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ADAPTATION OF A *PSEUDOMONAS AERUGINOSA* POPULATION TO A BIOFILM LIFESTYLE: WHY IS POLYPHOSPHATE KINASE IMPORTANT?

BY

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B.S. IN BIOLOGY, UNIVERSITY OF MASSACHUSETTS BOSTON, 2010

THESIS

Submitted to the University of New Hampshire

in Partial Fulfillment of

the Requirements for the Degree of

Master of Science

in

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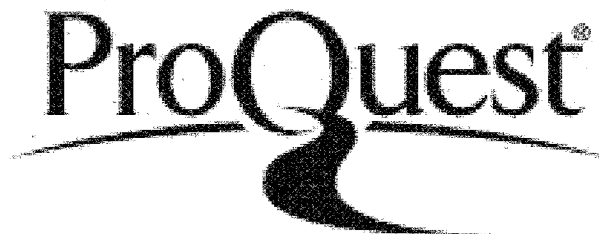

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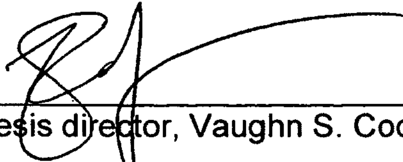

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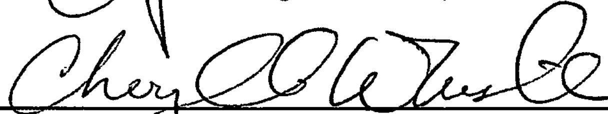

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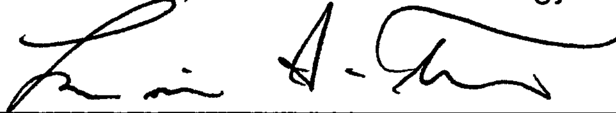

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ABSTRACT

ADAPTATION OF A *PSEUDOMONAS AERUGINOSA* POPULATION TO A BIOFILM LIFESTYLE: WHY IS POLYPHOSPHATE KINASE IMPORTANT?

By

Keith Ferguson

University of New Hampshire, December, 2012

In the environment, bacterial populations often exist as communities called biofilms. During this sessile state, bacteria excrete exopolysaccharides that form a sticky, protective matrix. This protective matrix also presents a prominent medical challenge since chronic infections of cystic fibrosis (CF) patients are commonly biofilm associated. *Pseudomonas aeruginosa* is one bacterium that can colonize the CF lung and persist in a biofilm community. During chronic infections, *P. aeruginosa* adapts to its lung environment and displays genetic and phenotypic diversification. To model evolution during chronic infections, biofilm populations were experimentally evolved for 540 generations and the genetic variation was sampled at 100, 260 and 540 generations to identify adaptive alleles. Of multiple alleles that fixed within the B1 population, mutations in two genes, *ppk* and *rcsC*, were characterized since they were thought to be adaptive. The goal was to measure the fitness effects of these alleles and to identify the mechanisms that explain why they would be adaptive.

CHAPTER I

INTRODUCTION

Biofilms & Experimental Evolution

The biofilm lifestyle is thought to be a more common state of existence than the planktonic lifestyle for natural bacterial communities (McDougald et al., 2011) and as such the relevance of biofilms extends to medical, environmental and industrial fields of research. Biofilms can pose threats to human health as they form in the lungs of cystic fibrosis (CF) patients (Mowat et al., 2011) and on medical devices such as catheters (Fu et al., 2010) and prosthetic heart valves (Mashaqi et al., 2011). They can also have a positive or negative influence on industrial processes. For example, biofilms coating ships can cost the Navy up to $56 million per year as these bacterial communities cause frictional drag, increasing transportation costs (Shultz et al., 2011). Conversely, these communities can be used to help in wastewater treatment processes (Boelee et al., 2012). Biofilms are complex communities as they are usually comprised of more than one species in these various settings. Furthermore, community diversity can result in additive, antagonistic or synergistic interactions between members (Vial & Déziel, 2008). Because they are ubiquitous and have human health and economic consequences, it is important to understand how biofilms form, mature and disperse (Southey-Pillig, 2005; Kaplan, 2010; McDougald et al., 2011).

Experimental evolution provides a unique method of studying bacterial adaptation to a biofilm environment. This approach allows for real time observation of phenotypic and genetic diversity that depends on the selective pressures of surface attachment and dispersal over hundreds or thousands of generations. Lenski et al. (1991) have used experimental evolution to show that the rate of adaptation slowed across 2,000 generations of replicate populations of planktonic *E. coli* in a minimal medium. Fitness relative to the ancestor increased throughout the evolution where early adaptations were more beneficial than later ones. These bacterial lines have been continued through 40,000 generations and hundreds of beneficial mutations have been identified (Barrick et al., 2009). The fitness effects of individual or combinations of alleles can be studied by direct competition between evolved clones and the ancestor. Adaptive mutations can be detected if their frequencies rise within the population and confer a positive fitness effect. Other studies have used experimental evolution to study adaptive trends in predator-prey interactions of microorganisms (Gallet et al., 2009) and in pathogens (Ellis & Cooper, 2010). In combination with high-throughput sequencing, experimental evolution can be used as a nuanced mutant screen to identify beneficial mutations favored by selection under defined conditions.

***Pseudomonas aeruginosa* & Cystic Fibrosis**

Pseudomonas aeruginosa is a metabolically diverse organism and has a broad niche breadth as it is found in medical, environmental and industrial

settings (Wolfgang et al., 2003; Wainwright et al., 2011; Yan et al., 2011). The bacterium can cause infections in plants and animals and is thought to grow as biofilms when inside its host (Bjarnsholt et al., 2009). This opportunistic pathogen is commonly found in the lungs of patients with CF, a human genetic disorder caused by mutations in a transmembrane conductance regulator ion channel (Pissara et a., 2008). Bacteria can easily colonize and persist in this environment as thick mucus accumulates, causing severe morbidity and mortality.

P. aeruginosa isolates from chronic CF infections often share similar traits as they adapt to the host resulting as a 'chronic phenotype' (Yang et al., 2011). Mutations in *mucA* cause a mucoid phenotype due to an overproduction of alginate, an important constituent of biofilm exopolysaccharide (Smith et al., 2006). Excess alginate helps the bacterium evade detection by host immune cells, which makes clearing infection difficult (Song et al., 2003). Hypermutator populations are also commonly observed in *P. aeruginosa* CF infections. Clones acquire loss-of-function mutations in a DNA repair gene and rapidly accumulate mutations, beneficial and maladaptive. The gene encoding a methyl mismatch repair protein, *mutS*, is commonly targeted during later stages of chronic infections and leads to isolates with mutation rates higher than the ancestor (Oliver et al., 2002; Maciá et al., 2005; Hogardt et al., 2007). Because most mutations are detrimental, it remains unclear why *P. aeruginosa* clinical strains develop this phenotype. One possible explanation is that despite the risk of accumulating maladaptive alleles, hypermutation may allow access to large-benefit mutations under stressful conditions that overcome effects of maladaptive

alleles. For example, Giraud et al. (2001) have shown that an *E. coli* Δ*mutS* genotype is initially more competitive than its ancestor in a mouse gut model. However, when they restored the ancestral *mutS* allele in the mutator that was isolated days after inoculation, it was more fit than the Δ*mutS* genotype. Therefore, adaptive mutations were able to quickly occur on the hypermutator genetic backgrounds.

P. aeruginosa CF isolates also commonly have mutations producing a quorum sensing deficiency. The quorum sensing regulator, *lasR* is often mutated in clinical isolates that also have mutations in *mucA* (affecting mucoidy and biofilm production) and *mutS*. It has been hypothesized that *mutS* enables these two other phenotypes to emerge but a recent study has shown that mutators usually arise later during infections, well after mucoid, quorum sensing deficient strains have been established in the lung (Ciof et al., 2010). Another study (Mowat et al., 2011) which sampled 1,720 *P. aeruginosa* isolates from 10 chronic infections found that most of these populations comprised of mucoid, hypermutable, quorum sensing-altered haplotypes, further suggesting that these changes represent adaptations to a biofilm lifestyle within the CF lung.

Clinical isolates of *P. aeruginosa* from chronically infected CF lungs develop antibiotic resistance through various mechanisms (Livermore, 2002). Mutations associated with resistance are often associated with hypermutators suggesting that having an increased mutation rate allows for relatively quick evolution of resistance in the CF lung. Common genes altered during chronic infections include *mexAB-oprM*, *mexXY-oprM*, *mexR and mexZ* which encode

multidrug efflux pumps or regulators of these pumps (Llanes et al., 2004; Gorgani et al., 2009). These adaptations confer resistance to a wide class of antibiotics including β-lactams, fluoroquinolones, tetracyclines, macrolides and aminoglycosides. Because of this characteristic, late stage CF populations consist of 'persister' strains (Mulcahy et al., 2010) that are difficult to clear, increasing patient morbidity and mortality.

A Model of *P. aeruginosa* Biofilm Adaptation & Potential Mechanisms

To quantify genetic variation and to identify mutations that are adaptive in a biofilm lifestyle, *P. aeruginosa* PA14 was propagated for over 500 generations on a polystyrene bead in a minimal medium with galactose as a carbon source (Flynn, unpublished). This process involved daily transfers of biofilm coated beads into tubes containing fresh media and a new bead (Figure 1). Three replicate biofilm populations (B1,B2,B3) and three replicate planktonic populations (P1,P2,P3) were evolved. As the evolution progressed, samples of each population saw heritable changes in colony morphology different from the ancestral type, suggesting that they had acquired mutations and that the mutations would be adaptive. Isolates were grouped together based on colony morphology and we assigned letters to represent each distinct type (Flynn, unpublished, defined in Table 6). Metagenomes, or total community DNA, were extracted at 100, 260 and 540 generations from the B1 population and subjected to Illumina sequencing.

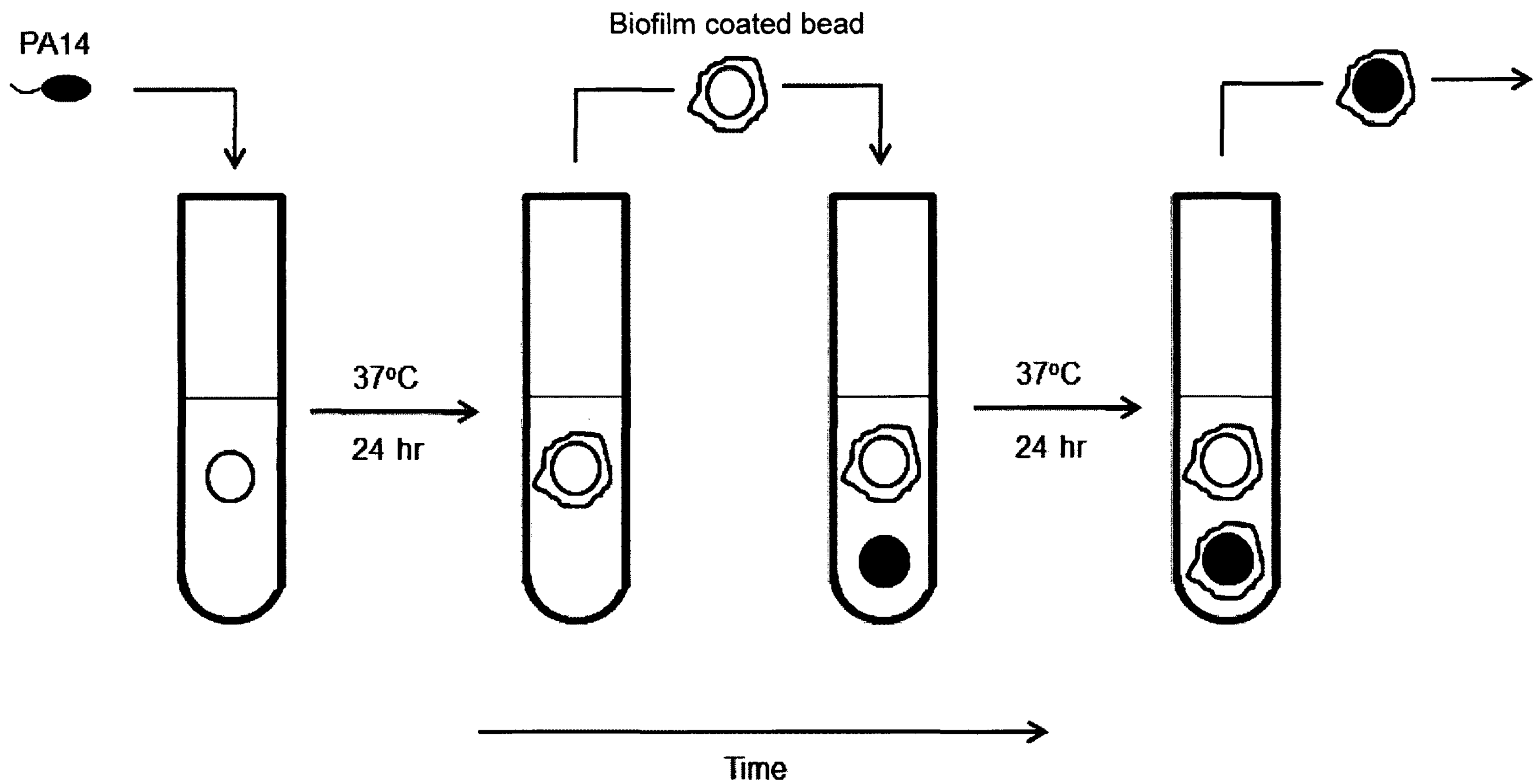

Figure 1 Experimental evolution of *Pseudomonas aeruginosa* PA14. Modified from Poltak and Cooper (2011).

After 100 generations in the B1 population, three nonsynonymous single nucleotide polymorphism (SNP) mutations were identified within three genes and verified by Sanger sequencing in some of the evolved isolates. The genes containing the SNPs are *ppk*, *mut*S and *rec*G. Analysis of sequence variation at intermediate frequencies also showed a two-nucleotide deletion in *argA*. Potential beneficial mutations and pertaining information are listed in Table 1. At this early time point, the *ppk* mutant allele frequency was 7% and the *mut*S, *argA* and *rec*G mutant allele frequencies were 13%, 14% and 16%, respectively. These four mutations fix in the population by 260 generations and remain fixed at the end of the evolution, suggesting that at least one of them is beneficial. Observed allele frequencies also suggest that the *argA*, *mutS* and *recG* mutations occurred before the *ppk* allele.

Table 1 Potential adaptive alleles detected in B1 metagenome

Locus	**Annotation**	**Mutation Effect**	**Significance**	**Generation [a] Detected**
PA14_17500	*mutS*	H112P	DNA mismatch repair	100 (12.8%)
PA14_59780	*rcsC*	L38P	rcsC sensor kinase, regulation of *cupD* fimbriae	260 (43%)
PA14_68740	*argA*	2 bp frameshift deletion	arginine biosynthesis	100 (14%)
PA14_69230	*ppk*	T443A	polyphosphate kinase	100 (6.8%)
PA14_70570	*recG*	A750V	ATP-dependent DNA helicase	100 (15.5%)

[a] Numbers in parentheses refer to allele frequencies

There are multiple mechanisms that may explain how each of the potentially adaptive alleles may benefit the evolved clones. The *mut*S and *rec*G

genes encode a DNA mismatch repair protein and an ATP-dependent DNA helicase, respectively. Preliminary studies show that the three biofilm populations are hypermutators (Flynn, unpublished), supporting the hypothesis that the mutations resulting in the *mutS*, *recG* or both genes result from loss-of-function or reduced function. Interestingly, *recG* is in an operon with *oxyR*, a regulator of the oxidative stress response in *P. aeruginosa*, and transcription of both genes were seen to increase in the presence of H_2O_2 (Ochsner et al., 2000).

The *argA* gene encodes an N-acetylglutamate synthase which is involved in arginine biosynthesis. The two nucleotide *argA* deletion could affect intracellular arginine concentrations which could promote a sessile lifestyle. Evolved clones with the mutation may produce high levels of c-di-GMP, which upregulates biofilm production. This may also reflect adaptation to the selective medium that was supplemented with 0.4% arginine. Physiological concentrations of arginine have been shown to be an important component in modulating biofilm production via the secondary messenger, cyclic di-GMP, in *P. aeruginosa* (Bernier et al., 2011).

The *ppk* gene encodes a polyphosphate kinase (Ppk) which can reversibly convert adenosine triphosphate (ATP) into adenosine diphosphate (ADP) and polyphosphate (polyP). Polyphosphate kinase, in addition to its role in polyP production, has been associated with RNA degradation (Blum et al., 1997), ribosomal protein degradation (Kurado et al., 1999), regulation of quorum sensing and motility (Rashid et al., 2000; Rashid & Kornberg, 2000) and regulation of stress responses (Shiba et al., 1997). The *ppk* mutation exists in

evolved isolates that also have the *mutS* or *recG* SNPs. If the *ppk* mutation is beneficial, the *argA*, *mut*S and *recG* alleles could have fixed because they are in association with this potentially adaptive step.

Since we were interested in understanding how PA14 adapts to a biofilm and because early beneficial mutations tend to have larger fitness effects than later beneficial mutations (Lenski et al., 1991) morphotypes isolated at 100 generations were chosen as the focus for this study. Our PA14 biofilm populations constantly consist of millions of cells, making it hard for beneficial alleles starting at low frequencies to fix. Therefore, in order to rise quickly, a mutation must have a significantly positive fitness advantage over its ancestral genotype, motivating my first hypothesis:

Hypothesis 1: Since the *ppk* allele fixes by 260 generations, I hypothesized that it is adaptive.

The protein structure of Ppk consists of an N-terminal domain responsible for binding ATP, a head domain that is involved in dimerization and two C-terminal domains that contain important sequences for catalytic activity. The *ppk* mutation results in a non-synonymous change in an amino acid within a putative active site in one of the C-terminal catalytic domains. The ancestral and evolved protein structures were constructed *in silico* and aligned using Phyre2 and FATCAT (Figure 2). The site of the mutation does not seem to alter nearby sequences in the evolved protein. However, the T443A change seems to influence the 3-dimensional conformation of residues 633-637. Within this five amino acid sequence, there are three putative active sites in the second catalytic

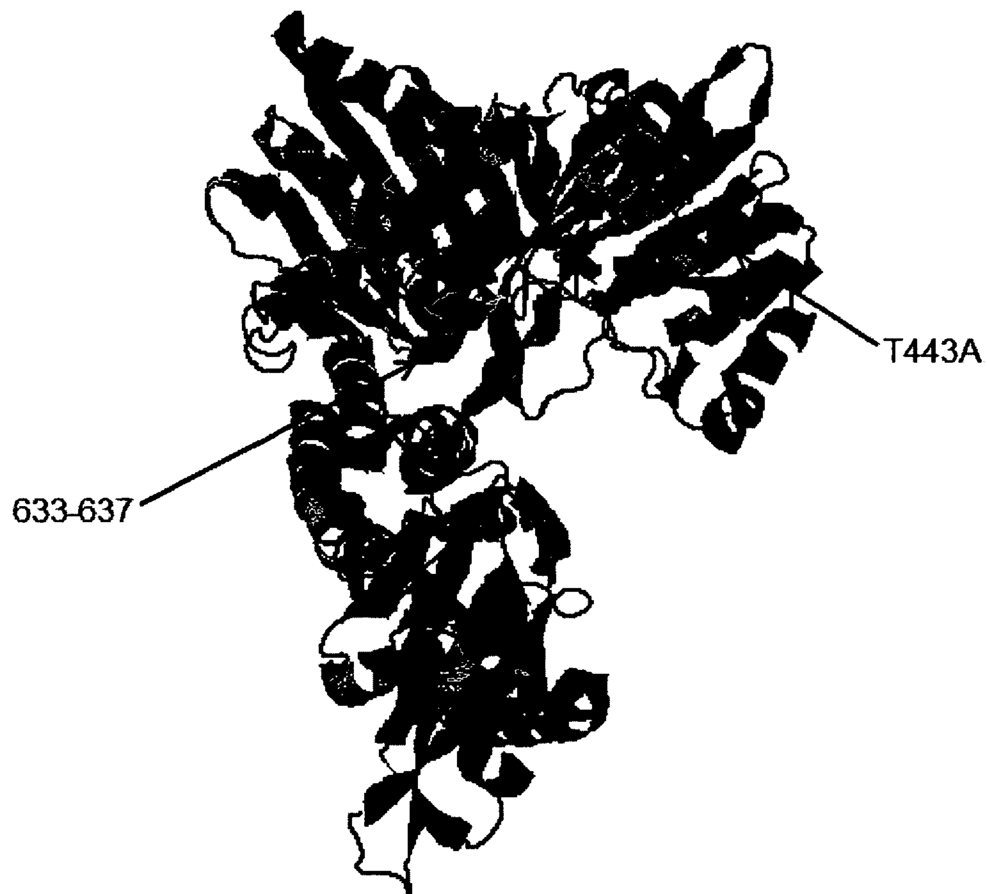

Figure 2 Computer generated structures of ancestral and evolved Ppk overlapped. Red/grey indicate where ancestral and evolved sequences share 3-dimensional conformation. Solid gray represents the evolved Ppk conformation. T443A is the site with the Ppk mutation and 633-637 are amino acid residues.

domain that is involved in polyP production. This functional organization led me to a second hypothesis:

Hypothesis 2: The amount of polyP produced by evolved clones with the SNP increases compared to the ancestor.

PolyP and Ppk can play multiple roles in prokaryotes (Figure 3) and an increase in polyP could be beneficial for *P. aeruginosa* in a biofilm for multiple

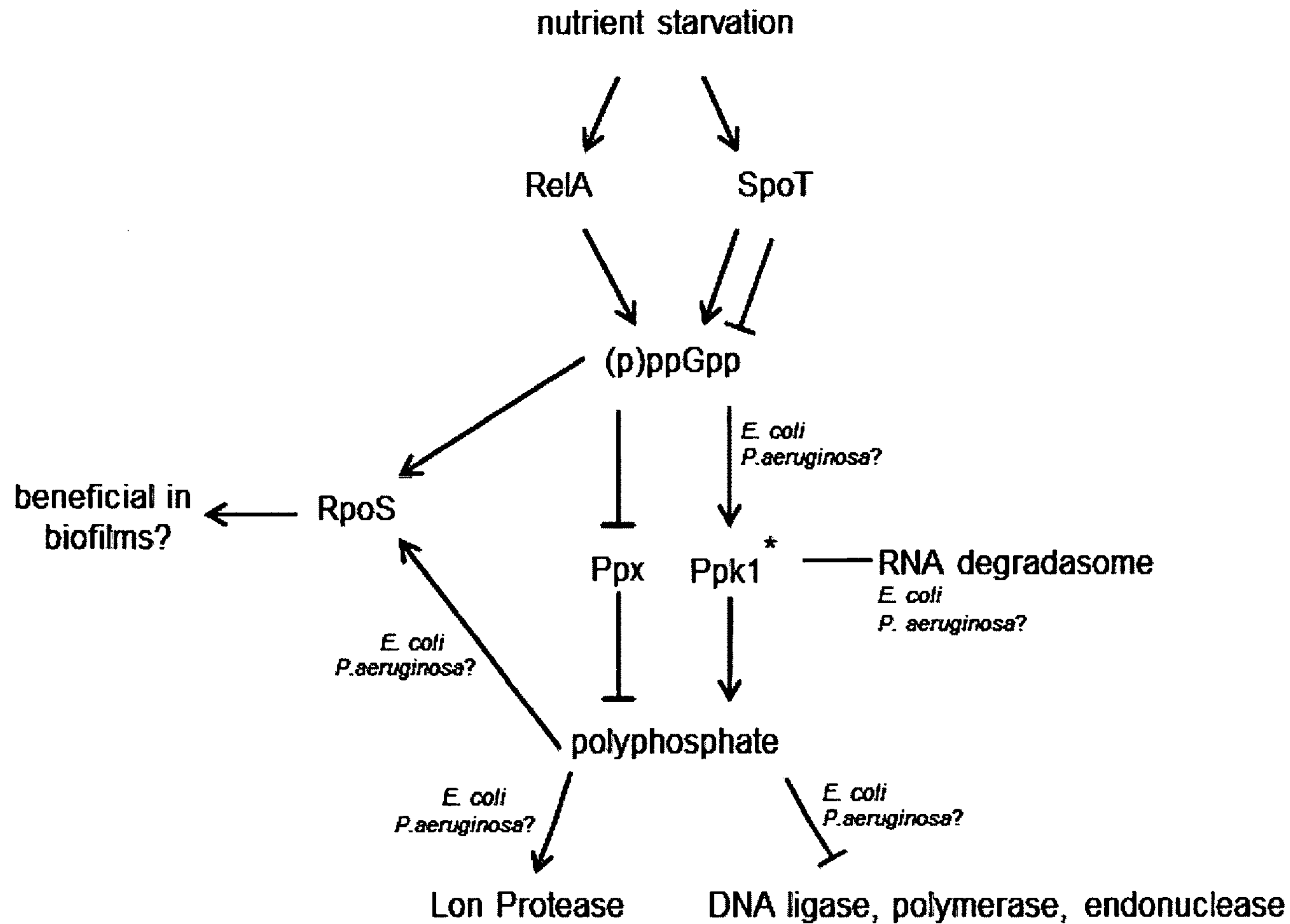

Figure 3 Roles of *ppk1* and polyP in *E. coli* and *P. aeruginosa*. Modified from Achbergerová & Nahalka (2011). Asterisk indicates where a mutation became detectable following 100 generations of selection and fixed by 260 generations.

reasons. Due to the thick exopolysaccharide matrix, concentration gradients of nutrients based on diffusion rates form (Stewart, 2003). Phosphate is essential for cell growth and metabolism and could be limiting within the *P. aeruginosa* biofilms. Therefore, the ability to create large reservoirs of this molecule in the form of polyP may be advantageous in this environment. PolyP can regulate expression of *rpoS* as removing polyP from *E. coli* cells significantly decreased expression of the alternative sigma factor (Shiba et al., 1997). In *E. coli*, the *rpoS* gene is expressed during stationary phase due to lack of nutrients, in response to extremes in pH and osmolarity and during exposure to temperature shock and hydrogen peroxide (Battesti et al., 2011). Although the role of RpoS differs somewhat between *P. aeruginosa* and *E. coli* (Venturi, 2003), there are similarities as the alternative sigma factor is also induced under starvation conditions, heat shock, high osmolarity and exposure to H_2O_2 in *P. aeruginosa* (Suh et al., 1999). However, studies are contradictory about the role that RpoS may play within bacterial biofilms. Some suggest that *rpoS* is upregulated and is necessary for mature biofilm development (Xu et al., 2001, Irie et al., 2010), whereas others propose that Δ*rpoS* mutants have enhanced biofilm phenotypes or are important only for early biofilm development (Corona-Izquierdo et al., 2002; Ferriéres et al., 2009). This role may be species-specific and could depend on the stage of biofilm development. These studies motivated my third hypothesis:

Hypothesis 3: The mutant *ppk* allele evolved in response to biofilm associated stress.

E. coli and *P. aeruginosa* differ in how they generate polyphosphate. Although both organisms have a *ppk1* gene, only *P. aeruginosa* has three *ppk2* enzymes that use GTP instead of ATP to add phosphates to a growing chain of polyP (Zhang et al., 2002). In addition, most of the molecular mechanisms determining the roles that PPK1 and polyP play in bacteria have been resolved in *E. coli* and less is known about them in *Pseudomonas*.

Within the focal evolved *P. aeruginosa* population, B1, a SNP in *rcsC* was detected at 260 generations at 43% frequency and rose to 92% by 560 generations, suggesting that it is also adaptive. In addition, according to metagenomic data and genotype screening, this seems to be the only mutation that significantly rises in frequency between these two time points. The non-synonymous mutation changed an alanine to valine towards the C terminal end between an active site and ATP-binding site. The *rcsC* gene may also modulate stress response in *P. aeruginosa* as it has been shown that a Δ*rcsC E. coli* strain displays a biofilm deficient phenotype due to elevated levels of RpoS which is regulated by the RcsC phosphorelay (Ferriéres et al., 2009). The Rcs phoshorelay is a two-component system consisting of a sensor kinase (RcsC), a histidine phosphotransfer protein (RcsD) and a response regulator (RcsB). RcsC and RcsD are membrane proteins that receive environmental cues that activate the pathway through a series of phosphotransfers. When cytoplasmic RcsB is phosphorylated, it binds to DNA to upregulate the Rcs regulon. Genes within the regulon are associated with virulence factors, motility, drug resistance and biofilm formation (Rogov et al., 2008).

Another potential role that the *rcsC* mutation could play in the biofilm lifestyle is the regulation of the chaperone-usher pathway (CUP) that is involved in fimbriae assembly (Nicastro et al., 2009) as shown in Figure 2. Fimbriae are important for cell-to-cell attachment and contribute to the development of biofilms (Ruer et al., 2007). Whereas most *Pseudomonas* strains contain three groups of *cup* genes, *cupA*, *cupB* and *cupC*, PA14 has these three as well as a fourth, *cupD*. The *cupD* gene cluster (consisting of *cupD*1-5) and *rcsBC* are located within a pathogenicity island, PAPI-1, unique to PA14, making it more virulent than other *P. aeruginosa* strains. The *cupA* clusters are modulated by the MvaT regulator, *cupB* and *cupC* are controlled by the RocASR two component system and *cupD* is regulated by the Rcs and Pvr two component pathways. Mikkelsen et al. (2009) found that *cupD* genes were upregulated and downregulated if *rcsB* and *pvrR* were overexpressed, respectively. Because a mutation was found in the *rcsC* gene in population B1, we isolated this allele in the ancestral background to directly test its fitness and phenotypic effects.

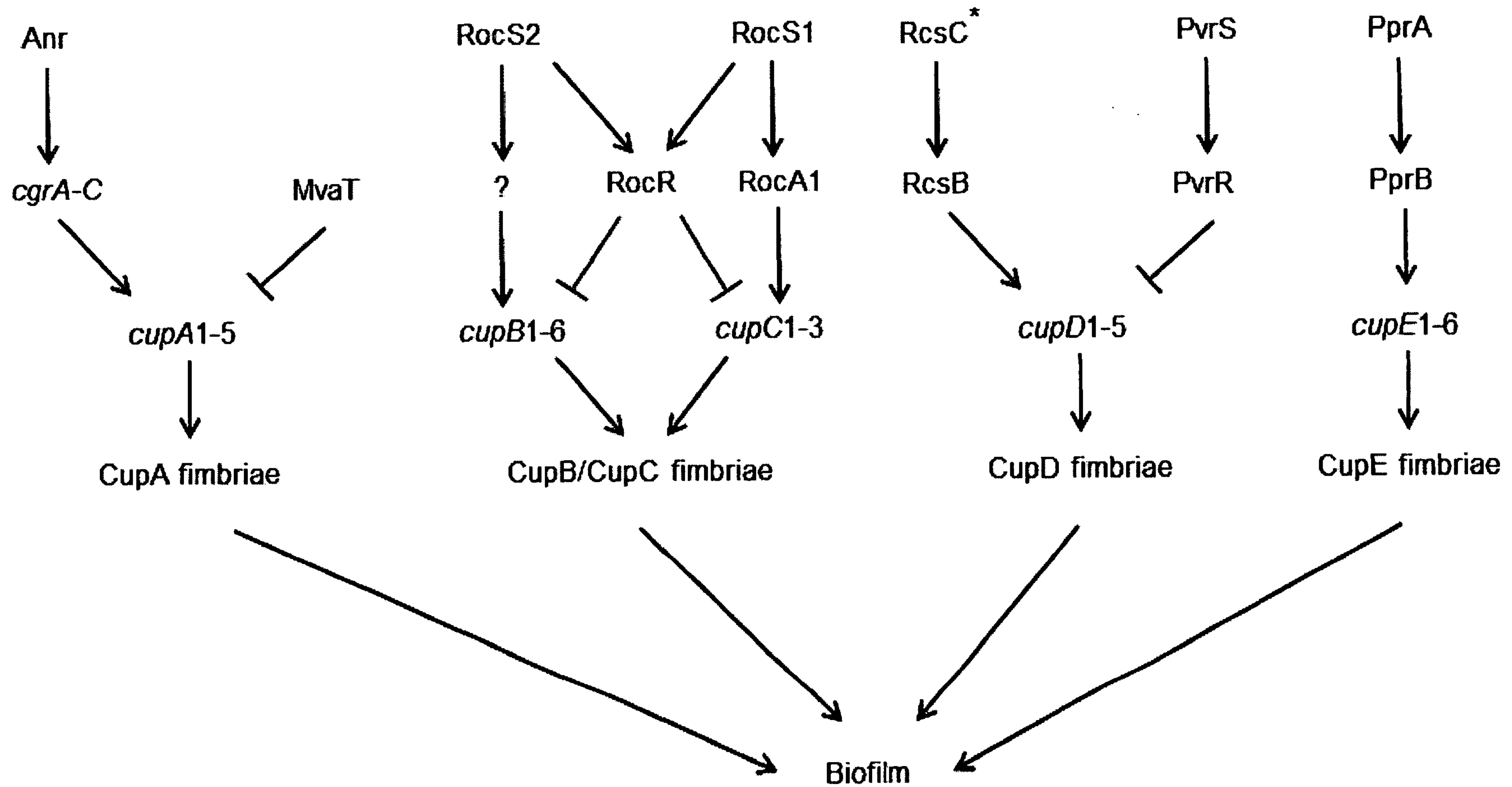

Figure 4 Regulation of CUP fimbriae in *Pseudomonas aeruginosa* PA14. Modified from Mikkelsen et al. (2011) and Giraud & Bentzmann (2012). Asterisk indicates the location of a mutation detected in population B1 at 260 generations.

CHAPTER II

METHODS

Growth Conditions

P. aeruginosa was evolved in 5 ml cultures of M63 (15 mM $(NH_4)_2SO_4$, 22 mM KH_2PO_4, 40 mM K_2HPO_4, 40 mM galactose, 1 mM $MgSO_4$, 25 µM $FeCl_2$, 0.4% arginine) with a polystyrene bead. After 24 hr, the bead was transferred to a new tube with fresh M63 and a second bead. This process was carried out for 90 days. Isolates were grown on Morph (1% w/v tryptone, 1.5% agar, 40 µg/ml Congo Red, 20 µg/ml Coomassie Blue) and VBMM plates (1.5% agar, 0.8 mM MgSO4•7H2O, 10 mM citrate, 60 mM K2HPO4, 4 mM NaNH4PO4) to observe changes in colony morphology.

Metagenomic Sequencing

Total community DNA was previously extracted at 100, 260, 540 Generations and Illumina sequencing was used to detect mutations (Flynn, unpublished). Using BWA, we aligned reads to the published *Pseudomonas aeruginosa* PA14 genome and we used SAMTOOLS to detect SNPs. Further details of this analysis are described by Flynn et al., unpublished).

Isolate Sequencing

From the population isolated after 100 generations, O2, P2 and P3 isolates were selected and subjected to Sanger sequencing. Clones were grown overnight in liquid LB and genomic DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen, 69504).

Isolate Genotyping

To verify mutations from metagenomic analysis, SNPs and indels were screened for by PCR amplifying genomic regions containing the putative mutations, sequencing amplicons and comparing them to a reference sequence. Templates for PCR were from cell lysates by suspending single colonies of evolved clones into 20 µl lysis buffer (10% SDS, 10 N NaOH). Samples were incubated at 95°C for 15 min. and pulse centrifuged. An aliquot of 180 µl water was added and samples were centrifuged at 13,000 rpm for 5 min. Lysates were stored at 4°C.

PCR was done in 25 µl volumes containing 1 X Standard Taq Reaction Buffer (New England BioLabs), 0.2 µM forward primer, 0.2 µl reverse primer, 0.2 mM dNTPs, 1 µl lysate. Cycles for each reaction were run as follows: 94°C for 30 sec.; 94°C for 30 sec., 45-68°C for 30 sec, 72°C for 1 min. per kb. Annealing and elongation temperatures were determined by T_m of primer and product length (Table 2). PCR products were analyzed on 1% agarose gels stained with Gel Red (Phenix). ExoSAP-IT (USB) was used to clean crude PCR

Table 2 Primers for mutation screening

Primer Name	Sequence 5' to 3'	T_m (°C)	Product Size (bp)	Reference
argASNPf	ACGCCGTGGGTAGTCTGC	58.8	895	This Study
argASNPr	GTGGTGAGCACGAACAGG	54.24		This Study
mutS F	CTCTCTCAGCACACGCCA	55.18	404	This Study
mutS R	TCGTCGCCGAGGATTG	55.74		This Study
ppkSNPf2	CCTGTCCGAAAGCGAGTTG	56.2	1077	This Study
ppkSNPr2	TCCAGTTCCTTCTTGACCCG	56.8		This Study
rcsC F	GAAATCCGCTTGCGTCCA	56.0	273	This Study
rcsC R	ATTCCCGCCAGGTTGTG	54.8		This Study
recGSNPf	GCGACGAGAACAAGCACAC	56.93	588	This Study
recGSNPr	GCCTGGCTGAAATGGTAGA	55.8		This Study

products before sequencing. Aliquots of 5 µl PCR reactions were added to 2 µl ExoSAP-IT solution. All samples were incubated at 37°C for 20 min. followed by 80°C for 15 min. Sequencing reactions consisted of 5 µl PCR products and 2 µl sequencing primer. Samples were sent to the Hubbard Center for Genomic Studies for sequencing.

Allelic Replacement

Amplifying the Mutant Allele

Because the *ppk* and *rcsC* mutations occur in clones harboring other mutations, these mutant alleles needed to be introduced in isolation to the ancestral clone. The *ppk* allele was synthesized by Genscript and cloned into pMQ30. For the *rcsC* allele, a cell lysate was prepared and used as templates in PCR. Evolved isolates were streaked onto Tryptone plates and incubated at 37°C overnight. Colonies were picked and suspended in 20 µl of lysis buffer (0.25% SDS, 0.05 N NaOH). Samples were incubated at 95°C for 15 minutes

and pulse centrifuged. An aliquot of 180 µl of water was added and samples were centrifuged at 13,000 rpm for 15 min. Allelic exchange primers, which contained 5' sequences homologous to sites flanking the *Sac*I restriction site of the suicide vector pMQ30, were made to amplify 1 kb on each side of the mutation (Table 3). Expand Long Template PCR System (Roche Applied Sciences) was used to amplify mutant sequences. Reactions of 15 µl contained 1 X Buffer 3, 350 µM each dNTP, 300 nM each primer and 1 µl lysate. Thermocycler parameters were as follows: 94° for 5 min, 20 cycles of 94°C for 30 s, T_m for respective primer pair for 30 s, 68°C for 2 min, and a final extension at 68° for 7 min. Products were resolved on a 1% agarose gel and stained with Gel Red.

<u>Yeast Recombineering</u>

Yeast recombineering was used to clone PCR fragments into pMQ30 (Shanks et al, 2006). Vector (1 µg) was digested for 16 hrs in a reaction containing 1 X NEBuffer 1, 100 µg/ml BSA and 20 U *Sac*I. Amplicons and cut vector were transformed into *Saccharomyces cerevisiae* InVSc1. See table 4 for strains and plasmids. Yeast was grown overnight at 30°C in 5 ml YPD (0.01% Yeast Extract, 0.02% Bacto peptone, 0.02% dextrose) and 0.5 ml aliquots of culture were pulse centrifuged. Pellets were washed once in T.E. (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) and suspended in 0.5 ml Lazy Bones Solution (40% Polyethylene glycol [MW3350; Sigma P 3640], 0.1 M Lithium Acetate, 10 mM

Tris-HCl [pH 7.5], 1 mM EDTA). Single stranded salmon sperm (0.08mg), 20-200 ng of cut pMQ30 and 50-500 ng amplicon were added to yeast suspensions, samples were vortexed for 1 min and incubated at room temperature overnight. Cells were heat shocked at 42°C for 11 min washed twice with T.E. and plated onto SD Medium-URA (MP Biomedicals). Cultures were incubated at 30°C until colonies formed.

Plasmid Extraction From Yeast

Colonies from SD Medium-URA agar were started in 10 ml liquid SD Medium-URA and incubated at 30°C overnight. Entire cultures were pelleted and suspended in 200 µl Smash and Grab Buffer (1% SDS, 2% Triton X-100, 100 mM NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA), 200 µl phenol:chloroform:isoamyl alcohol (25:24:1) and 0.3g of 0.5 mm glass beads(Next Advance, Inc.). Samples were vortexed for 2 min and centrifuged at 13,000 x g for 5 min. The aqueous layer was mixed with 200 µl chloroform and centrifuged for 5 min. Isopropyl alcohol (140 µl) was added to the aqueous layer, samples were inverted and incubated at room temperature for 5 min. Samples were centrifuged for 2 min and washed twice with 500 µl 70% ethanol. DNA was dried by incubating at room temperature overnight and pellets were suspended in 100 µl water.

Table 3 Allelic exchange primers

Primer Name	Primer Sequence 5' to 3'[a]	T_m (°C)[b]	Product Size (bp)	Reference
rcsClongForward	TGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTACCGAGCT TGACCCAGGATGACCAGCA	59.72	2147	This Study
rcsClongReverse	TTCACACAGGAAACAGCTATGACCATGATTACGAATTCG TCTTTCTCACAAACTCCAGCG	58.04		This Study

[a] Smaller letters are homologous to the sequence upstream/downstream of the SacI restriction site on pMQ30. Larger letters correspond to gene sequence

[b] Melting temperatures correspond to gene sequences

Table 4 Strains and plasmids

Strain	Relevant Characteristics	Reference
*Escherichia coli*S17.1	λ pir, used in biparental matings	ATCC 47055
Pseudomonas aeruginosa PA14	ancestral strain in evolution	Rahme et al., 1995
Pseudomonas aeruginosa PA14 *attB::lacZ*	constitutively expresses *lacZ*	Hogan et al., 2004
P. aeruginosa PA14*ppk*T443A	ancestor with *ppk* SNP	This study
P. aeruginosa PA14*rcsC*A750V	ancestor with *rcsC* SNP	This study
P. aeruginosa PA14 *ppk*::Tn	*ppk* transposon mutant	Liberati et al., 2006
Saccharomyces cerevisiae InVSc1	*ura3-52*	Shanks et al., 2006
Plasmid		
pMQ30	allelic replacement vector, R6K origin of replication, GmR, Ura3	Shanks et al., 2007
pMQ30*ppk*SNP	allelic replacement vector with ppkSNP	This Study
pMQ30*rcsC*SNP	allelic replacement vector with rcsC SNP	This Study

Transformation of *E. coli* S17

Constructed pMQ30ppk and pMQ30rcsC vectors were electroporated into *E. coli* S17.1. To prepare electrocompetent cells, 500 ml of SOB (2% tryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$) was inoculated with 200 µl of overnight cells and incubated at 37°C until the

OD_{600} reached 0.5. Cultures were centrifuged at 4,000 x g for 10 min at 4°C and washed twice with cold water. Cells were suspended in 50 µl 10% glycerol and stored at -80°C. For electroporation, cells were thawed on ice and 20 µl of yeast DNA was added and mixed. Samples were electroporated using 2 mm cuvettes at 2.5 kV. One ml of SOB was added to cells and samples were shaken at 37°C for 1 hr before plated on T-soy agar with 10 µg/ml gentamicin. Inserts were verified by sequencing using primers in Table 5.

Bacterial Conjugation

Suicide vectors were conjugated into PA14 using *E. coli* S17.1. Strains were grown overnight in LB (1% Bacto Tryptone, 0.5% Yeast Extract, 10 mM NaCl) at 37°C with appropriate antibiotic. Cultures were washed twice with LB and mixed at a 1:1, 2:1, 3:1 or 4:1 ratios of donor to recipient. Cultures were heat shocked at 42°C for 10 min and 50 µl were spot plated onto LB agar. Plates were incubated overnight at 30°C, spots were suspended in 1 ml LB and plated onto LB agar with 10 µg naladixic acid and 50 µg gentamicin. Plates were incubated at 30°C overnight and *P. aeruginosa* colonies were picked to start 5 ml LB cultures that were incubated overnight at 30°C. Cultures were plated onto 5%

Table 5 Sequencing Primers for Insert

Primer Name	Sequence 5' to 3'	T_m (°C)	Reference
pMQ30seq F	TAACGCCAGGGTTTTCCCAG	62.4	This study
pMQ30seq R	AGGCACCCCAGGCTTTAC	57.2	This study
rcsC F	GAAATCCGCTTGCGTCCA	56.0	This study
rcsC R	ATTCCCGCCAGGTTGTG	54.8	This study
rcsCseqF1	CGGATTCGCCAGGTGCTCA	60.4	This study
rcsCseqR1	CGCTCGCCCTCTTCTCGCA	62.6	This study

sucrose LB agar without NaCl and LB agar. Colonies were screened to verify allelic replacement by PCR and sequencing as described above. The ancestor and constructed mutants were plated on morphology (1% w/v tryptone, 1.5% agar, 40 µg/ml Congo Red, 20 µg/ml Coomassie Blue) and VBMM plates (1.5% agar, 0.8 mM $MgSO_4 \bullet 7H_2O$, 10 mM citrate, 60 mM K_2HPO_4, 4 mM $NaNH_4PO_4$) to test effects of mutations on colony morphology.

Fitness Assays

To measure fitness effects of mutations, evolved isolates or constructed mutants were competed against a neutrally marked *lacZ*+ ancestor (Hogan et al., 2004). Five biological replicates of each strain were propagated in 5 ml liquid medium consisting of 4 ml M63 and 1 ml T-soy containing a polystyrene bead. Cultures were shaken for 24 hrs at 37°C. Beads containing biofilm were removed and sonicated in 1.5 mL PBS for 10 s. Competitor and ancestor were mixed in 5 ml fresh M63 containing a new bead in a 1:1 ratio. $Day_{t=0}$ mixtures were plated on Tryptone agar with X-gal (40 µg/ml) and incubate for 24 hrs at 37°C. Beads were removed and sonicated in 1.5 ml PBS and $Day_{t=1}$ mixtures were plated on Tryptone agar with X-gal. Plates were incubated at 37°C for 24 hrs and let sit at room temperature for 24 hrs to develop.

The number of colonies for competitor and marked ancestor were used to calculate CFU/ml. To determine fitness of the competitor, the selection rate constant was calculated as:

$\ln(competitor_{t=1}/competitor_{t=0}) - \ln(ancestor_{t=1}/ancestor_{t=0})$

Polyphosphate Extraction & Measurement

Polyphosphate was extracted and measured according to Silby et al. (2009) with modifications. All strains were grown in 5 ml LB overnight and 100 ul of these cultures was added to 5 ml K10Tπ (50 mM Tris-HCl [pH 7.4], 0.2% [wt/vol] Bacto tryptone, 0.15% [vol/vol] glycerol and 0.61 mM $MgSO_4$) and incubated for 24 hr at 37°C. An aliquot of 200 µl was removed from each culture and OD_{600} was taken. For polyP extraction, 1 ml of each culture was centrifuged for 2 min. at 13,000 x g and pellets were suspended in 500 µl pre-warmed (95°C) guanidine thiocyanate lysis buffer (4 M guanidine thiocyanate in 500 mM Tris-HCl[pH7]). Samples were incubated at 95°C for 5 min. Aliquots of 30 µl 10% SDS, 500 ul 95% ethanol and 10 µl glassmilk were added to lysates and were centrifuged for 15 sec. Pellets were suspended in 500 µl New Wash Buffer (5 mM Tris-HCl [7.5], 50 mM NaCl, 5 mM EDTA, 50% ethanol) and centrifuged. This wash was repeated two more times. Pellets were suspended in 100 µl of a solution containing 50 mM Tris-HCl (pH7.4), 10 mM $MgCl_2$ and 1 mg/ml DNase I and RNase A. Samples were incubated at 37°C for 30 min. Glassmilk was suspended in 150 µl guanidine thyiocyanate lysis buffer and 150 µl 95% ethanol. Pellets were suspended in 300 µl New Wash Buffer and centrifuged. This wash was repeated once more. PolyP was eluted by suspending glassmilk in 50 mM Tris-HCl (pH8.0). This was repeated once more and elutions were pooled together.

To measure polyP, 10, 25 and 50 µl aliquots of elution was added to 900 µl of toluidine blue-O dye (6mg/liter in 40 mM acetic acid) and volume was brought to 1 ml. Samples were inverted to mix and incubated at room temperature for 15 min. Aliquots of 200 µl were loaded into 96-well polystyrene plates and absorbance was taken at 530 nm and 630 nm. PolyP levels were reported as the ratio A_{530}/A_{630}.

Biofilm Assay

The ability to form biofilms in monocultures of *P. aeruginosa* strains was measured by a modified crystal violet assay (O'Toole & Kolter, 1998). Strains were grown in 4 ml M63 and 1 ml T-soy at 37°C overnight. Cultures were diluted to OD_{600} = 0.01 in fresh M63 and transferred to sterile 96 well polystyrene plates which were incubated at 37°C for 24 hrs with orbital shaking. Cell density was measured at OD_{600} and unbound cells were removed by inverting the plate. Plates were incubated at 80°C for 30 min to heat-fix cells which were stained with 0.01% crystal violet for 20 min at room temperature. Stain was removed by washing with water 4 times and plates were dried at room temperature for 2 min. Wells were destained with an 80% ethanol, 20% acetone solution for 15 min and OD_{600} was measured.

Measuring Oxidative Stress

To test if the *ppk* mutation is altering oxidative stress tolerance via RpoS in *P. aeruginosa* (Jørgensen et al., 1999), strains were exposed to H_2O_2. Cultures of 5ml K10Tπ (50 mM Tris-HCl [pH 7.4], 0.2% [wt/vol] Bacto tryptone,

0.15% [vol/vol] glycerol and 0.61 mM $MgSO_4$) were incubated for 24 hrs at 37°C and were standardized by OD_{600}. Strains were plated on K10Tπ agar and sterile filter discs saturated with 3% H_2O_2 were placed in the center. Plates were incubated for 24 hrs and zones of inhibition were measured to determine sensitivity.

Heat Shock Assay

Because the RpoS general stress response can confer resistance to heat shock in *Pseudomonas aeruginosa* (Suh et al., 1999) strains were grown in 5 ml K10Tπ at 37°C for 24 hrs and exposed to 50°C for 4, 12, 20 and 45 min. Cultures were diluted in PBS, plated on T-soy agar before and after exposure and incubated for 24-48 hrs at 37°C. Response to heat shock was determined by calculating percent survival.

CHAPTER III

RESULTS

Morphotypes Are Genetically Diverse

Evolved isolates were initially grouped by colony morphology (Flynn, unpublished) when plated on tryptone agar containing Coomassie Blue and Congo Red dyes (morph plates), which bind to proteins and exopolyssacharides respectively. The O morph was small, dark red and had irregular edges. The M types were large, dark pink colonies usually with a bleb near the edge of the colony which may indicate M types that acquired a new mutation as the colony grew. P types were small, light pink, irregular colonies that have a wrinkly surface. H mutants displayed large, light brown colonies with highly irregular

Table 6 Early Allele Identification*

	O 1	O 2	O 3	M 1	M 2	M 3	P 1	P 2	P 3	H 1	H 2	H 3	WTL 1	WTL 2	WTL 3
argA	+	+	N/A	+	-	+	-	-	+	NA	NA	NA	NA	NA	NA
mutS	+	+	+	+	-	+	+	+	+	NA	+	+	NA	NA	NA
ppk	+	+	+	+	-	+	-	-	+	NA	-	-	-	-	-
recG	NA	NA	NA	+	-	NA	+	+	+	NA	NA	NA	-	NA	NA

*Isolates have (+) or lack (-) the respective mutation, NA = not available. Blank spaces indicate mutations that have not been screened in an isolate. Pictures represent colony morphologies of isolates (left to right:O,M,P,H,WTL) on tryptone plates containing Coomassie Blue and Congo Red dyes.

edges consisting of lighter colored blebs. The WTL colonies shared similar morphology with the ancestor. They consisted of large, pink colonies with irregular and diffuse edges. Three representatives of each morphotype at 100 generations were chosen for genetic and phenotypic analyses. Based on a prior metagenomic analysis (Flynn, unpublished), mutations in the *argA*, *mutS*, *ppk* and *recG* genes were detected approximately at 14, 13, 7 and 16% allele frequencies espectively at 100 generations and all fix within the population by 260 generations. Interestingly, isolates within a morphotype group did not necessarily share the same alleles (Table 6). For example, the O and H morphotypes shared the same mutations; however, isolates within the M and P morphotypes were not genetically identical.

The PA14*ppk*T443A strain, which has the *ppk* SNP isolated in the ancestral background) had a different morphology than the ancestor when plated on solid media (Figure 5). On VBMM, the ancestor had a larger colony morphology and its edges are more diffuse than the mutant. Differences between the strains on morphology plates were subtler as the ancestral colony was

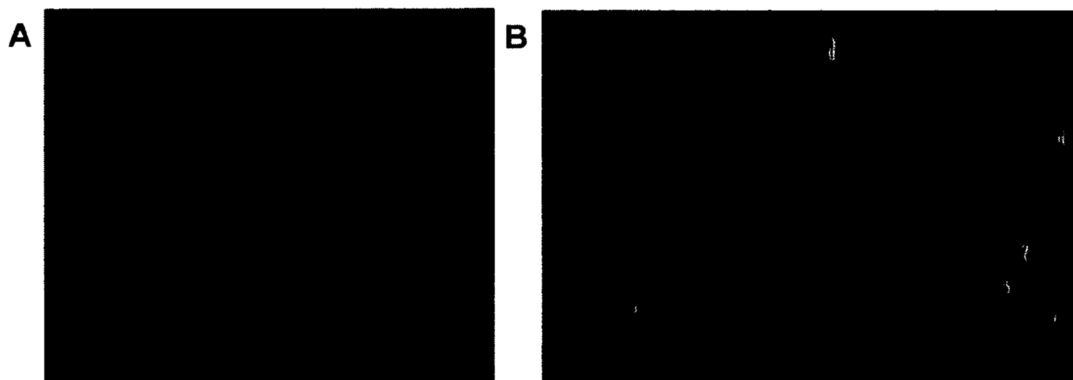

Figure 5 Colony morphologies of ancestor and the *ppk*T443A mutant on VBMM (A) and Morphology plates (B).

slightly larger and had a darker center than the *ppk* mutant. However, the SNP did not directly associate with any one of the evolved morphotypes. The *rcsC* allele did not alter colony morphology (not shown).

Evolved Isolates Have More Mutations Than Expected

The O2, P2 and P3 isolates from 100 generations were selected for Sanger sequencing to identify low frequency mutations that may not have been detected by the metagenome analysis and SNP screening. Each clone aquired additional mutations other than the ones found at high frequency in the population by 260 generations (Table 7). The O2 isolate had eight of the thirteen early mutations that rise to a high frequency whereas the P2 and P3 clones had two and four respectively. All three contain mutations that were located in genes that have not yet been annotated and in non-coding regions. Surprisingly, P3 shared more mutations with O2 than P2. However, all three sequenced isolates had the *mutS* and *recG* alleles.

Table 7 Mutations detected by sequencing of individual clones

O2	Mutation Type	P2	Mutation Type	P3	Mutation Type
aprA	SNP	*cupA4*	SNP	*argA*	DEL
argA	del	*eutB*	SNP	*envZ*	**SNP**
groES	SNP	*mutS*	SNP	*fliH*	SNP
mdoG	SNP	*oprC*	**SNP**	*glnS*	SNP
metR	**SNP**	*recD*	SNP	*mntH2*	INS
mutS	SNP	*recG*	SNP	*mutS*	SNP
ppk	SNP	*rhlA*	SNP	*ppk*	SNP
recC	SNP	*rnr*	SNP	*rbsA*	**SNP**
recG	SNP	*PA14_00650*	**SNP**	*recG*	SNP
PA14_02990/PA14_03000	NC	*PA14_00910*	**SNP**	*sdaB*	SNP
PA14_03350	SNP	*PA14_02530*	SNP	*trkA*	SNP
PA14_07260	SNP	*PA14_03166*	SNP	*PA14_02990/PA14_03000*	NC
PA14_10660	SNP	*PA14_06260*	SNP	*PA14_03350*	SNP
PA14_11160	**SNP**	*PA14_12350*	SNP	*PA14_07260*	SNP
PA14_11650	SNP	*PA14_19065/dcd*	NC	*PA14_09910/PA14_09920*	NC
PA14_14060	SNP	*PA14_21020*	SNP	*PA14_10660*	SNP
PA14_16800	SNP	PA14_25800	SNP	*PA14_21770*	SNP
PA14_17510	SNP	*PA14_31080*	SNP	*PA14_29560*	SNP
PA14_21160	**SNP**	*PA14_35080*	DEL	*PA14_33170/PA14_33190*	**SNP**
PA14_34320	**SNP**	*PA14_41420*	SNP	*PA14_39000*	**SNP**
PA14_45970	**SNP**	*PA14_44340*	SNP	*PA14_48470*	**SNP**
PA14_46440	SNP	*PA14_44650*	SNP	*PA14_48760*	SNP
PA14_47900/PA14_47910	NC	*PA14_45010*	SNP	*PA14_53570/PA14_53580*	SNP
PA14_49010/PA14_49020	NC INS	*PA14_45470*	SNP	*PA14_56090/PA14_56100*	**SNP**

PA14_63310	SNP	*PA14_46510*	SNP	*PA14_56890*	**SNP**
PA14_65400	SNP	*PA14_50850*	SNP	*PA14_61080*	**SNP**
		PA14_51000/PA14_51010	NC DEL		
		PA14_55490	**SNP**		
		PA14_61830/PA14_61840	NC		
		PA14_68390/PA14_68400	NC DEL		
		PA14_72540	**SNP**		

Underlined genes denote alleles that rise to high frequency from 100 to 260 generations
SNP = single nucleotide polymorphism, NC = non-coding SNP, INS = insertion, DEL = deletion
Bold type indicates synonymous SNPs

The *ppk* SNP Confers a Fitness Advantage

We compared the fitness of the *ppk* SNP strain and a *ppk* transposon mutant to determine if the SNP produced a loss of function mutation. Isolates from the 100-generation sample containing or lacking the *ppk* mutation were competed against the ancestral clone in a biofilm to determine its effect on biofilm adaptation (Figure 6). Strains with the SNP were more fit than those without it (F (1,23) = 87.3909, P<0.001). Isolated in the ancestral genetic background, the *ppk*

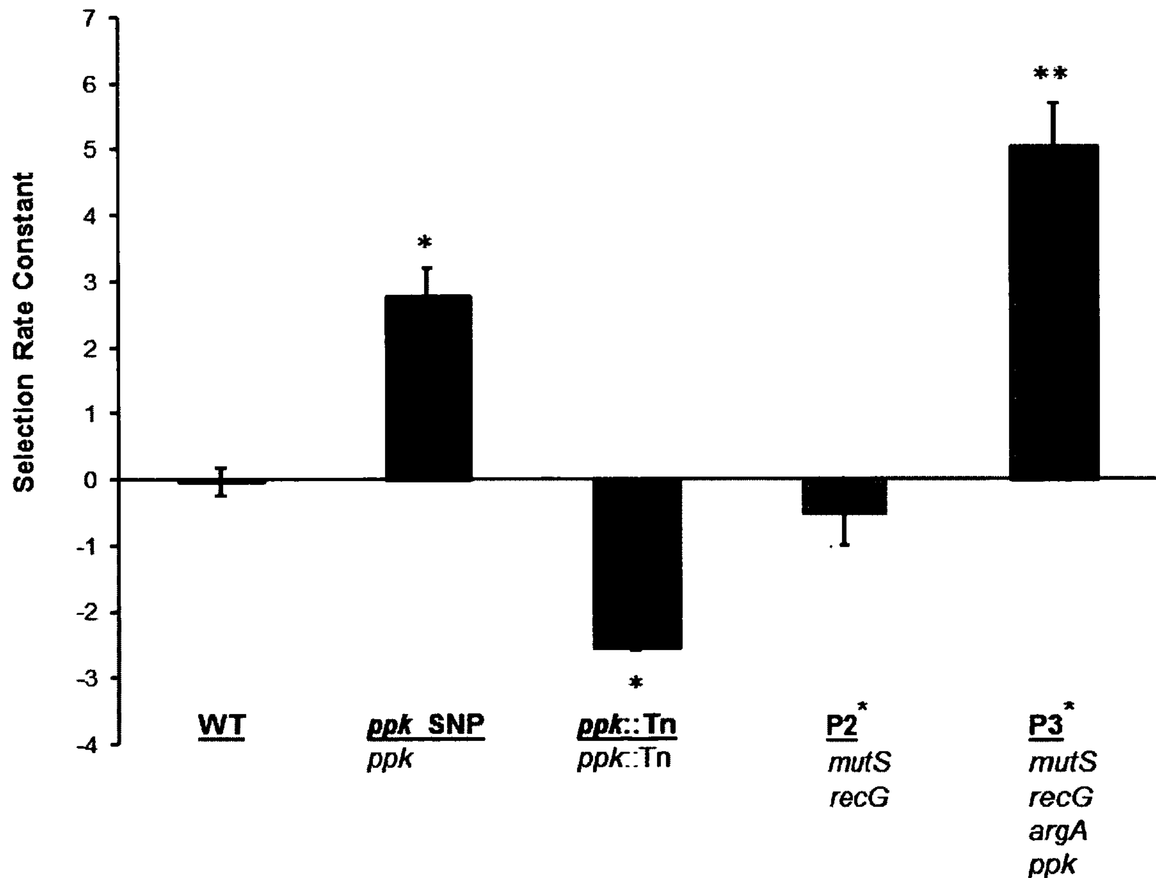

Figure 6 Biofilm associated fitness effects of the *ppk* SNP in different genetic backgrounds. Error bars represent 95% confidence intervals, n=5. Asterisks associated with bars indicate significant differences to WT, P<0.05. Strain names are underlined with genotypes below. Asterisks associated with strain names signify there are other mutations in these backgrounds (Table 7).

mutation conferred a selection rate constant of 2.78 (CI = 0.416), (*F* (1,8) = 140.630, *P*<0.001). The transposon mutant of *ppk* showed reduced fitness compared to the ancestor (*F* (1,7) = 252.628), *P*<0.001). Evolved isolate P2 (SNP-) was as fit as WT and P3 (SNP+) had a higher fitness than ancestor and the *ppk* SNP strain (*F* (1,7) = 35.085, *P*<0.001). The same strains were also competed in planktonic conditions to test if the *ppk* mutation was a biofilm specific adaptation (Figure 7) and a similar trend was observed. The *ppk* SNP

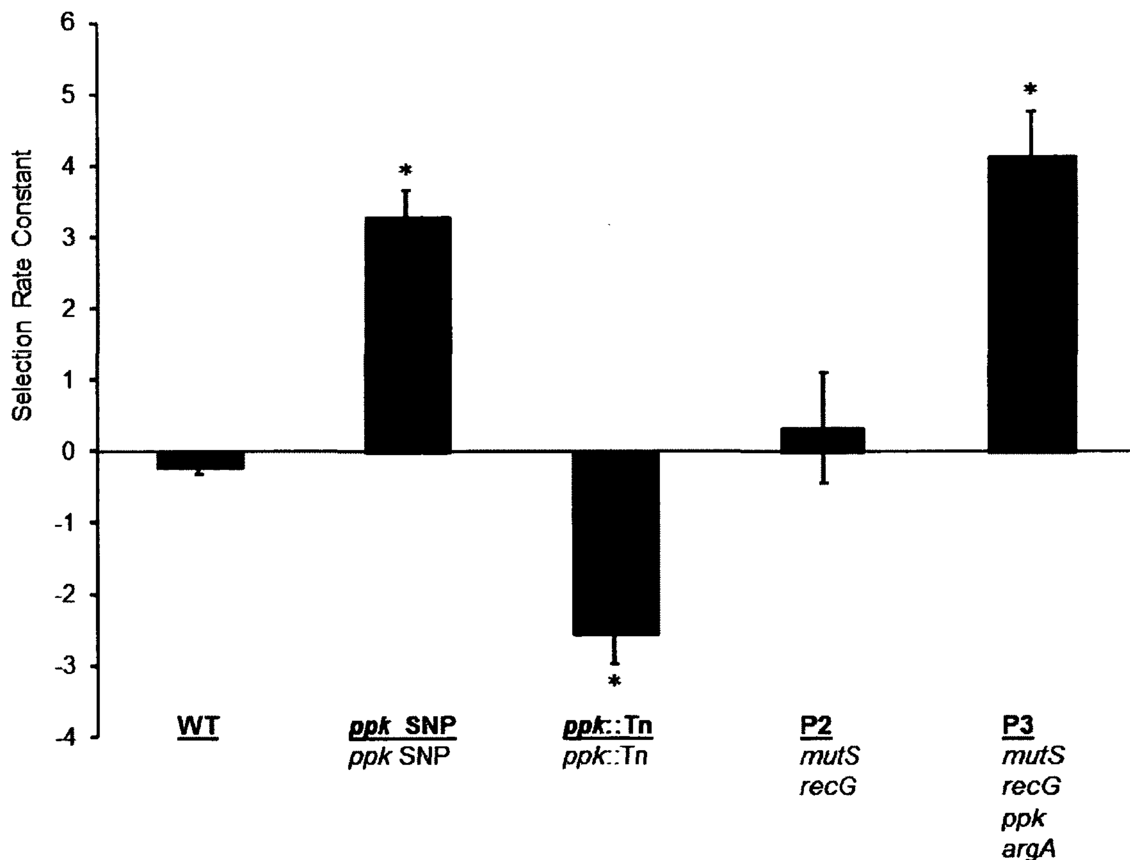

Figure 7 Planktonic fitness effects of the *ppk* SNP in different genetic backgrounds. Error bars represent 95% confidence intervals, n=5. Asterisks indicate significant differences, *P*<0.05.

mutant had a selection rate constant of 3.27, making it more fit than the ancestor ($t_{(4)} = 2.364$, $P<0.0001$) and the transposon mutant was significantly less fit than wildtype ($t_{(4)} = 2.306$, $P<0.0001$). P2 showed equal fitness to wildtype and P3 was more fit than the ancestor ($t_{(4)} = 2.306$, $P<0.0001$) and had equal fitness to the *ppk* SNP construct. Additional evolved isolates were also competed against ancestor to test the hypothesis that the *ppk* mutation confers an adaptive advantage (Figure 8). Although most isolates that have the *ppk* SNP appeared to

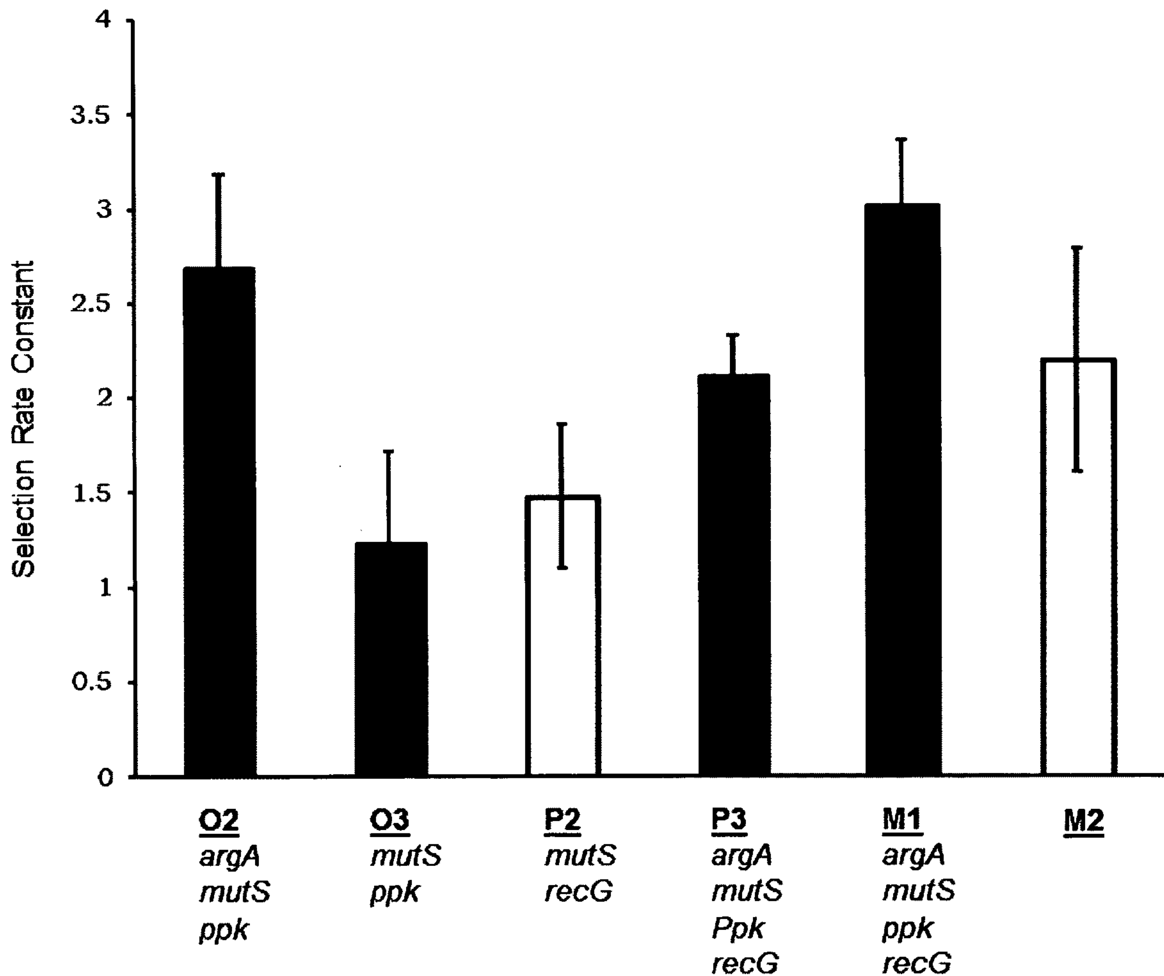

Figure 8 Comparison of selection rate constants for generation 100 morphotypes in M63 under biofilm conditions. Black and white bars represent isolates that have and lack the *ppk* SNP respectively. Error bars represent standard deviation, n = 5.

be more fit than those without it, there is not a significant difference. Despite having the SNP, O2 seemed to have similar fitness to isolates that did not have the mutation.

Morphotypes were also competed in a biofilm against ancestor in various concentrations of $FeCl_2$ to test if clones with the *ppk* mutation are more fit when exposed to potentially toxic levels of ferrous iron since polyphosphate is known to

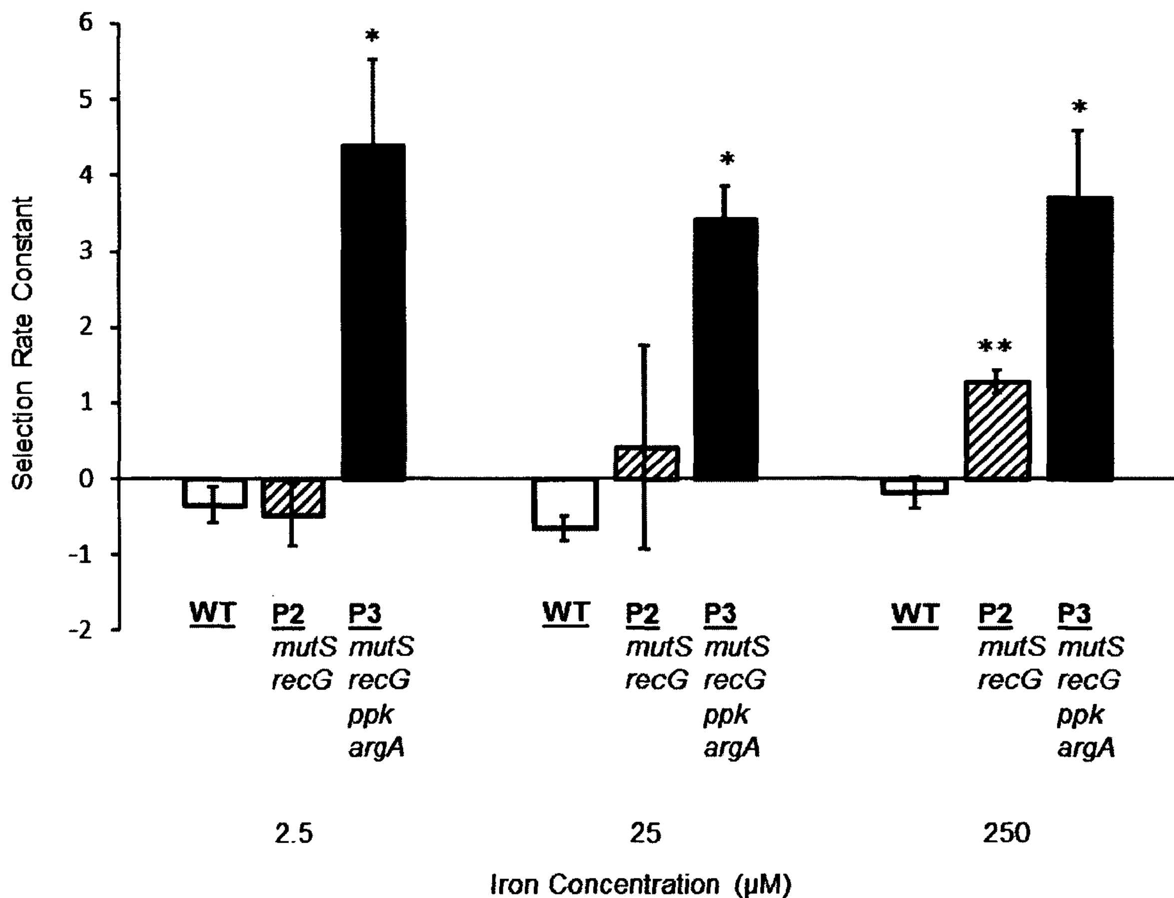

Figure 9 Fitness of isolates with and without the *ppk* SNP in different $FeCl_2$ concentrations in biofilm. Error bars are standard deviations, n = 5. Asterisk indicates significant difference, $P < 0.05$.

chelate divalent cations (Hossain et al., 2008). Fitness was measured in 2.5, 25 (evolved conditions) and 250 µM FeCl2 (Figure 9). At each concentration, P3

was more fit than both the ancestor and P2 (2.5 μM, $t_{(4)}$=2.178, P<0.0001; 25 μM, $t_{(4)}$=2.009, P<0.0001; 250 μM, $t_{(4)}$=2.178, P<0.0001). There was also a larger difference in fitness between P2 and P3 in M63 with 2.5 μM iron than 250 μM $FeCl_2$. P2 was more fit than ancestor in 250 μM $FeCl_2$ ($t_{(4)}$=2.502, P=0.0008).

Evolved Clones With the *ppk* Mutation Produce More PolyP

Since polyphosphate kinase 1 (ppk1) is the primary enzyme that synthesizes polyP in *P. aeruginosa* (Zhang et al, 2002), polymer concentrations were measured to determine if the mutation resulted in an increase or decrease of intracellular polyP (Figure 10). Overall, the isolates with the mutation produce more polyP than those that lack it (F (1, 59) = 13.398, P = 0.0005) Surprisingly, the transposon mutant produced levels of polyP similar to the ancestor. Early isolates with the mutation produced up to 18 times more polyP than the ancestor. Interestingly, M3, despite having the mutation, produced levels of the polymer comparable to WT. Early clones without the SNP showed slightly higher polyP than their ancestor but less than *ppk* SNP^+ clones. H, V, A, P, M, D, O are colony types from the 540 generation (late) time point which all have the *ppk* SNP. Late isolates, except D, made more polyP than ancestor; however, they also produced more of the polymer than *ppk* SNP^- isolates and less than early isolates with the allele.

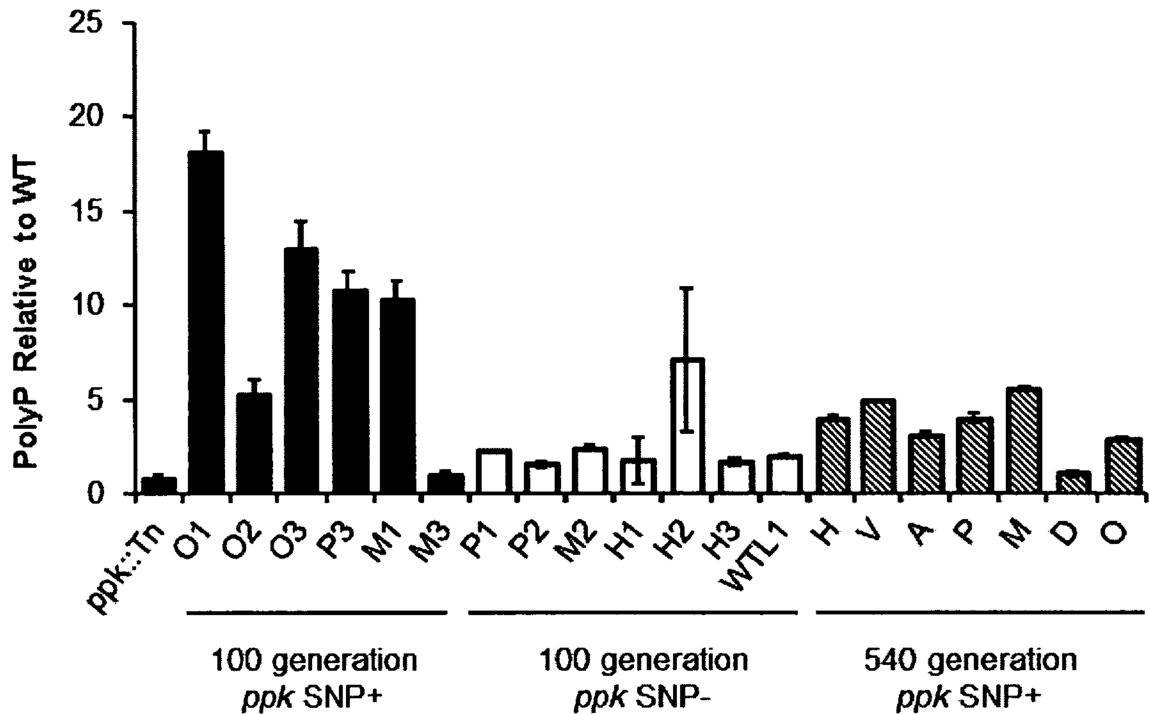

Figure 10 Amount of polyP produced by isolates relative to the ancestor. *ppk* transposon mutant (black), early isolate that have the *ppk* SNP (grey), early isolates that lack the *ppk* SNP (white) and 540 generation isolates that all have the *ppk* SNP (striped). Error bars represent standard deviation, n = 3.

The *ppk* Allele Alone Does not Increase PolyP

After isolating the *ppk* SNP in the ancestor, polyP was measured after 24 hrs to test whether this mutation alone was responsible for the increased production of the polymer and increased fitness observed in the evolved isolates with the mutation (Figure 11). Interestingly, production in the constructed strain, the transposon mutant and P2 is similar to the ancestor and there is an increase in P3 (F (1, 59) = 54.5135, P < 0.0001). While there may be a subtle fitness effect associated with high levels of polyP in P3, there is likely another reason why the *ppk* SNP alone is adaptive.

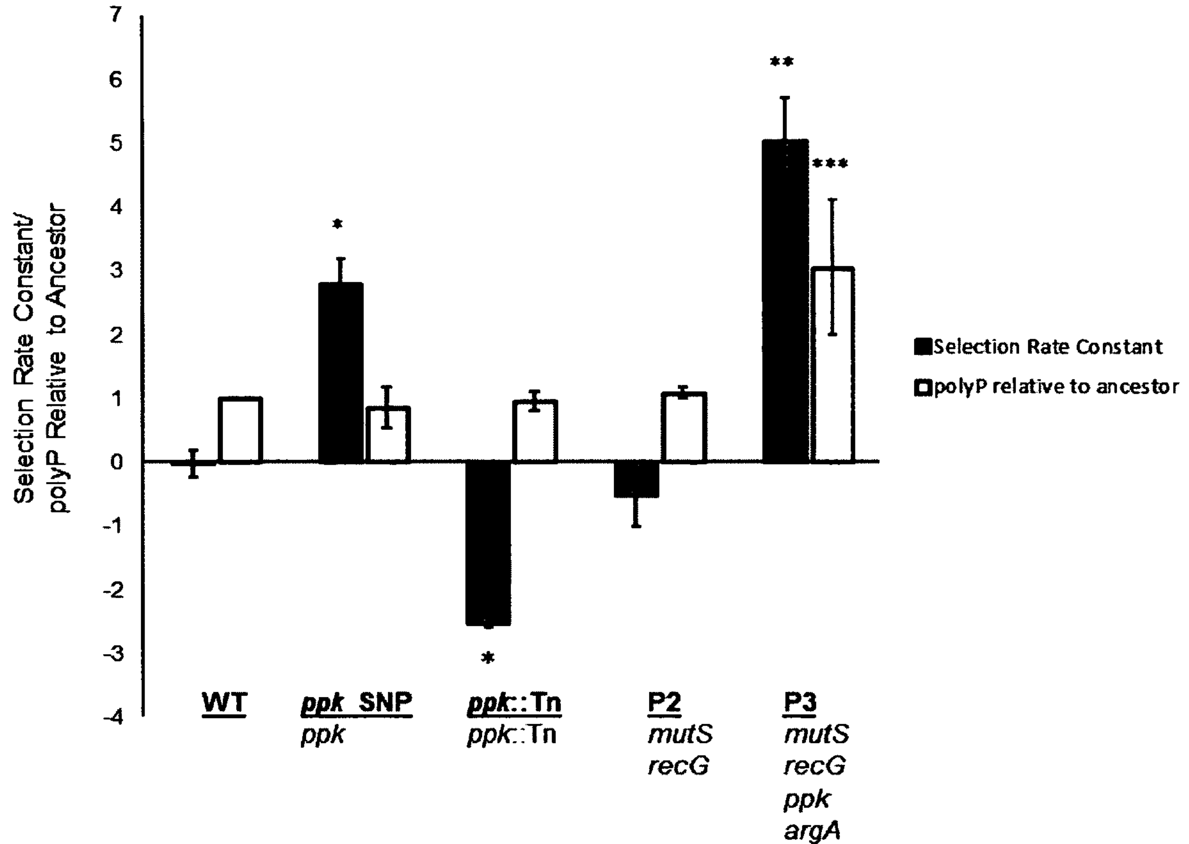

Figure 11 Amount of polyP synthesized compared to fitness in a biofilm. White bars represent selection rate constant and grey bars represent polyP production relative to ancestor. Single asterisk denotes a significant difference in fitness to ancestor. Double asterisk indicates significant fitness difference between P3 and the *ppk* SNP. Triple asterisk indicates significant difference in polyP (n=3) production from ancestor. Error bars represent 95% confidence intervals.

Evolved Isolates With the *ppk* SNP Are Not More Resistant to Hydrogen Peroxide But May Be More Resistance to Heat Shock

Because polyphosphate has been shown to regulate the RpoS general stress response in *E. coli* (Shiba et al., 1997), resistance to hydrogen peroxide and heat shock was measured. Many of the evolved morphotypes were more resistant to hydrogen peroxide than the ancestor regardless of having or lacking the *ppk* SNP (Figure 12). To test the effect of the *ppk* mutation on heat-shock, an

isolate with and without the SNP were exposed to 50°C over a 22 minute period (Figure 13). The P1 morphotype, lacking the mutation, shows a trend similar to the ancestor where both strains have an 8% survival after 22 minutes. The O1 isolate (*ppk* SNP +), however, has a 40% survival at the endpoint (F (2, 9) = 63.025, P<0.0001).

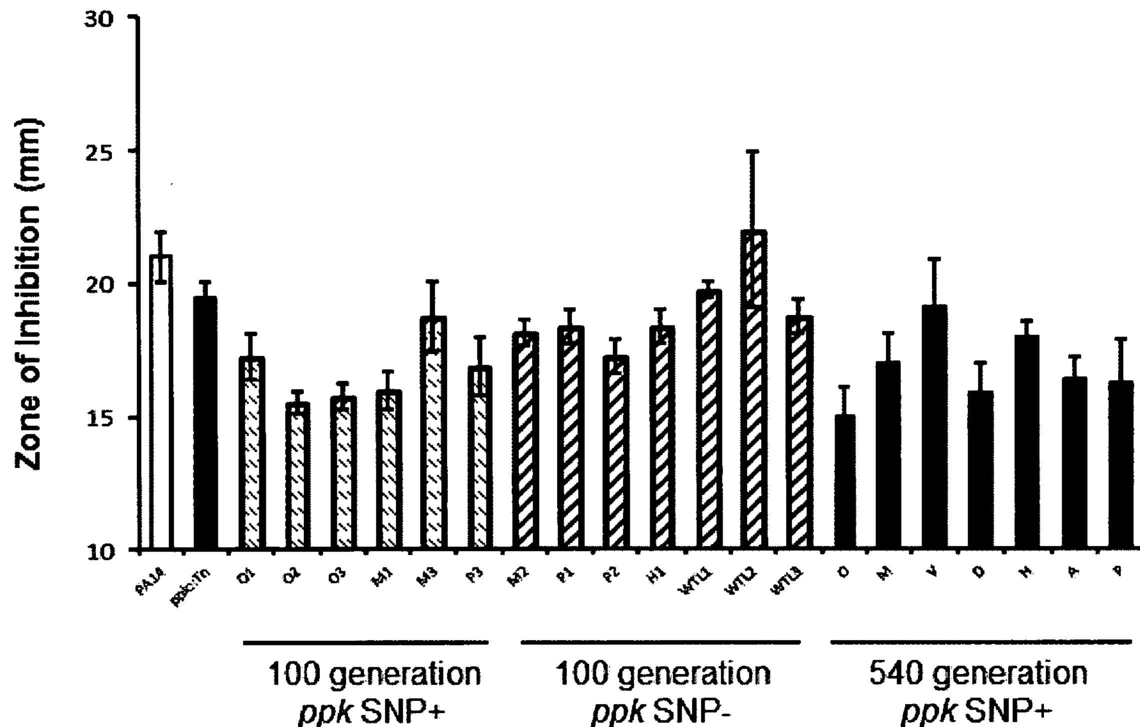

Figure 12 Hydrogen peroxide resistance by *P. aeruginosa* isolates: PA14 (white), *ppk*::Tn (black), generation 100 isolates with *ppk* SNP (small hatches), 100 generation isolates without *ppk* SNP (large hatches), generation 540 isolates (grey). Error bars are standard deviation, n = 4.

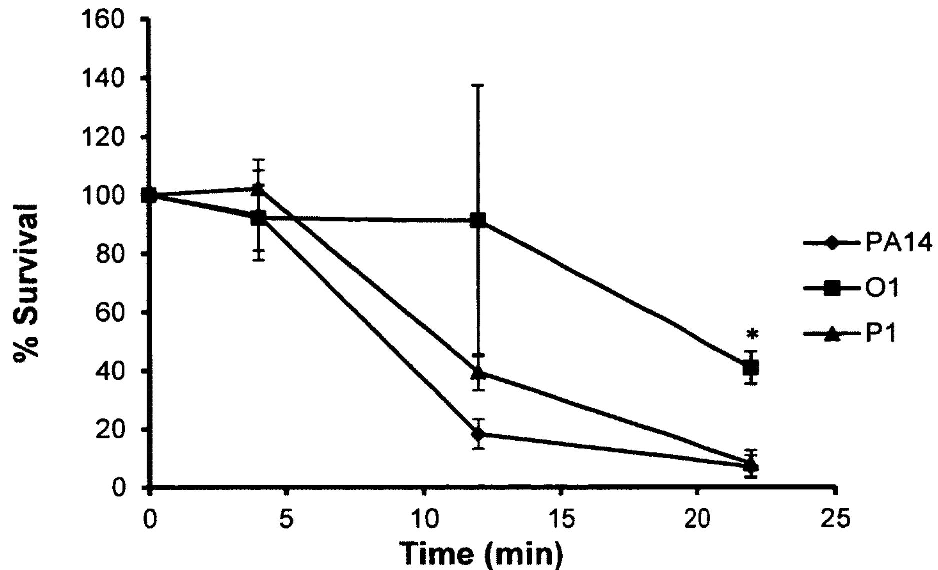

Figure 13 Effects of *ppk* mutation survival to high temperature. PA14 (black), O1 (red, *ppk* SNP+), P1 (blue, *ppk* SNP-). Error bars represent standard deviation, n = 5. Asterisk indicates significant difference, $P < 0.05$.

The *rcsC* SNP May Enhance Biofilm Formation

Since *rcsC* has been associated with the development of biofilms (Ferriéres et al., 2009), biofilm was assayed to measure the ability of *P. aeruginosa* strains to form these sessile communities (Figure 14). Throughout the time course, the ancestor and *rcsC* mutant show similar abilities to form biofilm. Interestingly, they both have less biofilm after 24 hr than 8 hr. After 4 and 8 hr, the O2 isolate containing the *rcsC* SNP forms less biofilm than the O3 clone lacking the mutation. However, at 24 hr, O2 forms more biofilm than O3, which seems to lose biofilm associated growth (F (1, 12) = 15.148, P = 0.0021).However, the fitness effect of the *rcsC* allele was measured in the PA14 genetic background and the mutation did not significantly affect fitness on its own (data not shown).

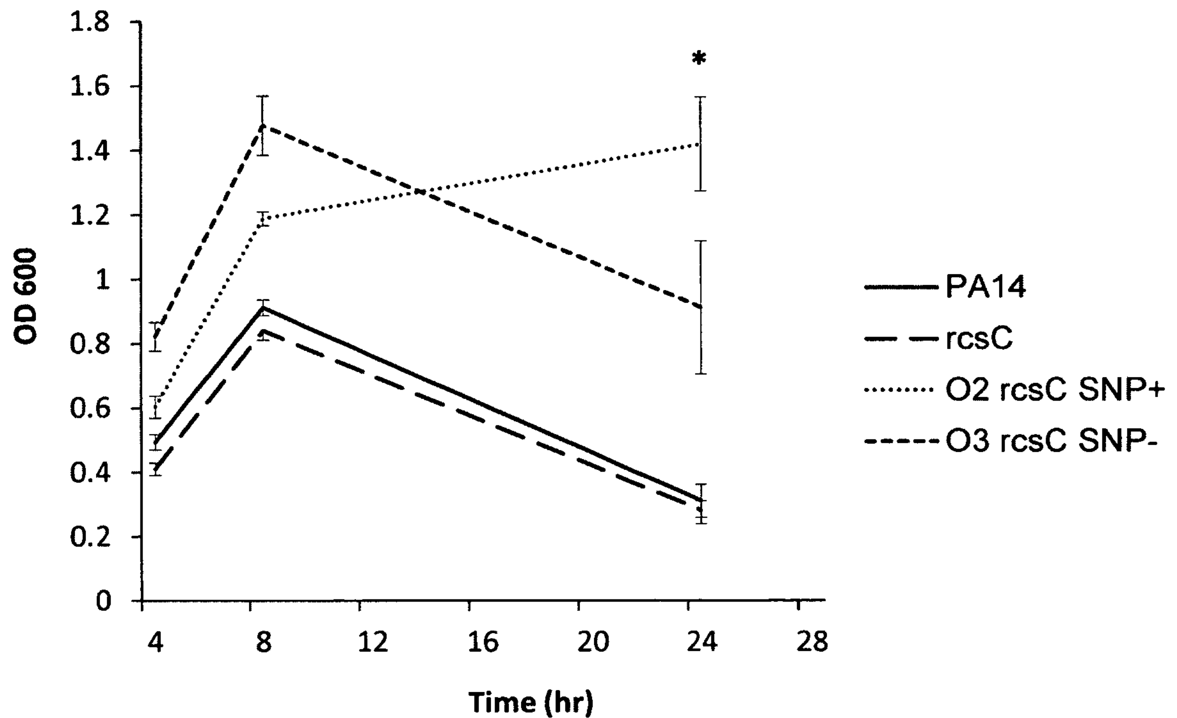

Figure 14 Biofilm formation measured by crystal violet assay over 24 hr for ancestor (black), *rcsC* SNP mutant (red) and 260 generation isolates O2 (green) and O3 (blue). Error bars represent confidence intervals, n=8. Asterisks indicate a significant difference, $P<0.05$.

CHAPTER IV

DISCUSSION

PA14 Biofilm-Adapted Populations Are Complex Communities

Isolates of biofilm-adapted *P. aeruginosa* from the CF lung and *in vitro* studies display broad genotypic and phenotypic diversity (Sauer et al., 2002; Kirisits et al., 2005; Lee et al., 2005; Smith et al., 2006). Hypermutability seems to be an important phenotype for *P. aeruginosa* in biofilms since a loss-of-function *mutS* allele commonly arises in many CF clones and was found in the B1 biofilm adapted community of this study. Although there is a mutation in *mutS* within the B1 population, no mutations were detected in the *lasR*, *mucA*, or *mex* genes, other frequently mutated loci in clinical isolates (Smith et al., 2006; Ciof et al., 2010). This could be a consequence of adapting to *in vitro* conditions instead of the CF lung, where PA14 adapts to a more complex environment with other microbes, antibiotics and the host immune system. However, the mutations acquired by the B1 population clones produced a range of colony morphologies and phenotypic diversity.

Identifying which gene(s) determine colony morphology seems to be complex since O2 shares more mutations with P3 than P2 does. Although the

ppk allele subtly changed morphology, it did not account for any of the morphologies observed in the 100-generation community, suggesting that another allele or combination of alleles define colony types. Having the *mutS* and *recG* alleles may define the P morphology since these are the only mutations exclusively shared by the two P clones. Even though O2 also had these mutations, it also had eighteen mutations independent of P2 and P3 that could result in the O morphology.

It was unexpected that we would find such genetic diversity within similar colony types after sampling three replicates of the five morphotypes. Although the complete genetic profiles of every morphotype were not reconstructed, we found that isolates within morphotypes are not necessarily identical. Though the O, H and WTL replicates seem to be genetically identical, genetic differenceswere found within the M and P morphotypes. Surprisingly, M2 lacks all of the four mutations that fix by generation 260, suggesting that there is at least one other mutation (associated with the M colony type) in this background not identified by our methods. An additional mutation in *groES* that arose at low frequency in early-evolved isolates and fixed by generation 260 was detected during the metagenomic analysis but its presence was not verified by Sanger sequencing probably because this allele was at 3% frequency. GroES is a co-chaperonin that associates with another chaperonin, GroEL, and this complex helps fold and assemble proteins (Sipos et al., 1991). However, mutations in chaperone genes from CF infections are not common in the literature.

Despite being apparently isogenic at the three loci I screened, fitness differences between the O1 and O2 clones suggested the presence of one or more mutations. Complete genome sequences of three representative clones showed that there is significant genetic diversity within a morphotype (Table 7), though many of these mutations may be neutral or slightly maladaptive. Based on sequencing data (Flynn, unpublished), the B1 and B2 populations become hypermutators between 100 and 260 generations whereas the B3 community become a hypermutator between 260 and 540 generations. One of the B3 clones was isolated at 260 generations and sequenced and revealed only four mutations, one of which is a synonymous SNP. This suggests that a relatively low number of mutations may be sufficient to enhance biofilm formation and dispersal and that many of the mutations in the B1 100 generation isolates may not be beneficial.

The fitness effects of some of the confirmed mutations were measured by competing them against the ancestral genotype. When the *ppk* mutation was introduced into the ancestral clone it conferred a significant positive fitness benefit. Furthermore, having a functional ancestral *ppk* allele in our system was important as the transposon knockout of this gene displayed significant negative fitness. However, this SNP did not occur alone in any of the evolved isolates and was always linked to the *mutS* allele and in some cases to the *recG* SNP. P3 had a higher fitness than the constructed *ppk* SNP mutant suggesting that at least one of the other mutations identified in P3 results in a fitness advantage. Comparing growth curves (data not shown) of the *ppk* SNP, *ppk* transposon, P2

and P3 isolates to that of ancestor suggested that P3 is capable of faster growth and higher yield while the transposon mutant grows more slowly (area under the curve data not shown). This further suggests that another mutation in P3 besides the *ppk* and *mutS* provides this advantage. Interestingly, the planktonic head-to-head competition between ancestor and the *ppk* constructed strain showed that the mutant had a significant fitness advantage; a result not predicted from individual growth curves. This could be a result of growing in 96-well plates instead of tubes or that there is a competitive interaction between wildtype and the PAT443A strain.

Mechanisms Not Accounting For Fitness Differences

Once positive fitness effects associated with mutations were identified, we wanted to explain why these alleles were adaptive. PolyP can chelate potentially toxic levels of iron as well as other divalent cations. Iron in the CF lung has been estimated to be over 10 µM (Reid et al., 2009) whereas the medium used for the evolution contains 25 µM. Concentrations of 10-100 µM are considered iron replete conditions since requirements for many bacteria are approximately 1 µM (Vasil and Ochsner, 1999). The data, however, did not support the hypothesis that the *ppk* mutation was adaptive by providing a means, increased polyP, of iron chelation (Figure 5). If iron was being sequestered, the difference in fitness between P2 and P3 should increase as iron concentration increases. However, this was not observed.

Another potential mechanism we tested was that polyP increased resistance to oxidative stress. Bacteria are often exposed to reactive oxygen species such as hydrogen peroxide during aerobic respiration (Hassett et al., 1999) and polyP is known to control the RpoS stress response at the transcription level in *E. coli*. Furthermore, several studies have suggested that sessile bacteria are in a stationary phase state and that RpoS is important for biofilm formation (Adams and McLean, 1999; Xu et al., 2001; Schembri et al., 2003). Many of the 100 generation clones were more resistant to hydrogen peroxide than the wildtype regardless of having the *ppk* SNP, which did not support my original hypothesis that increased polyP upregulated *rpoS* in PA14. To confirm this conclusion, *rpoS* expression could be measured in the constructed *ppk* SNP mutant. Also, competing a Δ*rpoS* PA14 strain against ancestor in our bead model could also help show whether the sigma factor is integral for biofilm formation or fitness in our biofilm model. Interestingly, a clone with the *ppk* mutation showed a higher tolerance to heat shock than the ancestor and a clone lacking the SNP. Of the major alternative sigma factors in *P. aeruginosa*, RpoS and RpoH can confer resistance to heat shock (Suh et al., 1999; Potvin et al., 2008). Polyphosphate could regulate *rpoH* instead of *rpoS*, however, it would be unclear how this would be beneficial in our biofilm model since cultures are not exposed to heat stresses.

Alternative Mechanisms of Fitness Advantages of Evolved Mutations

Although the hypotheses regarding the mechanism of the *ppk* mutation were not supported, several alternative explanations may be explored. With ancestral levels of polyP, the *ppk* SNP still produced a large fitness advantage. The mutation could affect the stability of global transcripts as Ppk in *E. coli* comprises part of the RNA degradosome (Blum et al, 1997). However, polyP levels in P3 were elevated and this isolate had a higher fitness than the *ppk* construct. Kuroda et al. (1999) found that during amino acid starvation, excessive levels of polyP can activate the Lon protease in *E. coli* resulting in the catabolism of free ribosomes. *P. aeruginosa* may experience an amino acid depleted environment in our bead model as the medium used during the evolution was supplemented with only one amino acid, arginine. In addition, there are chemical and metabolite gradients across biofilms due to the thick matrix of exopolysaccharides (Stewart, 2003). Cells deeper within the biofilm could be nutrient-deprived and have adapted to this nutrient deficiency.

The crystal structure of Ppk1 in *E. coli* has been characterized by Zhu et al. (2005). The protein forms a dimer with each monomer, which consists of four domains. In the C1 domain, His435 is a residue thought to be autophosphorylated as the first step in polyP synthesis. The *ppk* SNP results in a T443A change 39 amino acids upstream of the corresponding His in *P. aeruginosa*. Though the mutation does not occur directly at a phosphorylation site, the threonine to alanine change could affect the conformational shape (Figure 2), indirectly influencing the active site. The T443 is a residue shared not

only among the *Pseudomonas* genus but among many other bacteria. A change in such a conserved amino acid could explain such a large fitness effect and the increase of polyP. The mutation could also affect the binding property between Ppk and the RNA degradasome in PA14. However, the interaction between this protein and the degradasome has not yet been explored in *Pseudomonas*. In terms of identifying the mechanistic reason why this mutation is beneficial, comparing RNA or protein profiles of the ancestor, constructed mutants and evolved isolates may support the putative role that Ppk and polyP regulate transcript and protein degradation in PA14.

A Possible Role for Epistasis Among Evolved Mutations

Although the *rcsC* mutation alone did not show a fitness effect, it could be conferring a fitness advantage when another allele is present. Since there are at least four mutations that fix from 100 to 260 generations and the *rcsC* allele rises to high frequency from 260 to 540 generations, its benefits through epistasis should be considered. The fitness effects observed in the 100 and 260 generation isolates are only partly attributed to the *ppk* mutation and could also be a result of this allele in association with others. Therefore, constructing PA14 strains with individual and combinations of the putative adaptive alleles will show if fitness is determined by additive effects of these mutations or perhaps by antagonistic/synergistic epistasis.

The seemingly neutral effect of the *rcsC* mutation may also be a reflection of the fitness assays, which do not completely mimic the evolution environment.

To succeed during the evolution, isolates had to attach to a bead surface, survive within the biofilm, disperse and attach to a new bead within 48 hours. The competition assay used to measure fitness only requires that the bacteria attach and survive for 24 hours. Attachment is only part of the selective pressure as dispersal is another important component that needs to be considered as a proxy for fitness. Pairwise competitions are also not an accurate reflection of the bead model. During the evolution, individual strains are not only encountering the ancestor but are also competing for space and resources against a community of genetically diverse clones that may play specific roles within the biofilm.

Future Considerations

An interesting observation was that an increase in polyP was only seen in evolved clones with the *ppk* SNP and not in the strain with the mutation in the ancestral background. Also, there was no correlation between polyP and fitness. This suggests that the polymer is not important, the SNP requires another mutation or differences in polyP among the ancestor, transposon mutant and the *ppk* SNP were not detected by our methods. To increase total polyP, we are currently cloning the wildtype and evolved alleles into a multicopy plasmid with an inducible promoter, which may provide enough polyP to distinguish any differences between the two proteins' processivity. In addition, since PA14 has multiple copies of *ppk* genes, we want to overexpress the exopolyphosphatase, the enzyme that degrades polyP, in the same inducible vector to determine if having polyP is important.

Assuming that the *mutS* and *recG* neutral or maladaptive, the *argA* mutation may be beneficial since the *ppk* SNP does not account for all the fitness of the P3 clone. Arginine metabolism seems to be an important factor to *P. aeruginosa* biofilm formation (Müsken et al., 2010; Schobert & Jahn, 2010). Due to the production of EPS, PA14 is thought to be in an anaerobic or microaerophilic environment. Because the bacteria may not be aerobically respiring, they have to find an alternative route to generate ATP. During anaerobic growth, *P. aeruginosa* can use arginine as a source of ATP through the arginine deiminase (ADI) pathway (Wauven et al., 1984). Increases in transcription and protein levels associated with this pathway have been found in CF hypermutator isolates (Hoboth et al., 2009) but mutations in ADI genes were not identified in our PA14 evolution. ArgA, however, is the first enzyme in the biosynthesis of arginine (Lu et al., 2004) and was one of the genes targeted during the B1 evolution. We do not know why the nature of this mutation would be beneficial. The two nucleotide deletion is located at the C-terminal end of the protein not near any known active sites.

The PA14 B1 evolved population shares some traits with CF adapted isolates. Both show extensive morphological diversity and a hypermutable phenotype. The *in vitro* evolved community also contains mutations in genes that are not found in clinical strains but could be important for the general fitness of *P. aeruginosa*. For example, the *ppk* gene seems to be integral for PA14 survival in our bead model and the fitness of a true knockout strain could potentially be

tested *in vitro*. Since this gene is found only in prokaryotes, it could be a potential target for antimicrobial therapeutics. Additional considerations for understanding *P. aeruginosa* adaptation to a sessile lifestyle could include metagenomic and fitness analysis of the B2 and B3 populations and their clones. Comparing the genetics of these communities would reveal genes or pathways commonly targeted for biofilm adaptation, including those that may interface with the metabolism of polyphosphate.

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