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Character displacement in Burkholderia biofilm communities and the effect of adaptive history on the evolution of niche breadth

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CHARACTER DISPLACEMENT IN *BURKHOLDERIA* BIOFILM COMMUNITIES AND THE EFFECT OF ADAPTIVE HISTORY ON THE EVOLUTION OF NICHE BREADTH

BY

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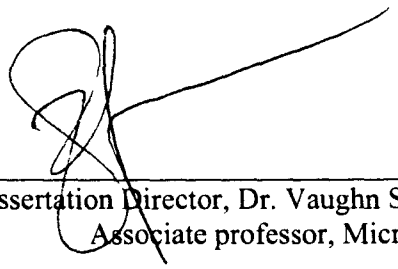

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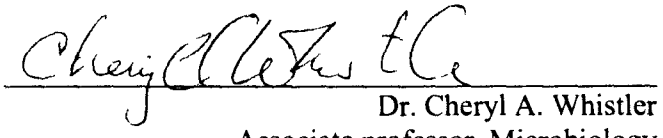

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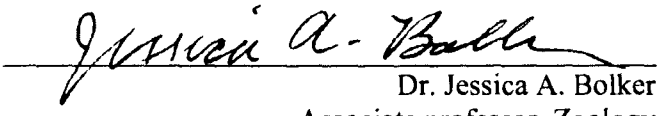

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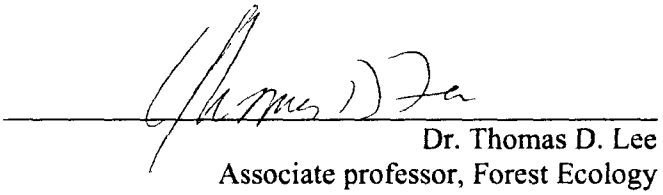

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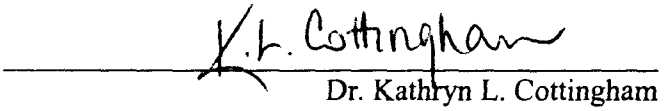

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

CHARACTER DISPLACEMENT IN *BURKHOLDERIA* BIOFILM COMMUNITIES AND THE EFFECT OF ADAPTIVE HISTORY ON THE EVOLUTION OF NICHE BREADTH

By

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Ecologists agree that biodiversity is essential for maintaining highly productive and stable ecosystems, yet the mechanisms that generate and preserve diversity are unknown in many habitats. Competition for shared resources may cause selection to favor niche specialization, which reduces competition and reinforces diversity. If the environment is significantly altered, the ecological history of the remaining colonizer may influence fitness and capacity for diversification in new environments. In many cases, specialists have limited adaptive potential due to trade-offs generated by niche-specific adaptation; however, generalists may be adaptable in many habitats, which would make them good pioneer species for colonization. We investigated the role of character displacement and facilitation in maintaining diversity in a synergistic, biofilm-adapted population of *Burkholderia cenocepacia*, an opportunistic pathogen of cystic fibrosis patients that infects by producing diverse biofilms. Additionally, we examined effects of adaptive

history on the ability of biofilm generalists and specialists to adapt to altered environmental conditions. Our model biofilm population consisted of phenotypically and genetically distinct ecotypes each fulfilling a separate ecological role. We found that diversification in the biofilm was associated with altered resource, as each ecotype occupied distinct spatial niches. We then evolved each ecotype in isolation in planktonic conditions to study whether adaptive history determined the potential to revert to a planktonic lifestyle. Following adaptation, each ecotype evolved into morphologically uniform populations phenotypically resembling the biofilm generalist type, yet fitness of these populations was constrained by prior niche specialization. Lastly, we modeled habitat colonization by evolving each ecotype in isolation in the biofilm environment and tested whether specialization limited the capacity for niche expansion in the absence of competitors. Each biofilm specialist evolved considerable functional diversity, yet the generalists expanded their niche without diversification, which suggested that their adaptive potential exceeded that of the specialists. In summary, diversification within biofilm communities generates competition that favors character displacement and facilitation. Furthermore, when environmental conditions are altered, generalists evolve more productive populations than specialists with less diversification and pleiotropic cost.

CHAPTER ONE

INTRODUCTION

Currently, the National Science Foundation's "Tree of Life" project predicts that there are approximately 5 to 100 million total species on Earth, with only about 1.7 million of them described by taxonomists (The World Conservation Union, 2010). Even more staggering is the proposal that this many species originated from very few founding ancestors (Freeman, 2001; Day, 2004). Ecologists agree that this abundant biodiversity is the key for maintaining highly productive and stable ecosystems, yet the mechanisms that generated and maintained this diversity has been the subject of much debate since the first publication of Charles Darwin's *Origin of Species* (Grinnell, 1917; Hutchinson, 1959; MacArthur, 1967; Chase, 2003; Isbell, 2009). Why are there 1 million species of insects (The World Conservation Union, 2010)? Why are there 400,000 million varieties of beetles alone (Hammond, 1992)? How did so much diversity evolve and what limits diversity in some ecosystems? Much of the described diversity on Earth is the product of natural selection and evolution by this mechanism is closely tied to the concept of the ecological niche (Darwin, 1859; Grant, 2002; Schluter, 1996; MacArthur, 1964).

The Ecological Niche: Definitions and Concepts

Characterizing a species' niche is critical for understanding many ecological properties of that species, such as morphology and physiology, and also for understanding its functional role in the ecosystem. Yet, this task is daunting since just defining the term "ecological niche" has been controversial among ecologists since its introduction in 1917. Classically, the term niche has been defined in two ways. The first was developed by Grinnell (1917), in which a species' niche was defined as the habitat it occupies and the total environmental requirements that allows survival, persistence, and reproduction. This is sometimes now defined as the realized niche (Hutchinson, 1957; Futuyma, 1988). Hutchinson (1957) expanded this definition into a broader one termed the fundamental niche, which was described as the multidimensional space of environmental factors affecting the welfare of a species beyond that which is actually being used in the field, or the "n-dimensional hypervolume" (Hutchinson, 1957). The second definition of niche was developed by Elton (1927) who characterized it as an ecological role in a community, namely its functional role in the food chain. The opposing viewpoint between Elton's focus on the effect of a species on the environment and Grinnell's focus on the effect of the environment on a species fuels the ongoing debate between ecologists concerning the need for a definition of ecological niche (Chase, 2003). Many contemporary ecologists have conceded on a definition which combines both of these concepts, which has allowed for a more complete measurement of a species niche in the field (Chase, 2003; Colwell, 1971; Schluter, 1992; Schluter, 1994; Dayan, 2005).

Proper measurement of a species' niche is no trivial task, but it is generally measured with reference to discrete resource states that can be quantified (Colwell,

1971). Grinnell (1917) stated that the resources being measured in a niche must influence the physiological tolerances, morphological limitations, feeding habits, and interactions between other species in the community. For example, Boag and Grant (1981) measured differences in niche between *Geospiza* finch species on the Galapagos islands as differences in food source, namely the sizes and shapes of different seeds found on the islands. Differences in seed size corresponded with differences in beak morphology and physiological tolerance to other seeds, which limited the survival of some species in the absence of a particular food source (Boag, 1981; Price, 1987). Schluter and McPhail (1992) measured the niches of sympatric stickleback fish species as a difference in foraging grounds, and linked a difference in habitat to morphological distinctions. As classical and contemporary ecologists measured niche boundaries between species, they often observed a link between differences in niche and differences in morphology, which contributed to the idea that the number of species in a community is largely determined by the number of available niches (Dobzhansky, 1951; MacArthur, 1964; Armstrong, 1980). This model inspired further studies into the role of competition as a driving force of diversity (MacArthur, 1964; MacArthur, 1967).

Experiments that investigated competitive interactions between species in communities explored the connection between competition and niche theory. Using laboratory populations of two yeast species, Gause (1932) observed that when they occupied the same niche in the experiment, one species consistently dominated the other by production of a high concentration of ethanol. This lead to the development of the competitive exclusion principle, which states that two species occupying the same niche and competing for the same resources cannot stably coexist; one of the two competitors

acquires an advantage and out-competes the other, leading either to its extinction or a shift to an alternative ecological niche (Gause, 1932; Gause, 1934). Later, Brown and Wilson (1956) observed that in some cases when niches overlapped between species, competition relaxed when one of the competitors favored an alternative resource, and differences in morphology that allowed exploitation of the alternative resource were reinforced by natural selection (Brown, 1956). MacArthur and Levins (1964) supported this observation and developed the principle of limiting similarity which stated that there is a limit to the amount of overlap that can occur between the resource requirements of two species (i.e. niche) before competition will ensue and either promote extinction or a niche shift. Ultimately, many investigators began to explore the role of competition as a driving force of adaptive radiation and speciation (Day, 2004; Schluter, 1992; Grant, 2002; MacArthur, 1964; Dayan, 1989; Dayan, 2005).

Character Displacement: Competition and Natural Selection Promote Diversification by Specialization

Competition between species who share a resource tends to reduce community production; therefore, selection favors phenotypes that allow exploitation of an alternative resource, which reduces competition caused by niche overlap and increases diversity (Brown, 1956; Schluter, 1985; Schluter, 1992; Day, 2004). Brown and Wilson (1956) first termed this process character displacement, which they described as a common process of speciation in areas of sympatry. They examined many taxonomic studies that characterized morphological differences within plant, insect, and bird species

(Brown, 1956). They observed that when two species occurred in allopatry, they were morphologically indistinguishable, but in sympatry, the populations were morphologically divergent, i.e., they displaced each another in one or more genetically based characteristics (Brown, 1956). This sparked interest by other investigators researching the role of competition in speciation and soon evidence of character displacement was reported in several natural populations.

The evolution of diversity by character displacement has been described in several natural populations including birds (Ficken, 1968; Grant, 1972; Schluter, 1985), lizards (Schoener, 1970; Losos, 1990), and mammals (Hutchinson, 1959; Schaffer, 1968; Dayan, 1989). Schluter et al. (1985) described a notable example of character displacement in natural populations of *Geospiza* ground finches on the Galapagos Islands. They measured phenotypes related to the birds' resource use, beak sizes and shapes, and found that differences in these characteristics were exaggerated between two species living in sympatry and more subtle between those living in allopatry. Based on these findings, Schluter et al. concluded that in sympatry, competition for a shared seed source increased selection for morphological traits that allowed one species to use an alternative seed; therefore, both species had increased fitness using distinct resources and overall competition relaxed. However, in allopatry, a lack of competitors allowed the birds to use all seeds, which relaxed selection for specialized traits and resulted in beak sizes and shapes that were intermediate between the two extremes. Dayan et al. (1989) observed similar patterns of character displacement in species of weasels; different feeding behaviors correlated with differences in skull and upper canine length, which were exaggerated in areas of sympatry. However, these investigators admitted a lack of

critical data to fully support their hypothesis. Unfortunately, this was common in many early studies of character displacement in natural populations as experimental data were typically based on observations and lacked universal criteria for measuring key phenotypes.

To add rigor to studies of character displacement, Schluter and McPhail (1992) identified six criteria that must be met experimentally in order to describe diversity in a population as the result of character displacement: 1) differences in traits must have a genetic basis, 2) differences in traits are greater than would be expected by chance, 3) differences in traits are related to resource use, 4) the resources within the community are limiting, 5) differences between sympatric and allopatric communities are not related to differences in resource availability between sites, and 6) differences in traits must have evolved *in situ.* They used these criteria to examine the role of character displacement in the speciation of three-spined stickleback fish that inhabited lakes in British Columbia (Schluter, 1992). In a later study, Schluter (1996) experimentally tested competition as a driving force for diversification of sticklebacks. He produced a hybrid population of fish that ranged in phenotypes specific to limnetic and benthic species, which varied in two phenotypes related to resource use, gill raker and body length. In man-made ponds similar to the stickleback's natural habitat, he introduced a strictly limnetic species to the hybrid population and observed a fitness decrease in fish that closely resembled the limnetic competitor. He also found that fish resembling the benthic species in the target population had increased fitness. This study was the first to show experimental evidence that resource competition promoted phenotypic diversification in a species undergoing radiation, and many investigators used it as a foundation for studying character

displacement in other populations (Rundle, 2000; Robinson, 1994; Smith, 1996). The testable criteria outlined by Schluter and McPhail (1992) have been applied in recent studies focusing on the potential role of character displacement in microbial biofilm systems where experimentation is more controlled and repeatable (Brockhurst, 2006).

Ecological and Genetic Limits of Adaptation

An ecosystem's potential for recovering lost diversity after a major disturbance depends on the adaptability of the remaining inhabitants. Adaptability is often constrained by the course of prior genetic and ecological events that contributed to specific adaptation, which is referred to as adaptive history (Travisano, 1995). Using a natural population of goby fishes, Caley and Munday (2003) investigated how adaptive history and habitat specialization restricted niche breadth. In one variety of goby fish that was specialized to growth in a coral reef environment, they observed low fitness in a large range of alternative habitats; however, they were unable to identify the genetic mechanism responsible for this trade-off. Laboratory studies of microbial populations have been instrumental in identifying the genetic mechanisms that cause trade-offs associated with adaptive history. Crill et al. (2000) investigated the effects of specific host adaptation in the bacteriophage ϕX174 by alternately adapting replicate populations to a *Salmonella* bacterial host and an *Escherichia* bacterial host. Using growth rate to measure fitness, they found that adaptation to the *Salmonella* host decreased the phage's ability to grow in the *Escherichia* host. They subsequently identified mutations in the major capsid protein responsible for recognition and attachment to host cells, which resulted in increased growth in the *Salmonella* host and decreased growth in *Escherichia*

host (Crill, 2000). Therefore, in studies of both natural and experimental populations, adaptive history restricted adaptation in alternative environments.

Ecological role, or the function of an organism in a community, may restrict future adaptation in alternative or disturbed environments. Ecological roles may be simplified into two basic lifestyles: habitat generalists and habitat specialists. Futuyma and Moreno (1988) defined a generalist as an organism with a wide physiological tolerance capable of using a broad range of resources and occupying many different types of habitats. The cockroach is a perfect example of a habitat generalist, as it is one of the most adaptable and prolific species: with the exception of Polar Regions, 3,500 different species occupy every ecosystem on Earth and consume an enormous variety of carbon sources (Miller, 2008). Generalist populations tend to occupy heterogeneous environments and due to high amounts of genetic variability, they are capable pioneer populations that colonize disturbed habitats (Carson, 2008; Kassen, 2002). Despite their wide habitat capacity and tolerance for change, generalists are often competitively inferior to specialists, which are organisms with high fitness on a narrow range of resources that occupy defined niches (Futuyma, 1988; Marvier, 2004). Specialist populations occupy homogeneous environments and low genetic variability tends to limit the niche expansion potential of these populations in the face of ecological disturbance (Futuyma, 1988; Kellermann, 2009). The giant panda is an example of a specialist and it remains an endangered species as the result of habitat loss and a specialized diet of bamboo (Miller, 2008). Researchers using laboratory populations of bacteria confirmed these trends related to generalist and specialist lifestyles and in some cases were able to

pinpoint the genetic mechanisms responsible (Bennett, 1992; Dykhuizen, 1980; Zhong, 2009).

The ecological role of an organism could influence its evolvability, defined by Wagner and Altenberg (1996) as an organism's ability to produce and maintain potentially adaptive genetic variants. Low evolvability is often the result of ecological and genotypic niche specialization, which may slow the rate of adaptation (Monro, 2009; Wagner, 1996). For example, as beneficial mutations increase fitness during adaptation to a specific environment, interactions between them or between acquired mutations and other genes in the ancestral background may decrease fitness in alternative environments. Organisms that are evolvable are typically robust, defined by resistance to antagonistic trade-offs, and phenotypically plastic, which allows them to persist during environmental fluctuation (Baquero, 2009; Gilchrist, 2007; Kitano, 2004; Lenski, 2006; Wagner, 2008; Masel, 2007). The concepts of evolvability and robustness are used to explain why some communities persist in the face of change and others go extinct, yet many models are purely theoretical and cannot be used to identify the mechanisms that contribute to these differences.

Epistatic interactions between acquired mutations are also factors that can limit adaptation. Epistasis is a term used to describe how a phenotype caused by one gene is modified by the activity of one or several other genes. Epistasis can be synergistic, which enhances the effect of a gene, or antagonistic, which hinders the effect of a gene (Elena, 2001). For example, Trindade et al. (2009) performed genetic experiments related to multidrug resistance in *E. coli* and found that many of the mutations that conferred resistance to an antibiotic were more beneficial than expected in genetic

backgrounds containing other resistance mutations, which is evidence of positive epistasis. However, Khan at el (2011) reported evidence of negative epistasis in a laboratory evolved population of *E. coli*. In this study, Khan et al. measured the expected and observed fitness benefit of five mutations that occurred during the course of serial passage by creating each mutation individually in the ancestral background and then in every combination, totaling 32 genotypes. They confirmed that each mutation was beneficial on its own and also that each sequential step was beneficial as long as the mutations appeared in the order that they were originally acquired. However, some combinations of the mutations resulted in lower than expected fitness measurements, indicating negative epistasis. They also observed that as each new mutation was acquired, the effect of negative epistasis was magnified, which they suggested may be the reason why the evolutionary rate of the *E. coli* population slowed down over time. Therefore, negative epistasis may hinder future adaptation in some cases, especially if a population has evolved to fixed conditions for an extended period of time.

Microbial Biofilms: A Model for Studying Phenotypic Diversification

Bacterial biofilms are often diverse communities and the ecological mechanisms that maintain diversity within them are poorly understood. Bacterial biofilms are highly structured, complex communities surrounded by a polysaccharide matrix produced by its physiologically distinct inhabitants (Stoodley, 2002). Biofilms are typically produced in stages and there is evidence that bacteria in each stage of biofilm development are physiologically different from cells in other stages (Sauer, 2002). The stages of biofilm development vary widely among species, but there four basic stages based on

Pseudomonas aeruginosa biofilms (Sauer, 2002; Stoodley, 2002; O'Toole, 1998). The first stage is reversible attachment, where cells come in contact with a surface and produce small amounts of extracellular polymeric substances (EPS) made up of polysaccharides, proteins, and nucleic acids (Stoodley, 2002). These cells are capable of movement and easily revert back to a planktonic state (O'Toole, 1998). The second stage is irreversible attachment in which intercellular bonds and connections to the substrate become strengthened and motility is lost (Stoodley, 2002). The third stage is biofilm maturation in which a robust matrix of EPS surrounds bacterial microcolonies (Stoodley, 2002). Robust EPS production confers resistance to antibiotics and the channels allow deeply set cells access to essential nutrients and oxygen, although cells in the center of the biofilm still experience slower growth due to diminished nutrient availability (Sternberg, 1999). The fourth stage is detachment by reversion of attached cells to a motile, planktonic state in which cells swim away from the mature biofilm (Stoodley, 2002). Depending on the stage of biofilm development, cells become physiologically specialized in response to localized niche conditions (Stoodley, 2002). The process of adaptation to a specific niche is accomplished by differential gene regulation of cells in different stages of biofilm development (Sauer, 2002, Whiteley, 2001). In *P. aeruginosa* biofilms, Whiteley, et al. (2001) detected altered gene expression in 73 genes compared to planktonic cells. Furthermore, Sauer et al. (2002) reported that the protein production between cells in different stages of *P. aeruginosa* biofilm development differed by an average of 35%, which may indicate the presence of different ecotypes within the biofilm (Cohen, 2001). Among the genes that are differentially expressed are those involved in metabolism, lipopolysaccharide biosynthesis (LPS), membrane transport, and secretion

(Whiteley, 2001). Additionally, Davies et al. (1998) observed evidence of cell density-dependant gene regulation by acyl HSL autoinducers within biofilms. Recently, the intracellular molecule c-di-GMP was implicated in regulation of genes that control biofilm formation (Jonas, 2009; Romling, 2005).

Niche specialization within a biofilm could result from differences in production of bis-(3'-5')-cyclic dimeric guanosine monophosphate, or c-di-GMP, which is a global second messenger in bacteria that regulates multiple phenotypes, including motility, biofilm formation, and autoaggregation (Romling, 2005). Synthesis and degradation of c-di-GMP is facilitated by conserved protein sequence motifs, GGDEF and EAL respectively, which are found in numerous proteins distributed widely throughout the bacterial phyla. GGDEF (Gly-Gly-Asp-Glu-Phe) domains have diguanylate cyclase activity (DGC) that catalyzes the joining of two guanosine triphosphate molecules to create one c-di-GMP molecule and two diphosphate molecules. In *E. coli* and *P. fluorescens*, intracellular accumulation of c-di-GMP via activation of GGDEF domain-containing proteins results in overproduction of extracellular matrix components and adhesive fimbriae, which in turn results in wrinkly or rugose colony morphologies (Zogaj, 2001; Spiers, 2002; Bantinaki, 2007). EAL (Glu-Ala-Leu) domains have phosphodiesterase (PDE) activity which hydrolyzes c-di-GMP by breaking the phosphodiester bond that joins the guanosine phosphate molecules (Romling, 2005). Activation of EAL domain-containing proteins decreases EPS production and autoaggregation while simultaneously activating swimming motility (Spiers, 2002). Many proteins in bacterial genomes contain GGDEF and EAL domains, which indicates

that regulation of biofilm production by c-di-GMP is modular and controlled by the activity of several genes (Romling, 2005).

Genetic diversity within the *yciR* gene may explain phenotypic diversity within bacterial biofilms, since it has been implicated in regulation of c-di-GMP. The *yciR* gene contains both GGDEF and EAL domains and controls intracellular levels of c-di-GMP in some Gram-negative bacteria (Weber, 2006; Traverse et al., MS). In vitro, *yciR* shows DGC and PDE activity which depends on the stress response regulator σ^S (Weber, 2006). In *E. coli*, induction of σ^S occurs in a cell density-dependent manner during stationary phase that in turn facilitates the DGC activity of *yciR* resulting in production of biofilm-associated curli fimbriae. The PDE activity of *yciR* reverses this effect. When a *yciR* homolog is deleted in *B. cenocepacia*, intracellular c-di-GMP concentration increases; this indicates that either the EAL activity of *yciR* in *B. cenocepacia* is overwhelming the DGC activity or the DGC activity is naturally suppressed in this bacteria by other unknown mechanisms (Traverse et al., MS).

Mutations within the Wsp signal transduction pathway could also generate diversity within biofilm communities. The Wsp pathway is a group of seven genes that regulates cellular c-di-GMP in *Pseudomonas* bacteria (Bantinaki, 2007; Hickman, 2005). Four genes, *wspA*, *wspB*, *wspD*, and *wspE* make up a membrane bound receptor signaling complex (Bantinaki, 2007). The *wspA* gene produces a membrane-associated methyl-accepting chemotaxis protein which senses environmental signals via its chemotactic transducer receptor. Methylation of WspA by the methyltransferase, WspC, causes a conformational change in the scaffold proteins WspB and WspD. This change activates the histidine kinase response regulator WspE, which phosphorylates the DGC response

regulator WspR. Phosphorylation of WspR drives its DGC activity which produces c-di-GMP molecules. The methyltrasferase, WspC, is constitutively expressed and continually methylates WspA; however, the methylesterase, WspF, removes methyl groups from WspA, which resets the histidine kinase, WspE, to an inactive state. Deletion of *wspF* in *P. fluorescens* causes constitutive activation of WspE and subsequently WspR, resulting in wrinkly colony morphology. The Wsp signal transduction pathway together with the activity of a *yciR* homolog are both implicated in regulating biofilm production in *B. cenocepacia*, an opportunistic, respiratory pathogen.

Infectious biofilm communities are typically diverse, especially those produced by the opportunistic respiratory pathogen *B. cenocepacia,* Gram-negative bacteria that form biofilm in the lungs of cystic fibrosis (CF) patients (Coenye, 2003). 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 Caucasians, which results in decreased immunity to invasive bacteria (Cystic Fibrosis Foundation, www.cff.org). CF patients often acquire *B. cenocepacia* from the environment and transmission via aerosol droplet occurs quickly by patient-to-patient contact and use of contaminated medical devices (Govan, 1993; Holmes, 1999). Infections caused by *B. cenocepacia* can result in a condition known as 'cepacia syndrome,' which is characterized by high fever, rapid pulmonary deterioration, sepsis and often fatal pneumonia (Huber, 2004; Isles, 1984; LiPuma, 1998). Because *B. cenocepacia* inhabits the lungs, the blood, and the sinuses of CF patients, those who test positive for *B. cenocepacia* infection are permanently removed from lung transplant lists, which is a major therapeutic technique used to treat CF (Saiman, 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, 2000; Saiman, 2004). Clinical studies of highly fatal *B. cenocepacia* biofilms revealed the presence of diverse cell types within the lung that vary in colony morphology and biofilm formation (Haussler, 2003). This suggests that *B. cenocepacia* forms complex communities that harbor cell types occupying distinct ecological niches. In light of these findings, Poltak and Cooper (2010) used experimental evolution of *B. cenocepacia* populations to serve as a model for understanding mechanisms that maintain diversity.

An Experimental Model of *B. cenocepacia* Biofilm Evolution

Poltak and Cooper (2010) developed an experimental evolution model to investigate the genetic and ecological mechanisms that contributed to *B. cenocepacia* biofilm evolution (Figure 1.1).

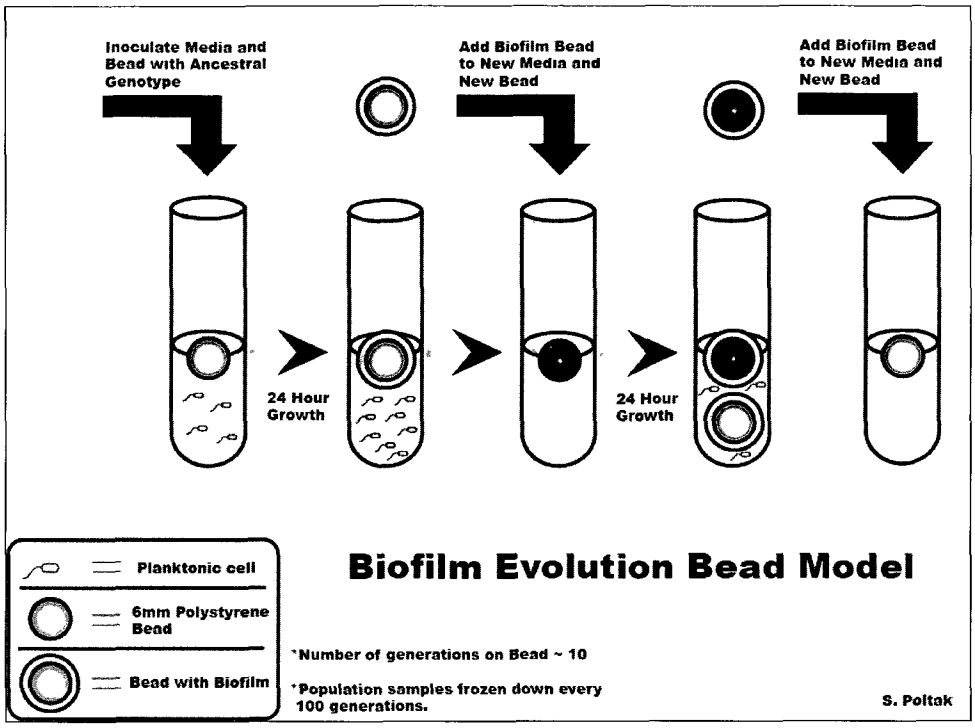

Figure 1.1: Biofilm evolution model by serial bead transfer as described by Poltak and Cooper (2011). Figure adapted from Poltak and Cooper (2011) with permission.

In this system, replicate populations of *B. cenocepacia* founded by single clones were adapted to a biofilm lifestyle by passaging populations for 1,500 generations in test tubes containing a single nutrient source and a surface for adherence in the form of a polystyrene bead. After 1,500 generations, these populations not only developed increased fitness and biofilm production, but they also became diverse, with the evolution of three morphologically distinct cell types: a Wrinkly type (W), a Rough type (R), and a Studded type (S). These cell types resembled clinical isolates, both in phenotype and genotype, which supported the clinical relevance of Poltak and Cooper's model. Each cell type shared a core set of mutations, but each also harbored unique mutations in genes controlling biofilm regulation (Traverse et al., MS). The core mutations were in genes

implicated in iron uptake, sugar metabolism, biofilm regulation by c-di-GMP pooling, and oxidative stress. Mutations unique to R and W were typically in different *wsp* genes, which indicated specialized biofilm regulation, although R harbored other mutations in genes controlling RNA degradation and cell wall synthesis. Differences in genotype, fitness and occupation of the bead space suggested that each cell type adapted to different environmental conditions within the biofilm, which defines each as a separate ecotype (Cohan, 2001). Additionally, there were differences in ecological role between some ecotypes, as a trade-off existed between forming biofilm and growth in galactose media. W is the biofilm specialist since it uses the galactose sugar to form large amounts of exopolysaccharide in the biofilm and does not metabolize it for growth (Table 1.1).

Table 1.1: Biofilm generalist and specialist phenotypes. Numbers represent means of each measured phenotype based on three replicates.

Ecotype	Generation	Role	Biofilm	Vmax	Lag phase
Studded	Early	Generalist	0.823	0.023	14.582
Wrinkly	Early	Specialist	1.040	0.006	18.083
Studded	Late	Generalist	0.972	0.022	11.082
Wrinkly	Late	Specialist	1.777	0.014	15.499

S is the biofilm generalist since it uses the galactose sugar for fast metabolism and growth, but forms weak, flat biofilms containing small amounts of exopolysaccharide (Table 1.1). Community role and frequency of each ecotype was stably maintained after 1,500 generations of adaptation to a biofilm lifestyle, yet the ecological mechanisms that maintained the diversity over time are unknown.

Research Goals and Objectives

Using Poltak and Cooper's model of biofilm evolution, I investigated the role of character displacement in maintaining biofilm diversity and its affect on community production (Chapter 2). The aim of this chapter was to quantify the degree of niche overlap between the three ecotypes and determine if the interactions that maintained each ecotype were facilitated by character displacement. Specifically, I hypothesized that population diversity and stability in a previously characterized biofilm population (B1) was maintained by the evolution of neutral interactions from previously competitive interactions. This predicts that inter-specific competition between ecotypes decreases over time as each becomes more adapted to their own niches. Subsequently, community production becomes additive, equal to the sum of each individual's production. It is important to note that this prediction assumes that displacement happens early after the diversification event, but is not responsible for the diversification event. An alternative to this hypothesis is that diversity and stability is maintained by mutualistic (positive) interactions. This predicts that competition between ecotypes decreases and positive interactions increase, causing community production to be more than the addition of each individual's production. The specific aims of Chapter 2 were:

1. Determine if the B1 population satisfied each of the criteria for character displacement detailed by Schluter and McPhail (1992).

2. Visualize the shift in niche overlap within the biofilm between early and late points in the archived fossil record of the B1 population.

3. Quantify the affect of ecotype interactions on community fitness and determine if observed community production was greater than the sum of the individual's production.

In chapter 3 of this dissertation, I examined the effects of niche specialization on the ability to revert back to a planktonic lifestyle by using experimental evolution to adapt each biofilm ecotype in isolation to a planktonic environment (no bead) (Figure 1.2).

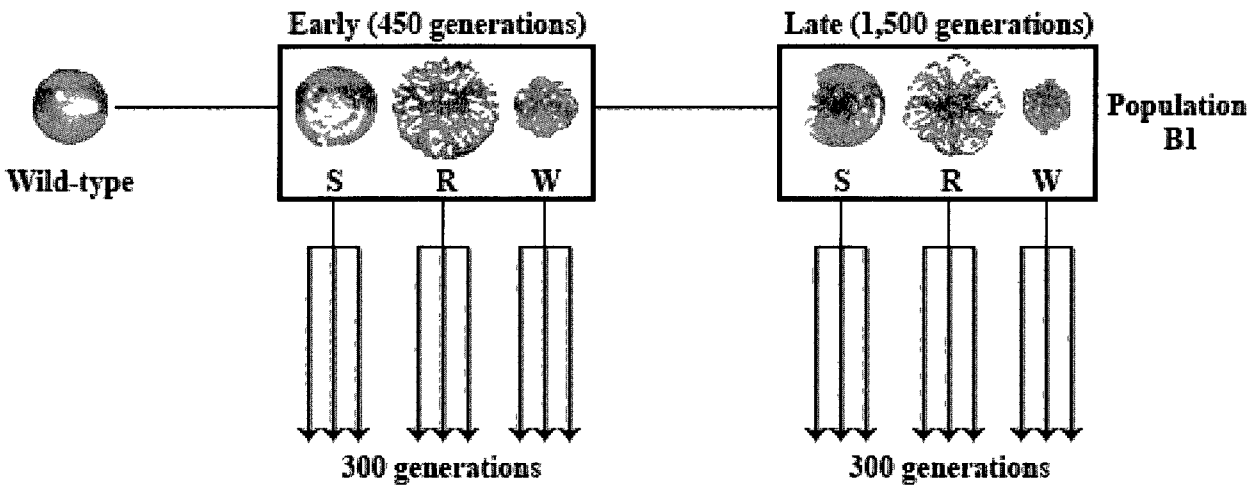

Figure 1.2: Experimental evolution model used to perform serial transfer of B1 ecotypes in isolation.

I hypothesized that long term adaptation and niche specialization produced ecotypes that were not adaptable. This predicts that more specialized ecotypes would produce populations that were less fit in a planktonic environment than populations founded by generalized ecotypes. The specific aims of chapter 3 were:

1. To quantify fitness of ecotypes from early and late points in the archived fossil record of the B1 population as they adapt to a planktonic evolution model.

2. To measure phenotypes in the derived populations to determine if increased planktonic fitness correlated with changes in growth rate, biofilm production, motility, or c-di-GMP production.

3. To sequence cell types derived from W ancestors to determine the genetic mechanisms of reversion to a planktonic environment following long term adaptation to a biofilm.

In chapter 4 of this dissertation, I modeled ecological disturbance by experimentally evolving the biofilm ecotypes to the bead model in isolation and investigated the role of adaptive history on the capacity for niche expansion (Figure 1.2). I hypothesized that specialized ecotypes would be unable to occupy empty niches and generalized ecotypes would expand their niche breadth. This predicts that R and W would undergo phenotypic diversification as mutants that could fill empty niches would be favored by selection, whereas S would not diversify since its physiology allows it to persist under heterogeneous conditions. The specific aims of chapter 4 were:

1. To characterize diversity and measure the production of populations derived from ecotypes isolated from early and late points in the archived fossil record of the B1 population.

2. To quantify the niche breadth of derived cell types and determine if the ability to expand and persist in a disturbed environment correlated with the ecological role of the founding ancestor.

CHAPTER TWO

COMPETITION IS REDUCED BY CHARACTER DISPLACEMENT AND FACILITATION IN EXPERIMENTALLY – EVOLVED *B. CENOCEPACIA* BIOFILMS

Introduction

Abundant biodiversity is a key attribute of productive ecosystems, and production frequently depends on interactions between co-existing species (Day, 2004; Isbell, 2009; Zhang, 2003). In areas where species' niches overlap, facilitative interactions often boost community production, while competitive interactions tend to reduce it (Day, 2004; Schluter, 1992; Tilman, 1994). In some cases, resource competition can cause divergence between species in a particular physical trait (Brown, 1956). Natural selection can reinforce the differences between competing species and may alter resource use, which reduces niche overlap and competition and improves community production (Grant, 2006; Brown, 1956; Schluter, 1985; Schluter, 1992; Dayan 1989). Minimized competition can also lead to facilitation between species, which positively affects community production and also maintains diversity (Bruno, 2003; Turner, 1996;

Travisano, 2000). Although many models have been developed to study how species interactions contribute to diversity and community productivity, most can only explain present day diversity and cannot identify its specific genetic basis. Furthermore, these models fail to address how diversity is maintained over time and if it changes due to altered species interactions.

Diversity is maintained in some communities through character displacement, first described by Brown and Wilson (1956) as a change in a species' resource use that reduces niche overlap as a consequence of competition. Competition is high in areas where several species compete for a limiting resource; therefore, a mutation in a physical trait that allows a species to use an alternative resource would be highly beneficial because it reduces niche overlap and competition. Furthermore, the once limiting resource becomes less limiting over time since one species no longer requires it for survival. Reduced competition usually correlates with increased productivity, so natural selection tends to favor these mutations, thus promoting character divergence and maintaining diversity. Character displacement maintains diversity in several natural populations such as in ground finch populations on the Galapagos Islands where competition for a shared seed source induced differences in beak width, or in aquatic isopod populations where competition for a substrate produced differences in body size (Frier, 1979; Grant, 2006; Dayan, 1989; Dayan, 2005; Abramsky, 1990; Stewart, 2002). Unfortunately, early studies reporting character displacement in natural populations were based on observations and lacked experimental evidence of the process itself. Therefore, Schluter and McPhail (1992) identified six criteria that can be met experimentally in order to demonstrate that character displacement is maintaining diversity in a population:

1) differences in traits must have a genetic basis, 2) differences in traits are greater than would be expected by chance, 3) differences in traits are related to resource use, 4) the resources within the community are limiting, be they food, space, or other shared resources, 5) differences between sympatric and allopatric communities are not related to differences in resource availability between sites, and 6) differences in traits must have evolved *in situ*. These testable criteria have been applied to several systems (Bolinick, 2003; Robinson, 1994; Rundle, 2000; Smith, 1996; Nagel, 1998; Cavender-Bares, 2004) and generally improved empirical testing for character displacement.

Diversity can also be maintained by facilitation, which is a positive interaction between species in which the presence of one species enhances the fitness of another, thus improving community productivity (Day, 2004; Whittaker, 1977; Bruno, 2003). Positive interactions improve fitness by reducing physical or biotic stresses in the habitat, or by creating new habitats or resources. Examples of facilitation are described in several natural populations (Thompson, 1991; Davidson, 1984; Bertness, 1993; Li, 1998) but it is also observed in many experimental bacterial populations, typically in the form of cross-feeding (Turner, 1996; Travisano, 2000). For example, in experimentally-evolved populations of *E. coli* in which only a single glucose carbon source was present, new mutants arose that specialized to growth on acetate, a byproduct of glucose metabolism produced by the wild-type bacteria. In this case, the wild-type bacteria facilitated the growth of the acetate specialists by introducing a new resource into the system. Structural facilitation has also been observed in oral bacterial biofilms, in which some species are responsible for primary binding on the dental surface which in turn facilitates

binding by secondary species (Straight, 2009). Other recent studies have also focused on the importance of facilitation in bacterial biofilms (Brockhurst, 2006).

Microbial biofilms are ubiquitous, diverse communities living on both biotic and abiotic surfaces (Huang, 2008; Stoodley, 2002) and may be the predominant lifestyle for microbes. Bacteria in biofilms typically secrete a thick exopolysaccharide (EPS) layer that provides protection from environmental challenges such as shear forces from waters, heavy metal presence, or in the case of infection, protection from the host immune response and antibiotic therapy (Stoodley, 2002). Infections by *Burkholderia cenocepacia*, an opportunistic pathogen of cystic fibrosis patients (CF), are often exacerbated by production of cepacian, an EPS that aids in establishment of thick biofilms, colonization, and persistence in the CF lung (Cunha, 2004). *Burkholderia* infections can be chronic, invasive, and often lead to a poorer prognosis for the patient, longer hospital stays, and an increased risk of early death by septicemia and necrotizing pneumonia (Govan, 1996; Mahenthiralingam, 2005). Interestingly, *B. cenocepacia* isolates recovered from the lungs of infected CF patients are often diverse, with cell types varying in colony morphology and biofilm formation (Haussler, 2003). *B. cenocepacia* biofilms harboring these diverse cell types are more difficult to treat and are associated with higher fatality (Haussler, 2003). This suggests that *B. cenocepacia* biofilms are complex communities whose members adapt to distinct ecological niches and could serve as a model for understanding the mechanisms that maintain diversity. Therefore, in order to understand the pathology of infectious biofilms, we would benefit from understanding the interactions that maintain diversity and productivity within them. In particular, we ask if an *in vitro* model of *B. cenocepacia* biofilm communities can be used to investigate

the role of competition for shared resources, character displacement, or facilitation and how each contributes to community yield and the maintenance of diversity.

I investigated the role of character displacement in sympatric diversification using a model of *B. cenocepacia* biofilm evolution developed by Poltak and Cooper (2010). Six replicate populations of *B. cenocepacia* founded by a single clone were adapted to form biofilm by passaging populations for 1,500 generations in test tubes containing only galactose, the main constituent of lung mucin, and a polystyrene bead surface for adherence. After 1,500 generations, these populations not only developed increased biofilm production, but they also became diverse, with the evolution of three genetically and morphologically distinct cell types: Wrinkly (W), Rough (R), and Studded (S). Differences in biofilm capabilities and occupation of bead space suggest that each cell type has adapted to different environmental conditions within the biofilm, which defines each as a separate ecotype (Cohan, 2001) (Figure 2.1). Each ecotype is maintained at a stable frequency, yet the nature of the interactions that maintain this diversity and frequency over time is unknown.

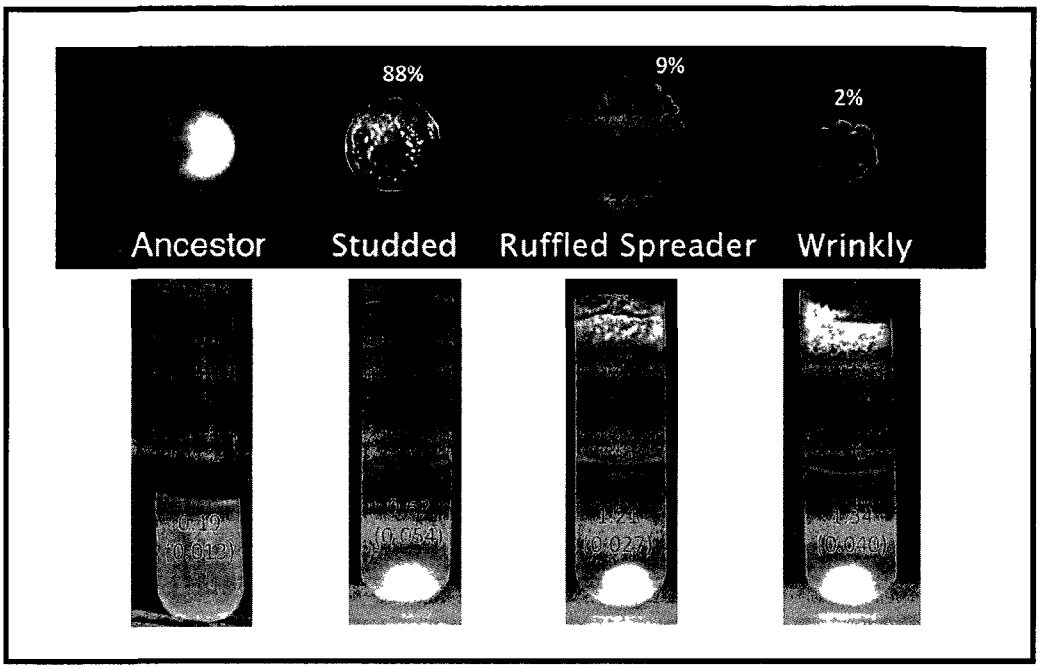

Figure 2.1: Representative colony morphologies of each biofilm ecotype. Percentages represent their frequency in the population. Each test tube contains a 24h culture of an individual ecotype. Red numbers represent the amount of biofilm produced by each as determined by a crystal violet staining assay and numbers in parentheses are 95% confidence intervals. Top image adapted from Poltak and Cooper (2011) with permission.

This model biofilm community satisfies five of the six criteria used as evidence of character displacement (Schluter, 1992). First, the phenotypes that differentiate the three ecotypes have a genetic basis; although each ecotype eventually evolved to share a core set of mutations, each also harbors unique, defining mutations (Traverse et al., MS). Second, the observed patterns of morphology and biofilm formation did not occur by chance, since the same three ecotypes also evolved in five other replicate populations, offering strong evidence that the ecotypes are adaptive (Poltak, 2010; Schluter, 1992). Third, the characters that obviously differ between each ecotype, morphology and the extent of biofilm formation, relate to how each exploits the limiting resource, adherence space. Lastly, the differences in biofilm formation and morphology evolved *in situ*. The

only criterion that is not met by our model requires the existence of sympatric (where species occur together) and allopatric (where species occur separately) communities, the latter of which does not exist in our system.

Our model *B. cenocepacia* biofilm population can help answer key questions about how diversity in infectious biofilms is maintained over time, what types of interactions maintain that diversity, and which genetic mechanisms potentially contribute to diversity. The aims of this study were to investigate if character displacement maintained diversity generated by resource competition in a biofilm and to determine if competition weakened over time as each player adapted to specific conditions. Additionally, since these ecotypes have been previously sequenced and their adaptive mutations characterized, we can identify the genetic mechanism responsible for changes in ecotype interactions over time. Community yield was measured at several time points during the biofilm evolution and was consistent with relaxed competition between all ecotypes for adherence space, the limiting resource in our system. Consistent with character displacement, microscopic images of the evolved biofilms revealed differences in how each ecotype occupied space and those differences correlated with ecotype morphology and biofilm production. Additionally, at the endpoint of the evolution, the S ecotype was more productive than expected when grown in the presence of its co-evolved partners, indicating that facilitative interactions likely evolved between S and the others after competition was relaxed by character displacement.

Materials and Methods

Strains, media, culture conditions and fluorescent marking. Mixed population samples were frozen every 50 generations at 80°C in glycerol during long-term serial passage of *B. cenocepacia* in the selective environment, which was a 7mm polystyrene bead suspended in 5ml of 3% GMM, an M9 minimal medium (40 mM Na_2HPO_4, 20 mM KH_2PO_4, 9 mM NaCl, 20 mM NH_4Cl, 1 mM $MgSO_4$, 1 mM CaCl2) containing 3% (1M) galactose (Poltak, 2010). A Studded (S), Rough (R), and Wrinkly (W) clone from 450 (Early) and 1,500 (Late) generations was isolated by streaking frozen cultures onto 50% T-soy agar and incubating at 37°C for 24h., and 48h. at room temperature. Clones were then picked and frozen individually at 80°C in glycerol. All *Escherichia coli* strains harboring vectors were recovered from frozen stocks and streaked to isolation on T-soy agar containing 50ug/ml chloramphenicol (Cm). Plasmids pSPR, which harbors the red fluorescent protein gene from DsRedExpress, and pSPY, which harbors the EYFP yellow fluorescent protein gene, were used to label the isolated clones from the Early and Late populations (Poltak, 2010). Plasmids were introduced to ecotype clones by electroporation and selection on T-soy agar containing 100ug/ml Cm.

Limiting resource experiment. To determine if nutrients were a limiting resource, yield (final CFU/ml) of the Early and Late populations was quantified from a single bead after 24h of bacterial growth at 37°C in 5ml M9 minimal medium containing 0.01%, 0.1%, 1%, 3% (selective concentration). To determine if space was a limiting resource, yield (final CFU/ml) of the Early and Late populations was quantified from either one or two beads after 24h of bacterial growth at 37°C in 5ml 3% GMM (selective medium). To

remove and quantify adhered cells, the beads were vortexed in 1X PBS for 2 periods of 10s and plating on half-strength T-soy agar.

Confocal microscopy. Ecotypes harboring pSPR or pSPY were recovered from frozen stocks by propagation in T-soy broth supplemented with 50ug/ml Cm for 24h. at 37°C with shaking at 130 rpm. Early and Late populations were constructed by diluting differentially-marked ecotypes 1:100 into 5ml 3% GMM, which is an M9 minimal medium (40 mM Na_2HPO_4, 20 mM KH_2PO_4, 9 mM NaCl, 20 mM NH_4Cl, 1 mM $MgSO_4$, 1 mM CaCl2) containing 3% (1M) galactose. A 7mm polystyrene bead was added to these cultures for adherence. Following overnight incubation at 37°C, beads were removed from the cultures, and vortexed in buffered saline. The supernatants were used to construct community mixtures in a 1:1:1 ratio; the mixtures were then cultured with a bead for 24h. Overnight beads were transferred to 5ml 3% GMM and a 7.5mm x 7.5mm glass coverslip to mimic the surface area of a new bead. Images of biofilm adhering to the glass coverslip were captured with a Zeiss LSM510 Meta microscope using the 20x objective lens, a 0.8 aperture, and the HFT UV/488/543/633, NFT 635 VIS, and NFT 545 lasers at 488nm (3%) and 543nm (50%). Z-stack images were captured from the top of the biofilm to the bottom. The three-dimenstional biofilm images were quantified based on mean thickness, roughness, substratum coverage and surface to volume ratio using the program COMSTAT (Heydorn, 2000). The quantity of S relative to R and W together in each biofilm was determined as the quantity of red fluorescent protein (S) to yellow fluorescent protein (R and W).

Yield and fitness experiments. Individual ecotypes were recovered from frozen stocks by growing overnight in T-soy broth at 37°C with shaking at 130 rpm.

Monocultures for measuring individual fitness and yield were constructed by diluting overnight cultures 1:100 into 5ml 3% GMM with a bead (selective environment), and grown for 24h. at 37°C with shaking. Mixed communities of the Early and Late clones were constructed by growing 70(S):20(R):10(W) combinations of each ecotype under the same conditions as the monocultures. This allowed cultures to acclimate to the original selective conditions of the long-term biofilm evolution at the same ecotype frequency observed at 1,500 generations (Poltak, 2010). After conditioning, community and individual observed yield was measured as final CFU/ml from a single bead colonized after 24h of bacterial growth by vortexing the beads in 1X PBS for 2 periods of 10s and plating on half-strength T-soy agar. To investigate if altering the amount of adherence space weakened community interactions, yield was quantified from two beads or from planktonic culture (in the absence of a bead) after bacterial growth in 3% GMM under the same growth conditions as the single bead experiments. Expected yield of individual ecotypes in mixture was calculated as the observed monoculture yield after 24h multiplied by the starting proportion of the ecotype in mixture (Loreau, 2001). Expected community yield is the sum of individual ecotype yields. Absolute fitness was quantified from vortexed beads and measured as the Malthusian parameter, or $\ln(N_{t=24} / N_{t=0})$, where $N_{t=24}$ is CFU/ml after 24h. of growth and $N_{t=0}$ is CFU/ml at 0h.

Statistics. Data was analyzed using JMP 9.0.2 for all statistical analyses. To compare the effects of galactose concentration, number of beads and time (Early or Late) on community yield, all response variables were $\log_{10}$ transformed and a two-way analysis of variance (ANOVA) was performed. The previous analysis was also used to determine if the cost of competition and benefit of adding extra bead space varied as a

function of time and ecotype. We used $\log_{10}$ transformed data and a one-way ANOVA to determine if expected and observed yield of the B1 populations significantly varied as a function of time. For all ANOVAs, we used Tukey's HSD post hoc tests to compare pairs of means and differences were significant when $p < 0.05$. To determine if S occupied a larger fraction of the biofilm in the Early population than the Late population, a two-tailed t test assuming unequal variance was used and the difference was significant if $p < 0.05$. We also used two-tailed t tests to determine significant differences between Early and Late community yield and fitness, although in these comparisons, both response variables were $\log_{10}$ transformed (significant if $p < 0.05$).

Results

Measurement of the limiting resource.

During adaptation to the biofilm model, we predicted that character displacement would relax competition for two finite resources in the system: 1) galactose, since it was the sole carbon source in the selective environment, and 2) space on the bead, since in order to persist during serial transfer, bacteria must attach to the bead. We tested if galactose was limiting by measuring the cellular production (hereafter referred to as yield) of the Early and Late populations in varying concentrations of this resource. For both the Early and Late populations, a two-way ANOVA supported that yield significantly varied as a function of galactose concentration ($F = 102.57$, $p = 4.84 \times 10^{-20}$, $df = 9$) (Figure 2.2 A). According to Tukey's HSD tests, yield increased with the addition of galactose, however, when the concentration changed from 3% to 5%, yield decreased significantly

in both populations ($p < 0.001$) (Figure 2.2 A). It appeared that adding extra galactose to the system reduced yield instead of increasing it. We speculate that yield was low at high sugar concentrations because solutions containing 5% galactose exerted high osmotic pressure on the bacterial cells, which would inhibit growth (Chirife, 1983). With this in mind, we calculated osmotic pressure in 5% GMM as 15.691 kg/cm^2, which is high compared to the osmotic pressure of neutral 1X phosphate-buffered saline (7.130 kg/cm^2). Toxic inhibition by the environment does not qualify as a resource limitation; therefore, for the Early population, competition for food is likely too weak to be a driving force for diversity. Interestingly, cellular yield under the selective condition (3% galactose) was 1.7 times higher in the Late than in the Early population ($p = 0.08$) (Figure 2.2 A). It appears that the optimum sugar concentration for growth shifted as a consequence of adaptation to 3% GMM and the Late population was able to adapt and overcome the toxicity of this environment. Therefore, it is possible that galactose becomes more limiting over the course of evolution as resource competition intensifies.

We then examined whether space on the bead surface was a limiting resource by measuring the yield of Early and Late populations on one bead and on two beads. If the population occupies a single bead to full capacity within 24h, yield will rise if a second bead is added. For both the Early and Late populations, yield significantly varied as a function of bead amount, which was supported by a two-way ANOVA ($F = 11.31$, $p = 8.00 \times 10^{-4}$, $df = 3$) (Figure 2.2 B). Post hoc Tukey's HSD tests were used for detecting significant pair-wise differences. Yield of the Early population nearly doubled when a second bead was added to the system, indicating that space on the bead indeed limits growth of this population ($p = 0.002$) (Figure 2.2 B). Therefore, for the Early population,

competition for bead space was likely strong enough to encourage diversification. Yield of the Late population was not statistically different when a second bead was added, suggesting that bead space is less limiting to this population ($p = 0.90$). It appears that competition for space relaxed as the evolving population became more efficient at bead attachment, which could be the result of either character displacement or facilitation between the three ecotypes.

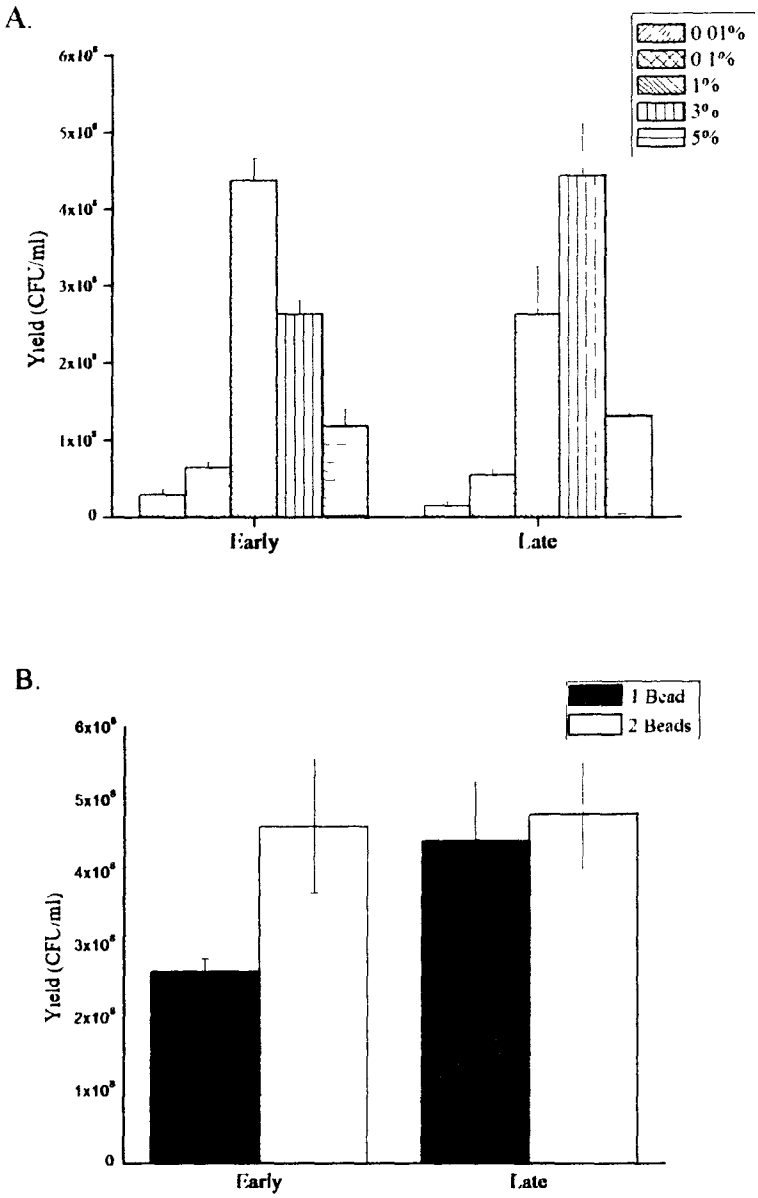

Figure 2.2: Determination of resource limitation, carbon source or space via yield (CFU/ml) of the Early (450 generations) and Late (1,500 generations) *B. cenocepacia* populations isolated from a 1,500 generation serial passage experiment (3% galactose was the selective environment). Error bars are 95% confidence intervals, (df = 3). A) Cellular yield on a single bead in the presence of varying concentrations of galactose. High concentrations of galactose were generally associated with low yield. B) Productivity in 3% GMM in the presence of one or two beads. For the Early population, adding bead space increased cellular yield, but for the Late population, yield on one bead and two beads is equivalent, which may reflect the population's ability to partition space more efficiently.

Visualization of the Early and Late biofilms.

The process of character displacement predicts that as a species adapts to a particular, preferred niche, the niche overlap between neighboring, competing species will decrease to minimize negative effects of competition (Schluter, 1994). Since we determined that competition was likely strongest for space, we predicted that the overlap in spatial distribution between ecotypes in the biofilm would decrease over time. We fluorescently labeled ecotypes and examined the structures of the biofilms formed by Early and Late populations using confocal microscopy (Figure 2.3). Two types of biofilm were observed adhering to the glass slide in both populations: tall, heterogeneous clumps of biofilm with mixtures of all three ecotypes and flat, homogeneous films produced primarily by the S ecotype. In the Early biofilm, the S ecotype occupied 84.5% of the mixed clumps but it only occupied 50.4% of the clumps in the Late biofilm ($t = -2.71$, $p = 0.05$, $df = 3.79$) (Figure 2.3), where it also was observed growing as a thin film around the EPS produced by the R and W ecotypes. Little difference in how R and W colonized the biofilm was observed at either time point, indicating that their niche overlap was maintained. In previous studies of the Late population, confocal microscopy revealed some discrete differences in how R and W form biofilms (Poltak and Cooper, 2010), which will be addressed below. To determine if effects of the fluorescent labels caused these results, we repeated these experiments using alternative markers and also measured fitness of every ecotype expressing each fluorescent protein (data not shown). No effects of marker were observed, however.

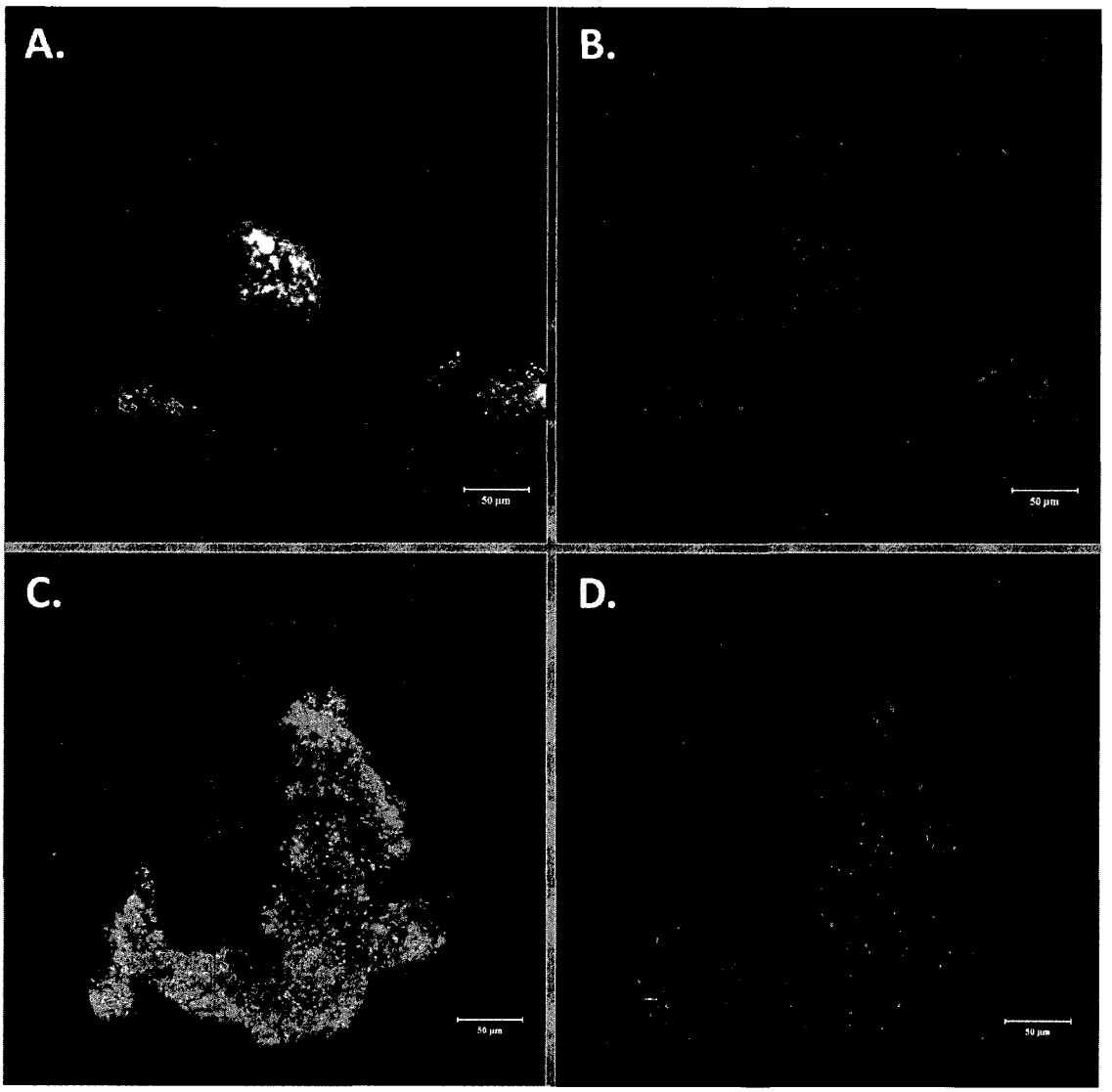

Figure 2.3: Representative images of Early and Late B1 biofilm populations after 24h of growth on a glass coverslip captured with confocal scanning laser microscopy. A) In this Early biofilm, the S ecotype carries pSPR and is displayed in red (1024 x 1024 pixels). The R and W ecotypes both carry pSPY and are displayed in yellow. B) Same image as in (A) with only the S type represented. In the Early population, S occupied a large portion of the biofilm. C) Image of the Late biofilm represented using the same color scheme as in (A) (1732 x 1732 pixels). D) Image of the Late biofilm with only the S ecotype represented. Compared to the Early population, S occupied a smaller portion of the biofilm produced by the R and W type.

Productivity and fitness of Early and Late populations.

Character displacement predicts that decreased niche overlap should correlate with decreased competition between ecotypes, so ecotype fitness and productivity in mixed communities should increase over time. We measured fitness (realized growth rate) and productivity (final CFU/ml) of the ancestor, the Early population, and the Late population in the selective environment with a single bead (Figures 2.4 A and B). Fitness and yield of the individual ecotypes was measured from growth in mixed culture. Mixed yield of the Late population was 1.69 times higher than mixed yield of the Early population ($t = 3.68$, $p = 0.04$, $df = 2.52$) (Figure 2.4 B). Usually yield positively correlates with fitness; however, absolute fitness of the Late population was only 1.11 times higher than the Early population ($t = 2.84$, $p = 0.06$, $df = 3.10$) (Figure 2.4 A). Despite the observed disconnect between fitness and productivity, these data suggest that competition has relaxed over time and as a consequence, productivity increased. However, increased yield could also reflect facilitation between the ecotypes, not displacement. The next several experiments are aimed at separating these two hypotheses.

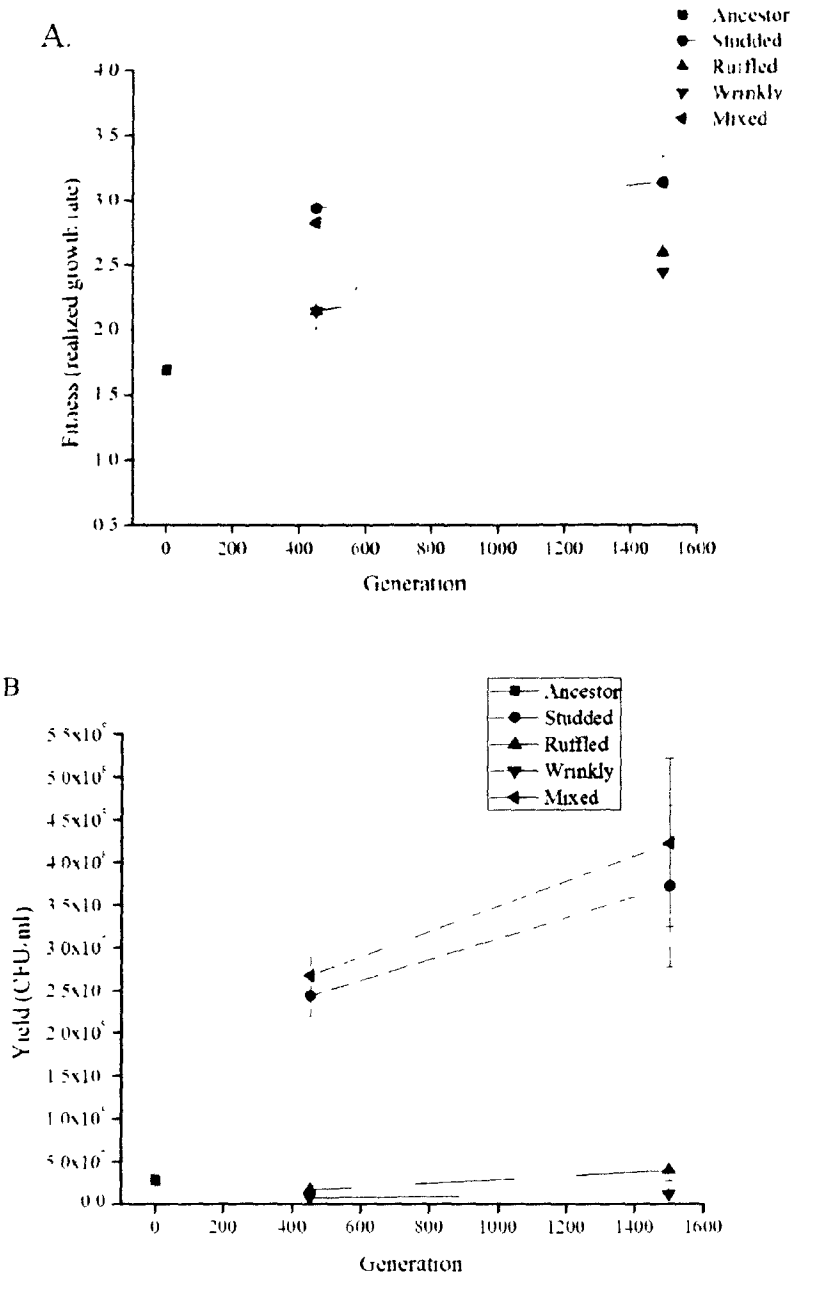

Figure 2.4: Adaptation of a *B. cenocepacia* population and its individual ecotypes to bead adherence during a 1,500 generation serial passage experiment. Error bars are 95% confidence intervals (df = 3). A) Absolute fitness of the mixed community and each ecotype measured as the Malthusian parameter over 24 hours (See Methods). Fitness of the mixed community increases dramatically between 0 and 450 generations and only slightly between 450 and 1,500 generations mostly due to the improvement of the Studded ecotype. B) Yield (CFU/ml) of the mixed community and each ecotype over 24 hours. Yield of the mixed community increases between both time points, indicating decreased competition.

A disconnect between yield and fitness can sometimes be explained by the presence of positive or negative interactions between ecotypes. We compared observed to expected yield in the Early and Late populations to determine if ecotype interactions affected community yield (Figure 2.5). In this comparison, lower than expected yield indicates competition between ecotypes, and higher than expected yield indicates positive interactions. If they are equal, interactions are generally neutral and indicate displacement.

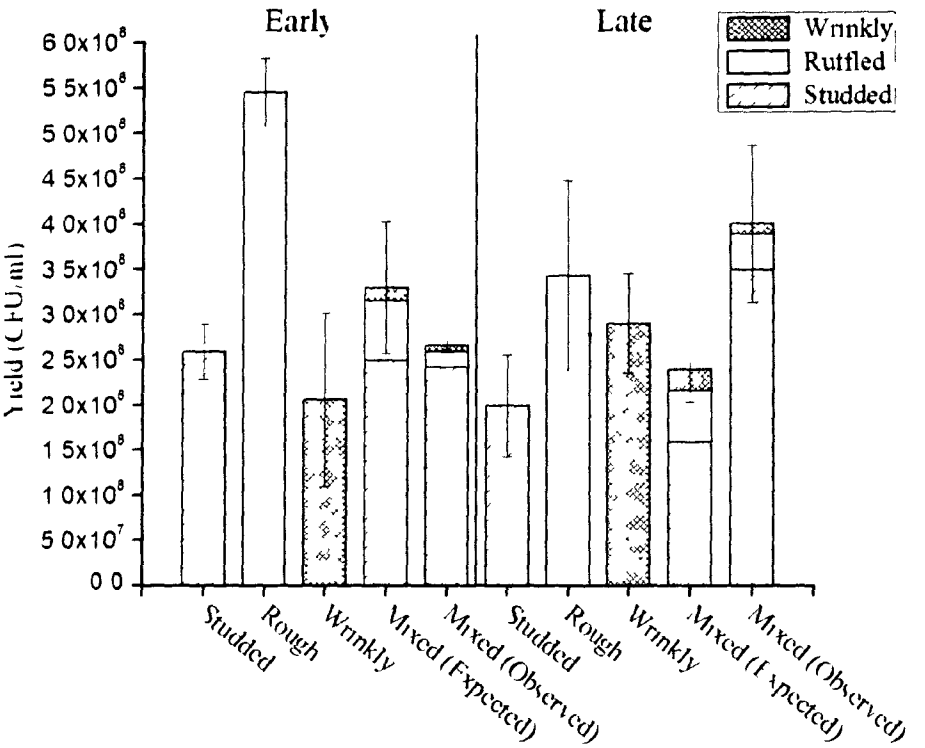

Figure 2.5: Yield (CFU/ml) of a *B. cenocepacia* population and its individual ecotypes measured at an Early (450 generations) and a Late (1,500 generations) time point during a 1,500 generation serial passage experiment. "Mixed" indicates that all three ecotypes were grown together and "Smooth," "Rough," and "Wrinkly" represent ecotypes grown in monoculture, all for 24 hours. Expected yield in mixture was calculated based on yield in monoculture at 24 hours and the ecotype fraction seeded relative to the total seeded in mixture at 0 hours (See Methods). Error bars are 95% confidence intervals (df = 3). Expected and observed mixed yield is essentially equal at the early time point, but at the late time point, observed mixed yield is higher than expected.

A one-way ANOVA supports the model that expected and observed yield varies significantly as a function of time (Early or Late) ($F = 9.26$, $p = 0.006$, $df = 3$). For the Early population, expected and observed community yield were not significantly different ($p = 0.19$), yet observed yield tended to be less than expected (Figure 2.5). It seemed that R and W occupied slightly lower fractions than expected, indicating competition; yet, observed yield of S was equal to what was expected, which means that S may have become displaced early. In contrast, observed yield in the Late population was higher than expected ($p = 0.007$), largely due to greater than expected S productivity (Figure 2.5). Expected and observed yield of R and W were statistically equivalent, indicating neutral interactions and possible displacement between these two types. These data suggest that interactions among ecotypes were initially competitive but evolved to become neutral or positive over time, as a consequence of displacement and perhaps facilitation.

Quantification of community interactions.

Character displacement predicts that the cost of competition in mixture should decrease over time for all ecotypes. We quantified the cost of competition for each individual ecotype in the Early and Late populations as the ratio of ecotype yield when grown in mixture to ecotype yield when grown alone (Table 2.1). Additionally, we quantified the benefit of growing with additional space, the limiting resource, which should change the nature of interactions among types.

Table 2.1: Yield of each ecotype in a mixed community relative to its yield in a monoculture. Yield was measured as final CFU/ml after 24 hours of growth in 3% GMM with a single bead for adherence.

Ecotype	Early	Late	Interpretation
Studded (S)	0.75	1.65	S evolved a benefit in mixture over time.
Ruffled (R)	0.03	0.13	R performs better alone, but is improving in mixture over time.
Wrinkly (W)	0.02	0.04	W performs better alone and doesn't improve significantly in mixture over time.

A two-way ANOVA supported our overall model that the cost of competition decreased for each ecotype as a function of time (Early or Late) ($F = 83.93$, $p = 6.53 \times 10^{-9}$, $df = 5$); however, the interaction term of Time*Ecotype was not significant in this analysis ($p = 4.97 \times 10^{-5}$). In the Early population, all ecotypes grew better alone than in mixture, which indicated that all ecotypes experienced competition for space when grown together. The S ecotype experienced the lowest cost of mixture, suggesting that character displacement in the S type may have occurred early in the evolution. Over time, the cost of mixture significantly decreased for the S and R ecotypes ($p = 0.003$) (Table 2.1) and the small cost of growing in mixture for S may have evolved into a benefit, indicating facilitation by the R and W types. However, R and W ecotypes did not experience any benefit from mixture despite reduced effects of competition over time. Since competition for space appears to relax over time, the benefit of adding extra space to the system should decrease over time (Table 2.2).

Table 2.2: The effect of additional quantities of the limiting resource, bead space, on ecotype yield. Yield (CFU/ml) was measured after 24 h of growth in 3% galactose minimal media in the presence of two, one or zero beads. Numbers represent the ratio of ecotype yield in mixture relative to monoculture under the three bead treatments. P values the result of a two-way nested ANOVA and are based on Tukey's HSD post hoc tests (df = 11).

Ecotype	Early	Late	P value	Interpretation
Two bead treatment relative to one bead treatment:[a]				
Studded (S)	0.99	0.33	3.97×10^{-5}	Space becomes less limiting over time.
Ruffled (R)	2.33	0.76	4.52×10^{-3}	Space becomes less limiting over time.
Wrinkly (W)	1.32	0.51	0.04	Space becomes less limiting over time.
One bead treatment relative to no bead treatment:[b]				
Studded (S)	1.19	2.92	0.32	Space tends to become more limiting over time and may enable overyielding.
Ruffled (R)	0.52	1.07	0.16	Space tends to become more limiting over time.
Wrinkly (W)	0.48	0.57	0.99	Space is likely not limiting regardless of time.

[a] (Yield (CFU/ml) in mixture relative to monoculture, 2 beads) / (Yield in mixture relative to monoculture, 1 bead).

[b] (Yield (CFU/ml) in mixture relative to monoculture, 1 bead) / (Yield in mixture relative to monoculture, 0 bead).

The benefit of adding extra space decreased over time for each ecotype according to a two-way ANOVA ($F = 16.92$, $p = 1.02 \times 10^{-8}$, $df = 11$), when space (extra or less) was nested within the interaction between time (Early or Late) and ecotype (S, R, or W). In the Early population, R and W benefited from additional space (Table 2.2), which is not surprising since both are less productive in mixture than expected (Figure 2.5). This

benefit of additional space decreases for all ecotypes in the Late population because space is less limiting to growth. When we compared growth on a single bead to growth in liquid culture, we found that space tended to become more limiting over time for the S and R ecotypes, but no significant differences were found with Tukey's HSD tests (Table 2.2). The presence of space likely enhanced yield for S and R, however, W yield was low in the presence of a bead and competition for space remained constant throughout time for this ecotype.

Discussion

Community productivity increases with added biodiversity when competition between species is relaxed by character displacement or the evolution of facilitation. The effect of these forces on community production has been examined in mammal (Dayan et al., 1989), bird (Grant, 2006), and fish populations (Schluter, 1992), but more recently Brockhurst et al. (2006) reported the evolution of cooperative interactions resulting from decreased niche overlap in bacterial biofilms. Using experimental evolution, Poltak and Cooper (2010) recently modeled and described the evolution of biofilm diversity by *B. cenocepacia*, an opportunistic pathogen of CF patients. We examined the roles of character displacement and facilitation in maintaining the diversity observed in this model system, and found that both of these forces led to increased biofilm community productivity over time. Moreover, competition between all ecotypes diminished over time as neutral interactions between the biofilm specialist types and the S type evolved into facilitative interactions.

Consistent with character displacement, competition for space between all ecotypes relaxed over time by reduced niche overlap in the biofilm (Schluter, 1992). Previously, Tilman (1994) demonstrated that trade-offs in exploitation of a limiting resource are required for many species to coexist in a small habitat. This pattern was mirrored in our biofilm model, as high bead colonization efficiency evolved at a cost to growth rate and likely reinforced differences between each ecotype. Specifically, S reached the highest frequency in the population because it competes for galactose best, yet it is inferior to R and W in surface adherence (see Introduction, Table 1.1). As selection reinforced this trade-off during the evolution, different ecotypes occupied different areas of the biofilm, which relaxed competition over time and increased yield, especially for S. Furthermore, as displacement reinforced differences in biofilm specialization between ecotypes, competition for space relaxed over time which reduced the benefit of additional amounts.

Improved community production as the result of decreased competition between co-evolved partners was also observed in experimentally-evolved *P. fluorescens* biofilms (Brockhurst, 2006). Brockhurst (2006) surmised that diverse populations maintained by character displacement were more productive because the community as a whole used more of the available resources; exploitation of the air-broth interface came at a cost for one type in this system, but conferred a group benefit since the whole community gained better access to a limiting resource, oxygen. We suspect this may be why our mixed biofilm communities are more productive than individual monocultures. Using microscopy, our study and a previous study (Poltak and Cooper, 2010) observed S growing as a thin film around or atop the biofilm created by R and W; therefore, the

whole population exploits more of the available adherence space if the biofilm created by R and W increases the surface area for S. Although we suspect that decreased competition was responsible for the initial increase in community yield, our results were also consistent with the evolution of facilitative interactions, which also maintains diversity in some populations (Loreau, 2001; Day, 2004).

Later in the evolution, we observed higher community yield than expected which may have resulted from the evolution of facilitative interactions between S and the others (Loreau, 2001). Facilitation may have evolved for several reasons. Futuyma and Slatkin (1983) reported that facilitation can result from modulation of resource abundance caused by some species in a population: for example, if one species produces a waste product another may use it as a resource. This is common in bacterial populations and is referred to as cross-feeding (Saxer, 2009). Previous studies of this *B. cenocepacia* population showed that the W ecotype is cross-fed by the S ecotype with an unknown byproduct of galactose metabolism (Poltak, 2010). Furthermore, we observed modification of bead space by R and W, since their biofilm increased surface area for S, which could explain why cost of mixture for S was small and eventually evolved into a benefit. Another reason why facilitation may have evolved in the biofilm is that these communities have greater resistance to invasion by cheaters due to a lack of available resources (Brockhurt, 2006; Loreau, 2001; Tilman, 1996). In evolved *P. fluorescens* biofilms, cheaters, or a colony types that used resources at the expense of biofilm integrity and stability, evolved less in mixed populations than in monoculture ones (Brockhurst, 2006). Also, Tilman (1996) observed similar trends in grassland communities: ecosystem productivity increased with plant biodiversity because more of the available nitrogen was consumed

which reduced availability for invasive weed species. This could explain the evolution of facilitation in our biofilm population, since we did not observe the evolution of cheats and all biofilm ecotypes positively contributed to community yield, yet this remains an area for future investigation.

The observed differences in community interactions and the subsequent effects on yield almost certainly relates to genotypic differences between the Early and Late populations (Traverse et al., MS). Illumina re-sequencing of the ecotypes in the Early and Late populations revealed different mutations known to regulate biofilm formation. In the Early population, both the S and R sequenced clones have mutations in *yciR*, a gene known to regulate biofilm formation in *E. coli* by controlling intracellular concentration of c-di-GMP. Additionally, the Early W type has a mutation in *wspA*, which is part of a *wsp* operon that also regulates c-di-GMP levels (Bantinaki, 2007). In many bacteria, transitions between a motile and a sessile lifestyle are mediated by changes in c-di-GMP concentration. When c-di-GMP concentrations increase, exopolysaccharide (EPS) production increases and motility is reduced. Degradation of c-di-GMP restores motility and EPS production decreases (Romling, 2005). Although similar mutations were found in clones from the Late population, their combinations differ: the S ecotype is a more derived version of the Early S clone, whereas the R and W ecotypes are new derivatives of this lineage combining new *yciR* and *wsp* alleles. We speculate that the differences in community dynamics between the Early and Late populations are the result of differential pooling of c-di-GMP, which in turn leads to differences in surface attachment. We are currently investigating differential pooling of c-di-GMP as a mechanism for character displacement.

In this study, we modeled biofilm evolution and characterized the community dynamics and genetic changes that increased production, which has many implications for understanding how diversity is maintained in both natural and infectious biofilm populations. Natural biofilms, such as those that occur on marine surfaces, are necessary for colonization by marine invertebrates in areas of low carbon availability and the integrity of these biofilms are influenced by abiotic factors such as temperature (Johnson, 1997). The biofilm communities in our model are also influenced by abiotic stimuli, such as the availability of adherence space, and manipulation of those stimuli in a controlled setting may help us understand what factors affect biofilm stability in natural ecosystems. For example, Cardinale et al. (2002) showed that substrate heterogeneity was essential for productivity and metabolism by benthic biofilm communities in natural stream ecosystems, and when heterogeneity was reduced, ecosystem function decreased. Here we observed similar results when we manipulated the availability of the substrate, revealing how substrate availability or composition may change the productivity of natural biofilms. We also suggest that this model could be used to identify abiotic factors that influence community interactions and reduce productivity of diverse, harmful biofilms, such as those that develop in membrane bioreactors used in wastewater treatment (Huang, 2008). Lastly, diverse *B. cenocepacia* biofilms isolated from the lungs of CF patients are phenotypically and genotypically similar to those evolved in our biofilm model (Poltak, 2010; Haussler, 2004). Therefore, it is likely that productivity of clinical biofilms is influenced by the nature of the interaction between diverse types and chemical factors, such as changes in c-di-GMP concentration (Haussler, 2004; Traverse et al., MS). Current systems that model the predictions made by character displacement

cannot predict how these processes are maintained or disrupted through time. This system may allow us to identify different chemical factors that change or reduce productivity of diverse biofilms over time, which may improve the effectiveness of antibiotic therapy as a treatment strategy for infectious biofilms.

CHAPTER THREE

ADAPTABILITY OF *B. CENOCEPACIA* BIOFILM SPECIALISTS IN A PLANKTONIC ENVIRONMENT

Introduction

An organism's potential to genetically adapt to new environments depends on its access to beneficial genetic variation and its ecological role in a community. In studies of natural and experimental populations, many researchers show that adaptive history, or the course of prior genetic and ecological events that contributed to specific adaptation, restricts adaptation in alternative environments (Travisano, 1995; Bennett, 1992; Dykhuizen, 1980; Zhong, 2009; Crill, 2000; Caley, 2003). Ecological role, which reflects adaptive history and is the function of an organism in a community, may also restrict future adaptation depending on whether the organism is a generalist or a specialist (Futuyma, 1988; Zhong, 2009). A generalist is a phenotypically plastic organism that may be more adaptable owing to wide physiological tolerance and the capacity to use a broad range of resources and habitats (Futyuma, 1988). Specialists, however, may be less adaptable because high fitness in distinct niches and specialization to a narrow range

of resources restricts their capacity to tolerate change. Given prolonged adaptation to a particular set of conditions, specialists may also be less tolerant of environmental fluctuation (Futuyma, 1988; Marvier, 2004).

Adaptive history may also restrict evolvability, defined as the ability of an organism to produce and maintain potentially adaptive genetic variants (Wagner, 1996). For example, specialization in resource use could result in low evolvability, as mutations beneficial to growth on one resource could be antagonistic to growth on alternative resources (pleiotropy), or alternatively, interactions among beneficial mutations could change in the presence of different resources (epistasis) (Cooper and Lenski 2000, Cooper et al 2001, Cooper 2006). Both pleiotropy and epistasis could therefore decrease a specialist's ability to produce adaptive genetic variants (Hansen, 2006; Monro, 2009; Reisinger, 2004; Wagner, 1996). Generalists, on the other hand, may be more genotypes that are resistant to antagonistic trade-offs; high robustness may be why generalists often persist in the face of environmental fluctuation (Baquero, 2009; Gilchrist, 2007; Kitano, 2004; Lenski, 2006; Wagner, 2008; Masel, 2007).

Recently, natural and experimentally-evolved bacterial populations have been used to study effects of adaptive history on adaptability and to identify the genetic mechanisms that influence adaptability (Shade, 2010; Lenski, 2006; Masel, 2007; Baquero, 2009; Trindade, 2009; Khan, 2011; Woods, 2011). In an experimentally-evolved population of *Escherichia coli,* Woods et al. (2011) isolated four different adaptive mutants from a sample early in the population's history and determined that two of them, despite their competitive inferiority to the others, eventually fixed in the population because they had greater potential for further adaptation. The genetic

mechanism responsible for the decreased evolvability of the eventual losers was a negative epistatic interaction between a subsequent beneficial mutation and other mutations present in the genetic background of the eventual losers. No such negative epistasis was observed in the eventual winners, which prevailed. These studies and others demonstrate the value of using microbial systems for investigating the mechanisms that influence adaptability.

Understanding adaptability in more complex bacterial communities is essential given that growth in multispecies biofilms may be the predominant mode of microbial life (Stoodley 2002). Moreover, understanding how members of these communities adapt critical for treatment of human respiratory infections (Huang, 2008; Stoodley, 2002). Biofilm residents typically secrete a thick exopolysaccharide layer that protect against host immune response and antibiotics (Cunha, 2004). Often, switching between a biofilm and a planktonic lifestyle is driven by changes in bis-(3'-5')-cyclic dimeric guanosine monophosphate, or c-di-GMP, a global second messenger in bacteria that regulates multiple phenotypes, including motility, biofilm formation, and autoaggregation (Romling, 2005). In *E. coli* and *P. fluorescens*, intracellular accumulation of c-di-GMP results in overproduction of extracellular matrix components and adhesive fimbriae, and degradation of this molecule decreases EPS production, autoaggregation, and stimulates swimming motility (Zogaj, 2001; Spiers, 2002; Bantinaki, 2007; Spiers, 2002). Dispersal from biofilms, such as during contagious transmission, likely requires reversion to a planktonic lifestyle by increasing motility (Costerton, 1999). If the fitness of dispersed bacteria relates to prior niche specialization in the biofilm community, highly specialized types may struggle to disperse to new, uncolonized surfaces. Likewise, adaptation to

planktonic conditions could also produce trade-offs that reduce fitness in biofilms. These dynamics raise an important question about the adaptability of biofilm communities: can a population that is specialized to a biofilm lifestyle still produce adaptive variants in a planktonic environment? If so, by what mechanisms?

In this study, we examined the adaptability of Early (~450 generation) and Late (~1500 generation) ecotypes that evolved in a previously described *B. cenocepacia* biofilm population (Poltak, 2010). Infectious biofilms formed by *B. cenocepacia*, an opportunistic pathogen of cystic fibrosis (CF) patients, often become diverse with cell types varying in colony morphology and biofilm formation (Haussler, 2003). Poltak and Cooper (2010) observed the evolution of diverse communities containing phenotypes similar to clinical isolates in experimentally evolved biofilm populations. Six replicate populations of *B. cenocepacia* founded by a single clone underwent selection for a daily cycle of surface colonization, biofilm formation, and dispersal for ~1,500 generations in test tubes containing a floating polystyrene bead. Each population evolved increased biofilm production and diversified into three ecologically, genetically and morphologically distinct types: a Wrinkly biofilm specialist type (W), a Rough biofilm specialist type (R), and a Studded generalist type (S) (Table 1.1). Differences in how each type colonized the plastic surface suggested that each ecotype adapted to different conditions within the biofilm. Moreover, each ecotype acquired distinct mutations in *yciR* and *wsp* genes, which likely influence biofilm production by controlling intracellular concentrations of c-di-GMP (Poltak, 2010; Table A1). In this study, we isolated generalist and specialist ecotypes from an Early and Late biofilm population and evolved them in isolation to planktonic culture to examine if adaptive history limited adaptation.

Populations founded by Early ecotypes rapidly adapted to planktonic conditions whereas those founded by Late ancestors adapted more slowly. The more ecologically specialized the ancestor, the less fit the progeny, and the more extensive the physiological trade-offs. As predicted, planktonic adaptation by both Early and Late populations likely occurred by mutations in genes controlling metabolism and intracellular concentration of c-di-GMP.

Methods:

Experimental evolution. Adaptation of *B. cenocepacia* HI2424 populations to a planktonic environment proceeded by serial transfer of bacterial cultures as outlined previously (Lenski, 1991). Populations were founded from three Studded, Rough, and Wrinkly clones from "Early" and "Late" points in the archived samples of an evolved biofilm population B1 (Poltak, 2010). Three populations were founded from each clone in 18 x 150 mm test tubes containing 5 ml M9 media (40 mM Na_2HPO_4, 20 mM KH_2PO_4, 9 mM NaCl, 20 mM NH_4Cl, 1 mM $MgSO_4$, 1 mM $CaCl_2$) supplemented with 3% galactose (this media is referred to as 3% GMM), resulting in nine total populations. Cultures were propagated daily by 1:100 dilutions every 24 hours at 37°C at 130 rpm for 300 generations of growth. Approximately 6.6 generations occurred per 24 hours (50 days), and every 50 generations 1ml samples from each whole population were stored in 30% glycerol at –80°C. Every 50 generations, cultures were diluted and grown on half strength T-Soy agar and incubated at 37°C for 24 hours and then at room temperature for 48 hours. CFUs with different morphologies were isolated and frozen for storage.

Production of each population and individual clones was determined as CFU/ml on half strength T-Soy agar after growing for 24 hours under the evolution parameters. For each population, the frequency of individual cell types was determined by conditioning mixed populations under the evolution parameters for 48 hours and enumerating CFU/ml on half strength T-Soy agar.

Fitness assays. Fitness of evolved cell types relative to their ancestors was determined as outlined previously (Lenski, 1991). All evolved clones and their ancestors were oppositely marked with fluorescent plasmids pSPY and pSPR to enable direct competition between clones of similar morphology; reciprocal marking was conducted to control for vector effects on fitness (Poltak, 2010). We conducted pair-wise competitions by pre-conditioning evolved clones and their ancestors to the selective environment and then mixing them 1:1 prior to 24h of growth (Lenski, 1991). Similar competition assays were also conducted in the ancestral environment (GMM with a polystyrene bead). Initial and final densities (CFU/ml) of the two competitors were calculated by plating diluted samples on half strength T-Soy agar. For biofilm competitions, beads were removed from the cultures, vortexed in buffered saline and the mixture was plated for enumeration on half strength tryptic soy agar plates. Agar plates were incubated at 37°C for 24 hours and then at room temperature for 48 hours; following incubation, absolute fitness was determined as the natural log of growth rate over 24 hours or the Malthusian parameter: $m_i = \ln(N_{t=24} / N_{t=0})$, where $N_{t=24}$ is CFU/ml after 24h. of growth and $N_{t=0}$ is CFU/ml at 0h. Selection rate constants were calculated as the difference in the two competitors' Malthusian parameters: $r_{ij} = m_i - m_j$, where m_i is the Malthusian parameter of the evolved cell type and m_j is the Malthusian parameter of the ancestral cell type.

Phenotypic assays. Biofilm production was measured by crystal violet staining of stationary phase cultures grown in 3% GMM in 96-well plates as described by O'Toole et al. (1999). Swimming motility was measured as diameters of spread on 0.3% T-soy agar following 24 hours of incubation at 37°C. Growth curves of individual colony types were performed by growing monocultures in 3% GMM in 96-well plates and measuring OD_{600} every 15 minutes for 24 hours. Maximum growth rate, or V_{max}, (OD_{600}/hour) was determined as the slope of the growth curve in exponential phase (OD_{600} @ Time 2 – OD_{600} @ Time 1 / Time 2 – Time 1). The length of lag phase (hours) was determined by plotting the growth curve on a log axis and measuring the time prior to the start of exponential growth. Intracellular c-di-GMP concentration was measured from nucleotides extracted from mid-log phase bacterial cultures (OD_{600} = 0.4) by incubation with extraction buffer (40% methanol, 40% acetonitrile, 0.02 N formic acid) for 1 hour at -20°C. c-di-GMP was quantified using liquid chromatography tandem mass spectrometry as previously described (Bobrov, 2011) in collaboration with C. Waters, Michigan State University.

Statistics. Data was analyzed using JMP 9.0.2 for all statistical analyses. A linear regression was performed with c-di-GMP concentration as a dependent variable and motility (mm), maximum growth rate (Vmax), and length of lag phase (hours) as independent variables. To determine if Vmax, lag phase, c-di-GMP concentration, and motility varied significantly between ecotypes, a one-way analysis of variance (ANOVA) was performed. Two-way ANOVAs were used to determine if fitness of the derived mutants in the planktonic environment or the biofilm environment varied as a function of ancestral lineage (Early or Late). A two-way ANOVA was also used to determine if

biofilm production or motility of the derived mutants varied as a function of ancestral lineage (Early or Late). To determine if population yield varied between different time points in the planktonic evolution, a two-way ANOVA was used in which ecotype was nested within ancestral lineage (Early or Late). For all ANOVAs, we used log10 transformed response variables and Tukey's HSD post hoc tests to compare pairs of means (differences were significant when $p < 0.05$).

Genome resequencing and the detection of adaptive mutations. Genomic libraries for Illumina re-sequencing were prepared according to Illumina standard procedures. Briefly, 5 μg of purified DNA was sheared using a nebulizer kit (Invitrogen). The sheared fragments were then blunted using an END-IT DNA repair kit (Qiagen) and A-tailed using a Klenow Exo-minus kit (Epicentre). The custom oligos DL131 and DL137 designed by Lazinski and Camilli (Tufts University) were ligated onto the A-tailed DNA fragments using a Fast Link kit (Epicentre). The resulting DNA was separated and imaged using standard gel electrophoresis and DNA ranging from 400-450bp was excised and purified using a gel purification kit (Qiagen). The resulting purified fragments were PCR amplified using a PfuUltra II Fusion HS DNA polymerase kit (Stratagene) and 0.6 μM of forward primer DL139 and reverse primer DL140 (Tufts University) under the following cycling conditions: 1X (95°C, 1 min.), 14X (95°C, 50 sec.; 65°C, 1 min.; 72°C, 30 sec.), 1X (72°C, 5 min.). Amplicons were purified using a gel purification kit (Qiagen), quantified by nanodrop, and diluted to a 20 nM concentration. The resulting library was sequenced at the Tufts University Core facility. The program MAQ was used to perform library re-construction, assembly against the reference genome *B. cenocepacia* HI2424 (NCBI) and SNP detection at the UNH

Hubbard Center for Genome Studies. Standard PCR and sequencing (UNH) was used to confirm SNP mutations using a *Taq* DNA polymerase kit (5 Prime), 0.08 μM of primers, and the conditions shown in Table 3.1.

Table 3.1: Specific gene primers and conditions used in this study.

Primer Name	Sequence	Annealing Temp. (°C)	Elongation Time (sec)
ABC F	5' CGTCACCTGTATCGCACTCT	55.7	40
ABC R	5' TTCACGACGCTCTGGTATTC	55.7	40
Sulf F	5' CTGTATCGCACTCTCCACGG	60.5	95
Sulf R	5' ACGCTCTGGTATTCGGGCAC	60.5	95
S-Trans F	5'CGACTGGATGGAAGTGCTG	56.6	30
S-Trans R	5'CGCCTGCTCGAACAGAAT	56.6	30

Results

Direct effects of planktonic evolution and phenotypes associated with reversion.

To determine if long-term specialization to the biofilm environment decreased adaptability, we founded three replicate populations from representative clones of each ecotype (S, R, and W) from an Early (450 generations) and a Late (1,500 generations) population and experimentally evolved them for 300 generations in a planktonic environment. Most populations produced a dominant S colony type regardless of the adaptive history of the ancestral founder (Figure 3.1).

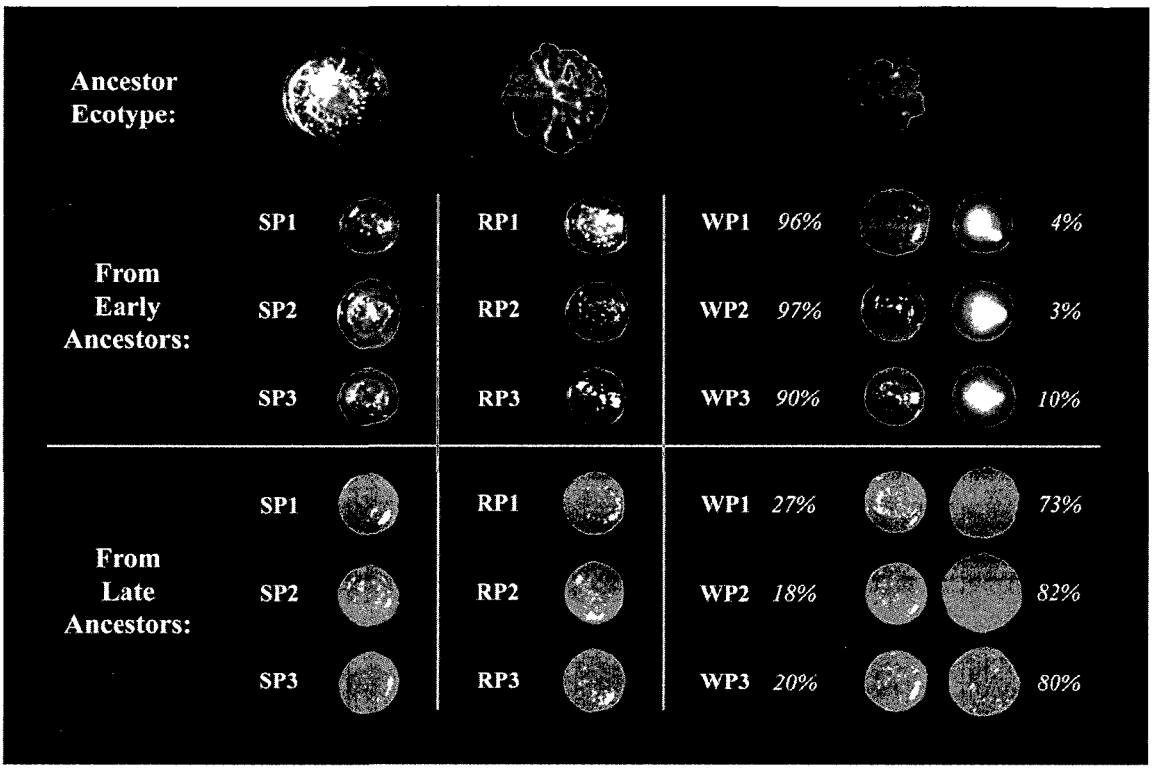

Figure 3.1: Colony morphologies of variants from populations that were evolved by planktonic serial passage for 300 generations. The ancestral ecotypes (top) from the Early and Late biofilm-adapted populations were each used to found three replicate populations for serial transfer, resulting in 18 total planktonic populations. All derived populations were monocultures containing studded colony types with the exception of populations founded by wrinkly, which consisted of two colony types. Percentages indicate the frequency of each colony type in the population.

S and R ecotypes isolated Early or Late consistently evolved into populations composed only of S (monocultures) following planktonic adaptation. Early W consistently evolved populations with a dominant S colony type and a minority mucoid (M) type. However, Late W evolved populations with a dominant R type and a minority S type. Therefore, all ancestors from the Early and Late populations were capable of producing an S colony type, but not all S types became the majority in their populations, suggesting unequal fitness among S colony types and alternative genetic causes of the S morphology. Also,

the evolution of an S colony type in every population suggested that this morphology was associated with increased fitness in a planktonic environment.

We hypothesized that adaptation to planktonic growth related to increased growth rate in galactose medium and altered intracellular concentrations of c-di-GMP. For all evolved derivatives of the S and W ancestors, we measured four phenotypes: 1) c-di-GMP concentration, 2) maximum growth rate (Vmax), 3) the length of lag phase, and 4) motility. As expected, most of the derived mutants exhibited increased Vmax and decreased lag phase compared to their founding ancestors, with the exception of the S colony type evolved from Late S (Table 3.2).

Table 3.2: Phenotypes associated with S colony types and their ancestors. A one-way ANOVA and post hoc Tukey's HSD tests were used to determine differences between means ($p < 0.05$). Numbers in parentheses are 95% confidence intervals.

Sample	c-di-GMP[1] (μM per cell)	Vmax[2] (OD_{600}/h)	Length of Lag Phase[3] (hours)	Motility[4] (mm)
Early S	0.23 (0.05)	0.023 (0.002)	14.58 (0.43)	1.73 (0.05)
S evolved from S	**0.58** (0.32)	0.022 (0.001)	9.92 (0.43)	1.7 (0.04)
Early W	0.12 (0.02)	0.006 (0.003)	18.08 (0.91)	0.97 (0.04)
S evolved from W	**0.69** (0.06)	0.013 (0.002)	14.00 (0.29)	**1.67** (0.04)
M evolved from W	**0.3** (0.07)	**0.030** (0.003)	9.50 (0.49)	**2.26** (0.09)
Late S	0.68 (0.19)	0.021 (0.001)	11.08 (0.16)	1.44 (0.07)
S evolved from S	0.77 (0.11)	0.012 (0.001)	13.00 (0.28)	1.39 (0.05)
Late W	0.27 (0.03)	0.014 (0.001)	15.50 (0.49)	0.85 (0.02)
S evolved from W	**0.65** (0.11)	**0.016** (0.001)	12.08 (0.16)	**1.67** (0.06)
R evolved from W	**0.66** (0.18)	**0.019** (0.001)	11.33 (0.59)	**1.84** (0.08)

Bold numbers represent a significant increase compared to the ancestor.
Underlined numbers represent a significant decrease compared to the ancestor.
[1] $F = 9.04$, $p = 2.46 \times 10^{-5}$, $df = 9$
[2] $F = 47.64$, $p = 1.46 \times 10^{-11}$, $df = 9$
[3] $F = 121.90$, $p = 1.75 \times 10^{-15}$, $df = 9$
[4] $F = 172.81$, $p = 1.76 \times 10^{-34}$, $df = 9$

Most evolved mutants became more motile than the ancestor, but this correlated with increased intracellular c-di-GMP concentration ($r^2 = 0.639$, $p = 0.017$), which was the opposite of the trend we expected based on previous literature (Bantinaki, 2009). In general, evolution in the planktonic environment produced mutants with increased intracellular c-di-GMP, increased motility, increased Vmax and a decreased lag phase.

Because more fit S variants evolved in all planktonic-evolved populations, adaptability may be measured as the time required for S types to become detectable, which is a function of both the supply of S-defining mutations and their relative selective advantage. We tested this prediction by measuring morphotype frequency, population productivity and time to extinction of the ancestral morphotype throughout the frozen archives of each evolved population (Figure 3.2). Productivity in each population varied significantly when S mutants were detected ($F = 16.35$, $p = 8.43 \times 10^{-13}$, $df = 8$). Not surprisingly, populations evolved from S never lost this morphotype. However, populations evolved from Late S became less productive between 250 and 300 generations of adaptation ($p = 0.004$), while the yield of Early S-derived populations remained constant throughout time. This suggests that populations evolved from Late S may have lacked access to beneficial mutations that improve yield in the planktonic environment. Populations founded by Early R produced S mutants by 50 generations that increased community yield and drove R extinct in two of three populations by 150 generations; however, in Late R populations, S appeared later in the evolution and despite an initial increase in yield, these populations eventually became less productive after fixation of the S mutant. In Early W-derived populations, S reached a majority by 150 generations, though there was no significant increase in yield when it was detected. The

S variant was not present in the Late W-derived populations until 250 generations despite its association with increased production. S mutants are not detectable in populations founded by Late R and W until late in planktonic evolution, which suggests that mutations producing S are either limited or highly pleiotropic. Taken together, the S morphotype fixed in the derived populations at different rates depending on the ancestral ecotype and extent of prior biofilm evolution, which suggested that i) fitness effects of alleles capable of producing the S type were heterogeneous and ii) such alleles may have been less accessible to certain genotypes (e.g. Late W).

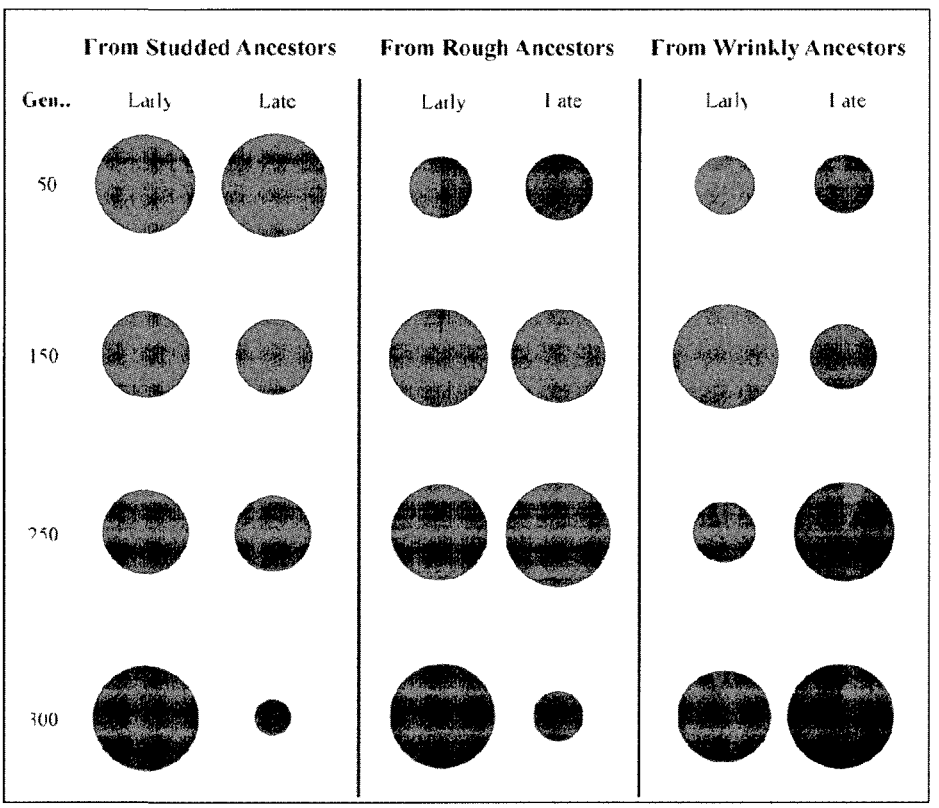

Figure 3.2: Community composition of populations evolved by planktonic serial passage from biofilm-adapted ancestors. Each pie chart shows the average frequencies of variants from three replicate populations sampled at four different generation timepoints in the frozen archives of the planktonic evolution. The size of the pie chart indicates the average cellular productivity from the three replicate populations. Red represents studded phenotypes, purple represents rough phenotypes, green represents mucoid phenotypes, and blue represents wrinkly phenotypes.

To measure fitness differences among the planktonic-derived S colony types, we competed them directly versus their immediate ancestors (Table 3.3). Overall, the mutants evolved from Early ancestors were more fit than those evolved from Late ancestors ($F = 63.21$, $p = 1.81 \times 10^{-10}$, $df = 7$). Within the Early populations, mutants evolved from the specialists (W and R) were more fit than those evolved from the generalist (S). However, this pattern did not occur within the Late populations. The S variants evolved from Early ancestors had the highest fitness (Table 3.3) which likely explains why they appeared earlier and fixed more rapidly in their populations (Figure 3.2). Additionally, the M variant had lower competitive fitness than the S variant from the same population (Table 3.3), which explains why it only occupied 3% of the population after 300 generations (Figure 3.2).

Table 3.3: Fitness of planktonic-derived colony types in the planktonic environment in the biofilm environment. Fitness is shown as the selection rate constant quantified by direct competition with the founding ancestor. "Early" ancestor ecotypes were isolated at 450 generations and "Late" ancestor ecotypes were isolated at 1,500 generations from the biofilm-evolved population. Numbers in parentheses are standard error (d.f. = 2).

Evolved Colony Type	Ancestor Ecotype	Selection Rate Constant
In planktonic environment:		
S	Early S	0.21 (0.03)
S	Early R	1.63 (0.11)
S	Early W	2.69 (0.10)
M	Early W	1.18 (0.15)
S	Late S	0.51 (0.16)
S	Late R	0.17 (0.11)
S	Late W	0.66 (0.02)
R	Late W	0.29 (0.12)
In biofilm environment:		
S	Early S	0.39 (0.10)
S	Early R	0.33 (0.01)
S	Early W	1.11 (0.05)
M	Early W	(-)1.00 (0.10)
S	Late S	(-)0.05 (0.09)
S	Late R	(-) 1.27 (0.12)
S	Late W	(-)0.17 (0.05)
R	Late W	(-)0.39 (0.06)

Mutants of W specialists were better adapted to the planktonic environment than those produced by the S generalists, relative to their immediate ancestors; however, this could simply reflect the lower fitness of the W ancestor in the planktonic environment. To evaluate this possibility, we competed colony types derived from S and W ancestors versus the original wild-type HI2424 clone (Table 3.4). The S evolved from Early S was more fit than S evolved from Early W, consistent with Vmax and the length of lag phase of these mutants (Table 3.2). The M colony type evolved from Early W was more fit than its S partner, which was also consistent with the phenotype data shown in Table 3.2.

The relative fitness values of the Late-derived colony types were higher than those of the Early-derived colony types ($F = 5.71$, $p = 3.26 \times 10^{-4}$, $df = 10$), likely because the Late ancestors were more adapted to galactose, not because they were more adaptable than the Early ancestors. Furthermore, fitness of all Late derived colony types was equivalent to that of wild-type, which indicates that all Late ancestors were equally adaptable.

Table 3.4: Summary of ancestor and derived colony type fitness values relative to the wild-type HI2424 clone.

Colony Type	Relative Fitness	95% Confidence Interval	Interpretation*
		In Planktonic	
Early S	1.189	0.020	S competes better than W.
S evolved from S	1.197	0.184	This type competes better than S evolved from W, but there is no difference from the S ancestor.
Early W	0.933	0.028	W is outcompeted by WT.
S evolved from W	0.765	0.053	This type is outcompeted by WT and it competes slightly worse than the W ancestor. This variant did not adapt to the planktonic environment.
M evolved from W	1.176	0.049	M competes better than its W ancestor. This type is adapted to the planktonic environment.
Late S	1.175	0.078	See Late W interpretation.
S evolved from S	1.390	0.151	This type competes better than types evolved from W, though the result is not significant.
Late W [1]	1.516	0.032	W competes better than S in planktonic phase. This is unusual and contradicts other experiments performed with these ecotypes.
S evolved from W	1.250	0.088	This type is less competitive than R evolved from W, which may explain its minority in the population. However, this result is not significant.
R evolved from W	1.354	0.505	See S evolved from Late W interpretation.

* Unless otherwise stated, indicates significance based on two-tailed t-tests, $p < 0.02$

[1] Due to this unexpected result, the evolved colony types were compared to each other, but not to their ancestors.

Correlated effects of planktonic evolution.

We hypothesized that a genotype was robust if adaptation to a new environment did not compromise fitness in the old environment. To test this hypothesis, we quantified fitness of the planktonic-evolved variants in the biofilm environment versus their founding ancestor (Table 3.3). Fitness among the Early-derived S types did not significantly differ from their respective ancestors in the biofilm environment and therefore experienced no fitness trade-offs as a consequence of planktonic adaptation. However, all variants derived from Late ancestors became less fit in biofilm conditions, particularly for the S variant derived from Late R ($F = 94.06$, $p = 8.45 \times 10^{-12}$, $df = 7$). Therefore, Late-derived variants became less fit in their ancestral environment during adaptation to the planktonic environment, whereas the Early-derived variants did not.

We predicted that adaptation to planktonic conditions would decrease biofilm and increase motility compared to the founding ancestors. When we measured these two inversely related phenotypes, Early ecotypes experienced increased motility ($F = 139.28$, $p = 2.62 \times 10^{-44}$, $df = 12$) with fewer trade-offs in biofilm production ($F = 157.08$, $p = 4.42 \times 10^{-46}$, $df = 12$) compared to Late ecotypes (Figure 3.3). The Early and Late S generalists produced mutants that were not significantly different in either biofilm formation or motility compared to their ancestors, indicating that they are robust to change. All other ancestral ecotypes produced mutants that were more motile, yet those evolved from Late specialists (R and W) experienced a trade-off in biofilm production. These findings were consistent with the mutants' prior fitness measurements: the Early ancestors evolved mutants that were motile (Figure 3.3 A) and fit in the planktonic environment (Table 3.4) while remaining fit in the biofilm environment (Table 3.4).

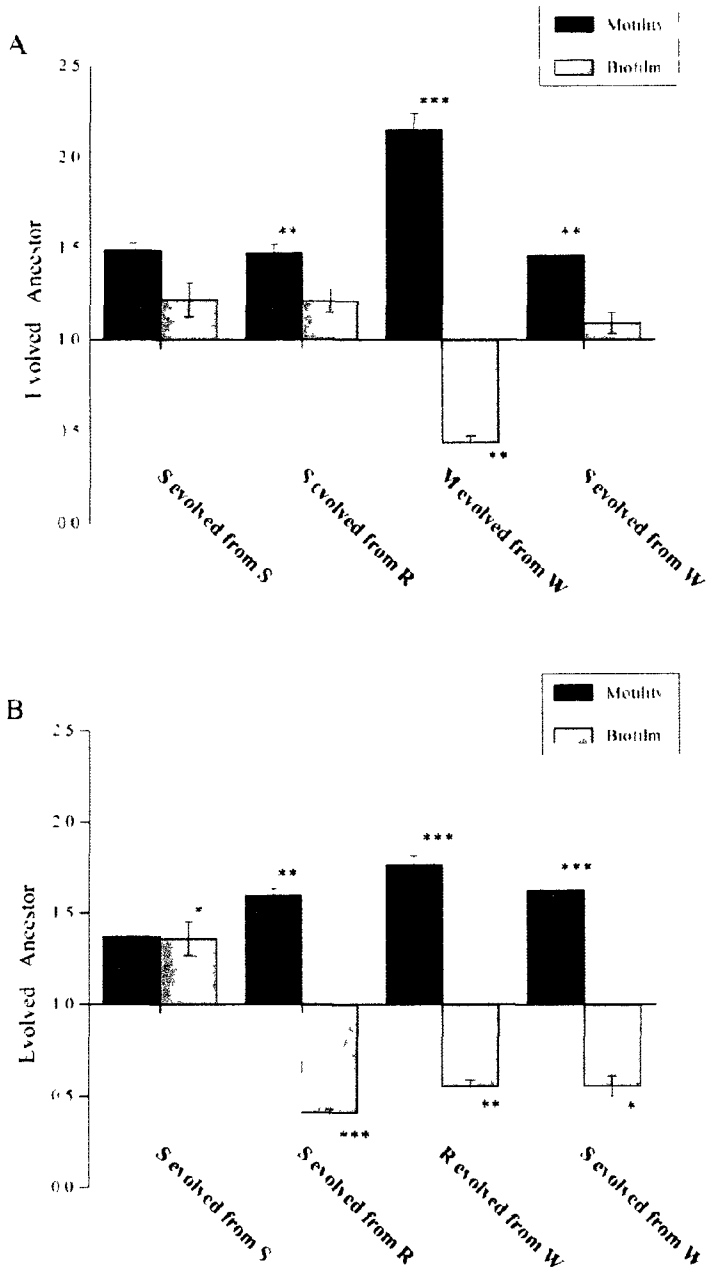

Figure 3.3: Biofilm production (gray) and motility (black) of planktonic-evolved variants represented as a ratio against their founding ancestors: A, founded by Early ancestors; B, founded by Late ancestors. S = studded, R = rough, W = wrinkly, and M = mucoid. Bars below 1.0 on the X axis represent a cost versus the ancestor and bars above 1.0 represent an improvement. Error bars represent standard error (df = 7). P values are the result of Tukey's HSD tests: * $p < 0.001$, ** $p < 1e10^{-5}$, *** $p < 1e10^{-9}$.

Genetic mechanisms of reversion.

Early and Late W are genotypically distinct, with mutations in genes that affect intracellular c-di-GMP concentration, oxidative stress, iron acquisition, and sugar metabolism (Table A1). We predicted that reversion of these ecotypes to a planktonic lifestyle would require different mutations depending on the genetic architecture of the ancestral background. We used Illumina sequencing of S mutants evolved from Early and Late W in order to identify the genetic mechanisms responsible for adaptation to the planktonic environment (Table 3.5). Annotations for all gene mutations can be found in Table A1.

Table 3.5: Presence and absence of mutations in Early and Late W ancestors and the derived S colony types. Gene annotations are in Table A1.

	Early W	S evolved from Early W	Late W	S evolved from Late W
rpoC	√	√*	x	x
*wspA*1	√	x	x	x
Sulfatase	x	√	x	x
glutamine ABC transporter	x	√	x	√
*wspA*2	x	x	√	x
mcsS	x	x	√	x
2-oxoglutarate	x	x	√	√
bacterioferritin, promoter	x	x	√	√
95 gene deletion with *yciR*	x	x	√	√
glutathione S-transferase, promoter	x	x	x	√

* Unconfirmed.

The ancestral *wspA* mutations reverted to the wild-type allele in both of the sequenced S colony types. Additionally, the *mcsS* mutation (R135R) was undetected in the S colony type evolved from Late W and is indicative of a reversion, which is especially surprising for a synonymous mutation. Both of the S mutants gained the same mutation (A170N) in a gene expressing a glutamine ABC transporter substrate-binding protein, which is responsible for transport of amino acids, peptides, and sugars across the cell membrane. The S colony type evolved from Early W also acquired a mutation in a sulfatase gene (M98I), which produces enzymes that hydrolyze sulfate groups from sulfated saccharides. Lastly, the S colony type evolved from Late W gained a mutation in the likely promoter (-10bp) of a glutathione S-transferase gene, which is involved in cellular detoxification by joining glutathione with alkylating agents produced by oxidative stress. Therefore, the W ancestors reverted to a planktonic state through mutation in shared and unique genes.

Discussion:

Early population ecotypes are more adaptable than Late population ecotypes.

In this study, one way we measured adaptability was the time required to reach the phenotypic optimum (Pigliucci, 2008; Quayle, 2006). Almost all of the biofilm ecotypes were supplanted by an S colony type with higher Vmax and a shorter lag phase when evolved under planktonic conditions. We concluded that mutants exhibiting these qualities were moving towards the phenotypic optimum and often they exhibited the S colony morphology. This result was not surprising given previous characterization of S in the ancestral B1 population (Poltak and Cooper 2010). In the biofilm environment, S

maintained a high growth rate in galactose and had the highest fitness in the planktonic phase (Poltak and Cooper, 2010). We concluded that both Early and Late ecotypes were adaptable since most of the colony types derived from these clones had increased Vmax and a shortened lag phase. Yet, the Early ecotypes were more adaptable than the Late ones since adaptive variants appeared sooner and fixed faster in all of these populations regardless of the ancestor. We concluded that prolonged adaptation to the biofilm environment limited adaptability in a planktonic environment.

Another way we measured adaptability was the capacity of the biofilm ecotypes to produce and maintain an adaptive S colony type (Wagner, 1996). Initially, we intended to compare fitness of each derived colony type across Early and Late populations, but since adaptation to the biofilm environment was coupled with increased fitness in the galactose found in the planktonic media, we were unable to make this comparison. Therefore, we compared the adaptability of specialists versus generalists, since the two lifestyle extremes existed within both Early and Late populations. From this comparison, we concluded that the S generalist was more adaptable than the W specialist, however, W produced a fit M colony type that was initially dominant. Since the S mutant competed better against the W ancestor, which was detected throughout the evolution, selection favored S over M. An alternative hypothesis is that selection favored the S mutant because it was more evolvable. Woods et al. (2011) reported second order selection for bacteria that were more evolvable than those that eventually went extinct in experimentally-evolved populations of *E. coli*. The more evolvable cells that eventually swept these populations had a genetic architecture that allowed better access to beneficial mutations (Woods, 2011). Therefore, it is possible that the first mutation that occurred

on the S background allowed access to more beneficial mutations later on, whereas the mutation that produced the M type did not.

Within the Late ecotypes, all derived colony types appeared equally adapted with a few exceptions. The S generalist produced mutants that appeared maladaptive compared to its ancestor but the W specialist produced mutants that were more fit than their ancestor. We speculate that Late S was unable to successfully adapt to the planktonic environment because it was unable to access certain beneficial mutations given its genetic background. Late S and Late W share a 95 gene deletion, yet Late S has an additional 49 genes deleted from its genome, which may have hindered adaptation by Late S. For example, several genes involved in sugar metabolism were deleted as part of the 49 gene deletion (Traverse et al, MS), therefore, natural selection could never favor mutations in those genes that may increase fitness in the planktonic environment. Regardless, prolonged adaptation to the biofilm resulted in a community of generalists and specialists that produced equally adapted colony types in the planktonic environment.

Generalists tend to have less specialized genomes, which makes them more adaptable in the face of environmental fluctuation (Carson, 2008; Kassen, 2002). High adaptability may be due to a flexible physiology that allows them to exploit a wider range of resources and habitats than specialists (Futuyma, 1988). In this study, we report that among Early populations, the generalists were more adaptable in a new environment than the specialists, which is consistent with other studies (Bennett, 1992; Dykhuizen, 1980; Zhong, 2009). However, within the Late population, the S generalist was equivalently adaptable as the specialists, which suggests that mutations granting higher fitness in the

biofilm environment hindered adaptation in the planktonic environment. We discuss the genetic mechanisms potentially responsible for this result further below.

Mucoid phenotypes of *B. cenocepacia* are sometimes isolated from CF lungs during infection and some recent studies have elucidated a putative role for this phenotype (Zlosnik, 2008; Chung, 2003; Conway, 2004). The mucoid phenotype is well-characterized in *Pseudomonas aeruginosa* infections and is associated with higher virulence, yet for *Burkholderia* mucoid types, this is not the case. Mucoid variants of *B. cenocepaica*, which are non-motile and produce no biofilm, are rarely isolated from the lung and are more frequently isolated from the environment (Zlosnik, 2008). In a murine model, infections by mucoid variants are more difficult to clear due to overproduction of EPS and are associated with prolonged persistence (Conway, 2004). In our study, we identified and characterized a mucoid colony type (M) that was similar in phenotype to those isolated from clinical studies. Although our M type was motile, unlike clinical mucoid isolates, it persisted at low numbers throughout the planktonic evolution and was unable to produce biofilm. This mucoid type, while dominant early in the evolution, was outcompeted by its co-evolved S type shortly after its detection. Additionally, mucoid colony variants are produced by all Wrinkly ecotypes after 24 hours of monoculture planktonic growth (data not shown). Therefore, we postulate an additional role for the mucoid phenotype: in addition to persistence in fluctuating environments, mucoid variants may be associated with biofilm transmission, in which low numbers of cells within the biofilm become motile and disperse to other environments. As evidence, we have recovered many spontaneous mucoid mutants from W genotypes following their brief culture in the laboratory in isolation from other community members.

Less derived ecotypes are adaptable and more robust to change.

Often, adaptation and robustness are opposing forces, since in order to adapt an organism must change in response to selective pressures and to be robust, an organism must resist change (Masel, 2007; Lenski, 2006). In this study, we showed that Early ecotypes adapted to the planktonic environment and remained robust, whereas Late ecotypes adapted with a cost to biofilm fitness. For example, most of the Early ecotypes persisted during planktonic adaptation whereas most of the Late ecotypes went extinct early. The switch to a planktonic lifestyle often associates with a loss of biofilm production, so we measured robustness as the ability to retain ancestral biofilm production and fitness during planktonic evolution (Costerton, 1999). Early ecotypes were robust to change since they produced S mutants that were fit in both the planktonic and biofilm environments; however, Late ecotypes were less robust since they produced S mutants that were fit in the planktonic environment but lost biofilm formation.

Adaptability in biofilm populations depends on modification of genes that alter metabolism and c-di-GMP concentration.

We hypothesized that reversion to a planktonic lifestyle would result in decreased c-di-GMP caused by mutations that suppress the phenotypes produced by mutations in *yciR* and *wsp* genes. Consistent with our hypothesis, we observed reversion of the *wspA* mutations in both the S colony types derived from the Early and Late W ancestors; yet we measured a consistent increase in c-di-GMP production and motility. Previous literature supports that reversion of the *wspA* mutations should have resulted in reduction of c-di-GMP, not increases (Bantinaki, 2007). Regulation of c-di-GMP in bacterial cells differs

depending on the growth phase of the cell (Romling, 2005). We quantified c-di-GMP during the early exponential phase of bacterial growth; therefore, we speculate that patterns of c-di-GMP degradation may match our expectations if we isolate molecules during late exponential or stationary phase, a possibility currently under investigation. Alternatively, our assumption that c-di-GMP is high in the biofilm environment and low in the planktonic environment could be invalid given the dynamics of Poltak and Cooper's (2010) biofilm populations. Serial passage of the bead did not select exclusively for biofilm formation; rather, it selected for reversible biofilm formation, since every 24 hours the cells had to detach from the bead when transferred to a new culture. Therefore, c-di-GMP production may be a transient phenotype in our system and it may be difficult to correlate c-di-GMP concentration with changes in biofilm or planktonic phenotypes.

In this study, we observed increased Vmax and a shortened lag phase resulting from adaptation to the planktonic environment, which were likely the cause of mutations in genes that controlled galactose metabolism. In the S colony types evolved from Early and Late W, we observed a shared mutation in a glutamate substrate binding protein which is part of a periplasmic ABC transporter system in *B. cenocepacia* HI2424. The substrate binding protein binds to amino acid or sugar substrates and transports them across the cell membrane via a membrane bound channel complex (Higgins, 1992). A mutation in the substrate binding protein may have increased affinity for binding sugars such as galactose, which would result in better access to nutrients and an increased growth rate. Since this mutation is shared by both of the sequenced S colony types, we

speculate that this may be the key mutation for increasing fitness in the planktonic environment.

In addition to the shared mutation in an ABC transporter gene, the S colony type evolved from Early W has a unique mutation. The mutation is in a sulfatase gene, which degrades sulfated saccharides such as sulfated N-acetylgalactosamine (di Ferrante, 1978). Sulfated sugars are typically found in the EPS of bacterial biofilms produced by *Pseudomonas* and *Burkholderia* species (Tobacman, 2003). A mutation in a sulfatase enzyme may increase degradation of sulfated sugars for use in metabolism rather than for biofilm structure. The S colony type may have increased fitness in the planktonic environment due to increased degradation of the sulfated sugars within the W biofilm. This, coupled with the mutation in the ABC transporter, would increase uptake of galactose and thus increase growth in the planktonic media. Interestingly, CF patients typically secrete an abundance of sulfated glycoaminoglycans and Tobacman (2003) reported that CF patients are more likely to be colonized by sulfatase-producing respiratory pathogens such as *Pseudomonas* and *Burkholderia* species. Therefore, the mutation in the sulfatase gene is clinically relevant and our model of planktonic reversion could be useful in exploring population dynamics of clinical biofilms.

The S colony type evolved from Late W also acquired a unique mutation likely involved in protection against oxidative stress. The mutation was in the promoter region 10bp upstream of a gene encoding glutathione S-transferase, which is a cytosolic dimeric protein that promotes binding between glutathione and alkylating agents produced during oxidative stress (Vuilleumier, 1997). Glutathione is a reducing agent that binds reactive oxygen species through the action of transferase enzymes, which helps to reduce

oxidative stress during energy metabolism (Vuilleumier, 1997). We speculate that if the Late W ecotype acquired the ABC transporter mutation first, galactose metabolism increased, which increased the production of reactive oxidative species. Assuming a promoter mutation in glutathione S-transferase increased expression of this enzyme, oxidative stress caused by increased sugar metabolism was reduced which increased fitness in the planktonic environment. Additionally, our results showed that the S colony type did not appear until 250 generations, which suggested that mutations that increased fitness in the planktonic environment may have interacted negatively with the other mutations contained on the Late W genetic background. Khan et al. (2011) demonstrated that the effect of negative epistasis increases as more mutations are acquired during adaptation. Therefore, negative epistasis likely slowed adaptation by Late W, but not Early W since it had only a two mutations at the start of planktonic evolution.

Summary and implications of this study.

We determined differences in adaptability and robustness between Early and Late biofilm-adapted *B. cenocepacia* populations by measuring 1) the time it took for a population to generate and fix a given phenotypic optimum, (Quayle, 2006), 2) the time to extinction of the ancestral ecotype (Grimm, 2004), 3) the fitness of evolved colony types in the new environment, and 4) the subsequent trade-offs experienced by the evolved variants. We also pinpointed the chemical and genetic mechanisms that explained the magnitude of each ecotype's adaptability, and described potential mechanisms for reversion from a biofilm to a planktonic lifestyle. Here, we demonstrated the usefulness of bacterial systems in modeling complex evolutionary processes and how they can be applied to understanding the disease ecology of *B.*

cenocepacia biofilm infections. Furthermore, our system can be used to study the evolvability of biofilm specialists by measuring rates of adaptation by these ecotypes and their capacity for generating variation, which is a subject for future study.

This study has strong implications for understanding dispersal of *B. cenocepacia* lung infections. Currently, the dominant treatment options for CF patients affected by *B. cenocepacia* infections are aggressive antibiotic therapy and isolation from other CF patients. Here, we demonstrate a relationship between adaptability and the likelihood of successful transmission by reversion to a planktonic state. Early populations adapt quickly to new conditions without losing to their capacity to form biofilm, whereas Late populations are slow to adapt and usually lose their ability to form biofilm when they revert to a planktonic state. Therefore, acute infections, as modeled by the Early populations, and chronic infections, as modeled by the Late populations, are markedly different in their potential for dispersal in a planktonic form and may require different treatment strategies.

CHAPTER FOUR

ADAPTIVE HISTORY DETERMINES THE POTENTIAL FOR NICHE EXPANSION DURING BIOFILM DISTURBANCE.

Introduction:

Habitat disturbance by natural means or by human interference is the major cause of species extinction and reduced diversity (Tilman, 1994; Barbero, 1990; Attiwill, 1994; Ibekwe, 2007; Staley, 1982). The term 'disturbance' refers to a distinct event in time that disrupts ecosystem, community, or population structure resulting in resource changes, alteration of the physical environment, or the destruction of biomass (Pickett, 1985; Grime, 1979; van der Maarel, 1993). On a large scale, volcanic eruptions and other natural disasters, deforestation, use of insecticides and poaching are all discrete events that disrupt ecosystem and community stability (Distefano, 2003; Ibekwe, 2007; Staley, 1982; van der Maarel, 1993; Wallace, 1986; Ives, 1984). Due to the often negative effects on ecosystem diversity and stability, researchers monitor disturbed habitats and use theoretical models to design management strategies for species recovery (Tilman, 1994; Marvier, 2004; Grime, 1979; Attiwill, 1994). However, these theoretical models

do not predict how specific characteristics of the surviving species, such as adaptive history or genotype, will affect recovery of diversity in a disturbed ecosystem.

The likelihood that an ecosystem will recover from a major disturbance depends on the potential of the surviving species to adapt to new conditions. Adaptive potential may be enhanced or constrained by an organism's history, which is the course of prior events that contributed to its genetic and developmental profile (Travisano, 1995). In heterogeneous environments where disturbance is common, selection is thought to favor generalists with broad physiological tolerance, the ability to use a wide range of resources, and the capacity to occupy multiple niches (Futuyma, 1988; Gilchrist, 1995; Jansson, 2002; Kassen, 2002). In homogenous environments where disturbance is rare, selection is thought to favor the evolution of specialists that are most competitive on a narrow range of resources and occupy defined niches (Futuyma, 1988; Kassen, 2002). Following ecosystem disturbance, specialists may be less capable of recovery and persistence, but generalists may be plastic enough to occupy many vacant niches (Manthey, 2011). Therefore, after a disturbance event, history and ecological role may negatively affect adaptability of some species and dramatically affect the ability of an ecosystem to recover lost diversity.

The study of ecological disturbance often focuses on large scale events and how they affect macro-scale communities; yet disturbance also has major consequences for smaller, complex communities such as microbial biofilms (Stoodley, 2002). Biofilms are communities of microbes that adhere to and persist on biotic and abiotic surfaces (Huang, 2008; Stoodley, 2002). Biofilm bacteria secrete a thick exopolysaccharide layer that provides protection from environmental stress and, for infectious biofilms, evasion of a

host's immune response (Stoodley, 2002; Costerton, 1999). Bacteria within biofilms are often phenotypically diverse, varying in colony morphology, biofilm formation, and resistance to antibiotics (Haussler, 2003; Broun, 2000; Stoodley, 2002; Hoiby, 2010). This suggests that biofilms are comprised of multiple ecological niches that are occupied by phenotypically diverse bacteria with distinct adaptive histories. Environmental and host-associated biofilms often experience ecological disturbance, either by environmental change or through the use of antibiotic treatment. Therefore, biofilms could serve as a small-scale model for understanding ecological disturbance and recovery in a community consisting of organisms with varying adaptive histories. Recent use of microbial laboratory models has allowed researchers to investigate the effects of ecological role on future adaptation in a controlled setting (Gilchrist, 1995; Kassen, 2002; Travisano, 1995; Vasi, 1994).

Using an experimentally-evolved *B. cenocepacia* biofilm community, we modeled ecological disturbance and investigated how adaptive history affected the capacity for niche expansion in the absence of other community members. Infections by *B. cenocepacia*, an opportunistic pathogen of cystic fibrosis patients (CF), are characterized by a robust, complex biofilm forming in the lung (Chung, 2003; Cunha, 2004). Poltak and Cooper (2010) developed a biofilm model system to investigate mechanisms that promoted diversification and niche specialization in a controlled laboratory setting. Populations of *B. cenocepacia* HI2424 were founded by single clones and adapted to a biofilm lifestyle by serial passage for 1,500 generations in test tubes containing galactose as a sole carbon source and a polystyrene bead for adherence (Poltak, 2010). Early in the experiment, three genetically and phenotypically distinct

colony types arose: Studded (S), Rough (R), and Wrinkly (W) (Traverse et al., MS) (Table A1). Each colony type was classified as a different ecotype since they occupied discrete areas of the biofilm and displayed differences in biofilm production and growth rate (Table 1.1 and Figure 2.3) that relate to their ecological role. S is a generalist because it produces moderate amounts of biofilm and retains a high planktonic growth rate in galactose media, whereas R and W are biofilm specialists because they produce copious biofilm at the expense of planktonic growth rate. These ecotypes persisted throughout the evolution of the biofilm community, which eventually became synergistic because of facilitative cross-feeding and biofilm segregation (Poltak, 2010; Chapter 2 of this dissertation).

In this study, we investigated if niche specialization and dependence on community synergy affected the ability of individual biofilm ecotypes to colonize an empty surface. The first aim of this study was to model community disturbance by evolving each ecotype in isolation from the other members of their community. The second aim was to quantify niche expansion of a biofilm generalist and a biofilm specialist and determine if it was affected by adaptive history. Because the genotypes of these mutants are known, we were able to determine which mutations limited niche expansion by some ecotypes. When ecotypes evolved alone on a surface, we observed two different colonization patterns which depended on the adaptive history of the founding ecotype. Specialists typically underwent phenotypic diversification (adaptive radiation) and generalists underwent niche expansion, and these mutants influenced community yield and synergy in various profound ways.

Methods:

Strains, media, culture conditions and fluorescent marking. All archived samples of *B. cenocepacia* HI2424 ancestral and derived clones were recovered from a frozen state by growth in 5 ml T-Soy broth for 24 hours at 37°C on a roller drum (130 rpm). Bacterial cultures were enumerated (CFU/ml) by growth on half strength T-Soy agar for 24 hours at 37°C, and then at room temperature for 48 hours. *Escherichia coli* DH5α harboring pSPY (yellow fluorescence) or pSPR (red fluorescent) were recovered from frozen stocks on T-soy agar containing 50 μg/ml chloramphenicol (Cm) (Poltak, 2010). Plasmids were isolated from their *E. coli* hosts using a plasmid purification kit according to manufacturer's instructions (Qiagen). The plasmids were delivered into washed, mid-log phase *B. cenocepacia* cultures using electroporation at 2.5 kV (Ausubel, 1990). Successful delivery was confirmed using antibiotic selection on 100 μg/ml Cm and fluorescence on a Zeiss LSM510 Meta confocal microscope.

Experimental evolution. Populations of *B. cenocepacia* HI2424 evolved in a biofilm evolution model by 24 hour serial transfer as outlined previously (Poltak, 2010). Three replicate populations were each founded by Studded, Rough, and Wrinkly clones from "Early" (450 generations) and "Late" (1,500 generations) time points in the archived samples of an evolved biofilm population B1 (Poltak, 2010). Each culture was propagated in 18 x 150 mm test tubes containing a 6 mm polystyrene bead and 5 ml M9 media (40 mM Na_2HPO_4, 20 mM KH_2PO_4, 9 mM NaCl, 20 mM NH_4Cl, 1 mM $MgSO_4$, 1 mM $CaCl_2$) supplemented with 3% galactose (referred to as 3% galactose minimal media, or GMM). After 24 hours of growth at 37°C and 130 rpm, the colonized bead was transferred to 5 ml 3%GMM with a new, oppositely-marked bead. Colonized beads were

transferred every 24 hours, with approximately 10 generations occurring per day; every 50 generations, 1 ml samples from each whole (mixed) population were stored in 30% glycerol at -80°C. After 300 generations of transfer (30 days), the mixed populations were diluted and grown on half strength T-Soy agar at 37°C for 24 hours, and then at room temperature for 48 hours to determine changes in morphology from the founding ancestor. For each population, representative clones of each colony type with a distinct morphology were digitally imaged, isolated, and frozen for storage. Yield of populations and representative clones was determined as CFU/ml of bead supernatant on half strength T-Soy agar following 24h of conditioning and then 24h of growth in 5ml 3%GMM.

Replacement experiments. All ancestral and derived clones were marked with plasmids expressing yellow (pSPY) or red (pSPR) fluorescent proteins to allow distinction between clones of similar morphology and to control for any effects of the marker on yield or fitness. Individual clones were recovered from a frozen state as described above. Cultures were then individually conditioned for 24 hours at 37°C in 3% GMM with a 6 mm polystyrene bead. Following incubation, the beads were removed, vortexed in 1X phosphate buffered saline (PBS), and standardized to an OD_{600} of 0.07. Ancestral clones were combined in 5 ml 3% GMM with a bead at a frequency of 70% Studded, 20% Rough, and 10% Wrinkly, which are the frequencies of each ecotype in the B1 biofilm population after 1,500 generations of evolution. Derived clones replaced their founding ancestor in the ancestral context at the same frequency. For example, clones derived from the Studded ancestor were combined with the Rough and Wrinkly ancestors at a frequency of 70% derived, 20% Rough, and 10% Wrinkly. Before incubation for 24 hours at 37°C, the mixtures were diluted and enumerated on half strength T-Soy agar.

Following incubation, the beads were removed from the mixtures and vortexed in 1X PBS. The bead supernatant was diluted and enumerated on half strength T-Soy agar. Yield and frequency of each clone in the populations were determined as CFU/ml. Absolute fitness of clones and whole populations was determined as the natural log of growth rate over 24 hours or the Malthusian parameter: $\omega = \ln [N_t (1) / N_t (0)]$ (Lenski, 1991).

Biodiversity experiment. The Late B1 Studded, Rough, and Wrinkly clones as well as the clones derived from the Late Wrinkly ancestor (population WB3: Studded evolved, Rough evolved, and Wrinkly evolved) were recovered from a frozen state as described above. The individual cultures were conditioned the same as in the replacement experiments. Following incubation, the beads were removed, vortexed in 1X PBS, and standardized to an OD_{600} of 0.07. Monocultures and mixed cultures were prepared using the standardized bead supernatant with the same final cellular density. The ancestral mixture was constructed using a frequency of 70% Studded, 20% Rough, and 10% Wrinkly; the WB3 mixture was constructed using a frequency of 2% Studded evolved, 13% Rough evolved, and 85% Wrinkly evolved, which was the frequency of these clones in population WB3 at 300 generations. Another WB3 mixture was constructed using the ancestral B1 frequencies of 70% Studded evolved, 20% Rough evolved, and 10% Wrinkly evolved. The mixtures and monocultures were diluted and enumerated on half strength T-Soy agar before incubation for 24 hours at 37°C. Following incubation, the beads were removed from all cultures and vortexed in 1X PBS. The bead supernatant was diluted and enumerated on half strength T-Soy agar. Observed yield of individuals and whole populations was determined as CFU/ml after 24 hours of

growth. Expected yield of individual colony types was calculated as the monoculture CFU/ml after 24 hours multiplied by the starting proportion of the colony type in mixture (Loreau, 2001). Expected yield of whole populations was the sum of individual colony type . Absolute fitness was determined as in the replacement experiments.

Phenotypic measurements. Biofilm production was measured by crystal violet staining of stationary phase cultures grown in 3% GMM in 96-well plates as described by O'Toole et al. (1999). Swimming motility was measured as diameters of spread on 0.3% T-soy agar following 24 hours of incubation at 37°C. Both biofilm production and motility of the derived clones were represented as a ratio versus its founding ancestor. Images of cultures in 18 x 150 mm test tubes were captured using a digital camera following 24 hours of growth at 37°C in 3% GMM with a bead.

Statistics. Data was analyzed using JMP 9.0.2 for all statistical analyses. To compare the effects of time (Early or Late) or ecotype on community yield and fitness, all response variables were $\log_{10}$ transformed and a two-way analysis of variance (ANOVA) was used. Pairwise comparisons were performed using post hoc Tukey's HSD tests and differences were significant if $p < 0.05$. To determine differences between observed and expected yield of mixed communities, the same analyses were used. Differences in biofilm formation and motility between ecotypes were also determined using the same procedure, except the values for motility were not transformed.

Results:

Experimental evolution and quantification of community production.

We were interested in how disturbance of a biofilm community and elimination of competitors affected future adaptation by the remaining inhabitants. Furthermore, we hypothesized that after disturbance a biofilm generalist would expand its niche to use all available resources whereas a specialist would be incapable of niche expansion. To model disturbance, I founded monoculture populations using the Studded (S), Rough (R), and Wrinkly (W) ecotypes isolated from a biofilm-adapted community (B1) and experimentally evolved them to the biofilm model. I used ecotypes isolated from both an Early (450 generations) and a Late (1,500 generations) population to determine if length of time spent adapting to a biofilm changed adaptive potential of the founder. After 300 generations of adaptation, all of the monoculture populations became diverse, except for those evolved from Early S (Figure 4.1).

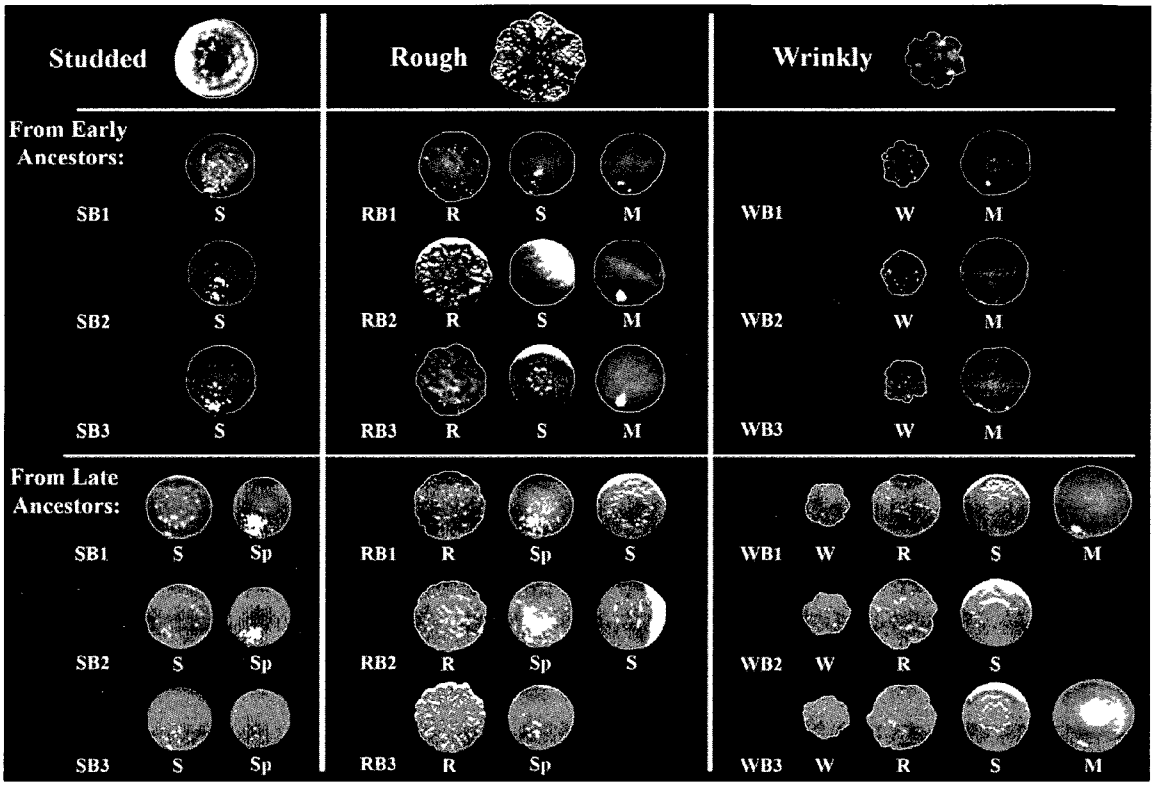

Figure 4.1: Colony morphologies of variants from populations that were evolved by serial passage in the biofilm model for 300 generations. The ancestral ecotypes (top) from the Early and Late B1 populations were each used to found three replicate populations for serial transfer, resulting in 18 total re-evolved populations. S = studded, R = rough, W = wrinkly, Sp = speckled, and M = mucoid. Most derived populations became mixtures of colony types resembling the original ancestors.

All replicate populations founded by the same ancestor evolved into communities comprised of the same morphotypes, which shows that diversification from the same genotype is repeatable. Additionally, many of the derived colony types mimicked those of the original B1 population, except for two new types that we termed Mucoid (M) and Speckled (Sp). Populations founded by biofilm specialists (R and W) became more diverse than populations founded by the biofilm generalist (S). Early S, which is the least evolved generalist, never gave rise to a new morphotypes, whereas Late W, which is the most specialized, consistently produced many diverse colony types during adaptation.

Furthermore, populations founded by Early ancestors were on average less diverse than those founded by Late ancestors. These patterns suggest that prolonged adaptation to a biofilm and niche specialization restricted the ability of some ecotypes to colonize vacant niches. Mutants of specialized ecotypes capable of invading vacant niches rapidly rose to high frequency. For the remainder of this study, we restricted our analysis to populations founded by S and W ancestors to represent the two extremes of ancestral niche breadth.

Often, greater ecosystem diversity correlates with greater community output (Isbell, 2009; Tilman, 1997; Tilman, 1996; Cardinale, 2004), so to investigate this hypothesis we measured the cellular yield of each derived population after 300 generations of biofilm adaptation. A two-way ANOVA showed that yield significantly varied as a function of time (Early or Late) and founding ancestor (S or W) ($F = 508.15$, $p = 1.57 \times 10^{-13}$, $df = 5$). Using post hoc Tukey's HSD tests, we determined that on average populations founded by Early ecotypes had a higher yield than their ancestral population (Figure 4.2). However, despite evolving greater morphological diversity, populations founded by Late ecotypes had low yield compared to their ancestral population and were less able to re-colonize an empty surface (Figure 4.2). W specialists evolved into populations that produced significantly less yield than those founded by S generalists ($p = 2 \times 10^{-10}$), which showed that specialists established more diverse, yet less productive, communities than generalists. Interestingly, the W colony type was always the most productive, which suggests that the other mutants may have facilitated its persistence.

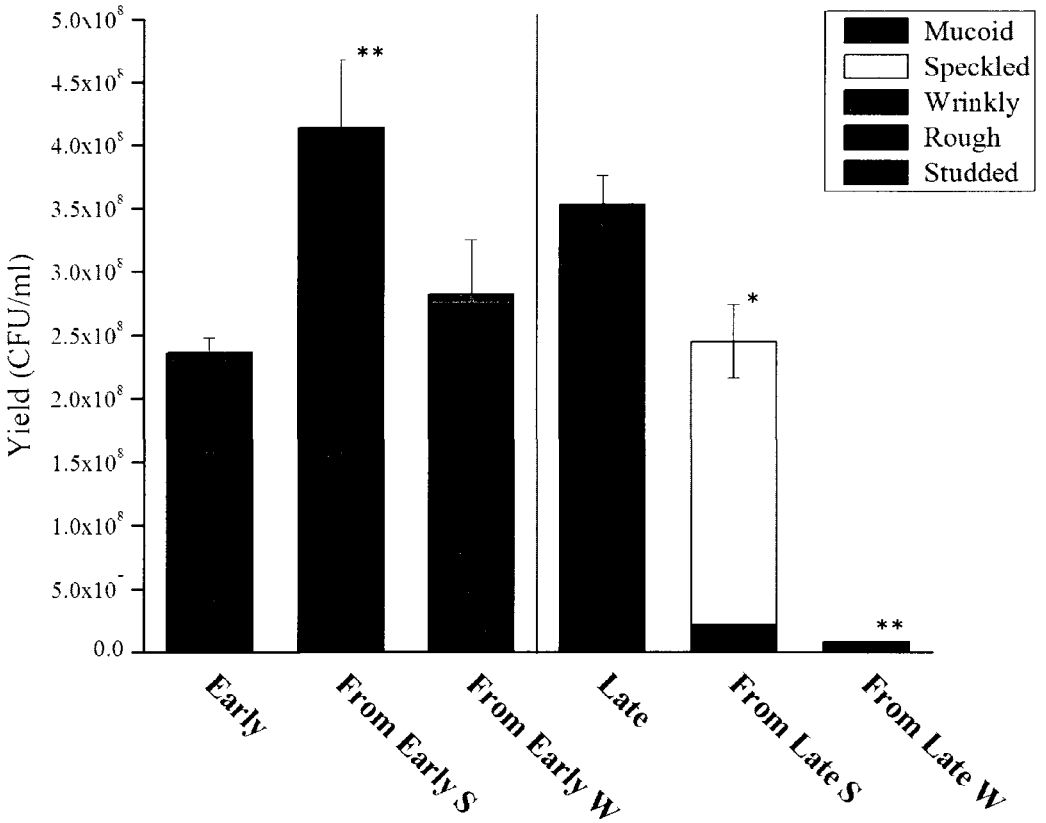

Figure 4.2: Yield (CFU/ml) of *B.cenocepacia* populations following serial passage in the biofilm evolution model. "Early" represents the B1 population at 450 generations, "Late" represents the B1 populations at 1,500 generations, and the others were populations evolved from B1 ecotypes for an additional 300 generations in isolation. Error bars are 95% confidence intervals (df = 2). Evolved populations were compared to their direct ancestral populations and significance was determined using a two-way ANOVA and post hoc Tukey's HSD tests: * $p<0.05$; ** $p<0.001$.

Niche breadth of derived colony types is influenced by adaptive history.

When grown in isolation, most of the B1 ecotypes diversified as they adapted to biofilm selection, which suggested that variation was required to fill multiple niches. We examined biofilm production and motility to determine if ecotypes isolated from the same communities were filling similar or different niches.

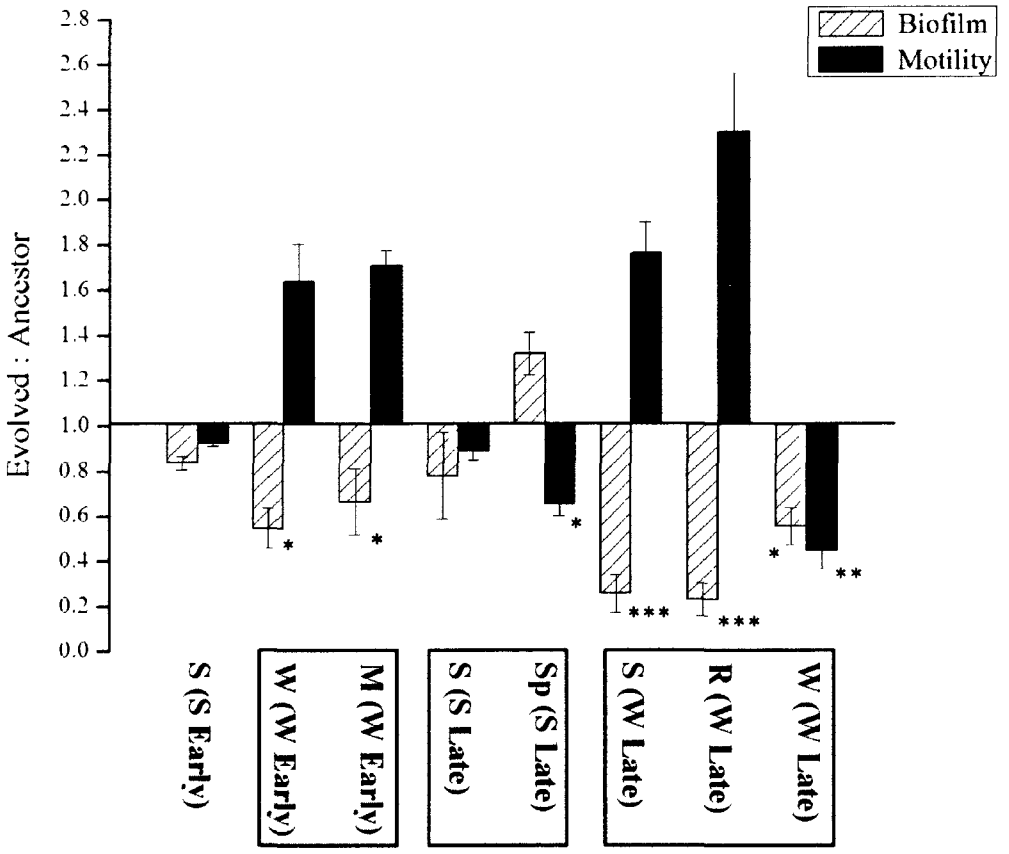

Figure 4.3: Biofilm production (dashed) and motility (black) of colony types evolved in isolation to the biofilm model represented as a ratio against their Early or Late founding ancestors. "S = studded, R = rough, W = wrinkly, and M = mucoid. Bars below 1.0 on the X axis represent a cost versus the ancestor and bars above 1.0 represent an improvement. Error bars are 95% confidence intervals (df = 2). Pairwise comparisons in which evolved ecotypes were compared to their direct ancestors were done using post hoc Tukey's HSD test performed as part of a two-way ANOVA: * $p < 0.05$, ** $p < 5 \times 10^{-3}$, *** $p < 5 \times 10^{-7}$.

Altered motility and biofilm production compared to the founding ancestor is consistent with a novel pattern of colonization and distinct niche dimensions for most ecotypes (Figure 4.3). A two-way ANOVA in which time (Early and Late) is nested within ecotype supports that biofilm and motility both vary as a function of Ecotype[Time] ($F = 34.36$, $p = 5.43$ x 10-2, $df = 11$). However, new colony types evolved from the same

Early ancestor did not differ in motility or biofilm and may inhabit the same niche. For example, populations founded by Early W consisted of W and M morphotypes with statistically equivalent motility and biofilm production (Biofilm: $p = 0.98$; Motility: $p = 1.00$). Conversely, colony types derived from Late ancestors differed in these traits and likely inhabited different niches (Figure 4.3). S and R derived from Late W were similar to one another but differed from their W partner ($p < 3.0 \times 10^{-4}$). Therefore, diversity arising from Late ancestors likely reflected ecological diversificiation.

To determine if colony types derived from the B1 ecotypes were able to colonize their ancestral niche, we measured production and fitness of Early and Late ancestral populations when a derived colony type replaced its ancestor in the community (Figure 4.4). Colony types evolved from generalists filled their ancestral niche and tended to enhance fitness of the other members, which was supported by a two-way ANOVA ($F = 19.74$, $p = 2.22 \times 10^{-11}$, $df = 14$). In the ancestral niche, Sp had higher fitness than Late S ($p = 1.98 \times 10^{-5}$), and fitness of R and W increased in the presence of Sp ($p = 2.71 \times 10^{-3}$ and $p = 7.66 \times 10^{-3}$ respectively). Therefore, it is likely that the presence of Sp increased fitness of R and W, which resulted in higher community yield (Figure 4.4 A). Conversely, colony types evolved from the Late W specialist had low fitness in the ancestral niche (significant below $p < 5.00 \times 10^{-5}$) and tended to decrease community production (Figure 4.4 B). These results show that while occupying the ancestral niche, generalists evolved into mutants that facilitated other biofilm ecotypes whereas specialists evolved into mutants that occupied other niches.

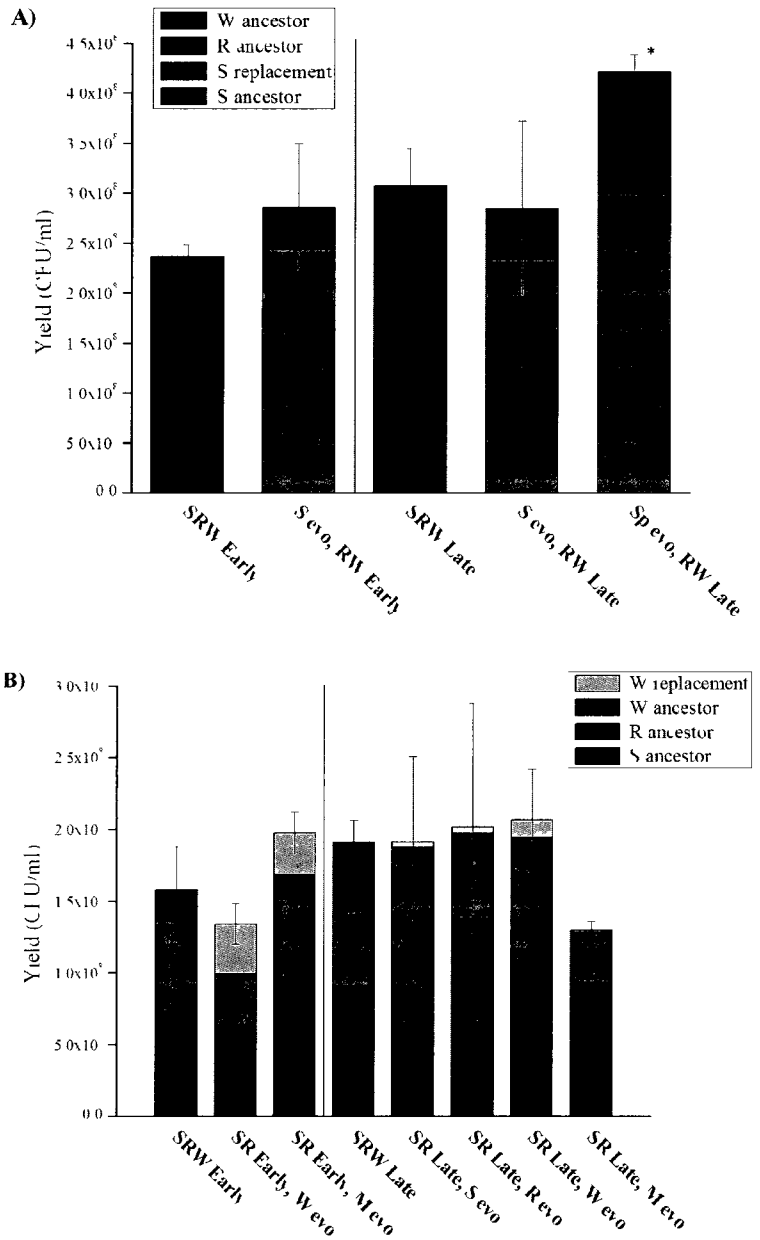

Figure 4.4: Production of Early and Late ancestral populations measured from replacement experiments in which an evolved colony type replaced its ancestor in the B1 community. Error bars are 95% confidence intervals (df = 2). Mixtures containing evolved ecotypes were compared to the ancestral mixtures and significant differences were determined based on a two-way ANOVA ($F = 5.81$, $p = 1.06 \times 10^{-4}$, $df = 10$) with post hoc Tukey's tests: * $p < 0.06$. A) Experiments in which a colony type evolved from the B1 S ecotype replaced its ancestor. Colony types evolved from the S generalist filled their ancestral niche and tended to increase community production. B) Experiment in which a colony type evolved from the B1 W ecotype replaced its ancestor. Colony types evolved from the W specialist could not fill their ancestral niche and some tended to decrease community.

Facilitative interactions are not innate during re-colonization of a surface.

A population derived from a long-term evolved Late W clone (WB3, and likely most ecologically specialized) diversified into colony types similar to the B1 ancestral population (Figure 4.1). There colony types colonize the surface differently (Figure 4.5 A) and also produced varying amounts of biofilm (Figure 4.5 B) consistent with the B1 ancestral community.

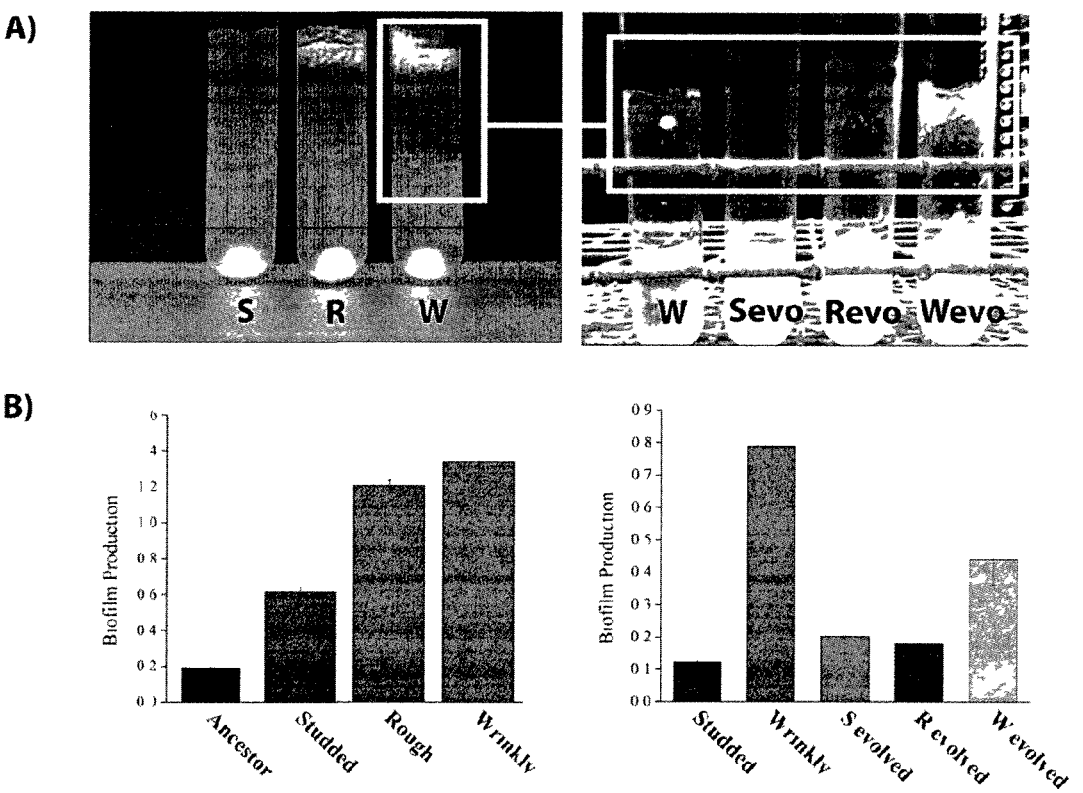

Figure 4.5: Phenotypic characterization of population WB3 compared to the ancestral B1 population. A) Left: image of each B1 ecotype grown in monoculture for 24 hours. Note the differences in how each occupies space on the glass wall of the tubes. Right: image of each WB3 colony type and their B1 ancestor W grown in monoculture for 24 hours. Each evolved colony type occupies space on the glass wall of the tubes similarly to the B1 ecotypes. B) Left: Biofilm production of the wild-type ancestor and the B1 ecotypes. Right: Biofilm production of the S and W ecotypes from B1 and the three evolved colony types isolated from population WB3. Error bars are 95% confidence intervals (df = 3). The colony types from WB3 have differences in the ability to produce biofilm similarly to the differences observed between the B1 ecotypes.

The B1 community is more productive than expected in mixture (Chapter 2), so we tested whether the derived WB3 population was also synergistic. If interactions between mutants are synergistic, observed community production will be greater than expected but, if interactions between mutants are competitive, the converse will occur. We constructed mixtures of mutants from population WB3 at their final evolved frequency (2% S, 13% R, 85% W) and determined observed and expected yield. We hypothesized that if the WB3 colony types were not synergistic at the evolved frequency, they may be synergistic when mixed at the B1 ancestral ecotype frequency (70% S, 20% R, 10% W). A two-way ANOVA supported our model that observed and expected yield significantly varied as a function of starting frequency ($F = 11.13$, $p = 3.56 \times 10^{-4}$, $df = 5$) (Figure 4.6). Observed community yield was lower than expected within mixtures comprised of WB3 ecotypes, which suggests competition and negative effects of growth in mixture. Based on post hoc Tukey's HSD tests, this difference was significant for WB3 ecotypes grown at the B1 frequency ($p = 0.02$). Therefore, even though Late W was isolated from a synergistic community, it evolved a community that was competitive in nature.

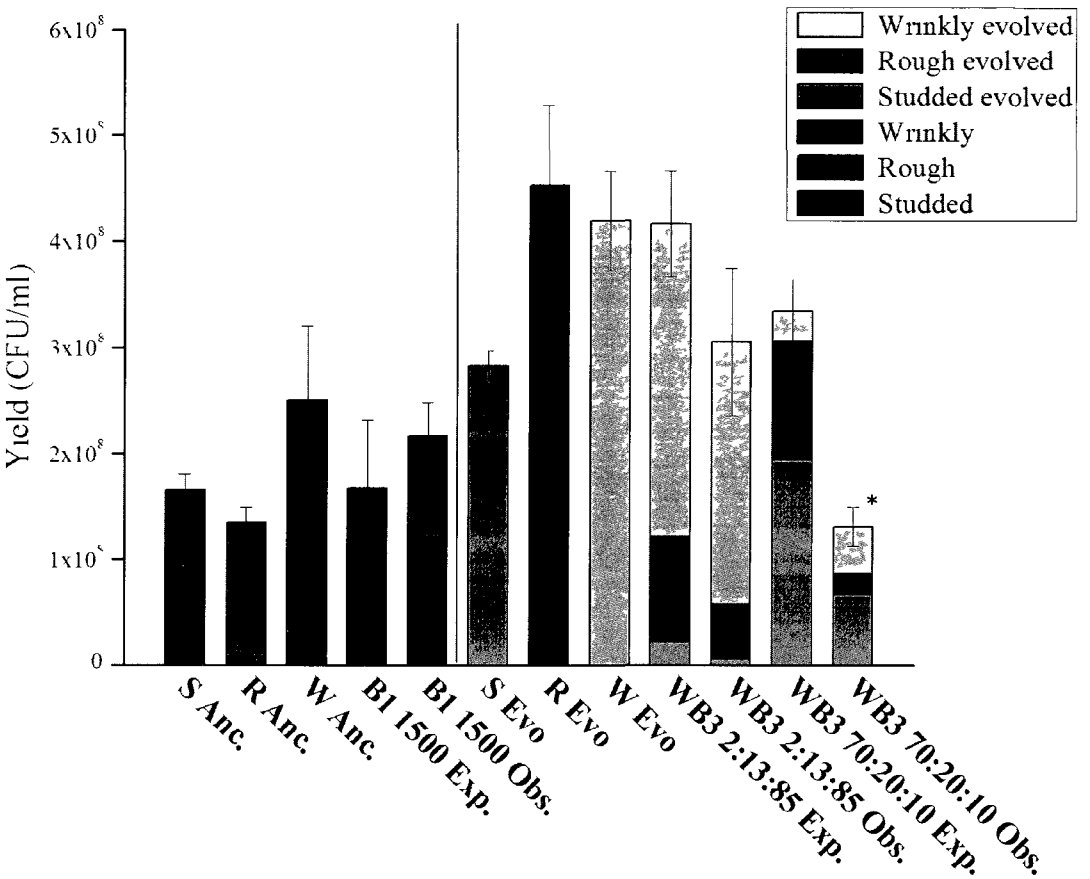

Figure 4.6: Expected and observed yield (CFU/ml) of the B1 and WB3 *B. cenocepacia* populations. WB3 was grown at two different starting frequencies indicated in parentheses: 2:13:85 = native frequency, 70:20:10 = B1 ancestral frequency. Populations labeled Anc. or Evo. were grown individually for 24h: Anc. = B1 ancestral ecotype and Evo. = WB3 evolved colony type grown. Obs. and Exp. populations are mixtures and were grown for 24h: Obs. = observed production and Exp. = expected production in mixture, which was calculated based on the ecotype fraction planted relative to the total planted in mixture at 0 hours and production in monoculture at 24 hours. Error bars are 95% confidence intervals (df = 2). P values are based on a two-way ANOVA and post hoc Tukey's HSD tests: * $p < 0.05$. Since observed yield is always less than expected, interactions between WB3 ecotypes are competitive regardless of ecotype frequency.

Discussion

Adaptive history, and especially the extent of niche specialization, likely determines how populations respond to major ecological disturbance. Theory suggests

that in heterogeneous environments selection tends to favor generalists with broad physiological tolerance (Futuyma, 1988; Jansson, 2002) and in homogenous environments, specialists to particular set of conditions (Futuyma, 1988). This implies that after major habitat destruction that eliminates other competitors, generalists would colonize vacant niches whereas specialists would not (Manthey, 2011). In general, re-colonization of vacant niches is accomplished in one of two ways: a generalist with a wide habitat tolerance will occupy all vacant niches non-specifically, or a large number of specialists will occupy each vacant niche individually (Van Valen, 1965; Roughgarden, 1972; Baur, 1987; Lister, 1976; Futyuma, 1988). Using biofilm-adapted bacteria, we witnessed both patterns of re-colonization and the evolution of one strategy over another depended on the adaptive history of the founding ancestor.

Generalists expand their niche breadth in the absence of competitors.

Generalist species tend to expand their niche in the absence of competitors because they have a broad capacity to occupy many habitats (Lister, 1976). In our system, the S type is a generalist due to its moderate biofilm production and retention of high planktonic growth rate. When isolated, populations founded by Early (less derived) S produced other generalist mutants that not only colonized the ancestral niche but also occupied the empty bead better than populations founded by specialists. We concluded that the S generalist did not diversify during biofilm selection because its moderate ability to form biofilm allowed it to occupy the bead surface alone. Our results were consistent with previous studies where colonization of disturbed or heterogeneous environments was achieved by generalists with high physiological tolerance to new conditions (Marvier, 2004; Parsons, 1982). Furthermore, our results may also be

consistent with ecological release, in which organisms evolve more intermediate traits to exploit all resources in the absence of competitors. (Losos, 1997; Cox, 1977; Brown, 1956; Robinson, 1994).

Specialization tends to limit niche expansion and may explain why selection favored diversification in populations founded by Late S (Bolnick, 2003; Futyuma, 1988). Continued adaptation to the biofilm eventually limited the capacity of S to produce generalist types. This was evident when colony types in these populations differed in motility, which likely means they were specializing to different areas of the biofilm. Still, they did not specialize at the cost of filling their old niche. Therefore, although Late S was more tolerant to changing environmental conditions than other Late ecotypes, its capacity to colonize every niche alone was slightly limited by adaptive history.

Specialists produce other specialists in order to fill vacant niches after ecological disturbance.

Vacant niches resulting from ecological disturbance may be subsequently occupied by an array of phenotypic variants each specializing to particular conditions rather than by a single generalist variant (Lister, 1976). In this case, selection favors the evolution of polymorphism from the remaining inhabitants of a disturbed ecosystem especially if they are niche specialists limited by physiological constraints (Futyuma, 1988). We observed rapid and consistent evolution of phenotypic diversification by the W specialists when evolved in isolation to the biofilm model. We hypothesize that these variants arose because their ancestors could not fill vacant niches as they were limited by

specialization (Bolnick, 2003; Futyuma, 1988). Since the W specialist could not expand its niche breadth, selection favored mutants that could successfully colonize the vacant niches (Marvier, 2004). Buckling (2003) showed that specialization limited adaptive radiation by *P. fluorescens*, yet we did not observe this pattern during our study. This may be because different chemical cues, such as quorum-sensing molecules or c-di-GMP, regulate biofilm formation in *B. cenocepacia* through the action of several gene pathways, such as the *cep* or the *wsp* operons (Conway, 2002; Huber, 2001; Bantinaki, 2007; Weber, 2006; Romling, 2005). In fact, several genes in the genome contain the necessary domains for synthesis or degradation of c-di-GMP, the overproduction of which up-regulates biofilm production (Bantinaki, 2007; Weber, 2006; Romling, 2005). Therefore, a mutation in one gene that affects a particular pathway may facilitate niche specialization without altering other pathways, which would allow for flexibility during adaptation (Griswold, 2006; Galis, 2002).

Diversifying selection typically promotes the evolution of a variety of different phenotypes each exploiting a different range of resources, which may lead to specialization (Lister 1976; Buckling, 2002). In our study, the Late W specialist evolved into multiple colony types that differed in biofilm production and motility, which indicates that they may be specializing to different niches within the biofilm. Furthermore, the colony types can no longer occupy the ancestral niche. However, as previously stated, we defined a biofilm specialist as a colony type with a decreased growth rate in galactose media and increased biofilm production compared to the generalists (Table 1.1). Therefore, in order to confirm that the diversity produced by the

Late W specialists is the result of niche specialization, we need to examine the growth rate of each colony type to confirm a trade-off.

Late W is a biofilm specialist with more limited capacity for niche expansion as a consequence of prolonged adaptation to the biofilm model, yet the less derived Early W did not experience this trade-off. The populations founded by Early W, which were less diverse than those founded by Late W, consisted of colony types that were phenotypically similar to those produced by the S generalists. This suggests that prolonged adaptation changes how a specialist responds to ecological disturbance. For a more derived specialist, selection favors the evolution of several niche specialists whereas for a less derived specialist, selection favors the evolution of generalists with intermediate phenotypes. This difference in evolutionary potential is likely linked to increasing epistatic interactions between beneficial mutations that accumulated during specialization to the biofilm model, which we address below.

Ecological context determines the nature of community interactions.

Fitness of the ancestral R and W specialists increased in the presence of the colony types derived from Early and Late S, which suggests that the S generalists produce colony types that are facilitative and improve overall community production; however, there was no evidence of facilitation in the presence of colony types produced by the Late W specialist (Loreau, 2001; Day, 2004). Studies of species succession demonstrate that generalists are typically the primary colonizers in areas of high ecological disturbance and they often facilitate secondary colonization by other more specialized species (Parsons, 1982; Nara, 2006; Hågvar, 2010). This has been observed

during habitat recovery where primary colonization is accomplished by fungal generalists capable of utilizing a wide variety of resources and occupying many niches. Once established, they alter the environment physically and chemically which conditions the habitat for secondary colonization by plant specialists (Nara, 2006; Molina, 1992). Straight and Kolter (2009) observed similar patterns in oral biofilms, where primary colonizers were receptors for adherence or producers of chemical cues that conditioned the biofilm for colonization by secondary specialists. Poltak and Cooper (2010) described evidence of cross-feeding between the Late biofilm ecotypes: S likely produces an unknown byproduct as the result of galactose metabolism which enhances growth of R and W in mixed culture. In our study, it is possible that the colony types produced by the S generalists are conditioning the biofilm environment to facilitate better adherence by the other members of the biofilm community, either by a chemical cue or by physically changing the attachment surface.

The population founded by Late W (WB3) consisted of colony types that were competitive in nature, despite phenotypic similarities with the synergistic, ancestral B1 population (Poltak, 2010). Therefore, synergistic ecotypes do not produce cooperative colony types despite the fact that they may be fulfilling similar ecological roles as their ancestors. In the B1 population, selection reinforced the differences between each ecotype, which reduced niche overlap and competition, and facilitation of S developed as the result of modification of the biofilm environment by R and W (Chapter 2). The colony types that evolved in the WB3 population were selected for because they could fill vacant niches. However, the colony types were likely very similar since they were founded by the same specialist ancestor and evolved for only a short period of time.

Therefore, niches between each type likely overlapped considerably and Gause's competitive exclusion principle (1932), which states that two species occupying the same niche cannot stably coexist, explains the observed competitive interactions.

Capacity for niche expansion is linked to changes in genes controlling c-di-GMP concentration.

A niche specialist experiences genotypic optimization during adaptation to a particular set of conditions, which maximizes fitness in their particular niche but may produce pleiotropic costs that restrict its physiological breadth (Futuyma, 1988; Poisot, 2011). Epistatic interactions between beneficial mutations in a specialized genome can improve fitness in the adaptive environment but also lead to physiological constraints that limit the niche breadth of adapting organisms (Blount, 2008; Lenski, 1988; Cooper, 2008; Remold, 2004; Flynn et al., MS). In our system, niche specialization in a biofilm was mediated by changes in genes that regulated production of the intracellular molecule c-di-GMP (Jonas, 2009; Romling, 2005; Traverse et al., MS). The *yciR* gene contains a GGDEF and an EAL domain which synthesizes and degrades c-di-GMP respectively (Weber, 2006). Deletion of *yciR* increased pooling of c-di-GMP and was associated with transition to a sessile lifestyle (Traverse et al., MS; Weber, 2006). Additionally, c-di-GMP production and degradation is controlled by the Wsp signal transduction pathway mediated by a group of seven *wsp* genes (Bantinaki, 2007). The Early ecotypes each contain a mutation in either the *yciR* gene or a *wsp* gene: Early S has a deletion in *yciR* and Early W has a SNP in the *wspA* gene, which is a membrane-associated methyl-accepting chemotaxis protein whose methylation drives construction of c-di-GMP (Bantinaki, 2007; Traverse et al., MS). Mutations in these genes are likely responsible

for increased biofilm formation by both ecotypes and differences in niche specialization. The Late W ecotype, however, contains a mutation in *wspA* and a deletion in *yciR*. Mutations in both genes, and possibly interactions between them, may have maximized fitness of Late W as a biofilm specialist. Late W is using galactose primarily for biofilm production mediated by c-di-GMP pooling and has decreased its overall growth rate, a trade-off characteristic of the biofilm specialists in our model system. Therefore, it is likely that epistatic interaction between the *yciR* and *wspA* mutations limited niche expansion by Late W, but did not limit expansion by Early S or Early W. To confirm epistasis between *wspA* and *yciR*, we will create single and double knockout mutants of these genes in the wild-type strain and quantify fitness in the biofilm environment to determine if the affects of the mutations are additive.

Implications for disease ecology and modeling habitat destruction.

Biofilms are complex communities of bacteria and developing effective treatment options for the control of infectious biofilms depends on understanding the community dynamics and ecology of the bacteria that occupy them (Donlan, 2002; Marsh, 2006; Davey, 2000; Stoodley, 2002; Haussler, 2003). Currently, rigorous antibiotic therapy is the most commonly used method for eradication of infectious biofilms; yet, antibiotics may only target some members of the biofilm community, as different ecotypes may have different resistance profiles (Poltak and Cooper, MS; Drenkard, 2002). In this study, we characterized the differences in the response to community disturbance between ecotypes of varying adaptive histories and how the ecological role of a founding ancestor dramatically affects future adaptation and community production. To better predict how *Burkholderia* biofilm infections will respond to antibiotic treatment, we recommend that

closer attention be paid to the ecology and community dynamics of these biofilms when designing treatment options for infected patients. For example, if Early populations are analogous to acute biofilm infections, our model predicts that disturbance of acute biofilms with antibiotics could result in the evolution of productive, facilitative populations from a antibiotic-resistant founder, which would be exceptionally difficult to treat.

Through the use of laboratory modeling with a microbial system, we observed the influence of adaptive history and prolonged niche specialization on future adaptation of biofilm bacteria. Using this system, we were able to investigate the adaptive potential of generalists and specialists, which allowed us to model succession in a disturbed environment. Microbial biofilms are often primary colonizers that facilitate occupation by other species in disturbed ecosystems and many species depend on them for proper ecosystem function. For example, Johnson et al. (1997) showed that colonization of marine surfaces by invertebrates was mediated by biofilms, with organisms responding to specific bacterial strains (Steinberg, 2002). Major disturbance of biofilms could alter the integrity of other biological communities that depend on them for maximum production. In the face of changing climate, heterogeneous environmental conditions could dramatically alter the ecology of pioneering biofilm communities and subsequently alter ecosystem stability. The use of laboratory microbial models allows us to understand the role of adaptive history in the habitat recovery and may help us develop better methods for risk analysis and control in disturbed ecosystems (Marvier, 2004; Staley, 1982; Ibekwe, 2007).

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APPENDIX

Table A1: Mutations of B1 W ancestor ecotypes from Early and Late populations and of S colony types derived from the W ancestor as determined by Illumina re-sequencing. Mutations in bold are new mutations in the dervived colony types. * deletion is part of a 98,089 bp deletion in chromosome 2 which includes 95 ORFs. ** unconfirmed.

Colony Type	Annotation	Gene ID	Mutation	Predicted function
Early W	Bcen2424_0341	*rpoC*	A1318V	DNA directed RNA polymerase, beta subunit
	Bcen2424_3786	*wspA1*	I196N	Membrane associated methyl accepting chemotaxis protein
S evolved from Early W	Bcen2424_0341	*rpoC*	A1318V*	DNA directed RNA polymerase, beta subunit
	Bcen2424_5072	**4452956**	**M98I**	**Choline sulfatase, conversion of choline-O-sulfate to choline.**
	Bcen2424_4339	**4452698**	**A170N**	**Amino acid ABC transporter substrate-binding protein**
Late W	Bcen2424_3786	*wspA2*	A407V	Membrane associated methyl accepting chemotaxis protein
	Bcen2424_5267	*mcsS*	R135R	Mechanosensitive ion channel
	Bcen2424_1509	4448628	R204S	2-oxoglutarate dehydrogenase
	Non-coding	Non-coding	Non-coding	37 bp upstream of bacterioferritin, Bcen2424_2195
	Bcen2424_3554	4452823	deletion**	c-di-GMP phosphodiesterase; Rnase II stability modulator
S evolved from Late W	Bcen2424_1509	4448628	R204S	2-oxoglutarate dehydrogenase
	Non-coding	Non-coding	Non-coding	37 bp upstream of bacterioferritin, Bcen2424_2195
	Bcen2424_3554	4452823	deletion**	c-di-GMP phosphodiesterase; Rnase II stability modulator
	Bcen2424_4339	**4452698**	**A170N**	**Amino acid ABC transporter substrate-binding protein**
	Non-coding	**Non-coding**	**Non-coding**	**10 bp upstream of glutathione S-transferase domain-containing protein, Bcen2424_3946**