Sociality in bacteria: Signaling, private, and public goods in *Pseudomonas aeruginosa* and *Escherichia coli*

Rebecca Lynn Scholz

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Reading Committee:

E. Peter Greenberg, Chair

Caroline Harwood

Matthew Parsek

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ABSTRACT

Sociality in bacteria: Signaling, private, and public goods in *Pseudomonas aeruginosa* and *Escherichia coli*

Rebecca Lynn Scholz

Chair of the Supervisory Committee:
Professor E. Peter Greenberg
Department of Microbiology

Bacteria are social organisms, and they are known to engage in cell-cell signaling, resource sharing, and construction of multicellular structures. These behaviors are important from both ecological and medical perspectives. In this thesis, we study *Pseudomonas aeruginosa* and *Escherichia coli*, which both demonstrate social behaviors. *P. aeruginosa* is well-known for its ability to engage in quorum sensing (QS), a form of cell-cell signaling that allows cells to regulate gene expression based on local cell density. We study a common regulatory component of QS, a positive autoregulatory loop, and work to understand its role in QS activation in populations of *P. aeruginosa*. We find that positive autoregulation in *P. aeruginosa* QS is not necessary, but does tightly synchronize QS gene expression across a population. This organism is a devastating pathogen for immunocompromised individuals, and QS regulates numerous virulence factors. Improving our understanding of QS regulation will benefit both medicine and sociomicrobiology studies. In *E. coli*, we study whether the siderophore enterochelin is shared within well-mixed populations of cells. Sharing of secreted products is an oft-studied form of bacterial cooperation, and studies of this behavior have revealed many mechanisms that bacteria use to stabilize cooperation. We find that enterochelin-producing cells have a growth advantage over non-producing cells when producing cells are scarce. This

supports the conclusion that enterochelin is partially privatized in *E.coli*, and may reveal how enterochelin production has been maintained through evolutionary time. Characterizing secreted products and analyzing the degree of privatization is relevant to evolutionary ecology, and characterizing *E. coli* iron acquisition is valuable both for treating pathogenic *E. coli* infections and for understanding the role that *E. coli* plays in the gut commensal community. Finally, we also construct a synthetic QS strain in *E. coli* using QS regulatory genes from *P. aeruginosa*, and we hope to use this strain to study the costs and benefits of using QS to control a single secreted product.

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DEDICATION

This thesis is dedicated to my parents, Ken and Wendy Scholz. I love you so much.

Thank you for everything, always.

CHAPTER 1: Introduction

Bacteria are social organisms

Humans have long viewed bacteria as solitary organisms, each a microscopic factory collecting nutrients and building molecules independently of the other cells around them. However, research over the last few decades has shown that bacteria display many canonically social characteristics, such as being able to sense and respond to signals from neighboring cells, build multicellular structures, and cooperate in the production of shared resources (1-3). These and other social behaviors are both medically- and environmentally-relevant, and the study of sociomicrobiology has expanded rapidly (4).

One form of bacterial social behavior is cooperation. In sociomicrobiology, cooperation is defined as a behavior which "provides a benefit to another individual (recipient), and which is selected for because of its beneficial effect on the recipient" (5). That single-celled organisms cooperate can sometimes be difficult to reconcile with the idea of "survival of the fittest." If a cell is cooperating, it is providing a benefit to other cells at a detriment (often a metabolic burden) to itself. This should make the cooperating cell less fit. "Cheaters", which do not cooperate but can benefit from others cooperating, forgo the metabolic burden and can instead direct more resources into reproduction. Cheaters may thus outgrow cooperators, leading to a loss of the cooperative behavior. This scenario is known as a tragedy of the commons (6). However, because cooperation is observed in bacteria, it must at times be an evolutionarily-winning strategy, and there are several mechanisms that may explain how cooperation evolved and is maintained (7, 8). We focus on cooperative behaviors within populations of *Pseudomonas aeruginosa* and *Escherichia coli*, specifically signaling and secretion of costly products. We will also briefly discuss some mechanisms that may stabilize bacterial cooperation, and we will

touch on the intersection of signaling and secretion by building a synthetic signaling system that controls a single secreted product.

P. aeruginosa and *E. coli*

P. aeruginosa and *E. coli* are gram-negative, rod-shaped bacteria with both pathogenic and non-pathogenic lifestyles. *P. aeruginosa* is a versatile species that lives in soil and water and is also frequently the cause of life-threatening infections in immunocompromised humans (9, 10). These infections can be acute, as in patients with burn wounds, or chronic, as in patients with cystic fibrosis (CF), and while many of these infections are nosocomial, they may also be caused by environmental isolates (11-13). Patients chronically infected with *P. aeruginosa* can carry these infections for decades, and bacterial isolates from these patients often accumulate extensive genetic mutations, highlighting the remarkable adaptability of this bacterium (12, 14-16).

P. aeruginosa is a particularly threatening pathogen because of this genetic versatility (17). Isolates are often resistant to many known antibiotics, may encode numerous virulence factors, and can rapidly form robust biofilms that can increase antibiotic tolerance and prevent eradication (13, 18-20). Relevant to our study, *P. aeruginosa* has several intercellular signaling systems which allow the bacteria to monitor the social and physical characteristics of their environment (21, 22). We will elaborate on this signaling in later sections.

E. coli is predominantly host-associated, although it can survive for a period in the environment, and is typically isolated from the mammalian gut commensal community (23, 24). Some strains are pathogenic and cause urinary tract infections as well as food borne illnesses and other diarrheal conditions (25). We are particularly interested in the iron acquisition systems of this organism. As free iron is usually limiting in host environments, *E. coli* needs high affinity iron acquisition systems to survive in the host, and pathogenic strains of *E. coli* often acquire

additional iron uptake systems (26). Many of these systems depend on siderophores, which are high-affinity ferric iron-binding molecules that many microbes produce to acquire iron (27). We study cooperation with respect to one of these siderophores, and we also use *E. coli* as a chassis to study a simple synthetic signaling system.

Although we will be studying these bacteria in single-species batch cultures, some of the mechanisms that we characterize here may be applicable to the interactions of these bacteria in other environments. Both *E. coli* and *P. aeruginosa* are often isolated from multi-species communities. Sequencing studies of mammalian gut communities, of which *E. coli* is a small percentage, have revealed incredible diversity, and we are only just beginning to understand the basic interactions occurring in this habitat (28). *P. aeruginosa* undoubtedly interacts with different microbes in the environment, and CF patients are often colonized with multiple bacterial species in addition to *P. aeruginosa* (29). The sputum from CF patients can support bacterial cell densities up to approximately 10^{12} colony forming units (CFUs) per mL of sputum, again demonstrating that *P. aeruginosa* cells live in dense communities and engage in inter- and intra-species interactions (30). Signaling and iron acquisition must play a role in these large communities.

Quorum Sensing

Quorum sensing (QS) is a signaling system in bacteria that allows cells to monitor population density, cooperativity, and/or the diffusion characteristics of their environments using small signaling molecules (1, 31-35). There are several types of characterized QS systems. The best-studied of these, and the system that we focus on in this work, have acyl-homoserine lactone (AHL) signals. Other QS signals include small peptides, quinolones, and autoinducer molecules AI-2 and AI-3. AHL QS was first discovered and characterized in *Vibrio fischeri*, where the *lux* QS system controls production of bioluminescence and may have evolved as a mechanism to differentiate between host-associated and free-living bacterial lifestyles (36-38).

Since the discovery of this system in *V. fischeri*, numerous other AHL systems have been characterized in other organisms. The basic regulatory framework remains the same: a LuxI homolog (named for the *lux* system in *V. fischeri*) synthesizes the AHL signal, and when this signal reaches a threshold concentration, the LuxR homolog binds this signal and transcriptionally regulates downstream targets (1). There is also a positive autoregulatory loop in every characterized AHL system that uses a LuxR homolog transcriptional activator; transcription of the *luxI* homolog is activated by signal-bound receptor (39-41). Once the threshold signal concentration is reached, this positive autoregulatory loop leads to an increase in signal production.

We specifically focus on the LasI/LasR QS system in *P. aeruginosa*. Like the *lux* system, the *las* system includes a signal synthase, LasI, and a transcriptional activator, LasR (42, 43). The AHL signal for this system is 3-oxo-C12-homoserine lactone (3OC12-HSL) (44). In the lab strain of *P. aeruginosa* PAO1, the *las* system is at the top of an AHL QS hierarchy. The *las* system regulates activity of the *rhl* system (which includes the RhlI signal synthase, the RhlR transcriptional activator, and C4-HSL signal) and *qscR* (an orphan AHL regulator that also binds 3OC12-HSL) (21). The *las* system, like the *lux* system, also includes a positive autoregulatory loop, in which QS activation increases production of QS signal (45). In Chapter 2 we study the positive autoregulatory loop in the *las* system of *P. aeruginosa* to understand its role in QS regulation, and in Chapter 3 we construct a synthetic *las* QS system in *E. coli*.

Cooperative secretion in *Pseudomonas aeruginosa*

Transcriptomic analyses indicate that many secreted products are controlled by QS in *P. aeruginosa* (46, 47). Secreted products are often thought to be public goods, as they are metabolically costly and available to all members of the population (48), and some have been shown to be “cheatable” (3). For example, mutants that have stopped production of the protease elastase regularly arise and increase to significant frequencies in cultures of *P. aeruginosa* that

are grown in a medium in which elastase is required for growth (49, 50). Additionally, mutants in production of pyoverdine, an iron-scavenging molecule or siderophore, can outgrow pyoverdine producers in environments where iron is limiting (51). Similar mutations have been identified in *P. aeruginosa* strains isolated from the chronically infected lungs of CF patients (15, 52). The advantage of cheaters over cooperators is dependent on many factors, including the degree of cooperation (magnitude of public goods production), the structure (or lack thereof) of the environment, the availability of other essential nutrients, and the timing of cooperation (53-58).

Density-dependent regulation and cheating

Cheating may be controlled in populations of *P. aeruginosa* by limiting expression of secreted products to situations of high cell density (58, 59). At high densities, the population-level production of a public good would be sufficient to produce a measurable benefit. At low cell densities, public good production would be less beneficial to cooperators, as public goods would rapidly diffuse away from cells, and with few cells cooperating, the overall quantity of public good in culture would be low. Controlling the timing of public goods expression allows populations to regulate the cost/benefit ratio of public good production and limit the opportunity for cheaters to cheat. This is, presumably, why many secreted products are under control of a cell density-dependent regulatory system such as QS.

Although density-dependent regulation improves the efficiency of public goods production, it does not completely remove the opportunity for cheating (60). QS mutants have been shown to arise in populations of *P. aeruginosa* growing in casein medium where the QS-controlled product elastase is required for growth (49, 50). In addition to stopping elastase production, these QS mutants also forgo production of dozens of other products under QS control. We are still studying how QS regulation is maintained in the face of this cheating, but at least two mechanisms within *P. aeruginosa* QS regulation appear to prevent complete take-over by QS mutants in a cooperative population. In one example, it was shown that co-regulation of an

intracellular enzyme (Nuh) with the public good elastase could constrain cheaters under conditions where Nuh provides some benefit (50). Additionally, it has been shown that a *P. aeruginosa* QS mutant is more susceptible to hydrogen cyanide intoxication than QS cooperators (61). Since QS cooperators also produce hydrogen cyanide, this mechanism ensures that strains that have mutated QS will be less fit than cooperators when grown together. These mechanisms have been termed “metabolic incentives to cooperate” and “policing” mechanisms. Interestingly, QS mutants are also regularly isolated from patients with CF who are chronically colonized with *P. aeruginosa* (15). However, the social environment, genetic regulatory networks, and consequences of QS mutations have yet to be fully characterized in the CF lung, so it is not clear if these clinical mutants are cheaters or simply well-adapted to their environment (62). In Chapter 3, we build a simple, synthetic QS system in an attempt to study how QS affects the costs and benefits of public goods production and to characterize policing mechanisms. This study is incomplete, but may be pursued in the future.

Cheating can be constrained by privatization

Other organisms may use different strategies to prevent cheating. One way in which cooperators may limit cheating is by privatizing goods, or retaining at least partial possession of a secreted product and thereby limiting the opportunity for non-producers to cheat. There are some examples of this strategy in microbial communities. For example, some marine bacteria produce amphiphilic siderophores, which remain embedded in the outer membrane of cells even after secretion (63, 64). This gives producers a biased opportunity to re-acquire their own siderophores. We explore privatization of the siderophore enterochelin in Chapter 4.

Microbial structures too allow for privatization of secreted products, as they can limit diffusion and preferentially direct secreted products to genetic relatives (65-68). Bacteria often grow in biofilms, both in the laboratory and in clinical and environmental settings. Although our experiments are in well-mixed cultures, it is useful to mention these structures to put our

experiments into context. Biofilms are communities of bacteria living within a self-produced matrix (18, 69). These structures have been shown to limit diffusion, and cells in biofilms likely experience varying degrees of nutrient availability (70, 71). Nutrient limitation can induce dormancy in cells and lead to increased antibiotic resistance (71-73). As noted above, a decrease in diffusion within a biofilm may allow cells to retain secreted products (privatization), and only cooperate with close genetic relatives, thereby limiting cheating.

Additional factors that influence bacterial cooperation

There are additional explanations for how cooperation is stabilized in bacteria. We will not address these in our experiments, but it is useful to remember that they may play a role in clinical or environmental conditions, and thus they provide evolutionary context to our observations.

An important assumption that underlies the definition of cooperation is that a population of strict cooperators will be more fit than a population of non-cooperators. For example, when secreted proteases are required for growth (such as when cells are required to grow on large peptides like albumin), populations that cooperatively secrete elastase will have a higher growth yield than those that do not (60). Cheaters may arise within population of cooperators, but if the environmental conditions lead to dispersion, the populations that were initiated with cooperators will be more successful than those initiated with cheaters. Put another way, although cheaters might outgrow cooperators when in close proximity, dispersion separates the competing strains and allows the cooperating populations to flourish without direct competition from cheaters (74). The described phenomenon, where a strain may decrease in frequency when in direct competition with a cheater but increase in frequency overall, is an example of Simpson's paradox.

Cooperation in complex microbial systems

The study of cooperation in microbes is expanding and will contribute to our understanding of complex bacterial communities. For example, recent work has identified a cooperative interaction in the mouse gut. The gut symbiont *Bacteroides ovatus*, which is capable of intracellular metabolism of the polysaccharide inulin, feeds neighboring cells by extracellular break-down of inulin at a metabolic cost to itself. This feeding induces a reciprocal benefit to *B. ovatus* when it is grown with *B. vulgatus* in the mouse gut (75). It is not yet clear what this reciprocal benefit is or how this benefit is directed to cooperating *B. ovatus*. This study, however, is a rare example of strict cross-species cooperation. In this example, the cooperative activity is costly to the cooperator in isolation, which indicates that the activity was selected for because of its effects on the recipient. This is a characteristic of strict cooperation (5). Broadening the definition of cooperation to include scenarios where the presence of one strain results in increased productivity of the community, regardless of the cost of the cooperative activity, would allow us to include many other examples of mixed species cooperation (76, 77). It is still unclear whether bacterial communities have evolved strict cooperation or if individuals are simply maximizing their own fitness by becoming metabolic specialists or filling an empty niche (78, 79). This will be an important area of further exploration and will be informed by studies performed on single-species or small mixed-species communities.

Cooperation in synthetic biology

Synthetic biology will both contribute to and benefit from the study of cooperation and QS (80-82). Cooperative synthetic communities have been developed that can cross-feed amino acids and other resources (83-86), and numerous synthetic signaling circuits have been designed and implemented that make use of QS components including those for LuxI/LuxR signaling (87-91). These systems provide simple frameworks to test theories in cooperation and signaling, and they have great potential to produce stable microbial communities and single-species sensors for medical treatments, environmental monitoring, and resource production (80, 91-94). We built

a synthetic QS system in *E. coli* to study how QS affects cooperation for a single secreted product (Chapter 3). Although the study is incomplete due to interesting characteristics of the secreted product (Chapter 4), we show that we can use a synthetic QS system to control production of the siderophore enterochelin in *E. coli* (Chapter 3).

Controversy in cooperation

While the literature on microbial cooperation indicates that cooperation may be widespread, we must be cautious in labeling systems as cooperative and mutants as cheaters. We will briefly discuss one example, where the labels of cooperation and cheating may only be applicable under specific conditions.

Pyoverdines are a family of structurally-related siderophores produced by *Pseudomonas* species (95). For most siderophores, it is generally assumed that the siderophore is secreted from the cell, binds iron in the extracellular mileau, and is then re-acquired by a cell that has the appropriate uptake machinery. Therefore, siderophores are often considered cheatable public goods, as cells with uptake machinery can use them even if they do not express the appropriate biosynthetic machinery. In laboratory experiments of *P. aeruginosa*, pyoverdine biosynthesis mutants can rapidly overtake pyoverdine producing strains in low-iron medium where siderophores are required for growth (51). This indicates that pyoverdine production is costly and that there may be a benefit to cheating. However, in iron-replete environments where pyoverdine production is not required for growth, pyoverdine mutants also arise, and these cannot be classified as cheaters, as they do not require the presence of producing strains to grow well (96, 97). In this case, the reduction in pyoverdine production may be advantageous for reasons other than simple exploitation. Genetic analyses of bacterial isolates from environmental samples as well as from CF patients have revealed many strains that encode siderophore uptake receptors without the accompanying biosynthetic genes (52, 98). These might initially be classified as cheaters, however the precise social and environmental

conditions from which clinical and natural strains are isolated are hard to quantify, and the pyoverdine studies demonstrate that we cannot assume that any particular siderophore biosynthesis mutant, or any public goods mutant in general, is a cheater. These studies also reveal the importance of choosing relevant laboratory conditions when attempting to characterize a cooperative system. We hope to perform our experiments in more biologically-relevant conditions in the future, as this would be a valuable extension of our work.

Objectives of this thesis

We are interested in studying cooperation in *P. aeruginosa* and *E. coli*. In *P. aeruginosa*, we work to understand how positive autoregulation in the LasI/LasR QS system affects the regulation of downstream targets. QS is known to be involved in *P. aeruginosa* cooperation, and further research into the regulation of this system will improve understanding of QS evolution and may inform continued efforts to treat *P. aeruginosa* infections. In *E. coli*, we study the siderophore enterochelin, and find that enterochelin is not a true public good as we first assumed. Understanding the characteristics of this molecule may also improve our understanding of the environment in which *E. coli* lives and evolves. At the intersection of these two projects is the question of why QS often controls secreted products. We present a third, short project where we design a system to study the costs and benefits of using QS to control a single secreted product. This work would be informative for both the fields of synthetic biology and QS.

CHAPTER 2: Autoregulation of an acyl-homoserine lactone quorum sensing circuit synchronizes the population response

Abstract

Many Proteobacteria utilize acyl-homoserine lactones as quorum sensing signals. At low population densities cells produce a basal level of signal and when sufficient signal has accumulated in the surrounding environment it binds to its receptor and quorum-sensing-dependent genes can be activated. A common characteristic of acyl-homoserine lactone quorum sensing is that one of the activated genes codes for acyl-homoserine lactone synthesis itself. Signal production is positively autoregulated. We have examined the role of the positive signal autoregulation in growing populations of *Pseudomonas aeruginosa*. We compared population responses and individual cell responses in populations of wild-type *P. aeruginosa* to responses in a strain with the signal synthase gene controlled by an arabinose-inducible promoter so that signal was produced at a constant rate per cell regardless of cell population density. At a population level, responses of the wildtype and the engineered strain were indistinguishable, but when we examined the responses of individual cells in a population we found that the wildtype response showed greater synchrony than the response of the engineered strain. Although sufficient signal is required to activate expression of quorum-sensing-regulated genes, it is not sufficient for activation of certain genes, the late genes, and their expression is delayed until other conditions are met. We found that late gene responses were reduced in the engineered strain. We conclude that positive signal autoregulation is not a required element in acyl-homoserine lactone quorum sensing but it functions to enhance synchrony of the responses of individuals in a population. Synchrony might be advantageous in some situations whereas a less coordinated quorum sensing response might allow bet-hedging and be advantageous in other situations.

Introduction

Quorum sensing (QS) allows bacterial cells to monitor population density, relatedness and diffusivity (1, 31-33, 35, 99). QS systems have been shown to control cooperative bacterial behaviors, and virulence of a number of pathogens is attenuated by mutations in QS genes (46, 49, 50, 60, 100) reviewed in (101, 102). We are interested in acyl-homoserine lactone (AHL)-mediated QS. The basic mechanism of AHL QS was first described for the luminescent marine bacterium *Vibrio fischeri*, and was originally termed autoinduction (36). Autoinduction serves to activate the luminescence (*lux*) genes at sufficiently high *V. fischeri* densities. The autoinduction response requires two regulatory genes, *luxI*, which codes for an AHL synthase, and *luxR*, which codes for an AHL-dependent transcriptional activator (103). Each cell in the population constitutively produces basal levels of the AHL signal, which is freely permeable through cell membranes (104, 105). When the AHL reaches a sufficient concentration in the local environment, cells can activate expression of the *lux* genes including *luxI* (37, 39, 40).

We have focused on related QS circuits in the pathogenic species *Pseudomonas aeruginosa*. This bacterium has two LuxI-LuxR-like genetic control circuits, LasI-LasR and RhlI-RhlR. Together these circuits activate hundreds of genes in *P. aeruginosa* (46, 47). Like the *V. fischeri* circuit, the *P. aeruginosa lasI* and *rhlI* genes are positively autoregulated by their cognate AHLs and LuxR homologs (45, 106). Positive autoregulation is a common characteristic of AHL-LuxR-type activator circuits (41). We are interested in exploring the costs and benefits of this QS positive autoregulatory loop.

The original term for *V. fischeri* QS was autoinduction, and the autoinduction of luminescence was described prior to our understanding that *luxI* itself is positively autoregulated (36). Perhaps because of the similarity of the terms autoinduction and autoregulation, it is not uncommon to read that positive autoregulation of autoinducer synthesis is an essential element in QS (60, 107). We sought to use our model *P. aeruginosa* to test the essentiality hypothesis with LasR

and LasI. We demonstrate that populations of *P. aeruginosa* engineered to produce the AHL signal at a steady rate show autoinduction responses similar to populations of cells with the wildtype (WT) positively autoregulated *lasI*. Analysis of individual cells in populations revealed that positive *lasI* autoregulation is more tightly synchronized than the responses of individuals in the population.

Materials & Methods

Bacterial strains, plasmids and culture conditions. The *P. aeruginosa* and *Escherichia coli* strains used are listed in Table 1. Bacteria were grown in Luria-Bertani (LB) broth (10 g tryptone, 5 g yeast extract, 5 g NaCl per liter) with 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS) pH 7.0 (LB-MOPS broth), or on LB-MOPS agar (LB-MOPS broth plus 1.5% agar) and supplemented as noted. Antibiotics were used for plasmid maintenance of selection at the following concentrations as appropriate: for *E. coli*, 100 µg per mL ampicillin (Ap), 10 µg per mL gentamicin (Gm), and 10 µg per mL tetracycline (Tc), and for *P. aeruginosa*, 100 µg per mL Gm, 100 µg per mL Tc, 150 µg per mL carbenicillin (Cb). Where indicated L-arabinose (final concentration, 0.5% unless otherwise indicated), or the QS signal 3OC12-HSL at the indicated concentration were added to the growth medium. The QS signal was dissolved in ethyl acetate, and the solution was dried onto the bottom of the culture vessel prior to addition of cultures. Bacteria were grown in 3-mL volumes in 18 mm tubes, 15-mL volumes in 125 mL baffled flasks, or 75-mL volumes in 500 mL baffled flasks, as noted, and grown at 37°C with shaking (250 rpm). Starter cultures of *P. aeruginosa* were from single colonies obtained by streaking from a frozen stock.

Strain and plasmid construction. We used Qiagen kits for routine DNA purification. QIAprep Spin Miniprep kits were used for plasmid preparation, and QIAquick PCR Purification kits and QIAquick Gel Extraction kits were used for PCR product purification and gel extraction, respectively. A chromosomal deletion of *lasI* (PAO-SC5) was constructed by mating pEXG2-

ΔlasI, which includes DNA homologous to the flanking regions of *lasI*, into PAO1 following standard protocols (108, 109). To construct a strain with a chromosomal copy of an arabinose-inducible *lasI* we first assembled pSW196-RBS-*lasI*. To make this plasmid, a PCR fragment with engineered *Not*I sites flanking bp −20 to +612 relative to the *lasI* translational start was ligated to *Not*I-digested pSW196 (110) following the *araBAD* promoter. This plasmid was built on a mini-CTX2 backbone, which was designed to integrate into the *attB* site of *P. aeruginosa* (110, 111). To construct a *P. aeruginosa* mutant with a single copy of *lasI* controlled by the arabinose promoter, we mated *E. coli* strain S17-1 carrying pSW196-RBS-*lasI* with the *ΔlasI P. aeruginosa* strain PAO-SC5. We used antibiotic selection to obtain an isolate with pSW196-RBS-*lasI* in the *attB* site. We then introduced pFLP2 and used Flp recombination to remove the antibiotic resistance cassette from the integrated plasmid (112). Finally, we used sucrose to counterselect *sacB* and cure the strain of pFLP2 (113).

To construct the pBS-series LasR-inducible promoter reporter plasmids described in Table 1 we cloned PCR-generated DNA fragments with *Sal*I and *Bam*HI overhangs into *Sal*I-*Bam*HI-digested pPROBE-GT (114) by using standard procedures. All plasmid and mutant constructs were confirmed by DNA sequencing.

Growth curves and population fluorescence measurements. Starter cultures for growth curves were grown from a single colony inoculated into 3 mL of LB-MOPS broth. When cultures reached an optical density at 600 nm (OD_{600}) of between 0.05 and 0.2 they were used to start experiments at an initial OD_{600} of 0.001. Optical densities were measured by using a Genesys spectrophotometer with a 1-cm path-length cuvette. Either 3OC12-HSL, L-arabinose or both were included as noted. Relative fluorescence units (RFUs) (485 nm excitation, 535 nm emission) and OD_{600} in 20 μL samples in black-walled transparent-bottom 384-well plates were measured by using a Biotek Synergy H1 microplate reader.

Flow cytometry. We used flow cytometry to measure individual cell responses in populations of *P. aeruginosa.* Cells were grown in 15 mL of LB-MOPS broth. The cultures were sampled at the times indicated, cells were pelleted by centrifugation and washed twice in phosphate-buffered saline, pH 7.4 (PBS), and then suspended in 2.5% paraformaldehyde in PBS and fixed at 37^{o}C with shaking for 10 min to fix. The fixed cells were washed three times in PBS and then stored in PBS at 4^{o}C. Cells were analyzed by using a BD™ LSR II flow cytometer and BD FACSDiva™ Software. A trigger was set based on forward scatter (FSC) and side scatter (SSC), and 20,000 counts were measured for each sample. To measure GFP fluorescence, we used a blue 100 mW laser set to 488 nm for excitation, and emission was detected as light passing a 530/30 bandpass filter. Analysis was performed using FCS Express 4 software.

Autoinducer bioassays. We used a bioassay to measure 3OC12-HSL as described elsewhere (115, 116). Briefly, we extracted the QS signal 3OC12-HSL from culture fluid in ethyl acetate acidified with 0.01% glacial acetic acid, and concentrated the autoinducer 10-fold. We used an *E. coli* bioassay strain containing *lasR* and a LasR-inducible promoter fused to *lacZ* (117, 118) to measure 3OC12-HSL and we prepared a standard curve by using synthetic 3OC12-HSL (RTI International) to calculate signal concentrations in cultures as described elsewhere (116).

Results

***Pseudomonas aeruginosa* PAO-SC6 produces 3OC12-HSL constitutively in LB-MOPS with 0.5% L-arabinose**. The LasI-LasR circuit in *P. aeruginosa* produces and responds to the autoinducer 3-oxo-C12-homoserine lactone (3OC12-HSL). In the WT strain PAO1, *lasI* is positively autoregulated. Strain PAO-SC6 has a deletion of the native *lasI* and an arabinose-inducible *lasI* inserted at the neutral *att* site on the chromosome. We first needed to measure 3OC12-HSL during growth of PAO-SC6 to determine whether it was produced at a constant level per cell. We also needed to determine whether cultures of PAO-SC6 and PAO1 reached a threshold autoinducer concentration at about the same time during growth. Growth of the two

strains with or without L-arabinose was indistinguishable (Fig. 2.1A). Figure 1B shows concentrations of the autoinducer in culture fluid over the growth curve. There was a steep increase in autoinducer concentration in the WT cultures over a period between about 5 and 6 h followed by a plateau in autoinducer concentration as cells entered stationary phase. The WT autoinducer synthesis was not affected by L-arabinose. Strain PAO-SC6 did not make detectable levels of autoinducer in the absence of L-arabinose. In the presence of L-arabinose, the increase in autoinducer concentration paralleled the increase in cell mass, as expected if autoinducer synthesis per cell remained constant throughout growth. To further analyze the data shown in Fig. 2.1B we calculated the rates of autoinducer synthesis over time between time points in the WT and the PAO-SC6 cultures (the difference in autoinducer concentration/the difference in cell density (OD_{600})/the difference in time between two time points) (Fig. 2.1C). In the WT PAO1 there was a sharp increase in the rate of synthesis between 5 and 6 h, and in strain PAO-SC6 the derived rate remained unchanged during logarithmic growth. These experiments confirm the positive autoregulation of signal production in the WT and the constitutive signal production in strain PAO-SC6.

For strain PAO-SC6, there is a small accumulation of autoinducer between 3 and 4 h of growth whereas autoinducer concentrations in WT cultures remain low during this time period. Later in growth, the peak autoinducer concentration for the WT PAO1 is about three times that of PAO-SC6 grown in the presence of L-arabinose (Fig. 2.1B). To probe the importance of these differences in autoinducer levels and to explore the sharpness of the QS threshold, we performed an autoinducer titration experiment. We used a QS-responsive reporter pBS351, which contains a *plasI-gfp* transcriptional fusion, and measured the autoinducer concentration required to saturate the reporter response in a *lasI*-deficient strain (PAO-SC5). We chose the *lasI* promoter because *lasI* is among the earliest QS-dependent genes to respond to 3OC12-HSL (46). The minimum concentration at which we could detect a response was about 10 nM

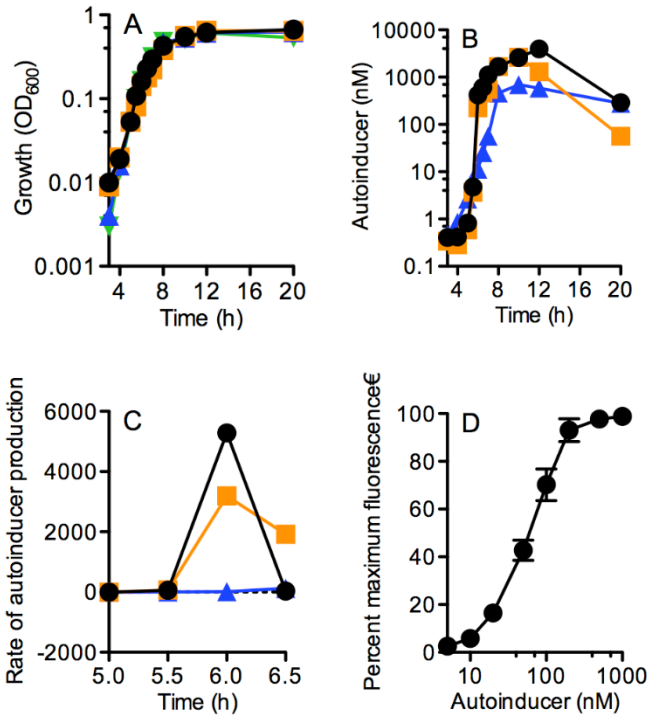

Figure 2.1 Production of 3OC12-HSL in *P. aeruginosa* PAO1 and PAO-SC6, and sensitivity of *P. aeruginosa* to 3OC12-HSL.

A. Growth curves of PAO1 and PAO-SC6 B. 3OC12-HSL levels during growth, and C. calculated rates of 3OC12-HCL production per unit OD600 in PAO1 and PAO-SC6 culture fluid. PAO1 with 0.5% L-arabinose (black circles), PAO1 without L-arabinose (orange squares), PAO-SC6 with 0.5% L-arabinose (blue up-pointing triangles), and PAO-SC6 without L-arabinose (green down-pointing triangles). PAO-SC6 without L-arabinose does not produce detectable levels of 3OC12-HSL and has been omitted from panels B and C. These are representative data for an experiment repeated at least 3 times with similar results. Cultures were grown in 75 mL of LB-MOPS broth with L-arabinose added as indicated. D. Percent maximum fluorescence for the ΔlasI strain PAO-SC5 with pBS351, 3OC12-HSL added at the indicated concentrations. Cells were grown in 160 mL medium without signal. At 3 h, 15 mL volumes were transferred to 125 mL baffled flasks containing 3OC12-HSL to give the desired final concentration of this autoinducer. Relative fluorescence units were measured at 6 h of growth. Error bars show the SEM of three independent experiments.

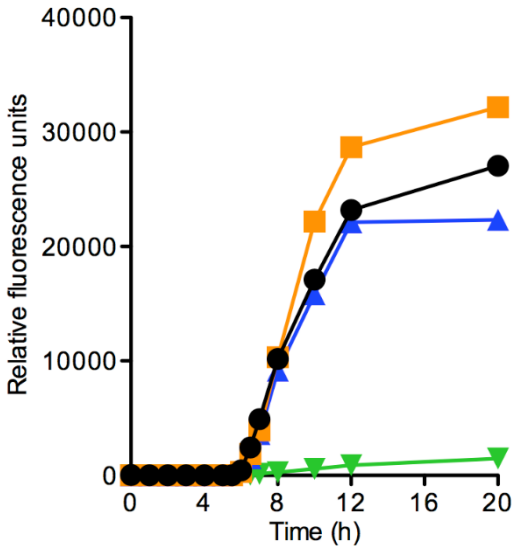

Figure 2.2 Positive 3OC12-HSL autoregulation is not necessary to induce a LasR-controlled promoter.

Relative fluorescence units for PAO1 with 0.5% L-arabinose (black circles), PAO1 without L-arabinose (orange squares), PAO-SC6 with 0.5% L-arabinose (blue up-pointing triangles), and PAO-SC6 without L-arabinose (green down-pointing triangles). Both strains contained the *plasI-gfp* reporter. Cells were grown in 75 mL of LB-MOPS broth as described in the Materials & Methods.

and the response was saturated by about 100-200 nM 3OC12-HSL (Fig. 2.1D). Thus we assume there is some cost associated with positive autoregulation of signal production.

Autoinduction in the absence of positive *lasI* autoregulation. To further study the role of positive autoregulation of QS signal production, we monitored GFP fluorescence along the growth curve of PAO1 and PAO-SC6 populations carrying pBS351 and growing in LB-MOPS broth with or without L-arabinose (Fig. 2.2). There was a sharp increase in GFP fluorescence in the WT with or without L-arabinose and in PAO-SC6 with L-arabinose. The control, PAO-SC6 without L-arabinose showed a very modest increase in fluorescence as population numbers increased over the growth curve. There may have been small difference in the responses of WT and PAO-SC6 in the first several hours of growth that were beyond the limits of our detection. Regardless, it is clear that autoinduction of *plasI-gfp* occurred in strain PAO-SC6 and that the response was quite similar to the response in WT.

We next wanted to test the effects of higher or lower basal rates of autoinducer production on activation of the *lasI* promoter. We did this by varying the concentration of L-arabinose in the growth medium (Fig 2.3A). With 1% L-arabinose autoinduction commenced somewhat earlier than it did with 0.5% L-arabinose, and at 0.2% L-arabinose autoinduction was somewhat delayed. This shows that the basal activity of LasI affects the onset of autoinduction and the signal is the trigger for activation of *lasI*.

Autoinduction of late QS-controlled promoters in the absence of positive *lasI* autoregulation. Sufficient *P. aeruginosa* LasR QS signal is required but not sufficient for activation of many genes in the LasR regulon (46, 119). The so-called late genes show an induction delay in WT as compared to early genes like *lasI,* presumably because their expression depends on additional regulatory inputs. We reasoned that late genes in particular should respond similarly in WT and PAO-SC6. Because of their delay in expression until late vs mid-logarithmic growth phase, sufficient autoinducer should have accumulated in the culture

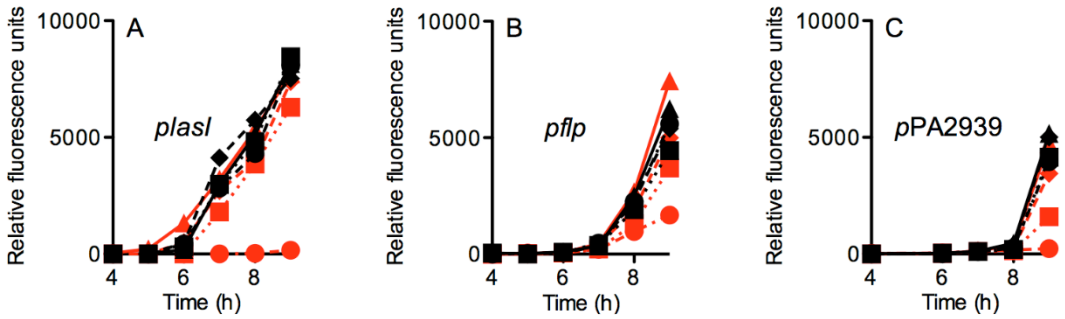

Figure 2.3 Multiple LasR-responsive promoters can be activated in the absence of positive 3OC12-HSL autoregulation.

Activation of the promoters for A. *lasI*, B. *flp*, or C. PA2939 in PAO1 and PAO-SC6. PAO1 (black) and PAO-SC6 (red) were grown with 1.0% L-arabinose (solid lines, triangles), 0.5% L-arabinose (dashed lines, diamonds), 0.2% L-arabinose (dotted lines, squares), or no L-arabinose (dot-dashed lines, circles). Cells were grown in 3 mL volumes as described in the Materials & Methods.

fluid of either bacterial strain to saturate LasR. Results with two late gene promoter-*gfp* fusions, *pflp-gfp* (PA4306) and *p*PA2939-*gfp* are shown in Figs. 2.3B & C. Whereas *plasI-gfp* induction in the WT commences at about 5 to 6 h of growth, *pflp-gfp* induction commences between 6 and 7 h and *p*PA2939-*gfp* induction commences after 7 h. Induction of either *flp* or PA2939 was not advanced by increasing the L-arabinose concentration from 0.5% to 1% and it was not delayed by decreasing the L-arabinose concentration from 0.5% to 0.2%. In agreement with our previous publication (46, 119), autoinducer concentration is not the trigger for activation of these two genes.

Positive autoregulation of signal production tightly synchronizes responses of individual cells in a population. To examine the autoinduction of *plasI-gfp* more closely, we followed the responses of individual cells in populations by using flow cytometry (Fig. 2.4). For the WT PAO1 very few cells showed induced levels of GFP until about 5.5 h in culture when a small fraction of cells were expressing *plasI-gfp*. Thirty min later at 6 h almost all of the cells were expressing *plasI-gfp*; the autoinduction response was tightly synchronized. In contrast, about 20% of PAO-SC6 cells showed partial GFP induction as early as 3.5 h in culture. The peak of induction occurred somewhere between 5.5 and 6 h, and at 6 h cell fluorescence of WT and PAO-SC6 were similar (Fig. 2.4A). As a control, we also measured single-cell GFP induction in populations of the WT and PAO-SC6 to which we added 3OC12-HSL (final concentration 1 μM) at 3.5 h post inoculation (Fig. 2.4B). In this experiment both the WT and PAO-SC6 responses were tightly synchronized. Thus the relaxed synchronization during autoinduction in PAO-SC6 cannot be attributed to factors unrelated to signal accumulation. We conclude that the autoinduction response in PAO-SC6 was less tightly synchronized than the response in the WT.

We note that there may be variations in *lasI* expression from the *paraBAD* promoter in PAO-SC6. However, AHLs can diffuse out of and into cells (104, 120). We do not expect the reduction in synchrony in PAO-SC6 to result from differences in *lasI* expression between cells.

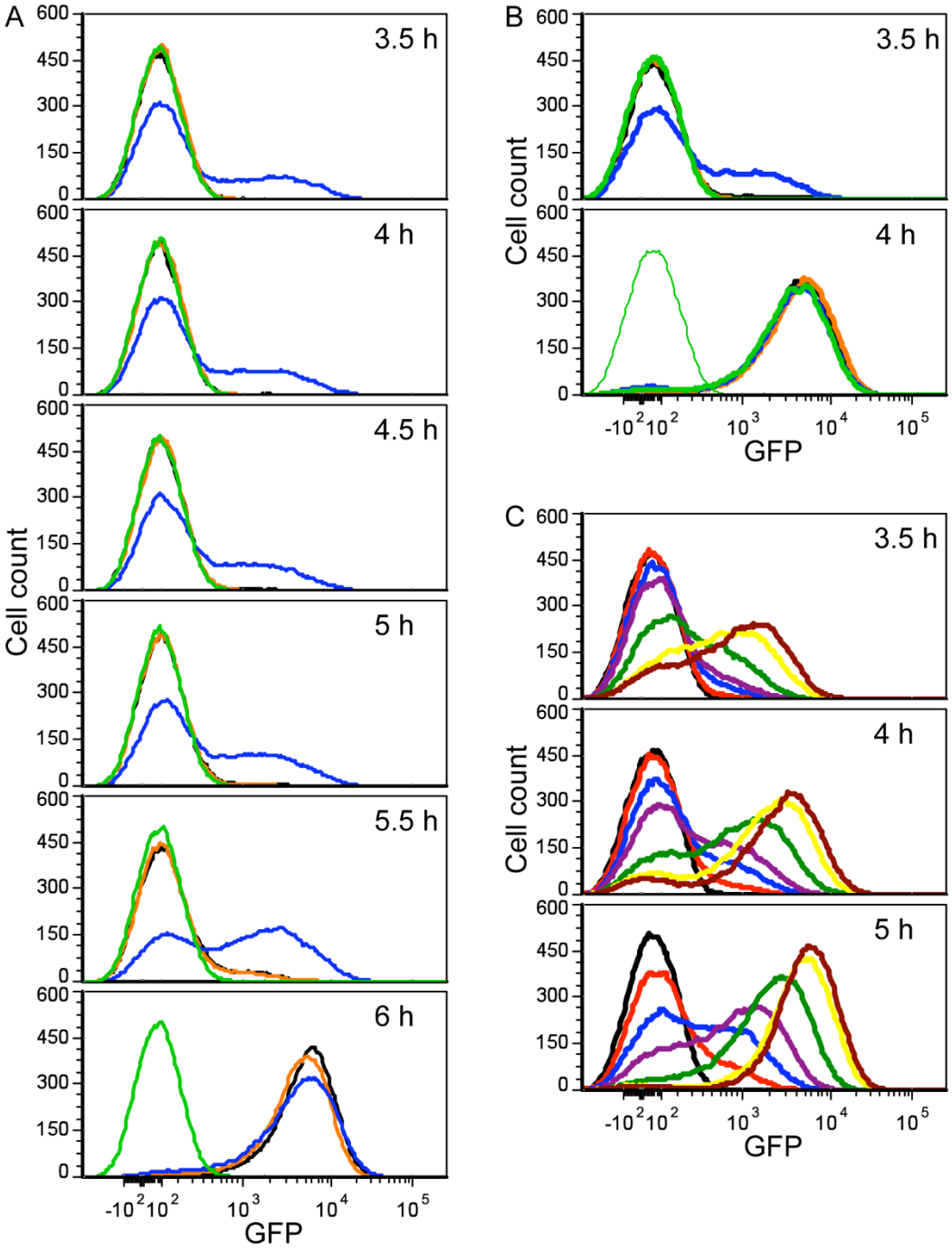

Figure 2.4 Positive 3OC12-HSL autoregulation synchronizes *plasI-gfp* expression.

GFP expression in single cells was measured as described in the Materials & Methods. All panels show cell counts with respect to GFP fluorescence. A. PAO1 and PAO-SC6 with the *plasI-gfp* reporter: PAO1 with 0.5% L-arabinose (black), PAO1 without L-

arabinose (orange), PAO-SC6 with 0.5% L-arabinose (blue), and PAO-SC6 without L-arabinose (green). B. As in A except 1 μM 3OC12-HSL was added at 3.5 h. PAO-SC6 without signal or L-arabinose was included as a negative control (green, thin line). C. The *P. aeruginosa ΔlasI* mutant with the *plasI-gfp* reporter grown with added 3OC12-HSL at 3 h: No 3OC12-HSL added (black), 5 nM (red) 10 nM (blue), 20 nM (purple), 50 nM (green), 100 nM (yellow), and 200 nM (rust) 3OC12-HSL. Cells exposed to sub-saturating levels of 3OC12-HSL show diminished synchrony of the response. Cells were grown in 15 mL of LB-MOPS broth as described in the Materials & Methods.

We also measured the response of the *plasI-gfp* reporter to sub-saturating levels of 3OC12-HSL in the *ΔlasI* mutant PAO-SC5 and found that low levels of 3OC12-HSL early in growth also induce heterogeneous expression of this reporter within a population (Fig. 2.4C). Thus, the slow accumulation of 3OC12-HSL that we observed in PAO-SC6 populations (Fig. 2.1B) should be sufficient to produce the population heterogeneity we observed.

Analysis of a late gene response in WT and PAO-SC6 by flow cytometry. We hypothesized that late gene induction is tightly synchronized in both the WT and PAO-SC6. This hypothesis is based on our findings that activation of late genes is delayed until after autoinducer concentrations in culture fluid have surpassed those required to saturate the *plasI-gfp* response (Figs. 2.1D & 3). To test this idea we examined the responses of *p*PA2939-*gfp* in the WT and in PAO-SC6 by flow cytometry (Fig. 2.5). With WT or PAO-SC6, GFP was uninduced at 6 h, at 6.5 h there was a small but detectable increase in fluorescence, and by 7 h, there was some autoinduction of the reporter in most cells. This comports with our hypothesis that autoinduction

of a late gene will be tightly synchronized regardless of whether *lasI* is positively autoregulated or not, however, the situation is complicated. Although cells show autoinduction, *p*PA2939-*gfp* is not as strongly activated in PAO-SC6 cells as it is in WT cells at 7 and 7.5 h. By 10 h the PAO-SC6 cells are almost as fluorescent as WT (Fig. 2.5A). As a control we added 1 μM 3OC12-HSL to cultures after 3.5 h. This autoinducer concentration is roughly equivalent to that in WT cultures after 6 h of growth and exceeds that in 6-h PAO-SC6 cultures by about 2 logs (Fig. 2.1B). In this control experiment GFP induction in PAO-SC6 cells is equivalent to that in WT (Fig. 2.5B). Thus the increased time for PAO-SC6 cells to fully activate the *p*PA2939-*gfp* reporter can be attributed to the linear kinetics of autoinducer production by cells of this strain.

One simple explanation for the synchronized but slow induction of *p*PA2939-*gfp* in PAO-SC6 is that higher levels of autoinducer are required to achieve maximal induction, however, there are also other explanations. One way that we tested the idea that full activation of the PA2939

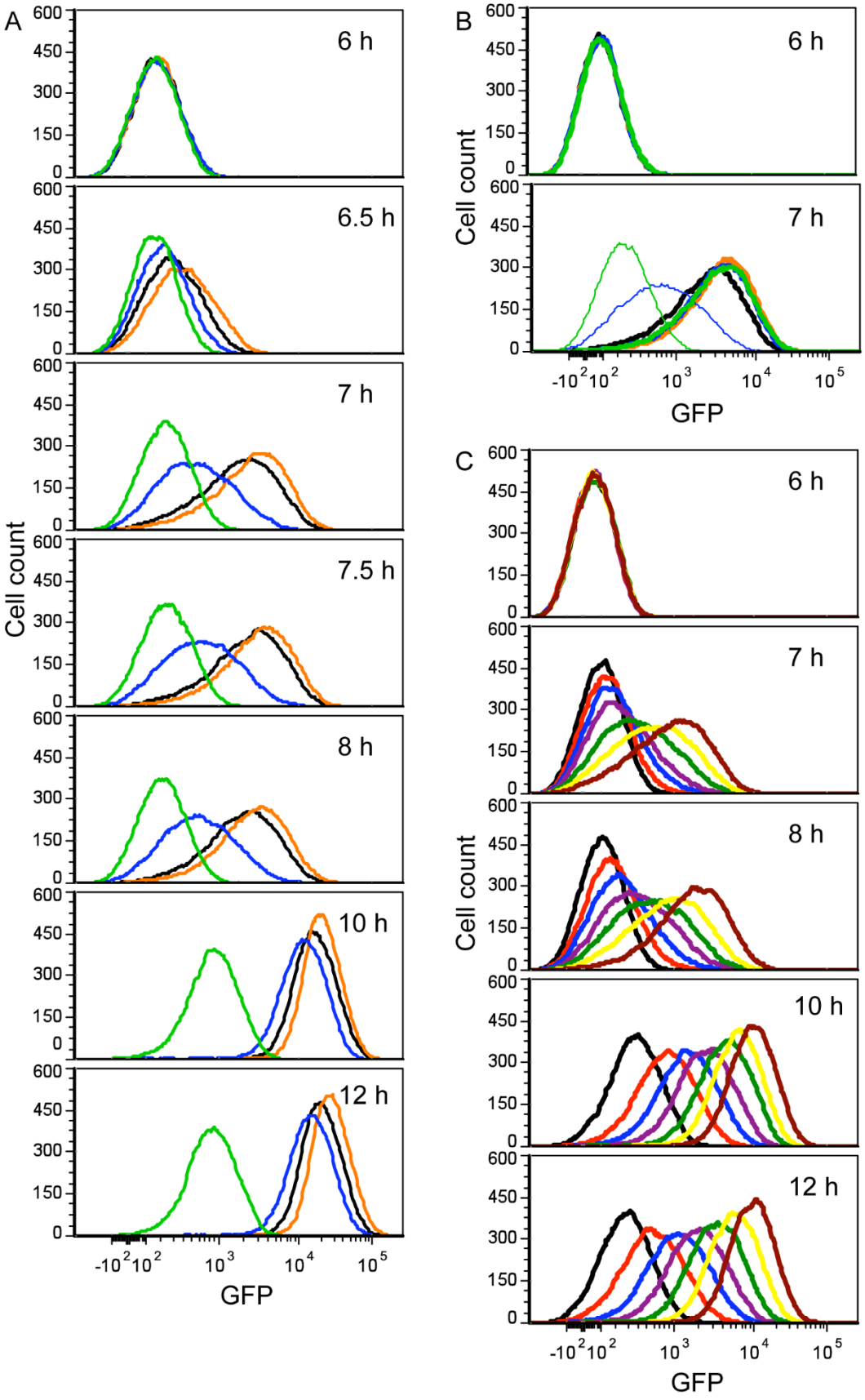
A
B
C
6 h
6.5 h
7 h
7.5 h
8 h
10 h
12 h
Cell count
GFP
600
450
300
150
0
-10^2
10^2
10^3
10^4
10^5

Figure 2.5 Positive *lasI* autoregulation facilitates maximal *p*PA2939-*gfp* expression.

GFP expression in single cells was measured by flow cytometry. A. PAO1 and PAO-SC6 with the *p*PA2939-*gfp* reporter grown with or without 0.5% L-arabinose. PAO1 with 0.5% L-arabinose (black), PAO1 without L-arabinose (orange), PAO-SC6 with 0.5% L-arabinose (blue), and PAO-SC6 without L-arabinose (green). B. When 1 μM 3OC12-HSL was added at 3.5 h (black, orange, blue, and green; thick lines*) gfp* expression was not advanced, but fluorescence of PAO1 and PAO-SC6 at 7 h was similar. PAO-SC6 cultures with 0.5% L-arabinose (blue, thin line) and without signal or L-arabinose (green, thin line) were included as controls. C. 3OC12-HSL was added to cultures of the *ΔlasI* mutant PAO-SC5 with the *p*PA2939-*gfp* reporter at 3.5 h of growth: No 3OC12-HSL added (black), 20 nM (red) 50 nM (blue), 100 nM (purple), 200 nM (green), 500 nM (yellow), and 1 μM (rust) 3OC12-HSL. Cells were grown in 15 mL of LB-MOPS as described in the Materials & Methods.

promoter requires high levels of autoinducer in comparison to activation of *lasI* was to add various amounts of 3OC12-HSL to populations of the PAO1 *lasI* mutant PAO-SC5 containing the *p*PA2939-*gfp* reporter plasmid and then follow GFP fluorescence over time (Fig. 2.5C). In fact maximum fluorescence per cell required high concentrations of autoinducer in the range of 500 to 1000 nM.

Discussion

Positive autoregulation of autoinducer synthesis is a characteristic of AHL QS systems governed by LuxR-family transcriptional activators, and it is common to many peptide signaling autoinduction systems in Gram-positive species (41, 121, 122). Positive signal autoregulation is not, however, a universal feature of all quorum sensing systems. There are a few examples of AHL quorum sensing systems where the LuxR-family, AHL-responsive transcription factor is a repressor rather than an activator, and in these systems the cognate *luxI* homolog is not positively autoregulated (123-126). For example, in *Pantoea stewartii* the LuxR homolg EsaR binds to target DNA in the absence of its cognate AHL and blocks transcription. At sufficient AHL concentrations EsaR is released from the target DNA (124). Because of the commonality of positive autoregulation of signal synthesis by LuxR-type transcriptional activators, and perhaps because the terms autoinduction and autoregulation can be confused, positive autoregulation has been described as an essential part of autoinduction by these transcriptional activators. Our experiments with *P. aeruginosa* demonstrate this is not the case (Figs. 2.1 & 2.2).

If positive autoregulation of AHL signal synthesis is not a required element of the autoinduction response, then why is it a common characteristic of the LuxR activated circuits? We examined the responses of individual *P. aeruginosa* cells in populations undergoing autoinduction of LasR-dependent-promoter-controlled *gfp* reporters and uncovered two consequences of positive signal autoregulation. First, for a so-called early quorum-sensing-responsive gene

(activation of early genes can be advanced by inclusion of autoinducer in the growth medium; the autoinducer is the trigger for gene activation), *lasI*, positive autoregulation enhanced synchronization of the response (Fig. 2.4). Second, so-called late genes, for which autoinducer is required but not sufficient for activation (the autoinducer is not the trigger for activation) are synchronized tightly but cells show a relatively slow response in the absence of positive autoregulation (Figs. 2.3 & 2.5). Improved synchronization seems intuitive: when signal is positively autoregulated, the length of time that the population is exposed to intermediate autoinducer concentrations (above the threshold for activation but below the saturating level) is short. Intrinsic heterogeneity amongst cells would be overridden by the quickly saturating signal concentrations. We did not anticipate the late gene response. The signal levels have saturated the *plasI-gfp* response by the time late-gene expression commences (Fig. 2.4A), which led us to believe that LasR activity is maximized by about 6 h post inoculation. We do not know the mechanistic basis for this slow cellular increase in late gene expression, but there are several possible explanations. For example, it may have to do with LasR binding affinities and competition with early QS-dependent genes for LasR.

We note that positive autoregulatory loops are not unique to QS systems. Experimentation and modeling show that such autoregulatory loops can reduce noise, create a bistable state, and decrease cost (127-131). With AHL responsive LuxR-family transcriptional activators, positive autoregulation of signal production can affect stability in populations. There should be hysteresis with the cell density required to achieve a threshold level of autoinducer being much higher than the density required to deactivate the system. We have not addressed this possibility experimentally.

It is of interest to determine whether responses of quorum-sensing-dependent genes governed by repressors like that of *P. stewartii* for example (124) show tight synchrony in their response. We also note that the autoinducer-2 activation of *Vibrio harveyi* luminescence, which does not

involve a LuxR homolog shows bistability (132). Bistability is thought of as a bet-hedging strategy that can facilitate success in an environment where conditions are variable. A more tightly synchronized response, which is perhaps further committed by positive autoregulation of autoinducer synthesis might be particularly well suited to situations where uninduced individuals are at risk from extrinsic or intrinsic factors, or where cooperation is essential for success.

Table 2.1. Bacterial strains and plasmids

Strain or plasmid	Description or relevant genotype	Source or reference
Strains		
P. aeruginosa PAO1	Wild-type prototroph	(133)
P. aeruginosa PAO-SC5	*lasI* deletion mutant of PAO1	This study
P. aeruginosa PAO-SC6	PAO1 *ΔlasI paraBAD-lasI*	This study
E. coli DH5α	*E. coli* cloning vehicle	Invitrogen
E. coli S17-1	*thi pro hsdR recA* RP4-2 (Tet::Mu) (Km::Tn7)	(134)
Plasmids		
pJN105L	*paraBAD-lasR*; Gm^r	(117)
pSC11	*plasI-lacZ* reporter; Ap^r	(118)
pEXG2-*ΔlasI*	Gene-replacement vector; contains *lasI* flanking regions to delete codons 31–191 of *lasI*; *sacB*; Gm^r	(109)
pSW196	mini-CTX2 with *paraBAD* promoter; Tet^r	(110)
pSW196-RBS-*lasI*	*attB* Integration plasmid with arabinose-inducible *lasI*; Tet^r	This study
pFLP2	Expresses Flp recombinase, *sacB*; Ap^r	(112)
pPROBE-GT	Promoterless *gfp*; Gm^r	(114)
pBS351	pPROBE-GT with *plasI;* Gm, -282 to +223 [a]	(62)
pBS383	pPROBE-GT with *pflp;* Gm, -283 to +100 [a]	This study
pBS347	pPROBE-GT with *p*PA2939*;* Gm^r, -290 to +223 [a]	This study

[a]The numbers indicate the coordinates of the *P. aeruginosa* gene fragments relative to translational start site of the relevant gene.

CHAPTER 3: Construction of a synthetic quorum sensing system to control enterochelin production in *Escherichia coli*

Abstract

Communication and cooperation are widespread in microorganisms. One mechanism that regulates cooperative behavior is quorum sensing (QS), a bacterial communication system that enables density-dependent gene expression. QS commonly controls many secreted products, such as proteases and other virulence factors, and allows bacteria a production delay of these products until cells are at a sufficient density to benefit from exoproducts. Secreted products are often considered public goods, as they are costly to produce and can benefit all members of the population, and public goods production is thought to be a cooperative activity. QS in *Pseudomonas aeruginosa* is a well-studied model, and its primary QS system is LasI/LasR. LasI synthesizes a diffusible signal which LasR senses, and above a threshold signal level, LasR can activate transcription of QS target genes. As in many cooperative systems, some individuals called cheaters will forgo production of public goods while still benefiting from those produced by others. In *P. aeruginosa*, cheaters emerge in populations of QS cooperators, and these cheaters are LasR signal receptor mutants. We assembled a synthetic QS system in *Escherichia coli* with a single secreted product under QS regulation to study how QS affects the costs, benefits, and stability of public goods production. We use this synthetic system because QS in *P. aeruginosa* is very complex. The synthetic QS system uses the LasI/LasR system from *P. aeruginosa* to control production of the siderophore enterochelin. This system shows robust QS-controlled regulation of growth in low-iron medium. We hope to assay the inherent stability of this system under conditions that require cooperation, create strains with metabolic restraints over cheating, and assess the inherent costs of QS.

Introduction

Quorum sensing (QS) is a bacterial signaling system that is thought to control cooperation (60). There are several known classes of quorum sensing (QS) systems in bacteria, and these are grouped based on the signals they use: oligopeptides, acyl-homoserine lactones (AHLs), quinolones, and a furanosyl borate diester (AI-2). We focus on AHL QS. As noted in Chapter 1, one of the best-studied models of AHL QS is *Pseudomonas aeruginosa*, an opportunistic pathogen known for causing serious nosocomial infections, particularly in immunocompromised individuals and cystic fibrosis (CF) patients (9). QS in *P. aeruginosa* controls hundreds of genes, and secreted products are overrepresented in the QS regulon (46, 47). Secreted products are often considered public goods, as they are costly to produce and are available to producing and nonproducing individuals alike (3). In populations of producers, “cheaters” may emerge which stop producing public goods but still use those produced by others. When grown in a medium where QS is necessary for growth, *P. aeruginosa* cheaters have been observed to arise *in vitro*, and these cheaters are almost invariably QS mutants, presumably because mutating QS is an efficient way to stop producing a large number of goods (49, 50). QS mutants also arise in the CF lung and may be social cheaters, although this is still an open question (15, 62). *In vitro*, cheaters have a growth advantage over producers because they do not pay the metabolic cost of public good production. If cheaters reach a great enough frequency in the population, public goods may be depleted such that population growth is no longer supported – a tragedy of the commons (6, 50, 135). We are interested in studying the costs and benefits of QS-controlled public good production. Additionally, we are interested in identifying metabolic restraints on cheaters and in characterizing mutations that disrupt these restraints and allow cheaters overrun a population of cooperators. Unfortunately, QS in *P. aeruginosa* is extremely complex and is deeply embedded in the overall regulatory networks of the organism (21, 46, 55, 118,

119, 136). We assembled a simple, synthetic QS system in *Escherichia coli* to determine how QS affects competition for a single secreted product and to study the stability and cost of QS.

The synthetic QS system uses the LasI/LasR system from *P. aeruginosa*. We chose to use *Escherichia coli* as a chassis because of its ease of manipulation and because it does not naturally make AHLs or respond to those produced by *P. aeruginosa* (137, 138). In LasI/LasR QS, LasI synthesizes a diffusible signal, and at a threshold signal concentration, LasR binds the signal and activates transcription of QS target genes. The signal is 3-oxo-C12-homoserine lactone (3OC12-HSL) (44). The secreted product controlled by the synthetic QS system is enterochelin, which is a siderophore, or iron scavenging molecule, that is normally produced by *E. coli* under iron-limiting conditions (139). Enterochelin is synthesized through non-ribosomal peptide synthesis by the *ent* genes (140, 141). A deletion mutant in the *entF* gene is unable to synthesize enterochelin (142) (Chapter 4). We constructed our synthetic system by introducing an arabinose-inducible LasR and *plasI*-controlled *lasI* and *entF* onto the chromosome of an *entF* mutant. With this arrangement, the signal receptor LasR could be produced constitutively at experimenter-controlled levels, and both *lasI* and *entF* would be transcribed in a QS-dependent manner.

Studies of siderophores and other public goods have already contributed greatly to the field of social evolution and cooperation (49, 51, 53, 60, 98, 143, 144). Additionally, some studies have investigated the value of simple QS systems (145) and density-dependent gene expression (58). Our synthetic system has the potential to add to this knowledge by allowing for direct competition between strains with and without QS control of enterochelin production.

Materials and Methods

Bacterial growth media. We use Luria-Bertaini broth (LB) with added kanamycin sulfate (30 μg/mL), chloramphenicol (25 μg/mL), gentamicin sulfate (10 ug/mL), or ampicillin sodium salt

(100 µg/mL) as appropriate for routine bacterial growth. The low-iron minimal medium for these experiments is based off of a modified M9 (MM9) salts medium from Wells *et al*. (146). This medium consists of MM9 salts with 2 mM MgSO4, 0.1 mM CaCl2, and 1% glycerol. 10x MM9 salts includes 5 g NaCl, 10 g NH_4Cl, 100.95 g 3-(N-morpholino)propanesulfonic acid (MOPS), 0.59 g $Na_2HPO_4{\cdot}2H2O$, and 0.45 g KH_2PO_4 in 1 liter of water, final solution adjusted to pH 7. We added 2,2'-bipyridyl (Sigma) to 100 µM to ensure iron limitation in the absence of enterochelin. Where noted, L-arabinose (Sigma) was added to 0.1% and 3OC12-HSL (RTI International) was added to 1 µM.

Bacterial strains and strain construction. We constructed the synthetic QS strain in derivatives of *E. coli* BW25113 (147). We obtained an *entF::kan* mutant from the Keio collection (142) and moved the mutation into *E. coli* BW25113 by using P1 transduction. To make the synthetic QS strain, we started with the plasmid pJN105L (117), which contains an arabinose-inducible *lasR* (*paraBAD-lasR*). Into this plasmid, we cloned the *plasI* promoter and the coding region of *lacZ* from pSC11 (118) by introducing *Apa*I and *Xho*I restriction sites up- and down-stream of the region, respectively, and cloning into pJN105L at those sites. This produced the plasmid pJN105L-*PlasI-lacZ*. *plasI* includes DNA from -282 to +223 bp relative to the *lasI* translational start site in *P. aeruginosa* strain PAO1. We then replaced *lacZ* with *entF* by PCR amplifying the coding region of *entF* from *E. coli* genomic DNA and introducing *Hind*III and *Xho*I restriction sites up- and down-stream, respectively. We digested *lacZ* out of pJN105L-*plasI-lacZ* and replaced it with *entF* using *Hind*III and *Xho*I restriction sites. This produced the plasmid pJN105L-*plasI-entF*. Next, we performed a 3-segment Gibson Assembly (148) to produce the final integration plasmid for the synthetic QS strain. We PCR amplified the *lasI* gene and its promoter region from *P. aeruginosa* genomic DNA, including -282 to +606 bp relative to the *lasI* translational start site. From pJN105L-*plasI-entF*, we PCR amplified the region from the beginning of *entF* to the end of *lasR* (which includes *entF* and *paraBAD-lasR*). We restriction

digested pCAH63 (149) at *Bam*HI and *Eco*RI sites and gel-purified the plasmid backbone. At the beginning of the *plasI-lasI* PCR product, we included ~30 bp of homology to pCAH63 before the *Bam*HI restriction site. At the beginning of the *entF-paraBAD-lasR* PCR product, we included ~30 bp of homology to the end of the *lasI* gene. At the end of the *entF-paraBAD-lasR* PCR product, we included ~30 bp of homology to pCAH63 after the *Eco*RI cut site. Using Gibson Assembly, we constructed the final integration plasmid pCAH63-*plasI-lasI-entF-paraBAD-lasR*. We cloned into DH5*αpir116* to maintain the pCAH63-derived plasmids (150). The plasmid was then integrated into the *entF::kan* mutant using pInt-ts following Haldimann and Wanner (149).

Results

We have a synthetic QS system in *E. coli*. This system, outlined in Figure 3.1, includes the LasI/LasR QS system from *P. aerugionsa* and is integrated into the *E. coli* genome at a λ phage integration site (*attB*) (149). A single secreted product, enterochelin, has been placed under QS-control by moving *entF,* an enterochelin biosynthesis gene, under control of *plasI*, a QS-responsive promoter. In this system, cooperators are cells that produce enterochelin, and the QS strain is a conditional cooperator because enterochelin production is dependent on population density.

In Figure 3.2, we show that the synthetic QS strain is indeed a conditional cooperator. The synthetic QS strain was grown in iron-limited MM9 medium with or without L-arabinose (which controls expression of *lasR*) and with or without 3OC12-HSL (which determines QS activation status). Without L-arabinose, the QS system is inactive, and we observe minimal growth regardless of 3OC12-HSL concentrations. When both L-arabinose and 3OC12-HSL are present, the strain grows robustly, as QS is active from the beginning of growth and enterochelin production is high. When only L-arabinose is present, the strain grows poorly at first and then well later, indicating that the strain can make 3OC12-HSL, but that it takes time for signal to

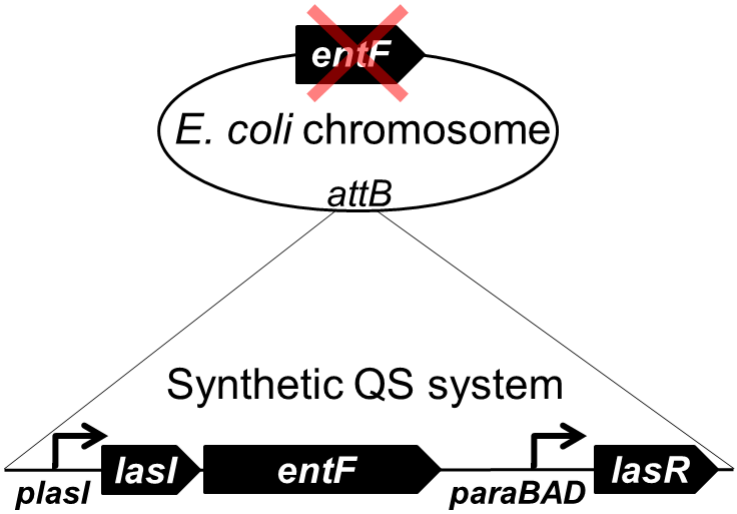

Figure 3.1 Orientation and components of the synthetic QS strain *entF::kan plasI lasI entF paraBAD lasR*.

This synthetic QS system was inserted in single copy into the chromosome at an *attB* site (149). *lasR* is under arabinose-inducible expression, and both *lasI* and *entF* are under control of QS-responsive *plasI*.

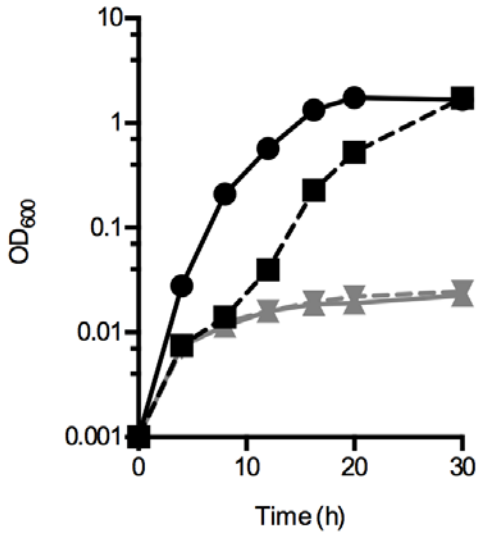

Figure 3.2 Synthetic QS strain *entF::kan plasI lasI entF paraBAD lasR* can grow in low iron medium only when QS is intact.

Growth curves of the synthetic QS strain in batch culture at 37°C. *lasI* and *entF* are under control of QS-responsive *plasI*, and *lasR* is under L-arabinose-inducible expression. Cultures were grown in MM9 medium supplemented with 0.1% arabinose (black lines) and/or 1 μM 3OC12-HSL (solid lines). Grey lines are without L-arabinose, and dashed lines are without added 3OC12-HSL.

reach the QS threshold concentration. Once signal levels are sufficient, the strain can induce enterochelin production to support growth.

Discussion

We have constructed a synthetic QS system in *E. coli* that controls production of enterochelin. We intended to use this strain to study how QS regulation affects cooperation and cheating for a single public good. Unfortunately, as we show in Chapter 4, enterochelin is not a true public good under the conditions we are using here. We will discuss this more in detail later, but here we will outline some strategies that could be used to circumvent the private/public nature of enterochelin and present some future directions for this work.

Although enterochelin may not be a true public good, as it may be retained and used in the periplasm of cells (privatized; Chapter 4), we may be able to study our synthetic QS system under conditions where enterochelin privatization is not beneficial. For example, if our experiments were performed in the presence of human transferrin, only the public portion of enterochelin, that which is fully secreted from cells, would provide a growth benefit. The private portion, which is retained in the periplasm, would not encounter iron, as transferrin-bound iron would be too large to diffuse into the periplasm of cells. Thus, the private portion of enterochelin would not provide a growth benefit, so we could specifically study the costs and benefits of public enterochelin production. These conditions would allow us to study the costs and benefits of using QS to control a single public good.

Enterochelin may also be a metabolically inexpensive product, which is not ideal for studying the costs and benefits of using QS to control a *costly* public good. To increase the metabolic cost of cooperation, we could introduce additional products, such as GFP, to the QS regulon of the synthetic strain. Furthermore, we could perform our experiments under carbon-limiting conditions, such as in chemostats. This should make enterochelin production relatively more

costly because it would force the cells to divert precious carbon and energy from pathways that support cell growth. We had previously attempted experiments using these strategies, but we had not yet addressed the problem of enterochelin privatization. Future attempts in the presence of transferrin may prove more successful.

QS cheaters arise in populations of *P. aeruginosa* cooperators under a variety of conditions. However, even when cheaters do reach a significant frequency in the population, their presence does not necessarily lead to a tragedy of the commons. It has been shown that QS cheaters can reach a stable frequency of approximately 40% of the population under conditions where QS is required to acquire carbon and energy (49, 50). The carbon and energy source in these experiments is casein, which must be extracellularly digested to be used by cells, and which can be broken down by the QS-controlled exoproduct elastase. Only when the cost of QS is increased, by forcing cells to use casein as a nitrogen source as well, do cheaters overtake and collapse the population (50). Dandekar et al. identified a mechanism that restrains cheaters to a low percentage of the population. A private activity, adenosine metabolism via Nuh, is also under QS regulation, and replacing a percentage of the casein with adenosine (≥75%) prevents QS cheaters from growing to high frequencies in culture, as they are unable to use adenosine for growth (50). If we were able to optimize the synthetic QS system to observe cheating, we would like to integrate a similar private good into the synthetic system and test if this system is also able to constrain cheaters.

CHAPTER 4: Sociality in *Escherichia coli*: Enterochelin is a private good at low cell density and can be shared at high cell density

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Abstract

Many bacteria produce secreted iron chelators called siderophores, which can be shared among cells with specific siderophore uptake systems regardless of whether the cell produces siderophores. Sharing secreted products allows freeloading, where individuals use resources without bearing the cost of production. Here we show that the *Escherichia coli* siderophore enterochelin is not evenly shared between producers and non-producers. Wild-type *Escherichia coli* grows well in low-iron minimal medium and an isogenic enterochelin synthesis mutant (Δ*entF*) grows very poorly. The enterochelin mutant grows well in low-iron medium supplemented with enterochelin. At high cell densities the Δ*entF* mutant can compete equally with the wild type in low-iron medium. At low cell densities the Δ*entF* mutant cannot compete. Furthermore, the wild type growth rate is unaffected by cell density. The wild type grows well in low-iron medium even at very low starting densities. Our experiments support a model where at least some enterochelin remains associated with the cells that produce it, and the cell-associated enterochelin enables iron acquisition even at very low cell density. Enterochelin that is not retained by producing cells at low density is lost to dilution. At high cell densities, cell-free enterochelin can accumulate and be shared by all cells in the group. Partial privatization is a solution to the problem of iron acquisition in low iron, low cell density habitats. Cell-free

enterochelin allows for iron-scavenging at a distance at higher population densities. Our findings shed light on the conditions where freeloaders might benefit from enterochelin uptake systems.

Introduction

Iron is an essential nutrient for the vast majority of bacteria (27, 151). However, under aerobic conditions at neutral pH, iron is found predominantly in insoluble Fe^{3+} compounds, resulting in extremely low iron concentrations in water (about 10^{-18} M) (152). Within a host, available iron is also limited, as many host organisms produce iron-binding molecules, which sequester iron away from bacteria (153). Thus, bacteria have evolved a variety of mechanisms to acquire this resource. One mechanism is to secrete molecules called siderophores that bind and solubilize extracellular iron. Different bacteria produce different high-affinity secreted siderophores and there are specific high-affinity uptake systems for the siderophores (154, 155).

Siderophores, by necessity, are secreted, and there has been interest in and debate over the cooperative nature of siderophore production (51, 53, 96, 97, 144, 156-161). In the simplest case, a siderophore, once secreted, is available to all members of a community. Consequently, a freeloading cell, which uses siderophores but does not produce them, may have a fitness advantage over a producing cell, as it does not incur the metabolic cost of siderophore production. Freeloading should destabilize siderophore production. However, competition for siderophores is often much more complex, as evidenced by numerous studies and the continued existence of siderophore-producing bacteria. The availability of siderophores and other extracellular products for use by freeloaders depends on siderophore diffusion as well as population density and structure (162-164). Some bacteria have also evolved mechanisms that appear to prevent or minimize siderophore freeloading, including maintaining variability in structure of siderophore-receptor pairs (diversifying selection) (16, 165), and privatizing siderophores through high hydrophobicity or association with membrane-bound receptors (54,

63, 64, 166). We are interested in siderophore maintenance through privatization, for which there is little direct evidence in microbes (166).

The *Escherichia coli* siderophore enterochelin (Ent) has been studied in detail. It is a catecholate produced not only by *E. coli* but also by several other Enterobacteriaceae (167-169). Ent synthesis and uptake genes are under control of the ferric uptake regulator (Fur) protein (170-172). Ent is produced in the cytoplasm by products of the *ent* genes via non-ribosomal peptide synthesis (140, 141, 173, 174). Following synthesis, Ent is secreted via EntS (175), and released from the cell. Upon binding Fe^{3+}, ferric enterochelin (FeEnt) enters the periplasm via the TonB-dependent receptor FepA (176, 177). In the periplasm, FeEnt binds FepB, a periplasmic binding protein (178, 179) and FeEnt then moves into the cytoplasm through an ABC transporter (180, 181). Finally, FeEnt is hydrolyzed by the cytoplasmic enterochelin esterase (Fes) and free iron is released (182). Each Ent molecule can only be used once, however the linear Ent breakdown products can be repurposed as lower-affinity siderophores (183).

It is of interest that *Pseudomonas aeruginosa* possesses the complete machinery for FeEnt uptake and utilization (184) but it cannot produce Ent itself. The general belief is that this enables *P. aeruginosa* to steal iron from Ent producers; it is capable of freeloading on enteric bacteria like *E. coli.* In fact, a variety of microbes possess Ent uptake systems but not *ent* biosynthesis genes (185). Here we present evidence that Ent is a partially privatized *E. coli* product. Although Ent mutants are unable to grow independently in iron-limited media, they can grow when co-cultured with Ent producers. However, growth of Ent mutants in these co-cultures is dependent on the overall frequency and density of Ent producers. Additionally, when alone, Ent-producer growth in low-iron media is independent of cell density. Our results are consistent with a model where some fraction of Ent must remain associated with the cells that produce it.

Materials and Methods

Bacterial strains and strain construction. All bacteria used were *E. coli* BW25113 (147) derivatives. We obtained an *entF::kan* mutant from the Keio collection (142) and moved the mutation into *E. coli* BW25113 by using P1 transduction. The kanamycin resistance marker was removed by using Flp-FRT recombination (186) to create *E. coli* RS100, which has a non-polar scar in place of *entF*. We constructed a *ΔentCEBAH* mutant by using the method of Datsenko et al. (147). Briefly, a kanamycin marker flanked by FRT sites was PCR amplified from pKD4. The amplification primers included DNA homologous to the regions upstream of *entC* and downstream of *entH*. The PCR product was introduced into the *E. coli* BW25113 chromosome by λ red recombination to yield a *ΔentCEBAH* operon mutant with a deletion extending from the *entC* translation start codon to the *entH* stop codon. We used pKD46 to express the λ red genes and removed the kanamycin marker using Flp-FRT recombination as described above. Where noted, strains also carried either kanamycin or chloramphenicol resistance markers. These markers were PCR amplified from pKD4 or pKD3, respectively, before insertion into the arsenate transporter (*arsB*) gene in the opposite orientation as described elsewhere (147). Again, we used pKD46 to express the λ red genes. The markers replaced most of *arsB*, leaving only the start codon, codons for the 6 C-terminal residues, and the stop codon. Previous work has shown that inserting a marker into this gene does not noticeably affect growth in normal laboratory conditions (142, 179, 187).

Bacterial growth media. We use Luria-Bertani broth (LB) with added kanamycin sulfate (30 μg/mL), chloramphenicol (25 μg/mL), or ampicillin sodium salt (100 μg/mL) as appropriate for routine bacterial growth. The low-iron minimal medium for our experiments consisted of 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS) pH 7, 50 mM NaCl, 40 mM NH_4Cl, 5 mM KH_2PO_4, 5 mM Na_2HPO_4, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$ and 1% glycerol. Where noted, $FeCl_3$ was added to a final concentration of 5 or 50 μM, apo-enterochelin to 5 μg/mL (Sigma Chemicals or Genaxxon BioScience), human apo-transferrin to 100 μg/mL (Sigma Chemicals),

and sodium bicarbonate to 20 mM. In some experiments we included siderophore extracts of *E. coli* culture fluid prepared as described below.

Preparation of siderophore extracts. Extracts from *E. coli* BW25113 growth medium were prepared as described previously (175). Briefly, spent medium from stationary-phase cultures grown in low-iron medium was centrifuged and filtered to remove cells. The pH of the culture fluid was then reduced to about 2.5 with 12 N hydrochloric acid and the siderophores were extracted with two equal volumes of ethyl acetate. The ethyl acetate extract was dried under a steady stream of nitrogen and the residue was dissolved in ethyl acetate. This extraction method should result in a solution containing Ent and recycled Ent degradation products (175, 188). Extracted siderophores were added to growth media as follows: the ethyl acetate preparation was added to sterile 18 mm tubes at the indicated level and the ethyl acetate was removed by evaporation under a stream of nitrogen gas. After removal of ethyl acetate the siderophores were dissolved in low-iron medium.

Growth and competition experiments. For starter cultures, bacteria were first grown overnight in LB broth, washed once in an equal volume of low-iron medium, and then used as an inoculum (1% v/v) in low-iron medium containing siderophore extract prepared as described above. The extract used was equivalent to the material extracted from a 1% volume of *E. coli* BW25113 culture fluid. The bacteria were then grown in 5 mL of medium in 18 mm tubes with shaking at 37^{o}C. When cultures reached mid-logarithmic phase (optical density at 600 nm of 0.2-0.3), cells were washed twice in low-iron medium, diluted to an optical density at 600 nm of 0.1, mixed at desired starting ratios for competition experiments, and then used as inocula for growth or competition experiments. The addition of siderophore extract allows equal growth of the *ΔentF* mutant and the wild type in the starter cultures. For growth curves, bacteria were grown in 2.5 mL of medium in 18 mm tubes with shaking at 37^{o}C.

To determine cell numbers in single strain growth experiments, we performed plate counts on LB agar. For competition experiments, cells were grown for 14 to 16 h. The frequencies of competing strains were measured before and after growth by plate counting on LB agar with kanamycin or chloramphenicol, and total cells per mL were calculated as the sum of the number of cells resistant to each antibiotic.

Our detection threshold for colony counting was 10^2 cells per mL. The counts for the *ΔentF* mutant at the lowest starting density in the density-dependent fitness experiments occasionally fell below this threshold. When this happened, we assigned a value equal to one-half of the detection threshold, or 50 cells per mL to these cultures. The competitive index (CI) is defined as the ratio of mutant to wild type in the output divided by the ratio of mutant to wild type in the input (172). To calculate generations, we used the formula: number of generations = $\ln(N_{final}/N_{initial})$ where N is the measured cells per mL. We used GraphPad Prism software to generate graphs and nonlinear semi-log fit curves for the wild type to wild type competitions. To test whether slopes were significantly different from 0, we used a sum-of-squares F test with $P < 0.05$.

Results

Growth of the *ΔentF* mutant in low-iron medium with and without added enterochelin. As expected, our *ΔentF* mutant showed very limited growth in low-iron medium as compared to the parent (Fig. 4.1). The final growth yield was <1% of the parent strain. The addition of 5 or 50 μM $FeCl_3$ to cultures failed to rescue *ΔentF* mutant growth. In the presence of added apo-Ent (5 μg/mL), however, growth of the *ΔentF* mutant was equivalent to growth of the wild type (Fig. 4.1). This is consistent with the general view that Ent can serve as a shared resource, which can be used by any cell with an Ent receptor system whether or not that cell produced Ent. To show that iron limited growth in unsupplemented low-iron medium we measured growth yields. Without added enterochelin yields were $2.4x10^9$ cells per ml without added iron and $4.6x10^9$

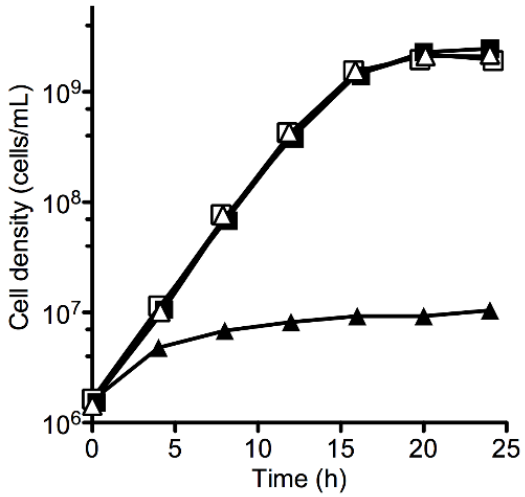

Figure 4.1 Growth curves of wild type and Ent mutant in low-iron medium with and without added Ent.

The wild-type *E. coli* BW25113 grown with (open squares) or without (closed squares) added Ent. The Δ*entF* mutant grown with (open triangles) and without (closed triangles) added Ent. We used low-iron medium for these experiments and supplemented with 5 μg/mL Ent where indicated. Cultures were run in duplicate and the range is within the symbols.

cells per ml with 50 μM $FeCl_3$, and with enterochelin yields were $2.1x10^9$ cells per ml without added $FeCl_3$ and $8.9x10^9$cells per ml with 50 μM $FeCl_3$. Wild type growth rates, however, were indistinguishable between the no iron added and 50 μM $FeCl_3$ added conditions (0.45 +/- 0.02 doublings per hour). These growth curve data indicate that Ent production is metabolically inexpensive.

The *ΔentF* mutant can grow in low-iron medium when co-cultured with the Ent-producing wild type. Co-culture with the wild type *E. coli* BW25113 supported growth of the *ΔentF* mutant (Fig. 4.2). When the *ΔentF* mutant to wild type starting ratio was 1:1 and starting density was 10^5 cells per mL, both strains grew equally well: the final cell yield was comparable to that of the wild type in pure culture and the competitive index (CI) was about 1. These findings indicate that the *ΔentF* mutant can freeload; it can use Ent produced by the wild type to acquire iron. The CI is a measure of the relative fitness of a mutant strain, with >1 indicating an advantage and <1 indicating a disadvantage. The CI of about 1 indicates that the cost of Ent production is low under the conditions of this experiment.

Enterochelin mutants have a negative frequency-dependent fitness in competition with wild type. To more thoroughly investigate whether production of Ent confers a burden to the wild type in co-culture with the *ΔentF* mutant, we measured the CI in experiments started at a wide range of *ΔentF* mutant to wild type ratios. Previous work with *P. aeruginosa* showed that a siderophore mutant can have negative frequency-dependent fitness in competition with a wild type: the siderophore mutant showed greater fitness when at low relative abundance than it did at higher relative abundance (156). We tested whether or not initial frequencies affect the competitive fitness of the *ΔentF* mutant. The starting cell densities for these experiments were about 10^6 cells per mL of low-iron medium and the starting ratios ranged from 1000:1 to 1:1000 *ΔentF* mutant to wild type. As a control, we performed a similar experiment in which the wild type with a kanamycin resistance marker was co-cultured with the wild type with the

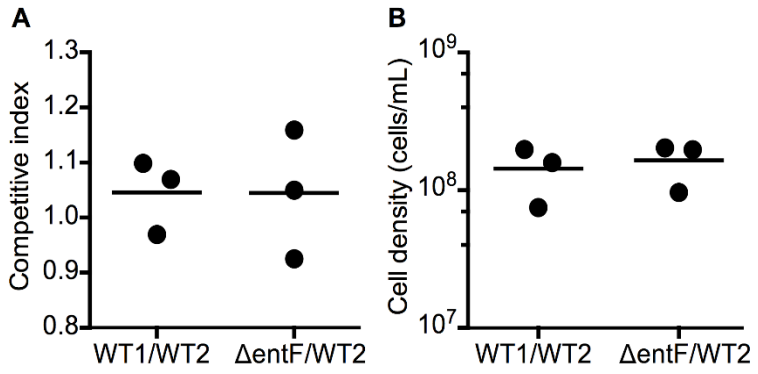

Figure 4.2 The Δ*entF* mutant can grow in low-iron medium when in co-culture with the wild type.

E. coli BW25113 *arsB::kan* (WT1) or Δ*entF arsB::kan* (Δ*entF*) were mixed at a 1:1 ratio with *E. coli* BW25113 *arsB::cat* (WT2). (A) The competitive index and (B) the final cell density. The initial cell density was about 10^5 cells per mL. Total cells per mL and strain frequencies were measured at the start of the experiment and after 14 h. The competitive index was calculated as described in the Materials and Methods. Three independent experiments are shown and the bars are the means.

chloramphenicol resistance marker. The control provides a measure of any burden from the antibiotic resistance marker. The control showed little effect of the antibiotic resistance markers and did not show frequency dependence. At low initial frequencies of the *ΔentF* mutant, the CI was about 1, indicating that neither strain has a significant growth advantage over the other. This supports the view that Ent production is inexpensive and therefore does not significantly reduce the growth rate of the Ent-producers (Fig. 4.3A). In contrast, at high *ΔentF* mutant starting frequencies, the CI was <1. That is, the wild type was more fit than the *ΔentF* mutant. The antibiotic markers are not responsible for this result, as the CIs in wild type control experiments remained about 1.

We performed similar experiments with a *ΔentCEBAH* mutant. This mutant is defective in conversion of chorismic acid to the aromatic substrate for Ent synthesis whereas EntF is required for incorporation of serine in Ent biosynthesis. Our results with the *ΔentCEBAH* mutant were consistent with the *ΔentF* mutant. For the *ΔentCEBAH* mutant, the competitive index is close to 1 at high wild type frequencies and rapidly decreases as the starting mutant frequency increases above 10%.

Additionally, we found that, although the overall culture growth was reduced with high *ΔentF* mutant starting frequencies (Fig. 4.3B), the approximate generations per h of the competing wild type was unaffected by *ΔentF* mutant frequency (Fig. 4.3C and D). Therefore, the reduced growth yield in these cultures is due solely to the reduced growth of the *ΔentF* mutant. We believe the results of the frequency-dependence experiments indicate that Ent is not a freely shared public good. Rather it is partially privatized by the wild type, and when wild type cells are scarce, they make sufficient Ent to support themselves but there is insufficient free Ent for iron acquisition by the *ΔentF* mutant.

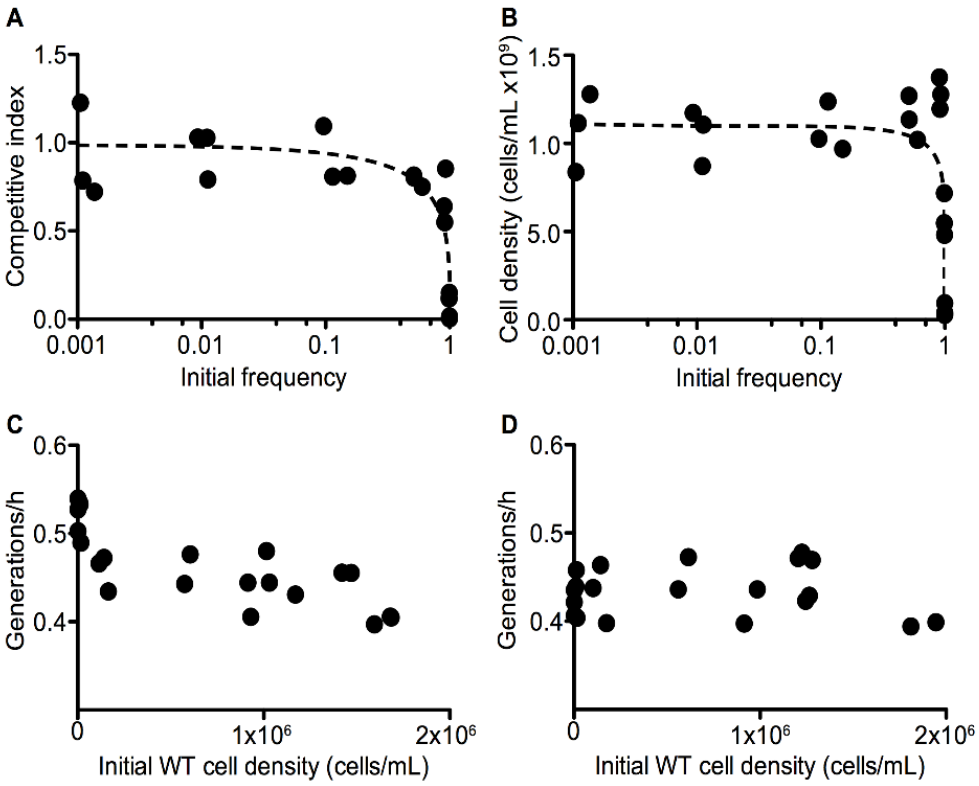

Figure 4.3 Negative frequency-dependent fitness of the Δ*entF* mutant.

Competition experiments were with WT1 or Δ*entF* against WT2. Initial ratios ranged from 1000:1 to 1:1000 and initial cell densities were about 10^6 cells per mL. Cells were grown in low-iron medium. (A) CI of competitions between the Δ*entF* mutant and WT2 and (B) total yield of the cultures. At high frequencies of the Δ*entF* mutant, its fitness and total culture yield fall off. Controls with WT1 and WT2 show a CI of about 1 regardless of initial frequency and total yields also remain constant. Frequencies were measured at the start of the experiment and after 14 to 16 h of growth. (C) Growth of WT2 in culture with the Δ*entF* mutant or (D) with WT1. Generations/h was calculated as generations over the growth period divided by the hours of growth.

Fitness of the *ΔentF* mutant depends on the initial cell density. Privatization of bacterial extracellular products has been demonstrated by showing density-dependent fitness during competition, although previous work has been done on surfaces where population structure

plays a large role (157). By measuring density-dependent fitness, we can determine how wild type cell density affects fitness of the *ΔentF* mutant. For these experiments the initial wild type to *ΔentF* mutant ratios were 1:1, and initial cell densities were varied between 10^1 and 10^5 cells per mL (Fig. 4.4). The *ΔentF* mutant showed density-dependent fitness in low-iron medium. The greater the initial cell density, the greater the fitness of the *ΔentF* mutant. These data, in agreement with the frequency-dependent fitness data, indicate that Ent is partially privatized by Ent-producing *E. coli*. We interpret the results to mean that at sufficiently high densities of wild-type *E. coli* the fraction of Ent in the culture fluid is sufficient for growth of the mutant. Cell-free Ent concentrations are too low to be of value at lower cell densities where only the wild type with cell-associated Ent is capable of growth in low-iron medium. When Ent is added to the culture medium, the mutant and wild type grow well together regardless of the starting cell density. Thus the inability of the Ent mutant to compete with the wild type when the initial cell density is low is a direct consequence of the lack of sufficient cell-free Ent.

One possible explanation for our results is that a significant fraction of iron-free Ent partitions to the periplasm of producing cells and diffusion of Fe^{3+} into the periplasm allows growth on low-iron medium when cell density is low. We do not have conclusive evidence for or against this hypothesis, but it is worth noting that inclusion of the iron chelator transferrin (with bicarbonate) in the low-iron medium restricts the growth of the wild type at low starting cell densities but not at higher starting densities (Fig. 4.5). Exogenous enterochelin can support wild type growth in the presence of transferrin even at low starting cell densities, and the *ΔentF* mutant does not grow in the presence of transferrin regardless of starting density. One interpretation of these findings is that cell-associated Ent is within the periplasm where it cannot obtain iron bound to

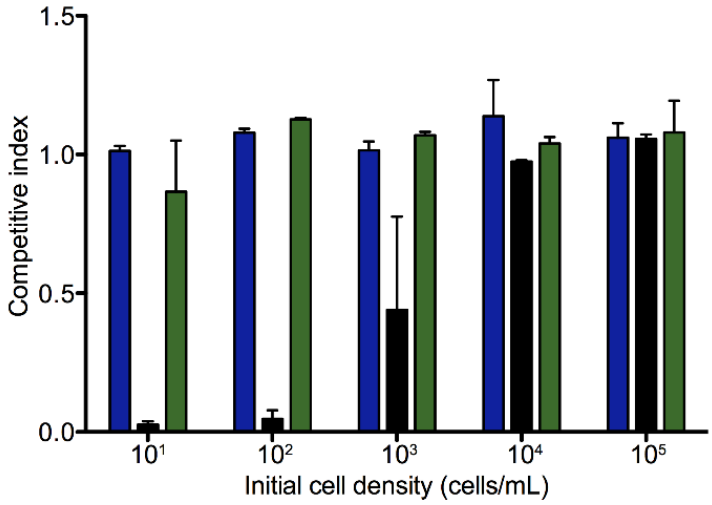

Figure 4.4 Fitness of the Δ*entF* mutant vs the wild type depends on initial cell density.

The *E. coli* BW25113 *arsB::cat* strain was grown together with the *E. coli* BW25113 *arsB::kan* strain or the *ΔentF* mutant (*E. coli ΔentF arsB::kan*) in low-iron medium with or without supplements as indicated. Starting ratios were 1:1 and initial cell densities are shown. (Blue bars) The two Ent wild type strains in unsupplemented low-iron medium. The CI was about 1 regardless of inoculum size. (Black bars) The *ΔentF* mutant and the wild type in unsupplemented medium. The *ΔentF* mutant is competitive only at initial cell densities of 10^3-10^4 or greater. (Green bars) The *ΔentF* mutant and wild type in medium supplemented with Ent (5 µg/mL). The mutant is competitive regardless of inoculum size. Cell numbers and frequencies were determined immediately after inoculation and at 14 h. The bars represent two independent experiments and error bars are the range.

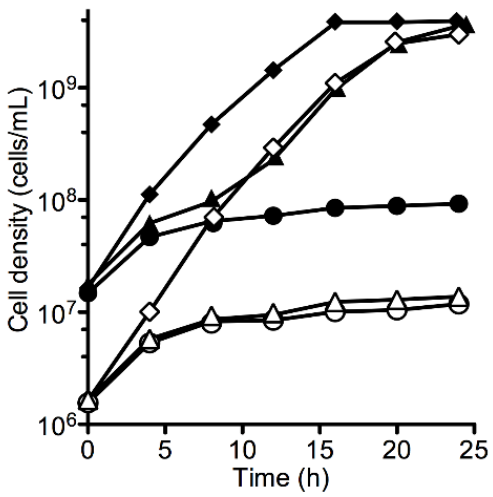

Figure 4.5 Growth of wild-type *E. coli* in the presence of transferrin is dependent on inoculum size.

E. coli BW25113 and the *ΔentF* mutant were grown in low-iron medium with human apo-transferrin and $NaHCO_3$ (sodium bicarbonate). (Open circles) the *ΔentF* mutant started from low density and (closed circles) the *ΔentF* mutant started from high density. (Open triangles) the wild type started from low density and (closed triangles) the wild type started from high density. (Open diamonds) the wild type started from low density with Ent added and (closed diamonds) the wild type started from high density with Ent added. Cultures were run in duplicate and the range is within the symbols**.**

transferrin. At high cell densities there is sufficient cell-free Ent for iron acquisition from transferrin.

Growth of Ent-producing wild-type *E. coli* in low-iron medium is not influenced by cell density. The ability of Ent-producing wild-type *E. coli* to grow at low cell density and the inability of the *ΔentF* mutant to grow together with the wild type at low cell density indicates that some fraction of Ent remains with producing cells. At low cell density there is insufficient public Ent for cell growth. To obtain further evidence that wild-type growth is similar at very low and higher cell densities, we serially diluted the wild type in low-iron medium and followed growth. Growth was observed even from starting densities of one or a few cells per mL (Fig. 4.6). Previous work on yeast invertase has shown that for predominantly shared products, growth rate is severely limited at low starting densities, as products rapidly diffuse away from producers (86). Therefore our finding that growth of *E. coli* in low-iron medium is unaffected by cell density is further evidence that a fraction of this siderophore remains bound to producing cells.

Discussion

We tested the hypothesis that the *E. coli* siderophore Ent is a secreted public good. Our results indicate that sufficient Ent or Ent breakdown products are released from cells to support the growth of non-siderophore producing mutants, but only at relatively high cell densities. At low densities, Ent producers grow well but they cannot support growth of non-producers. This leads to an alternate hypothesis that only a fraction of the Ent produced by wild-type cells is public, and the public Ent is not sufficient to support growth in low-iron medium when *E. coli* is at low population densities. Furthermore, we did not find that Ent mutants were cheating. Under any condition we tested they did not have a fitness advantage over wild type. Thus we conclude that EntF expression and Ent production are relatively inexpensive.

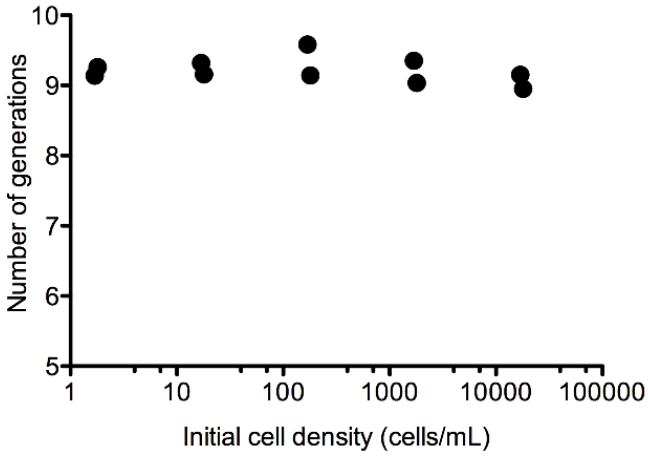

Figure 4.6 Growth rate of wild-type *E. coli* in low-iron medium is not affected by inoculum size.

E. coli BW25113 was grown in low-iron medium starting at various densities as indicated (Initial cell density). Cell numbers were determined by plate counting at the time of inoculation and at 18 h. In all cases growth was in logarithmic phase at 18 h. The number of generations is the number of doublings in 18 h. Cultures were run in triplicate. One of the lowest density starting cultures failed to grow, presumably because this culture did not receive any cells during inoculation; this culture was excluded from analysis.

The apparent partial privatization of Ent contrasts with The *P. aeruginosa* siderophore pyoverdine. Pyoverdine non-producers arise both in vivo and in vitro and they are social cheats; they can have a fitness advantage over pyoverdine producers in low-iron media (51-53). However, there are indications that, as we have found with Ent, pyoverdine too is somewhat privatized (96, 166). Mathematical models indicate that social cheating can occur even in the face of significant privatization, so the differences in competition between siderophore-producing and non-producing *E. coli* and *P. aeruginosa* may be due to siderophore cost and degree of privatization (159, 162-164).

Although it has long been suspected that many siderophores might remain cell-associated after secretion (54, 189-191), evidence for this notion is limited and restricted to a few bacterial species. Furthermore, aside from the few studies on amphiphilic siderophores and pyoverdine, the mechanisms for privatization appear to be largely unexplored (63, 64, 166). Apo-Ent might be retained in the periplasm, it might bind to FepA, and it might adhere to the cell surface non-specifically. Evidence indicates that adherence of FeEnt to the surface of *E. coli* is almost entirely mediated by FepA (192), but the mechanism for apo-Ent adherence is unclear, as studies typically measure adsorbance and transport of radiolabeled iron-siderophore complexes into cells, not apo-siderophores (193). Due to the hydrophobicity of apo-Ent, it is possible that it remains associated with the cell surface after secretion (54, 190). We have not addressed the mechanism of Ent privatization, but we have found that iron chelated with transferrin supports growth of *E. coli* only when cells are at sufficient population densities or if exogenous enterochelin is added. This result suggests that Ent may be sequestered in the periplasm. By studying salmochelins, which are a family of glycosylated enterochelins, it may be possible to assess the importance of siderophore hydrophobicity to privatization. Salmochelin glycosylation results in siderophores that are more hydrophilic than enterochelin (194).

Many microbes produce FeEnt uptake receptors despite their lack of Ent biosynthesis genes. The general view is that this allows these microbes to acquire iron at the expense of Ent-producing enteric bacteria. Our experiments suggest that this will occur only when the density of Ent-producing cells is sufficiently high. Thus, Ent-production is stabilized in at least two ways: Ent-production is inexpensive and non-producers should have little to no growth advantage over producers, and Ent-producers will always have an initial head start over non-producing cells when growing from low densities such as after a dispersal event. What might be the advantage of making Ent publicly available at high cell densities? One might imagine that as cell density rises, local iron concentrations become increasingly limited. In this situation iron will come in contact with cells less often than at low cell densities. Here iron scavenging by diffusible Ent might provide a benefit to any cell in the group with an Ent uptake system. Ent may also serve purposes other than iron-acquisition. For example, it has been suggested that cell-free siderophores may be beneficial to producers because they can sequester iron from competitors or possibly to support mutualists (167, 195, 196).

CHAPTER 5: Significance and future directions

Our work has shown that positive autoregulation in *P. aeruginosa* synchronizes and ensures high expression levels for QS-controlled genes. We also show that we have constructed a synthetic QS system in *E. coli* that has enterochelin under QS control, and that enterochelin is a partially privatized product in *E. coli*. These studies have all been targeted towards understanding bacterial cooperation, particularly how bacteria coordinate population responses and prevent cheating by cells that do not cooperate. The results and extensions of these studies will be relevant for diverse applied fields, including medicine, bacterial ecology, and synthetic biology.

Our data in Chapter 2 provides several interesting follow-up projects, some of which are already being studied in the lab. One re-occurring question is: how does positive autoregulation affect populations growing in biofilms? As we show in Figure 2.1, the wild type PAO1 population rapidly reaches high signal levels after about 5 to 6 hours of growth in liquid cultures, and cells induce QS synchronously. PAO-SC6, which does not have positive autoregulation, shows less synchronized QS activation, and maximum signal levels are lower. We hypothesize that the higher overall signal production in the wild type compared to PAO-SC6 might correspond to a greater area of influence for a biofilm population; if more signal is produced, then populations that are further away may be able to sense it and respond. We will be testing this hypothesis in collaboration with the Whiteley and Shear labs at UT Austin (197). Additionally, cells in biofilms are subject to contact-dependent interactions. Type VI secretion (T6S) has been shown to mediate interbacterial contact-dependent (often antagonistic) interactions in *P. aeruginosa*, and some components of T6S are regulated by QS (46, 198-201). If T6S system genes are induced in a less-synchronized manner without positive autoregulation, then we may observe within-

population killing in biofilms. Both of these studies would provide biological explanations for why positive autoregulation is so common in QS.

We are also interested in testing why some natural AHL systems are not positively autoregulated. As mentioned in Chapter 2, some AHL systems which use LuxR-homolog transcriptional repressors do not show positive autoregulation (123-126). We would like to test the synchronization of QS activation in these systems, possibly by expressing the LuxR homologs and QS responsive reporters in *E. coli,* and compare their QS activation dynamics to the *las* system. This study will help clarify the environmental and genetic conditions that require and maintain positive autoregulation.

Additionally, we have the tools to study whether 3OC12-HSL is partially privatized in *P. aeruginosa*. AHLs have been shown to be freely diffusible into and out of cells (104), however a fraction of the signal produced by cells could be bound by internal LuxR homologs before the signal diffuses out of the cell. By monitoring fluorescent reporter expression in mixed populations of signal-producing and *ΔlasI* cells, we may be able to precisely determine if the signal-producers are activated before the *ΔlasI* cells, which would indicate a partial privatization of 3OC12-HSL in *P. aeruginosa*. Additional AHL systems could also be tested in this manner, as acyl chain lengths play a role in diffusion kinetics.

We have already discussed follow-up studies for our synthetic QS system presented in Chapter 3. Using these strategies, we may be able to optimize the conditions for growth of our synthetic QS system and study the costs and benefits of using QS to control a single public good. We initiated this study with the goal of understanding whether QS regulation is beneficial when only a single public good is controlled. We may still realize this goal, and this work would be informative for future synthetic biology efforts. It would outline conditions under which QS stabilizes cooperation, and may provide additional “policing” tools that could be used for future cooperative synthetic systems.

Our results in Chapter 4 fit into a rich literature regarding the ecological significance of private and public goods in bacteria. Privatization would be expected to influence the development of microbial communities (202), and using population structure to achieve privatization has been shown to influence the stability and evolution of cooperation (66, 68, 203-205). These structures may even be useful as early models of multicellularity (67). Public goods cooperation and privatization of secreted products both influence microbial community dynamics and are active in many niches, from oceanic particles to the human gut (75, 98, 206). Interestingly one study has interrogated siderophore privatization across known siderophores and correlated this information with the ecology of the siderophore-producing organisms (54). This study indicates that the diffusion properties of an environment are likely to influence the privatization strategies of a resident microbe.

Finally, our work in characterizing QS and public goods cooperation supports efforts to combat bacterial infections. *Pseudomonas aeruginosa* continues to be a difficult pathogen, and conventional antibiotics are rapidly becoming unusable as the organism develops antibiotic resistant mechanisms (207, 208). Last-line antibiotics are being used for some patients, and these often have worrisome toxicity to humans (209). Targeting QS, which *P. aeruginosa* uses to control expression of some virulence factors, may prove to be an effective approach to treating these infections. Not only would this approach limit virulence, and thereby limit damage to the host, but it may also be robust against the development of resistance (210, 211). Cells that develop resistance would begin producing virulence factors, and cells that remain sensitive would theoretically “cheat” off of the resistant cells, putting resistant cells at a disadvantage. Unfortunately, it is not that simple. As we and others have shown, secreted products may not always be strictly public goods, and population structure and other factors could limit “cheating” by sensitive strains (66, 204). Furthermore, QS in *P. aeruginosa* is very complex, and the social and nutritional environment of the lung is incompletely characterized, so we cannot precisely

predict the effects of inhibiting QS in the lung (212). For example, *lasR* mutants are regularly isolated from the chronically-infected lungs of CF patients (15). It is unclear whether these patients would benefit from a QS inhibitory drug, as QS mutants already exist and flourish (213). However, there have been attempts to develop QS inhibitors as therapeutics, and the benefits are promising in mouse models of infection (214, 215). By modifying QS activation in P. aeruginosa in PAO-SC6, we gain insight into the effects of partial QS activation within a population, and may learn why some treatments do not work or possibly how to develop new treatments that would encourage pathogenic bacteria to destroy themselves or their neighbors.

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CURRICULUM VITAE

Rebecca Lynn Scholz

3513 NE 95th St. Suite 5
Seattle, WA 98115

(541) 848-9439 (cell)
bscholz@u.washington.edu

Education and Training

University of Washington, Seattle	Ph.D.	2011 – 2017	Molecular and Cellular Biology
Marine Biological Laboratory	Student	Summer 2015	Microbial Diversity
California Institute of Technology	B.S.	2011	Biology
University of Copenhagen	Exchange Student	Fall 2009	Biology
Bend Senior High School	H.S.	2007	Valedictorian

Awards and Honors

ASM Student Travel Grant to attend Mechanisms in Interbacterial Cooperation and Competition	2017
ASM Student Travel Grant to attend the 5th Annual Conference on Cell-Cell Communication in Bacteria	2014
Cell and Molecular Biology Trainee Fellowship	2012 – 2014
Honorable Mention - National Science Foundation Graduate Research Fellowship	2012
Doris Everhart Summer Undergraduate Research Fellowship	2010
Hugh and Andy Lou Colvin Scholarship	2009, 2010
Amgen Summer Research Fellowship	2009
Thomas Hunt Morgan Summer Undergraduate Research Fellowship	2008
Wasserman Scholarship from the Wasserman Foundation	2008

Research Experience

PhD, University of Washington, Seattle WA — 2011 – 2017

E. Peter Greenberg Laboratory

Thesis: Studied bacterial cell-cell signaling and social behavior in *Pseudomonas aeruginosa* and *Escherichia coli*.

Biotechnology Externship, Omeros Corporation, Seattle WA — Summer 2012

Designed and built G protein expression plasmids to improve signaling through G protein-coupled receptors.

Undergraduate Research, California Institute of Technology, Pasadena CA — 2009 – 2011

David Baltimore Laboratory

Studied the role of microRNA-155 on immune cell function using both *in vivo* and *in vitro* methods.

Undergraduate Research, California Institute of Technology, Pasadena CA — 2008

Angelike Stathopoulos Laboratory

Identified transcription factor (Dorsal-Twist-Snail) targets within the genome of *Drosophila melanogaster.*

Chemistry Intern, Chemica Technologies, Bend OR 2006 – 2007

Research and QC Inspector, Grace Bio-Labs, Inc., Bend OR Summers 2004 – 2007

Teaching Experience

Science Education Partnership Mentor, University of Washington, Seattle WA 2013
Mentored a science teacher from a local high school and taught her basic laboratory techniques in molecular biology and microbiology.

Graduate Teaching Assistant, University of Washington, Seattle WA 2013
Worked with two professors and one other TA to run a Molecular and Cellular Biology course of approximately 120 junior and senior level undergraduate students. Led three discussion sections of 20 students each and helped write and grade homework and exams.

Volunteer Experience

Bio Expo Mentor, Judge, Northwest Assoc. for Biomedical Research, Seattle WA 2013 – 2016

Research Weekend Volunteer Scientist, Pacific Science Center, Seattle WA 2013, ’15, ‘16

Publications

Scholz, R.L. and Greenberg, E.P. Sociality in *Escherichia coli*: Enterochelin is a private good at low cell density and can be shared at high cell density. ***J Bacteriol*** (2015).

O'Connell, R.M., Kahn, D., Gibson, W.S.J., Round, J.L., **Scholz, R.L.**, Chaudhuri, A.A., Kahn, M.E., Rao, D.S., and Baltimore, D. MicroRNA-155 Promotes autoimmune inflammation by enhancing inflammatory T cell development. ***Immunity*** (2010).

Manuscripts

Scholz, R.L. and Greenberg, E.P. Autoregulation of an acyl-homoserine lactone quorum sensing circuit synchronizes the population response. *In revision*.

Conference Presentations

Oral

Mechanisms in Interbacterial Cooperation and Competition, Washington, D.C. 2017

Poster

5th Annual Conference on Cell-Cell Communication in Bacteria, San Antonio, TX 2014

BEACON Congress, East Lansing, MI 2013