H. Bryant.** 2004. CD-Search: protein domain annotations on the fly. Nucleic Acids Res **32:**W327-331.

136. **Marketon, M. M., M. R. Gronquist, A. Eberhard, and J. E. Gonzalez.** 2002. Characterization of the *Sinorhizobium meliloti sinR/sinI* locus and the production of novel N-acyl homoserine lactones. J Bacteriol **184:**5686-5695.

137. **Marolda, C. L., B. Hauroder, M. A. John, R. Michel, and M. A. Valvano.** 1999. Intracellular survival and saprophytic growth of isolates from the *Burkholderia cepacia* complex in free-living amoebae. Microbiology **145 (Pt 7):**1509-1517.

138. **Martin, D. W., and C. D. Mohr.** 2000. Invasion and intracellular survival of *Burkholderia cepacia*. Infect. Immun. **68:**24-29.

139. **McKenney, D., K. E. Brown, and D. G. Allison.** 1995. Influence of *Pseudomonas aeruginosa* exoproducts on virulence factor production in *Burkholderia cepacia*: evidence of interspecies communication. J. Bacteriol. **177:**6989-6992.

140. **McKevitt, A. I., S. Bajaksouzian, J. D. Klinger, and D. E. Woods.** 1989. Purification and characterization of an extracellular protease from *Pseudomonas cepacia*. Infect. Immun. **57:**771-778.

141. **Meng, A.** 1994. Simplified downward alkaline transfer of DNA. Biotechniques **17:**72-73.

142. **Meyer, J. M., and M. A. Abdallah.** 1978. The fluorescent pigment of *Pseudomonas fluorescens:* biosyntehsis, purification and physico-chemical properties. J. Gen. Microbiol. **107:**319-328.

143. **Meyer, J. M., D. Hohnadel, and F. Halle.** 1989. Cepabactin from *Pseudomonas cepacia*, a new type of siderophore. J Gen Microbiol **135:**1479-1487.

144. **Meyer, J. M., V. T. Van, A. Stintzi, O. Berge, and G. Winkelmann.** 1995. Ornibactin production and transport properties in strains of *Burkholderia vietnamiensis* and *Burkholderia cepacia* (formerly *Pseudomonas cepacia*). Biometals **8:**309-317.

145. **Michel, R., and B. Hauroder.** 1997. Isolation of an *Acanthamoeba strain* with intracellular *Burkholderia pickettii* infection. Zentralbl Bakteriol **285:**541-557.

146. **Middleton, B., H. C. Rodgers, M. Camara, A. J. Knox, P. Williams, and A. Hardman.** 2002. Direct detection of N-acylhomoserine lactones in cystic fibrosis sputum. FEMS Microbiol Lett **207:**1-7.

147. **Miller, M. B., and B. L. Bassler.** 2001. Quorum sensing in bacteria. Annu Rev Microbiol **55:**165-199.

148. **Miyano, N., S. Oie, and A. Kamiya.** 2003. Efficacy of disinfectants and hot water against biofilm cells of *Burkholderia cepacia*. Biol Pharm Bull **26:**671-674.

149. **Mohr, C. D., M. Tomich, and C. A. Herfst.** 2001. Cellular aspects of *Burkholderia cepacia* infection. Microbes Infect. **3:**425-435.

150. **Moore, R. A., S. Reckseidler-Zenteno, H. Kim, W. Nierman, Y. Yu, A. Tuanyok, J. Warawa, D. DeShazer, and D. E. Woods.** 2004. Contribution of gene loss to the pathogenic evolution of *Burkholderia pseudomallei* and *Burkholderia mallei*. Infect Immun **72:**4172-4187.

151. **Nasser, R. M., A. C. Rahi, M. F. Haddad, Z. Daoud, N. Irani-Hakime, and W. Y. Almawi.** 2004. Outbreak of *Burkholderia cepacia* bacteremia traced to contaminated hospital water used for dilution of an alcohol skin antiseptic. Infect Control Hosp Epidemiol **25:**231-239.

152. **Nelson, M. J., S. O. Montgomery, W. R. Mahaffey, and P. H. Pritchard.** 1987. Biodegradation of trichloroethylene and involvement of an aromatic biodegradative pathway. Appl Environ Microbiol **53:**949-954.

153. **O'Toole, G. A., and R. Kolter.** 1998. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. Mol. Microbiol. **30:**295-304.

154. **Oda, K., T. Takahashi, Y. Tokuda, Y. Shibano, and S. Takahashi.** 1994. Cloning, nucleotide sequence, and expression of an isovaleryl pepstatin-insensitive carboxyl proteinase gene from *Pseudomonas* sp. 101. J Biol Chem **269:**26518-26524.

155. **Park, J., I. Hwang, J. Kim, S. Lee, B. Conway, E. P. Greenberg, and K. Lee.** 2001. Characterization of quorum-sensing signaling molecules produced by *Burkholderia cepacia* G4. J. Microbiol. Biotechnol. **11:**804-811.

156. **Parke, J. L., and D. Gurian-Sherman.** 2001. Diversity of the *Burkholderia cepacia* complex and implications for risk assessment of biological control strains. Annu. Rev. Phytopathol. **39:**225-258.

157. **Parsek, M. R., A. L. Schaefer, and E. P. Greenberg.** 1997. Analysis of random and site-directed mutations in *rhII,* a *Pseudomonas aeruginosa* gene encoding an acylhomoserine lactone synthase. Mol. Microbiol. **26:**301-310.

158. **Passador, L., J. M. Cook, M. J. Gambello, L. Rust, and B. H. Iglewski.** 1993. Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to- cell communication. Science **260:**1127-1130.

159. **Pearson, J. P., K. M. Gray, L. Passador, K. D. Tucker, A. Eberhard, B. H. Iglewski, and E. P. Greenberg.** 1994. Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. Proc. Natl. Acad. Sci. U S A **91:**197-201.

160. **Pearson, J. P., L. Passador, B. H. Iglewski, and E. P. Greenberg.** 1995. A second N-acylhomoserine lactone signal produced by *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. U S A **92:**1490-1494.

161. **Pearson, J. P., C. Van Delden, and B. H. Iglewski.** 1999. Active efflux and diffusion are involved in transport of *Pseudomonas aeruginosa* cell-to-cell signals. J Bacteriol **181:**1203-1210.

162. **Pesci, E. C., J. P. Pearson, P. C. Seed, and B. H. Iglewski.** 1997. Regulation of *las* and *rhl* quorum sensing in *Pseudomonas aeruginosa*. J. Bacteriol. **179:**3127-3132.

163. **Piper, K. R., S. Beck Von Bodman, I. Hwang, and S. K. Farrand.** 1999. Hierarchical gene regulatory systems arising from fortuitous gene associations: controlling quorum sensing by the opine regulon in *Agrobacterium*. Mol. Microbiol. **32:**1077-1089.

164. **Platt, T. B., B. Muller-Hill, and J. H. Miller.** 1972. Analysis of the *lac* operon enzymes, p. 352-355. *In* J. H. Miller (ed.), Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

165. **Rasch, M., J. B. Andersen, K. F. Nielsen, L. R. Flodgaard, H. Christensen, M. Givskov, and L. Gram.** 2005. Involvement of bacterial quorum-sensing signals in spoilage of bean sprouts. Appl Environ Microbiol **71:**3321-3330.

166. **Riedel, K., C. Arevalo-Ferro, G. Reil, A. Gorg, F. Lottspeich, and L. Eberl.** 2003. Analysis of the quorum-sensing regulon of the opportunistic pathogen *Burkholderia cepacia* H111 by proteomics. Electrophoresis **24:**740-50.

167. **Riedel, K., M. Hentzer, O. Geisenberger, B. Huber, A. Steidle, H. Wu, N. Hoiby, M. Givskov, S. Molin, and L. Eberl.** 2001. N-acylhomoserine-lactone-mediated communication between *Pseudomonas aeruginosa* and *Burkholderia cepacia* in mixed biofilms. Microbiology **147:**3249-3262.

168. **Riedel, K., M. Kothe, B. Kramer, W. Saeb, A. Gotschlich, A. Ammendola, and L. Eberl.** 2006. Computer-aided design of agents that inhibit the *cep* quorum-sensing system of *Burkholderia cenocepacia*. Antimicrob Agents Chemother **50:**318-323.

169. **Romling, U., B. Fiedler, J. Bosshammer, D. Grothues, J. Greipel, H. von der Hardt, and B. Tummler.** 1994. Epidemiology of chronic *Pseudomonas aeruginosa* infections in cystic fibrosis. J Infect Dis **170:**1616-1621.

170. **Rutherford, K., J. Parkhill, J. Crook, T. Horsnell, P. Rice, M. A. Rajandream, and B. Barrell.** 2000. Artemis: sequence visualization and annotation. Bioinformatics **16:**944-945.

171. **Sajjan, U. S., L. Sun, R. Goldstein, and J. F. Forstner.** 1995. Cable (*cbl*) type II pili of cystic fibrosis-associated *Burkholderia* (*Pseudomonas*) *cepacia*: nucleotide sequence of the *cblA* major subunit pilin gene and novel morphology of the assembled appendage fibers. J. Bacteriol. **177:**1030-1038.

172. **Sambrook, J., Fritsch, E.F and Maniatis T.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor N.Y.

173. **Schuster, M., and E. P. Greenberg.** 2006. A network of networks: quorum-sensing gene regulation in *Pseudomonas aeruginosa*. Int J Med Microbiol **296:**73-81.

174. **Schuster, M., C. P. Lostroh, T. Ogi, and E. P. Greenberg.** 2003. Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis. J. Bacteriol. **185:**2066-2079.

175. **Schwab, U., M. Leigh, C. Ribeiro, J. Yankaskas, K. Burns, P. Gilligan, P. Sokol, and R. Boucher.** 2002. Patterns of epithelial cell invasion by different species of the *Burkholderia cepacia* complex in well-differentiated human airway epithelia. Infect. Immun. **70:**4547-4555.

176. **Schweizer, H. P.** 1992. Allelic exchange in *Pseudomonas aeruginosa* using novel ColE1-type vectors and a family of cassettes containing a portable *oriT* and the counter-selectable *Bacillus subtilis sacB* marker. Mol. Microbiol. **6:**1195-1204.

177. **Schwyn, B., and J. B. Neilands.** 1987. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. **160:**47-56.

178. **Shaw, P. D., G. Ping, S. L. Daly, C. Cha, J. E. Cronan, Jr., K. L. Rinehart, and S. K. Farrand.** 1997. Detecting and characterizing *N*-acyl-homoserine lactone signal molecules by thin-layer chromatography. Proc. Natl. Acad. Sci. U S A **94:**6036-6041.

179. **Shields, M. S., S. O. Montgomery, P. J. Chapman, S. M. Cuskey, and P. H. Pritchard.** 1989. Novel Pathway of Toluene Catabolism in the Trichloroethylene-Degrading Bacterium G4. Appl Environ Microbiol **55:**1624-1629.

180. **Sifri, C. D., J. Begun, and F. M. Ausubel.** 2005. The worm has turned--microbial virulence modeled in *Caenorhabditis elegans*. Trends Microbiol **13:**119-127.

181. **Singh, P. K., A. L. Schaefer, M. R. Parsek, T. O. Moninger, M. J. Welsh, and E. P. Greenberg.** 2000. Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. Nature **407:**762-764.

182. **Smith, K. M., Y. Bu, and H. Suga.** 2003. Induction and inhibition of *Pseudomonas aeruginosa* quorum sensing by synthetic autoinducer analogs. Chem Biol **10:**81-89.

183. **Smith, K. M., Y. Bu, and H. Suga.** 2003. Library screening for synthetic agonists and antagonists of a *Pseudomonas aeruginosa* autoinducer. Chem Biol **10:**563-571.

184. **Smith, R. S., and B. H. Iglewski.** 2003. *P. aeruginosa* quorum-sensing systems and virulence. Curr. Opin. Microbiol. **6:**56-60.

185. **Smith, R. S., and B. H. Iglewski.** 2003. *Pseudomonas aeruginosa* quorum sensing as a potential antimicrobial target. J. Clin. Invest. **112:**1460-1465.

186. **Sokol, P. A.** 1986. Production and utilization of pyochelin by clinical isolates of *Pseudomonas cepacia*. J Clin Microbiol **23:**560-562.

187. **Sokol, P. A., P. Darling, D. E. Woods, E. Mahenthiralingam, and C. Kooi.** 1999. Role of ornibactin biosynthesis in the virulence of *Burkholderia cepacia*: characterization of *pvdA*, the gene encoding L-Ornithine N^5 -oxygenase. Infect. Immun. **67:**4443-4455.

188. **Sokol, P. A., C. J. Lewis, and J. J. Dennis.** 1992. Isolation of a novel siderophore from *Pseudomonas cepacia*. J Med Microbiol **36:**184-189.

189. **Sokol, P. A., D. E. Ohman, and B. H. Iglewski.** 1979. A more sensitive plate assay for detection of protease production by *Pseudomanas aeruginosa*. J. Clin. Microbiol. **9:**538-540.

190. **Sokol, P. A., U. Sajjan, M. B. Visser, S. Gingues, J. Forstner, and C. Kooi.** 2003. The CepIR quorum-sensing system contributes to the virulence of *Burkholderia cenocepacia* respiratory infections. Microbiology **149:**3649-3658.

191. **Solis, R., I. Bertani, G. Degrassi, G. Devescovi, and V. Venturi.** 2006. Involvement of quorum sensing and RpoS in rice seedling blight caused by *Burkholderia plantarii*. FEMS Microbiol Lett **259:**106-112.

192. **Speert, D. P., M. Bond, R. C. Woodman, and J. T. Curnutte.** 1994. Infection with *Pseudomonas cepacia* in chronic granulomatous disease: role of nonoxidative killing by neutrophils in host defense. J Infect Dis **170:**1524-1531.

193. **Speert, D. P., M. E. Campbell, D. A. Henry, R. Milner, F. Taha, A. Gravelle, A. G. Davidson, L. T. Wong, and E. Mahenthiralingam.** 2002. Epidemiology of *Pseudomonas aeruginosa* in Cystic Fibrosis in British Columbia, Canada. Am. J. Respir. Crit. Care. Med. **166:**988-993.

194. **Speert, D. P., D. Henry, P. Vandamme, M. Corey, and E. Mahenthiralingam.** 2002. Epidemiology of *Burkholderia cepacia* complex in patients with cystic fibrosis, Canada. Emerg. Infect. Dis. **8:**181-187.

195. **Stover, C. K., X. Q. Pham, A. L. Erwin, S. D. Mizoguchi, P. Warrener, M. J. Hickey, F. S. Brinkman, W. O. Hufnagle, D. J. Kowalik, M. Lagrou, R. L. Garber, L. Goltry, E. Tolentino, S. Westbrock-Wadman, Y. Yuan, L. L. Brody, S. N. Coulter, K. R. Folger, A. Kas, K. Larbig, R. Lim, K. Smith, D. Spencer, G. K. Wong, Z. Wu, I. T. Paulsen, J. Reizer, M. H. Saier, R. E. Hancock, S. Lory, and M. V. Olson.** 2000. Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. Nature **406:**959-964.

196. **Summer, E. J., C. F. Gonzalez, T. Carlisle, L. M. Mebane, A. M. Cass, C. G. Savva, J. LiPuma, and R. Young.** 2004. *Burkholderia cenocepacia* phage BcepMu and a family of Mu-like phages encoding potential pathogenesis factors. J Mol Biol **340:**49-65.

197. **Sun, L., R. Z. Jiang, S. Steinbach, A. Holmes, C. Campanelli, J. Forstner, U. Sajjan, Y. Tan, M. Riley, and R. Goldstein.** 1995. The emergence of a highly transmissible lineage of *cbl*+ *Pseudomonas* (*Burkholderia*) *cepacia* causing CF centre epidemics in North America and Britain. Nat. Med. **1:**661-666.

198. **Swift, S., J. A. Downie, N. A. Whitehead, A. M. Barnard, G. P. Salmond, and P. Williams.** 2001. Quorum sensing as a population-density-dependent determinant of bacterial physiology. Adv. Microb. Physiol. **45:**199-270.

199. **Taga, M. E., and B. L. Bassler.** 2003. Chemical communication among bacteria. Proc Natl Acad Sci U S A **100 Suppl 2:**14549-14554.

200. **Taplin, D., D. C. J. Bassett, and P. Mertz.** 1971. Foot lesions associated with *Pseudomonas cepacia*. Lancet **ii:**568-571.

201. **Tomich, M., A. Griffith, C. A. Herfst, J. L. Burns, and C. D. Mohr.** 2003. Attenuated virulence of a *Burkholderia cepacia* type III secretion mutant in a murine model of infection. Infect. Immun. **71:**1405-1415.

202. **Tomlin, K. L., O. P. Coll, and H. Ceri.** 2001. Interspecies biofilms of *Pseudomonas aeruginosa* and *Burkholderia cepacia*. Can. J. Microbiol. **47:**949-954.

203. **Tomlin, K. L., R. J. Malott, G. Ramage, D. G. Storey, P. A. Sokol, and H. Ceri.** 2005. Quorum-sensing mutations affect attachment and stability of *Burkholderia cenocepacia* biofilms. Appl Environ Microbiol **71:**5208-5218.

204. **Tran Van, V., O. Berge, S. Ngo, J. Balandreau, and T. Heulin.** 2000. Repeated beneficial effects of rice inoculation with a strain of *Burkholderia vietnamiensis* on early and late yield components in low fertility sulphate acid soils of Vietnam. Plant Soil **218:**273-284.

205. **Tyler, S. D., K. R. Rozee, and W. M. Johnson.** 1996. Identification of IS1356, a new insertion sequence, and its association with IS402 in epidemic strains of *Burkholderia cepacia* infecting cystic fibrosis patients. J. Clin. Microbiol. **34:**1610-1616.

206. **Ulrich, R. L., D. Deshazer, E. E. Brueggemann, H. B. Hines, P. C. Oyston, and J. A. Jeddeloh.** 2004. Role of quorum sensing in the pathogenicity of *Burkholderia pseudomallei*. J. Med. Microbiol. **53:**1053-1064.

207. **Ulrich, R. L., D. Deshazer, H. B. Hines, and J. A. Jeddeloh.** 2004. Quorum Sensing: a Transcriptional Regulatory System Involved in the Pathogenicity of *Burkholderia mallei*. Infect. Immun. **72:**6589-6596.

208. **Ulrich, R. L., H. B. Hines, N. Parthasarathy, and J. A. Jeddeloh.** 2004. Mutational analysis and biochemical characterization of the *Burkholderia thailandensis* DW503 quorum-sensing network. J. Bacteriol. **186:**4350-4360.

209. **Valade, E., F. M. Thibault, Y. P. Gauthier, M. Palencia, M. Y. Popoff, and D. R. Vidal.** 2004. The PmlI-PmlR quorum-sensing system in *Burkholderia pseudomallei* plays a key role in virulence and modulates production of the MprA protease. J. Bacteriol. **186:**2288-2294.

210. **Van Delden, C., and B. H. Iglewski.** 1998. Cell-to-cell signaling and *Pseudomonas aeruginosa* infections. Emerg Infect Dis **4:**551-560.

211. **Van Delden, C., E. C. Pesci, J. P. Pearson, and B. H. Iglewski.** 1998. Starvation selection restores elastase and rhamnolipid production in a *Pseudomonas aeruginosa* quorum-sensing mutant. Infect. Immun. **66:**4499-4502.

212. **Vandamme, P., D. Henry, T. Coenye, S. Nzula, M. Vancanneyt, J. J. LiPuma, D. P. Speert, J. R. Govan, and E. Mahenthiralingam.** 2002. *Burkholderia anthina* sp. nov. and *Burkholderia pyrrocinia*, two additional *Burkholderia*

cepacia complex bacteria, may confound results of new molecular diagnostic tools. FEMS Immunol. Med. Microbiol. **33:**143-149.

213. **Vandamme, P., B. Holmes, T. Coenye, J. Goris, E. Mahenthiralingam, J. J. LiPuma, and J. R. Govan.** 2003. *Burkholderia cenocepacia* sp. nov.--a new twist to an old story. Res. Microbiol. **154:**91-96.

214. **Vandamme, P., B. Holmes, M. Vancanneyt, T. Coenye, B. Hoste, R. Coopman, H. Revets, S. Lauwers, M. Gillis, K. Kersters, and J. R. Govan.** 1997. Occurrence of multiple genomovars of *Burkholderia cepacia* in cystic fibrosis patients and proposal of *Burkholderia multivorans* sp. nov. Int. J. Syst. Bacteriol. **47:**1188-1200.

215. **Vandamme, P., E. Mahenthiralingam, B. Holmes, T. Coenye, B. Hoste, P. De Vos, D. Henry, and D. P. Speert.** 2000. Identification and population structure of *Burkholderia stabilis* sp. nov. (formerly *Burkholderia cepacia* genomovar IV). J. Clin. Microbiol. **38:**1042-1047.

216. **Venturi, V., A. Friscina, I. Bertani, G. Devescovi, and C. Aguilar.** 2004. Quorum sensing in the *Burkholderia cepacia* complex. Res. Microbiol. **155:**238-244.

217. **Vermis, K., T. Coenye, J. J. LiPuma, E. Mahenthiralingam, H. J. Nelis, and P. Vandamme.** 2004. Proposal to accommodate *Burkholderia cepacia* genomovar VI as *Burkholderia dolosa* sp. nov. Int. J. Syst. Evol. Microbiol. **54:**689-691.

218. **Vinion-Dubiel, A. D., and J. B. Goldberg.** 2003. Lipopolysaccharide of *Burkholderia cepacia* complex. J. Endotoxin. Res. **9:**201-213.

219. **Visser, M. B., S. Majumdar, E. Hani, and P. A. Sokol.** 2004. Importance of the ornibactin and pyochelin siderophore transport systems in *Burkholderia cenocepacia* lung infections. Infect Immun **72:**2850-2857.

220. **Wagner, V. E., D. Bushnell, L. Passador, A. I. Brooks, and B. H. Iglewski.** 2003. Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment. J. Bacteriol. **185:**2080-2095.

221. **Walsh, P. S., D. A. Metzger, and R. Higuchi.** 1991. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques **10:**506-513.

222. **Walsh, T. A., and D. P. Ballou.** 1983. Halogenated protocatechuates as substrates for protocatechuate dioxygenase from *Pseudomonas cepacia*. J. Biol. Chem. **258:**14413-14421.

223. **Waters, C. M., and B. L. Bassler.** 2005. Quorum sensing: cell-to-cell communication in bacteria. Annu Rev Cell Dev Biol **21:**319-346.

224. **Watson, W. T., T. D. Minogue, D. L. Val, S. B. von Bodman, and M. E. Churchill.** 2002. Structural basis and specificity of acyl-homoserine lactone signal production in bacterial quorum sensing. Mol Cell **9:**685-694.

225. **Weingart, C. L., C. E. White, S. Liu, Y. Chai, H. Cho, C. S. Tsai, Y. Wei, N. R. Delay, M. R. Gronquist, A. Eberhard, and S. C. Winans.** 2005. Direct binding of the quorum sensing regulator CepR of *Burkholderia cenocepacia* to two target promoters *in vitro*. Mol Microbiol **57:**452-467.

226. **West, S. E., H. P. Schweizer, C. Dall, A. K. Sample, and L. J. Runyen-Janecky.** 1994. Construction of improved *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19 and sequence of the region required for their replication in *Pseudomonas aeruginosa*. Gene **148:**81-86.

227. **Whitehead, N. A., A. M. Barnard, H. Slater, N. J. Simpson, and G. P. Salmond.** 2001. Quorum-sensing in Gram-negative bacteria. FEMS Microbiol. Rev. **25:**365-404.

228. **Wisniewski-Dye, F., J. Jones, S. R. Chhabra, and J. A. Downie.** 2002. *raiIR* genes are part of a quorum-sensing network controlled by *cinI* and *cinR* in *Rhizobium leguminosarum*. J. Bacteriol. **184:**1597-1606.

229. **Woods, C. W., A. M. Bressler, J. J. LiPuma, B. D. Alexander, D. A. Clements, D. J. Weber, C. M. Moore, L. B. Reller, and K. S. Kaye.** 2004. Virulence associated with outbreak-related strains of *Burkholderia cepacia* complex among a cohort of patients with bacteremia. Clin. Infect. Dis. **38:**1243-1250.

230. **Woods, D.** 1984. Oligonucleotide screening of cDNA libraries. Focus. **6:**1-2.

231. **Wopperer, J., S. T. Cardona, B. Huber, C. A. Jacobi, M. A. Valvano, and L. Eberl.** 2006. A quorum-quenching approach to investigate the conservation of quorum-sensing-regulated functions within the *Burkholderia cepacia* complex. Appl Environ Microbiol **72:**1579-1587.

232. **Wu, L., O. Estrada, O. Zaborina, M. Bains, L. Shen, J. E. Kohler, N. Patel, M. W. Musch, E. B. Chang, Y. X. Fu, M. A. Jacobs, M. I. Nishimura, R. E. Hancock, J. R. Turner, and J. C. Alverdy.** 2005. Recognition of host immune activation by *Pseudomonas aeruginosa*. Science **309:**774-777.

233. **Zhou, H., F. Yao, D. P. Roberts, and T. G. Lessie.** 2003. AHL-deficient mutants of *Burkholderia ambifaria* BC-F have decreased antifungal activity. Curr Microbiol **47:**174-179.

234. **Zhu, J., J. W. Beaber, M. I. More, C. Fuqua, A. Eberhard, and S. C. Winans.** 1998. Analogs of the autoinducer 3-oxooctanoyl-homoserine lactone strongly inhibit activity of the TraR protein of *Agrobacterium tumefaciens*. J. Bacteriol. **180:**5398-5405.