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The experimental evolution of host adaptation of the emerging pathogen *Burkholderia cenocepacia*

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THE EXPERIMENTAL EVOLUTION OF HOST ADAPTATION OF THE
EMERGING PATHOGEN *Burkholderia cenocepacia*

BY

CRYSTAL NICOLE ELLIS

B.S., Stonehill College, 2004

THESIS

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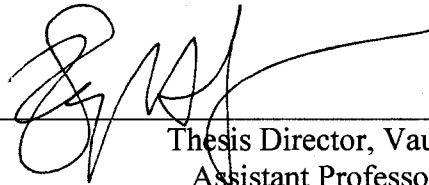
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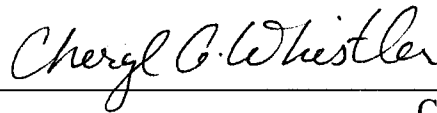
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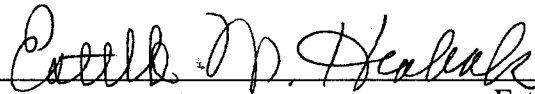
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TABLE OF CONTENTS

LIST OF TABLES.....	v
LIST OF FIGURES.....	vi
ABSTRACT.....	viii

CHAPTER	PAGE
I. INTRODUCTION.....	1
The <i>Burkholderia cepacia</i> Complex.....	1
Laboratory Models for the Study of the Bcc.....	8
Host Adaptation: Theories and Supporting Experiments.....	10
Research Goals and Objectives.....	18
II. BROAD HOST RANGE MINI-TN7-BASED MARKING SYSTEMS FOR USE WITH <i>BURKHOLDERIA</i> SPECIES AND EXPERIMENTAL EVOLUTION.....	21
Introduction.....	21
Methods.....	27
Results.....	39
Discussion.....	42
III. EXPERIMENTAL EVOLUTION OF <i>BURKHOLDERIA CENOCEPACIA</i> TO A NOVEL HOST, <i>ALLIUM CEPA</i> AND ITS EFFECTS ON VIRULENCE IN AN ALTERNATIVE HOST.....	45
Introduction.....	45
Methods.....	49
Results.....	56

Discussion.....	76
LIST OF REFERENCES.....	84

LIST OF TABLES

Table 1.1: Summary of probable virulence phenotypes expressed in <i>Burkholderia cepacia</i> complex species.....	5
Table 2.1: Bacterial strains used and plasmid vectors derived in this study.....	28
Table 2.2: Specific gene primers used in this study.....	31
Table 3.1: Summary of statistical analysis performed on evolved and ancestral mean fitness values	59
Table 3.2: Summary of statistical analysis performed on evolved and ancestral mean maximum growth rates.....	61
Table 3.3: Mean nematode virulence index of all 12 evolved populations and the ancestral clone.....	66
Table 3.4: Summary of mean area-under-the-curve (AUC) calculations obtained from analyzing the diet breadth of the <i>B. cenocepacia</i>	75

LIST OF FIGURES

Figure 1.1: Summary of the broad host range and niche characteristics of species in the <i>Burkholderia cepacia</i> complex	3
Figure 1.2: Experimental design of a serial passage evolution experiment.....	13
Figure 1.3: Diagram summarizing the stated hypothesis.....	20
Figure 2.1: Components required for Tn7 transposition.....	23
Figure 2.2: A model of Tn7 insertion into chromosomal DNA.....	24
Figure 2.3: Vector map of pCElacZ.....	33
Figure 2.4: Vector maps of pcrmLRFP and pcrmLYFP.....	35
Figure 2.5: Vector maps of pCERFP and pCEYFP.....	36
Figure 3.1: Competitive fitness data of evolved genotypes.....	57
Figure 3.2: Maximum growth rates of evolved and ancestral populations.....	60
Figure 3.3: Two separate onion virulence assays of six Lac ⁺ populations	63
Figure 3.4: Nematode killing in monoxenic liquid culture.....	65
Figure 3.5: Radii of <i>B. cenocepacia</i> HI2424 and 12 onion-evolved populations motility through 0.3% swim agar.....	67
Figure 3.6: Summary of results obtained from a crystal violet biofilm assay of all 12 evolved populations and the ancestral clones	69
Figure 3.7: Phase contrast microscopy images of evolved and ancestral populations.....	70
Figure 3.8: Image of a <i>B. cenocepacia</i> HI2424 Lac ⁺ ancestral sample	71

Figure 3.9: Image of a *B. cenocepacia* HI2424 Lac⁺ evolved sample.....71

Figure 3.10: An image summarizing the results of a gel electrophoresis procedure
that was performed to visualize the amplicons produced by vPCR.....73

ABSTRACT

THE EXPERIMENTAL EVOLUTION OF HOST ADAPTATION OF THE EMERGING PATHOGEN *Burkholderia cenocepacia*

By

Crystal Nicole Ellis

University of New Hampshire. May, 2008

I investigated the ability of *Burkholderia cenocepacia*, an opportunistic bacterial pathogen, to adapt to a host. Studies have identified trade-offs associated with environmental adaptation, but few have investigated host adaptation. Consequently, I studied effects of adaptation by *B. cenocepacia* to onions (*Allium cepa*) on the ability to kill *Caenorhabditis elegans*. I hypothesized that adaptation to onions would reduce virulence in *C. elegans*. I evolved twelve populations of bacteria in onion tissue medium for 500 generations. Then, I quantified fitness differences between evolved and ancestral populations by direct competition, having developed molecular marking techniques to discriminate among competitors. Competitions revealed fitness increases in nine populations. Next, I measured virulence against *C. elegans* of each population and observed a reduced worm killing ability. I also quantified pleiotropic effects of adaptation related to virulence. In conclusion, I supported that adaptation of *B. cenocepacia* to one host resulted in decreased virulence in another host.

CHAPTER I

INTRODUCTION

Sudden expansion of host range has been the cause of numerous bacterial outbreaks and epidemics and continues to threaten both the immunocompromised and healthy human populations (Woolhouse *et al.*, 2001). For this reason, pathogenic microbes that are capable of persisting in multiple hosts and environments are under continued investigation (D'Argenio *et al.*, 2001; Kuiken *et al.*, 2006; Woolhouse *et al.*, 2001). By identifying factors, genetic or phenotypic, that allow for the expansion of host range, investigators can begin to understand what allows for adaptation to new hosts (Rahme *et al.*, 1995). One common approach is experimental evolution, which has been useful for evaluating general models of adaptation and for quantitative evaluation of evolutionary theories (Elena and Lenski, 2003). Here, I describe the use of experimental evolution to examine the effects of adaptation to a specific host by the emerging human pathogen *Burkholderia cenocepacia*, a member of the *Burkholderia cepacia* complex.

The *Burkholderia cepacia* Complex

The *Burkholderia cepacia* complex (Bcc) consists of at least 10 non-spore forming, Gram-negative, bacterial species that are not only ubiquitous in the environment but are able to use a wide variety of carbon sources (Coenye and Vandamme, 2003; Levy

et al., 2008). These species are found in numerous environments including soils, freshwater, seawater, plant rhizospheres, human hosts, and animal hosts (Parke and Gurian-Sherman, 2001) (Figure 1.1). Coupled with an ability to occupy many environmental niches, they also have a wide catabolic potential (Coenye and Vandamme, 2003). The metabolic diversity of members of the Bcc has encouraged their use as bioremedial agents (Coenye and Vandamme, 2003). They can break down and utilize many environmental pollutants such as herbicides, gasoline additives, and polycyclic aromatic hydrocarbon compounds, which are common constituents of crude oil (Coenye and Vandamme, 2003). Their metabolic diversity may be an artifact of their large genomes, which contains 2-3 chromosomes of 6-9 Mbp (Lessie *et al.*, 1996). Bcc genomes are also peppered with insertion sequences, which have been linked disproportionately to microevolutionary adaptation in bacteria (Schneider *et al.*, 2000).

Because Bcc bacteria are ecologically and metabolically diverse, they are useful for biocontrol and bioremediation purposes (Coenye *et al.*, 2001; Parke *et al.*, 1991). Bcc species have been isolated as plant symbionts due to their nodulation and nitrogen fixation capabilities, making them a good candidate for increasing commercial crop yields (Parke *et al.*, 1991). Bcc bacteria also prevent root infections caused by the fungi *Pythium aphanidermatum* and *Aphanomyces euteiches* and have been used in place of chemical pesticides to control seedling and root diseases by colonizing the rhizospheres of agricultural crops of corn, maize, rice and pea plants (Parke *et al.*, 1991). Lastly, they are useful as bioremedial agents due to their ability to break down gasoline additives and crude oil constituents, which are often two sources of environmental pollution (Mahenthiralingam *et al.*, 2005). Unfortunately, due to the disease-causing capabilities

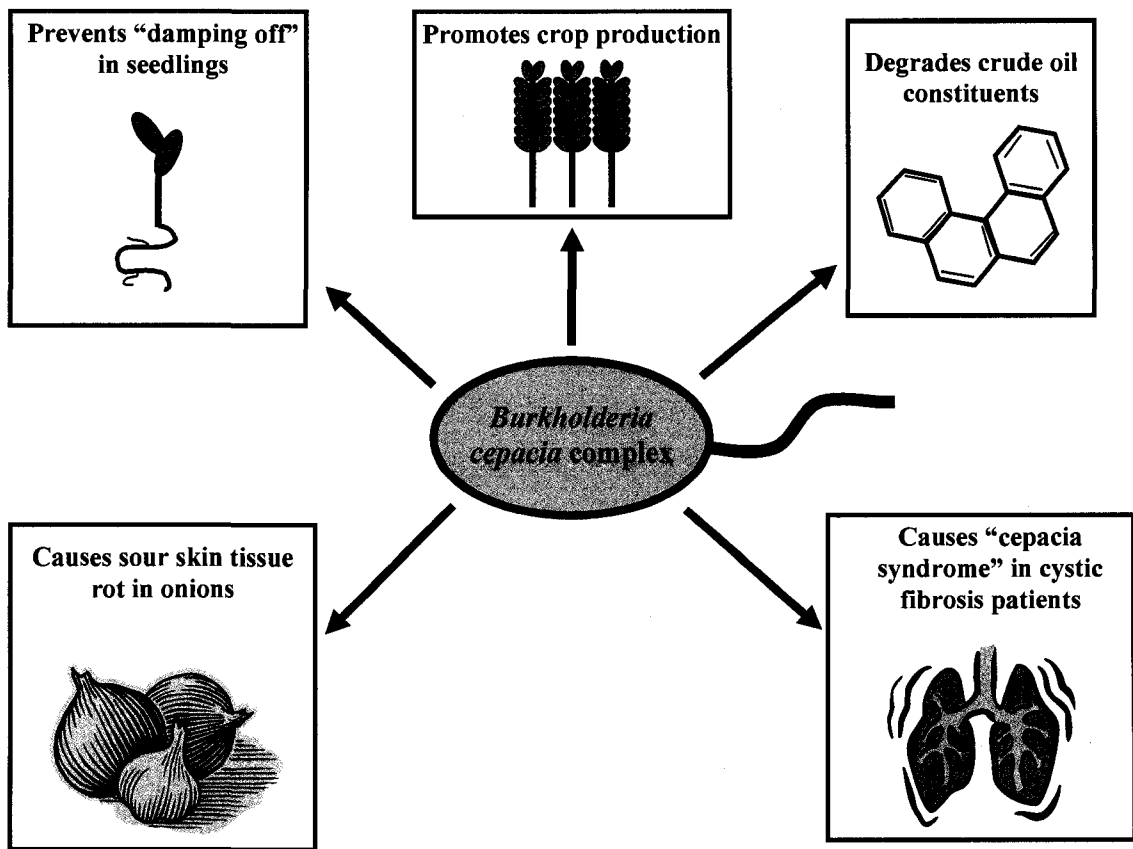


Figure 1.1: Summary of the broad host range and niche characteristics of species in the *Burkholderia cepacia* complex. The top three arrows indicate beneficial interactions of Bcc species with the host/ environment, whereas bottom two arrows indicate detrimental interactions of species with known natural hosts.

of some members of the Bcc and their close relatives, they are no longer used as biocontrol or bioremedial agents on a large scale (Coenye *et al.*, 2001; Parke and Gurian Sherman, 2001). However, a better understanding of their capabilities and limits might allow for the future use of certain members as bioremedial or biocontrol agents.

Despite the numerous benefits that could come from using Bcc members commercially, Bcc have raised concerns for the agricultural industry and also the medical field. One indirect concern is their close relatedness to *Burkholderia mallei* and *Burkholderia pseudomallei*, two highly infectious pathogens classified as CDC category B agents of biological warfare (Holden *et al.*, 2004; Nierman *et al.*, 2004; O'Quinn *et al.*, 2001). These two agents cause the diseases glanders and melioidosis, respectively, and are highly resistant to treatment, not to mention extremely contagious when aerosolized (O'Quinn *et al.*, 2001). Both glanders and melioidosis lead to septicemia and without treatment, they can be fatal (O'Quinn *et al.*, 2001). Because Bcc members are closely related to these two pathogens, the use of Bcc members on a large-scale was discouraged. In addition to relatedness to dangerous pathogens, Bcc members have raised increasing concern due to their capabilities as plant and human pathogens coupled with the presence of a wide variety of virulence phenotypes (Baldwin *et al.*, 2004; Conway *et al.*, 2002; Corbett *et al.*, 2003; Engledow *et al.*, 2004; Gonzalez *et al.*, 1997; Kothe *et al.*, 2003; Nzula *et al.*, 2002; Tomich *et al.*, 2003; Tomich and Mohr, 2003; Urban *et al.*, 2004; Visser *et al.*, 2004) (Table 1.1).

Table 1.1: Summary of probable virulence phenotypes expressed in *Burkholderia cepacia* complex species (adapted from Mahenthiralingham *et al.*, 2005).

Virulence Factor	Notes on phenotype characteristics
Biofilm production	Most members form biofilms, but <i>B. cenocepacia</i> and <i>B. multivorans</i> in particular form thick biofilms; induced by high cell density and provides protection from host immune responses (Conway <i>et al.</i> , 2002).
Resistance to antibiotics	Resistance to multiple antibiotics results in difficult treatment of infections.
Secretion systems	<i>B. cenocepacia</i> and <i>B. cepacia</i> harbor Type III and Type IV secretion systems. Type III systems are required for toxin-mediated sepsis in a mouse model of infection. Type IV systems are required for plant tissue water-soaking phenotype (Engledow <i>et al.</i> , 2004; Tomich <i>et al.</i> , 2003).
Cable pili and adhesin	<i>B. cenocepacia</i> requires these for host cell adherence (Tomich <i>et al.</i> , 2003).
Quorum sensing	All Bcc species encode CepIR systems; facilitates cell-to-cell communication and has been implicated in <i>B. cenocepacia</i> as an inducer of virulence phenotypes (Venturi <i>et al.</i> , 2004).
Siderophore production	All species express siderophore; required for iron acquisition and infection persistence in mouse models.
Cenocepacia pathogenicity island	Found only in <i>B. cenocepacia</i> ; expresses many virulence genes as well as metabolism genes.
Flagella	Found in all Bcc species; motility is often required for cellular invasion (Urban <i>et al.</i> , 2004).

The first identified species of the Bcc, *B. cepacia*, was isolated as a pathogen of onion bulb and leaf tissue (Burkholder, 1950; Cother and Dowling, 1985). It was classified as the cause of a tissue rot in onions called sour skin disease, which is characterized by a yellow or brown discoloration coupled with severe tissue maceration and odor (Burkholder, 1950). The specific onion pathogenic isolate, *B. cepacia* ATCC25416, has been responsible for the destruction of large commercial onion crops and accomplishes this using a plasmid-encoded pectate hydrolase (Cother and Dowling, 1985; Gonzalez *et al.*, 1997). This enzyme degrades pectin molecules located in the cell walls of plant tissue, subsequently compromising the structure of the plant cell (Ulrich, 1975). Removal of the gene encoding for pectate hydrolase, *pehA*, halted tissue maceration in *B. cepacia* ATCC25416, yet the bacteria still exhibited a plant tissue watersoaking (PTW) phenotype, a phenotype characteristic of onion plant disease (Gonzalez *et al.*, 1997). PTW appears as softening of onion tissue and secretion of liquid by the infected onion bulb (Gonzalez *et al.*, 1997). The PTW phenotype is attributed to plant-cell cytotoxic effector molecules delivered by a type IV secretion system; the effector molecules themselves remain uncharacterized (Engledow *et al.*, 2004). In addition to *B. cepacia* ATCC25416, other Bcc environmental and clinical species exhibit the PTW phenotype, including the species used in this study, *B. cenocepacia*. This raises concerns addressing the host range capacity and pathogenic potential of the Bcc (Engledow *et al.*, 2004).

In addition to causing disease in plants, Bcc bacteria have been isolated as pathogens from the lungs of immunocompromised individuals, especially cystic fibrosis (CF) patients. Cystic fibrosis, characterized by an overproduction of thick, sticky mucus

in the lungs, is a genetically inherited condition that affects the lungs and digestive tracts of thousands of children and adults (Cystic Fibrosis Foundation, www.cff.org). This disorder is caused by a mutation in the human CFTR gene, which encodes an apically localized epithelial chloride ion channel (Davidson and Rolfe, 2001). Other symptoms include salty sweat, insufficient pancreatic function, intestinal blockages, male infertility, and chronic inflammation of the lungs caused by bacterial infection (Davidson and Rolfe, 2001). Life expectancy for CF patients has increased to a median age of 37 years due to more efficient treatments, which include inhaled anti-inflammatory medications, intense antibiotic therapy, and lung transplant surgery (Cystic Fibrosis Foundation, www.cff.org). Because treatment has increased life span, however, a new ecological niche has been created in CF patients for invasion by Bcc members and other opportunistic bacteria such as *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*.

Today, CF patients most often acquire Bcc infections from the environment, but in the past they have been acquired in hospitals (Holmes *et al.*, 1999). This can occur through patient-to-patient contact and sometimes by contaminated medical devices (Holmes *et al.*, 1999). Infections can be acquired through patient-to-patient contact outside of a hospital setting as well, mainly through aerosol droplets and exchange of infected secretions (Govan *et al.*, 1993; Saiman and Siegal, 2004). Due to the high genetic diversity of Bcc members, infectious strains can be difficult to diagnose and are often confused with another CF pathogen, *Pseudomonas aeruginosa* (Baldwin *et al.*, 2005). Bcc infections can range from mild to severe, and can seriously compromise the life of a CF patient (Huber *et al.*, 2004).

Infections caused by Bcc members can result in a condition known as ‘cepacia syndrome,’ which affects CF patients both physically and socially (Huber *et al.*, 2004; Saiman and Siegal, 2004). ‘Cepacia syndrome’ is characterized by high fever, rapid pulmonary deterioration, sepsis and often fatal pneumonia (Huber *et al.*, 2004; Isles *et al.*, 1984; LiPuma, 1998). Because Bcc bacteria not only inhabit the lungs of CF patients, but also the blood and the sinuses, infected patients are permanently removed from lung transplant lists, which is a major therapeutic technique used to treat CF (Saiman and Siegal, 2004). Also, because Bcc infections spread rapidly and are so difficult to treat due to a high antibiotic resistance, the most often employed methods of Bcc disease control involve rigorous prevention strategies that often remove infected patients from the CF community (Aaron *et al.*, 2000; Saiman and Siegal, 2004). These measures include disbanding of CF summer camps for children, limited contact of infected patients with outpatient CF clinics, and their exclusion from CF conferences (Saiman and Siegal, 2004).

Laboratory Models for the Study of the Bcc

Several laboratory models exist for the study of Bcc pathology and virulence in both animal and plant hosts. Mouse models include burned mouse tissue models and models that simulate chronic infections, such as the mouse agar bead model (Chu *et al.*, 2002; Stover *et al.*, 1983; Tomich and Mohr, 2003). In one study, strains of *B. cepacia* were shown to adhere to and invade murine respiratory epithelial cells, with adherence being an important phenotype for establishment of infection (Chiu *et al.*, 2001). Some murine models involve mice that have been genetically altered by disrupting the homolog

of the same gene that is mutated in CF patients, the CFTR gene (Davidson and Rolfe, 2001). Unfortunately, these models do not completely represent all of the symptoms of the human disease (Davidson and Rolfe, 2001). Also, mice are not as genetically tractable as some invertebrate models, and there are numerous ethical and cost constraints that prevent many researchers from using this model.

Tissue culture models have been developed for the study of cell invasion by Bcc members. Some studies use pulmonary macrophage cells and alveolar epithelial cells, both derived from human cell lines, to show invasion and intracellular survivability of *B. cepacia* (Carterson *et al.*, 2005; Martin and Mohr, 2000). Tissue culture models can be used to detect virulence factors required for invasion and persistence without using live animals, which allows more laboratories to ethically and cost-effectively study the effects of Bcc infections. Unfortunately, most tissue cultures are derived from tumorigenic lung epithelia and monogenic cell lines, which cannot fully represent important temporal and structural cell changes that may occur in the actual host in response to invasion (Carterson *et al.*, 2005). These changes include factors such as differentially regulated protein expression and cell signaling (Carterson *et al.*, 2005). For these reasons, live animal models remain more informative for investigating *in vitro* interactions between a pathogen and an infected host.

Caenorhabditis elegans, a natural soil invertebrate, is a bacteria-feeding nematode and a laboratory model organism that has been used in many studies to examine virulence of bacterial pathogens (Joshua *et al.*, 2003; Maadani *et al.*, 2007; Wareham *et al.*, 2005). The maintenance of these organisms is easy and inexpensive compared to murine models for many reasons (Tan and Ausubel, 2000). Firstly, *C. elegans* requires more simple

growth conditions than mice (Tan and Ausubel, 2000). Also, they can be raised aseptically and they have a rapid generation time (Hope, 1999). Furthermore, they are genetically tractable due to their hermaphroditic nature (Hope, 1999; Tan and Ausubel, 2000). Both acute and chronic infections can be simulated in the *C. elegans* host model, which allows for the study of phenotypes necessary for Bcc bacterial invasion and infection (Huber *et al.*, 2004; Kothe *et al.*, 2003). Lastly, many existing genetic tools can be used in this system, in particular the use of fluorescent bacterial markers for determining the localization of a bacterial infection (Huber *et al.*, 2004).

Because members of the Bcc associate with plants, examining pathology or symbiosis in plant models is also possible. Varying levels of virulence exist among strains of *B. cepacia* in an alfalfa infection model (Bernier *et al.*, 2003). Both tissue maceration and PTW phenotypes were observed in this model, making it useful for studying plant-specific virulence phenotypes (Bernier *et al.*, 2003). Alternatively, an onion-rot model has also been used to examine virulence factors required specifically for onion infection (Aguilar and Venturi, 2003). For example, Aguilar and Venturi (2003) examined the contribution of quorum sensing (QS) to virulence by infecting onion bulbs with QS⁻ mutants of *B. cepacia* ATCC25416. The onion-rot model is useful for studying plant pathogens responsible for destruction of large agricultural onion crops, as it replicates tissue maceration and PTW phenotypes in a natural host, *Allium cepa*.

Host Adaptation: Theories and Supporting Experiments

Ecologists have long been interested in the adaptation of organisms to specific environmental niches and hosts (Futuyma and Moreno, 1988). In studies that

investigated either host adaptation or niche adaptation, trade-offs associated with either type of adaptation have been identified (Caley and Munday, 2003; Cooper and Lenski, 2000; Crill *et al.*, 2000; Duffy *et al.*, 2006; Lenski *et al.*, 1991). For this reason, the study of niche adaptation is comparable to the study of host adaptation and both types of studies provide background for the current research.

Natural populations of organisms adapting to a specific environment have been used to study whether this adaptation is accompanied by changed function in other environments. Caley and Munday (2003) tested this trade-off model by studying diet breadth of goby fishes. They hypothesized that adaptation to one particular niche should result in a trade-off when introduced to an alternative niche. Caley and Munday (2003) observed and manipulated natural goby populations, one that was adapted for growth in a particular niche within a coral reef and another that displayed a wider niche range. Fitness, defined as reproductive success of a natural population, is the outcome of natural selection in evolving populations and Caley and Munday (2003) used growth rate as an indicator of fitness. They observed that adaptation to one habitat strongly correlated with a decrease in the ability to grow in a broader range of habitats. This study was instrumental in demonstrating trade-offs associated with niche adaptation in a natural population; however, some limitations remained which included a lack of investigator control of these populations and the fact that the genetic identities of the populations could not be determined. In order to obtain more conclusive results regarding fitness trade-offs, experiments must be done in controlled laboratory settings using easily manipulated organisms, such as microbes.

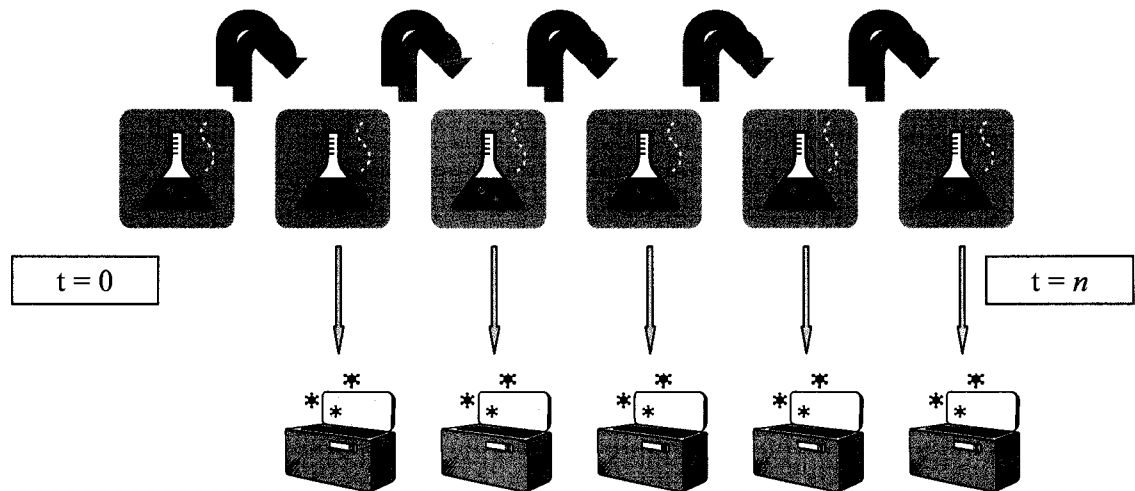


Figure 1.2: Experimental design of a serial passage evolution experiment (only a single replicate population is shown). Inoculation of a population occurs using a single clone (represented at $t = 0$) and subsequent daily passage proceeds until a chosen number of generations is reached ($t = n$). Periodically, samples are frozen to create a fossil record of the evolving population, which is essential for tracking genetic and phenotypic changes over time.

The design of the long-term evolution experiment conducted by Lenski *et al.* (1991) required daily passage of bacterial populations, allowing them to reach stationary phase of growth once every 24 hours (Croizat *et al.*, 2005; Lenski *et al.*, 1991). After quantifying fitness at 2,000, 10,000 and 20,000 generations, all 12 *E. coli* populations showed a significant increase in fitness when compared to the ancestral clone, indicating adaptation to this environment had occurred (Cooper and Lenski, 2000). Because frequent changes in growth phase have been shown to influence DNA supercoiling and topology, specific genes associated with these phenotypes were examined in all twelve populations (Croizat *et al.*, 2005). It was also found that DNA supercoiling changed significantly compared to the ancestor in 10 of 12 populations, and that this change occurred early in the adaptive process (Croizat *et al.*, 2005). Two common mutations were identified in two genes that control DNA supercoiling, *topA* and *fis* (Croizat *et al.*,

Due to their ease of propagation and genetic tractability, microbes have been essential for the study of host adaptation and characterization of genetic changes that occur during the process of evolution (Cooper *et al.*, 2001a; Crill *et al.*, 2000; Crozat *et al.*, 2005). Experimental evolution, the process of culturing populations in a controlled laboratory environment for an extended period of time, is a common practice utilized by evolutionary microbiologists for studying the patterns of genetic changes associated with niche adaptation (Cooper *et al.*, 2001a; Cooper *et al.*, 2001b; Korona, 1996; Lenski *et al.*, 1991) (Figure 1.2). Because phenotypic changes directly related to the adaptive process can be characterized using these types of experiments, two central questions can be addressed: 1) what are the phenotypes responsible for adaptation and 2) are there trade-offs associated with adaptation?

Some experiments involving the experimental evolution of microbes have been conducted in order to seek more definitive answers to these questions. One such set of experiments involved long-term laboratory adaptation of 12 populations of *E. coli* to a single carbon source, glucose (Lenski *et al.*, 1991). These populations were serially passaged through this environment for 20,000 generations (Cooper and Lenski, 2000). The ongoing study of these populations has not only led to the identification of many of the specific genetic changes directly associated with adaptation to the glucose environment, but they also identified the presence of trade-offs associated with specific adaptation (Cooper and Lenski, 2000; Cooper *et al.*, 2001b; Crozat *et al.*, 2005; Lenski *et al.*, 1991).

2005). These two mutations were identified as those directly responsible for an increase in fitness in the glucose minimal environment.

In part to identify other mutations responsible for adaptation in the Lenski system, two of the 12 evolved *E. coli* populations were analyzed using expression arrays by Cooper *et al.* (2003). Many of the changes in gene expression occurred in parallel between the two examined populations; furthermore, most of the genes that showed a difference in expression were known to be regulated by the metabolite guanosine 3',5'-bispyrophosphate (ppGpp) (Cooper *et al.*, 2003). This led Cooper *et al.* (2003) to target specific genes known to regulate ppGpp, in particular, *spoT* which was shown previously to degrade ppGpp in the cell (Cooper *et al.*, 2003). After examining the sequences of the *spoT* genes in the two evolved populations, a point mutation was identified (Cooper *et al.*, 2003). To assess the effect of this mutation on fitness in the glucose environment, the mutated *spoT* gene was introduced into the ancestor clone; this resulted in an increase in ancestor fitness when it was allowed to grow in the glucose environment; this confirmed that the identified *spoT* mutation was a beneficial mutation acquired as the result of specific adaptation to the glucose environment (Cooper *et al.*, 2003). The studies conducted by Crozat *et al.* (2005) and Cooper *et al.* (2003) not only demonstrate that experimental evolution of microbial laboratory populations leads to adaptation, but that experimental evolution also allows for the identification of some specific genetic changes responsible for that adaptation.

In addition to identifying the genetic changes associated with adaptation, the population genetic mechanisms underlying trade-offs associated with adaptation were also studied. The diet breadth of all 12 evolved populations was assessed after 20,000

generations of laboratory adaptation to glucose (Cooper and Lenski, 2000). The 12 populations were grown on 64 carbon sources not represented in the selective environment (Cooper and Lenski, 2000). Most change was the result of parallel growth reductions on a common subset of resources, which strongly supports a mechanism of antagonistic pleiotropy, and not random accumulation of mutations, in reducing diet breadth (Cooper and Lenski, 2000).

The previously mentioned studies are important for the support of niche adaptation, but what of host adaptation specifically? The study of host adaptation is important for many reasons including understanding the evolution of host range and virulence in pathogens. Past studies of parasites have shown that the ability of a pathogen to cause disease is dependent on its host range, in that multi-host parasites tend to exhibit intermediate virulence whereas single-host parasites exhibit higher virulence (Combes, 1997; Garamszegi, 2006; Regoes *et al.*, 2000). A mathematical model developed by Regoes *et al.* (2000) supported this interaction between a parasite population and a heterogeneous host population. This model was then tested empirically using populations of *Plasmodium falciparum*, the causative agent of malaria (Garamszegi, 2006). When a population of *Plasmodium* exhibited a narrow host range, virulence of that parasite was high in that host, whereas when a population of *Plasmodium* exhibited a wide host range, virulence decreased significantly in all hosts (Garamszegi, 2006). This suggests that there is a trade-off associated with adaptation to a single host that limits virulence in alternative hosts.

Another reason for studying host adaptation involves the use of attenuated, avirulent pathogens as vaccine candidates. Often, when designing vaccines, virulent viral

populations are forced to undergo evolutionary adaptation in a specific animal host, which may result in decreased virulence in a human host (Ebert, 1998). Once attenuated, a viral population could be introduced to a human population, allowing for antibody production and subsequent immunity without causing disease (Crill *et al.*, 2000; Ebert, 1998). Unfortunately, some studies have shown that viruses can undergo rapid reversal in attenuated virulence, sparking further interest in the study of viral host adaptation (Crill *et al.*, 2000; Ebert, 1998). In addition, phylogenetic studies have shown that while a single viral species may infect a single host type, other closely related viruses infect a large range of hosts (Crill *et al.*, 2000; Gibbs *et al.*, 1995). This raised the question of what barriers keep viruses restricted from infecting a wider range of hosts. In addition to these findings, other studies investigating viral host range have shown that viruses are capable of shifting their host range rapidly and this has historically been the cause of many human and animal epidemics (Crill *et al.*, 2000; Gao *et al.*, 1999; Kuiken *et al.*, 2006).

Many studies that investigate trade-offs associated with host adaptation have been conducted using viral populations (Crill *et al.*, 2000; Duffy *et al.*, 2006; Ebert, 1998). Crill *et al.* (2000) investigated the effects of host adaptation of the bacteriophage ϕ X174. In this study, replicate populations of ϕ X174 were allowed to adapt alternately to a *Salmonella* bacterial host and an *Escherichia* bacterial host (Crill *et al.*, 2000). Using individual growth rates to represent fitness, they found that adaptation to the *Salmonella* host resulted in a decreased ability to grow in the *Escherichia* host, indicating a trade-off associated with host adaptation. They subsequently identified that mutations in the major capsid protein responsible for recognition and attachment to host cells resulted in the

observed decrease in growth rate in *Escherichia* (Crill *et al.*, 2000). These mutations were also responsible for the fitness increase in the selective host (Crill *et al.*, 2000). Surprisingly, adaptation to the *Escherichia* host did not significantly affect growth of ϕ X174 in the *Salmonella* host (Crill *et al.*, 2000). This asymmetry is puzzling and demonstrates the importance of investigating viral host barriers and its implications in viral attenuation.

Duffy *et al.* (2006) explored the consequences of host adaptation in the bacteriophage ϕ 6. Thirty mutants of ϕ 6 with expanded host range were isolated from populations of virus growing on lawns of the original host, *Pseudomonas syringae* pv. *phaseolicola* (Duffy *et al.*, 2006). These mutants were able to infect 15 other *Pseudomonas* strains but showed a significant growth defect in the original host when compared to the ancestral clone (Duffy *et al.*, 2006). Conversely, the ancestral clone showed a poor ability to infect the 15 other *Pseudomonas* strains but was more fit than any of the mutants in the original host (Duffy *et al.*, 2006). Therefore, there is a trade-off associated with specific host adaptation: a narrow host range.

Trade-offs associated with host adaptation are well studied in viral populations, but do these same patterns exist when bacterial populations become adapted to a specific host? Studies have shown that there are trade-offs associated with niche adaptation, but are there similar patterns associated with host adaptation? These questions and others were addressed during this study.

Research Goals and Objectives

My research focused on examining the effects of host adaptation on growth in an alternative host. Specifically, I used experimental evolution to adapt *B. cenocepacia* HI2424 to an *Allium cepa* onion model and characterized subsequent decreases in virulence in a *C. elegans* infection model. I used experimental evolution because it remains an effective approach for studying phenotypes associated with adaptation to a specific host, as opposed to other methods that involve heavy interference by the researcher such as a candidate gene approach (Cooper, 2007). By allowing the onion environment to exert selective pressures on bacterial populations, any changes occurring during the experiment are the direct result of adaptation in that environment (Cooper, 2007). Bacteria are ideal candidates for experimental evolution mostly due to the easy construction of a frozen fossil record. The fossil record allows the investigator to examine changes in adaptation and genetic profiles over time, a benefit that is lacking when examining higher organisms' fossil records (Cooper, 2007; Lenski *et al.*, 1991).

Our chosen strain, *B. cenocepacia* HI2424, was recovered in upstate New York from agricultural soil as a normal member of the soil microbial community. Macrorestriction digestion with pulsed-field gel electrophoresis and multilocus sequence typing classified this isolate as belonging to the PHDC strain lineage within the species *B. cenocepacia* (LiPuma *et al.*, 2002). This strain, named for its initial identification as the cause of an outbreak in the Philadelphia-DC area of the United States, is widely distributed as a human CF pathogen in 24 US states and parts of Europe (LiPuma *et al.*, 2002). In addition to its relevant strain characterization, the species *B. cenocepacia* is the

Bcc species most often recovered from the lungs of CF patients (Coenye and LiPuma, 2002).

In addition to its clinical relevance, *B. cenocepacia* HI2424 displays an ability to initially infect both animal and plant hosts. In preliminary experiments, this strain macerated plant tissue in an onion half model and kill nematodes in a liquid *C. elegans* chronic model of infection (both models are described fully in Chapter 3). These characteristics make this strain a good candidate for further investigation.

Using experimental evolution modeled after Lenski *et al* (1991), I hypothesized that adaptation of *B. cenocepacia* to a specific host environment would compromise virulence in an alternative host environment (Figure 1.3). During this thesis, I addressed the following objectives:

1. Design of neutral broad host range genetic markers for use in experimental evolution projects and other experiments that require stable genetic markers.
2. Experimental evolution of *B. cenocepacia* HI2424 populations in a liquid onion model and quantification of adaptation to this host-related environment.
3. Examination of side effects associated with adaptation to the onion model, in particular, those related to virulence in the *C. elegans* liquid model of infection.

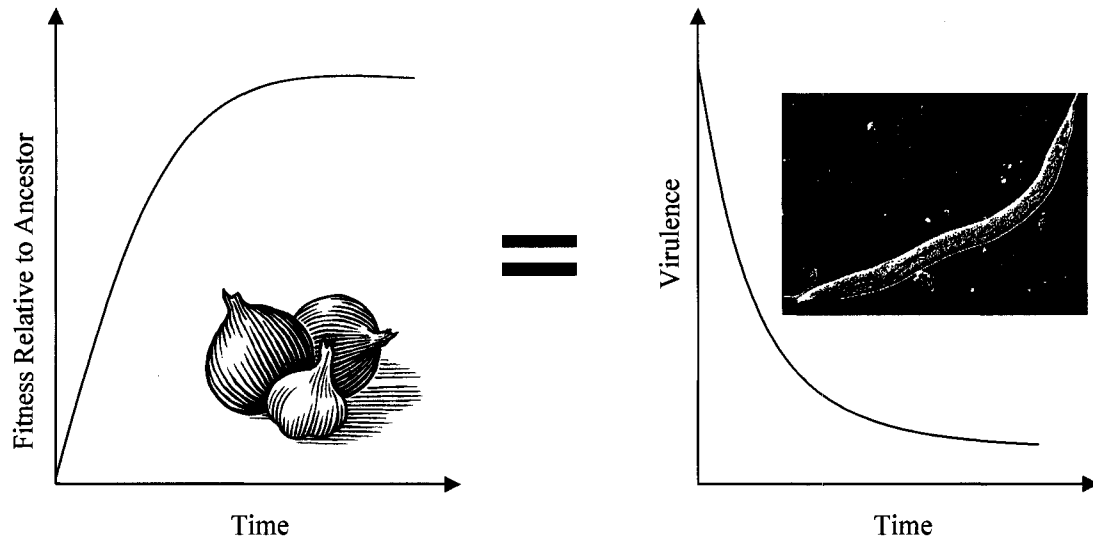


Figure 1.3: Diagram summarizing the stated hypothesis. As adaptation to the novel host, onions, increases, virulence in the alternative host, *C. elegans*, decreases.

CHAPTER II

BROAD HOST RANGE MINI-TN7-BASED MARKING SYSTEMS FOR USE WITH *BURKHOLDERIA* SPECIES AND EXPERIMENTAL EVOLUTION

Introduction

When marking bacteria that are to be grown for multiple generations, genetic tools that produce chromosomal insertions are preferred over methods that introduce plasmids. Some plasmids can be lost in the absence of antibiotic selection, whereas chromosomal insertions are stably maintained through many rounds of vertical transmission (Lambertson *et al.*, 2004). In addition, high copy number plasmids can inhibit bacterial growth (Valenzuela *et al.*, 1996). Also, markers that avoid antibiotic resistance are highly preferred; many types of bacteria including *Pseudomonas* and *Burkholderia* species are already highly resistant to commonly-used antibiotics and it is necessary to avoid introducing extra resistance that could compromise future genetic studies (Choi *et al.*, 2005; Hoang *et al.*, 1998). Furthermore, studies have shown that during experimental evolution, the introduction of antibiotic resistance markers may interfere with the experiment throughout passaging procedures (Riley *et al.*, 2001). Because head-to-head competitions are the preferred means of measuring performance of one population relative to another during experimental evolution, systems that introduce neutral mutations are especially preferred (Cooper, 2007). Lastly, a good marking system should result in phenotypes that allow for easy screening and detection (Lenski *et al.*, 1991; Cooper, 2007).

Transposons have been instrumental for genetic manipulation of Gram negative bacteria and especially Tn7 transposable elements whose biology is well understood (Choi *et al.*, 2005). Tn7 transposons are used to modify bacterial genomes and using Tn7s has provided researchers with many benefits (Choi *et al.*, 2005). One benefit is that Tn7 transposons are effective in a wide range of bacteria including members of the genera *Agrobacterium*, *Vibrio*, *Pseudomonas*, *Rhizobium*, and *Caulobacter* (McKown *et al.*, 1988). Another benefit to using Tn7 elements is that they can be delivered in target cells using suicide vectors, one containing the Tn7 element itself and the other containing the necessary transposase protein complex (Figure 2.1) (Choi *et al.*, 2005). Suicide vectors are used to improve positive selection of transconjugants, due to the inability of these vectors to replicate in target organisms; after selecting for cells containing the Tn7 element using antibiotic resistance, colonies that grow are ones that harbor a chromosomal insertion, and not a vector containing the Tn7 element (Choi *et al.*, 2005). Lastly, a benefit to using Tn7 elements is that they are site-specific transposons and their mechanisms of insertion are well understood.

Tn7 genetic elements insert site-specifically with high frequency at an attachment site termed the *attTn7* site (Figure 2.2) (Choi *et al.*, 2005). This site is located downstream of a highly conserved gene encoding the essential glucosamine-6-phosphate synthase (*glmS*) (Choi *et al.*, 2005). Tn7 transposons require portions of the 3' region of the *glmS* gene to insert, but do so without interrupting any open reading frames (Choi *et al.*, 2005). In order for insertion to occur, Tn7 requires the Tn7 left and right ends, which are transposase binding sites measuring 150 base pairs (bp) and 90 bp, respectively (Hauer and Shapiro, 1984) (Figure 2.1). Tn7L and Tn7R sequences ensure that

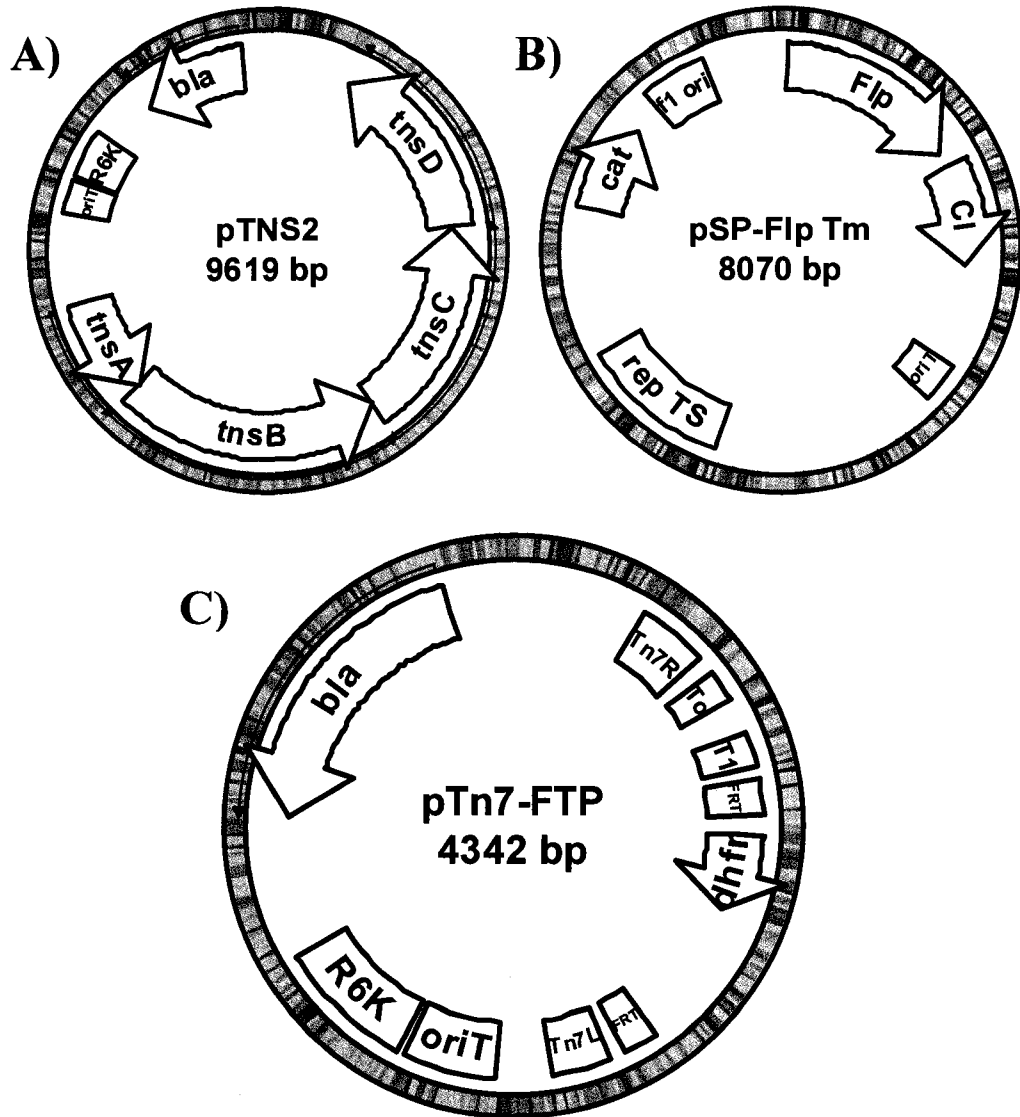


Figure 2.1: Components required for Tn7 transposition. A) The Tn7 vector, pTn7-FRT, contains the following: required Tn7R and Tn7L recognition sites for chromosomal insertion, terminators T₀ and T₁ to prevent readthrough of upstream genes, FRT recognition sites required for Flp recombinase-mediated excision of the *dhfr* trimethoprim resistance cassette, a multiple cloning site (MCS) for insertion of the target gene into the vector, a *bla* gene conferring resistance to ampicillin, a λ -pir dependent R6K origin of replication, and an *oriT* to facilitate transfer during conjugation. B) The vector pTNS2 contains the following: four transposase genes required for transposition to occur (*tnsA*, *tnsB*, *tnsC*, and *tnsD*), a λ -pir dependant R6K origin of replication, and an *oriT* to facilitate transfer during conjugation. C) The Flp recombinase vector pSP-Flp^{TS} contains the following: F1 ori(+) bacteriophage origin of replication, the *cat* gene which grants chloramphenicol resistance, a temperature sensitive origin of replication *rep*^{TS} that allows curing of the plasmid by incubation at 42°C, a *flp* gene encoding Flp recombinase, and an *oriT* origin of transfer to facilitate transfer during conjugation.

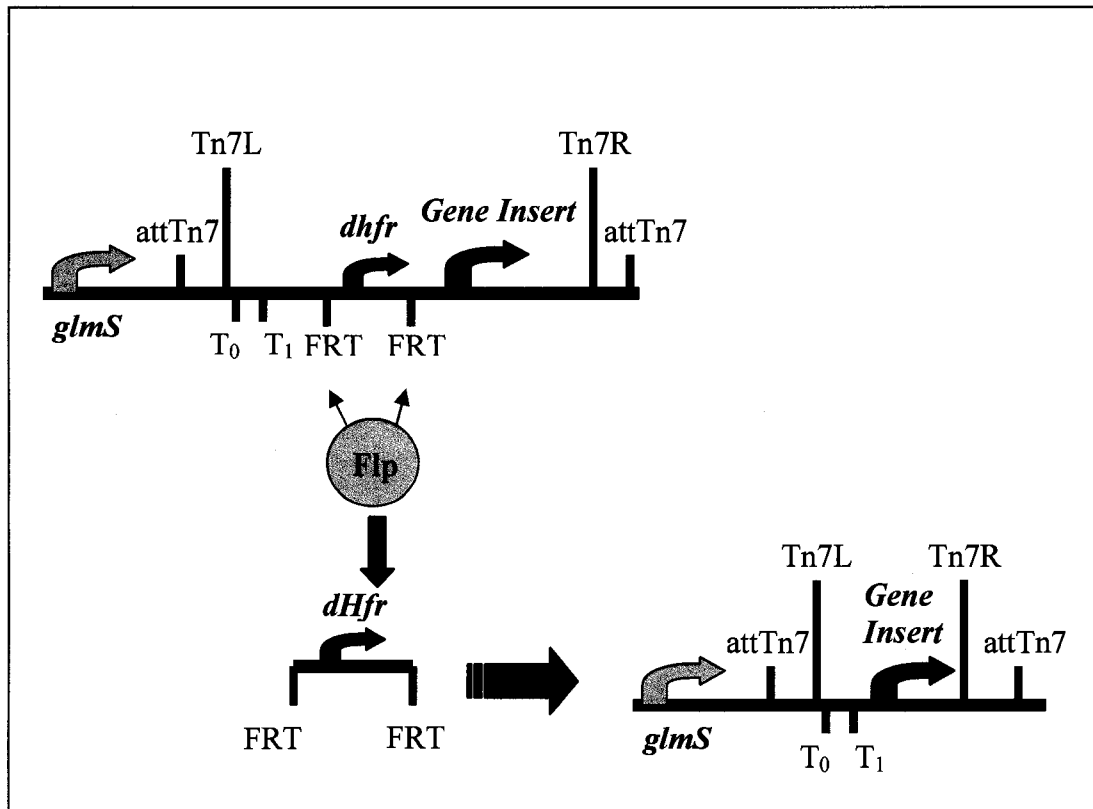


Figure 2.2: A model of Tn7 insertion into chromosomal DNA. Insertion of genes flanked by Tn7L and Tn7R transposase recognition sites occurs downstream of the highly conserved *glmS* gene at *attTn7* attachment sites. Addition of pSP-Flp^{TS} results in expression of Flp and subsequent recognition of FRT sites flanking the *dhfr* cassette. Flp then removes the cassette and re-ligates the target chromosome.

transposons insert site-specifically and in a consistent orientation (Hauer and Shapiro, 1984). One drawback to using Tn7 elements is that it is sensitive to target immunity; that is, if the target organism contains regions of the Tn7 left or right sequences at the *attTn7* attachment site as the result of previous insertion by another Tn7 element, the frequency of transposition is reduced 100-1000 fold (Stellwagen and Craig, 1997).

There are two possible transposase complexes that can be used to mobilize Tn7 elements. One complex, TnsABC+D encourages a high frequency of insertion at the *attTn7* site specifically, while the other complex, TnsABC+E encourages a low frequency of insertion non-specifically throughout the genome and in conjugative plasmids (Stellwagen and Craig, 1997). The proteins TnsA and TnsB encode for transposases that recognize the Tn7 end sequences (Sarnovsky *et al.*, 1996). TnsAB together remove the Tn7 from the donor vector using double-stranded DNA breaks and join the exposed transposon ends to the chromosomal DNA within the target genome (Sarnovsky *et al.*, 1996). TnsA acts like a type II restriction enzyme and requires interaction with TnsB, a member of the retroviral integrase superfamily, in order to facilitate DNA recognition and excision from the donor vector (Peters and Craig, 2001). TnsAB forms a complex with TnsC, an ATP-hydrolyzing protein that binds non-specifically to target DNA (Peters and Craig, 2001). In the presence of ATP and target site selecting proteins TnsD or TnsE, TnsC activates the transposase activity of TnsAB to allow insertion of Tn7 elements (Bainton *et al.*, 1993). TnsD or TnsE is also responsible for recruitment of the TnsABC machinery to the target DNA (Bainton *et al.*, 1993).

In this study, we modified mini-Tn7 vectors previously engineered by Choi *et al.* (2005) for use in experiments requiring a broad-host range genetic marker that avoids

antibiotic selection (Figure 2.1). Different Tn7 vectors were modified to include a β -galactosidase-encoding *lacZ* gene, a red fluorescent protein (RFP)-encoding *mCherry* gene, or a yellow fluorescent protein (YFP)-encoding *Venus* gene. The vector containing *lacZ* allows for easy screening of marked populations on 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal). A *lacZ* marker is especially useful for experimental evolution projects because visualization of mixed evolved and ancestral populations can be achieved on a single agar surface.

The vectors containing fluorescent proteins are useful for marking populations that can then be visualized using standard fluorescence readings, confocal scanning, or flow cytometry (Tomlin *et al.*, 2004). Since the excitation and emission wavelengths of RFPs and YFPs are sufficiently different, detection of both proteins is achieved independently, making this system especially useful for study of mixed populations (Shaner *et al.*, 2005). The *mCherry* RFP is the best general purpose red monomer owing to its high photostability and low toxicity (Shaner *et al.*, 2005). The *Venus* YFP is a weak dimer, rendering it less toxic than other dimers or tetramers and is also UV excitable (Shaner *et al.*, 2005). Both have been modified to fold tightly at 37°C, a common optimal temperature among human pathogens (Shaner *et al.*, 2005). Fluorescing bacteria are useful for studying the structures of biofilms in mixed or single species populations and for studying infections of model host organisms (Kothe *et al.*, 2003; Tomlin *et al.*, 2004). Also, marking with fluorescence holds an advantage over marking with *lacZ* because the methods used to detect fluorescence of individual cells are more sensitive.

We also included the use of FRT excision sites flanking the antibiotic cassettes that were specifically engineered by Hoang *et al.* (1997) to allow for use of the yeast F1p

recombinase excision system (Figure 2.1). This allows for insertion of Tn7 elements driven by antibiotic selection and subsequent removal of the antibiotic cassette by a *sacB* curable vector containing Flp recombinase. Flp recombinase is a site-specific recombinase that recognizes a 13 bp site in a 65 nucleotide region termed an FRT site (Hoang *et al.*, 1997). Because sucrose curing cannot be used in any bacterial background that contains an endogenous *sacB* gene, the Flp recombinase vector was modified so that curing could be accomplished using temperature sensitivity (S. R. Poltak, unpublished data) (Figure 2.1).

Methods

Bacterial Strains, Media and Growth Conditions

Strains and plasmids are listed in Table 2.1. *Burkholderia* species were grown in Luria-Bertani broth (LB: 1% tryptone, 0.5% yeast extract, 1% NaCl) at 32°C with orbital shaking at 130 rpm. *E. coli* was grown similarly at 37°C with orbital shaking. Antibiotic concentrations for *B. cenocepacia* were 100 µg/ml trimethoprim (Tp), and 30 µg/ml polymyxin B sulfate (PMB) in LB. Antibiotic concentrations for *E. coli* were 50 µg/ml Tp, 50 µg/ml kanamycin (Km), and 50 µg/ml ampicillin (Ap) in LB. For β-galactosidase screening, 0.04% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was added to appropriate media. For red fluorescent protein (RFP) screening, fluorescence was read on a Tecan Infinite 200 multimode scanning plate reader with an excitation wavelength of 587 nm and an emission wavelength of 610 nm (Shaner *et al.*, 2005). For yellow fluorescent protein (YFP) screening, fluorescence was read with an excitation wavelength of 515 nm and an emission wavelength of 528 nm (Shaner *et al.*, 2005).

Table 2.1.

Bacterial strains used and plasmid vectors derived in this study		
Strain	Description	Source
<i>E. coli</i> strains:		
DH5 α	F- RecA1 endA1 hsdRi7 supE44 thi-1 gyrA96 relA1 Δ (argF-lacZYA) U169 ϕ 80lacZ Δ M15 λ -.	Gibco-BRL, Inc.
DH5 α λ pir	F- RecA1 endA1 hsdRi7 supE44 thi-1 gyrA96 relA1 Δ (argF-lacZYA) U169 ϕ 80lacZ Δ M15 λ - with λ pir .	Gibco-BRL, Inc.
<i>E. coli</i> 10G	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) <i>endA1 recA1</i> ϕ 80d <i>lacZ</i> Δ M15 Δ <i>lacX74araD139</i> Δ (<i>ara,leu</i>)7697 <i>galU galK rpsL nupG</i> λ <i>tonA</i> .	Lucigen
K12	MG1655 F- lambda- <i>ilvG- rfb-50 rph-1</i> ; derived from W1485.	Laboratory stock
<i>B. cenocepacia</i> HI2424	Natural soil isolate recovered from agricultural soil in upstate NY, USA; PMB ^R .	Laboratory stock
Plasmids:		
pFTP1	Source of Tp antibiotic resistance cassette flanked by FRT recognition sites.	Choi <i>et al.</i> , 2005
pUC18 R6KT-mini-Tn7T	Ap ^R ; R6K replicon; oriT origin of transfer; contains Tn7 mini transposable element.	Choi <i>et al.</i> , 2005
pTn7-FTP	Ap ^R ; Tp ^R ; derived from pUC18R6KT-mini-Tn7T	This study
pcrSMART	Km ^R ; pUC origin of replication; contains blunt cloning site	Lucigen
pcrSMART <i>lacZ</i>	Km ^R ; derived from pcrSMART; contains <i>lacZ</i> gene with pLac promoter amplified from <i>E. coli</i> K12.	This study
pCELacZ	Ap ^R ; Tp ^R ; contains <i>lacZ</i> gene from pcrSMART <i>lacZ</i> ; derived from pTn7-FTP.	This study
pHC02	Km ^R ; Ap ^R ; pUC origin of replication; contains promoterless mCherry RFP; source of pmHC02.	Shaner <i>et al.</i> , 2005

Table 2.1. continued.

pHC04	Km ^R ; Ap ^R ; pUC origin of replication; contains promoterless Venus YFP; source of pmHC04.	Shaner <i>et al.</i> , 2005
pmHC02	Km ^R ; Ap ^R ; derived from pHC02; contains a modified RBS that matches the RBS of a <i>recA</i> gene from <i>B. cenocepacia</i> HI2424.	This study
pmHC04	Km ^R ; Ap ^R ; derived from pHC04; contains a modified RBS that matches the RBS of a <i>recA</i> gene from <i>B. cenocepacia</i> HI2424.	This study
pcrmLRFP	Km ^R ; derived from pcrSMART; contains modified RBS and mCherry RFP expressed off a pLac promoter.	This study
pcrmLYFP	Km ^R ; derived from pcrSMART; contains modified RBS and VenusYFP expressed off a pLac promoter.	This study
pCERFP	Ap ^R ; Tp ^R ; derived from pTn7-FTP; contains modified RBS, and mCherry RFP expressed off a pLac promoter.	This study
pCEYFP	Ap ^R ; Tp ^R ; derived from pTn7-FTP; contains modified RBS, and Venus YFP expressed off a pLac promoter.	This study
pTNS2	Ap ^R ; R6K replicon; encodes the TnsABC+D transposition pathway.	Choi <i>et al.</i> , 2005
pEVS104	Km ^R ; F+ conjugal helper plasmid.	Stabb <i>et al.</i> , 2002

Plasmid and Transposon Constructions

Standard molecular methods were used throughout (Lund *et al.*, 1996; Sambrook *et al.*, 1989). Cloning using polymerase chain reaction (PCR) was done using *Taq* DNA polymerase (5 Prime) according to manufacturer's protocols. The oligonucleotide primers (Integrated DNA Technologies) used for cloning are listed in Table 2.2. All plasmids were isolated from bacterial cells using the Qiaprep Spin Miniprep kit (Qiagen) according to manufacturer's protocols. All restriction enzymes were from New England Biolabs (NEB) and restriction digest protocols were followed according to manufacturer's instructions (NEB).

To confer Tp resistance to bacteria harboring a Tn7 vector, I derived pTn7-FTP from pFTP1 and pUC18 R6KT-mini-Tn7T (Figure 2.1). The Tp cassette with flanking FRT recognition sites (860 bp) was removed from pFTP1 using the *Xma*I sites on either side of the desired region and cloned into the *Xma*I site of pUC18 R6KT-mini-Tn7T using T4 DNA ligase (NEB) (Figure 2.1). Details, including the source, for pFTP1 are listed in Table 2.1. The resulting ligation mix was transformed into chemically competent *E. coli* DH5 α λ pir and Tp^R clones were cultured. Post culturing, the plasmids were isolated and the insertion was verified by cutting with *Xma*I; the DNA fragments were separated on a 1.2% agarose gel and the presence of a 860 bp band was detected.

To clone *lacZ* from *E. coli*, I derived pcrSMART *lacZ*. The *lacZ* gene with the pLac promoter (3.0 kb) was PCR-amplified from *E. coli* K12 (genome sequence available online at the DOE Joint Genome Institute: <http://www.jgi.doe.gov>) using the gene-specific primers EcolilacZ F and EcolilacZ R. Then, I cloned *lacZ* into pcrSMART[®] (Lucigen) to produce pcrSMART *lacZ* according to manufacturer's protocols.

Table 2.2.

Specific gene primers used in this study	
Primer Name	Sequence
EcolilacZ F	5' ATTCGAAATGCTTCCGGCTCGTATGTTGTGT
EcolilacZ R	5' ATTGTACAACATGGCCTGCCCGGTTATTATTA
FP mutation F	5' ACCTCTAGAAGGGAAGGACCCCGAATGGTG
FP mutation R	5' CACCATTTCGGGGTCCTTCCCTTCTAGAGGT
HCplacF	5' CAGGTTTCCCGACTG
HCplacR	5' GCCAGTGTGATGGAT
HCplacF Seq	5' GCAGCGAGTCAGTGAGCG

Confirmation of the *lacZ* insertion was accomplished by cutting pcrSMART *lacZ* with *Bam*HI and separating fragments on a 1.2% agarose gel; the presence of a 3.0 kb band was detected.

To construct a Tn7 vector that would allow insertion of *lacZ*, I cloned the *lacZ* gene from pcrSMART *lacZ* into pTn7-FTP to produce pCElacZ by the following methods (Figure 2.3). The *lacZ* gene (3.0 kb) was cut from pcrSMART *lacZ* using *Eco*RI sites that flanked the desired region and cloned into the *Eco*RI site of pTn7-FTP using T4 DNA ligase (NEB). Confirmation of the *lacZ* insertion was done by cutting pCElacZ with *Eco*RI and separating fragments on a 1.2% agarose gel; the presence of a 3.0 kb band was detected.

In order to improve binding efficiency by *Burkholderia* ribosomes, I generated pmHC02 and pmHC04 by mutating the RBS upstream of each fluorescent protein-encoding gene on pHC02 and pHC04. The RBSs were mutated using the Qiagen QuikChange® Mutagenesis kit and mutagenic primers FPmutation F and FPmutation R according to manufacturer's protocols. Details, including the sources, for pHC02 and pHC04 are listed in Table 2.1. The pHC02 and pHC04 RBSs were mutated to match the RBS found upstream of the *recA* gene in *B. cenocepacia* HI2424 (genome sequence available online at the DOE Joint Genome Institute: <http://www.jgi.doe.gov>). Confirmation of the mutation was accomplished at the Hubbard Genome Center Sequencing Core Facility (Durham NH) according to their standard sequencing protocols (available online: <http://dnacore.unh.edu>) using the primer HCplacF Seq.

To drive expression of the *mCherry* and *Venus* genes from a pLac promoter, I cloned the *lac* promoter with the mutated RBS and fluorescent protein-encoding gene

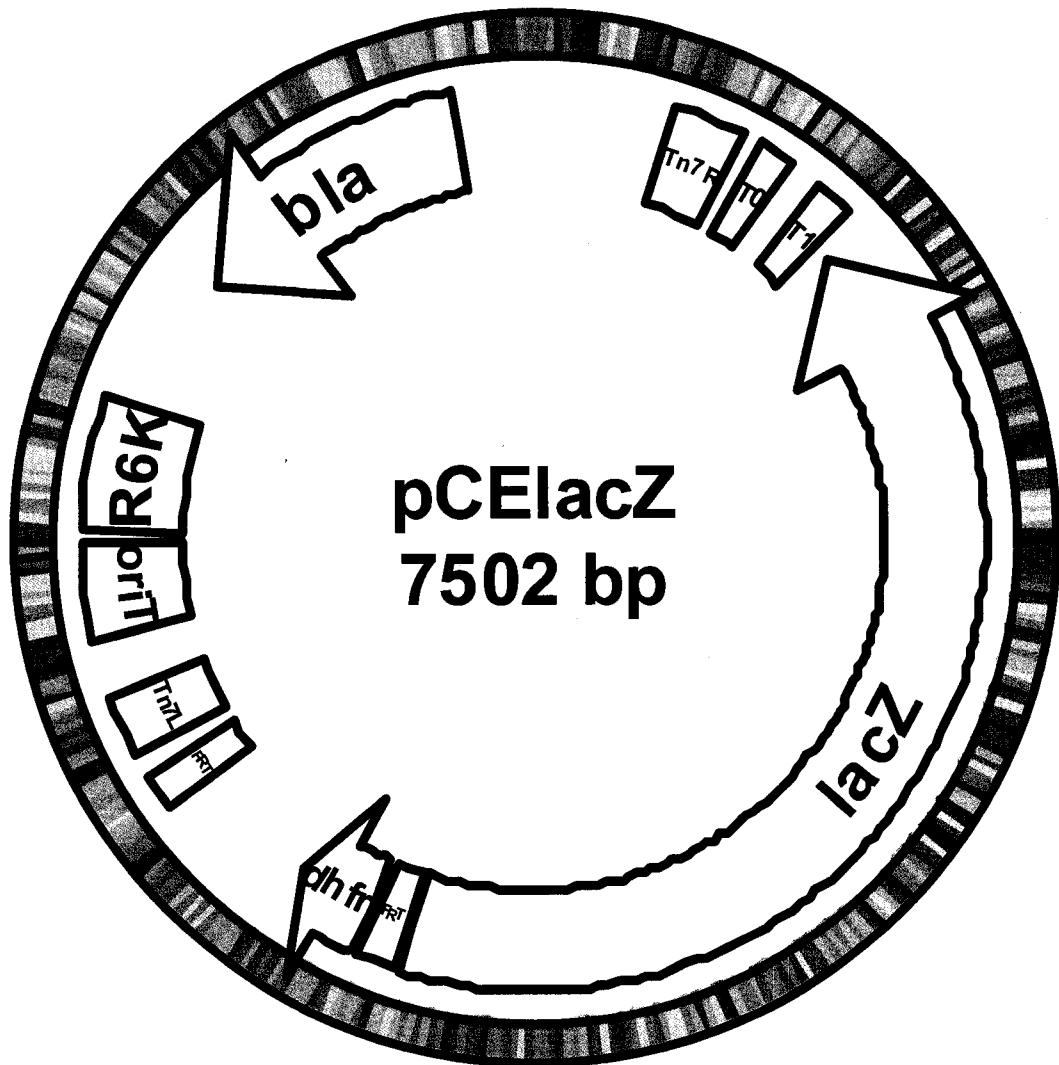


Figure 2.3: Vector map of pCElacZ, which contains a *lacZ* gene under control of its native promoter. Additionally, this vector contains required Tn7R and Tn7L recognition sites for proper chromosomal insertion, terminators T₀ and T₁ to prevent readthrough of upstream genes into the T^R dhfr gene, FRT recognition sites required for F1p recombinase-mediated excision of the *dhfr* trimethoprim resistance cassette, a *bla* gene conferring resistance to ampicillin, a λ -pir dependent R6K origin of replication, and an oriT to facilitate transfer during conjugation.

from pmHC02 and pmHC04 into pcrSMART vectors using the following methods. The *lac* promoter with the mutated RBS and fluorescent protein encoding gene was PCR-amplified (0.8 kb) using the gene-specific primers HCplacF and HCplac R. The amplicons were cloned into pcrSMART® according to manufacturer's protocols to generate pcrmLRFP and pcrmLYFP (Figure 2.4). Confirmation of RFP and YFP insertions was accomplished by measuring fluorescence of bacterial cultures harboring the vectors in a 96-well plate using a Tecan Infinite 200 multimode scanning plate reader (Shaner *et al.*, 2005).

To improve efficiency of further cloning procedures, I removed the restriction digest sites (*HindIII-KpnI-SacI-BamHI-SpeI*) between the pLac promoter and the open reading frames encoding a fluorescent protein on the plasmids pcrmLRFP and pcrmLYFP. The plasmids pcrmLRFP and pcrmLYFP were cut with *HindIII* and *SpeI* to linearize the vectors. The 3' DNA overhangs were removed using DNA polymerase I, large (Klenow) fragment according to manufacturer's protocols (NEB) and the resulting blunt-ended vectors were self-ligated using T4 DNA ligase (NEB). The resulting ligation mixes were transformed into chemically competent *E. coli* DH5 α λ pir and Km^R clones were cultured. Post culturing, the plasmids were isolated and analyzed by restriction digest to verify the removal of the *HindIII*, *KpnI*, *SacI*, *BamHI*, and *SpeI* sites.

In order to construct a Tn7 vector that would allow insertion of *mCherry* and *Venus*, I derived pCERFP and pCEYFP (Figure 2.5). pcrmLRFP, and pcrmLYFP were cut with *EcoRV* to remove the desired region (pLac promoter, AGGA RBS and fluorescent protein coding gene) and the 0.8 kb segment was blunt cloned into the *EcoRV* site on pTn7-FTP using T4 DNA ligase (NEB). The resulting ligation mix was

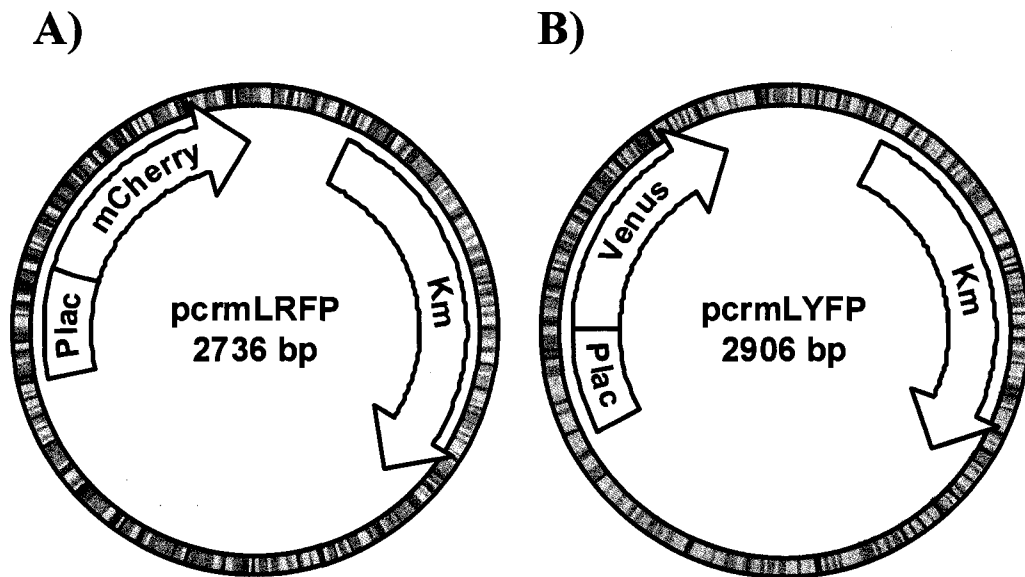


Figure 2.4: Vector maps of pcrmLRFP and pcrmLYFP. A) pcrmLRFP contains an *mCherry* RFP-expressing gene driven by a pLac promoter and a *B. cenocepacia recA* RBS. This vector also contains a Km^R gene. B) pcrmLYFP contains a *Venus* YFP-expressing gene driven by a pLac promoter and a *B. cenocepacia recA* RBS. This vector also contains a Km^R gene.

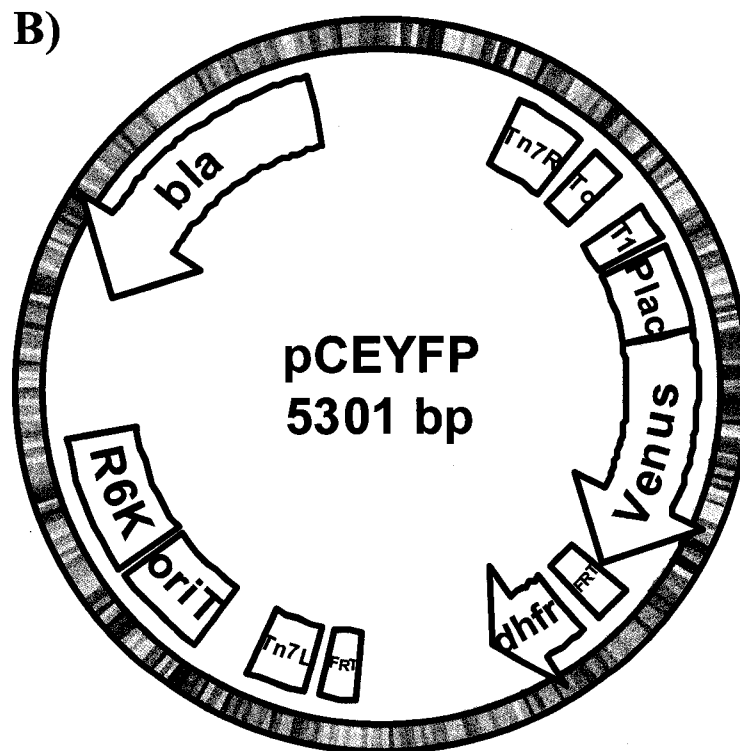
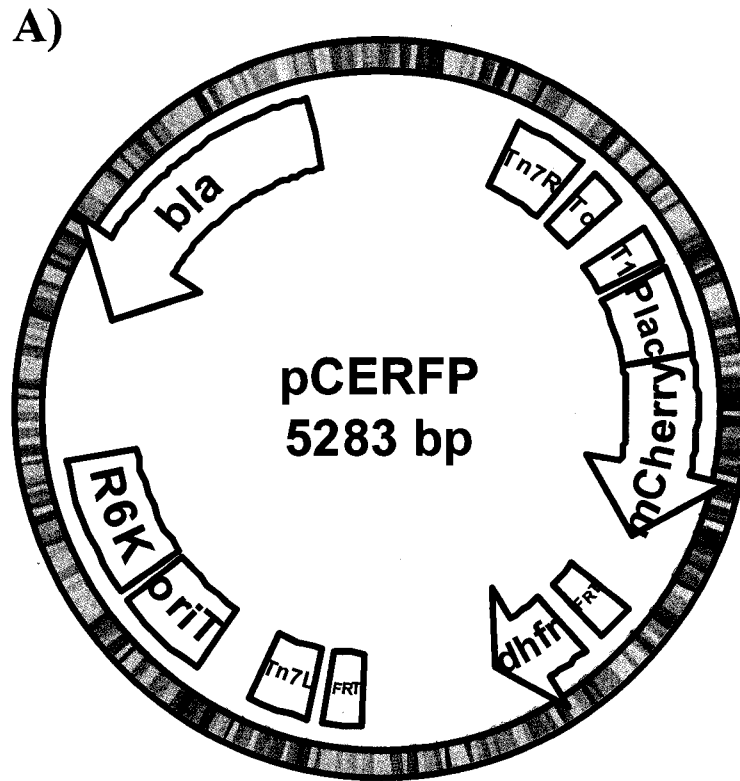


Figure 2.5: Vector maps of pCERFP and pCEYFP. These vectors contain required Tn7R and Tn7L recognition sites for proper chromosomal insertion, terminators T₀ and T₁ to

prevent readthrough of upstream genes into the Tp^R dhfr gene, FRT recognition sites required for Flp recombinase-mediated excision of the *dhfr* trimethoprim resistance cassette, a *bla* gene conferring resistance to ampicillin, a λ -pir dependent R6K origin of replication, and an oriT to facilitate transfer during conjugation. A) pCERFP contains an *mCherry* RFP gene under the control of a pLac promoter. B) pCEYFP contains a *Venus* YFP gene under the control of a pLac promoter.

transformed into chemically competent *E. coli* DH5 α λ pir and Tp^R clones were cultured. Post culturing, the plasmids were isolated and analyzed by restriction digest to verify the insertion. Plasmids were cut with *EcoRV* and fragments were separated on a 1.2% agarose gel; a 0.8 kb fragment was detected. The plasmids were also analyzed using fluorescence readings (Shaner *et al.*, 2005). The Tn7 L and Tn7 R sites on the pCERFP and pCEYFP, which are necessary for transposition to occur were sequenced using the Tn7 Forward and Reverse Sequencing primers. Sequencing of the completed vectors was performed at the Hubbard Genome Center Sequencing Core Facility (Durham NH) according to their standard sequencing protocols.

Delivery and Confirmation of Transposition

To mark *B. cenocepacia* HI2424, the Tn7-FTP derived vectors pCElacZ, pCERFP, and pCEYFP were individually delivered into *B. cenocepacia* HI2424 by four-parental conjugation as previously described (Choi *et al.*, 2006). Each conjugation procedure required a Tn7 vector (pCElacZ, pCERFP, or pCEYFP), the transposase donor pTNS2, the F pilus-expressing helper vector pEVS104 and the *B. cenocepacia* HI2424 recipient. The bacterial helper and donors and were cultured overnight in LB supplemented with the appropriate antibiotic (*E. coli* DH5 α λ pir/ pTNS2 in Ap; *E. coli* DH5 α λ pir/ pEVS104 in Km; *E. coli* DH5 α λ pir/ Tn7-FTP derived vectors in Tp). *B.*

cenocypacia HI2424 was also cultured overnight but in the absence of antibiotics. Equal amounts of each culture were combined and washed twice in 10 mM MgSO₄. The mixture was spotted onto a 0.45 μm nitrocellulose filter on an LB agar plate (2% tryptone, 1% yeast extract, 2% NaCl, 1.6% agar) supplemented with 10 mM MgSO₄. Post incubation, the mixture was plated on LB agar plates containing 100 μg/ml Tp and 15 μg/ml PMB. After 48 hours of incubation, Tp^R colonies were picked and subsequently screened for the Tn7 insertion by using blue/white screening on X-gal to detect a *lacZ* insertion or by using fluorescence to detect a RFP or YFP insertion.

To mark *E. coli* DH5α, the Tn7-FTP derived vectors were delivered into chemically competent *E. coli* DH5α λ pir using standard procedures (Sambrook *et al.*, 1989). Briefly, equal concentrations (50 ng) of pTNS2 and the Tn7-FTP derived vector (pCElacZ, pCERFP, or pCEYFP) were heat shock transformed into *E. coli* DH5α λpir and cell mixtures were plated on LB agar containing Tp. Tp^R colonies were picked and subsequently screened for the Tn7 insertion by using blue/white screening on X-gal to detect a *lacZ* insertion) or by using fluorescence to detect a RFP or YFP insertion.

Verification of Neutrality

To test if insertions by Tn7 transposons derived in this study were neutral, fitness of *B. cenocypacia* Lac⁺ relative to their Lac⁻ counterparts was estimated as outlined previously (Lenski *et al.*, 1991). Briefly, a Lac⁺ single derivative and a Lac⁻ single derivative were recovered from a frozen state and individually acclimated to LB broth. Each Lac⁺ clone was combined with its oppositely-marked Lac⁻ clone in a 1:1 ratio and allowed to grow in LB broth for 24 hours. Initial and final densities (colony-forming

units/ml or CFU/ml) of the two competitors were calculated by plating diluted samples on tryptic soy (T-Soy: 1.5% tryptic soy, 0.75% agar) containing 0.04% X-gal, which distinguished them by their β -galactosidase encoding gene marker *lacZ*. The net growth of each competitor was determined colony counts and the relative fitness (W_{ij}) of the Lac^+ clones was expressed as the log-ratio of their realized growth over one day using the following equation:

$$W_{ij} = \frac{\ln [N_i (1) / N_i (0)]}{\ln [N_j (1) / N_j (0)]}$$

A relative fitness ratio close to one ($\pm 1\%$) was considered neutral.

Results

Vector Construction

In this study, I constructed three suicide mini-Tn7 transposon vectors from the source vector pTn7-FTP that contain the λ -*pir* dependent R6K origin of replication. The three vectors pCElacZ, pCERFP, and pCEYFP require the *pir* protein for maintenance as a replicating plasmid in an *E. coli* background (Figures 2.3 and 2.5). Movement of the vectors into host backgrounds that do not contain the *pir* gene results in loss of the vector through subsequent cell doublings. Therefore, clones exhibiting the phenotypes granted by the Tn7 vectors are the result of chromosomal insertions of the plasmid and not a maintained vector.

The vectors pCElacZ, pCERFP, and pCEYFP also contain oriT origins of transfer, which allow for movement of these vectors by conjugative mating procedures, rather than by electroporation, which is limiting due to its mutagenic properties (Figures

2.3 and 2.5) (Myers and Tisa, 2003). Also, electroporation of pathogens in particular is discouraged due to the aerosolization of cells during this procedure (Choi *et al.*, 2006).

The first vector derived from pTn7-FTP was pCElacZ (Figure 2.3). This vector contains a repressible β -galactosidase encoding gene, *lacZ* that was originally amplified from the *E. coli* K12 genome using specific primers (Table 2.2). Expression of this gene is driven by the native *E. coli* pLac promoter. β -galactosidase is commonly used in molecular biological techniques due to its ease of quantification and phenotypic screening. When β -galactosidase cleaves the molecule X-gal, a blue color is seen in colonies harboring the *lacZ* gene. Bacteria harboring the inserted pCElacZ vector contain the *lacZ* gene as well as the *dhfr* trimethoprim-resistance cassette flanked by FRT recognition sites. Subsequent removal of antibiotic resistance can be accomplished using the pSP-Flp^{TS} vector, which encodes Flp recombinase. Flp recombinase recognizes the FRT sites, excises the *dhfr* cassette, and rejoins the chromosomal DNA. Following recombination, pSP-Flp^{TS} can be cured using temperature sensitivity (S. R. Poltak, unpublished data).

Two additional vectors were derived from pTn7-FTP to contain genes expressing fluorescent proteins. The vector pCERFP contains a *mCherry* red fluorescent protein (RFP) and pCEYFP contains a *Venus* yellow fluorescent protein (YFP) and both are under control of a repressible pLac promoter and a *B. cenocepacia recA* ribosomal binding site (RBS) to improve expression in our strain. Once inserted into the target bacterial chromosome, the fluorescent proteins can be detected using wavelengths previously described (Shaner *et al.*, 2005). Since the excitation and emission ranges of the RFP and YFP used in this study are sufficiently separated, oppositely marked

bacterial populations can be detected in a mixed culture, a requirement for many experimental evolution procedures and other competition experiments.

Delivery of mini-Tn7 Vectors

Delivery of vectors derived from pUC18 R6KT mini-Tn7T into a broad range of Gram (-) bacteria by conjugal mating procedures and chemical transformation was documented previously (Choi *et al.*, 2006; Choi *et al.*, 2005). In this study, the mini-Tn7 vector pCElacZ (Figure 2.3) and the transposase helper vector pTNS2 (Figure 2.1) were co-delivered into *E. coli* DH5 α by chemical transformation. Delivery by this method yielded an average efficiency of ~300-400 putative transformants per 10⁶ cells and verification of insertion was easily accomplished by plating transformants on LB agar containing trimethoprim and X-gal. The novelty of these derived mini-Tn7 vectors lies in their ability to insert genes in a large range of Gram (-) bacteria. With this in mind, I tested the Tn7 insertion ability of pCElacZ in *B. cenocepacia*. Traditional methods for chemical and electrical competence cannot be used on this bacterial species, making it notoriously difficult to manipulate (Choi *et al.*, 2005). Instead, four-parental conjugal mating of *B. cenocepacia* HI2424 was used by mixing this target species in an equal ratio (1:1:1:1) with *E. coli* harboring the transposase helper pTNS2, *E. coli* harboring the mini-Tn7 vector pCElacZ, and *E. coli* harboring a conjugative helper plasmid pEVS104 that expresses pilus formation genes. This mating procedure yielded ~50 putative transformants per 10⁶ cells. The delivery of pCERFP and pCEYFP into a host bacterium was not conducted during this study.

Verification of Neutral Insertions

Many researchers not only require their marking systems to be versatile, but may also require them to mark genomes without disrupting endogenous genes (Cooper, 2007). The *lacZ-dhfr* insertion delivered by CElacZ into *B. cenocepacia* HI2424 was examined for neutrality by allowing a head-to-head competition between this genotype and the wildtype. Realized growth of the competing bacterial populations was assessed by analyzing colony counts on LB agar plates containing X-gal at 0 hours and at 24 hours. To represent fitness, the colony counts were log transformed and represented as a ratio of marked to unmarked (Lenski *et al.*, 1991). A ratio of 1.0 represents equal fitness of the two competitors (Lenski *et al.*, 1991). Results of this analysis showed a neutral fitness ratio of *lacZ-dhfr* marked *B. cenocepacia* to unmarked *B. cenocepacia* in LB, yielding an average fitness of 1.008 (± 0.053 standard deviation).

Discussion

The Tn7 system designed in this study is useful for marking bacterial genomes for many reasons. Because the vectors derived in this study mark by Tn7 insertion, they not only insert site-specifically downstream of an evolutionarily conserved gene, but results of competition assays between marked and unmarked bacteria also show that the insertions do not affect fitness. Additionally, detection of β -galactosidase, RFP, and YFP proteins were rapid and easy. Therefore, this particular system provides a useful method for marking bacterial backgrounds without disrupting endogenous genes. Because the three Tn7-derived vectors cannot be replicated outside a λ pir background due to the R6K origin of replication, easier screening of bacteria harboring a Tn7 insertion can occur,

resulting in a higher recovery of desired clones. The *oriT* origin of transfer on each vector facilitates easy delivery of all mini-Tn7 and transposase helper vectors. Though not performed in this study, other researchers have shown that Flp-mediated excision by pSP-Flp^{TS} resulted in marked genomes lacking the presence of the *dhfr* antibiotic resistance cassette (S. R. Poltak, unpublished data). Lastly, insertion of these vectors has been documented as stable, and insertion by pCElacZ in particular was shown to be stable for up to 500 generations of bacterial passages (see Chapter 3 of this thesis). These general features make this system ideal for use in experimental evolution procedures.

While pCElacZ insertions allow for useful detection of marked bacteria using traditional plating methods, a more sensitive detection method may be necessary depending on the demands of the experiment being conducted. The use of fluorescent proteins for marking bacterial genomes has been widely used in molecular biology and microbiology (Shaner *et al.*, 2005; Tomlin *et al.*, 2004). By marking bacteria with the two Tn7-derived vectors that contain two fluorescent proteins with non-overlapping spectral ranges, pCERFP and pCEYFP, we can conduct mixed population competition experiments, experimental evolution studies, *in vivo* biofilm analysis, or *in vivo* co-infection studies.

The Tn7 insertion system described in this study exhibits many useful qualities for genetically-marking bacterial populations. However, it should be noted that there are a few caveats when using Tn7-based marking systems. First, target immunity caused by previous acquisition of Tn7 transposons can prevent further insertion by the system described in this study. Presence of Tn7R and L recognition sites in this region have been shown to prevent further insertion of Tn7 elements (Stellwagen and Craig, 1997). It

is advisable to examine the genomic content downstream of the *glmS* gene in the desired target genome before using the system described in this study (Stellwagen and Craig, 1997). Second, the presence of multiple *glmS* genes in a bacterial genome can lead to multiple Tn7 insertions rather than a single insertion when using this system; therefore, it is advisable to check target bacterial genomes for the presence of multiple *glmS* genes (Choi *et al.*, 2005). If multiple *glmS* genes are present, this system can still be used as long as a single insertion can be verified by sequencing downstream of each *glmS* gene in the positively-marked bacterial genome. The location of the *lacZ* gene that was inserted into *B. cenocepacia* HI2424 during this study was not confirmed because it did not affect the fitness of bacteria. Yet, because *B. cenocepacia* HI2424 contains multiple *glmS* sites, future work will include identification of the *lacZ* insertion in this bacteria.

Despite a few minor limitations, this Tn7-based marking system offers new and improved methods for inserting genetic material into a genome. This Tn7 system will serve as a valuable set of tools for researchers working with difficult-to-manipulate bacteria.

CHAPTER III

EXPERIMENTAL EVOLUTION OF *BURKHOLDERIA CENOCEPACIA* TO A NOVEL HOST, *ALLIUM CEPA*, AND ITS EFFECTS ON VIRULENCE IN AN ALTERNATIVE HOST.

Introduction

The study of how parasites adapt to hosts is a topic of increasing interest among epidemiologists, agricultural biologists, ecologists, and evolutionary biologists. Some microbial species, such as *Pseudomonas* and *Burkholderia* species, show the ability to infect a wide variety of hosts, whereas others, such as *Mycobacterium leprae*, have a far more specific host range (Coenye and Vandamme, 2003; Cole *et al.*, 2001; Rahme *et al.*, 1995; Tan *et al.*, 1999). Studies of evolving viral populations have demonstrated substantial increases in host adaptation, yet trade-offs associated with adaptation of a viral population to a specific host have also been identified, including a decreased ability to infect a wide range of hosts (Crill *et al.*, 2000; Duffy *et al.*, 2006). Unfortunately, studies of how bacteria adapt to hosts and the consequences of adaptation are limited.

Identifying the mechanisms by which bacterial pathogens adapt to new host environments would serve as a general model for understanding adaptation of various bacterial species, but would also increase understanding of how pathogens emerge. Some emergent pathogens persist in a wide range of plant and animal hosts, which suggests that the virulence factors needed to infect plants and animals are similar (Rahme *et al.*, 1995). What allows some bacterial populations to infect a wide host range, while others infect

such a narrow one? Are there trade-offs associated with bacteria becoming adapted to a specific host?

Members of the *Burkholderia cepacia* complex (Bcc), which are ubiquitous in the environment, were once used as biocontrol and bioremedial agents, yet due to the potentially pathogenic nature of some members, they are no longer eligible to serve this purpose on a large scale (Parke and Gurian-Sherman, 2001). In particular, *B. cenocepacia*, a species frequently recovered from environmental soil samples as a normal soil bacterium, is of interest due to its ability to cause infection in both plant hosts and human hosts (Carvalho *et al.*, 2007; Coenye *et al.*, 2001; Isles *et al.*, 1984). *B. cenocepacia* causes infection in the common yellow onion, *Allium cepa*, and closely related *Burkholderia* species were the cause of large commercial onion crop destruction (Gonzalez *et al.*, 1997). *B. cenocepacia* onion infection is classified as a tissue rot characterized by yellow or brown discoloration, severe tissue maceration and odor (Burkholder, 1950). *B. cepacia* ATCC25416, which is the classified Bcc onion pathogen, breaks down onion tissue by secreting a pectate hydrolase enzyme (Cother and Dowling, 1985; Gonzalez *et al.*, 1997). This enzyme degrades pectin molecules located in the cell walls of plant tissue, subsequently compromising the structure of the plant cell (Ulrich, 1975). *B. cenocepacia* does not produce pectate hydrolase, yet the bacteria still exhibits a plant tissue watersoaking (PTW) phenotype responsible for onion tissue maceration (Gonzalez *et al.*, 1997). PTW appears as softening of tissue and secretion of liquid by the infected onion bulb (Gonzalez *et al.*, 1997). The PTW phenotype is attributed to plant-cell cytotoxic effector molecules delivered by a type IV secretion system; the effector molecules themselves remain uncharacterized (Engledow *et al.*, 2004).

B. cenocepacia can also cause serious infection in the lungs of cystic fibrosis (CF) patients (Coenye *et al.*, 2001; Isles *et al.*, 1984). *B. cenocepacia* infections called ‘cepacia syndrome’ are highly contagious among CF patients and infections result in many negative impacts on an already poor quality of life, including longer hospital stays, removal from lung transplant lists, blood poisoning, and eventual death (Huang *et al.*, 2001). Some putative virulence factors were identified as contributing to lung infection, which include phenotypes such as biofilm formation, adherence to tissue, motility, and extracellular toxin production delivered by type III secretion systems (Carvalho *et al.*, 2007; Kothe *et al.*, 2003; Mahenthiralingam *et al.*, 2005). *B. cenocepacia* has been a threat to the CF community since its discovery and treatments for cepacia syndrome are limited due to an inherent high antibiotic resistance of the pathogen (Mahenthiralingam *et al.*, 2005).

The mechanisms by which *B. cenocepacia* cause disease or adapt to human and plant hosts are unclear, despite the presence of many virulence genes that were identified by transposon mutagenesis or candidate gene knockout experiments (Carvalho *et al.*, 2007; Mahenthiralingam *et al.*, 2005). These candidate gene approaches relied heavily on preconceptions that the chosen phenotypes under examination were required for virulence in human beings; yet, these were *a priori* assumptions based on the function of these same virulence factors in other plant and human respiratory pathogens (Huber *et al.*, 2004; Sokol *et al.*, 2003). It is unknown whether changes in these particular phenotypes were what allowed *B. cenocepacia* to infect plant and human hosts. In contrast to gene knockout experiments, the knowledge learned from experimental evolution offers a complementary alternative to finding the means by which pathogens

adapt to new hosts (Cooper, 2007). Experimental evolution involves growth and passage of a population through a controlled setting, allowing the environment to select for traits that are necessary to improve fitness in that setting (Cooper, 2007). Fitness, the key measurement of adaptation, is determined as the ratio of the growth rates of evolved versus ancestral populations in head-to-head competition experiments (Lenski *et al.*, 1991). Because populations can be frozen throughout the experiment, characterization of differences occurring over time is possible, and the process, rather than the endpoint, of adaptation can be more thoroughly studied (Lenski *et al.*, 1991). Therefore, experimental evolution is an ideal method for characterizing mechanisms responsible for host adaptation in a laboratory setting.

Using the soil isolate *B. cenocepacia* HI2424, a member of the clinically relevant PHDC strain lineage, we studied the extent to which host adaptation to the common yellow onion *Allium cepa* affected subsequent virulence in the alternative host *Caenorhabditis elegans* using experimental evolution. If adaptation to a specific host does not affect the host range of *B. cenocepacia*, as virulence in the onion increases, virulence in the worm should also increase. Yet, we predicted that adaptation of *B. cenocepacia* to an onion model would reduce virulence in the *C. elegans* worm host model due to trade-offs associated with becoming adapted to the onion. We also characterized several phenotypes associated with adaptation to the onion and changes in the nematode model to determine if there were common virulence factors for pathogenicity of onions and nematodes.

Methods

Experimental Evolution

Adaptation of *B. cenocepacia* to the onion host proceeded by serial transfer of bacterial populations, which has been outlined previously (Lenski *et al.*, 1991). Briefly, six populations of *B. cenocepacia* HI2424 Lac⁺ and six populations of *B. cenocepacia* HI2424 Lac⁻ were founded from a single clone and propagated daily by 1:100 dilution into 2% onion media (2% macerated sterile *Allium cepa* tissue, 40 mM Na₂HPO₄, 20 mM KH₂PO₄, 9 mM NaCl, 20 mM NH₄Cl, 1 mM MgSO₄, 1 mM CaCl₂) at 32°C. The populations were maintained in this manner for 500 generations (75 days), with log₂100 = ~6.6 generations occurring per 24 hours; every 100 generations, 750 µl samples from each whole population were stored at -80°C.

Fitness Assays

Fitness of evolved strains relative to their ancestors was determined as outlined previously (Lenski *et al.*, 1991). Briefly, 50 µl mixed samples of the evolved populations and the ancestral clones were recovered from a frozen state and allowed to grow in Luria-Bertani broth (LB: 1% tryptone, 0.5% yeast extract, 1% NaCl) at 32°C with orbital shaking at 130 rpm. Following incubation, each population was diluted 1:100 into 2% onion media and grown for 24 hours at 32°C with orbital shaking at 130 rpm. This allowed the individual populations to acclimate to the host environment. Following incubation, each evolved population was combined with its oppositely-marked ancestor in a 1:1 ratio and allowed to grow in the conditions experienced by the evolving population during the evolution experiment. Initial and final densities (colony-forming

units/ml or CFU/ml) of the two competitors were calculated by plating diluted samples on tryptic soy (T-Soy) containing 0.04% X-gal that allowed them to be distinguished by their β -galactosidase encoding gene marker *lacZ*. The net growth of each competitor was determined from colony counts and the relative fitness (W_{ij}) of the evolved populations (N_i) and the ancestral populations (N_j) was expressed as the log-ratio of their realized growth over one day:

$$W_{ij} = \frac{\ln [N_i (1) / N_i (0)]}{\ln [N_j (1) / N_j (0)]}$$

Each experiment was performed with five replicates. A relative fitness ratio close to one ($\pm 1\%$) is considered neutral.

Growth Curves

Estimation of single population growth rates was determined by performing standard growth curves. Bacterial populations were recovered from a frozen state by growing 50 μ l samples of each population in LB for 24 hours at 32°C with orbital shaking at 130 rpm. Following incubation, each population was diluted 1:100 into 2% onion media and grown for 24 hours at 32°C with orbital shaking at 130 rpm. Overnight cultures were then diluted 1:100 in fresh 2% onion media in five replicates in a 96-well plate. The plate was incubated at 32°C without shaking for 24 hours and optical density readings were taken at 600 nm (OD_{600}) every 15 minutes by a Tecan Infinite M200 plate reader. Maximum growth rate during log phase was calculated as the change in optical density over the change in time between hours 12 and 13 of exponential growth.

***C. elegans* Virulence Assays**

Estimation of bacterial virulence to *C. elegans* was performed using a liquid model of *C. elegans* infection. *C. elegans* strain N2 was recovered from a frozen state and propagated on nematode growth medium (NGM: 0.3% NaCl, 0.25% peptone, 1 mM MgSO₄, 1 mM CaCl₂, 5 µg/ml cholesterol, 25 mM KH₂PO₄, 1.5% agar, 12.5 µg/ml nystatin) seeded with *E. coli* OP50 as a food source. The worms were washed and lysed to harvest their eggs; the eggs were allowed to hatch and grow aseptically in *C. elegans* habitation and reproduction medium (CeHR: Clegg, E. D., LaPenotiere, H. F., French, D. Y. and Szilagyi, M., Abstr. East Coast Worm Meeting, abstr. 91, 2002). Bacterial populations were recovered from a frozen state by growing 50 µl samples of each population in LB for 24 hours at 32°C with orbital shaking at 130 rpm. Following incubation, each population was diluted 1:100 into filtered 2% onion media and grown for 24 hours at 32°C with orbital shaking at 130 rpm. To begin the virulence assay, samples of approximately 100 synchronized worms were each placed in 3 wells of a 6-well plate with 5 ml S medium (0.1 M NaCl, 5.7 mM K₂HPO₄, 44 mM KH₂PO₄, 5 µg/ml cholesterol, 10 mM K₃C₆H₅O₇, 3 mM CaCl₂, 3 mM MgSO₄, 1% trace metals solution (5.5 mM disodium EDTA, 4.5 mM FeSO₄, 1.6 mM MnCl₂, 1.8 mM ZnSO₄, 0.1 mM CuSO₄)). Following a wash in 1X phosphate-buffered saline (PBS: 0.8% NaCl, 0.14% Na₂HPO₄, 0.02% KH₂PO₄), 500 µl samples of each bacterial population previously grown in filtered 2% onion media and standardized to an OD₆₀₀ of 1.0 was added to the worms in S medium. The mixtures were incubated at 24°C for nine days. Percent worm death was monitored by counting the number of dead worms per 50 total worms. The OD₆₀₀ of each mixture was monitored using a Tecan Infinite M200 plate reader.

***Allium cepa* Virulence Assays**

Whole onions were washed with 95% ethanol and cut in half with a sterile knife. Halves were dipped in an onion wash solution (40 mM Na₂HPO₄, 20 mM KH₂PO₄, 9 mM NaCl, 20 mM NH₄Cl, 1 mM MgSO₄, 1 mM CaCl₂, 2 µg/ml nystatin, 2.5 µg/ml gentamycin, 50 µg/ml tetracycline) and inoculated with 200 µl bacterial culture that was previously grown overnight in 2% onion media. The overnight cultures were diluted and plated on T-Soy containing 0.04% X-gal to determine CFU/ml of the initial inoculums. The halves were incubated at 32°C for 72 hours. Post incubation, the halves were diluted in onion wash, blended aseptically and plated on T-Soy containing 0.04% X-gal to determine the final CFU/ml. Virulence was calculated as a ratio of final CFU/ml to initial CFU/ml.

Biofilm Assays

Detection of biofilm formation was performed as outline previously (O'Toole *et al.*, 1999). Bacterial populations were recovered from a frozen state by growing 50 µl samples of each population in LB for 24 hours at 32°C with orbital shaking at 130 rpm. Following incubation, each population was diluted 1:100 into filtered 2% onion media and grown for 24 hours at 32°C with orbital shaking at 130 rpm. Overnight cultures were then diluted 1:100 in fresh 2% onion media in five replicates in a 96-well plate and incubated for 24 hours at 32°C with orbital shaking at 130 rpm. OD₆₀₀ readings were taken with a Tecan Infinite M200 plate reader before the planktonic cells were removed by inversion. The remaining biofilm was stained with 0.01% crystal violet and

subsequently destained in 95% ethanol. The optical density at 595 nm was recorded using a Tecan Infinite M200 plate reader.

Motility Assays

Bacterial populations were recovered from a frozen state by growing 50 µl samples of each population in LB for 24 hours at 32°C with orbital shaking at 130 rpm. Following incubation, each population was diluted 1:100 into filtered 2% onion media and grown for 24 hours at 32°C with orbital shaking at 130 rpm. Bacterial swimming was detected by inoculating T-swim plates (0.3% agar, 1% tryptone, 0.5% NaCl) with 2 µl of overnight bacterial cultures. The plates were incubated at 32°C for 18 hours before the radius of each swimming pattern was determined in millimeters.

Vectorette PCR

All primer sequences are found in Table 2.2. Vectorette PCR analysis was performed as previously outlined (Zhong *et al.*, 2004). Briefly, 25 µg of genomic DNA was digested overnight with 10 units of *RsaI* in 1X New England Biolabs (NEB) buffer #1 at 37°C. Digested fragments were ligated to 2 µl of vectorette bubble units in a reaction containing 1X T4 DNA ligase reaction buffer (NEB) and 800 units T4 DNA ligase (NEB). The reaction was incubated for five cycles: 20°C for 1 hour, followed by incubation at 37°C for 30 minutes. PCR was then performed in 25 µl reactions containing 0.2 µM IS605 forward primer or 0.2 µM IS605 reverse primer, 0.2 µM 224 vectorette primer, 2 ng DNA from ligation reaction, 1 mM dNTP mix, 1X PCR enhancer (5 Prime), 1X Taq buffer containing 15 mM Mg²⁺ (5 Prime), and 2.5 units *Taq* DNA

polymerase (5 Prime). The PCR profile consisted of three cycles: 1(x), 95°C for 15 minutes; 35(x), 94°C for 30 seconds, 57°C for 1 minute and 30 seconds, 72°C for 2 minutes; 1(x), 72°C for 10 minutes. Products were separated in a 1.5% agarose gel at 60 volts/cm for 120 minutes.

Determination of Diet Breadth

The total catabolic diet breadth of the Lac⁺ evolved populations and the Lac⁺ ancestor clone was determined as described previously (Cooper and Lenski, 2000). Briefly, bacterial populations were recovered from a frozen state by growing 50 µl samples of each population in LB for 24 hours at 32°C with orbital shaking at 130 rpm. Following incubation, each population was diluted 1:100 into fresh LB and grown for 24 hours at 32°C with orbital shaking at 130 rpm. Overnight cultures washed in 1X PBS and standardized to an OD₆₀₀ of 1.0. Assays were run in three replicates for each population in Biolog (Hayward, California) ES plates by taking OD₆₀₀ readings at 0, 2, 4, 6, 8, 10, 12, 24, and 48 hours using a Tecan Infinite M200 plate reader. The nine measurements for each well were integrated into one value that arithmetically approximates the area-under-the-curve of catabolic function. The values for the three replicates were averaged to represent one value for each carbon source tested. These values were averaged across all Lac⁺ evolved populations to represent one value for each carbon source tested. These averages were summed to represent the total catabolic usage for the Lac⁺ evolved populations as one value. The same procedure was done for the Lac⁺ ancestor to represent the total catabolic usage as one value.

Phase Contrast Microscopy

Stationary phase bacterial cultures were grown in Luria-Bertani (LB) broth and 3 μ l samples were smeared onto microscope slides containing an agar solution (2% agar, 0.1 M NaCl, 10 mM Na₂HPO₄, 1 mM KH₂PO₄). Mounts were visualized using standard light microscopy at 400x and 1000x.

Confocal Microscopy

A single clone isolated from a Lac⁺ evolved population (D6) and a Lac⁺ ancestral clone were marked with pBBR1-RFP, a stably maintained plasmid expressing a *DsRed* fluorescent protein from a Lac promoter (Poltak, S. P., unpublished data). This marking proceeded by mating procedures previously described (Choi *et al.*, 2006). Briefly, the recipient *Burkholderia* clones were each grown in LB broth for 24 hours at 32°C with orbital shaking at 130 rpm. *Escherichia coli* harboring the mating helper vector pEVS104, a plasmid expressing genes for pilus formation, was grown in LB broth supplemented with 50 μ g/ml kanamycin for 24 hours at 37°C with orbital shaking at 130 rpm. *E. coli* harboring the donor vector pBBR1-RFP was grown in LB broth supplemented with 50 μ g/ml chloramphenicol (Cm) for 24 hours at 37°C with orbital shaking at 130 rpm. Equal amounts of each culture were combined and washed twice in 10 mM MgSO₄. The mixture was spotted onto a 0.45 μ m nitrocellulose filter on an LB agar plate (2% tryptone, 1% yeast extract, 2% NaCl, 1.6% agar) supplemented with 10 mM MgSO₄. Post incubation, the mixture was plated on LB agar plates containing 100 μ g/ml Tp and 15 μ g/ml PMB. After 48 hours of incubation, Cm^R colonies were picked and subsequently screened for pBBR1-RFP by detecting fluorescence at an excitation

wavelength of 515 nm and an emission wavelength of 528 nm using a Tecan Infinite M200 plate reader. Bacterial cultures positive for RFP expression were grown overnight in LB medium and were subsequently washed with 1X PBS. The washed cultures were combined with *C. elegans* nematodes as described previously. After three days of incubation, sample nematodes were mounted on a glass slide containing agar medium. Mounts were visualized using confocal laser scanning microscopy at 200X magnification and 543 nm emission with a BP 560-615 nm filter.

Results

Direct Effects of Experimental Evolution

In order to quantify the fitness changes experienced by the *B. cenocepacia* HI2424 evolved populations after 500 generations of serial passage in an onion host, head-to-head competition assays were performed. Mean fitness was represented as a logarithmic ratio of the evolved Malthusian parameter versus the ancestral Malthusian parameter. Deviations from a ratio of 1.0 indicated a change in fitness, with values less than 1.0 indicating reduced fitness and values greater than 1.0 showing an increase in fitness (Lenski *et al.*, 1991). The individual fitness ratios were plotted (Figure 3.1).

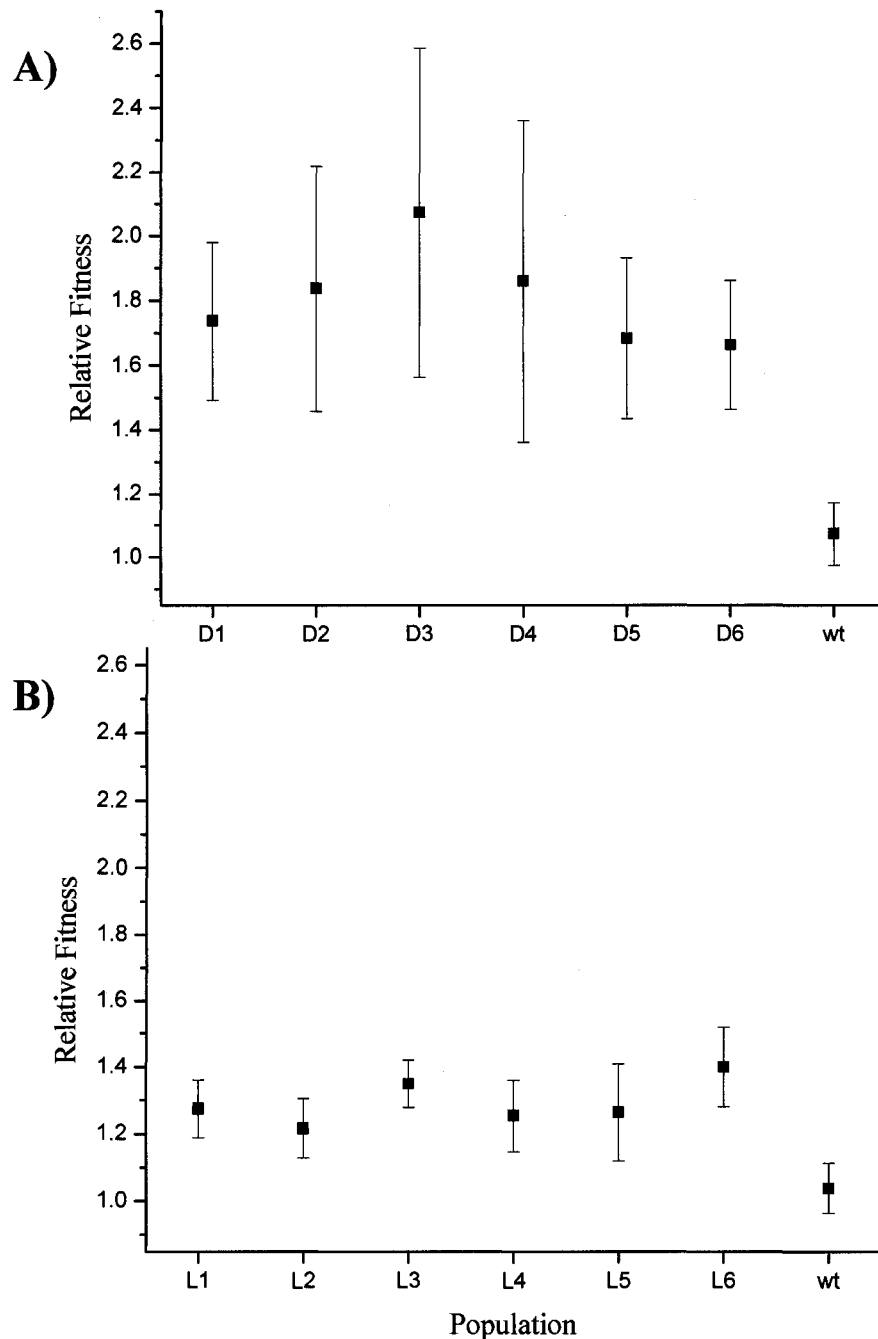


Figure 3.1: Competitive fitness data of evolved genotypes relative to their oppositely marked ancestor in 2% liquid onion medium. Error bars are \pm 95% confidence intervals with d.f. = 4. *A)* Six *B. cenocepacia* HI2424 Lac⁺ populations (labeled “D”) evolved for 500 generations in 2% liquid onion media were competed against their oppositely marked (Lac⁻) ancestor in a 1:1 ratio. The genotype designated “wt” is the fitness of the oppositely marked ancestors competed against each other in a 1:1 ratio. All six evolved populations had significantly increased fitness in the selective environment when compared to wild-type ($p \leq 0.05$). *B)* Six *B. cenocepacia* HI2424 Lac⁻ populations

(labeled “L”) evolved for 500 generations in 2% liquid onion media were competed against their oppositely marked (Lac^+) ancestor in a 1:1 ratio. Three of the six evolved populations had significantly increased fitness in the selective environment when compared to wild-type ($p \leq 0.05$).

Mean fitness of nine populations significantly increased compared to the ancestral clone in the selective environment (Table 3.1 A). The average mean fitness for all 12 populations was 1.551. A one-way analysis of variance (ANOVA) showed significant variation in fitness between populations (Table 3.1 B).

Individual growth curve experiments were conducted on the evolved and ancestor populations in 2% liquid onion medium to determine any difference in maximum growth rate (V_{\max}) during logarithmic phase. Maximum growth rates of evolved and ancestor populations were plotted (Figure 3.2). Mean V_{\max} of 10 of 12 populations was significantly greater than the ancestor (Table 3.2 A). The average mean V_{\max} value for all 12 populations was 0.0135 units/hour ($\Delta\text{Optical density} / \Delta \text{hours}$). A one-way ANOVA was performed that supports an overall significant variation in V_{\max} across all 12 populations (Table 3.2 B).

Table 3.1: Summary of statistical analysis performed on evolved and ancestral mean fitness values obtained from head-to-head competition assays. A) Mean fitness of 12 evolved populations and the ancestral clone (WT), including 95% confidence intervals. B) One-way ANOVA with the dependent variable as fitness and the independent variable as population.

A.

Population	Mean Fitness	95% Confidence Interval
D1	1.7363	± 0.3458
D2	1.8375	± 0.5373
D3	2.0738	± 0.7232
D4	1.8601	± 0.7091
D5	1.6821	± 0.3514
D6	1.6618	± 0.2819
L1	1.2743	± 0.1221
L2	1.2165	± 0.1259
L3	1.3490	± 0.1011
L4	1.2536	± 0.1513
L5	1.2633	± 0.2050
L6	1.2633	± 0.2050
WT	1.0557	± 0.0685

B.

Source	Sum of Squares	df	Mean Square	F	Sig.
Population	6.791	12	0.566	6.893	0.000
Replicate	4.679	57	0.082		
Total	11.470	69			

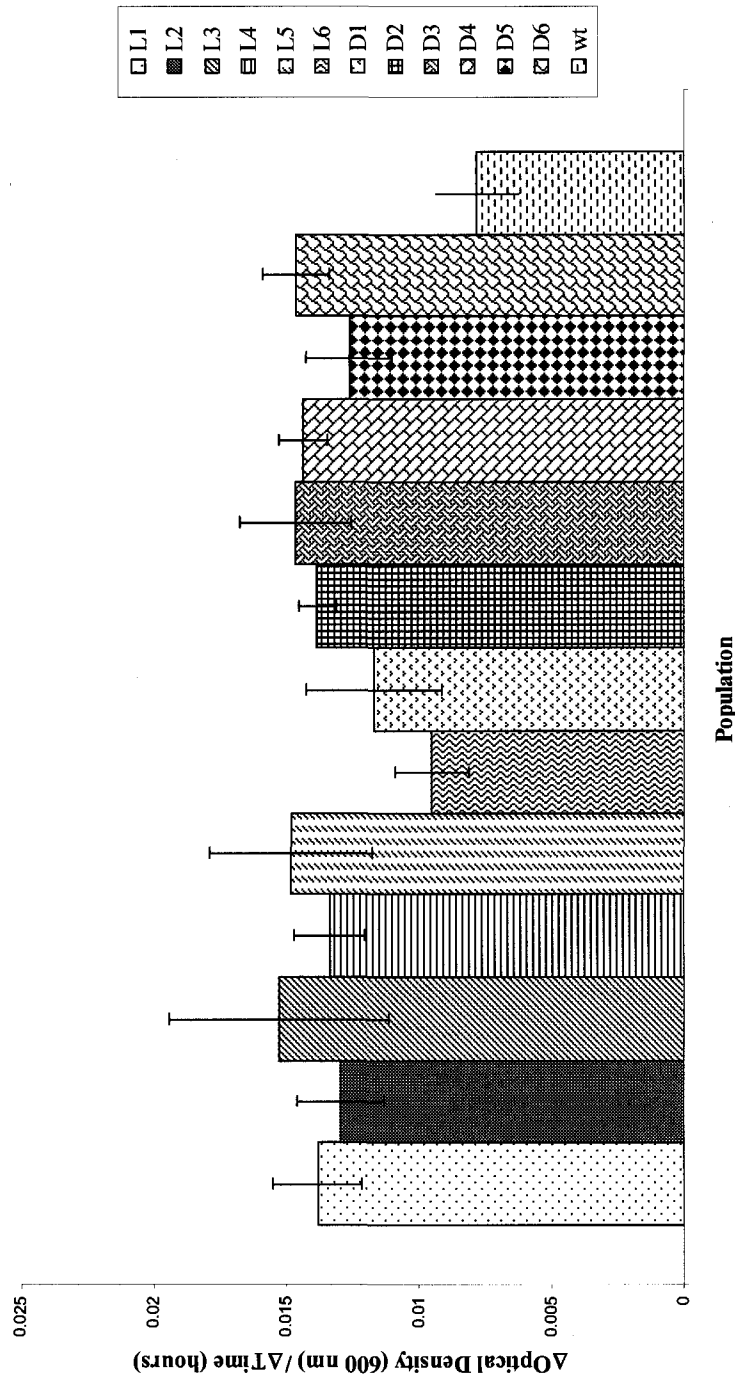


Figure 3.2: Maximum growth rates of evolved and ancestral populations in 2% liquid onion medium. Error bars are $\pm 95\%$ confidence intervals, with d.f. = 4. Populations designated “D” were Lac^+ , whereas populations designated “L” were Lac^- . Populations marked with (*) are significantly different from the ancestor (wt). Ten of the twelve evolved populations showed a significant increase in maximum growth rate compared to the ancestral clone.

Table 3.2: Summary of statistical analysis performed on evolved and ancestral mean maximum growth rates obtained from individual growth curves. A) Mean growth rate during logarithmic phase of all 12 evolved populations and the ancestral clone (WT), including 95% confidence intervals. B) One-way ANOVA with the dependent variable as growth rate and the independent variable as population.

A.

Population	Mean V_{max}	95% Confidence Interval
L1	0.0138	± 0.0023
L2	0.0130	± 0.0024
L3	0.0153	± 0.0059
L4	0.0134	± 0.0019
L5	0.0148	± 0.0043
L6	0.0095	± 0.0019
D1	0.0117	± 0.0031
D2	0.0138	± 0.0010
D3	0.0147	± 0.0030
D4	0.0144	± 0.0013
D5	0.0126	± 0.0023
D6	0.0147	± 0.0018
WT	0.0078	± 0.0018

B.

Source	Sum of Squares	df	Mean Square	F	Sig.
Population	0.000	12	0.000	6.124	0.000
Replicate	0.000	57	0.000		
Total	0.001	69			

Virulence on intact onion halves was assessed to determine if an increase in fitness in 2% liquid onion medium correlated to an increase in growth yield on onion halves. Growth yield was represented as a ratio of final CFU/ml to initial CFU/ml as determined by traditional plate counting on agar surfaces. In one experiment, there was no significant difference in growth on onion halves between Lac⁺ evolved populations and ancestral populations (Figure 3.3). Yet, when this experiment was repeated, significant decreases in growth on the onion half were noted for all Lac⁺ evolved populations compared to the ancestor. Because repeated experimentation yielded inconsistent results between assays, virulence in onion halves as measured by growth rate could not be reliably determined (Figure 3.3). Tissue maceration and severe odor was observed for all evolved populations and also the ancestor clones; however, there was no qualitative difference detected in either tissue maceration or odor between evolved populations and the ancestor clones.

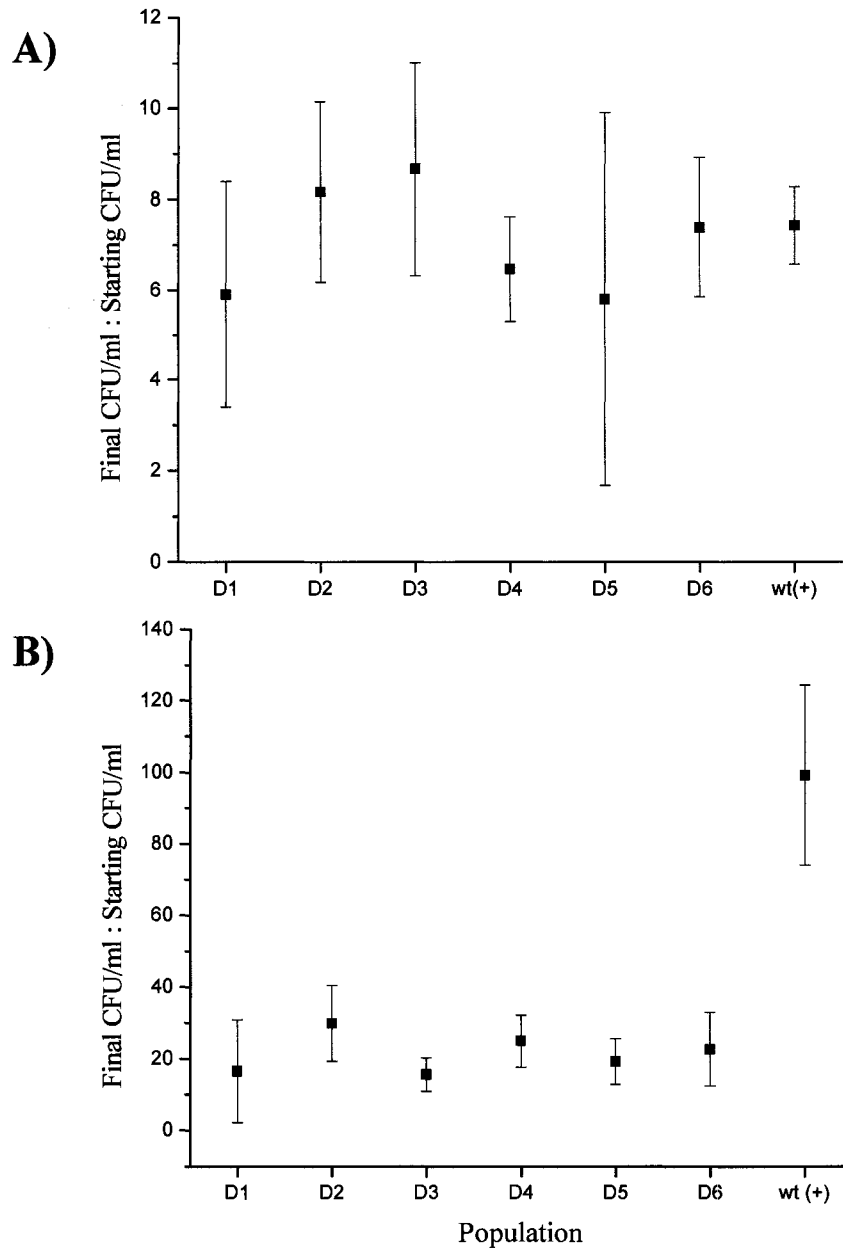


Figure 3.3: Two separate onion virulence assays of six Lac⁺ populations. Genotype designations refer to the bacterial population being tested, with wt (+) representing the Lac⁺ ancestral clone. Growth yield was measured as a ratio of Final CFU/ml to initial CFU/ml. *A)* Growth yield of all six populations did not differ significantly from wt. *B)* Growth yield of these same six populations showed a significant decrease compared to wt. Inconsistency between experiments prevented a reliable estimate of virulence on onion halves.

Correlated Effects of Experimental Evolution

Four separate phenotypes involved with virulence in plant and animal models were examined to determine whether the evolved populations diverged from the ancestor. These were: (1) virulence in liquid *C. elegans* killing assays, (2) motility, (3) biofilm production, and (4) autoagglutination ability. Virulence in liquid *C. elegans* killing assays was quantified for all evolved and ancestor populations to determine if adaptation to the onion host model correlated to a decrease in virulence in the worm host model. Percent of worm death was standardized to log-transformed OD₆₀₀ readings and plotted against time (Figure 3.4 and Table 3.3). All evolved populations showed a significant decrease in worm killing ability when compared to the ability of the ancestral clone. A one-way ANOVA was performed that showed a significant amount of variation between populations in worm killing ability (Table 3.3).

In order to detect any changes in bacterial motility, swimming ability through 0.3% agar was tested individually for all twelve evolved populations, the Lac⁺ and Lac⁻ ancestral clones, and a non-motile *E. coli* mutant. The radius of movement from a central point on the plates was measured in millimeters and plotted versus population (Figure 3.5). All evolved populations significantly increased in motility compared to its clonal ancestor based on 95% confidence intervals.

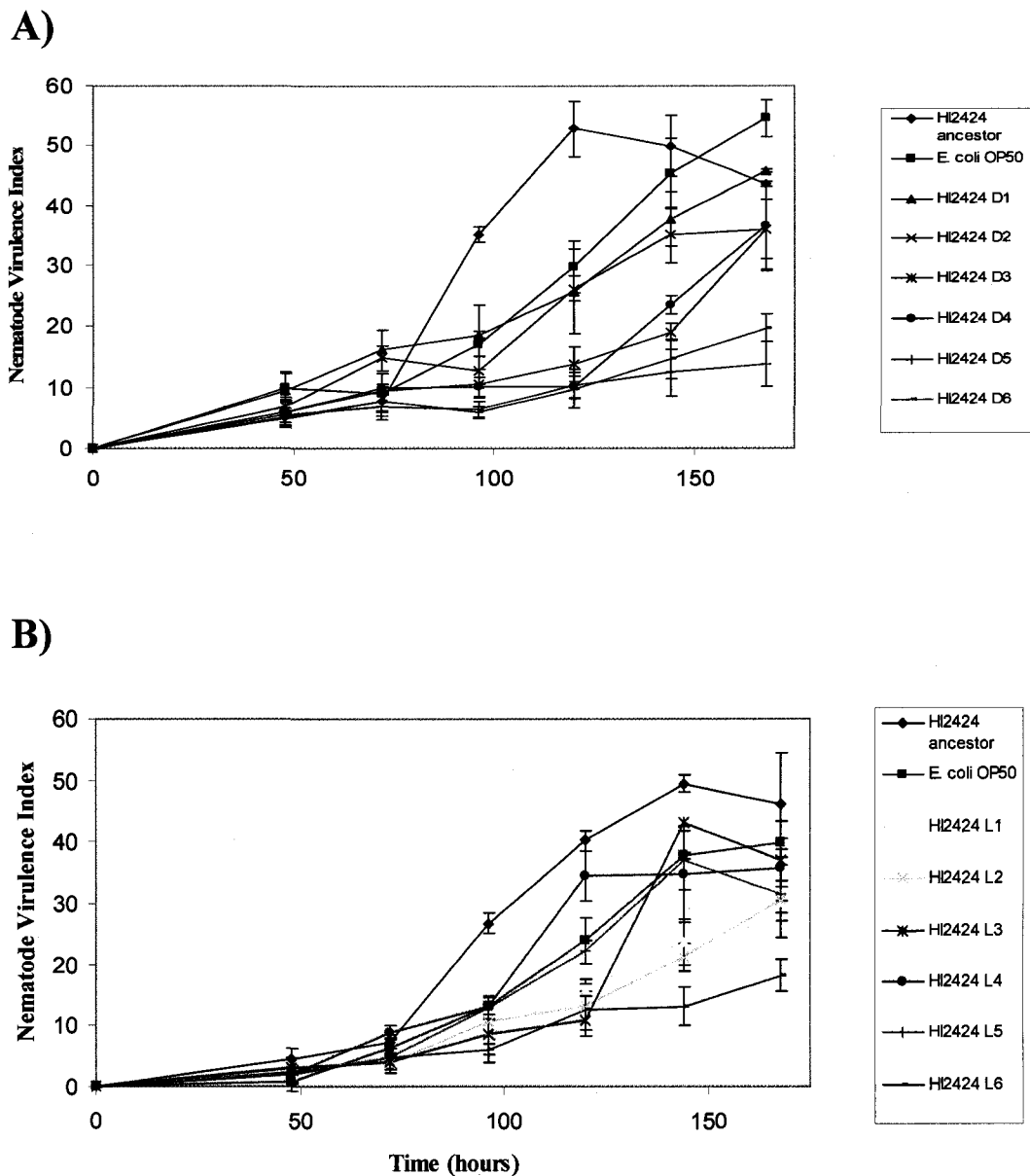


Figure 3.4: Nematode killing in monoxenic liquid culture. Nematode virulence index was expressed as % worms killed divided by the exponent of the optical density. Error bars are $\pm 95\%$ confidence intervals ($df = 2$). *A*) Six *B. cenocepacia* HI2424 Lac⁺ populations evolved for 500 generations in 2% liquid onion medium were introduced to axenically-raised nematodes for 7 days. *B*) Six *B. cenocepacia* HI2424 Lac⁻ populations evolved for 500 generations in 2% liquid onion medium were introduced to axenically-raised nematodes for 7 days. All 12 evolved populations were less virulent than the HI2424 ancestor and supported worm reproduction.

Table 3.3: A) Mean nematode virulence index at 120 hours of all 12 evolved populations and the ancestral clone, (WT) including \pm 95% confidence intervals (df = 2). The mean nematode virulence index was calculated as the percent of worm death divided by log-transformed OD₆₀₀ readings. B) One-way ANOVA, with the dependent variable as nematode virulence index and the independent variable as population.

A.

Population	Mean Virulence Index (120 hours)	95% Confidence Interval
D1	25.7582	\pm 15.5519
D2	26.1926	\pm 4.4565
D3	13.8030	\pm 6.0790
D4	10.0439	\pm 3.7921
D5	10.4445	\pm 4.7497
D6	9.7272	\pm 6.8953
L1	15.8176	\pm 2.1841
L2	13.0389	\pm 10.1006
L3	10.9657	\pm 2.1920
L4	34.4949	\pm 8.7174
L5	22.1475	\pm 4.1858
L6	12.4950	\pm 6.8107
WT	46.5591	\pm 7.7883

B.

Source	Sum of Squares	df	Mean Square	F	Sig.
Population	6635.952	12	552.996	33.335	0.000
Replicate	481.081	29	16.589		
Total	7117.033	41			

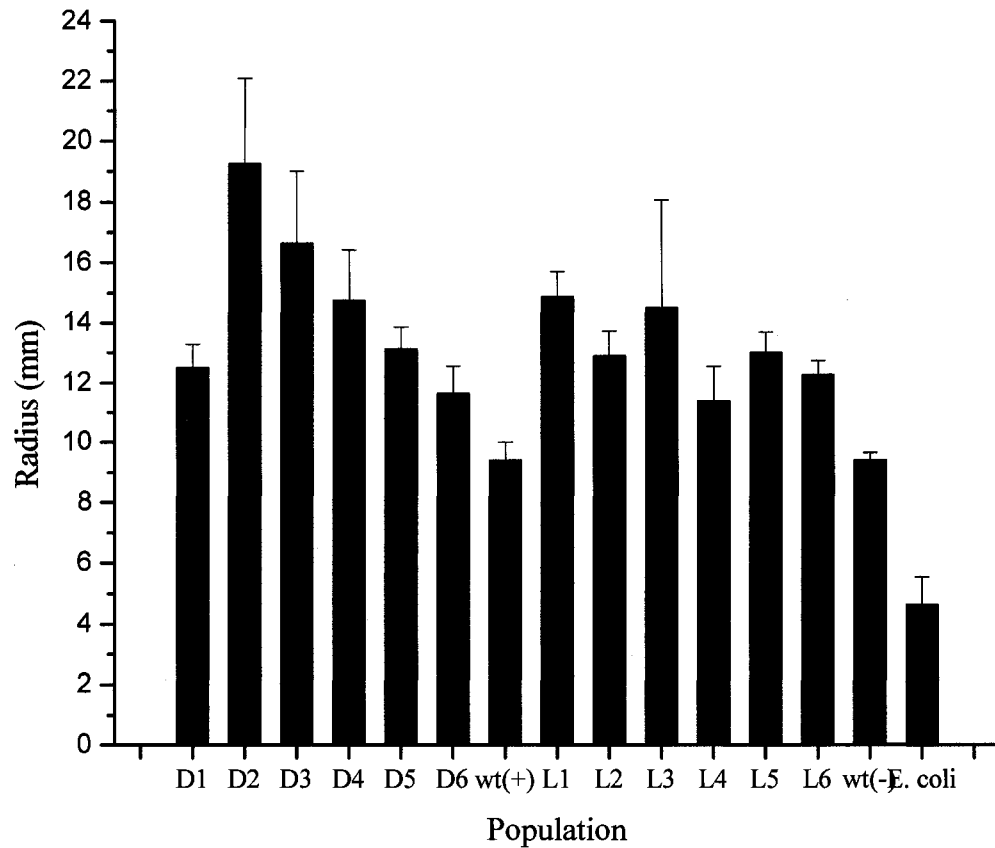


Figure 3.5: Radii of *B. cenocepacia* HI2424 and 12 onion-evolved populations motility through 0.3% swim agar. Genotype designations: L = Lac⁻ evolved populations, D = Lac⁺ evolved populations, wt (+) = Lac⁺ ancestor clone, wt (-) = Lac⁻ ancestor clone. All 12 evolved populations showed a significant increase in motility compared to the ancestral clones. Error bars are ± 95% confidence intervals (df = 2).

In order to detect any differences in biofilm forming ability, biofilm production for all evolved populations and the ancestral clones was assessed using the crystal violet assay. The amount of biofilm, measured as intensity of crystal violet, was plotted versus population designation (Figure 3.6). Six of the twelve evolved populations showed a significant increase in biofilm production when compared to the ancestral clones.

In order to detect any change in autoagglutination ability of evolved populations compared to the ancestral clones, cellular clumping was qualitatively assessed using phase microscopy at 400X and 1000X magnifications. The representative images were taken of the D6 evolved population, a Lac⁺ population that showed a significant decrease in worm killing ability in liquid *C. elegans* killing assays (Figure 3.4 and Figure 3.7). All evolved populations showed a significant decrease in autoagglutination. To complement this finding, *C. elegans* nematodes were individually infected with an RFP-marked clone from the Lac⁺ evolved D6 population and an RFP-marked Lac⁺ ancestral clone for three days. Confocal laser scanning imagery was taken of sample worms from each of these populations using a (Figure 3.8 and Figure 3.9). The wild-type population ingested by the *C. elegans* worm was highly concentrated behind the pharynx in the grinder region of the worm (Figure 3.8). However, the D6 evolved clone ingested by the nematode showed a uniform distribution throughout the body of the nematode, which is suggestive of normal digestion (Figure 3.9).

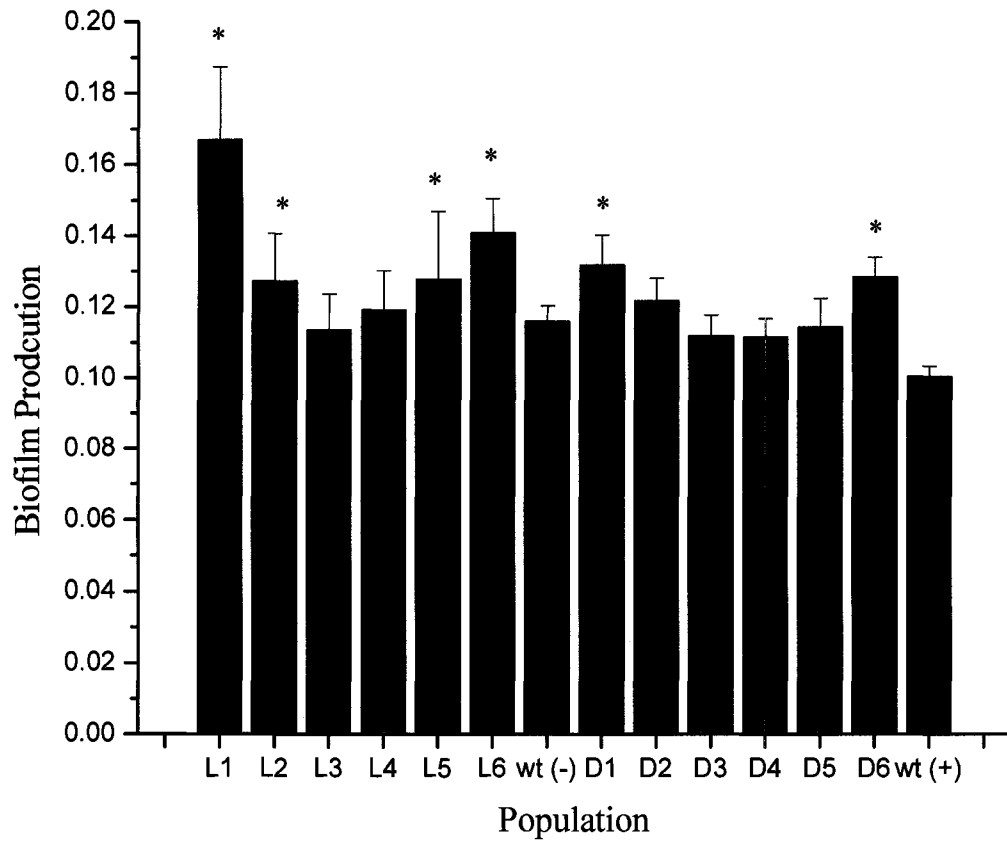


Figure 3.6: Summary of results obtained from a crystal violet biofilm assay of all 12 evolved populations and the ancestral clones. Error bars are \pm 95% confidence intervals (df = 4). Populations marked with (*) are significant from the ancestor (wt). Six (L1, L2, L5, L6, D1, D6) evolved populations show a significant increase in biofilm production compared to wild-type HI2424.

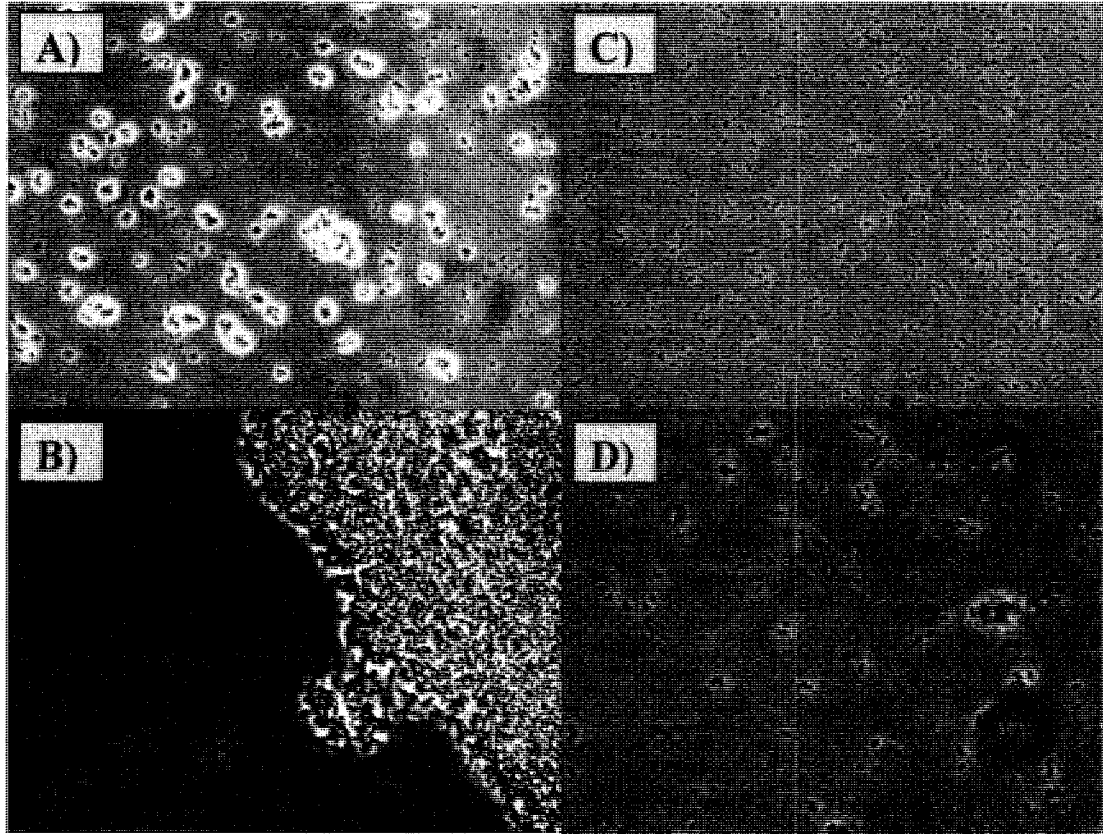


Figure 3.7: Phase contrast microscopy images of evolved and ancestral populations. *A/B*) *B. cenocepacia* HI2424 Lac⁺ ancestral sample at 400X (*A*) and 1000X (*B*) magnification. Frequent clumping of cells is present in these wild-type populations. *C/D*) *B. cenocepacia* HI2424 Lac⁻ D6 sample evolved for 500 generations in 2% liquid onion medium at 400X (*C*) and 1000X (*D*) magnification; this population was selected for microscopy because it showed a dramatic decrease in *C. elegans* worm virulence. Clumping of bacterial cells is absent in this evolved population.

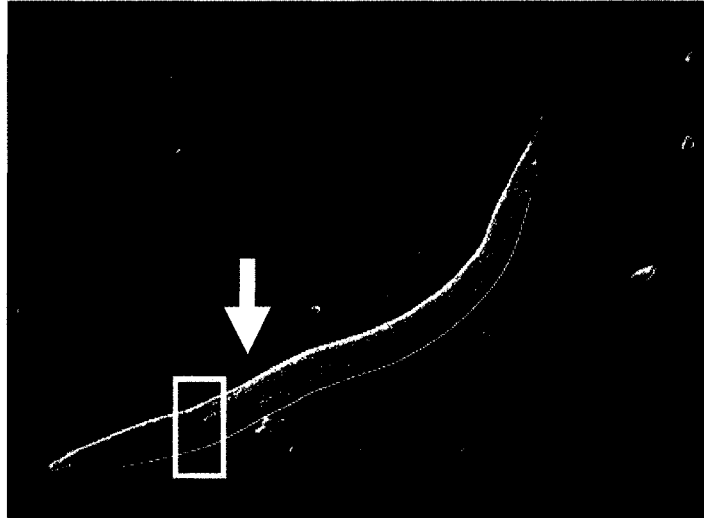


Figure 3.8: Image of a *B. cenocepacia* HI2424 Lac⁺ ancestral sample at 200X magnification. Confocal fluorescent image overlaid with a light microscopic image of RFP-marked bacteria in *C. elegans*. Localization of ingested bacteria, indicated by the white arrow, is highly concentrated behind the grinder region of the worm, indicated by the white box.

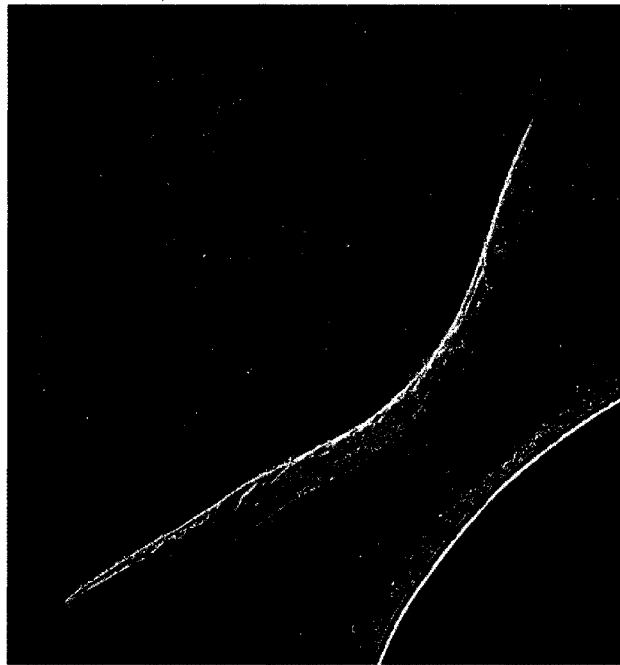


Figure 3.9: Image of a *B. cenocepacia* HI2424 Lac⁺ evolved sample (D6) at 200X magnification. Confocal fluorescent image overlaid with a light microscopy image of RFP-marked bacteria in *C. elegans*. Localization of ingested bacteria is non-specific throughout the intestine of the nematode.

Identification of Insertion Sequence Movement

Random movement of insertion sequences (IS) in the genome has been described as one mechanism behind rapid ecological specialization (Zhong *et al.*, 2004). In order to track the movement of IS605 within the genomes of the evolved populations, the PCR-based technique called vectorette PCR (vPCR) was used. Amplicons resulting from this analysis were visualized using agarose gel electrophoresis (Figure 3.10). Ancestral clones were treated in the same manner as the evolved populations. IS movement was seen in populations L1, L2, and L3 when compared to its ancestor and were each missing the 300 bp band that was present in the lane occupied by the ancestral clone. Also, IS movement was seen in population D3 when compared to its ancestral clone. This population experienced the addition of a 300 bp band that was not seen in the ancestral clone. The differences in the evolved populations' banding patterns compared to the ancestor banding patterns suggest IS movement within the evolved populations; however, the ancestor clones have different banding patterns than each other. This suggests inconsistency with the vPCR experiments performed and further optimization of this technique is needed before any conclusions can be drawn about IS movement in these populations.

Determination of Diet Breadth

In order to detect a difference in total diet breadth as a consequence of adaptation to the onion host model, the evolved and ancestor Lac⁺ populations were inoculated in 95 different carbon sources found on Biolog GN plates and function was represented as area-under-the-curve (AUC) calculations over 48 hours of incubation. The Lac⁺ evolved

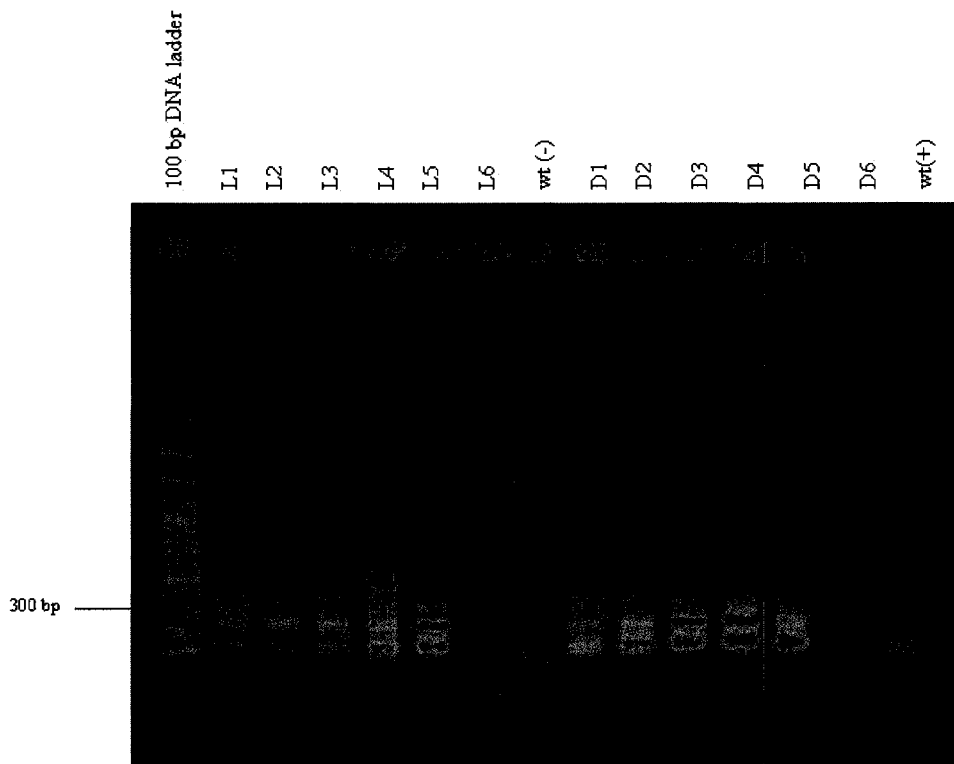


Figure 3.10: An image summarizing the results of a gel electrophoresis procedure that was performed to visualize the amplicons produced by vPCR of evolved populations and the ancestral clones. Lanes L1, L2, L3, L4, L5 and L6 were compared to lane WT(-). Some lanes contain bands that are dark in the center due to overexposure of the image. Lanes D1, D2, D3, D4, D5, and D6 were compared to lane WT(+). The 300 bp band present in WT(-) was not visualized in lanes L1, L2, and L3. Also, lane D3 contained a 300 bp band not seen in lane WT(+).

populations were selected for this assay because of their higher increase in fitness than the Lac⁻ populations (Figure 3.1). AUC values across the six Lac⁺ populations were averaged and grouped by carbon source. T-tests were performed to determine significant differences of evolved Lac⁺ populations from the ancestor Lac⁺ populations. At a *p* criterion of ≤ 0.05 , the evolved populations showed a significant decrease in catabolic usage of 14 carbon sources when compared to the ancestor clone; similarly, when examining the sum of the mean AUC values for all 95 carbon source, the evolved populations experienced an overall significant decrease in diet breadth when compared to the ancestor clone (Table 3.4). Therefore, the diet breadth of the Lac⁺ evolved populations experienced a narrowing of diet breadth as a consequence of passage through the onion host model.

Table 3.4: Summary of mean area-under-the-curve (AUC) calculations obtained from analyzing the diet breadth of the *B. cenocepacia* HI2424 Lac⁺ evolved populations and the Lac⁺ ancestor clone on 95 carbon sources. AUC units are in (Time x OD₆₀₀)². A t-test was performed to determine differences in carbon source catabolism from the ancestor mean. Only carbon sources that met a criterion of $p \leq 0.05$ in comparing evolved catabolic usage with ancestor catabolic usage are shown. Also shown is the sum of the mean AUCs for evolved and ancestor populations of all 95 carbon sources tested.

Carbon Source	Mean Evolved AUC	Mean Ancestor AUC	t-Test
á-D-Lactose	17.443	20.474	0.022
Formic	7.258	10.686	0.000
D-Gluconic	17.833	20.993	0.010
á-Keto Glutaric	18.483	24.554	0.025
D, L-Lactic	15.656	19.428	0.000
Malonic	10.177	15.178	0.001
Propionic	11.461	15.900	0.019
L-Alanine	18.156	20.443	0.005
L-Alanylglycine	18.133	25.708	0.004
L-Glutamic	18.756	21.026	0.028
á-Amino Butyric	21.368	23.222	0.007
Urocanic Acid	17.721	11.883	0.004
D,L-á-Glycerol-6-phosphate	12.406	18.771	0.007
D-Glucose-6 phosphate	12.860	16.520	0.034
Sum of AUC for all 95 Carbon Sources Tested	1057.052	1134.885	0.024

Discussion

Direct Effects of Onion Selection

Twelve populations of *B. cenocepacia* HI2424 underwent 500 generations of serial passage through 2% liquid onion medium and two direct effects were observed as a consequence. First, mean fitness of nine evolved populations increased compared to the ancestor. On average, the evolved populations experienced an increase in reproductive success that was 1.55 times greater than the ancestral clone in direct competition assays. Second, the mean maximum growth rate of eleven evolved populations was significantly faster than the ancestor. Therefore, it can be concluded that significant adaptation to the selective environment occurred as a result of serial passage for 500 generations.

Because the evolved populations that showed no significant difference in fitness and maximum growth rate compared to the ancestor were all Lac⁻ populations, it suggests an effect of the Lac marker. Evolved populations harboring a *lacZ* insertion due to the marking procedure experienced higher increases in fitness when compared to the evolved populations lacking a *lacZ* insertion. The *lacZ* gene encodes for β -galactosidase, which breaks down lactose and other glycoproteins (Skudlarek *et al.*, 1992). A chemical component of onion includes alliinase, a glycoprotein comprising 6% of the soluble proteins in onion bulbs (Lancaster *et al.*, 2000). Addition of the *lacZ* gene during the marking procedure may have provided six populations with a mechanism to increase fitness in the onion host model; *lacZ* may have increased their ability to break down alliinase in the medium. Therefore, it was assumed that the fitness bias experienced by the Lac⁺ populations was a result of the *lacZ* gene influencing subsequent evolutionary paths (Riley *et al.*, 2001). Also, when the diet breadth of the Lac⁺ evolved populations

was determined, they became significantly worse at lactose catabolism, which suggests that the β -galactosidase being expressed by the Lac⁺ populations was not increasing their ability to break down lactose (Table 3.4). Because of this bias towards Lac⁺ populations, it cannot be determined if the fitness of these populations increased as a result of natural selection alone, or as a compounding result of history and natural selection (Riley *et al.*, 2001).

Interpretation of Correlated Effects

Correlated effects are phenotypes observed that are indirect consequences of adaptation in the selective environment (Cooper *et al.*, 2001b). Correlated effects can also be trade-offs associated with specific adaptation. Parallelism of correlated effects can point to similar mutational paths taken by adapting populations (Cooper and Lenski, 2000; Crozat *et al.*, 2005).

A correlated effect was seen in this study that involved virulence in the *C. elegans* model of infection. Mean nematode killing ability decreased across all twelve evolved populations (Figure 3.4). Since a decrease in worm virulence at 500 generations was correlated with an increase in fitness at 500 generations in the onion environment, we concluded that adaptation to the onion host model resulted in a decrease in virulence in the *C. elegans* host model. This conclusion supports the pattern seen in evolving virus populations, that is, adaptation to one host compromises growth in an alternative host (Crill *et al.*, 2000). We also supported another trend seen in adapting viral populations: narrowing of host range as a consequence of specific adaptation to the onion host model resulted in decreased ability to infect an alternative host model (Duffy *et al.*, 2006).

Another correlated effect was seen in this study after examining the total diet breadth of the Lac⁺ evolved populations. Catabolic decay was seen in experimentally evolving populations of *E. coli* that were adapting to a mass-action glucose environment in the laboratory (Cooper and Lenski, 2000). As a consequence of this decay, the *E. coli* populations were unable to grow on certain carbon sources not represented in the selective environment (Cooper and Lenski, 2000). Since our experiment involved serial passage of bacteria in a mass-action environment, we suspected that catabolic decay would occur as a consequence. After quantifying growth on 95 carbon sources, we observed an overall decrease in diet breadth in the Lac⁺ evolved populations when compared to the Lac⁺ ancestor clone (Table 3.4). This catabolic decay may have accounted for the evolved populations' growth decrease observed in the worm killing assays, since they were unable to use as wide a variety of carbon sources as the ancestor. If their diet breadth is limited as a result of adaptation to the onion host model, they may have become unable to break down the carbon sources available to them in the worm host model.

Correlated effects relating to specific virulence phenotypes were analyzed during this study to get a better understanding of which factors are required for killing nematodes or macerating onion tissue and if there are similar virulence factors for killing both. Onion half virulence assays were conducted to determine if an increase in growth on an onion half corresponded to adaptation in the liquid onion host model. Unfortunately, growth yield measurements of evolved populations and ancestral populations varied widely between experiments (Figure 3.3). This inconsistency can be attributed to variable recovery efficiency of the bacteria contained within infected onions.

Also, indigenous bacterial and fungal contamination within the onion halves compounded recovery efforts. It is possible that a decrease in onion half virulence was a result of adaptation to a liquid environment. The mass-action environment created here did not require invasion properties for growth, whereas persistence in plant or animal host tissue requires some mode of tissue invasion in order to establish an infection (Engledow *et al.*, 2004; Tomich and Mohr, 2003). Detection of increased levels of tissue maceration or odor could not be effectively quantified using the assay presented in this study; therefore, a new measurement of onion virulence must be used, such as the previously described onion scale model (Gonzalez *et al.*, 1997), before any firm conclusion can be reached; this remains an area of future study.

Swimming ability was required for virulence of pathogenic bacteria in both animal and plant models in past studies (Eaves-Pyles *et al.*, 2001; Feldman *et al.*, 1998; Felix *et al.*, 1999; Urban *et al.*, 2004). In a study examining *B. cenocepacia* infectivity in a murine model, researchers performed gene knockout experiments of *fliCII*, a gene encoding flagellin production, and showed a decrease in C57/BL6 mouse killing ability (Urban *et al.*, 2004). Other studies have shown that motility is an important virulence factor required for establishment of infection specifically in *C. elegans* models by related *Burkholderia* species (O'Quinn *et al.*, 2001). In the present study, all 12 populations showed a significant *increase* in motility as a result of adaptation to the onion host model, but a *decrease* in worm killing ability was noted in the *C. elegans* host model. This suggests that motility was a factor associated with increased fitness in the onion host model, but not in the nematode model. Increases in motility, as is the case with hyperswimming bacterial mutants, may actually be antagonistic when establishing an

association with an animal host (Millikan and Ruby, 2002). This is consistent with our findings: increased motility is associated with a virulence trade-off in the nematode model as the result of specific adaptation to the onion host model.

Biofilm production is another phenotype required for establishment of bacterial infections in plants and animals (Ramey *et al.*, 2004; Van Alst *et al.*, 2007). Biofilm production has also been speculated as a virulence factor necessary for chronic *B. cenocepacia* infections (Huber *et al.*, 2004; Mahenthiralingam *et al.*, 2005). Mean biofilm production, as measured by a crystal violet biofilm assay, increased for six (L1, L2, L5, L6, D1, and D6) of the twelve evolved populations when compared to the ancestral clones (Figure 3.6). Our study used the liquid *C. elegans* model, which tests the ability of bacterial populations to establish a chronic infection. In acute infections, onset of virulence is rapid and the host often dies quickly, whereas in chronic infections, onset of virulence is gradual and the host dies slowly (Gilleland *et al.*, 1988; Hughes and Gilleland, 1995). In our model, onset of virulence in the nematode host is slow when compared to fast killing assays used in other studies (Tan *et al.*, 1999). During this study, we saw a decrease in virulence in our *C. elegans* liquid worm model for all twelve populations, but this decrease in virulence did not directly correspond to a decrease in biofilm formation (Figure 3.4; Figure 3.6). We would expect that decreases in virulence in this chronic infection model would correspond to decreases in biofilm production since biofilm production is necessary for establishment of a chronic infection, yet some increases in biofilm production were observed. It should be noted that biofilm production of respiratory pathogens increases once an infection has been established and increased

biofilm production before infection is established may hinder invasion ability (V. S. Cooper and S. R. Poltak, unpublished data).

Autoagglutination, which is also known as bacterial clumping, is a phenotype often required for establishing a bacterial infection in plant and animal models and has been specifically implicated in the virulence of *B. cenocepacia* in animal models (Whitby *et al.*, 2006). In related *Burkholderia* species, this phenotype is mediated by pilin production, expressed by the *pilA* gene, and a type IV secretion system (Essex-Lopresti *et al.*, 2005). Knockout deletion experiments were conducted on these gene targets and studies showed that a deletion mutant of *pilA* reduced virulence in the *C. elegans* model (Essex-Lopresti *et al.*, 2005). During this study, autoagglutination of all 12 evolved populations decreased when compared to the ancestral populations as a result of adaptation in the onion host model. This decrease in autoagglutination also correlated to the decrease in virulence observed in the worm killing assays. Further confocal imagery of *C. elegans* worms infected with a clone from the D6 evolved population, a population which showed a significant decrease in worm virulence, revealed a change in localization within the worms when compared to the ancestral clone (Figure 3.8 and Figure 3.9). Because a decrease in autoagglutination ability correlates with decreased virulence in the worm model, it suggests that bacterial clumping is needed for infection in the nematode model, but selected against in the onion model.

Movement of IS elements within a bacterial genome has been implicated as a mechanism of rapid adaptation in some experiments (Schneider *et al.*, 2000; Zhong *et al.*, 2004). IS elements are short 1-2 kb segments of DNA that can transpose within a genome and also across bacterial species (Zhong *et al.*, 2004). They are facilitators of

chromosomal rearrangements, duplications, and deletions driven by recombination events occurring between homologous regions. In this study, movement of IS605 was detected in four of the twelve evolving populations; therefore, IS movement could have contributed to the phenotypic changes observed in these populations (Figure 3.10). However, due to a difference in banding patterns detected between the two ancestral clones, the results obtained from this experiment are probably due to inconsistency with the vPCR method used in this study. Further optimization of this technique for use in this system is needed before any conclusions can be drawn about IS movement in the evolved populations.

During this study, we observed patterns of phenotypic evolution as a consequence of specific adaptation to a liquid onion host: (1) trade-offs common to all of the evolved populations (worm killing ability; motility; autoagglutination); (2) changes specific to the selective environment (increased fitness and maximum growth rate); (3) changes that appeared to be random (biofilm production); and (4) changes that depended on the genetic marker (effects of the *lacZ* insertion). We also observed phenotypes that were correlated with a fitness increase in the onion host model and a decrease in the nematode host model. This suggests that different virulence factors are required for destruction of onion tissue than for destruction of nematode tissue, which is contrary to past studies (Rahme *et al.*, 1995).

Because this study is examining the level of adaptation occurring over 500 generations, a very short amount of time, only 1-2 selective mutations were expected to occur (Lenski *et al.*, 1991). Yet, many phenotypic differences were observed across the 12 populations. In past studies, numerous phenotypic changes occurring in a short span

of time have been attributed to mutations in regulatory regions (Velicer *et al.*, 2006). Also, gene knockout experiments have shown that deletion of global regulators results in changes in expression of multiple virulence phenotypes and also phenotypes required for interaction with a host (Rahme *et al.*, 1995; Whistler *et al.*, 2007; Willis *et al.*, 2001). Because global regulators have been shown to mediate the expression of multiple virulence phenotypes in plant and animal bacterial pathogens, disruption of a global regulator may have been the cause of the phenotypic differences observed in these evolving populations (Rahme *et al.*, 1995; Willis *et al.*, 2001). Future studies will include identification of the mutations that lead to phenotypic differences observed in the onion model and the nematode model, and these studies will specifically target major regulatory pathways.

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