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Biofilm genetics of Burkholderia cenocepacia

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BIOFILM GENETICS OF BURKHOLDERIA CENOCEPACIA

BY

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BS, University of New Hampshire, 2005

THESIS

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ABSTRACT

BIOFILM GENETICS OF BURKHOLDERIA CENOCEPACIA

By

Laura C. Benton

University of New Hampshire, December 2009

*Burkholderia cenocepacia* is a soil bacterium and opportunistic human pathogen found to infect the lungs of cystic fibrosis (CF) patients. One factor that may allow *B. cenocepacia* to persist in the CF lung and continue to cause a decline in lung function is its ability to form biofilms. Little is known about the genetic mechanisms allowing this bacterium to transition from an acute to a chronic (biofilm) lifestyle. I used both transposon mutagenesis and positive laboratory selection to identify mutations that increase biofilm production. Mutations affecting capsule polysaccharide synthesis were identified, among others, by random mutagenesis to confer an increased biofilm phenotype, whereas mutations putatively resulting in increased intracellular levels of cyclic-di-GMP were identified in biofilm-adapted isolates.
CHAPTER 1

INTRODUCTION

The Transition from Acute to Chronic Infections

A phenomenon that occurs in cystic fibrosis (CF) lung infections is a transition of the infecting bacteria from a lifestyle associated with an acute infection to one associated with a chronic infection. An acute infection is more likely to be cleared by aggressive antibiotic therapy than a chronic infection associated with establishment of persistent biofilm (Hoiby et al., 2005). It has been proposed that the transition involves a series of key adaptive mutations in genes that govern these two lifestyles (Furukawa et al., 2006) and that the evolution of bacterial populations in the CF lung follows a predictable, repeated pattern (D’Argenio et al., 2007). Identifying the pattern of molecular changes that occurs in bacteria establishing a chronic, biofilm-forming lifestyle is crucial for developing methods to control such infections by preventing the switch from acute to chronic stages.

Biofilms

A biofilm is a complex community of bacteria anchored to a living or inert surface and protected from the ‘outside world’ by a secreted extracellular matrix of complex sugars, proteins, lipids, nucleic acids and decaying cell debris (reviewed by Vu et al., 2009). Biofilms can form on surfaces ranging from the inside of plumbing pipes to the surface of our heart valves (endocarditis). In medicine, biofilms forming on surfaces
such as catheters and open wounds are especially worrisome since they lead to
infections that are increasingly resistant to antibiotics and can often evade our immune
system (Donlan, 2001). Since infections caused by bacteria forming biofilms are so
difficult to clear from patients, many produce chronic infections that may become fatal
(Furukawa et al., 2006; Cunha et al., 2004). One such bacterium associated with a poor
prognosis when causing biofilm-related infections is *Burkholderia cenocepacia*, the
model organism for my studies.

**The *Burkholderia cepacia* complex**

*B. cenocepacia* is a soil bacterium and opportunistic pathogen of the *Burkholderia
cepacia* complex (Bcc). The Bcc is comprised of at least 17 species, most of which have
been found to infect the lungs of CF patients (Springman et al., 2009). These lung
infections can lead to ‘cepacia syndrome’, characterized by necrotizing pneumonia and
septicemia (Mahenthiralingam et al., 2008). Bcc-positive CF patients may be removed
from lung transplant lists in lung transplant centers (Alexander et al., 2008) and
commonly cohorted or segregated from the rest of the CF community to prevent
contagious spread (Lynch, 2009). Previous infection with *B. cenocepacia*, specifically,
correlates with a higher rate of death post-transplant than other members of the Bcc or
other infecting bacteria in CF patients receiving a lung transplant (Alexander et al.,
2008). In addition to its high correlation with infection and post-transplant fatality, this
species is important to the CF community because it has a nearly 50% prevalence in Bcc
CF lung infections and is frequently acquired from environmental reservoirs
(Mahenthiralingam et al., 2005; 2008).
An important virulence determinant of *B. cenocepacia* in the CF lung may be the ability to form a persistent biofilm (Moreau-Marquis et al., 2008; Savoia and Zucca, 2007; Romanova Lu et al., 2009). Previous work to identify genes controlling biofilm formation in *B. cenocepacia* has mainly involved screens for mutants defective in biofilm formation among transposon mutants (Huber et al., 2001; 2002; Tomlin et al., 2005; Ferreira et al., 2007). Another strategy to identify biofilm genes has involved the use of comparative genomics between *B. cenocepacia* and the CF pathogen *P. aeruginosa* to identify well-characterized biofilm gene homologs and subsequently manipulate them in the *B. cenocepacia* background (Saldias et al., 2008; Aubert et al., 2008). Though these genes may have important roles in the ability to form biofilms, there is still much to be discovered and characterized in *B. cenocepacia*, especially in defining the relative importance of these genes for biofilm production.

**Genetic Control of Biofilm Production in *B. cenocepacia***

A variety of approaches have identified biofilm genes in the CF lung pathogen *Pseudomonas aeruginosa* but the genes that control biofilm production in *B. cenocepacia* are less well known. The sigma factor, RpoN, is known to control biofilm in *P. aeruginosa* (Boucher et al., 2000), and Saldias et al. (2008) identified its homolog in *B. cenocepacia* J2315. When inactivated by a transposon insertion, the *rpoN* homolog in *B. cenocepacia* K56-2 reduced biofilm production by 40%. The RetS protein in *P. aeruginosa* also controls biofilm formation (Goodman et al., 2004). Aubert et al. (2008) identified a *Pseudomonas* RetS homolog called AtsR in *B. cenocepacia* J2315, which they knocked out in *B. cenocepacia* K56-2, resulting in a 50% increase in biofilm yield.
The majority of known biofilm genes have been identified through random mutagenesis. For example, the genes \textit{bceD} and \textit{bceF} were initially identified in a random mutagenesis of \textit{B. cepacia} IST408 and further characterized as genes governing exopolysaccharide (EPS) production. Knockouts of \textit{bceD} and \textit{bceF} decreased cepacian (EPS) production and 48hr biofilm yield (Ferreira et al., 2007).

Huber et al. (2002) performed a random transposon mutagenesis of \textit{B. cenocepacia} H111 and identified the genes \textit{tolA}, \textit{bap}, \textit{recR}, \textit{yciR}, \textit{gspE}, \textit{suhB}, \textit{rodA} and \textit{ycil} that when knocked out exhibited biofilm defects. Two of these genes, \textit{gspE} and \textit{yciR}, will be discussed later in this thesis. The \textit{gspE} gene has a putative role in a general secretory pathway, perhaps affecting secretion of biofilm components. The \textit{yciR} gene has a putative role in regulating the bacterial second messenger, cyclic-di-GMP, since the predicted YciR protein contains both GGDEF and EAL amino acid motifs, which generally are found in diguanylate cyclase and phosphodiesterase enzymes, respectively, that synthesize or break down this second messenger (reviewed by Tamayo et al., 2007).

The Role of Cyclic-di-GMP
Secondary messengers, such as cyclic-di-GMP (Figure 1.1), cyclic-AMP and guanosine pentaphosphate (pppGpp) and tetraphosphate (ppGpp), respond to and transduce signals from stimuli such as environmental oxygen, light, or intracellular changes and cell-cycle signals, turning them into different cellular responses (Pesavento and Hengge, 2009). Cyclic-di-GMP is a bacterial secondary messenger widely distributed among prokaryotes that was first described as an allosteric activator of cellulose synthase in \textit{Gluconacetobacter xylinus} (Ross et al., 1987). It has since been identified as
an important regulator of biofilm formation, motility, and production of virulence factors in *Pseudomonas aeruginosa*, *Xanthomonas campestris*, and *Vibrio cholerae* (Kulasekara et al., 2005, Lim et al., 2007, Ryan et al., 2006a, Tischler and Camilli, 2005). Cyclic-di-GMP also regulates phase changes in *V. cholerae* from ‘smooth’ to ‘rugose,’ a phenotype that is less susceptible to bacteriophage infection (Beyhan et al., 2007, Beyhan and Yildiz, 2007).

![Figure 1.1: Chemical Structure of Cyclic-di-GMP](image)

Figure 1.1: Chemical Structure of Cyclic-di-GMP (bis-(3’-5’)-cyclic dimeric guanosine monophosphate)

Two enzymes, diguanylate cyclase and phosphodiesterase A, control intracellular levels of cyclic-di-GMP by synthesizing and degrading the dinucleotide, respectively. These enzymes are proteins with conserved amino acid motifs: a GGDEF motif in diguanylate cyclase and EAL or HD-GYP motifs in phosphodiesterase. In hybrid proteins with both predicted enzyme functions, the action of the synthesizing GGDEF protein domain is often countered by either an EAL or HD-GYP domain that hydrolyzes cyclic-di-GMP (reviewed by Ryan et al., 2006b; Hengge, 2009).

In *Pseudomonas*, as in many other bacteria, intracellular pooling of cyclic-di-GMP leads to a sessile, biofilm lifestyle and the absence or decrease of cyclic-di-GMP leads to
a more motile lifestyle (Merritt et al., 2007). Increases in cyclic-di-GMP levels in general up-regulate or down-regulate different virulence traits of *P. aeruginosa* (Kulasekara et al., 2005). Low levels of cyclic-di-GMP promote acute virulence traits, specifically, in *V. cholerae* (Tamayo et al., 2007). High levels of cyclic-di-GMP in *P. aeruginosa*, which promote a sessile, biofilm lifestyle, may also lead to attenuated acute virulence since a mutant resulting in high cyclic-di-GMP levels is impaired in acute virulence (Chung et al., 2008).

Quorum sensing in the genus *Burkholderia* has also been implicated in regulation of biofilm formation as well as regulation of virulence factors (Eberl, 2006). The CepIR Quorum Sensing (QS) system was identified in screens of random mutants of *B. cenocepacia* H111 and K56-2 as a two-component regulatory system controlling biofilm production (Huber et al., 2001; Tomlin et al. 2005). In the genus *Burkholderia*, QS systems employ *N*-acyl-homoserine lactone (AHL) molecules to communicate and regulate expression of certain genes in a cell-density dependent manner. In many strains of *B. cenocepacia*, the QS system is composed of the CepI/CepR two-component system that produces octanoyl homoserine lactone (C8-HSL) as its main AHL, and produces hexanoyl homoserine lactone (C6-HSL) to a lesser degree (Eberl 2006). The YciR protein identified by Huber et al. (2002) was described as a possible higher-level regulator of the CepIR quorum sensing (QS) system based on the results that a *yciR* mutant behaved similarly to a *cepR* mutant in biofilm defect and loss of QS molecule production but could not be complemented with a functional *cepR* in trans (Huber et al., 2002).
In the CF pathogen *P. aeruginosa*, some chronic infection-associated mutations have been identified from clinical isolates of CF lung infections (Smith et al., 2006), but little has been done in *B. cenocepacia* to identify adaptively relevant biofilm mutations. It is clear that *Pseudomonas* undergoes many genetic changes in transitioning from an acute to a chronic infection, often losing the ability to produce acute virulence factors (Smith et al., 2006). The common mutations occurring in chronic isolates of *P. aeruginosa* are in *lasR* (quorum sensing) and *mex* (multidrug efflux) genes (Smith et al., 2006). *B. cenocepacia* may also adapt to the CF lung in a similar manner since the two pathogens are in the same selective environment, however the molecular basis of biofilm adaptation in members of the Bcc has yet to be identified.

**CF Lung Pathogens Produce Small-Colony Variants (SCVs) or Rugose SCVs (RSCVs)**

Small colony variants (SCVs), or rugose SCVs (RSCVs), recovered from CF patients with chronic lung infections support the hypothesis that similar and predictable mechanisms of adaptation to a biofilm lifestyle exist between *P. aeruginosa* and *B. cenocepacia*. Phenotypes of SCVs of Bcc members isolated from chronic CF lung infection are similar to those of *P. aeruginosa* in that these colony variants are highly adherent biofilm producers (Haussler et al., 2003; Haussler, 2004). Clinical RSCVs from a CF lung have similar colony and biofilm phenotypes to a laboratory manipulated *Pseudomonas wspF* mutant, which leads to constitutive activation of a diguanylate cyclase and results in intracellular pooling of cyclic-di-GMP (D’Argenio et al., 2002). Though similar in colony appearance and cell adherence, not all *Pseudomonas* RSCVs (laboratory or clinical isolates) are caused by the same mutations. Indeed, in a study by
Starkey et al. (2009), genetic complementation of the *Pseudomonas* RSCV phenotype with a functional *wspF* gene *in trans* was successful only in a portion of laboratory biofilm derived RSCVs, returning colony morphology and biofilm production to wild-type appearance and levels, respectively. In contrast, complementation with a native phosphodiesterase was successful in all RSCVs. The clinical RSCV from the same study was only complemented with a native phosphodiesterase *in trans*, which reversed the RSCV phenotype to wild-type colony and biofilm production by reducing intracellular cyclic-di-GMP pooling.

In a recent study by Poltak et al. (2009), populations of *B. cenocepacia* were experimentally evolved for about 1500 generations in an environment favoring biofilm formation. Each of six replicate populations adapted to the biofilm lifestyle in part by production of wrinkly or rugose colony variants, some very similar to RSCVs, that are highly adherent hyper-biofilm formers. Control populations selected for planktonic growth in similar media did not produce the same variation seen in the biofilm-adapted populations nor did they increase their biofilm production. The genomes of three colony variants from one population (termed Studded [S], Ruffled Spreader[R] and Wrinkly [W]) were fully sequenced and revealed mutations shared by all three variants that likely have consequences for iron metabolism, general efflux, and sugar metabolism (Poltak et al., 2009). One shared mutation, a *yciR* deletion, as well as a *wspA* mutation in the W variant and a *wspD* mutation in the R variant, all have potential for increasing intracellular cyclic-di-GMP levels in the adapted mutants. I focus on one of these mutations, the *yciR* deletion, shared by all three evolved isolates from 1500 generations
as a potential genetic basis for the RSCV phenotype in biofilm-adapted isolates of *B. cenocepacia* because this mutation likely leads to increased intracellular pooling of cyclic-di-GMP through inactivation of a phosphodiesterase.

Little is known about the specific genetics of the RSCV isolates from chronic respiratory infections, but the presence of these biofilm-adapted colony variants results in a poorer patient prognosis (Starkey et al., 2009). Starkey et al. (2009) confirmed that *P. aeruginosa* RSCV strains produced elevated concentrations of cyclic-di-GMP, either as a consequence of a *wspF* mutation or due to different but functionally similar mutations. Thus, increased intracellular cyclic-di-GMP concentration appears to be the cause of the RSCV phenotype in all *P. aeruginosa* isolates sampled whether they originated from a chronic CF lung infection or were produced in the laboratory. The same mechanism may also occur in laboratory produced and chronic CF lung isolates of *B. cenocepacia*.

In the following chapters I present research on the genes involved in biofilm production in *B. cenocepacia*, specifically those that when mutated increase biofilm production. My approach to identify these genes is two-fold. First, I study mutants created by random transposon mutagenesis that exhibit enhanced biofilm production. Second, I study evolved mutants that rose to high frequency in populations selected for enhanced biofilm production over ~1500 generations and identify their molecular bases. These mutations highlight both the potential mutational paths to increased biofilm production as well as those that are actually favored during adaptation to a biofilm lifestyle.
CHAPTER 2

IDENTIFYING MUTATIONS RESULTING IN INCREASED BIOFILM PRODUCTION

Introduction
Bacterial biofilms are commonly difficult to clear from patients with maladies ranging from ear infections to chronic lung infections; therefore, a better understanding of the mechanisms controlling a persistent biofilm lifestyle is invaluable for treatment. Often, the lungs of cystic fibrosis patients become chronically colonized with *Pseudomonas aeruginosa* and, detected in increasing frequency, with members of the *Burkholderia cepacia* complex (Mahenthiralingam et al., 2008).

Adaptive phenotypes of the CF pathogens, *B. cenocepacia* and *P. aeruginosa*, produced from biofilm populations include a wide range of colony morphologies, from mucoid to wrinkly or rugose, as well as a range of biofilm production and adherence abilities (Haussler et al., 2003; Haussler 2004; Poltak et al., 2009; Smith et al., 2009). Earlier studies utilizing random mutagenesis identified a handful of structural and regulatory genes involved with biofilm formation in *B. cenocepacia* (Huber et al., 2001; 2002; Tomlin et al., 2005; Ferreira et al., 2007; Aubert et al., 2008; Saldias et al., 2008) but it remains an open question whether these mutations are those that produce the adaptive phenotypes seen in bacterial biofilm populations. One critical adaptive mechanism leading to increased biofilm formation in *Burkholderia* may be intracellular pooling of cyclic-di-GMP, which has been identified in *P. aeruginosa* to be responsible
for both colony morphology change and increased biofilm production. Mutations leading to increased intracellular cyclic-di-GMP pooling have been identified both by random mutagenesis (Hickman et al., 2005) and by sequencing of lung-adapted isolates of *P. aeruginosa* (Smith et al., 2006).

The majority of biofilm genes identified to date in *B. cenocepacia* negatively affect biofilm production when inactivated (Saldias et al., 2008; Ferreira et al., 2007; Huber et al., 2001 and 2002). However, it is known that mutations occurring in a portion of chronic *Pseudomonas* isolates from CF patients may enhance biofilm production (Haussler, 2004). Some of the genetic mechanisms conferring this phenotype, often associated with small colony variants (SCVs), are known in *Pseudomonas* (Starkey et al., 2009). Based on the similar *Burkholderia* clinical SCV phenotype of increased biofilm production and strong cell-cell adherence (Haussler et al., 2003), I hypothesize that genetic mechanisms leading to increased intracellular levels of cyclic-di-GMP are also similar between these two CF pathogens.

We do not know the possible mutations that improve biofilm production in *B. cenocepacia*, nor do we know which of these possible pathways underlie the adaptation that occurs in a biofilm-selective environment such as the CF lung. These gaps in our knowledge of *B. cenocepacia* and how it adapts in chronic infections led me to two questions:

1. **What mutations may increase biofilm production in *B. cenocepacia***?

2. **Of the possible mutations contributing to this phenotype, what fraction improve fitness in an environment favoring biofilm production?**
To answer the first question, I created a random transposon mutant library and screened each mutant for increased biofilm production, possibly selecting mutations in genes involved in biofilm regulation. This is the first gain-of-function biofilm screen to be performed with *B. cenocepacia*, though at least one such study was recently performed on *P. aeruginosa* (Ueda and Wood, 2009). Ueda and Wood (2009) described *tbpA*, a tyrosine phosphatase controlling a diguanylate cyclase (*tbpB*). *TbpB* is overexpressed in the *tbpA* mutant and leads to increased intracellular levels of cyclic-di-GMP, increased biofilm, wrinkly colony morphology and decreased motility. I predicted that I would find mutations leading to increased intracellular pooling of cyclic-di-GMP since this would increase biofilm production. I also expected to find gain-of-function biofilm mutations in genes yet to be characterized in *B. cenocepacia* since the gain-of-function biofilm screen has not been utilized yet in this organism.

I wondered how many of the genes identified by my random screens also would acquire mutations in an adaptive model of laboratory biofilm evolution. Replicate populations of *B. cenocepacia* HI2424 were evolved in an environment selecting for increased biofilm production over 1500 generations and produced colony variants resembling RSCV’s that also exhibited greatly increased biofilm production (Poltak et al., 2009). To determine the genetic mechanisms underlying these variants, Poltak et al. (2009) fully sequenced three representative isolates (termed Studded [S], Ruffled Spreader [R] and Wrinkly [W]) from one laboratory-evolved population after 1500 generations of adaptation to the biofilm-selective environment and identified putative adaptive mutations by comparison to the ancestral HI2424 genome. I would be able to
compare the mutations identified in my random screen to those identified in the biofilm-adapted isolates by Poltak et al. (2009) and look for overlapping mutations.

As highlighted by Smith et al. (2006), adaptive mutations frequently arise in the CF lung in chronic *Pseudomonas* isolates in general, the most common being mutations in *mex* (multidrug efflux) genes and *lasR* (QS). Though they are not the most common isolates, SCVs or RSCVs are often recovered from CF patients just as they are experiencing a significant decline in lung function. The genetic basis of the SCV phenotype is not known for most isolates but it is clear that mutations leading to pooling of cyclic-di-GMP occur in all tested *Pseudomonas* RSCVs, which suggests that adaptation to a biofilm lifestyle in the lung predictably follows this path to produce this phenotype (Starkey et al., 2009). Biofilm-adapted populations of *Burkholderia cenocepacia* in our laboratory produced colony variants that resemble clinical RSCVs isolated from chronic CF lung infections and are highly adherent hyper-biofilm producers (Poltak et al., 2009). Since RSCVs in *Pseudomonas* are all linked by a common mechanism (pooling of cyclic-di-GMP), it is likely that the similar phenotypes from our laboratory have adapted using a similar genetic mechanism. Predictability of the outcome and genetic mechanisms of adaptation to a biofilm lifestyle is important knowledge for researchers exploring novel therapeutics to eradicate CF pathogens such as *P. aeruginosa* and *B. cenocepacia* by preventing the transition from an acute to a chronic infection. This leads to my third question:

3. Do bacterial populations adapt to a biofilm lifestyle in a predictable manner?
To answer this question, I looked for mutations shared by laboratory-evolved isolates of *B. cenocepacia* across replicate populations. From the identified mutations in the three fully sequenced biofilm-adapted mutants, at least one mutation per mutant was predicted to result in cyclic-di-GMP pooling; these include mutations in the genes *yciR*, *wspA* and *wspD*. As mentioned previously, pooling of cyclic-di-GMP is a predictable adaptive trait found to occur in all of the studied *P. aeruginosa* RSCVs (Starkey et al., 2009). The finding that mutations occur that may be relevant to cyclic-di-GMP pooling in the three representative evolved mutants prompted me to look at the many other collected mutants from replicate biofilm-evolved populations and start by searching for mutations in the *yciR* gene by targeted sequencing.

**Methods**

**Strains and Culture Conditions**

*E. coli* DH5αλpir and GT115 and *B. cenocepacia* HI2424 and derivatives were routinely propagated at 37°C in T-Soy liquid (30g/L T-Soy powder) or on T-Soy solid agar (14g/L agar).

**Constructing a Tp-Resistant EZ-Tn5 Vector**

The EZ-Tn5™ pMOD™-5<R6Kyor'/MCS> Transposon Construction Vector kit from Epicentre Biotechnologies was used to construct a trimethoprim (Tp) resistant transposon for random mutagenesis of *B. cenocepacia*. The dihydrofolate reductase (*dhfr*) cassette conferring Tp resistance from plasmid pFTP1 (Choi et al., 2005) was cloned into pMOD™-5<R6Kyor'/MCS>. Plasmids were digested with HindIII and the 1kb *dhfr* fragment was isolated, gel-purified, and ligated into pMOD-5. Ligation products
were electroporated into *E. coli* DH5αλpir; recovered cells were plated on T-Soy agar (50μg/ml Tp) and incubated overnight. Tp resistant colonies were screened by colony PCR using MasterTaq (Eppendorf) to verify presence of the *dhfr* cassette using the following cycling parameters and primers:

\[
\begin{align*}
\text{ptnmoddhfr-F:} & \quad 5'\text{-TGGGTCAAAGTAGCGATGAAGCCA-3'} \\
\text{ptnmoddhfr-R:} & \quad 5'\text{-ACGTTCAAGTGCAAGCCACAGGATA-3'} \\
1 \text{ cycle} & \quad 95^\circ C \text{ 4min initial denaturation} \\
30 \text{ cycles} & \quad 95^\circ C \text{ 30sec denaturation} \\
& \quad 60^\circ C \text{ 30sec annealing} \\
& \quad 72^\circ C \text{ 30sec elongation} \\
1 \text{ cycle} & \quad 72^\circ C \text{ 7min final elongation}
\end{align*}
\]

PCR was routinely performed using the MasterTaq kit from Eppendorf according to manufacturer’s directions, unless otherwise stated. The desired construct was verified by restriction digestion with *PvuI* and named pEZTnTp.

**Preparing the Transposome Mix**

The transposome mix for electroporation into target cells was prepared as per manufacturer’s instructions (Epicentre). Instead of the PCR-based protocol I used the restriction digest method of liberating the Tn5Tp portion of the pEZTnTp plasmid. Concentrated plasmid DNA (~10μg) was digested with *PvuI* and the 1.38kb TnTp fragment was isolated from the 1.6kb vector fragment by gel purification. DNA was eluted in TE buffer to avoid contaminating free magnesium to promote stability of the transposon:transposase complex (transposome) to be introduced into target cells. TnTp DNA was mixed with the kit transposase; the resulting transposome mix was incubated at 4°C overnight before storing at -20°C.
Preparing Electrocompetent Cells

Bacterial cells were made electrocompetent by growing to mid-log phase (OD_{600}=0.5) in liquid broth, harvesting by centrifugation at 4°C and rinsing with sterile deionized water twice, followed by one rinse in 10% (v/v) glycerol. Cells were finally pelleted, resuspended in 10% glycerol, aliquotted and stored at -80°C.

Mutagenesis

The TnTp transposome (1µl) was introduced into B. cenocepacia HI2424 by electroporation, spread plated on T-Soy (100µg/ml Tp) and incubated 48hrs at 37°C. Resulting putative transposants were grown overnight in T-Soy (100µg/ml Tp). Glycerol was added to a final concentration of 30% (v/v) and mixed before storing at -80°C. About 3,300 putative transposants were collected in this manner.

Screening for Mutants with Increased Biofilm Production

Biofilm production by all mutants was screened using a crystal violet microplate assay adapted from O’Toole et al. (1999). Initial biofilm assays were performed by replica-plating from freezer stock into T-Soy broth containing 150µg/ml trimethoprim and growing overnight. Saturated cultures were sub-cultured into fresh broth by replica-plating and readings were recorded for optical density at 600nm (OD_{600}). After 24hr incubation, final cell density (OD_{600}) was recorded and liquid removed from wells by inversion. Remaining biofilm was heat-fixed at 80°C for 40min. and stained with 0.01% (v/v) crystal violet for 20min. Stain was removed by inversion and samples were gently rinsed five times with deionized water. Samples were allowed to dry inverted for 2min and were destained with a mixture of 80% (v/v) ethanol and 20% acetone for
15min. The OD$_{600}$ of the remaining crystal violet was recorded and directly corresponded to biofilm yield.

Mutants with at least a 50% increase in total biofilm yield from the wild-type (WT) control of the same assay were selected. Biofilm assays were repeated on this subset of mutants in the same manner but with at least three replicate samples per mutant; 24 mutants were selected that met the 50% increase criterion. Significance of mutant biofilm increase compared to WT biofilm was evaluated by performing two-tailed t-tests assuming equal variance. The 0.05 significance level was corrected by dividing by the number of independent tests (Bonferroni correction).

**Phenotype Screens of Biofilm Mutants**

The 24 mutants with increased biofilm production were tested for altered protease production, swarming motility, exopolysaccharide (EPS) production and colony morphology as these phenotypes are often co-regulated with biofilm production (Cunha et al., 2004; Huber at al., 2001).

Protease production was evaluated by spot-plating 5µl of saturated overnight culture on skim milk agar (T-Soy agar with 10% (v/v) skim milk) and incubating overnight. Zones of clearing on milk plates indicated protease production; the radius of the clearing was measured from the edge of the clearing to the edge of the spotted culture. Swarming motility was evaluated by point inoculating saturated overnight cultures onto low agar T-Soy (0.4% agar). Swarming distances were measured to the furthest point traveled from the point of surface inoculation.
EPS production was evaluated in a qualitative assay by spot plating overnight cultures onto Congo Red (CR) agar [1.5% T-Soy, 0.0015% CR, 0.004% Coomassie blue (adapted from Friedman and Kolter, 2004)], incubating overnight, and evaluating differential dye uptake. This medium allows better visualization of colony morphology as well as relative EPS production than routine culture on T-Soy agar. The redder the colony on CR agar, the less EPS was produced. Light red or colorless colonies on this medium would suggest greater EPS production. Each quantitative mutant phenotype was compared to that of the ancestor by two-tailed t-tests assuming equal variance. The 0.05 significance level was corrected by dividing by the number of independent tests (Bonferroni correction).

Isolation of Genomic DNA and Verification of Source as Burkholderia

Genomic DNA (gDNA) from putative mutants was prepared using a mini-prep salting-out method (Sambrook and Russell, 2001). Briefly, cells from overnight cultures were suspended in Tris-EDTA (10mM EDTA, 10mM Tris-Cl pH8) and lysed overnight at 37°C with shaking in buffer (1% SDS, 50mM Tris-Cl pH8, 50mM EDTA pH8) containing 100μg/ml proteinase K. Saturated ammonium acetate was added and samples shaken vigorously and incubated 1hr at room temp. Nucleic acids were extracted with chloroform to remove the proteins and lipids and then precipitated from the aqueous phase by adding 3 volumes of 100% ethanol and mixing by inversion. DNA was spooled and washed with 70% ethanol and dissolved in TE (10mM Tris, 1mM EDTA, pH8) containing 50μg/ml RNase A. PCR targeting the 16S rDNA sequence was performed to
verify the source of the gDNA as *Burkholderia* using the following primers (LiPuma et al., 1999):

\[
\text{RHG-F: 5’-GGGATTCTTTCCCTTAGTAAC-3’}
\]
\[
\text{RHG-R: 5’-GCGATTACTAGCGATTCCAGC-3’}
\]

Cycling parameters were the same as for the PCR to amplify the *dhfr* gene except the annealing temperature was 55°C with 1 min elongation.

**Rescue Cloning for Location of Insertion Mutations**

Genomic DNA (2 μg) from putative mutants was digested with *Bsa*HI, an enzyme that cuts about once every 1 kb within the HI2424 genome but not within the Tn5Tp sequence, and heat inactivated. Digested gDNA was self-ligated with T4 DNA ligase. Ligation products were transformed by electroporation into *E. coli* GT115 (Invivogen). Recovered cells were incubated overnight on T-Soy (50 μg/ml Tp). Plasmid DNA was harvested from Tp resistant colonies and was prepared for sequencing by mixing 5.5 μl of varying concentration (at least 28 ng/μl) template DNA with 0.5 μl of 10 μM sequencing primer: EZ-Tn5™ pMOD™<MCS> Forward Sequencing Primer (5’-GCCAACGACTACGC ACTAGCCAAC-3’). and sent to the Hubbard Genome Center for sequencing.

**Sequence Analysis**

Sequences were aligned to the *B. cenocepacia* HI2424 reference sequence (http://img.jgi.doe.gov/cgi-bin/w/main.cgi) using BLAST to identify locations of Tn5Tp insertions. The initial sequence generated from each sequencing reaction was that of transposon DNA since the primer anneals to the transposon facing out; the junction of
the transposon and the target gDNA was recognized by the 19-bp mosaic end sequence of the transposon (AGATGTGTATAAGAGACAG). The ‘IMG Top Homologs’ option was used to find annotations of domains of unknown function or hypothetical proteins.

Whole-Genome Sequencing of Evolved Mutants
Genomic DNA was isolated (as described above) from three representative isolates (wrinkly, ruffled spreader, studded) from one population following ~1500 generations of experimental evolution favoring biofilm formation (Poltak et al., 2009). DNA was sent to the University of Houston for Solexa/Illumina sequencing; sequence was aligned to the reference \textit{B. cenocepacia} HI2424 sequence and SNP’s were identified using the programs ELAND (Illumina, San Diego) and MAQ (http://maq.sourceforge.net/).

PCR Screen for Presence or Absence of \textit{yciR} Homolog
A PCR-based screen was performed to determine the presence or absence of the \textit{yciR} homolog, Bcen2424\_3554, in three evolved mutants from all six populations at 1500 generations and biofilm population 1 at 450 generations. The template DNA was isolated as described above for most samples but a boiling lysis preparation was used instead for the 450-generation isolates because it required less time to perform. For those samples prepared by the lysate method, a small sample of a single colony was added to 20\mu l of lysis buffer (0.25% SDS, 0.05N NaOH) and boiled at 95°C for 15min. Boiled samples were cooled and centrifuged and the supernatant was diluted 1:10 with sterile deionized water and centrifuged again before using as template DNA. PCR to amplify the 16S rDNA sequence was run to confirm that all template DNA comes from
Burkholderia before performing PCR to amplify a portion of the yciR homolog. The primers used in the PCR screen for presence/absence of the yciR homolog were:

\[
\text{yciR F: } 5'-\text{AGCAACATCACGGGCTTCTTC-3'} \\
\text{yciR R: } 5'-\text{GCGACCTTCTGGTTCACTCTC-3'}
\]

Cycling parameters were the same as those used for PCR amplification of the dhfr gene except the annealing temperature was 57°C, with a 45sec. elongation and 5min. final elongation.

Results

Biofilm Gain-of-Function Mutant Phenotypes

Out of 3,300 B. cenocepacia HI2424 TnTp insertion mutants, 24 exhibited increased (by at least 50%) biofilm production compared to wild-type HI2424 when evaluated in a crystal violet biofilm assay. These 24 mutants (numbered M1-M24, Table 2.1) were also evaluated for a range of phenotypes often associated with altered biofilm production in other bacteria. The purpose of performing these additional phenotype screens was to further characterize the effects of the insertion mutations and the possible normal function of each disrupted gene or region of DNA in B. cenocepacia. Mutants were assigned a number preceded by ‘M’ (for mutant) from 1 to 24 based on the location in the genome or locus tag.

The 24 mutants were screened for altered protease production, swarming motility, EPS phenotype and colony morphology (Table 2.1). In addition to Table 2.1, complete biofilm, protease production and swarming motility results are graphed for quantitative detail of each phenotype (Figures 2.1, 2.2 and 2.3).
**Table 2.1: Mutants with Increased Biofilm Production**

Each mutant corresponding to the insertion site identified from rescue cloning is listed along with the locus tag assigned by JGI, as well as the available annotation and homolog information from *Pseudomonas aeruginosa* or other *Burkholderia* species. Bold locus tags indicate insertion sites that in the same chromosome region. Numbers following the *wcbR* gene are the base-pair distance from translational start where insertion occurred. An asterisk (*) denotes a darker colony (maroon color), indicating decreased EPS production on CR agar. Absence of an asterisk implies that the colony color was similar to the WT phenotype (pink colony). Scaled biofilm is the result of dividing the OD$_{600}$ value of crystal violet by the OD$_{600}$ value of the cell density after 24hr growth. N/C=No Change

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Locus Tag</th>
<th>Annotation and Homolog</th>
<th>Protease</th>
<th>Swarming Motility</th>
<th>Colony Appearance on CR Agar</th>
<th>% Increase in Scaled Biofilm over WT Scaled Biofilm</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>Bcen2424_0051</td>
<td>hypothetical protein, general secretion pathway N protein, type II secretion, <em>gspN</em></td>
<td>Decreased</td>
<td>N/C</td>
<td>Matte</td>
<td>63</td>
</tr>
<tr>
<td>M2</td>
<td>Bcen2424_0770</td>
<td>glycosyl transferase, group 1, <em>wcbB</em></td>
<td>N/C</td>
<td>N/C</td>
<td>Matte*</td>
<td>141</td>
</tr>
<tr>
<td>M3</td>
<td>Bcen2424_0771</td>
<td>polysaccharide export, <em>wcbC</em></td>
<td>N/C</td>
<td>N/C</td>
<td>Matte</td>
<td>72</td>
</tr>
<tr>
<td>M4</td>
<td>Bcen2424_0775</td>
<td>glycosyl transferase, LPS biosynthesis</td>
<td>N/C</td>
<td>N/C</td>
<td>Matte*</td>
<td>60</td>
</tr>
<tr>
<td>M5</td>
<td>Bcen2424_0778</td>
<td>LPS biosynthesis, <em>wcbD</em></td>
<td>N/C</td>
<td>N/C</td>
<td>Matte*</td>
<td>81</td>
</tr>
<tr>
<td>M6</td>
<td>Bcen2424_0779</td>
<td>insert 5' before <em>wcbO</em> capsule polysaccharide biosynthesis protein, export protein</td>
<td>N/C</td>
<td>Increased</td>
<td>Matte</td>
<td>96</td>
</tr>
<tr>
<td>M7</td>
<td>Bcen2424_0781</td>
<td>sulfatase, Cell wall/membrane/envelope biogenesis, <em>wcbQ</em></td>
<td>Increased</td>
<td>N/C</td>
<td>Matte</td>
<td>58</td>
</tr>
<tr>
<td>M8</td>
<td>Bcen2424_0782</td>
<td>beta-ketoacyl synthase, <em>wcbR 3868</em></td>
<td>N/C</td>
<td>Decreased</td>
<td>Matte*</td>
<td>24</td>
</tr>
<tr>
<td>M9</td>
<td>Bcen2424_0782</td>
<td>beta-ketoacyl synthase, <em>wcbR 7088</em></td>
<td>N/C</td>
<td>N/C</td>
<td>slightly mucoid</td>
<td>92</td>
</tr>
<tr>
<td>M10</td>
<td>Bcen2424_0782</td>
<td>beta-ketoacyl synthase, <em>wcbR 777</em></td>
<td>N/C</td>
<td>N/C</td>
<td>slightly mucoid</td>
<td>43</td>
</tr>
<tr>
<td>M11</td>
<td>Bcen2424_0782</td>
<td>beta-ketoacyl synthase, <em>wcbR 9950</em></td>
<td>N/C</td>
<td>N/C</td>
<td>Matte*</td>
<td>41</td>
</tr>
<tr>
<td>M12</td>
<td>Bcen2424_0949</td>
<td>NADP oxidoreductase coenzyme F420-dependent</td>
<td>N/C</td>
<td>N/C</td>
<td>Matte</td>
<td>113</td>
</tr>
<tr>
<td>M13</td>
<td>Bcen2424_1592</td>
<td>rhodanese domain protein, ATPase, tRNA 2-selenouridine synthase</td>
<td>N/C</td>
<td>N/C</td>
<td>slightly mucoid</td>
<td>51</td>
</tr>
<tr>
<td>M14</td>
<td>Bcen2424_1623</td>
<td>Efflux transporter, HAE1Family</td>
<td>N/C</td>
<td>N/C</td>
<td>Matt</td>
<td>85</td>
</tr>
<tr>
<td>M15</td>
<td>Bcen2424_1747</td>
<td>DUF81</td>
<td>N/C</td>
<td>N/C</td>
<td>slightly mucoid*</td>
<td>69</td>
</tr>
<tr>
<td>M16</td>
<td>Bcen2424_1917</td>
<td>glucose 6 phosphate isomerase, Embden Meyerhoff pathway</td>
<td>N/C</td>
<td>N/C</td>
<td>slightly mucoid*</td>
<td>178</td>
</tr>
<tr>
<td>M17</td>
<td>Bcen2424_2694</td>
<td><em>phoH</em> family protein, phosphate starvation inducible, predicted ATPase</td>
<td>N/C</td>
<td>N/C</td>
<td>mucoid</td>
<td>55</td>
</tr>
<tr>
<td>M18</td>
<td>Bcen2424_2985</td>
<td>hypothetical protein, <em>algP</em> homolog</td>
<td>N/C</td>
<td>N/C</td>
<td>slightly mucoid*</td>
<td>66</td>
</tr>
<tr>
<td>M19</td>
<td>Bcen2424_4356</td>
<td>DUF6</td>
<td>N/C</td>
<td>N/C</td>
<td>very mucoid</td>
<td>95</td>
</tr>
<tr>
<td>M20</td>
<td>Bcen2424_4436</td>
<td>transcriptional regulator, <em>marR</em> family</td>
<td>N/C</td>
<td>N/C</td>
<td>slightly mucoid*</td>
<td>86</td>
</tr>
<tr>
<td>M21</td>
<td>Bcen2424_5271</td>
<td>amino acid permease-associated region</td>
<td>Increased</td>
<td>N/C</td>
<td>Matte</td>
<td>131</td>
</tr>
<tr>
<td>M22</td>
<td>Bcen2424_6217</td>
<td>hypothetical protein, PQQ enzyme repeat, arylsulfotransferase superfamily, <em>S</em> to uracil/xanthine permease</td>
<td>N/C</td>
<td>N/C</td>
<td>matte</td>
<td>36</td>
</tr>
<tr>
<td>M23</td>
<td>Bcen2424_6794</td>
<td><em>traB</em> pilus assembly protein</td>
<td>Increased</td>
<td>N/C</td>
<td>matte, granular*</td>
<td>130</td>
</tr>
<tr>
<td>M24</td>
<td>Bcen2424_6851</td>
<td><em>oprB</em> superfamily carbohydrate-selective porin</td>
<td>N/C</td>
<td>N/C</td>
<td>matte, slightly granular*</td>
<td>86</td>
</tr>
</tbody>
</table>
Figure 2.1: Increase in Mutant Scaled Biofilm Over WT Scaled Biofilm

Original scaled biofilm values were calculated by dividing the OD\textsubscript{600} value of crystal violet by the OD\textsubscript{600} value of the cell density after 24hr growth in a biofilm assay to standardize biofilm production per cell density. The percent increase of mutant biofilm production was calculated relative to WT biofilm production.
The relative protease activity of biofilm mutants was evaluated on skim milk agar plates. Radii of clearings (from the edge of growth to the outer edge of the clearing) were measured for each mutant with three replicates per plate. Error bars represent 95% confidence intervals. Mutant M13 was tested separately from the rest of the mutants and has its own WT reference for comparison, denoted by darker gray bars. Protease production of mutant M1 was significantly different from WT (Supplementary Table 1).
Figure 2.3: Swarming Motility of Biofilm Mutants
Overnight cultures were point inoculated in three replicates onto 0.4% agar T-Soy. Swarming motility was measured from the point of inoculation to the furthest distance traveled from that point. Error bars represent 95% confidence intervals. Only M6 was significantly different from WT for swarming motility.
In *B. cenocepacia*, mutants defective in biofilm may also be defective in protease production and swarming motility if quorum sensing is altered (Huber et al., 2001; 2002). Altered EPS production has been linked to changes in biofilm (Ferreira et al., 2007) and colony morphology changes may correlate with altered biofilm production as well (Friedman and Kolter, 2004).

Phenotypes of transposon mutants were compared to the wild-type *B. cenocepacia* HI2424 control and significance was evaluated as described in Methods (Supplementary Table 1). Mutant M1 produced no protease (Figure 2.2) but significantly more biofilm than wild-type (WT) HI2424 (Supplementary Table 1). Mutants M7, M21 and M23 exhibited elevated protease production but were not significantly different from WT HI2424 (Figure 2.2; Supplementary Table 1). Mutant M6 exhibited significantly higher swarming motility than WT, while M8 appeared to have a modest but not statistically significant swarming defect (Figure 2.3; Supplementary Table 1). Three mutants appeared to be significantly higher biofilm producers but did not exceed the Bonferroni-corrected p-values: M9, M22, and M23 (Figure 2.1; Supplementary Table 1).

Interestingly, there was a range of colony characteristics, from WT (slightly mucoid) to ‘matte’ (not mucoid, not shiny). Only one mutant, M23, appeared to have a granular or studded colony surface on CR agar (Table 2.1). M24 did have a slightly granular or studded colony appearance but this was very subtle while M17 and M19 each produced a more mucoid colony than WT.
Rescue-Cloned Transposon Mutants

I determined the locations of each Tn insertion site by rescue cloning and sequencing (Table 2.1). Ten of the 24 insertion mutations mapped to the same region of chromosome 1 containing the *wcb* genes previously described (Cuccui et al., 2007) as capsular polysaccharide synthesis and export genes in *B. pseudomallei* (Figure 2.4). Cuccui et al. (2007) found that insertions in these homologs resulted in attenuated virulence in a mouse model of infection; a subset of the virulence mutants also had loss of capsule polysaccharide. However, these genes have not been previously characterized as having a role in biofilm formation in *B. cenocepacia* or other members of the Bcc. Four of the insertions from my study are in a large 7.6kb gene annotated as *wcbR* in *B. pseudomallei*, a capsule polysaccharide biosynthesis gene coding for a polyketide synthase, which did not result in virulence attenuation when mutated by Cuccui et al. (2007).

The eight protein domains in *wcbR* in HI2424 are shown in Figure 2.5. Each insertion mutation in this gene mapped to a different functional region. Mutant M8 had the transposon insertion between the acyltransferase and the methyltransferase domains, while the insertion in M9 is mutated between the last two domains, polyketide synthase and PPP binding. Mutant M10 received the Tn5Tp insertion 5' to the beta-ketoacylsynthase C terminal domain and the insertion was found within the alcohol dehydrogenase zinc-binding domain in M11. Each of the protein domains of *wcbR* has a putative role in fatty acid chain synthesis for capsule formation; interrupting any of these domains would theoretically disrupt fatty acid chain synthesis and hinder
Figure 2.4: Chromosome View of \textit{wcb} Region

Shown here is the JGI chromosome view of the region where ten insertion events occurred. The majority of these are in the \textit{wcb} genes annotated as encoding capsular polysaccharide synthesis proteins. Four insertions mapped to the \textit{wcbR} gene (dark gray).

Figure 2.5: Protein Domains of \textit{WcbR}

The beta-ketoacylsynthase, \textit{wcbR}, is comprised of eight main protein domains. The active site of this protein is located between the N and C beta-ketoacylsynthase terminal domains. In order, the domains are: beta-ketoacylsynthase (N-terminal), beta-ketoacylsynthase (C-terminal), acyltransferase, methyltransferase type 12, alcohol dehydrogenase (GroES-like), alcohol dehydrogenase (zinc-binding), polyketidesynthase (KR), phosphopantetheine-binding. Triangles indicate TnTp insertion locations within \textit{wcbR}. Domains are not to scale.
capsule production. Fatty acid-based lipids are important structural components of many bacterial capsules, such as variations produced by *E. coli* and *Neisseria meningitidis* (reviewed by Whitfield, 2006). The precise structure of the *B. cenocepacia* HI2424 capsule is unknown.

**Sequencing of Experimentally-Evolved Mutants Adapted to Biofilm Growth**

Our lab (Poltak et al., 2009) sequenced three laboratory-evolved mutants of *B. cenocepacia* HI2424 which displayed 1-fold to 4-fold increases in biofilm production to identify the genetic bases of biofilm adaptation. These mutants (S, R and W) were isolated from one population and each contained mutations related to cyclic-di-GMP turnover, as well as several other mutations in pathways such as iron sequestration, general efflux, polysaccharide synthesis and general metabolism. Here I will discuss mainly those mutations with potential for affecting cyclic-di-GMP turnover (Table 2.2): the *yciR* homolog deletion occurring in all three morphotypes (S, R and W), the *wspA* mutation in W, and the *wspD* mutation in R.

**Adaptive Loss of *yciR* in Evolved Mutants**

A PCR screen of S, R, and W isolates from six replicate biofilm-evolved populations revealed that most, 16 of 21 isolates, lost the *yciR* gene, though biofilm population 1 from 450 generations produced a W isolate that still has the *yciR* gene (Table 2.3). All R isolates lost *yciR* but two S isolates and two W isolates from populations from 1500 generations retained the gene. Only one of the replicate planktonic populations lost its *yciR* gene (Table 2.3).
**Table 2.2: Mutations in Fully Sequenced Biofilm-Evolved Isolates from 1500 Generations**

Columns are the fully sequenced morphological variants of one population of *B. cenocepacia* adapted to the biofilm environment over 1500 generations of evolution and rows are the mutation(s) thought to be associated with cyclic-di-GMP turnover and leading to increased biofilm in each isolate. An 'X' indicates that the mutation occurs in the isolate at the top of the column.

<table>
<thead>
<tr>
<th></th>
<th>Studded</th>
<th>Ruffled Spreader</th>
<th>Wrinkly</th>
</tr>
</thead>
<tbody>
<tr>
<td>95 gene deletion: <em>yciR</em></td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>SNP in <em>wspA</em> homolog</td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>SNP in <em>wspD</em> homolog</td>
<td></td>
<td>X</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.3: *yciR* Screen of Planktonic and Biofilm Isolates

The presence or absence of *yciR* was determined by PCR in isolates from all adapted populations after 1500 generations and one population after 450 generations of biofilm evolution, including the six planktonically evolved populations. An "yciR-" indicates that the *yciR* gene was no longer present in the isolate. A '+' symbol indicates that a portion of the *yciR* gene was successfully amplified from the specified isolate. Gray boxes indicate that the respective morphology was not present in the population. The three isolates from biofilm population 1 were fully sequenced after 1500 generations of evolution.

<table>
<thead>
<tr>
<th>Population*</th>
<th>Ancestral Morphology</th>
<th>Studded</th>
<th>Ruffled Spreader</th>
<th>Wrinkly</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biofilm 1 (450 generations)</td>
<td></td>
<td>yciR-</td>
<td>yciR-</td>
<td>+</td>
</tr>
<tr>
<td>Biofilm 1 (1500 generations)</td>
<td></td>
<td>yciR-</td>
<td>yciR-</td>
<td>yciR-</td>
</tr>
<tr>
<td>Biofilm 2</td>
<td>+</td>
<td>yciR-</td>
<td>yciR-</td>
<td>yciR-</td>
</tr>
<tr>
<td>Biofilm 3</td>
<td>+</td>
<td>yciR-</td>
<td>yciR-</td>
<td>yciR-</td>
</tr>
<tr>
<td>Biofilm 4</td>
<td>yciR-</td>
<td>yciR-</td>
<td>yciR-</td>
<td></td>
</tr>
<tr>
<td>Biofilm 5</td>
<td>yciR-</td>
<td>yciR-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Biofilm 6</td>
<td>yciR-</td>
<td>yciR-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Planktonic 1</td>
<td>yciR-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Planktonic 2</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Planktonic 3</td>
<td>+</td>
<td></td>
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<td>Planktonic 4</td>
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<td>Planktonic 5</td>
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</tr>
<tr>
<td>Planktonic 6</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*1500 generations unless otherwise noted*
Discussion

Mutations Increasing Biofilm Production in *B. cenocepacia*

I sought to identify mutations that positively influence biofilm production in *B. cenocepacia* and found mutations in 21 unique genes in 24 mutants from a transposon library of 3,300. This approach has not been previously used in *B. cenocepacia*; biofilm genes have traditionally been identified by random screening for biofilm defects. Although more mutants could have been collected to identify other biofilm regulatory genes, 24 mutants were sufficient to identify genes previously uncharacterized as having a role in biofilm formation in *B. cenocepacia*.

One-third of these genes (7) are in homologs of the *wcb* operon previously characterized in *B. pseudomallei* (Cuccui et al., 2007), where it controls capsular polysaccharide biosynthesis. Absence of capsule production in *B. pseudomallei* results in attenuated virulence in mouse models of intraperitoneal and intranasal infection (Cuccui et al., 2007). The study by Cuccui et al. did not test these mutants for biofilm production, but absence of capsule production has been shown to increase biofilm production in the pathogens *Vibrio vulnificus* (Joseph and Wright, 2004), *Porphyromonas gingivalis* (Davey and Duncan, 2006), and *Neisseria meningitidis* (O’Dwyer et al., 2009). It is noteworthy that a loss of capsule in *N. meningitidis* also correlated with colony variation; rough, acapsular small colony variants frequently are produced in *N. meningitidis* biofilms (McEllistrem et al., 2009). *N. meningitidis* is a member of the β-proteobacteria, as is *B. cenocepacia*, and is therefore a close relative whose capsule functions and biofilm regulation may be similar.
It is interesting that 10 of the 24 gain-of-function biofilm mutants (~42%) from my screen have insertions in the same region of chromosome 1, the \( wcb \) operon. This region was also found to be a Tn5 insertional hotspot in \( B.\ pseudomallei \) (Cuccui et al., 2007). Cuccui et al. selected 39 virulence-attenuated signature-tagged mutagenesis mutants from 892 mutants screened. Of those, 22 insertions (~57%) were in the \( wcb \) operon. The investigators proposed that this region was more prone to insertion by Tn5 since it had a 10% lower G+C content (58% G+C) than the rest of the genome (67% G+C).

Upon investigating the homologous region in \( B.\ cenocepacia \) HI2424 containing the \( wcb \) operon, I have found that the region in also has a lower GC content (50-58% G+C) than the rest of the genome (67% G+C) except for the \( wcbR \) homolog, which is higher (71% G+C) in G+C content than the genome average. My random approach may have been biased toward transposon insertion in the \( wcb \) operon for the same reason proposed by Cuccui et al. (2007), but the four insertions in \( wcbR \) do not support this argument since its G+C content is actually greater than the genome average. The chances of four insertion events occurring in the same gene from only 3,300 mutants are low, especially given that the genome of \( B.\ cenocepacia \) HI2424 contains 7,050 genes. However, \( wcbR \) is a large target of 7.6 kb, representing 0.1% of the total genome, so finding at least one mutant is not improbable.

Other genes harboring transposon mutations that increased biofilm production in my study include \( gspN, phoH, algP, marR, traB \) and \( oprB \). The \( gspN \) gene encodes a protein in the general secretory pathway II, which was previously identified as having a role in biofilm production by \( B.\ cenocepacia \) in a screen of mutants defective in biofilm
production (Huber et al., 2002). The gspE insertion mutant characterized in that study caused a biofilm defect, whereas the gspN insertion in my study increased biofilm production.

The PhoH protein is annotated as a phosphate starvation-inducible protein and as a predicted ATPase. PhoH was first characterized in E. coli by Kim et al. (1993) and has a putative role in the transport and use of various forms of combined or free phosphate. The algP gene has been identified in P. aeruginosa as an important regulatory gene in alginate production (EPS), a key pathogenic factor important in chronic CF lung infections (Konyecsni and Deretic, 1990). B. cenocepacia is not known to produce alginate, and thus this pathway likely has another function in this organism.

The MarR family of proteins is annotated as transcriptional regulators, which could possibly control the transcription of any number of factors important to biofilm formation. A mutation in a gene encoding a protein belonging to the MarR family was identified in S. aureus that resulted in increased biofilm formation (Tamber and Cheung, 2009). The TraB protein is annotated as having a role in pilus assembly but traB has no previously known connection to biofilm formation. OprB is annotated as a carbohydrate-selective porin and has been characterized in B. pseudomallei as a component of an efflux pump that is necessary for optimal biofilm production and virulence (Chan and Chua, 2005). It is noteworthy that the insertion in oprB from the B. pseudomallei mutant screen has the opposite effect on biofilm production of the oprB mutant in my study. Insertions in different locations within the same gene may result in
either knockout or overexpression of the gene, depending on the promoter associated with the transposon and whether or not it also contains a stop codon.

Though I expected that mutations resulting in increased intracellular pooling of cyclic-di-GMP would be identified in this screen, I do not have enough information to rule out a role for any of the 24 mutations in cyclic-di-GMP turnover. None of the transposon insertions mapped to genes coding for predicted phosphodiesterases or diguanylate cyclases that may be involved in cyclic-di-GMP turnover. However, that does not mean that the genes identified cannot have a regulatory role or be regulated themselves by cyclic-di-GMP such as the \textit{tbpA} mutation in \textit{P. aeruginosa} (Ueda and Wood, 2009) that leads to increased levels of cyclic-di-GMP through overexpression of a diguanylate cyclase. It may be necessary to perform measurements of intracellular levels of cyclic-di-GMP to answer the question of whether or not this mechanism increases biofilm production in any of these mutants.

\textbf{Genes Identified by Random Mutagenesis not Implicated in Adaptation to Biofilm Lifestyle}

Of the 21 genes that I have identified as regulating biofilm formation in \textit{B. cenocepacia}, none acquired mutations in the three mutants that rose to high frequency during prolonged biofilm selection (Poltak et al., 2009). However, defective capsule production may be a common mechanism for increased biofilm formation between the two groups. Indeed, loss of capsule is known to positively affect biofilm formation in \textit{N. meningitidis} and some rough small colony variants of this organism are defective in capsule production (McEllistrem et al., 2009). In the future we plan to characterize the
capsule production of both the transposon mutants and the evolved biofilm system mutants by either simple staining or immunostaining and confocal microscopy.

**Predictability of Adaptation to a Biofilm Lifestyle**

In each biofilm-adapted population produced by Poltak et al. (2009), a mutant featuring a deletion of the *yciR* gene was found, supporting the hypothesis that populations of *B. cenocepacia* predictably adapt to a biofilm lifestyle. Fixation of this mutation within these biofilm-adapted populations may be due to a selective advantage conferred in the biofilm environment by increased intracellular pooling of cyclic-di-GMP, since this gene may function as a phosphodiesterase that would normally break down the bacterial second messenger.

The importance of studying a CF pathogen such as *B. cenocepacia* and identifying and characterizing genes involved in biofilm regulation is that we know that adaptation occurs in the CF lung (Haussler, 2004; Haussler et al., 2003, Starkey et al., 2009) but we do not know the genetic mechanisms of this adaptation. I am particularly interested in how biofilm-adapted populations of *B. cenocepacia* produce small colony variants (SCVs) that are highly adherent and exhibit increased biofilm production as well as altered colony morphology because clinical SCVs of *B. cenocepacia* and *P. aeruginosa* in the CF lung lead to a poor prognosis for patients and correlate with post-transplant fatalities in the CF population (Haussler et al., 2003). It is critical for development of future treatment of clinical SCVs that we know the genetic basis of the biofilm-adapted SCV in *B. cenocepacia* and whether this phenotype that occurs reproducibly in a laboratory biofilm evolution arises from a predictable genetic mechanism.
**Supplementary Table 1: Phenotypic Changes in Biofilm Mutants Over WT**

The observed phenotypes for protease and swarming are shown along with the t-test statistic for each mutant compared to WT, including the results for t-tests of scaled biofilm values. The Bonferroni corrected significance level (0.05/24) is 0.002; any statistic above this value is not considered significant. Gray highlighted values indicate a significant difference from WT. Mutants M9, M22 and M23 were not significantly different from WT in biofilm production.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Protease</th>
<th>T-test Statistic</th>
<th>Swarming</th>
<th>T-test Statistic</th>
<th>T-test Statistic for Biofilm Increase</th>
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</thead>
<tbody>
<tr>
<td>M1</td>
<td>negative</td>
<td>0.000</td>
<td>0.398</td>
<td>0.001</td>
<td></td>
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<tr>
<td>M2</td>
<td></td>
<td>0.184</td>
<td></td>
<td>0.700</td>
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<tr>
<td>M3</td>
<td></td>
<td>0.624</td>
<td></td>
<td>0.381</td>
<td>0.001</td>
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<tr>
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<td></td>
<td>0.180</td>
<td></td>
<td>0.732</td>
<td>0.000</td>
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<tr>
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<td></td>
<td>0.098</td>
<td>0.000</td>
</tr>
<tr>
<td>M6</td>
<td></td>
<td>0.252</td>
<td>higher</td>
<td>0.002</td>
<td>0.000</td>
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<tr>
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<td></td>
<td>0.163</td>
<td>0.000</td>
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<tr>
<td>M8</td>
<td></td>
<td>0.050</td>
<td>lower</td>
<td>0.018</td>
<td>0.002</td>
</tr>
<tr>
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<td></td>
<td>0.879</td>
<td></td>
<td>0.037</td>
<td>0.004</td>
</tr>
<tr>
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<td></td>
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<td></td>
<td>0.357</td>
<td>0.000</td>
</tr>
<tr>
<td>M11</td>
<td></td>
<td>0.463</td>
<td></td>
<td>0.404</td>
<td>0.000</td>
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<tr>
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<td>0.329</td>
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<td></td>
<td>0.934</td>
<td>0.000</td>
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<tr>
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<td></td>
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<tr>
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<td></td>
<td>0.050</td>
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<td>0.000</td>
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<tr>
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<td>0.204</td>
<td>0.002</td>
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<td>0.242</td>
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<tr>
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<td></td>
<td>1.000</td>
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</tr>
<tr>
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<td></td>
<td>0.463</td>
<td></td>
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</tr>
<tr>
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<td>higher</td>
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<td>0.000</td>
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<tr>
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<td></td>
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<td>0.219</td>
<td>0.029</td>
</tr>
<tr>
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<td></td>
<td>0.067</td>
<td></td>
<td>0.381</td>
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</tbody>
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CHAPTER 3

GENETIC MECHANISMS OF INCREASED BIOFILM PRODUCTION in B. CENOCEPACIA

Introduction
Populations of B. cenocepacia evolved in the laboratory under conditions favoring biofilms produce significantly more biofilm than the ancestral isolate (Poltak et al., 2009). We hypothesize that these evolved populations adapted to growth in biofilms by similar mechanisms to mutants recovered from the lungs of CF patients in chronic stages of infection. Our preliminary data on the physiology and genetics of the evolved populations support this hypothesis, but the precise genetic mechanisms remain to be confirmed. We suggest that these laboratory-evolved populations could potentially be used to model bacterial evolution during chronic infections.

I have attempted to determine some of the key molecular mechanisms by which B. cenocepacia has adapted to life in a biofilm in a laboratory system. Whole genome sequencing of three mutants from one population that evolved for 1500 generations shows that at least 12 mutations have occurred. Seven of these mutations were nonsynonymous substitutions, three were in promoter regions and two involved large, multiple gene deletions (Poltak et al., 2009). I focused on one of these genes, yciR, to characterize its function and relevance to the adaptation to a biofilm lifestyle. The predicted YciR protein (Figure 3.1) has both EAL and GGDEF domains that are found in
phosphodiesterase and diguanylate cyclase enzymes, respectively, that break down or synthesize cyclic-di-GMP (Hengge, 2009). Low levels of cyclic-di-GMP are known to promote a motile lifestyle and high levels elicit the opposite response, promoting biofilm production in *P. aeruginosa* and other bacteria (Hengge, 2009).

**Figure 3.1: Protein Domains of YciR**
The PAS domain may function as a signal receiver, while the GGDEF domain is a predictor of diguanylate cyclase function. The EAL domain likely functions as a phosphodiesterase that breaks down cyclic-di-GMP.

Intracellular pooling of cyclic-di-GMP is responsible for colony morphology changes from smooth to wrinkled colonies as well as increased biofilm formation and decreased motility in *P. aeruginosa*, *Vibrio*, and more (Kulasekara et al., 2005, Lim et al., 2007, Ryan et al., 2006b, Tischler and Camilli, 2005). These phenotypes associated with cyclic-di-GMP are exhibited by each of three distinct variants that arose during 1500 generations of laboratory selection for biofilm formation (Poltak et al., 2009). These phenotypes, taken together with our sequencing data that show mutations in genes predicted to be relevant to cyclic-di-GMP turnover (*yciR*, *wspA* and *wspD*), suggest that pooling of cyclic-di-GMP is occurring in the three mutants of interest, S, R and W.

In order to show that the *yciR* deletion produced the observed phenotypes (wrinkly colonies, increased biofilm and decreased motility), I provided biofilm-adapted mutants with a functional copy of the mutated gene *in trans* based on the prediction that genetic complementation would reverse some or all of the observed phenotypes to
wild-type HI2424. The yciR deletion occurs in all three sequenced W, R and S isolates, whereas the wspA and wspD SNPs occur in only W and R, respectively.

I genetically complemented yciR in R, S and W isolates from two time points in the laboratory evolution, 450 and 1500 generations, and screened for changes in biofilm production, colony morphology and motility, as well as changes in colonization efficiency (fitness) in the biofilm environment to which the mutants had been adapted. I initially complemented yciR in S, R and W from 1500 generations of biofilm evolution and subsequently complemented the S, R and W isolates from 450 generations of biofilm evolution, even though the genomes of these earlier isolates have not yet been fully sequenced. It is likely that fewer mutations had accumulated by 450 generations than at 1500 generations. Therefore, I expected better (closer to wild-type) complementation of colony morphology, biofilm production, motility and fitness in the biofilm environment by the introduction of intact yciR in trans into the 450 generation isolates than the 1500 generation isolates due to epistatic effects of additional mutations after 1500 generations.

Methods

Strains and Culture Conditions
E. coli GT115 (Invivogen) and TOP10 (Invitrogen) and B. cenocepacia HI2424 and derivatives were routinely propagated at 37°C in T-Soy liquid (30g/L T-Soy powder) or on T-Soy solid agar (14g/L agar).

Cloning yciR for Genetic Complementation
After a series of cloning steps, an intact copy of yciR was cloned into a stably maintained vector, pBBR1MCS (Kovach et al., 1994), for genetic complementation, as
described below. The wild-type yciR gene from *B. cenocepacia* HI2424 was amplified by PCR with MasterTaq (Eppendorf) using the following primers and cycling parameters:

\[
\begin{align*}
\text{yciR comp F: } & 5'-\text{TCACGGCAATCACGGCAG-3'} \\
\text{yciR comp R: } & 5'-\text{ATCTACACGGGCTTCGGCAC-3'} \\
1 \text{ cycle} & 95^\circ C \text{ 4min initial denaturation} \\
25 \text{ cycles} & 95^\circ C \text{ 30sec denaturation} \\
& 58.6^\circ C \text{ 30sec annealing} \\
& 72^\circ C \text{ 2min 30sec elongation} \\
1 \text{ cycle} & 72^\circ C \text{ 5min final elongation}
\end{align*}
\]

PCR was routinely performed using the MasterTaq kit from Eppendorf according to manufacturer’s directions, unless otherwise stated.

PCR products were cloned into pCR®-XL-TOPO (Invitrogen). Cloning products were transformed by electroporation into One Shot® TOP10 Electrocompetent *E. coli* (Invitrogen) at 1.8Kv in a 1mm gap cuvette. Cells were recovered and spread-plated onto T-Soy agar containing 50μg/ml kanamycin (Kan). Plasmid DNA was isolated from Kan-resistant colonies using the Wizard® Plus SV Minipeps DNA Purification System. Plasmid DNA was screened for presence of yciR by PCR using the yciRcomp primers as described in Chapter 2 and one correct clone was named pyciRTOPO.

The yciR gene was excised from pyciRTOPO by double digestion with *XbaI* and *HindIII* (NEB) and gel purified using the Wizard® SV Gel and PCR Clean-up System. The broad-host range vector pBBR1MCS was also double digested with *XbaI* and *HindIII*. The digested and gel purified yciR gene and double digested pBBR1MCS were ligated at a 6:1 (suggested by manufacturer) insert:vector ratio with T4 DNA ligase (NEB) at 16°C overnight.
Ligation products were transformed by electroporation into *E. coli* GT115 (Invivogen) as described in Chapter 2. Plasmid DNA was isolated as described above and screened for presence of *yciR* by PCR; one correct clone was named pyciRcom. The complementation vector, pyciRcom, was electroporated into electrocompetent evolved isolates of *Burkholderia*, prepared and electroporated as described in Chapter 2. The pBBR1MCS vector served as a positive control and a cell only 'no DNA' control was also included as a negative control. Recovered cells were plated on T-Soy agar containing 100μg/ml chloramphenicol (Cm) and incubated overnight. Cm-resistant colonies were screened by colony PCR using the yciRcom primers.

**Phenotype Screens for Effects of Genetic Complementation**

Biofilm, swarming motility, colony morphology and protease production were evaluated to determine the effects of genetic complementation with *yciR* on biofilm adapted isolates of *B. cenocepacia* HI2424. Biofilm assays were performed as described in Chapter 2 except that 24-well plates were used for the 450 generation samples in addition to the 96-well plate assay since error was high for these samples in the smaller volume wells perhaps due to biofilm clumps detaching from the inside well surface. Swarming motility of 450 generation samples was evaluated by point-inoculating saturated overnight cultures onto 0.4% agar T-soy and measuring distance traveled from inoculation point after 24 hours. Protease production of 450 generation isolates was evaluated by spotting 5μl of saturated overnight culture onto 2% skim milk T-Soy agar plates and incubating overnight. Zones of clearing indicating protease production were measured as the distance from the edge of the colony to the outer edge of the clearing.
Colony morphology of all isolates was evaluated by isolating colonies on Congo Red (CR) agar [1.5% T-Soy, 0.0015% CR, .004% Coomassie blue (adapted from Friedman and Kolter 2004)].

**Assays of Mutant Fitness**

The fitness of evolved mutants and mutants complemented with pyciRcom was measured in the laboratory biofilm evolution environment. Cultures were started from single colonies on T-Soy with or without 100µg/ml Cm and grown for 24hrs in evolution medium (3% galactose M9 minimal medium) with a 3mm polystyrene bead. Beads were removed with bent inoculating loops and vortexed in 1.5ml of PBS (0.765% NaCl, 0.0724% Na$_2$HPO$_4$, 0.021% KH$_2$PO$_4$) twice for 10sec. Replicate samples were taken from the vortexed bead broth and diluted 1:50 in fresh evolution medium with a fresh bead. Samples from each replicate were diluted and plated onto 1.5% T-Soy agar and incubated overnight to estimate culture density at t=0 and observe colony morphology. The 1:50 dilutions in fresh medium with a fresh bead were incubated for 24 hrs; cells were harvested from beads in the same manner as the initial t=0 cultures. Harvested cells were diluted and plated onto 1.5% T-Soy agar and incubated overnight for t=24hrs cell density calculations. Biofilm fitness was calculated as the logarithm of the increase in cells attached to the bead over 24h, as follows: $\ln[\text{CFU/ml}_{24\text{hrs}}/\text{CFU/ml}_{0\text{hrs}}]$.

**Assay of Relative Quorum-Sensing Molecule Production**

A GFP-based reporter assay was performed on evolved isolates to determine changes in production of C8-homoserine lactones (C8 HSL) as described (Steidle et al., 2001). Evolution medium cultures (with and without bead) were started from single
colonies and grown overnight. Cultures were centrifuged and supernatants were filter-sterilized (0.22μm cellulose acetate column filter, Corning). Cell-free supernatants were diluted 1:11 in sterile deionized water and dilutions mixed 1:1 with log phase sensor strain, *Pseudomonas putida* F117 pAS-C8, that had been grown overnight at 28°C in liquid T-Soy containing 20μg/ml gentamycin and sub-cultured on day of assay. Sensor strain and cell-free supernatants were incubated overnight at 28°C with shaking. Fluorescence intensity readings were taken on the plate reader (Tecan) in a 96-well black plate with clear well bottoms using the settings for optimal gain with excitation at 474nm and emission at 515nm. Negative controls included *E. coli* OP50 as well as the HI2424-R cepR knockout strain that is defective in QS molecule production (Chapter 3); the blank was a 1:1 mix of sensor strain and liquid T-Soy.

**Scanning Electron Microscopy (SEM)**

Cultures for SEM were grown in evolution medium with bead (started from single colonies) overnight (~23hrs) at 37°C. Beads were removed with a bent inoculating loop and suspended in 3% gluteraldehyde at 4°C for 3hrs. Beads were washed twice in PBS before a series of ethanol washes, each for 10min: 30%, 50%, 70%, 95%, and two 100% washes. Two final hexamethyldisilazane (HMDS) washes were performed before allowing beads to air dry. Dry beads were mounted on adhesive tape for transport before further drying and coating with gold and palladium. SEM images were taken on the Amray 3300FE field emission SEM at magnifications ranging from 200-20,000X.
Results

Genetic Complementation of yciR

Evolved isolates of *B. cenocepacia* HI2424 from 450 and 1500 generations of biofilm adaptation containing no introduced vector, containing the control vector pBBR1MCS, or containing the complementation vector, pyciRcom, were tested for phenotypic changes in a variety of settings. Phenotypes of complemented isolates were compared to phenotypes of isolates harboring the empty control vector, pBBR1MCS (Table 3.1). None of the evolved isolates were fully restored to wild-type phenotypes when complemented with the yciR gene. Wild-type *B. cenocepacia* HI2424 did not show any phenotypic changes when provided an extra copy of yciR in trans except that increased protease production was observed (Table 3.1; Figure 3.2).

Fewer phenotypic changes were observed in the yciR complemented isolates at 1500 generations than the yciR complemented 450 generation isolates (Table 3.1). The isolates from 1500 generations did not undergo changes in their colony morphologies when complemented with yciR (data no shown), but did show some changes in biofilm production, fitness, and yield when possessing the complementation vector pyciRcom. Complemented W<sub>1500</sub> (W isolate from 1500 generations) exhibited significantly reduced biofilm and increased fitness (Figure 3.3). R<sub>1500</sub> also had significantly reduced biofilm, but no other phenotype tested was changed. S<sub>1500</sub> did not undergo changes in biofilm or fitness, but was the only 1500 generation isolate to exhibit a significant reduction in C8-HSL production (Figure 3.4). I chose to include relative C8-HSL QS molecule production in this analysis because a yciR mutant in *B. cenocepacia* H111 was previously shown to be defective in QS molecule production (Huber et al., 2002) and I predicted
Table 3.1: Summary of _yciR_ Complemented Phenotypes for Isolates Containing _pyciRcom_ Relative to Empty Vector Control

The complemented phenotype data for each biofilm-adapted isolate is compared to empty vector data for isolates. 'Unchanged' denotes no significant difference from empty vector; 'Increased' or 'Reduced' denotes a significant phenotype change relative to the empty vector control. N/A is listed for samples not evaluated for the given phenotype.

<table>
<thead>
<tr>
<th>Control Isolate*</th>
<th>Biofilm</th>
<th>Bead Fitness</th>
<th>Bead Yield</th>
<th>Relative C8-HSL Production</th>
<th>Protease Production</th>
<th>Swarming Motility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-Type HI2424</td>
<td>Moderate</td>
<td>Low</td>
<td>Low</td>
<td>Moderate</td>
<td>High</td>
<td>Moderate</td>
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<tr>
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<td>High</td>
<td>High</td>
<td>High</td>
<td>Low</td>
<td>Moderate</td>
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<tr>
<td>Ruffled Spreader 450gen.</td>
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<td>High</td>
<td>High</td>
<td>Low</td>
<td>Low</td>
<td>Moderate</td>
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<tr>
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<td>High</td>
<td>Low</td>
<td>Low</td>
<td>Moderate</td>
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<tr>
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<td>N/A</td>
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<td>High</td>
<td>Moderate</td>
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<td>N/A</td>
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<tr>
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<td>N/A</td>
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<td>Complemented Isolate**</td>
<td>Biofilm</td>
<td>Bead Fitness</td>
<td>Bead Yield</td>
<td>Relative C8-HSL Production</td>
<td>Protease Production</td>
<td>Swarming Motility</td>
</tr>
<tr>
<td>Wild-Type HI2424</td>
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<td>Unchanged</td>
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<td>Reduced</td>
<td>Reduced</td>
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<td>Unchanged</td>
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<tr>
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<td>Reduced</td>
<td>Unchanged</td>
<td>Reduced</td>
<td>Increased</td>
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</tr>
<tr>
<td>Studded 450gen.</td>
<td>Reduced</td>
<td>Unchanged</td>
<td>Unchanged</td>
<td>Increased</td>
<td>Unchanged</td>
<td>Reduced</td>
</tr>
<tr>
<td>Wrinkly 1500gen.</td>
<td>Reduced</td>
<td>Increased</td>
<td>Unchanged</td>
<td>Unchanged</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Ruffled Spreader 1500gen.</td>
<td>Reduced</td>
<td>Unchanged</td>
<td>Unchanged</td>
<td>Unchanged</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Studded 1500gen.</td>
<td>Unchanged</td>
<td>Unchanged</td>
<td>Unchanged</td>
<td>Reduced</td>
<td>N/A</td>
<td>N/A</td>
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*Transformed with Empty Vector  
**Transformed with _pyciRcom_
Figure 3.2: Swarming Motility and Protease Production of yciR-Complemented 450 Generation Isolates

For each isolate tested for biofilm production there is the test strain containing pyciRcom, a control strain containing pBBR1MCS, and the original strain. ‘WT’ represents *B. cenocepacia* HI2424; ‘S’ ‘R’ and ‘W’ represent the studded, ruffled spreader and wrinkly morphotypes, respectively. Zones of clearing on milk agar were measured for relative protease production for each sample and swarming motility was measured on 0.4% agar T-Soy as the farthest distance traveled from point of inoculation. Error bars represent 95% confidence intervals, n=3. Significant differences between transformation with pyciRcom and pBBR1MCS are seen for WT, W450 and R450 for protease production. There is a significant difference between the two transformed samples of S450 for swarming motility.
Figure 3.3: Bead Yield, Fitness and Scaled Biofilm of yciR-Complemented Isolates at 1500 generations

For each isolate there is an isolate containing yciRcom, a control containing pBBR1MCS, and the original strain. ‘WT’ refers to B. cenocepacia HI2424; ‘S’ ‘R’ and ‘W’ represent the studded, ruffled spreader and wrinkly morphotypes, respectively. Bead yield (A), fitness (B) and scaled biofilm (C), were calculated. Scaled biofilm values were calculated by dividing the OD$_{600}$ values of crystal violet per sample by the OD$_{600}$ of the 24hr cell growth. Error bars represent 95% confidence intervals, n=3 for bead assays; n=7 for biofilm assay. Light gray bars indicate a significant difference between the phenotype indicated for that isolate with yciRcom versus the empty vector control, pBBR1MCS.
Figure 3.4: Relative C8-HSL Production of yciR-Complemented 450 Generation Isolates

For each isolate tested for biofilm production there is the test strain containing pycIiRcom, a control strain containing pBBR1MCS, and the original strain. "WT" represents *B. cenocepacia* HI2424; 'S' 'R' and 'W' represent the studded, ruffled spreader and wrinkly morphotypes, respectively. Fluorescence intensity was measured for each supernatant sample mixed with sensor strain. Relative C8-HSL production is calculated by dividing the fluorescence intensity by log transformed OD_{600} of test sample pre-filter. Error bars represent 95% confidence intervals, n=3 from one assay. Light gray bars indicate a significant difference between the relative QS molecule production indicated for that isolate with pycIiRcom versus the empty vector control, pBBR1MCS.
that providing *yciR in trans* would increase QS production in the defective biofilm isolates.

All three morphological variants (S, R and W) from 450 generations exhibited phenotype changes when complemented with the *yciR* complementation vector, pyciRcom. W_{450} showed the most changes after complementation, though this is the only isolate that still contains the *yciR* gene (Table 2.3) as verified by a PCR screen. Reductions were observed in biofilm (Figure 3.5), bead fitness (Figure 3.6A), and C8-HSL production (Figure 3.4), and an increase was seen in and swarming motility (Figure 3.2). Colony morphology of W_{450} reverted back to the WT slightly mucoid appearance but remained small in size (Figure 3.7). Scanning electron microscopy revealed that biofilm formation on a bead is altered in the complemented W_{450}, which covers the bead more evenly than the clumping seen in the controls (Figure 3.8). Cell size of W_{450} changed when complemented; cells appeared longer and more like WT (Supplementary Figure 1).

The R_{450} isolate had reduced biofilm (Figure 3.5) and bead yield (Figure 3.6B) with an *in trans* copy of *yciR*, though bead fitness was unchanged. Relative quorum sensing molecule production was increased (Figure 3.4), as was protease production (Figure 3.2), though swarming motility remained the same. Colony morphology did not revert to WT, but did change from tight wrinkles to a more spreading, less wrinkly colony (Figure 3.7). Coverage on the bead also changed from many biofilm clumps to fewer clumps (Figure 3.8).
Figure 3.5: Biofilm Production of yciR Complemented 450 Generation Isolates

For each isolate tested for biofilm production there is the test strain containing yciRcom, a control strain containing pBBR1MCS, and the original strain. ‘WT’ represents *B. cenocepacia* HI2424; ‘S’ ‘R’ and ‘W’ represent the studded, ruffled spreader and wrinkly morphotypes, respectively. Scaled biofilm values were calculated by dividing the OD_{600} values of crystal violet per sample by the OD_{600} of the 24hr cell growth prior to performing the staining portion of the biofilm assay. Error bars represent 95% confidence intervals, n=4. Light gray bars indicate a significant difference between the scaled biofilm for that isolate with yciRcom versus the empty vector control, pBBR1MCS.
Figure 3.6: Fitness and Bead Yield of Complemented 450 Generation Isolates
For each isolate tested for absolute fitness on a bead (A) and bead yield (B) there is the test strain containing pyciRcom, a control strain containing pBBR1MCS, and the original strain. 'WT' represents *B. cenocepacia* HI2424; 'S' 'R' and 'W' represent the studded, ruffled spreader and wrinkly morphotypes, respectively. The suffix 'yciR' implies introduction of pyciRcom and 'pBBR1MCS' the control vector pBBR1MCS. Error bars represent 95% confidence intervals n=3 except for S450, n=2. Light gray bars indicate a significant difference between the bead yield indicated for that isolate with pyciRcom versus the empty vector control, pBBR1MCS.
Figure 3.7: Colony Morphologies of 450 Generation Isolates
Digital photographs of each isolate were taken after allowing colonies to develop at room temperature for a week after initial overnight incubation at 37°C on CR agar. 'WT,' 'W450,' 'R450,' and 'S450' refer to *B. cenocepacia* HI2424, and three 450 generation isolates wrinkly, ruffled spreader and studded, respectively. All pictures are representative of the colonies from each sample and are shown at the same scale. Column labels of 'no vector,' 'pBBR1MCS control,' and 'pyciRcom' indicate the vector status of each morphological variant genotype.
Figure 3.8: Scanning Electron Micrographs of vciR-Complemented 450 Generation Samples on Beads
Composite pictures were assembled at 1,000X magnifications of the three evolved morphotypes at 450 generations.
The $S_{450}$ isolate had reduced biofilm (Figure 3.5), but unchanged bead fitness and yield (Figure 3.6). C8-HSL production increased (Figure 3.4), protease was unchanged and swarming motility was reduced (Figure 3.2). Colony morphology changed from many small wrinkles to a flatter, crater-studded colony with some wrinkles (Figure 3.7) but any change in bead coverage change was subtle, with the complemented $S_{450}$ appearing to have more sparse coverage (Figure 3.8).

**Discussion**

The goal of this study was to genetically complement one mutation, the $yciR$ deletion, acquired after laboratory biofilm adaptation in three fully sequenced isolates from 1500 generations of adaptation from one population. The $yciR$ gene is attractive to study because it contains an EAL amino acid motif that often identifies a phosphodiesterase that breaks down cyclic-di-GMP (Hengge, 2009). This deletion may lead to increased levels of intracellular cyclic-di-GMP, which has been shown to lead to increased biofilm production and wrinkly or rugose colony morphology (Hengge, 2009), similar to the phenotypes observed in the three genotype variants sequenced from 1500 generations.

It is noteworthy that the $yciR$ deletion is the starting point of a large 95-gene deletion that occurred in all three sequenced evolved isolates of *B. cenocepacia* HI2424 (Poltak et al., 2009). The deletion of $yciR$ in all three morphotypes of *B. cenocepacia* (S, R and W) at 1500 generations of evolution from an adaptive biofilm population, as well as the SNPs in the *wspA* gene in the W isolate and *wspD* in the R isolate (Poltak et al. 2009), suggests that selection to form more biofilm favored increased intracellular
concentrations of cyclic-di-GMP. These three mutations all have implications for increased intracellular pooling of cyclic-di-GMP. First, we know that a yciR deletion in *E. coli* (Weber et al., 2006) and *Salmonella* (Garcia et al., 2004) increased biofilm production and I predicted that the complete deletion of the gene, as occurred in the biofilm-adapted isolates, had the same effect in our system. However, a yciR transposon insertion mutant of *B. cenocepacia* H111 (Huber et al., 2002) was defective in biofilm formation and quorum sensing (QS) molecule production. This mutant did not revert its phenotype when complemented with a functional cepR, the regulator gene of the CepIR QS system, leading to the conclusion that YciR is an upper-level regulator of the CepIR QS system.

Though the *B. cenocepacia* H111 yciR insertion mutant did not behave similarly to the yciR deletion mutants of *Salmonella* and *E. coli*, it is possible that the insertion mutation did not abolish phosphodiesterase activity of the YciR protein or to cyclic-di-GMP pooling, whereas a complete deletion may have these effects. The transposon insertion in yciR in H111 (Huber et al., 2002) may not have inactivated phosphodiesterase activity of yciR, depending on where in the gene the transposon inserted. If the transposon inserted before the EAL domain, the downstream portion of yciR may still have been translated if there is a strong promoter associated with the transposon and no translational stop codon. Maintained phosphodiesterase function of yciR may explain the conflicting phenotypes observed between *B. cenocepacia* H111 and mutants in other bacteria.
To show that the loss of \( yciR \) affected biofilm production, I genetically complemented six isolates, three from 450 generations and three from 1500 generations of biofilm adaptation, to evaluate the effects on biofilm and related phenotypes (colony morphology, motility, fitness in a biofilm evolution environment). I found that providing an intact copy of \( yciR \) in \( \text{trans} \) alters many phenotypes of the evolved colony morphology variants, the most obvious of these being the loss of the wrinkly colony phenotype of the \( W_{450} \) mutant. Interestingly, this is the one isolate that retained the \( yciR \) gene.

Though \( W_{450} \) has retained the \( yciR \) gene, it may not have retained the \( yciR \) gene function. I plan to fully sequence \( yciR \) in \( W_{450} \) to determine if function may be altered due to a mutation within the gene itself. It is puzzling that providing an intact \( yciR \) in \( \text{trans} \) in this isolate had the most dramatic effects on colony morphology, biofilm, and fitness in the biofilm evolution environment. If the gene is not mutated, another way to determine if the isolate expresses \( yciR \) would be to perform expression studies, such as reverse transcription-PCR, to show that the gene is not being expressed in the mutant (Chan and Chua, 2005).

I chose to evaluate effects of the complementation vector on QS molecule production of the biofilm-adapted mutants because YciR is thought to be an upper-level regulator of the \( cep \) QS system (Huber, et al., 2002) and because initial data (S. Poltak, personal communication) suggested that the biofilm-adapted mutants were defective either in sensing or production of the QS signal. It is interesting that introducing the complementation vector into WT HI2424 did not induce increased relative C8-HSL.
Since the yciR mutant in B. cenocepacia H111 (Huber et al., 2002) abolished QS molecule production, additional yciR in HI2424 should have an opposite effect, if the functions are equivalent, and increase C8-HSL QS molecule production. Since I did not observe increased relative QS molecule production in WT HI2424 containing the complementation vector, pyciRcom, it seems that YciR either does not have the same function in strain HI2424 as in H111 or that the initial, limited characterization of function in strain H111 was incorrect. It is interesting that introducing the complementation vector actually reduced relative C8-HSL production in the W isolate from 450 generations and the S isolate from 1500 generations. Perhaps other mutations acquired by these generations exert epistatic effects on QS in the biofilm-adapted isolates or perhaps YciR truly does not function as previously thought. To better show that the reporter assay I adapted to determine relative increase of C8-HSL is a legitimate assay for this purpose, it would be necessary to create a standard curve of fluorescence intensity relative to known concentrations of C8-HSL as well as isolating HSLs rather than using conditioned medium.

It is also critical, for all isolates, to measure the intracellular levels of cyclic-di-GMP to determine if pooling of this secondary messenger correlates with the observed biofilm phenotypes, as has been observed previously in P. aeruginosa, E. coli, Vibrio, and Salmonella (Kulasekara et al., 2005, Garcia et al., 2004, Tischler and Camilli, 2005, Weber et al., 2006). We will perform this measurement via either TLC with radiolabelled P$^{32}$ (Hickman et al., 2005) or MALDI-TOF Mass Spectrometry analysis on HPLC-purified nucleotides (Simm et al., 2009).
I predict that quantitation of cyclic-di-GMP levels in S, R, and W will show that each has greater concentration than the ancestral strain, with the S isolate having the lowest pool of the di-nucleotide of the three and R and W having highest cyclic-di-GMP concentrations. This prediction is supported by the fact that only the yciR deletion in S is predicted to lower levels of cyclic-di-GMP whereas R and W each have additional mutations in one of the wsp genes that would likely add to the pool. Perhaps the initial loss of yciR leads to pooling of cyclic-di-GMP, the S colony phenotype and increased biofilm, and the secondary mutations in wsp genes lead to increased pooling, and exaggerated colony wrinkling and even higher biofilm production in W and R.

The single nucleotide polymorphisms (SNPs) in wspA and wspD in the W and R isolates, respectively, may lead to pooling of cyclic-di-GMP through constitutive phosphorylation of wspR, a diguanylate cyclase that would continue to catalyze the formation of cyclic-di-GMP, resulting in an abundance of this di-nucleotide. The proposed mechanism of the Wsp pathway, based on studies in P. aeruginosa, is shown in Figure 3.9. The non-synonymous SNP in wspA in the W1500 isolate may alter the WspF binding site in the translated WspA protein, preventing WspF from performing its methylesterase activity to demethylate conserved glutamate residues and thereby shutting down the Wsp system. Without the balancing function of WspF activity, the methyltransferase, WspC, will constitutively methylate WspA and cyclic-di-GMP will be constitutively produced (Guveneer and Harwood, 2007; Bantinaki et al., 2007). I predict that the non-synonymous SNP in wspD in the R1500 isolate may alter the WspA protein because the two are closely associated and would deny WspF access to its binding
The proposed mechanism of cyclic-di-GMP production by the Wsp pathway in *B. cenocepacia* based on the pathway in *P. aeruginosa* (adapted from Guvener and Harwood, 2007; Bantinaki et al., 2007), which is analogous to the Che pathway in *E. coli*. WspA is a predicted membrane-associated methyl-accepting chemotaxis protein; WspC is a predicted methyltransferase that methylates conserved glutamate residues on WspA. WspF is a predicted methylesterase with a CheY-like response regulator domain. When phosphorylated, WspF removes methyl groups from WspA. WspB and WspD are scaffold proteins. WspE is a histidine kinase that autophosphorylates when WspA becomes methylated, transferring phosphate groups to WspR, a diguanylate cyclase (DGC) with a response regulator domain. The activated DGC causes two molecules of GTP to combine and form cyclic-di-GMP. WspE also transfers a phosphate group to WspF, which demethylates WspA and 'turns off' the Wsp pathway. WspC constitutively methylates WspA (in *Pseudomonas*). If there is a defect in WspF methylesterase activity then the pathway remains 'on' and WspE continues to activate WspR, resulting in pooling of cyclic-di-GMP. The SNP in *wspA* in W1500 may affect the predicted binding site of WspF in the amino acid sequence, preventing WspF from 'shutting off' the pathway. Likewise, the SNP in *wspD* in R1500 may alter the protein structure enough that WspF cannot 'find' its binding site on WspA to demethylate the glutamate residues.
site on WspA, producing a similar predicted effect as the wspA mutation in W_{1500}.

Future work to continue this research will involve creation of a yciR deletion in the HI2424 ancestral background to evaluate effects of this single mutation on biofilm formation, motility and fitness in the biofilm evolution environment. Epistatic effects of additional mutations at both 450 and 1500 generations may mask or enhance the phenotypes of the loss of yciR. The evolved isolates from 1500 generations have almost certainly acquired more mutations than those at 450 generations because populations remained under strong selection to adhere to the bead and exhibit enhanced fitness in the selective environment (S. Poltak, personal communication). The more additional mutations that have accumulated, the more likely it is that epistatic effects of these mutations will contribute to the increased biofilm phenotypes in concert with the original mutation.

In addition, I propose creating mutations in wspA and wspD of the ancestral clone to evaluate their effects on biofilm fitness individually and also to complement the evolved isolates with functional copies of either wspA or wspD.

I propose that the initial loss of yciR is a key mutation along the path to biofilm adaptation. Though one isolate at 450 generations still has a yciR gene; by 1500 generations the loss of yciR has fixed in all three isolates. This lends support to the hypothesis that the yciR deletion gives evolved isolates a fitness advantage in the biofilm evolution environment since isolates with this mutation prevail and rise to high frequency by 1500 generations.
Though the \textit{yciR} deletion may be an important adaptive mutation, it is not the only adaptive mutation that may occur predictably in a biofilm-selective environment in the transition from a planktonic (acute) to a biofilm (chronic) lifestyle. However, mutations in three genes linked to cyclic-di-GMP turnover in three separate evolved isolates lend support to the hypothesis that cyclic-di-GMP pooling is a key adaptive mutation in our system.

There are several drawbacks to my genetic complementation approach that must be considered when making conclusions about the function of \textit{yciR} in \textit{B. cenocepacia} HI2424. One drawback is that we do not know the full genetic background of the biofilm-adapted isolates from 450 generations of evolution and can only speculate on the other mutations that have fixed at this timepoint. Genotyping of isolates from this time point is ongoing and full genome sequencing is on the horizon for these mutants. We also do not know whether the \textit{yciR} gene present in the W isolate from 450 generations is functioning; the first step would be a complete sequence of this gene. Though I cloned enough of the \textit{yciR} gene that I should have captured its promoter region, I also directionally cloned the gene into pBBR1MCS to create the complementation vector pyciRcom. One consequence of the cone construction is that the \textit{lac} promoter within pBBR1MCS may constitutively express \textit{yciR}, leading to anomalous phenotype results. I saved constructs of \textit{yciR} in pBBR1MCS that have the gene in the opposite direction and it would be interesting to test these for complementation in the biofilm-evolved isolates.
In conclusion, we believe that adaptation to the laboratory biofilm selective environment parallels adaptation to the cystic fibrosis lung. Indeed, mutations leading to cyclic-di-GMP pooling have been identified in clinical lung isolates of *P. aeruginosa* (Starkey et al., 2009; Smith et al., 2006). If cyclic-di-GMP pooling also occurs predictably in *Burkholderia* clinical lung infections, allowing *B. cenocepacia* to persist in the lung and continue to contribute to lung damage and functional decline, pathways of cyclic-di-GMP synthesis would be a target for novel therapeutics. The more we know about the genes and mechanisms that are altered in adapting to a biofilm or chronic lifestyle, the better prepared we may be to combat biofilm-associated infections through novel therapeutics. Through our laboratory model, I have identified putative cyclic-di-GMP pooling as an important adaptation to the chronic biofilm lifestyle and therefore potential target for disruption of chronic biofilm communities.
Supplementary Figure 1: Scanning Electron Micrographs of 450 generation Samples on Beads
Shown are the same samples as in Figure 1 but at 10,000X magnification. Colony pictures are included for reference.
APPENDIX

cepR KNOCKOUT

Introduction
The genus *Burkholderia* in general has been shown to employ quorum-sensing (QS) systems necessary for maximum virulence in animal host models. *B. cepacia* specifically employs the CepIR QS system, relying on C8-HSL, to regulate virulence and specific factors relevant to virulence, such as biofilm formation (Eberl, 2006). As reviewed in previous chapters, the YciR protein, which contains domains implicated in cyclic-di-GMP turnover, is thought to be an upper-level regulator exerting post-transcriptional control over CepR. The current model of biofilm regulation centers on the CepIR quorum-sensing system (Figure A1). A functional CepIR system is required for biofilm formation though QS is not implicated in initial surface attachment but rather controls biofilm maturation (Huber et al., 2001).

The QS molecule, C8-HSL, is produced at low levels by the CepI synthase until enough C8-HSL accumulates due to cell density that a threshold concentration is reached and the QS molecule binds to the CepR protein which then induces or represses certain traits such as biofilm production and ornibactin (siderophore) production, respectively. The CepR-C8-HSL complex binds specifically to the *cepI* promoter and upregulates expression of *cepI*, producing more QS molecule (Weingart et al., 2005).
Figure A1: Current Model of Biofilm and Virulence Factor Regulation in *B. cenocepacia*.
YciR is predicted to exert posttranscriptional control over the expression of CepR, which upregulates biofilm formation in this model, and activates transcription of *cepl*, producing C8-HSL, which binds to and activates transcription of *cepR*.
Kothe et al. (2003) demonstrated that individual cepl and cepR mutants of B. cenocepacia strain H111 are each attenuated in killing in a C. elegans worm model. Slow-killing in the C. elegans model resembles an infection-like process, which is why this nematode model is an accepted alternative to a mouse or cell culture model. The cep system also positively regulates factors associated with virulence, such as swarming motility, extracellular proteolytic and chitinolytic activity and represses the synthesis of ornibactin, the siderophore produced by B. cenocepacia (Lewenza et al., 1999; Huber et al., 2001; 2002; Lewenza and Sokol, 2001).

Our strain, B. cenocepacia HI2424, is highly virulent in the C. elegans model of infection, more so than other strains of B. cenocepacia tested by Cooper et al. (2009), such as AU1054. Some strains of B. cenocepacia have additional identified QS systems, such as CclR, located on a pathogenicity island (Baldwin et al., 2004), though this region of DNA is not present in HI2424. The HI2424 strain must also have additional factors contributing to virulence in the worm model since it differs significantly from its close relatives in worm virulence (Cooper et al., 2009). It was this fact that prompted me to mutate cepR, which tightly regulates cepl expression (Huber et al., 2004), to determine if the mutant would behave similarly to strain H111-R (a cepR mutant) that was attenuated in both slow and fast killing of C. elegans (dependent on growth medium osmolarity). If the cepR mutant of HI2424 does not behave similarly to H111-R, this would support the existence of another functional QS system or other regulator of biofilm and virulence traits.
I predicted that the cepR mutant in HI2424 would be defective in C8-HSL production, biofilm formation, protease activity and worm virulence, following the trend of cepR mutants in other strains as reviewed above. Interestingly, not all of these predictions came true, suggesting another virulence regulator in HI2424 in addition to the CepIR system.

**Methods**

**Cloning of cepR**

The wild-type cepR gene from *B. cenocepacia* HI2424 was cloned and inactivated by inserting the *dhfr* cassette from pFTP1 within cepR. This mutant copy was then introduced into WT HI2424 to replace the WT gene with the mutated copy.

The wild-type cepR gene was PCR amplified from HI2424 using the following primers:

- CepR F: 5’-GGCGAACAGCGACTTCAGCA-3’
- CepR R: 5’-GGACAGTTGCAGCACTCCGT-3’

Cycling conditions were the same as those in the *dhfr* PCR screen described in Chapter 2 except annealing was at 61°C and elongation was 3min. Amplified cepR was cloned into pCR®2.1-TOPO® and chemically transformed into One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen). Recovered transformants were plated on LB agar containing 50μg/ml Kan and IPTG and X-gal. White, Kan’ colonies were used to start overnight liquid cultures for plasmid isolation using the QIAprep spin mini-prep kit (Qiagen). Cloning of the cepR gene into pCR2.1-TOPO was verified by PCR and one correct clone was named pCepR.

The *dhfr* cassette from pFTP1 was cloned into pCepR by digesting both plasmids with *Sall* (NEB). The two desired fragments were ligated with T4 DNA ligase (NEB) at a
3:1 insert to vector ratio (NEB), without gel purifying the \textit{dhfr} cassette out of the digestion product. Ligation products were transformed into chemically competent \textit{E. coli} DH5\(\alpha\) and plated onto T-Soy agar containing 50\(\mu\)g/ml Tp and incubating overnight. Tp-resistant colonies were patched onto T-Soy containing 50\(\mu\)g/ml Kan. Doubly resistant colonies were screened by PCR for the \textit{cepR} gene with an elongation of 3min 30sec. \textit{CepR}-positive cultures were harvested for plasmid DNA as previously described and one correct clone was named pCepRTp.

The suicide vector pCM42 (Tomich and Mohr, 2004) and pCepRTp were digested with EcoRI (NEB) and the mutated \textit{cepR} was cloned into pCM42 by combining the EcoRI digested products at a 3:1 insert:vector ratio in a reaction with T4 DNA ligase (NEB). Ligation products were transformed into chemically competent \textit{E. coli} DH5\(\alpha\) \(\lambda\) pir and plated onto T-Soy with 50\(\mu\)g/ml Tp and incubated overnight. Tp-resistant colonies were patched onto T Soy containing 50\(\mu\)g/ml Kan or 50\(\mu\)g/ml Cm. Cm resistant, Kan sensitive colonies were screened in a \textit{cepR} PCR as described previously. \textit{CepRdhfr}-positive colonies were harvested for plasmid DNA using the Qiagen kit as described above and one correct clone was named pLCB8-84.

\textbf{\textit{cepR} Inactivation in HI2424}

The suicide vector, pLCB8-84 in \textit{E. coli}, was mated into \textit{Burkholderia cenocepacia} HI2424 in a tri-parental mating with \textit{E. coli} harboring the ‘helper’ plasmid pEVS104. For mating, cultures were grown overnight in liquid broth containing appropriate antibiotic. Cultures were mixed 2:3:3 (recipient:donor:helper) and diluted 3:1 with 10mM MgSO\(_4\). Mating mixture was pelleted, resuspended in 10mM MgSO\(_4\) and washed a second time.
Resuspended cells were spotted onto a filter paper square on T-Soy agar spread previously with 100μl 2M MgSO₄. Mating mix was incubated for 8hrs at 37°C and cells were harvested by vortexing the filter paper in PBS. Cells were diluted and plated onto T-Soy containing 15μg/ml polymixin B and 50μg/ml Tp and incubated for three days at 32°C. Resulting colonies (putative single-crossover events) were patched to T-Soy agar containing Cm 50μg/ml and T-Soy agar containing Tp 50μg/ml and incubated overnight. Cm-sensitive, Tp-resistant colonies (2nd crossover events) were screened by PCR for the *cepR* gene to determine if the wild-type *cepR* gene had been replaced by the *dhfr*-insertion containing *cepR*, creating *B. cenocepacia* HI2424-R. Positive cultures were verified to be *Burkholderia* PCR for 16S rDNA as described in Chapter 1.

**Phenotypic Screens for *cepR* Knockout**

Phenotypic screens were performed on HI2424-R to determine changes to virulence-associated traits (virulence factors). Biofilm assays were performed as described in Chapter 2, in 96-well microplate format. Protease production was quantified by spotting 5μl of overnight culture onto milk plates (T-Soy agar, 20g skim milk powder/L) and measuring zones of clearing after overnight incubation. The C8-HSL reporter assay with *P. putida* F117 (pAS-C8) was also performed as described previously (Steidle et al., 2001) except *B. cenocepacia* was grown in T-Soy broth. Liquid worm killing assays were performed to evaluate any change in pathogenicity conferred by the *cepR* mutation as described by Cooper et al. (2009). Significance of phenotype differences from wild-type was evaluated by performing two-tailed t-tests assuming equal variance; t-test values below 0.05 were considered significant.
Results
Most phenotypes of the cepR mutant, HI2424-R, that were tested were not significantly different from WT HI2424. However, in an initial AHL sensor assay, WT produces significantly more C8-HSL than does the cepR mutant (Figure A2) and protease production was significantly reduced versus WT (Figure A3). Worm virulence in the C. elegans model did not differ significantly from wild-type (Figure A4). Biofilm production was also not significantly different (Figure A5).
Figure A2: AHL Sensor Assay

_Pseudomonas putida_ F117 pAS-C8 sensor strain was grown overnight in the presence of filtered spent supernatant from either WT HI2424, _E. coli_ OP50, or HI2424 cepR mutant. The cepR mutant produces significantly less quorum sensing molecule as determined by lower relative fluorescence produced by the sensor strain than the _E. coli_ control and the medium blank. Error bars represent 95% confidence intervals, n=3.

Figure A3: Protease Plate Assay

The cepR mutant produced significantly less protease as determined by zones of clearing on skim milk agar than the WT HI2424 strain. Error bars represent 95% confidence intervals, n=3.
**Figure A4: Liquid Worm Virulence Assay**
Over 168 hours *C. elegans* worms were monitored for % death in liquid culture that grew more turbid as bacteria continued to thrive. Error bars represent 95% confidence intervals, n=3.

**Figure A5: Scaled Biofilm Values for the cepR Mutant and WT HI2424**
Scaled values were obtained by dividing the OD$_{600}$ value of crystal violet by the OD$_{600}$ value of the cell density after 24hr growth Error bars represent 95% confidence intervals, n=32.
Discussion
A functional $cepR$ gene does not appear to be required for virulence or for production of most associated virulence factors in $B.\ cenocepacia$ HI2424. Though C8-HSL production is abolished in HI2424-R, worm virulence is not affected, nor was biofilm production. These results contradict those found in studies of $cepR$ mutants in other strains of $B.\ cenocepacia$. However, Kothe et al. (2003) performed agar medium-based assays of worm virulence whereas I used a liquid medium model of virulence. I also performed agar medium killing assays but results were too variable to draw conclusions about virulence. My results suggest that there may be other virulence factors or global regulators acting in HI2424 in addition to the CepIR QS system.
References


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