Naomi Balaban

SPRINGER SERIES ON BIOFILMS

Control of Biofilm Infections by Signal Manipulation

Springer Series on Biofilms

Volume 2

Naomi Balaban
Editor

Control of Biofilm Infections by Signal Manipulation

Foreword by J. William Costerton

With 53 Figures, 13 in color

Springer

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ISBN 978-3-540-73852-7 e-ISBN 978-3-540-73853-4

DOI 10.1007/978-3-540-73853-4

Springer Series on Biofilms ISSN 1863-9607

Library of Congress Control Number: 2007941546

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Cover Design: Boekhorst Design BV, The Netherlands

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Foreword

In the well-watered groves of academe, most of us are content to gather worshipful students and technicians in a shady nook to contemplate the eternal verities and to plan extravagant feasts to celebrate our contributions to "knowledge" and to the gradual improvement of the human condition. As one convocation follows another, and as our funding agencies pump billions of dollars into incremental research that fills every possible pigeon-hole in which a gene makes a protein, a small number of intellectual athletes seize a pivotal concept and plunge into the real world. It is this small band of nimble and impossibly brave intellectual halfbacks who win games in the real world, and this book is the result of the drive and intellectual athleticism of its editor and several of her contributors.

Bacteria affect humans more than any other life forms with which we share the blue planet, but our understanding of these invisible companions has developed in a staggering pattern, crippled by our panic and consequent shifts of emphasis. When our race was threatened by epidemic diseases, we visualized bacteria as swarms of potentially lethal planktonic cells from which we must remain isolated by sanitation and which we had to kill by immunization and chemical antibacterial compounds. By the time this overriding threat had been obviated, we began to examine natural and pathogenic ecosystems by direct methods, and we were surprised to find that planktonic bacteria are comparatively rare and that most prokaryotes grow in matrix-enclosed biofilms. This discovery helped explain the failure of immunization and antibiotic therapy in controlling the device-related and other chronic bacterial diseases that replaced epidemic diseases, but it provided no new weapons to the clinical armamentarium.

The academic team that assembled to study these fascinating multicellular bacterial communities was well funded; a network of research centers sprang up around the world; and a clear picture of the structure and function of biofilms began to emerge. The academic biofilm community penetrated the societies that present microbiology to the world, and the American Society for Microbiology (ASM) soon incorporated biofilm symposia and (most important) biofilm poster sessions into its annual meetings. The "slime people" assembled at weeklong ASM-sponsored symposia and workshops (1996, 2000, 2003, and 2007), and selected gurus were invited to meetings of the whole gamut of medical and dental specialties impacted by biofilms. But no biofilm-based therapies emerged.

Intellectual athletes look at the facts and theories in a field, as Wayne Gretzky would survey the scared thugs of Broad Street, and they design an experiment that

asks a well-defined question. Pete Greenberg surveyed the facts and theories of the biofilm field, listened to my enthusiastic ramblings about the complexity of biofilms, and assembled Jim Pearson's acyl homoserine lactone (AHL) minus mutants to see whether they could form proper biofilms. They couldn't, and the rest is history. We had published hundreds of papers and dozens of reviews suggesting that some kind of "communication" must be involved in the development of architecturally complex biofilms, but one simple experimental design by Pete nailed down the fact that AHL signals enable biofilm formation by gram-negative bacteria. In the meantime, Naomi Balaban had explored the control of biofilm formation in gram-positive bacteria by polypeptide signals and had designed and synthesized an effective inhibitor of the TRAP and *agr* system in *Staphylococci*. Also in the meantime, Mike Givskov had joined Staffan Kjelleberg's team in the bucolic reaches of Coogee Bay in Sydney and had participated in the demonstration that natural and synthetic brominated furanones inhibit biofilm formation by a wide variety of bacteria. More recently, David Davies has focused hard on the phenomenon of natural detachment and has identified a signal that triggers this release of planktonic cells from established biofilms.

The original notion that chemical signals must be involved in the complexity of biofilms gradually emerged from our speculations concerning how the familiar "water channel/microcolony" pattern could be initiated and maintained in biofilms. We did not anticipate that certain signals could switch biofilm formation on and off, as the AHL signals and the polypeptide and furanone inhibitors clearly do, and we are still searching for less draconian signals with fewer far-reaching effects. But the burgeoning biofilm research community is moving inexorably towards a consensus that the most important discovery in modern microbiology is that the activities of both planktonic and sessile bacteria are controlled by chemical signals. When we saw bacteria as unicellular entities, each responding to its environment according to the dictates of its individual genome and associated mechanisms, our only option in controlling these minute creatures was either to kill them or to feed them and hope for their gratitude and cooperation. Now we see bacteria primarily as sentient members of complex communities, within which they communicate with each other to set up mutually beneficial associations and coordinate measures that protect the whole community from stress and attack. Paradoxically, their use of a chemical language in their pursuit of safety may prove to be their Achilles' heel. The academics amongst us have conjured up visions of "grow slow" signals that could be very effective in saving the lives of septic patients with overwhelming bacteremia, in which antibiotic-mediated killing and lysis of the planktonic bacteria are clearly contraindicated. Similarly, we suggest that the bacteria that cause device-related infections may be "locked" in the planktonic state by biofilm signal inhibitors, and thus rendered susceptible to antibiotics and host defense factors. More recently we have suggested, in the preambles of our health-related grants, that species-specific or general signals may be useful in causing the detachment of planktonic cells from established biofilms, with the resultant resolution of chronic biofilm infections.

The intellectual athletes amongst us have taken specific concepts from this swelling mass of biofilm data and speculation, and have asked and answered very specific

and very incisive questions. Mike Givskov has used cells of *E. coli* that turn on green fluorescent protein in response to AHL signals produced by *P. aeruginosa* to show that these organisms actually produce AHL signals when growing in biofilms. Then he showed that certain brominated furanones repress this formation of AHL signals by cells of *P. aeruginosa* in biofilms growing in vitro in flow cells and in vivo in the mouse. Then this consummate intellectual athlete established model pulmonary infections in mice, treated the mice with the most effective brominated furanone, and showed the first resolution of a biofilm infection in an animal.

When I present Mike's data to academic audiences, the most common question is "Yes, but aren't the brominated furanones toxic?" We all tend to be underwhelmed by data once they are published, but we are obliged by basic scientific morality to admire the hardnosed plunge straight at the basic questions that the less athletic amongst us dance around for decades. Bacteria in biofilms do actually produce AHL signals. These signals diffuse throughout the communities; their production is affected by signal inhibitors, and this inhibition of signal production affects the course of at least one biofilm disease. Why don't all of us do these kinds of incisive experiments? Why do very few people play nose tackle for the Pittsburgh Steelers?

Intellectual bravery is not the exclusive property of pugnacious Danish males, and certainly our mild-mannered friend Bob McLean cut straight to the chase when he found biofilm signals in the mixed-species biofilms in the river in San Marcos. But perhaps the toughest of our intellectual athletes is Naomi Balaban. She developed her RIP inhibitor of the RAP signal in the crowded milieu of the early days of polypeptide signaling, paused only briefly to confirm its activity in vitro, and proceeded directly to animal models in which she has shown its efficacy in preventing both colonization and infection by staphylococci. She has enlisted dozens of collaborators in the march towards commercialization of this biofilm inhibiting signal blocker, and she has approached any anomalies in the team data by offering to repeat the critical experiments using her own strains and conditions. She has bulldozed the entire field in the most charming and disarming manner, and she could actually play nose tackle for the Steelers if she were more muscular and less beautiful. Naomi has acted as the highly visible bellwether of signal inhibition, while frankly commercial corporate labs have operated in secret, and the eventual success of signal inhibitors in the prevention of biofilm infections by staphylococci must certainly be laid at her feet.

Every practical implementation of a scientific discovery in clinical medicine requires a partnership between intellectually athletic scientists and perceptive clinicians, and the biofilm community is very fortunate to have "converted" Randy Wolcott to our cause. Randy runs a chronic wound center in Lubbock, Texas, and he is passionately devoted to the welfare of his many patients, who come to him for the resolution of wounds that have been complicated by biofilm infections for months or years. Randy has acquired impressive skills in biofilm microbiology: He is a scientific partner in a National Institutes of Health wound center based at Montana State University (CBE) and the University of Washington (UWEB), and he uses modern molecular techniques to analyze bacterial wound populations. In this book, Randy reports on his use of the RIP inhibitor identified by Naomi and the lactoferrin identified by Pete Greenberg and Pradeep Singh as being pivotal in the control of

biofilm infections. But the full importance of Randy's involvement in this widening team of incisive intellectual athletes is that he is just as brave and just as committed as they are, and he is completely prepared to leap for any pass they can throw to help his patients.

Our policy in the production of the Springer Series on Biofilm books will be to offer the basic *Biofilm Primer* (Costerton), which was published in March of 2007, followed by a series of books on various aspects of these multicellular communities. We chose the topic of signal manipulation for the second book, with Naomi Balaban as author/editor, because we wish to capture and spotlight the area within the biofilm field that shows the greatest potential for the translation of biofilm science to medical, dental, and veterinary applications.

We will keep this book updated because this field is very active, and we will gradually add new books on specific biofilm infections and on the structure and function of these sessile communities that dominate the prokaryotic component of the biosphere.

Los Angeles, June 2007 Bill Costerton

Preface

The number of patients affected by and dying from what can be considered as a "biofilm diseases" is higher than that for heart disease and cancer combined, making medical biofilms the biggest single disease that the health care system is facing today. It is thus immensely important to better understand biofilms. When I first started studying bacterial pathogenesis, I was struck by the extent that bacteria need to communicate with one another in order to achieve functions necessary for their survival in the host. One can imagine that in a close-knit community such as that found in a biofilm, the bacteria can more effectively protect themselves as well as exploit the environment for their benefit. This book describes the molecular mechanisms of bacterial cell-to-cell communication, the development of anti-biofilm inhibitors, and the use of such inhibitors to suppress bacterial infections in animals and humans.

This book is the product of Dr. Bill Costerton's determination and inspiration to so many of us and to biofilm researchers throughout the world. I am extremely grateful to my co-authors who are carrying out such incredible work with dedication and often with great personal sacrifice, resulting in numerous contributions to science and humanity. Some of us work under the radar to make sure that scientific truth and medical advancement do not get confused with politics, fame, or monetary compensation. We hope this book will benefit all those who care to learn.

North Grafton, November 2007 Naomi Balaban

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List of Abbreviations

aa	amino acid
ABC	ATP-binding cassette
ADI	Urease and arginine deiminase
AHL	*N*-acyl homoserine lactone
AIP	Agr autoinducing peptide
CF	Cystic fibrosis
CFU	Colony forming unit
CSP	Competence stimulating peptide
CVC	Central venous catheter
EM	Electron microscopy
EPS	Extrapolymeric substances
GFP	Green fluorescent protein
HK	Histidine kinase
MRSA	Methicillin-resistant *Staphylococcus aureus*
MRSE	Methicillin-resistant *Staphylococcus epidermidis*
MSSA	Methicillin-sensitive *Staphylococcus aureus*
MSSE	Methicillin-sensitive *Staphylococcus epidermidis*
OHHL	N-3-(oxohexanoyl)-L-homoserine lactone
OD	Optical density
PBP2a	Penicillin-binding protein 2a
PBS	Phosphate buffered saline
PQS	*Pseudomonas* quinolone signal
QS	Quorum sensing
QSA	QS autoinducers
QSI	Quorum sensing inhibitor
QSIS	QSI selector
RAP	RNAIII activating protein
RIP	RNAIII inhibiting peptide
RPB	RAP-binding protein
SAM	S-adenosylmethionine
SEM	Scanning electron microscopy
SQS	Staphylococcus quorum sensing
SRB	Sulfate-reducing bacteria
TRAP	Target of RAP

TNF	Tumor necrosis factor
VISA	Vancomycin-intermediate *S. aureus*
VISE	Vancomycin-intermediate *S. epidermidis*
VRSA	Vancomycin-resistant *S. aureus*
VRSE	Vancomycin-resistant *S. epidermidis*

Introduction

Naomi Balaban[1], Dacheng Ren[2], Michael Givskov[3],
Thomas Bovbjerg Rasmussen[4]

Abstract This introductory chapter discusses the problem of drug resistance and persistent medical biofilm infections, emphasizing the need for alternative approaches to the prevention and treatment of biofilm infections. Such alternative approaches are described in subsequent chapters, culminating with clinical studies that describe treating otherwise untreatable wound infections with the aid of antibiofilm approaches.

1 The Problem: Untreatable Bacterial Infections[1–4]

The discovery of penicillin by Fleming in 1929 opened the era of antimicrobial chemotherapy, which has saved millions of lives by bringing many serious bacterial infections under control (Drews 2000; Fleming 1929). However, this medical miracle is being eroded by the emergence and spread of bacterial drug resistance. This problem has become a serious global issue. For instance, *Staphylococcus aureus* and *S. epidermidis* are leading causes of hospital-acquired infections, and the mortality associated with *S. aureus* bacteremia remains approximately 20–40% despite the availability of effective antimicrobials (Lowy 2003). Of the 2 million nosocomial infections each year, staphylococci cause over 90 000 deaths a year in the United

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N. Balaban (ed.) *Control of Biofilm Infections by Signal Manipulation*
Springer Series on Biofilms 2
doi: 10.1007/978-3-540-73853-4, © Springer 2008

States alone (Lowy 2003). The first effective antibiotic against *S. aureus*, penicillin, became available in the 1940s. Soon after, the bacteria evolved resistance to penicillin, and by the late 1950s, 50% of all *S. aureus* strains were resistant. Today, fewer than 10% of *S. aureus* infections can be cured with penicillin. The next weapons against *S. aureus*, methicillin and cephalosporins, became available in the 1960s and 1970s. By the late 1970s, some strains (2%) of *S. aureus* had evolved resistance to these drugs. Today, as much as 70% of *S. aureus* isolated from U.S. hospitals are resistant to methicillin (Fig. 1). The last effective defense against methicillin-resistant *S. aureus* (MRSA) is vancomycin. However, the increasing use of vancomycin has set the stage for the evolution of vancomycin-resistant *S. aureus* (VRSA) (Lowy 2003; Appelbaum et al. 2006). Over the past 20 years, MRSA infections have been limited primarily to patients in hospitals or long-term-care facilities. However, recent reports of "community-acquired" MRSA infections are alarming.

The same trend is observed for *S. epidermidis*. A study of hundreds of clinical *S. epidermidis* isolates derived from clinical orthopedic infections associated with prosthetic devices indicated that 37–38% were resistant to beta-lactams such as oxacillin and imipenem, while resistance to penicillin, ampicillin, cefazolin, and cefamandole was consistently observed in over 80% of the strains. Forty-one percent were resistant to erythromycin, 16% to clindamycin, 10% to chloramphenicol, 23% to sulfamethoxazole, and 26% to ciprofloxacin (Arciola et al. 2005).

Another example is *Pseudomonas aeruginosa* infections. *P. aeruginosa* is the fourth most commonly isolated nosocomial pathogen, accounting for 10% of all hospital-acquired infections. The gram-negative bacterium *P. aeruginosa* is adept at infecting many different organs and tissues. Because it causes disease primarily in persons whose health is compromised in some manner, it is considered an opportunistic pathogen. Mechanical ventilation, for instance, predisposes patients to pneumonia caused by *P. aeruginosa*. Likewise, the presence of a urinary catheter is associated with an increased risk of urinary tract infections. Patients with cancer who have neutropenia resulting from chemotherapy or hematologic malignan-

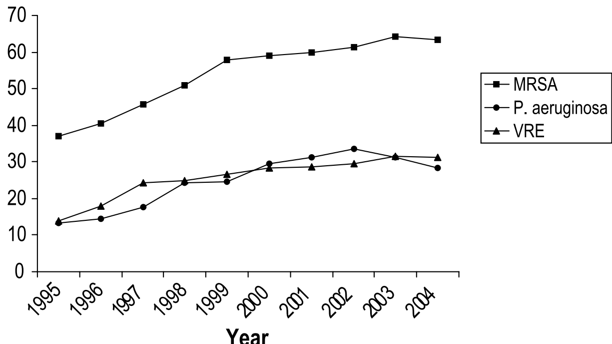

Fig. 1 Percentage of nosocomial infections caused by methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant enterococci (VRE), and fluoroquinolone-resistant *P. aeruginosa* in intensive-care patients in the United States in 1995–2004 (data source: National Nosocomial Infections Surveillance)

cies are prone to bacteremia, and burn patients often experience wound infections. Although each of these infections is most often categorized as hospital-acquired, *P. aeruginosa* frequently causes community-acquired infections in patients with cystic fibrosis (Hauser and Sriram 2005). *P. aeruginosa* is frequently resistant to many commonly used antibiotics. Although many strains are susceptible to gentamicin, tobramycin, colistin, and amikacin, resistant forms have developed; for example, fluoroquinolone-resistant *P. aeruginosa* strains have risen from 14% to 25% in the last 10 years (Fig. 1).

The rapid development of antimicrobial resistance could eventually lead to failure of most, if not all, of the currently available antibiotics. Hence, it poses a great threat to the economy and public health. While the problem is partially caused by overuse of antibiotics, it is also due to the inhibitory mechanisms of presently available antimicrobials. Most of these drugs were discovered for growth inhibition of individual cells in growing cultures—that is, in planktonic conditions (Stewart and Costerton 2001). However, the vast majority of bacteria exist within bacterial communities, otherwise known as biofilms (see below). The biofilm mode of growth plays an important role in antimicrobial resistance: Biofilm cells are up to 1000 times less susceptible to environmental stresses and disinfection treatments than planktonic (free-swimming) cells (Hoyle and Costerton 1991; LeChevallier et al. 1988). Whereas the planktonic cells are easily eliminated, the biofilm cells can survive and therefore provide a source of recontamination in both medical and engineering environments.

In clinical settings, biofilms are believed to be a common cause of persistent infections. The ability of biofilm-forming bacteria, such as *S. aureus*, *S. epidermidis*, and *P. aeruginosa*, to establish sessile communities on inert surfaces of medical devices or on dead as well as living tissue is now being recognized as a major problem (Costerton et al. 1999). Growing in biofilms, bacteria are protected against antibodies, leukocytes, and antibiotics. In addition, biofilms may spawn systemic infections by sloughing of planktonic bacteria, leading to dissemination, bacteremia, sepsis, and death.

2 Biofilm[3,4]

Costerton et al. (1999) proposed a basic definition of biofilm as “a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface.” The matrix components can be exopolysaccharides, proteins, nucleic acids, or other substances (referred to as extrapolymeric substances, or EPS) that are believed to provide the cells with an array of advantages as compared to planktonic cells (Costerton et al. 1987, 1999; Anwar et al. 1990; Matz et al. 2004). This is important, especially in the clinical context, where it is estimated that about 60% of all microbial infections involve bacterial biofilms (Lewis 2001). (Refer to the case studies in the chapter *Clinical Wound Healing Using Signal Inhibitors*.)

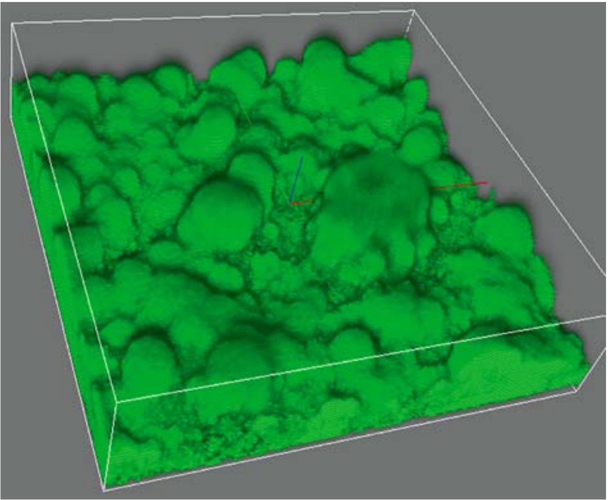

Fig. 2 Biofilm formed by Gfp-tagged *P. aeruginosa* in a continuous flow cell

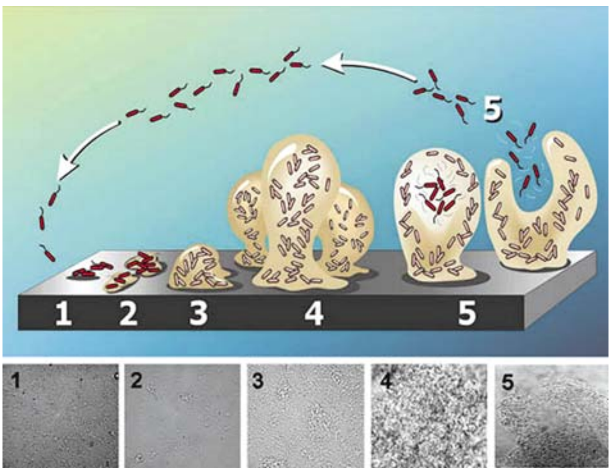

Fig. 3 Development of a *P. aeruginosa* biofilm. (1) Initial attachment. (2) Bacterial adherence. (3) Microcolony formation. (4) Biofilm maturation and development of three-dimensional structures. (5) Release/sloughing of cells able to form new biofilms. Reprinted, with permission, from the Annual Review of Microbiology, Volume 56 © 2002 by Annual Reviews www.annualreviews.org

Biofilms are not homogenous layers of cells; they are highly heterogeneous because they are comprised of patches of cells that are interspersed in the EPS matrix, which itself varies in density. This creates open areas where water channels are formed, allowing nutrients to enter the lower layers of the biofilm and, in addition, allowing waste products to be removed (Davey and O'Toole 2000; Dunne 2002). The bacteria found in a biofilm can either be of one species or it can, depending on the environment, be composed of multiple species.

In vitro biofilm formation by *P. aeruginosa* is one of the most intensively studied cases. After initial attachment of *P. aeruginosa* to a surface, microcolonies are formed, which in turn can grow to larger structures such as towers and mushrooms (Figs. 2 and 3). Recent analysis based on transcriptomics revealed that biofilm cells express their genes in a pattern that differs from that expressed by most stages of growth of planktonic bacteria, and the bulk of biofilm cells, even in the early stages, express genes in a pattern that is reminiscent of gene expression seen in the early stationary phase of planktonic cells (Hentzer et al. 2005). Although the experimental conditions would differ in the various experiments, the existence of a specific biofilm program would always require a core set of genes to be expressed, regardless of the experimental conditions. To date, transcriptomic studies such as of *P. aeruginosa* biofilms have not delivered such an outcome, and it strongly suggests that multiple pathways exist by which a biofilm can be built. Regardless, what is becoming evident is that bacterial cell-to-cell communication is required for a successful biofilm to form in vivo; this is discussed in subsequent chapters.

3 Resistance to Antibiotics[1–4]

3.1 Inherent Bacterial Resistance to Antibiotics

P. aeruginosa and *S. aureus* will be used here as examples of antibiotic resistance. Several factors contribute to the antibiotic resistance of *P. aeruginosa*. It appears that the bacterium has an intrinsic resistance conferred by lowered permeability of the outer membrane as well as efflux pumps that rapidly shuttle many different compounds out of the cell (Hancock 1998; Lee et al. 2000). Five different efflux systems have been identified in *P. aeruginosa*, but the sequence analysis by Stover et al. (2000) suggests that there may be up to 30. The identified systems include the MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexJK-OprM, and MexXY-OprM systems (Adewoye et al. 2002). The highly homologous efflux pump proteins consist of a cytoplasmic-membrane-associated drug-proton antiporter, a membrane channel-forming protein, and a periplasmic fusion protein. The pumps have broad specificity and transport varying molecules, including dyes, detergents, antibiotics, organic solvents, and secondary metabolites and signaling molecules such as *N*-acyl homoserine lactone (AHLs) (Poole and Srikumar 2001). The antibiotics to which the multidrug efflux pumps confer resistance include chloramphenicol, gentamicin, trimethoprim, imipenem, and tetracycline as well as other quinolones, macrolides, and beta-lactams (Kohler et al. 1997, 1999; Yoneyama et al. 1997; Pumbwe and Piddock 2000). Other compounds also affected by the action of the pumps include the heavy metal vanadium (Aendekerk et al. 2002). In addition, *P. aeruginosa* (and staphylococci; see below) produce beta-lactamases encoded on the chromosome, conferring enhanced resistance to beta-lactam-based antibiotics such as imipenem (Bagge et al. 2002).

Staphylococcal antibiotic resistance has been extensively reviewed (Lowy 2003). As described, staphylococcal resistance to penicillin is mediated by *blaZ*, the chromosomal gene that encodes beta-lactamase. Methicillin resistance (leading to MRSA strains) requires the presence of the chromosomally localized *mecA* gene Chambers 1997). *mecA* is responsible for synthesis of penicillin-binding protein 2a (PBP2a) (Hartman and Tomasz 1984; Utsui and Yokota 1985; Song et al. 1987). PBPs are membrane-bound enzymes that catalyze the transpeptidation reaction that is necessary for cross-linkage of peptidoglycan chains (Ghuysen 1994). PBP2a substitutes for the other PBPs and, because of its low affinity for all beta-lactam antibiotics, enables staphylococci to survive exposure to high concentrations of these agents. Thus, resistance to methicillin confers resistance to all beta-lactam agents, including cephalosporins. Resistance to quinolones results from the stepwise acquisition of chromosomal mutations. The quinolones act on DNA gyrase, which relieves DNA supercoiling, and topoisomerase IV, which separates concatenated DNA strands. Amino acid changes in critical regions of the enzyme DNA complex (quinolone resistance-determining region) reduce quinolone affinity for both of its targets. The ParC subunit (GrlA in *S. aureus*) of topoisomerase IV and the GyrA subunit in gyrase are the most common sites of resistance mutations; topoisomerase IV mutations are the most critical because they are the primary drug targets in staphylococci (Hooper 2002; Ng et al. 1996).

The reduced susceptibility to vancomycin appears to result from changes in peptidoglycan biosynthesis. The VISA (vancomycin-intermediate *S. aureus*) strains are notable for the additional quantities of synthesized peptidoglycan that result in irregularly shaped, thickened cell walls. There is also decreased cross-linking of peptidoglycan strands, which leads to the exposure of more D-Ala-D-Ala residues (Hanaki et al. 1998a, b). As a result, more D-Ala-D-Ala residues are available to bind and trap vancomycin. The bound vancomycin then acts as a further impediment to drug molecules reaching their target on the cytoplasmic membrane. The second form of vancomycin resistance results from the probable conjugal transfer of the *vanA* operon from a vancomycin-resistant *E. faecalis*. Resistance in these VRSA isolates is caused by alteration of the terminal peptide to D-Ala-D-Lac instead of D-Ala-D-Ala. Synthesis of D-Ala-D-Lac occurs only with exposure to low concentrations of vancomycin (Lowy 2003).

3.2 Resistance of Biofilms to Antibiotics

Generally, resistance to a drug or a heavy metal means that a bacterium can grow and form a culture or colony in the presence of that particular drug or heavy metal. Tolerance, on the other hand, refers to the situation in which a bacterial culture is not eradicated by treatments with that particular drug. Whether it is resistance or tolerance, both may contribute to the fact that the biofilm mode of growth enables the bacteria to survive the exposure to 1000-fold higher concentrations of a number of antibiotics compared with their growing counterparts (Anwar et al. 1990; Allison and Gilbert 1995; Teitzel and Parsek 2003). In other words, the underlying mechan-

ism responsible for biofilm resistance is multifactorial, and in the literature (as in the present book), authors usually do not discriminate between resistance and tolerance, because the underlying mechanism in the context of a biofilm is unknown.

Restricted penetration of antimicrobial compounds into the biofilm accounts for some of the resistance. This is especially true for some compounds such as aminoglycosides but not as much for others, such as fluoroquinolones. Because restricted penetration is based on binding of the molecules to, most probably, the EPS matrix, it is believed that at some point, the matrix becomes saturated, and penetration will eventually occur without delay. On the other hand, EPS is probably constantly being produced, creating new spots for antimicrobial binding (Lewis 2001; Campanac et al. 2002; Drenkard 2003; Teitzel and Parsek 2003).

Another factor adding greatly to biofilm tolerance is the very heterogeneous metabolic activity of the biofilm cells. In a biofilm, there exist gradients of nutrients and oxygen, which limit the growth rate of most of the cells (except for the cells on the surface of the film). Because antimicrobials mostly target metabolically active cells, the large slow or nongrowing parts of the biofilms are very difficult to target. Some antibiotics have reduced activity in oxygen-deprived environments, which also contributes to biofilm resistance as availability of oxygen is reduced in deeper levels of a biofilm (Lewis 2001; Drenkard 2003). Another option is the expression of certain genes in a biofilm, conferring enhanced resistance to antibiotics. The exact nature of these genes remains to be elucidated (Lewis 2001; Drenkard 2003; Sauer et al. 2002; Whiteley et al. 2001). A glimpse into this is provided by Drenkard and Ausubel (2002), who identified a gene, *pvrR* (phenotypic variant regulator), that is involved in conversion of wild type *P. aeruginosa* into a rough-colony phenotypic variant. The rough-colony variant has a highly elevated resistance to antibiotics (Drenkard and Ausubel 2002; Drenkard 2003). Mah et al. (2003) recently reported on the presence of a specific gene product that inactivates tobramycin by binding to the drug in *P. aeruginosa* PA14 is upregulated in biofilms. The locus identified, *ndvB*, is required for the synthesis of periplasmic glucans. These periplasmic glucans interact physically with drugs and therefore might prevent antibiotics such as gentamicin, ciprofloxacin, chloramphenicol, and ofloxacin from reaching their sites of action by sequestering these antimicrobial agents in the periplasm.

In staphylococci, persistence within a biofilm requires an adaptive response that limits the deleterious effects of the reduced pH associated with anaerobic growth conditions (Beenken et al. 2004). Several of the operons that were induced in biofilms have also been found to be important in acid tolerance in other bacterial species, including the oral bacteria *Streptococcus salivarius* (Li et al. 2000), and have been correlated with virulence in *Streptococcus pyogenes*. Bacteria can combat acidic environments by producing alkaline compounds such as ammonia. Two ways in which bacteria generate ammonia are through the urease and arginine deiminase (ADI) pathways. Multiple genes from both of these pathways [arginine deiminase (*arcA*), ornithine transcarbamylase (*arcB*), and carbamate kinase (*arcC*)] were indeed induced in *S. aureus* biofilms in comparison to both planktonic conditions (Beenken et al. 2004).

An additional gene upregulated in *S. aureus* biofilm is the arginine repressor encoded by *argR*. Under anaerobic conditions in the presence of arginine, ArgR represses anabolic ornithine carbamoyltransferase and induces the ADI pathway. Ammonia generated by the deimination of arginine can neutralize acids generated by bacterial glycolysis (Beenken et al. 2004).

Also included among the genes induced in biofilms are seven genes that comprise the urease operon (Beenken et al. 2004). Urease (urea amidohydrolase) is a nickel-containing enzyme that catalyzes the hydrolysis of urea to yield two molecules of ammonia and one molecule of CO_2. Ureases of most bacteria are composed of three distinct subunits encoded by three contiguous genes, *ureA*, *ureB*, and *ureC*. Urease activity is essential for colonization of the gastric mucosa by *Helicobacter pylori* and colonization of the urinary tract by both *Proteus mirabilis* and *Staphylococcus saprophyticus* (Eaton et al. 2002; Gatermann and Marre 1989; Jones et al. 1990). In addition, urease is thought to play a central role in the pathogenesis of *Ureaplasma urealyticum* urinary and respiratory tract infections (Hedelin et al. 1984; Ligon and Kenny 1991).

Several operons of the pyrimidine nucleotide biosynthetic (*pyr*) pathway (*pyrRPBC, carAB*, and *pyrFE*) are also induced in biofilms. The pathway for the de novo synthesis of pyrimidines consists of six enzymatic steps leading to the formation of UMP. This is important because the level of UMP in cells growing in a biofilm is severely limited. In addition, upregulation of the *pyr* operon may be required for synthesis of sufficient levels of arginine to be used by the ADI pathway during anaerobic growth (Beenken et al. 2004).

Taken together, as indicated by microarray studies (Beenken et al. 2004; Hentzer et al. 2005), mature biofilms grow anaerobically, and genes of the acid tolerance response are upregulated in response to an acidic environment. Global regulators such as the quorum-sensing sensor TRAP (Korem et al. 2005; see also below), sigma factor B (SigB), and staphylococcal accessory regulator (SarA) are involved (Beenken et al. 2004).

Although more work is needed to fully understand antimicrobial resistance in biofilms, it is clearly not caused by a single mechanism but by several factors acting in concert. First, as mentioned above, bacterial cells in biofilms produce a matrix of polysaccharide, which may retard or block antimicrobial agents from reaching the cell (Elvers and Lappin-Scott 2000). In addition, it was found that 40% of the cell wall proteins in biofilm cells are different from those of planktonic cells; therefore, the permeability of the cell membrane may change (Potera 1999), making it difficult for antimicrobials or immune factors to reach their targets. Recent studies have also shown that biofilm cells have profound changes in gene expression and cell physiology compared with planktonic cells, even though they have identical genotypes (Hall-Stoodley et al. 2004; Prigent-Combaret et al. 1999).

Most antimicrobials inhibit growth-related cellular activities, such as protein, DNA, and cell wall synthesis. Hence, they are not efficient against biofilm cells that have slow or even no growth (Potera 1999; Xu et al. 2000). Furthermore, the close cell-to-cell contact in biofilms provides a favorable environment for horizontal gene transfer (Li et al. 2001b), which results in easy spread of antimicrobial resistance.

Although several factors are responsible for antimicrobial resistance in biofilms, they are all related to the multicellular nature of biofilm communities (Stewart and Costerton 2001) because it has been shown that the biofilm cells lose their resistance rapidly after resuspension and planktonic growth (Anwar et al. 1989).

The next chapters will address how understanding bacterial cell-to-cell communication and bacterial response to the environment can lead to the development of novel therapeutics.

References

Adewoye L, Sutherland A, Srikumar R, Poole K (2002) The mexR repressor of the mexAB-oprM multidrug efflux operon in *Pseudomonas aeruginosa*: characterization of mutations compromising activity. J Bacteriol 184:4308–4312

Aendekerk S, Ghysels B, Cornelis P, Baysse C (2002) Characterization of a new efflux pump, MexGHI-OpmD, from *Pseudomonas aeruginosa* that confers resistance to vanadium. Microbiology 148:2371–2381

Allison DG, Gilbert P (1995) Modification by surface association of antimicrobial susceptibility of bacterial populations. J Ind Microbiol 15:311–317

Anwar H, Dasgupta MK, Costerton JW (1990) Testing the susceptibility of bacteria in biofilms to antibacterial Agents. Antimicrob Agents Chemother 34:2043–2046

Anwar H, van Biesen T, Dasgupta M, Lam K, Costerton JW (1989) Interaction of biofilm bacteria with antibiotics in a novel in vitro chemostat system. Antimicrob Agents Chemother 33:1824–1826

Appelbaum PC (2006) MRSA—the tip of the iceberg. Clin Microbiol Infect 12(Suppl 2):3–10

Arciola CR, Campoccia D, Gamberini S, Donati ME, Pirini V, Visai L, Speziale P, Montanaro L (2005) Antibiotic resistance in exopolysaccharide-forming *Staphylococcus epidermidis* clinical isolates from orthopaedic implant infections. Biomaterials 26:6530–6535

Bagge N, Ciofu O, Hentzer M, Campbell JI, Givskov M, Hoiby N (2002) Constitutive high expression of chromosomal beta-lactamase in *Pseudomonas aeruginosa* caused by a new insertion sequence (IS1669) located in *ampD*. Antimicrob Agents Chemother 46:3406–3411

Beenken KE, Dunman PM, McAleese F, Macapagal D, Murphy E, Projan SJ, Blevins JS, Smeltzer MS (2004) Global gene expression in Staphylococcus aureus biofilms. J Bacteriol 186:4665–4684

Campanac C, Pineau L, Payard A, Baziard-Mouysset G, Roques C (2002) Interactions between biocide cationic agents and bacterial biofilms. Antimicrob Agents Chemother 46:1469–1474

Costerton JW, Stewart PS, Greenberg EP (1999) Bacterial biofilms: a common cause of persistent infections. Science 284:1318–1322

Costerton JW, Cheng KJ, Geesey GG, Ladd TI, Nickel JC, Dasgupta M, Marrie TJ (1987) Bacterial biofilms in nature and disease. Annu Rev Microbiol 41:435–464

Davey ME, O'Toole GA (2000) Microbial biofilms: from ecology to molecular genetics. Microbiol Mol Biol Rev 64:847–867

Drenkard E (2003) Antimicrobial resistance of *Pseudomonas aeruginosa* biofilms. Microbes Infect 5:1213–1219

Drenkard E, Ausubel FM (2002) *Pseudomonas* biofilm formation and antibiotic resistance are linked to phenotypic variation. Nature 416:740–743

Drews J (2000) Drug discovery: a historical perspective. Science 287:1960–1964

Dunne WM Jr (2002) Bacterial adhesion: seen any good biofilms lately? Clin Microbiol Rev 15:155–166

Eaton KA, Gilbert JV, Joyce EA, Wanken AE, Thevenot T, Baker P, Plaut A, Wright A (2002) In vivo complementation of *ureB* restores the ability of *Helicobacter pylori* to colonize. Infect Immun 70:771–778

Elvers KT, Lappin-Scott HM (2000) Biofilms and biofouling, 2nd edn. Academic Press, San Diego

Fleming A (1929) On the antibacterial action of cultures of a *Penicillium*, with special reference to their use in the isolation of *B. influenzae*. Br J Exp Pathol 10:226–236

Gatermann S, Marre R (1989) Cloning and expression of *Staphylococcus saprophyticus* urease gene sequences in *Staphylococcus carnosus* and contribution of the enzyme to virulence. Infect Immun 57:2998–3002

Ghuysen JM (1994) Molecular structures of penicillin-binding proteins and beta-lactamases. Trends Microbiol 2:372–380

Hall-Stoodley L, Costerton JW, Stoodley P (2004) Bacterial biofilms: from the natural environment to infectious diseases. Nat Rev Microbiol 2:95–108

Hanaki H, Labischinski H, Inaba Y, Kondo N, Murakami H, Hiramatsu K (1998a) Increase in glutamine-non-amidated muropeptides in the peptidoglycan of vancomycin-resistant *Staphylococcus aureus* strain Mu50. J Antimicrob Chemother 42:315–320

Hanaki H, Kuwahara-Arai K, Boyle-Vavra S, Daum RS, Labischinski H, Hiramatsu K (1998b) Activated cell-wall synthesis is associated with vancomycin resistance in methicillin-resistant *Staphylococcus aureus* clinical strains Mu3 and Mu50. J Antimicrob Chemother 42:199–209

Hancock RE (1998) Resistance mechanisms in *Pseudomonas aeruginosa* and other nonfermentative gram-negative bacteria. Clin Infect Dis 27(Suppl 1):S93–S99

Hartman BJ, Tomasz A (1984) Low-affinity penicillin-binding protein associated with beta-lactam resistance in *Staphylococcus aureus*. J Bacteriol 158:513–516

Hauser AR, Sriram P (2005) Severe *Pseudomonas aeruginosa* infections. Tackling the conundrum of drug resistance. Postgrad Med 117:41–48

Hedelin H, Brorson JE, Grenabo L, Pettersson S (1984) Ureaplasma urealyticum and upper urinary tract stones. Br J Urol 56:244–249

Hentzer M, Eberl L, Givskov M (2005) Transcriptome analysis of *Pseudomonas aeruginosa* biofilm development: anaerobic respiration and iron limitation. Biofilms 2:37–61

Hooper DC (2002) Fluoroquinolone resistance among Gram-positive cocci. Lancet Infect Dis 2:530–538

Hoyle BD, Costerton JW (1991) Bacterial resistance to antibiotics: the role of biofilms. Prog Drug Res 37:91–105

Jones BD, Lockatell CV, Johnson DE, Warren JW, Mobley HL (1990) Construction of urease-negative mutant of *Proteus mirabilis*: analysis of virulence in a mouse model of ascending urinary tract infection. Infect Immun 58:1120–1123

Kohler T, Epp SF, Curty LK, Pechere JC (1999) Characterization of MexT, the regulator of the MexE-MexF-OprN multidrug efflux system of *Pseudomonas aeruginosa*. J Bacteriol 181:6300–6305

Kohler T, Michea-Hamzehpour M, Henze U, Gotoh N, Curty LK, Pechere JC (1997) Characterization of MexE-MexF-OprN, a positively regulated multidrug efflux system of *Pseudomonas aeruginosa*. Mol Microbiol 23:345–354

Korem M, Gov Y, Kiran MD, Balaban N (2005) Transcriptional profiling of target of RNAIII-activating protein, a master regulator of staphylococcal virulence. Infect Immun 73:6220–6228

LeChevallier MW, Cawthon CD, Lee RG (1988) Inactivation of biofilm bacteria. Appl Environ Microbiol 54:2492–2499

Lee A, Mao W, Warren MS, Mistry A, Hoshino K, Okumura R, Ishida H, Lomovskaya O (2000) Interplay between efflux pumps may provide either additive or multiplicative effects on drug resistance. J Bacteriol 182:3142–3150

Lewis K (2001) Riddle of biofilm resistance. Antimicrob Agents Chemother 45:999–1007

Li YH, Chen YY, Burne RA (2000) Regulation of urease gene expression by *Streptococcus salivarius* growing in biofilms. Environ Microbiol 2:169–177

Li YH, Lau PC, Lee JH, Ellen RP, Cvitkovitch DG (2001b) Natural genetic transformation of *Streptococcus mutans* growing in biofilms. J Bacteriol 183:897–908

Ligon JV, Kenny GE (1991) Virulence of ureaplasmal urease for mice. Infect Immun 59:1170–1171

Lowy FD (2003) Antimicrobial resistance: the example of *Staphylococcus aureus*. J Clin Invest 111:1265–1273

Mah TF, Pitts B, Pellock B, Walker GC, Stewart PS, O'Toole GA (2003) A genetic basis for *Pseudomonas aeruginosa* biofilm antibiotic resistance. Nature 426:306–310

Matz C, Bergfeld T, Rice SA, Kjelleberg S (2004) Microcolonies, quorum sensing and cytotoxicity determine the survival of *Pseudomonas aeruginosa* biofilms exposed to protozoan grazing. Environ Microbiol 6:218–226

Ng EY, Trucksis M, Hooper DC (1996) Quinolone resistance mutations in topoisomerase IV: relationship to the flqA locus and genetic evidence that topoisomerase IV is the primary target and DNA gyrase is the secondary target of fluoroquinolones in *Staphylococcus aureus*. Antimicrob Agents Chemother 40:1881–1888

O'Toole GA, Gibbs KA, Hager PW, Phibbs PV Jr, Kolter R (2000) The global carbon metabolism regulator Crc is a component of a signal transduction pathway required for biofilm development by *Pseudomonas aeruginosa*. J Bacteriol 182:425–431

Poole K, Srikumar R (2001) Multidrug efflux in *Pseudomonas aeruginosa*: components, mechanisms and clinical significance. Curr Top Med Chem 1:59–71

Potera C (1999) Forging a link between biofilms and disease. Science 19:1837–1838

Prigent-Combaret C, Vidal O, Dorel C, Lejeune P (1999) Abiotic surface sensing and biofilm-dependent regulation of gene expression in *Escherichia coli*. J Bacteriol 181:5993–6002

Pumbwe L, Piddock LJ (2000) Two efflux systems expressed simultaneously in multidrug-resistant *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 44:2861–2864

Sauer K, Camper AK, Ehrlich GD, Costerton JW, Davies DG (2002) *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. J Bacteriol 184:1140–1154

Song MD, Wachi M, Doi M, Ishino F, Matsuhashi M (1987) Evolution of an inducible penicillin-target protein in methicillin-resistant *Staphylococcus aureus* by gene fusion. FEBS Lett 221:167–171

Stewart PS, Costerton JW (2001) Antibiotic resistance of bacteria in biofilms. Lancet 358:135–138

Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrener P, Hickey MJ, Brinkman FS, Hufnagle WO, Kowalik DJ, Lagrou M, Garber RL, Goltry L, Tolentino E, Westbrock-Wadman S, Yuan Y, Brody LL, Coulter SN, Folger KR, Kas A, Larbig K, Lim R, Smith K, Spencer D, Wong GK, Wu Z, Paulsen IT, Reizer J, Saier MH, Hancock RE, Lory S, Olson MV (2000) Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. Nature 406:959–964

Teitzel GM, Parsek MR (2003) Heavy metal resistance of biofilm and planktonic *Pseudomonas aeruginosa*. Appl Environ Microbiol 69:2313–2320

Utsui Y, Yokota T (1985) Role of an altered penicillin-binding protein in methicillin- and cephem-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 28:397–403

Whiteley M, Bangera MG, Bumgarner RE, Parsek MR, Teitzel GM, Lory S, Greenberg EP (2001) Gene expression in *Pseudomonas aeruginosa* biofilms. Nature 413:860–864

Xu KD, McFeters GA, Stewart PS (2000) Biofilm resistance to antimicrobial agents. Microbiology 146:547–549

Yoneyama H, Ocaktan A, Tsuda M, Nakae T (1997) The role of mex-gene products in antibiotic extrusion in *Pseudomonas aeruginosa*. Biochem Biophys Res Commun 233:611–618

Bacterial Cell-to-cell Communication (Quorum Sensing)

Michael Givskov[1], Thomas Bovbjerg Rasmussen[2], Dacheng Ren[3], Naomi Balaban[4]

Abstract Within the biofilm, the bacteria use cell-to-cell communication systems to pool their activities and act in a multicellular organized manner. One such activity is to launch their arsenal of virulence factors at the strategically right moment, and hence coordinate the progressive attack on the host. This process is termed quorum sensing (QS), whereby bacteria produce diffusible chemical signals (autoinducers) that interact with specific receptors on itself and on neighboring cells, which in turn regulate the expression of specific target genes. By integrating this with other environmental stimuli, bacteria are capable of exhibiting complex responses and take part in sophisticated interactions, allowing them to survive in most adverse environments. This chapter describes the molecular mechanisms of QS in Gram-negative and gram positive bacteria, and QS in a biofilm, leading to what is described in subsequent chapters that QS is a highly attractive target for therapy against biofilm chronic infections.

Well within the biofilm, the bacteria use cell-to-cell communication systems to pool their activities and act in a multicellular organized manner. One such activity is to launch their arsenal of virulence factors at the strategically right moment and hence coordinate the progressive attack on the host. The view of bacterial biofilms as sanc-

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N. Balaban (ed.) *Control of Biofilm Infections by Signal Manipulation*
Springer Series on Biofilms 2
doi: 10.1007/978-3-540-73853-4, © Springer 2008

tuaries in a hostile environment has gained momentum over the last decade. Bacteria released from these protected areas are then able to spark systemic infections (Costerton et al. 2003). The ability of coordinating gene expression in accordance with population density and hence to act as a group is a process termed **quorum sensing** (QS) (Fuqua et al. 1994; Withers et al. 2001) (see Fig. 1). The amount of bacteria needed to activate QS-controlled genes are known as the "quorum" or the "quorum size," reflecting the number of individual cells needed to make a qualified decision.

The underlying mechanism of QS is the production of diffusible chemical signal molecules by the bacteria (autoinducers) that interact with specific receptors on self and on neighboring cells, which in turn regulate expression of specific target genes. By integrating this with other environmental signals and stimuli, bacteria are capable of exhibiting complex responses and taking part in sophisticated interactions (Gray 1997).

QS is implicated in the regulation of phenotypes that are also involved in interactions with higher organisms. These interactions can be beneficial to the host, or they can be pathogenic. From an evolutionary point of view, it makes sense that the underlying factors are produced only when the bacterial population is sufficiently large to confer a significant effect (Velicer 2003). QS systems form the command line of opportunistic pathogens such as *P. aeruginosa* and *S. aureus* (Winzer and Williams 2001). Expressing the battery of antigenic determinants such as host-damaging virulence factors only when the bacterial population has reached a high level is believed to be a "stealthy strategy"; by the time the host organism realizes it is under attack, it has been left with a poor possibility of mounting an effective defense against the intruder (Donabedian 2003; de Kievit and Iglewski 2000; Parsek and Greenberg 2000).

To date, several types of QS systems are known: one for gram-positive bacteria relying on polypeptides (Abraham 2006; Balaban et al. 1998; Waters and Bassler 2005) and another for gram-negative bacteria mediated by *N*-acyl homoserine lactone (AHL) derivatives (Eberhard et al. 1981; Nasser and Reverchon 2007). A third type of QS system, AI-2, has been proposed as a global signaling system common to all bacteria (Winans and Bassler 2002; Waters and Bassler 2005).

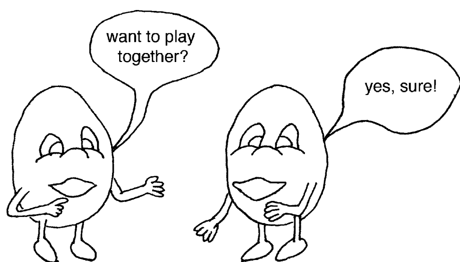

Fig. 1 Bacterial cell-to-cell communication (quorum sensing) (Illustration by Mike Beshiri, Tufts University, Cummings School of Veterinary Medicine, Department of Biomedical Sciences, Division of Infectious Diseases, North Grafton, MA, USA)

1 AHL-mediated Quorum Sensing in Gram-negative Bacteria1[1–4]

The core elements of all AHL-based QS systems are a gene termed the *luxI* homolog encoding an AHL synthetase and a *luxR* homolog encoding the signal receptor protein, which also acts as a response regulator. At low cell densities, the *luxI* homolog is expressed constitutively at a low level; hence, AHLs are synthesized in small quantities, which slowly accumulate in the environment around the bacteria, depending on the diffusion restraints (Fuqua and Greenberg 2002). Accordingly, there is a correlation between AHL concentration and population density – more cells results in more signal molecules per volume. Work on the *Vibrio fischeri* QS system has given rise to a "QS dogma," which states that when a sufficient population density has been attained, QS target genes become activated. The signal molecules bind to the LuxR homolog receptor proteins, inducing a conformational change and allowing the proteins to form dimers or multimers. This in turn enables the receptor multimer to bind to DNA and act as a transcriptional regulator. The activity of a QS-controlled gene is determined by the concentration of activated LuxR homolog multimer, which in turn is dependent on the concentration of AHL signal, which again is dependent on the population size/density. In other words, activation of QS-controlled genes relies on both the concentration of AHL signal molecules and the amount of available LuxR homolog receptor protein.

Gram-negative bacteria that are unable to synthesize signal molecules are still able to perceive and respond to the AHL signal molecules. These include *Salmonella typhimurium* and *Escherichia coli*, which both posses a gene, *sdiA*, that is a LuxR homolog. The SdiA receptor is responsive to 3-oxo-C6 and 3-oxo-C8 signal molecules, enabling the bacteria to sense the presence of other AHL-producing bacteria in a mixed community and to respond to the AHL signal molecules.

1.1 Multiple Quorum-sensing Systems Regulate Virulence

The model gram-negative bacterium *P. aeruginosa* produces two QS signals: *N*-(3-oxododecanoyl)-L-homoserine lactone [OdDHL, synthesized by LasI and sensed by LasR (Gambello and Iglewski 1991; Ochsner et al. 1994)] and *N*-butanoyl-L-homoserine lactone [BHL, synthesized by RhlI and sensed by RhlR (Pearson et al. 1994)]. With respect to function, they are organized in a hierarchical manner, the former controlling the expression of the latter. This allows for further fine-tuning of the responses of QS target genes. Sequence analysis suggests that these systems have been acquired independently rather than arising by endogenously gene duplication – the *las* system in *P. aeruginosa* shows no affiliation with the *rhl* system. This indicates that horizontal gene transfer is a mechanism for the spread of, and is important for the prevalence of, QS controllers.

1.2 AHL Signal Generation

The LuxI and homologs direct synthesis of the AHLs. The signal molecules consist of an invariable highly conserved lactone ring and a variable acyl side chain [Fig. 2a,b (AHL or AI-1)]. The side chains differ in length (2–18 carbon atoms), but all side chains contain a keto group on the C1 position and various degrees of substitution on C3. Molecules that carry a keto oxygen on the C3 carbon are referred

Fig. 2 Structures of representative quorum-sensing signals and brominated furanones. **a** *N*-butanoyl-L-homoserine lactone [encoded by *P. aeruginosa* RhlI (Pearson et al. 1994)). **b** *N*-(3-oxododecanoyl)-L-homoserine lactone [encoded by *P. aeruginosa* LasI (Gambello and Iglewski 1991; Ochsner et al. 1994)]. **c** (2S, 4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate [AI-2 of *V. harveyi* (Chen et al. 2002)]. **d** (2R, 4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran [AI-2 of *Salmonella typhinurium* (Miller et al. 2004)]. **e** (5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(5*H*)-furanone, natural furanone from *D. pulchra* (de Nys et al. 1993)

to as oxo-HSLs. A few rare AHL species contain a hydroxyl substitution on the C3 position, whereas others posses a double bond in the side chain.

The LuxI synthases use S-adenosylmethionine (SAM) as HSL donor and acyl-acyl carrier protein (acyl-ACP) or acyl-coenzyme A from the fatty acid synthesis complex as acyl donor (Parsek et al. 1999). The different side chains probably reflect variations in acyl-ACP specificity. Conversely, the specificity for SAM must be similar for all LuxI homologs as the HSL ring is invariable (Watson et al. 2002).

After synthesis, the signal molecules enter the surrounding environment, either by passive diffusion in the case of C4 HSL or active efflux in the case of 3-oxo-C12 HSL (Pearson et al. 1999; Waters and Bassler 2005).

1.3 Signal Reception and Response Regulation

Perception of the signal molecules and subsequent gene regulation are performed by the LuxR homologs. LuxR homolog proteins contain two functional domains, the AHL-binding N-terminal and a DNA-binding C-terminal (Hanzelka and Greenberg 1995; Egland and Greenberg 2001; Koch et al. 2005). In LuxR, it has been found that the C-terminal third part of the 250-residue protein has a helix-turn-helix motif able to bind to DNA and is able to activate LuxR-controlled genes independently of AHL (Choi and Greenberg 1991). This has led to the model that the N-terminal two-thirds of the protein quenches the DNA-binding activity. Another truncated LuxR, consisting of the N-terminal part, has been shown to bind AHL and in turn activates the C-terminal part (Hanzelka and Greenberg 1995). Activated LuxR homologs are thought to bind as dimers capable of interacting with the promoter regions of QS-controlled genes (Vannini et al. 2002; Zhang et al. 2002b; Ledgham et al. 2003). The *lux* box, where the dimer binds, is a palindromic sequence centered −42.5 bp upstream of the *luxI* start codon. The dimer overlaps the −35 region and acts as an ambidextrous activator of transcription (Egland and Greenberg 1999). Located at this position, the LuxR dimer interacts with the alpha-subunit C-terminal domain of RNA polymerase, where two residues of the subunit interact directly with the C-terminal part of LuxR (Finney et al. 2002; Johnson et al. 2003). Alanine mutation scanning of the N-termini of LuxR has provided evidence for a direct correlation between the binding of LuxR to the *lux* box and activation of QS-controlled target genes (Trott and Stevens 2001; Egland and Greenberg 2001). It appears that each LuxR homolog protein has its own *lux* box type of binding site, and similar binding sites have been identified for LasR and RhlR in *P. aeruginosa*. These *las* and *rhl* boxes are important for the expression of QS target genes such as *lasB, hcnA*, and others (Whiteley and Greenberg 2001). Two *las* boxes are located upstream of the *lasB* gene; one is located directly upstream of the transcriptional initiation site, whereas the other is placed 102 bp upstream. Both participate in controlling *lasB* expression (Rust et al. 1996; Fukushima et al. 1997).

1.4 AI-2 Signaling

AI-2 was initially identified for its control of the expression of bioluminescence in the marine bacterium *Vibrio harveyi* (Bassler et al. 1993) and was identified as a furanosyl borate diester. The AI-2 (Fig. 2c,d) and its synthase LuxS have been identified in a few bacterial species (Chen et al. 2002; Miller et al. 2004, Schauder et al. 2001; Xavier and Bassler 2005). However, the presence of *luxS* analogs in more than 55 species indicates that it is widely used in cell-to-cell signaling (Vendeville et al. 2005) to regulate genes specifying diverse functions, such as those encoding virulence factors in *Actinobacillus actinomycetemcomitans*, enterohemorrhagic *E. coli* (EHEC) O157:H7, *P. gingivalis*, *Streptococcus pyogenes*, *Vibreio cholerae*, and *V. vulnificus*; motility in *Campylobacter jejuni*, EHEC O157:H7, and enteropathogenic *E. coli* O127:H6; cell division in *E. coli* W3110 and EHEC O157:H7; antibiotic production in *Photorhabdus luminescens*; and biofilm formation and carbohydrate metabolism in *Streptococcus gordonii* (Xavier and Bassler 2005; Gonzalez Barrios et al. 2006; Ren et al. 2004b; Sperandio et al. 1999). In *S. aureus*, functional analysis of *luxS*/AI-2 reveals a role in metabolism but not quorum sensing, in which inactivation of *luxS* did not affect virulence-associated traits such as production of hemolysins and extracellular proteases, biofilm formation, and the *agr* QS signaling system (Doherty et al. 2006).

1.5 Other Quorum-signaling Systems

At least two additional QS systems have been identified in gram-negative bacteria. These include autoinducer 3 (AI-3), which is associated with virulence regulation in EHEC O157:H7 (Sperandio et al. 2003), and the *Pseudomonas* quinolone signal (PQS), which is associated with *P. aeruginosa* (Mashburn and Whiteley 2005). AI-3 is associated with *luxS* homologs in EHEC O157:H7, but the signal itself is hydrophobic and thus chemically distinct from the polar AI-2 signals (Sperandio et al. 2003). AI-3 is also biologically distinct from AI-2. During EHEC pathogenesis, both AI-3 and host epinephrine, but not AI-2, stimulate expression of the locus of enterocyte effacement (LEE) genes and thus provide evidence of bacteria and host cross-talk during this infection (Walters and Sperandio 2006). PQS molecules are quite hydrophobic and have been shown to be transported between cells by outer membrane vesicles. There is also strong evidence that the PQS actually induces the formation of these vesicles through interference with Mg^{2+} and Ca^{2+} ions in the outer membrane (Mashburn and Whiteley 2005). In a recent review (Mashburn-Warren and Whiteley 2006), it was suggested that membrane vesicles may represent a mechanism for interkingdom signaling in the plant rhizosphere.

1.6 AHL QS Interference with the Host Immune System

Eukaryotic cells communicate by means of hormones and prostaglandins that are structurally related to the bacterial AHLs, and it has been investigated whether the signal molecules produced by *P. aeruginosa* are able to interact directly with the human host cells (Smith and Iglewski 2003). Indeed, 3-oxo-C12 HSL was found to inhibit lymphocyte proliferation and tumor necrosis factor alpha (TNF-α) production by macrophages. In addition, IgE production, which is stimulated by interleukin-4, was found to be upregulated by 3-oxo-C12 HSL. Furthermore, the presence of the AHL signal molecule downregulated production of interleukin 12 (a Th-1 response promoting signal). As a consequence, a Th-2 response is encouraged (Telford et al. 1998). In contrast, other researchers found that 3-oxo-C12 HSL activates T-cells to produce interferon-γ, an inflammatory cytokine that promotes a Th-1 environment (Smith et al. 2002a). These discrepancies probably reflect biases in the underlying immune response. In C57B1/6 mice, which are Th-1 biased, 3-oxo-C12 HSL was found to increase interferon-γ, thereby promoting a Th-1-dominated response. Conversely, if BALB/C mice biased for Th-2 were used, the *P. aeruginosa* signal molecules increased production of interleukin-4, favoring a Th-2-dominated response (Moser et al. 1997). In both cases, the underlying immune response bias was accentuated by 3-oxo-C12 HSL (Ritchie et al. 2003). Production of cyclooxygenase 2 was markedly increased in human lung fibroblasts through stimulation of the transcription factor NF-κB. PGE_2, which induces mucus secretion, vasodilatation, and edema, was also produced in higher amounts when the cells were exposed to 3-oxo-C12 HSL (Smith et al. 2002b). AHL signal molecules also inhibit ATP and UTP-induced chloride secretion by submucosal tracheal serous gland cells from cystic fibrosis patients. Normally, the nucleosides lead to relaxation of the bronchia, which, in turn, promotes bacterial clearance. This ability of the 3-oxo-C12 HSL signal molecule to modulate the immune response has promoted research into generating analogs that can be used as treatment for TNF-alpha-driven immunological diseases such as psoriasis, rheumatoid arthritis, and type 1 diabetes (Chhabra et al. 2003).

Other eukaryotes also respond to the presence of AHL signal molecules. The model legume plant *Medicago truncatula*, a close relative of alfalfa, was found to produce elevated amounts of flavonoids in response to 3-oxo-C12 HSL. Interestingly, the plant also begins to secrete compounds that mimic AHL molecules when it encounters QS bacteria such as *P. aeruginosa* (Mathesius et al. 2003).

Similar relationships between QS and infection have been established for several opportunistic pathogens, including *Serratia liquefaciens* (Eberl et al. 1996, 1999), *Chromobacterium violaceum* (Brito et al. 2004), *Burkholderia cepacia* (Wopperer et al. 2006), and *Yersinia* species (Atkinson et al. 2006), all of which cause infections in humans. Other pathogens such as *V. anguliarum*, which causes the deadly infection vibriosis in fish, and the plant pathogens *Agrobacterium tumefaciens* (Sheng and Citovsky 1996) and *E. caratovora* (Whitehead et al. 2002) also employ QS to control infection and virulence. Bacteria such as *S. proteamaculans* B5a and *Enterobacter agglomerans* B6a, which causes food-quality deterioration, utilize QS

to control expression of exoenzymes that are involved in decay (Gram et al. 1999, Christensen et al. 2003).

Inhibition of QS would thus not only be beneficial in a clinical context but could possibly also be applied in aquaculture, agriculture, and food preservation.

2 Quorum Sensing in Gram-positive Bacteria[4]

Quorum sensing in gram-positive bacteria regulates a number of physiological activities, including those involving pathogenesis and biofilm formation. Examples are competence development in *Streptococcus pneumoniae* and *S. mutans*, antibiotic biosynthesis in *Lactococcus lactis* and virulence in staphylococci.

Gram-positive bacteria communicate using polypeptides as autoinducers and two-component or phosphorelay systems for signaling (Hoch and Varughese 2001). The release of the polypeptides from the cells is mediated in many cases by dedicated exporters. In most cases, signal processing and modification are concomitant with signal release. In many peptide QS systems, signals are cleaved from larger precursor peptides, which are then modified to contain lactone or thiolactone rings, lanthionines, or isoprenyl groups (Ansaldi et al. 2002; Mayville et al. 1999; Nakayama et al. 2001). QS in gram-positive bacteria has been well reviewed (e.g., Walters and Bassler 2005, Abraham 2006). Here we will focus on QS in staphylococci, whose inhibitors have extensively been tested in vivo (see the chapter *In Vivo Studies: Inhibiting Biofilm-Associated Bacterial Infections Using QSIs*). Refer to the chapter *Quorum Sensing in Streptococci* for information on that topic.

2.1 Quorum Sensing in S. aureus

S. aureus pathogenesis is regulated by two QS systems (Balaban et al. 2001; Gov et al. 2004, Korem et al. 2005). As in *P. aeruginosa*, the two QS systems are organized in a hierarchical manner, the former controlling the expression of the latter. This apparently allows for further fine-tuning of the responses of QS target genes.

The two QS systems that have been described to date for *S. aureus* (Balaban et al. 2001) will be referred to herein as staphylococcal quorum-sensing 1 (SQS 1) and staphylococcal quorum-sensing 2 (SQS 2). SQS 1 consists of the autoinducer RNAIII-activating protein (RAP) and its target molecule TRAP (Balaban et al. 1998, 2001). SQS 1 induces the synthesis of the second system, SQS 2, which consists of the components of the *agr* system, including autoinducing peptide (AIP) and its sensor AgrC (Lyon et al. 2000). The two systems interact with one another to collectively regulate the expression of virulence factors (Balaban et al. 2001; Korem et al. 2005).

The notion that more than one QS system regulates virulence in staphylococci had been controversial, and it was suggested that only one QS system regulates

S. aureus pathogenesis – the one encoded by *agr* (Novick 2003). It is now, however, very clear that this is not the case and that SQS 1 in fact regulates SQS 2 (Korem et al. 2005). This phenomenon is not surprising in view of the fact that multiple systems are known to regulate necessary biological functions both in gram-negative and gram-positive bacteria (e.g., Miller and Bassler 2001; March and Bentley 2004; Gambello and Iglewski 1991; Ochsner et al. 1994; Pearson et al. 1994).

2.1.1 Components of SQS 1

RAP is the autoinducer of SQS 1. RAP is a 277AA protein that activates the *agr* by inducing the phosphorylation of TRAP (Balaban et al. 1998, 2001; Korem et al. 2003; Yang et al. 2003). From its sequence, RAP is predicted to be an ortholog of the 50S ribosomal protein L2, which is encoded by the gene *rplB* found in all eubacterial genomes known to date. Recombinant RAP applied to the cells activates the synthesis of RNAIII (which is encoded by the *agr*) like the native RAP molecule that is secreted, confirming that L2 has extraribosomal functions (Korem et al. 2003). Inhibiting RAP by anti-RAP antibodies or by RAP-binding peptides suppresses infections in vivo (Balaban et al. 1998; Yang et al. 2003). (Refer to the chapter *In Vivo Studies: Inhibiting Biofilm-Associated Bacterial Infections Using QSIs*.)

TRAP is the target protein of RAP and is a master regulator of *S. aureus* pathogenesis. It is a 167-residue-long protein that is histidine-phosphorylated in the presence of RAP (Balaban et al. 2001). TRAP expression is constitutive, but its phosphorylation is regulated by RAP and reaches peak phosphorylation in the mid-exponential phase of growth. Phosphorylated TRAP then leads to *agr* expression, and the components of SQS 2 are made (Gov et al. 2004). (See below.) TRAP is highly conserved among staphylococcal strains and species and contains three conserved histidine residues that are phosphorylated and are essential for its activity (Gov et al. 2004; Korem et al. 2005). TRAP orthologs are found in other gram-positive bacteria including *Bacillus* (Ivanova1 et al. 2003) and *Listeria*. Although the sequence identity between TRAP orthologs is low, their predicted secondary structure is very similar, as is their gene organization (Kiran et al., unpublished data). TRAP-like proteins may thus represent a novel general class of signal transducers in gram-positive bacteria.

Functional genomics studies (Korem et al. 2005) indicate that in the absence of TRAP expression or phosphorylation (TRAP–), multiple regulatory systems are disrupted, such as the global regulatory locus *agr* (*agrABCD* and *hld* [RNAIII]); *sarH2*, otherwise known as *sarU* (Manna and Cheung 2003); and most, if not all, virulence factors known to date. Those include alpha, beta, gamma, and delta hemolysin; triacylglycerol lipase precursor; glycerol ester hydrolase; hyaluronate lyase precursor; staphylococcal serine protease (V8 protease); cysteine protease precursor; cysteine protease; staphopain-cysteine proteinase; 1-phosphatidylinositol phosphodiesterase; zinc metalloproteinase aureolysin precursor; holing-like proteins; and capsular polysaccharide synthesis enzymes (Korem et al. 2005). In the case of genes

involved in bacterial adhesion and consequent biofilm formation, the only over-expressed genes found when TRAP function is disrupted are those encoding for protein A, fibrinogen-binding protein, and Ser-Asp rich fibrinogen-binding bone-sialoprotein-binding protein. There is, however, no evidence that these proteins independently contribute to pathogenesis. No upregulation of other known genes encoding for adhesion molecules has been observed, such as fibronectin-binding protein, collagen-binding protein, elastin-binding protein, clumping factor A, extra-cellular fibrinogen-binding protein, and extracellular adherence protein.

Finally, in the absence of TRAP expression or phosphorylation, the level of expression of genes required for biofilm survival is reduced, such as ArcABC (arginine deaminase, ornithine transcarbamoylase, carbamate kinase), UreABC (urease alpha, beta, gamma subunits), UreDEFG (urease accessory proteins), PyrR (pyrimidine operon repressor), PyrP (uracil permease), PyrB (aspartate transcarbamoylase chain A), PyrC (dihydroorotase), CarA (carbamoyl-phosphate synthase small chain), and CarB (carbamoyl-phosphate synthase large chain) (Korem et al. 2005; Balaban et al. 2005). These proteins are necessary for the persistence of the bacteria within a biofilm, requiring an adaptive response that limits the deleterious effects of the reduced pH associated with anaerobic growth conditions (Beenken et al. 2004).

Functional genomics studies can easily explain that in the absence of TRAP phosphorylation, the ability of the bacteria to produce toxins, to attach to host cells or foreign material, to form a biofilm, and to survive within the host is seriously compromised; therefore, when TRAP is not expressed or phosphorylated (using anti-TRAP antibodies or peptides (see below and the chapter *In Vivo Studies: Inhibiting Biofilm-Associated Bacterial Infections Using QSIs*), the bacteria do not adhere, do not form a biofilm, do not express toxins, and do not cause disease (Dell'Acqua et al. 2004; Balaban et al. 2000, 2003a,b, 2004, 2005; Cirioni et al. 2003, 2006, 2007; Giacometti et al. 2003, 2005; Gov et al. 2004; Yang et al. 2005; Vieira-da-Motta 2001; Anguita-Alonso et al. 2006).

TRAP represents a novel class of signal transducers in gram-positive bacteria because it does not contain any conserved domains specific for two-component or phosphorelay systems (Han et al. 2005). QS in bacteria typically involves phosphorylation in a two-component system. The classic two-component system is composed of two proteins, the sensor kinase, which is histidine-phosphorylated, and the effector protein, which is aspartic-acid-phosphorylated. The sensor typically contains a transmembrane as well as a kinase domain (Perraud et al. 1999). Like typical sensors, TRAP is histidine-phosphorylated (Balaban et al. 2001; Gov et al. 2004), and studies indicate that it is membrane associated and can be used as a vaccine (Yang et al. 2005). However, TRAP lacks both a typical kinase domain and a predicted transmembrane region. This suggests that TRAP may be associated with the membrane by anchoring of hydrophobic surface residues or by binding to an integral membrane protein. A possible candidate may be OpuCA, discovered by a two-hybrid system (Kiran et al., unpublished data). OpuCA is encoded by the *opuC* operon that is highly conserved, is known as an ABC transporter, and thus is hypothesized to act upstream of TRAP. The type of interaction that OpuCABCD has with TRAP (anchoring?) or with the QS regulators RAP, AIP, and RIP is not yet known.

Interestingly, recombinant RAP or its inhibitor RIP (see the chapter *Quorum-Sensing Inhibitory Compounds*) were added to recombinant TRAP and shown to activate (RAP) or inhibit (RIP) the phosphorylation of TRAP in vitro, in the absence of any other cellular components. This further confirms the working hypothesis that RAP activates and RIP inhibits TRAP phosphorylation and further suggests that TRAP may in fact be a histidine kinase (Kim, personal communications).

One of the regulatory genes that is distinctly regulated by TRAP is *sarH2* (known also as *sarU*) (Balaban et al. 2005). SarU, a positive transcriptional activator of *agr* expression, encodes a 247-residue polypeptide and is a member of the SarA family of proteins. It has conserved basic residues within the helix-turn-helix motif and within the beta hairpin loop, which are two putative DNA-binding domains within this protein family (Manna and Cheung 2003). Of note is that insertions in *sarH2* have diminished the ability of the *S. aureus* strain Newman to kill worms (Bae et al. 2004), suggesting that its role in pathogenesis. SarH2 (SarU) may act as one of the downstream components in the TRAP system, regulating the expression of *agr* (see below). This, however, needs to be confirmed experimentally because *sarH2* is not conserved among strains.

Points of controversy

Lately it has been suggested that TRAP does not regulate the *agr* (Shaw et al. 2007; Tsang et al. 2007) because when the authors deleted *traP*, *agr* activity was still intact. These results will need to be confirmed as the authors have not yet shown that the mutants do not contain an active TRAP molecule (by standard in vivo phosphorylation assays and by western blotting). Interestingly, one of the two strains used, UAMS-1, is non-hemolytic although it is a virulent musculoskeletal isolate, suggesting that perhaps its virulence is not toxin related and maybe virulence of such strains does not involve TRAP. In fact, global transcriptional differences between these clinical isolates and laboratory strains have been documented (Cassat et al. 2006). It is too early to say whether these strains represent the norm or not. Luckily, virulence studies using laboratory strains have in fact resulted in clinical applications using clinical isolates (see the chapters *In Vivo Studies: Inhibiting Biofilm-Associated Bacterial Infections Using QSIs* and *Clinical Wound Healing Using Signal Inhibitors*).

It has also been suggested that the non-virulent *agr*– minus phenotype found in the TRAP– mutant are seen only because of a nonsense mutation in the *agrA* locus (Adhikari et al. 2007). This nonsense mutation was not found in TRAP– strains described in Korem et al. 2005, but other nonsense mutations were found in some of the freezer stocks (Balaban et al., in preparation), suggesting that when TRAP is inactivated, multiple nonsense mutations can occur in *agr* (and possibly other loci) more readily. Interestingly, ClpP is not expressed when TRAP or YhgC (the TRAP-like protein in Bacillus) are mutated (Kiran et al., in preparation). ClpP proteases were shown to be important for expression of various regulons involved in virulence (*agr*), oxidative stress response, autolysis, and DNA repair (Michel et al. 2006). Put

together, these results suggest that in the absence of TRAP, not only virulence is downregulated but also DNA repair is impaired, which can lead to deleterious multiple *agr* mutations (Adhikari et al. 2007). Such mutants cannot survive in vivo because of the lack of expression of virulence factors necessary for in vivo survival. But in vitro, in the absence of adverse conditions, such mutants can survive and be detected. Thus, TRAP not only regulates the production of virulence factors but also other genes necessary for bacterial survival in the host, making TRAP a very attractive target for therapy (see the chapters *Quorum-Sensing Inhibitory Compounds* and *In Vivo Studies: Inhibiting Biofilm-Associated Bacterial Infections Using QSIs*).

2.1.2 Components of SQS 2

SQS 2 encompasses the products of the *agr* system. The chromosomal *agr* operons, active from the midexponential phase of growth, encode two divergently transcribed transcripts, RNAII and RNAIII (Novick et al. 1993, 1995). RNAII is a polycistronic transcript that encodes AgrB, AgrD, AgrC, and AgrA (Ji et al. 1997; Qiu et al. 2005; Zhang and Ji 2004; Novick et al. 1995; Lina et al. 1998; Koenig et al. 2004). RNAIII is a polycistronic transcript, coding for delta hemolysin and acting as a regulatory RNA molecule that upregulates the expression of multiple exotoxins (Novick et al. 1993).

Agr-autoinducing Peptide (AIP)

AIP is processed from AgrD. AgrD sequences from various staphylococcal species are remarkably divergent, with only four identical amino acids (Qiu et al. 2005). The AIP sequence is in the middle of the AgrD sequence that is preceded by the N-terminal amphipathic helix and followed by a highly hydrophilic C-terminal region. The processing of AgrD to generate mature AIP involves the proteolytic cleavages at two processing sites, the thioester (or ester) bond formation, and the secretion of the mature AIP. The mature AIPs isolated so far from a number of staphylococcal species are seven to nine amino acids in length, and all are thiolactone molecules containing a 5-amino-acid ring linked by a thioester bond formed between the sulfhydryl group of a conserved cysteine residue and the carboxyl group of the C-terminal amino acid, except for the *Staphylococcus intermedius* AIP, in which a lactone molecule contains an ester bond formed between the hydroxyl group of a serine residue (in place of the cysteine residue that is conserved among other AIPs) and the carboxyl group of the C-terminal residue (Qiu et al. 2005; Lyon et al. 2002; Mayvelle et al. 1999).

A polymorphism in the amino acid sequence of the AIP and its corresponding receptor AgrC divides *S. aureus* strains into four major groups. Within a given group, each strain produces a peptide that can activate the *agr* response in the other strains, whereas the AIPs belonging to different groups are usually mutually inhibitory. Limited in vivo studies have been carried out using inhibitory AIPs (refer to the sec-

tion on inhibitors to SQS 2 in the chapter *Quorum-Sensing Inhibitory Compounds*), but their clinical significance remains unclear.

AgrB

AgrB protein is a putative cysteine endopeptidase and a transporter, facilitating the export of the processed AgrD peptide (Qiu et al. 2005). AgrB is a membrane protein with six transmembrane segments, including four transmembrane helices and two highly hydrophilic regions (Zhang et al. 2002a). Like AgrD and AgrC, AgrB sequenced from various staphylococcal species are also divergent, except for the N-terminal region located in the cytoplasm and the two highly hydrophilic regions that are proposed to be in the membrane (Zhang et al. 2002a). It is likely that all AgrBs are structurally and functionally similar and that the mechanisms of processing AgrD and of secreting the mature AIP by AgrBs are the same or similar, even though the AgrD propeptides are different and the interaction between AgrB and AgrD is specific (Zhang et al. 2004).

AgrC

AgrC is the receptor to AIP. AgrC is a membrane protein with its N-terminal half integrated into the cytoplasmic membrane and is the AIP binding site (Lina et al. 1998; Lyon et al. 2002). Its C-terminal half is located in the cytoplasm and possesses histidine kinase activity (Lina et al. 1998). The N-terminal halves are divergent, and the C-terminal halves are highly conserved. This reflects the fact that the AgrCs are activated only by their cognate AIPs but are inhibited by heterologous AIPs. Based on the AIP cross-activation and cross-inhibition activities, four specificity groups of *S. aureus* and three groups of *S. epidermidis* have been identified. Upon the binding of AIP, AgrC is autophosphorylated (Lina et al. 1998), the phosphoryl group of the phosphorylated AgrC is transferred to AgrA, and phosphorylated AgrA activates the transcription of RNAIII (Koenig et al. 2004) (see below).

AgrA

AgrA is the regulator that is part of the AgrC/AgrA two component system. Once phosphorylated, it shows high-affinity binding to the RNAIII-*agr* intergenic region, where binding is localized to a pair of direct repeats in the P2 and P3 promoter regions of the *agr* locus, consistent with the function of AgrA as a response regulator (Koenig et al. 2004) that activates the production of RNAIII (see below).

RNAIII

RNAIII is the actual regulator that activates the expression of genes encoding secreted virulence factors. RNAIII is a 512-nt-long mRNA, affecting expression of

multiple genes either directly or indirectly. RNAIII also encodes for the toxin δ-hemolysin, once translated at the postexponential phase of growth (Balaban and Novick 1995a). The commonly accepted dogma is that staphylococcal genes encoding secreted proteins are activated by the presence of RNAIII, whereas genes encoding surface proteins are repressed, leading to phase variation (Novick et al. 1993; Lowy 1998). However, this was proven only for alpha-hemolysin and protein A (see below).

The structure of RNAIII suggests that it is able to form 14 different hairpins (Benito et al. 2000). Specific domains of RNAIII control the expression of different targets: The 5′-end of RNAIII positively controls the translation of *hla* (encoding alpha-hemolysin) by competing directly with an inhibitory intramolecular RNA secondary structure that sequesters the *hla* ribosome-binding site. Hybridization of RNAIII to the *hla* mRNA frees the ribosome-binding site and enables translation of *hla* (Novick et al. 1993; Morfeldt et al. 1995).

Complementation analysis suggests that the 3′-end of RNAIII is important for repression of the *spa*-gene that encodes the well-known IgG-binding protein, protein A (Novick et al. 1993). In this case, RNAIII is believed to function either directly or indirectly at the transcriptional level, although it is possible that RNAIII affects the stability of the *spa* transcript.

RNAIII levels are evident from the midexponential phase of growth and reach a maximum in late logarithmic- and stationary-phase cultures. As mentioned above, RNAIII also encodes the small peptide δ-hemolysin in its 5′-end. Intriguingly, translation of the RNAIII transcript into δ-hemolysin is delayed 1 h after the appearance of RNAIII in the midexponential phase. This inhibitory mechanism seems to involve the 3′-end of RNAIII, possibly by blocking access of the ribosome to the ribosome-binding region (Balaban and Novick 1995a).

The mechanism by which RNAIII activates or inhibits expression of the other virulence factors remains unknown. It has been shown that transcriptional activation or repression preferentially occurs at the level of transcriptional initiation rather than by affecting transcript stability. A possible scenario would be that RNAIII functions as an antirepressor by directly binding global transcriptional regulators and then sequestering them, thereby regulating the initiation of transcription at target promoters (Arvidson and Tegmark 2001; Johansson and Cossart 2003).

agr in other Bacteria

An operon termed Fas (fibronectin/fibrinogen binding/hemolytic activity/streptokinase regulator) that shows similarity to the two-component system of *agr* was found in *Streptococcus pyogenes* (Kreikemeyer et al. 2001). As with *agr*, the effector molecule for virulence gene expression is a small, untranslated RNA molecule (fasX), although little is known of its mode of action (Kreikemeyer et al. 2001). In addition, an analogous case was found in *Clostridium perfringens*, where a small, untranslated RNA [VirR-regulated-RNA (VR-RNA)] is the effector molecule of a two-component system shown to be involved in virulence gene ex-

pression (Shimizu et al. 2002). In this case, the 3′-end of VR-RNA appears to be important in mediating virulence gene regulation (Johansson and Cossart 2003).

2.1.3 Interaction Between the two QS Systems in Staphylococci

The two QS systems in staphylococci SQS 1 and SQS 2 interact with one another (Fig. 3) as follows: As the cells multiply and the colony grows, the cells secrete RAP, inducing the histidine-phosphorylation of its target molecule TRAP (possibly via ClpP and OpuC). The phosphorylation of TRAP leads (possibly via SarH2) to the activation of the *agr* (Balaban et al. 2001; Gov et al. 2004; Korem et al. 2005) in the midexponential phase of growth and thus to the synthesis of RNAII and consequently to the production of AIP and AgrC (Novick et al. 1995). AIP downregulates TRAP phosphorylation in an unknown mechanism and upregulates the phosphorylation of its receptor, AgrC (Lina et al. 1998; Balaban et al. 2001).

Phosphorylation of AgrC causes the phosphorylation of AgrA, which together with SarA and SigB (Koenig et al. 2004; Manna and Cheung 2003; Chien et al. 1999; Ziebandt et al. 2001) results in the production of RNAIII (Novick et al. 1993; Lina et al. 1998). RNAIII leads to the expression of toxic exomolecules, resulting in dissemination and disease (Lowy 1998).

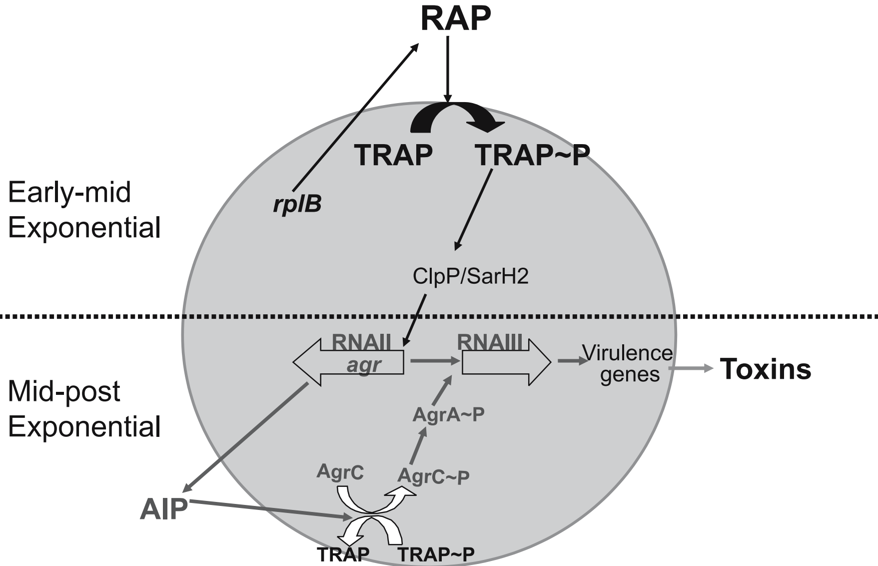

Fig. 3 Diagram showing regulation of toxin production in *S. aureus* via the quorum-sensing systems TRAP and *agr*

3 Quorum Sensing and Biofilm[1–4]

As stated above, QS refers to the ability of the bacteria to sense the density of the surrounding bacterial population. This is done by measuring and responding to the concentration of signal molecules. In order to build up a sufficient concentration of QS autoinducers (QSA), diffusion barriers are required. A dense, mature biofilm is not completely sealed off from the surroundings, but diffusion is certainly lowered compared with the situation in a planktonic culture (Hentzer et al. 2002). The timing of induction of QS-controlled genes probably depends on several factors. The diffusion rate will be dependent on the volume of the surrounding nonbiofilm phase, the flow rate (if any) of bulk fluid outside the biofilm. Furthermore, the chemical composition of the extrapolymeric substances will influence diffusion rates. As the cell density varies in the biofilm, different sets of QS-controlled genes may be expressed in different positions or niches of the biofilm. Hence, QS-controlled genes in biofilms also exhibit a spatial expression pattern (de Kievit et al. 2001).

Indeed, QS signals can be detected in diverse environments. For example, biofilms grown on rocks in the San Marcos River in Texas have been shown to produce AHL signals (McLean et al. 1997). In a completely different setting, in the lungs of cystic fibrosis patients, signal molecules have also been found (Collier et al. 2002, Singh et al. 2000).

In the protected biofilm environment, bacteria are free to produce and secrete a battery of virulence factors. In *S. aureus* and *P. aeruginosa*, for example, many of these virulence factors are controlled by QS (Korem et al. 2005; Dunman et al. 2001; Mittal et al. 2006; Joyce et al. 2004; Wagner et al. 2003). Virulence factors, in conjunction with immune complexes and phagocytic enzymes released by the immune system, cause extensive tissue destruction and inflammation. In the case of cystic fibrosis, this tissue destruction contributes significantly to the loss of pulmonary function (Costerton et al. 1999; Donlan 2002; Donlan and Costerton 2002; Parsek and Singh 2003).

Whether QS is involved in control of the developmental pattern of *P. aeruginosa* biofilms is still controversial (Kjelleberg and Molin 2002), but most of these studies were done in vitro and should be taken with caution. As reported by Charlton et al. (2000a), the concentration of QS signal OdDHL is significantly higher in *P. aeruginosa* biofilm (632 μM) than the effluent (14 nM). Consistent with this observation, QS has been found to play a critical role in the development of *P. aeruginosa* biofilms (Davies et al. 1998), showing that a *lasI* mutant formed flat, undifferentiated biofilms. In striking contrast, Heydorn et al. (2002) demonstrated, using Comstat-assisted image analysis, that a wild-type biofilm is indistinguishable from a biofilm formed by a *lasI* mutant. Again, these differences may be attributed to various strains and the experimental setups employed. When a biofilm of a *P. aeruginosa* QS mutant was grown on glucose as the carbon source, a difference in biofilm architecture could be found using image analysis. If the carbon source was changed to citrate, no difference could be detected (Heydorn et al. 2002).

In addition to AHL, AI-2 also plays a role in biofilm formation. Deletion of *luxS* has been found to influence the biofilm formation of *Streptococcus gordonii* (Blehert

et al. 2003) and *S. mutans* (Merritt et al. 2003), in which the bacteria no longer produced AI-2 and the biofilm had a more granular appearance. Direct addition of AI-2 induces biofilm formation in *E. coli* through a motility QS regulator (MqsR), which in turn regulates the two-component motility regulatory system (QseBC) and motility (Gonzalez Barrios et al. 2006).

In staphylococci, disruption of quorum sensing by mutagenesis or by inhibitory peptides (refer to section 2 in the chapter *In Vivo Studies: Inhibiting Biofilm-Associated Bacterial Infections Using SQIs* and to Fig. 4 in the chapter *Quorum-Sensing Inhibitory Compounds*) leads to suppression of biofilm formation in vivo. As shown in Fig. 4 in the chapter *Quorum-Sensing Inhibitory Compounds*, essentially no biofilm is formed in vivo by the TRAP– mutant (SQS 1 mutant). In comparison, only reduced biofilm is formed by *agr*– mutants (SQS 2 mutant), suggesting that TRAP, which acts upstream of *agr*, regulates multiple genes necessary for biofilm formation in vivo in addition to those regulated by *agr*. These studies are important because for years it had been suggested that *S. aureus* exists in two phases and that the switch between the two phases is regulated by RNAIII (*agr*). It has been suggested that in one phase, in low cell density, before *agr* is expressed, there is high expression of adhesion molecule, whereas in the other phase, in high cell density, after *agr* is expressed, there is reduced expression of adhesion molecules and, instead, increased expression of exotoxins (Lowy 1998; Novick et al. 1993). This meant that if quorum systems TRAP or *agr* are repressed, toxins will be repressed, but adhesion molecules will be expressed. This in turn was expected to enhance biofilm formation, thus making QS inhibitors inadequate for inhibiting biofilm-related infections in vivo and for future clinical use (Otto 2004; Vuong et al. 2000, 2003). However, functional genomics studies show that when TRAP or *agr* is mutated, multiple tox-

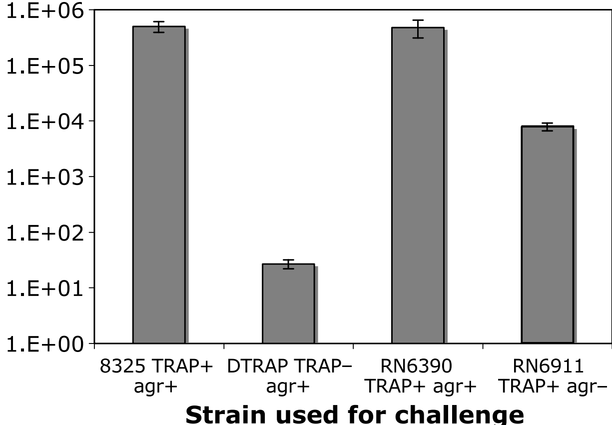

Fig. 4 Formation of a biofilm in vivo by TRAP– or *agr*– mutants. Under the rat graft model (see the chapter *Animal Models Commonly Used To Study Quorum-Sensing Inhibitors*), rats were challenged with *S. aureus* RN6390 (WT), RN6911 (*agr*–), 8325-4 (WT) or TRAP– strains. Grafts were removed 10 days later, and the bacterial loads on grafts were determined and expressed as colony-forming units (CFU)/ml

ins are repressed, but no significant increase in expression of adhesion molecules is observed (Beenken et al. 2004; Dunman et al. 2001; Korem et al. 2005). This is in accordance with the fact that TRAP mutants that do not express *agr* do have reduced ability to form a biofim or infection (Gov et al. 2004) in vivo (Fig. 4). The components of SQS 1 (RAP/TRAP) are very conserved, making them especially attractive as target sites for therapy (e.g., Balaban et al. 2005 and section 2 in the chapter *In Vivo Studies: Inhibiting Biofilm-Associated Bacterial Infections Using SQIs*).

Whether QS is involved in forming one type of biofilm or another is probably of less importance. More interesting are the properties or function of the biofilm. Davies et al. (1998), Hentzer et al. (2003), and Bjarnsholt et al. (2005a) found a link between biofilm tolerance against various antibiotics, biocides, peroxide, and QS. Biofilms formed by QS mutants or biofilms treated with inhibitors of QS were much more susceptible to the actions of these compounds (Davies et al. 1998; Hentzer et al. 2003; Rasmussen et al. 2005a,b; Bjarnsholt et al. 2005a; Dell'Acqua et al. 2004; Balaban et al. 2003a, 2004, 2005; Cirioni et al. 2003, 2006; Giacometti et al. 2003, 2005). These findings, in conjunction with the QS control of virulence factors, point out QS as a highly attractive target for chemotherapy against biofilm chronic infections!

References

Abraham WR (2006) Controlling biofilms of gram-positive pathogenic bacteria. Curr Med Chem 13:1509–1524

Adhikari RP, Arvidson S, Novick RP (2007) A nonsense mutation in agrA accounts for the defect in agr expression and the avirulence of Staphylococcus aureus 8325-4 traP::kan. Infect Immun 75:4534–4540

Anguita-Alonso P, Giacometti A, Cirioni O, Ghiselli R, Orlando F, Saba V, Scalise G, Sevo M, Patel R, Balaban N (2006) RNAIII-inhibiting peptide-loaded polymethylmethacrylate prevents in vivo *Staphylococcus aureus* biofilm formation. Antimicrob Agents Chemother, Nov 20 (Epub ahead of print)

Ansaldi M, Marolt D, Stebe T, Mandic-Mulec I, Dubnau D (2002) Specific activation of the Bacillus quorum-sensing systems by isoprenylated pheromone variants. Mol Microbiol 44:1561–1573

Arvidson S, Tegmark K (2001) Regulation of virulence determinants in Staphylococcus aureus. Int J Med Microbiol 291:159–170

Atkinson S, Sockett RE, Camara M, Williams P (2006) Quorum sensing and the lifestyle of *Yersinia*. Curr Issues Mol Biol 8:1–10

Bae T, Banger AK, Wallace A, Glass EM, Aslund F, Schneewind O, Missiakas DM (2004) Staphylococcus aureus virulence genes identified by bursa aurealis mutagenesis and nematode killing. Proc Natl Acad Sci USA 101:12312–12317

Balaban N, Novick RP (1995a) Translation of RNAIII, the Staphylococcus aureus agr regulatory RNA molecule, can be activated by a 3′-end deletion. FEMS Microbiol Lett 133:155–161

Balaban N, Goldkorn T, Nhan RT, Dang LB, Scott S, Ridgley RM, Rasooly A, Wright SC, Larrick JW, Rasooly R, Carlson JR (1998) Autoinducer of virulence as a target for vaccine and therapy against *Staphylococcus aureus*. Science 280:438–440

Balaban N, Collins LV, Cullor JS, Hume EB, Medina-Acosta E, Vieira-da-Motta O, O'Callaghan R, Rossitto PV, Shirtliff ME, Serafim da Silveira L, Tarkowski A, Torres JV (2000) Prevention of diseases caused by *Staphylococcus aureus* using the peptide RIP. Peptides 21:1301–1311

Balaban N, Goldkorn T, Gov Y, Hirshberg M, Koyfman N, Matthews HR, Nhan RT, Singh B, Uziel O (2001) Regulation of *Staphylococcus aureus* pathogenesis via target of RNAIII- activating protein (TRAP). J Biol Chem 276:2658–2667

Balaban N, Giacometti A, Cirioni O, Gov Y, Ghiselli R, Mocchegiani F, Viticchi C, Del Prete MS, Saba V, Scalise G, Dell'Acqua G (2003a) Use of the quorum-sensing inhibitor RNAIII-inhibiting peptide to prevent biofilm formation in vivo by drug-resistant *Staphylococcus epidermidis*. J Infect Dis 187:625–630

Balaban N, Gov Y, Bitler A, Boelaert JR (2003b) Prevention of *Staphylococcus aureus* biofilm on dialysis catheters and adherence to human cells. Kidney Int 63:340–345

Balaban N, Gov Y, Giacometti A, Cirioni O, Ghiselli R, Mocchegiani F, Orlando F, D'Amato G, Saba V, Scalise G, Bernes S, Mor A (2004) A chimeric peptide composed of a dermaseptin derivative and an RNA III-inhibiting peptide prevents graft-associated infections by antibiotic-resistant staphylococci. Antimicrob Agents Chemother 48:2544–2550

Balaban N, Stoodley P, Fux CA, Wilson S, Costerton JW, Dell'Acqua G (2005) Prevention of staphylococcal biofilm-associated infections by the quorum sensing inhibitor RIP. Clin Orthop Relat Res 437:48–54

Bassler BL, Wright M, Showalter RE, Silverman MR (1993) Intercellular signalling in *Vibrio harveyi*: sequence and function of genes regulating expression of luminescence. Mol Microbiol 9:773–786

Beenken KE, Dunman PM, McAleese F, Macapagal D, Murphy E, Projan SJ, Blevins JS, Smeltzer MS (2004) Global gene expression in Staphylococcus aureus biofilms. J Bacteriol 186: 4665–4684

Benito Y, Kolb FA, Romby P, Lina G, Etienne J, Vandenesch F (2000) Probing the structure of RNAIII, the Staphylococcus aureus agr regulatory RNA, and identification of the RNA domain involved in repression of protein A expression. RNA 6:668–679

Bjarnsholt T, Jensen PO, Burmolle M, Hentzer M, Haagensen JA, Hougen HP, Calum H, Madsen KG, Moser C, Molin S, Hoiby N, Givskov M (2005a) Pseudomonas aeruginosa tolerance to tobramycin, hydrogen peroxide and polymorphonuclear leukocytes is quorum-sensing dependent. Microbiology 151:373–383

Blehert DS, Palmer RJ Jr, Xavier JB, Almeida JS, Kolenbrander PE (2003) Autoinducer 2 production by Streptococcus gordonii DL1 and the biofilm phenotype of a luxS mutant are influenced by nutritional conditions. J Bacteriol 185:4851–4860

Brito CF, Carvalho CB, Santos F, Gazzinelli RT, Oliveira SC, Azevedo V, Teixeira SM (2004) *Chromobacterium violaceum* genome:molecular mechanisms associated with pathogenicity. Genet Mol Res 3:148–161

Cassat J, Dunman PM, Murphy E, Projan SJ, Beenken KE, Palm KJ, Yang SJ, Rice KC, Bayles KW, Smeltzer MS (2006) Transcriptional profiling of a Staphylococcus aureus clinical isolate and its isogenic agr and sarA mutants reveals global differences in comparison to the laboratory strain RN6390. Microbiology 152:3075–3090

Charlton TS, de Nys R, Netting A, Kumar N, Hentzer M, Givskov M, Kjelleberg S (2000a) A novel and sensitive method for the quantification of *N*-3-oxoacyl homoserine lactones using gas chromatography-mass spectrometry: application to a model bacterial biofilm. Environ Microbiol 2:530–541

Chen X, Schauder S, Potier N, Dorsselaer AV, Pelczer I, Bassler BL, Hughson FM (2002) Structural identification of a bacterial quorum-sensing signal containing boron. Nature 415:545–549

Chhabra SR, Harty C, Hooi DS, Daykin M, Williams P, Telford G, Pritchard DI, Bycroft BW (2003) Synthetic analogues of the bacterial signal (quorum sensing) molecule *N*-(3-oxododecanoyl)-L-homoserine lactone as immune modulators. J Med Chem 46:97–104

Chien Y, Manna AC, Projan SJ, Cheung AL (1999) SarA, a global regulator of virulence determinants in Staphylococcus aureus, binds to a conserved motif essential for sar-dependent gene regulation. J Biol Chem 274:37169–37176

Choi SH, Greenberg EP (1991) The C-terminal region of the *Vibrio fischeri* LuxR protein contains an inducer-independent *lux* gene activating domain. Proc Natl Acad Sci USA 88:11115–11119

Christensen AB, Riedel K, Eberl L, Flodgaard LR, Molin S, Gram L, Givskov M (2003) Quorum-sensing-directed protein expression in *Serratia proteamaculans* B5a. Microbiology 149:471–483

Cirioni O, Giacometti A, Ghiselli R, Dell'Acqua G, Gov Y, Kamysz W, Lukasiak J, Mocchegiani F, Orlando F, D'Amato G, Balaban N, Saba V, Scalise G (2003) Prophylactic efficacy of topical temporin A and RNAIII-inhibiting peptide in a subcutaneous rat Pouch model of graft infection attributable to staphylococci with intermediate resistance to glycopeptides. Circulation 108:767–771

Cirioni O, Giacometti A, Ghiselli R, Dell'acqua G, Orlando F, Mocchegiani F, Silvestri C, Licci A, Saba V, Scalise G, Balaban N (2006) RNAIII-inhibiting peptide significantly reduces bacterial load and enhances the effect of antibiotics in the treatment of central venous catheter-associated *Staphylococcus aureus* infections. J Infect Dis 193:180–186

Cirioni O, Giacometti A, Ghiselli R, Kamysz W, Silvestri C, Orlando F, Mocchegiani F, Vittoria AD, Kamysz E, Saba V, Scalise G (2007) The lipopeptides Pal-Lys-Lys-NH(2) and Pal-Lys-Lys soaking alone and in combination with intraperitoneal vancomycin prevent vascular graft biofilm in a subcutaneous rat pouch model of staphylococcal infection. Peptides 28:1299–1303

Collier DN, Anderson L, McKnight SL, Noah TL, Knowles M, Boucher R, Schwab U, Gilligan P, Pesci EC (2002) A bacterial cell to cell signal in the lungs of cystic fibrosis patients. FEMS Microbiol Lett 215:41–46

Costerton JW, Stewart PS, Greenberg EP (1999) Bacterial biofilms: a common cause of persistent infections. Science 284:1318–1322

Costerton JW, Veeh R, Shirtliff M, Pasmore M, Post C, Ehrlich G (2003) The application of biofilm science to the study and control of chronic bacterial infections. J Clin Invest 112:1466–1477

Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton JW, Greenberg EP (1998) The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science 280:295–298

de Kievit TR, Iglewski BH (2000) Bacterial quorum sensing in pathogenic relationships. Infect Immun 68:4839–4849

de Kievit TR, Gillis R, Marx S, Brown C, Iglewski BH (2001) Quorum-sensing genes in *Pseudomonas aeruginosa* biofilms: their role and expression patterns. Appl Environ Microbiol 67:1865–1873

de Nys R, Wright AD, Konig GM, Sticher O (1993) New halogenated furanone from the marine alga *Delisea pulchra* (cf. fimbriata). Tetrahedron 49:11213–11220

Dell'Acqua G, Giacometti A, Cirioni O, Ghiselli R, Saba V, Scalise G, Gov Y, Balaban N (2004) Suppression of drug-resistant staphylococcal infections by the quorum-sensing inhibitor RNAIII-inhibiting peptide. J Infect Dis 190:318–320

Doherty N, Holden MT, Qazi SN, Williams P, Winzer K (2006) Functional analysis of luxS in *Staphylococcus aureus* reveals a role in metabolism but not quorum sensing. J Bacteriol 188:2885–2897

Donabedian H (2003) Quorum sensing and its relevance to infectious diseases. J Infect 46:207–214

Donlan RM (2002) Biofilms: microbial life on surfaces. Emerg Infect Dis 8:881–890

Donlan RM, Costerton JW (2002) Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev 15:167–193

Dunman PM, Murphy E, Haney S, Palacios D, Tucker-Kellogg G, Wu S, Brown EL, Zagursky RJ, Shlaes D, Projan SJ (2001) Transcription profiling-based identification of *Staphylococcus aureus* genes regulated by the *agr* and/or *sarA* loci. J Bacteriol 183:7341–7353

Eberhard A, Burlingame AL, Eberhard C, Kenyon GL, Nealson KH, Oppenheimer NJ (1981) Structural identification of autoinducer of *Photobacterium fishcheri* luciferase. Biochemistry 20:2444–2449

Eberl L, Winson MK, Sternberg C, Stewart GS, Christiansen G, Chhabra SR, Bycroft B, Williams P, Molin S, Givskov M (1996) Involvement of N-acyl-L-homoserine lactone autoinducers in controlling the multicellular behaviour of *Serratia liquefaciens*. Mol Microbiol 20:127–136

Eberl L, Molin S, Givskov M (1999) Surface motility in *Serratia liquefaciens*. J Bacteriol 181:1703–1712

Egland KA, Greenberg EP (1999) Quorum sensing in Vibrio fischeri: elements of the luxl promoter. Mol Microbiol 31:1197–1204

Egland KA, Greenberg EP (2001) Quorum sensing in *Vibrio fischeri*: analysis of the LuxR DNA binding region by alanine-scanning mutagenesis. J Bacteriol 183:382–386

Finney AH, Blick RJ, Murakami K, Ishihama A, Stevens AM (2002) Role of the C-terminal domain of the alpha subunit of RNA polymerase in LuxR-dependent transcriptional activation of the *lux* operon during quorum sensing. J Bacteriol 184:4520–4528

Fukushima J, Ishiwata T, You Z, Ishii T, Shigematsu T, Kurata M, Chikumaru-Fujita S, Bycroft BW, Stewart GS, Kawamoto S, Morihara K, Williams P, Okuda K (1997) Dissection of the promoter/operator region and evaluation of *N*-acylhomoserine lactone mediated transcriptional regulation of elastase expression in *Pseudomonas aeruginosa*. FEMS Microbiol Lett 146:311–318

Fuqua C, Greenberg EP (2002) Signalling: listening in on bacteria: acyl-homoserine lactone signalling. Nat Rev Mol Cell Biol 3:685–695

Fuqua WC, Winans SC, Greenberg EP (1994) Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. J Bacteriol 176:269–275

Gambello MJ, Iglewski BH (1991) Cloning and characterization of the *Pseudomonas aeruginosa lasR* gene, a transcriptional activator of elastase expression. J Bacteriol 173:3000–3009

Giacometti A, Cirioni O, Gov Y, Ghiselli R, Del Prete DS, Mocchegiani F, Saba V, Orlando F, Scalise G, Balaban N, Dell'Acqua G (2003) RNAIII inhibiting peptide (RIP) inhibits in vivo biofilm formation of drug-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 47:1979–1983

Giacometti A, Cirioni O, Ghiselli R, Dell'Acqua G, Orlando F, D'Amato G, Mocchegiani F, Silvestri C, Del Prete MS, Rocchi M, Balaban N, Saba V, Scalise G (2005) RNAIII-inhibiting peptide improves efficacy of clinically used antibiotics in a murine model of staphylococcal sepsis. Peptides 26:169–175

Gonzalez Barrios AF, Zuo R, Hashimoto Y, Yang L, Bentley WE, Wood TK (2006) Autoinducer 2 controls biofilm formation in *Escherichia coli* through a novel motility quorum-sensing regulator (MqsR, B3022). J Bacteriol 188:305–316

Gov Y, Borovok I, Korem M, Singh VK, Jayaswal RK, Wilkinson BJ, Rich SM, Balaban N (2004) Quorum sensing in Staphylococci is regulated via phosphorylation of three conserved histidine residues. J Biol Chem 279:14665–14672

Gram L, Christensen AB, Ravn L, Molin S, Givskov M (1999) Production of acylated homoserine lactones by psychrotrophic members of the *Enterobacteriaceae* isolated from foods. Appl Environ Microbiol 65:3458–3463

Gray KM (1997) Intercellular communication and group behavior in bacteria. Trends Microbiol 5:184–188

Han YH, Kim YG, Kim DY, Ha SC, Lokanath NK, Kim KK (2005) The target of RNAIII-activating protein (TRAP) from *Staphylococcus aureus*: purification, crystallization and preliminary X-ray analysis. Biochim Biophys Acta 1748:134–136

Hanzelka BL, Greenberg EP (1995) Evidence that the N-terminal region of the *Vibrio fischeri* LuxR protein constitutes an autoinducer-binding domain. J Bacteriol 177:815–817

Hentzer M, Riedel K, Rasmussen TB, Heydorn A, Andersen JB, Parsek MR, Rice SA, Eberl L, Molin S, Høiby N, Kjelleberg S, Givskov M (2002) Inhibition of quorum sensing in *Pseu-*

domonas aeruginosa biofilm bacteria by a halogenated furanone compound. Microbiology 148:87–102

Hentzer M, Wu H, Andersen JB, Riedel K, Rasmussen TB, Bagge N, Kumar N, Schembri MA, Song Z, Kristoffersen P, Manefield M, Costerton JW, Molin S, Eberl L, Steinberg P, Kjelleberg S, Høiby N, Givskov M (2003) Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. EMBO J 22:3803–3815

Heydorn A, Ersboll B, Kato J, Hentzer M, Parsek MR, Tolker-Nielsen T, Givskov M, Molin S (2002) Statistical analysis of *Pseudomonas aeruginosa* biofilm development: impact of mutations in genes involved in twitching motility, cell-to-cell signaling, and stationary-phase sigma factor expression. Appl Environ Microbiol 68:2008–2017

Hoch JA, Varughese KI (2001) Keeping signals straight in phosphorelay signal transduction. J Bacteriol 183:4941–4949

Ji G, Beavis R, Novick RP (1997) Bacterial interference caused by autoinducing peptide variants. Science 276:2027–2030

Johansson J, Cossart P (2003) RNA-mediated control of virulence gene expression in bacterial pathogens. Trends Microbiol 11:280–285

Johnson DC, Ishihama A, Stevens AM (2003) Involvement of region 4 of the sigma70 subunit of RNA polymerase in transcriptional activation of the lux operon during quorum sensing. FEMS Microbiol Lett 228:193–201

Joyce EA, Kawale A, Censini S, Kim CC, Covacci A, Falkow S (2004) LuxS is required for persistent pneumococcal carriage and expression of virulence and biosynthesis genes. Infect Immun 72:2964–2975

Kjelleberg S, Molin S (2002) Is there a role for quorum sensing signals in bacterial biofilms? Curr Opin Microbiol 5:254–258

Kjelleberg S, Høiby N, Givskov M (2003) Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. EMBO J 22:3803–3815

Koch B, Liljefors T, Persson T, Nielsen J, Kjelleberg S, Givskov M (2005) The LuxR receptor: the sites of interaction with quorum sensing signals and inhibitors. Microbiology 151:3589–3602

Koenig RL, Ray JL, Maleki SJ, Smeltzer MS, Hurlburt BK (2004) *Staphylococcus aureus* AgrA binding to the RNAIII-agr regulatory region. J Bacteriol 186:7549–7555

Korem M, Sheoran A, Gov Y, Borovok I, Tzipori S, Balaban N (2003) Characterization of RAP, a quorum sensing activator in *Staphylococccus aureus*. FEMS Microbiol Lett 223:167–175

Korem M, Gov Y, Kiran MD, Balaban N (2005) Transcriptional profiling of target of RNAIII-activating protein, a master regulator of staphylococcal virulence. Infect Immun 73:6220–6228

Kreikemeyer B, Boyle MD, Buttaro BA, Heinemann M, Podbielski A (2001) Group A streptococcal growth phase-associated virulence factor regulation by a novel operon (Fas) with homologies to two-component-type regulators requires a small RNA molecule. Mol Microbiol 39:392–406

Ledgham F, Ventre I, Soscia C, Foglino M, Sturgis JN, Lazdunski A (2003) Interactions of the quorum sensing regulator QscR: interaction with itself and the other regulators of *Pseudomonas aeruginosa* LasR and RhlR. Mol Microbiol 48:199–210

Lina G, Jarraud S, Ji G, Greenland T, Pedraza A, Etienne J, Novick RP, Vandenesch F (1998) Transmembrane topology and histidine protein kinase activity of AgrC, the *agr* signal receptor in *Staphylococcus aureus*. Mol Microbiol 28:655–662

Lowy FD (1998) *Staphylococcus aureus* infections. N Engl J Med 339:520–532

Lyon GJ, Mayville P, Muir TW, Novick RP (2000) Rational design of a global inhibitor of the virulence response in *Staphylococcus aureus*, based in part on localization of the site of inhibition to the receptor-histidine kinase, AgrC. Proc Natl Acad Sci USA 97:13330–13335

Manna AC, Cheung AL (2003) sarU, a sarA homolog, is repressed by SarT and regulates virulence genes in *Staphylococcus aureus*. Infect Immun 71:343–353

March JC, Bentley WE (2004) Quorum sensing and bacterial cross-talk in biotechnology. Curr Opin Biotechnol 15:495–502

Mashburn LM, Whiteley M (2005) Membrane vesicles traffic signals and facilitate group activities in a prokaryote. Nature 437:422–425

Mashburn-Warren LM, Whiteley M (2006) Special delivery: vesicle trafficking in prokaryotes. Mol Microbiol 61:839–846

Mathesius U, Mulders S, Gao M, Teplitski M, Caetano-Anolles G, Rolfe BG, Bauer WD (2003) Extensive and specific responses of a eukaryote to bacterial quorum-sensing signals. Proc Natl Acad Sci USA 100:1444–1449

Mayville P, Ji G, Beavis R, Yang H, Goger M, Novick RP, Muir TW (1999) Structure-activity analysis of synthetic autoinducing thiolactone peptides from *Staphylococcus aureus* responsible for virulence. Proc Natl Acad Sci USA 96:1218–1223

McLean RJ, Whiteley M, Stickler DJ, Fuqua WC (1997) Evidence of autoinducer activity in naturally occurring biofilms. FEMS Microbiol Lett 154:259–263

Merritt J, Qi F, Goodman SD, Anderson MH, Shi W (2003) Mutation of luxS affects biofilm formation in *Streptococcus mutans*. Infect Immun 71:1972–1979

Michel A, Agerer F, Hauck CR, Herrmann M, Ullrich J, Hacker J, Ohlsen K (2006) Global regulatory impact of ClpP protease of Staphylococcus aureus on regulons involved in virulence, oxidative stress response, autolysis, and DNA repair. J Bacteriol 188:5783–5796

Miller MB, Bassler BL (2001) Quorum sensing in bacteria. Annu Rev Microbiol 55:165–199

Miller ST, Xavier KB, Campagna SR, Taga ME, Semmelhack MF, Bassler BL, Hughson FM (2004) *Salmonella typhimurium* recognizes a chemically distinct form of the bacterial quorum-sensing signal AI-2. Mol Cell 15:677–687

Mittal R, Sharma S, Chhibber S, Harjai K (2006) Contribution of quorum-sensing systems to virulence of *Pseudomonas aeruginosa* in an experimental pyelonephritis model. J Microbiol Immunol Infect 39:302–309

Morfeldt E, Taylor D, von Gabain A, Arvidson S (1995) Activation of alpha-toxin translation in *Staphylococcus aureus* by the trans-encoded antisense RNA, RNAIII. EMBO J 14:4569–4577

Moser C, Johansen HK, Song Z, Hougen HP, Rygaard J, Hoiby N (1997) Chronic *Pseudomonas aeruginosa* lung infection is more severe in Th2 responding BALB/c mice compared to Th1 responding C3H/HeN mice. APMIS 105:838–842

Nakayama J, Cao Y, Horii T, Sakuda S, Nagasawa H (2001) Chemical synthesis and biological activity of the gelatinase biosynthesis-activating pheromone of Enterococcus faecalis and its analogs. Biosci Biotechnol Biochem 65:2322–2325

Novick RP (2003) Autoinduction and signal transduction in the regulation of staphylococcal virulence. Mol Microbiol 48:1429–1449

Novick RP, Ross HF, Projan SJ, Kornblum J, Kreiswirth B, Moghazeh S (1993) Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. EMBO J 12:3967–3975

Novick RP, Projan SJ, Kornblum J, Ross HF, Ji G, Kreiswirth B, Vandenesch F, Moghazeh S (1995) The *agr* P2 operon: an autocatalytic sensory transduction system in *Staphylococcus aureus*. Mol Gen Genet 248:446–458

Ochsner UA, Koch AK, Fiechter A, Reiser J (1994) Isolation and characterization of a regulatory gene affecting rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*. J Bacteriol 176:2044–2054

Otto M (2004) Quorum-sensing control in Staphylococci – a target for antimicrobial drug therapy? FEMS Microbiol Lett 241:135–141

Parsek MR, Greenberg EP (2000) Acyl-homoserine lactone quorum sensing in gram-negative bacteria: a signaling mechanism involved in associations with higher organisms. Proc Natl Acad Sci USA 97:8789–8793

Parsek MR, Singh PK (2003) Bacterial biofilms: an emerging link to disease pathogenesis. Annu Rev Microbiol 57:677–701

Parsek MR, Val DL, Hanzelka BL, Cronan JE, Greenberg EP (1999) Acyl homoserine-lactone quorum-sensing signal generation. Proc Natl Acad Sci USA 96:4360–4365

Pearson JP, Gray KM, Passador L, Tucker KD, Eberhard A, Iglewski BH, Greenberg EP (1994) Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. Proc Natl Acad Sci USA 91:197–201

Pearson JP, Van Delden C, Iglewski BH (1999) Active efflux and diffusion are involved in transport of *Pseudomonas aeruginosa* cell-to-cell signals. J Bacteriol 181:1203–1210

Perraud AL, Weiss V, Gross R (1999) Signalling pathways in two-component phosphorelay systems. Trends Microbiol 7:115–120

Qiu R, Pei W, Zhang L, Lin J, Ji G (2005) Identification of the putative staphylococcal AgrB catalytic residues involving the proteolytic cleavage of AgrD to generate autoinducing peptide. J Biol Chem 280:16695–16704

Rasmussen TB, Bjarnsholt T, Skindersoe ME, Hentzer M, Kristoffersen P, Kote M, Nielsen J, Eberl L, Givskov M (2005a) Screening for quorum-sensing inhibitors (QSI) by use of a novel genetic system, the QSI selector. J Bacteriol 187:1799–1814

Rasmussen TB, Skindersoe ME, Bjarnsholt T, Phipps RK, Christensen KB, Jensen PO, Andersen JB, Koch B, Larsen TO, Hentzer M, Eberl L, Høiby N, Givskov M (2005b) Identity and effects of quorum-sensing inhibitors produced by *Penicillium* species. Microbiology 151:1325–1340

Ren D, Bedzyk LA, Thomas SM, Ye RW, Wood TK (2004b) Differential gene expression shows natural brominated furanones interfere with the autoinducer-2 bacterial signaling system of *Escherichia coli*. Biotechnol Bioeng 88:630–642

Ritchie AJ, Yam AO, Tanabe KM, Rice SA, Cooley MA (2003) Modification of in vivo and *in vitro* T- and B-cell-mediated immune responses by the *Pseudomonas aeruginosa* quorum-sensing molecule *N*-(3-oxododecanoyl)-L-homoserine lactone. Infect Immun 71:4421–4431

Rust L, Pesci EC, Iglewski BH (1996) Analysis of the *Pseudomonas aeruginosa* elastase (lasB) regulatory region. J Bacteriol 178:1134–1140

Schauder S, Shokat K, Surette MG, Bassler BL (2001) The LuxS-family of bacterial autoinducers: biosynthesis of a novel quorum-sensing signal molecule. Mol Microbiol 41:463–476

Shaw LN, Jonsson IM, Singh VK, Tarkowski A, Stewart GC (2007) Inactivation of traP has no effect on the agr quorum-sensing system or virulence of Staphylococcus aureus. Infect Immun 75:4519–4527

Sheng J, Citovsky V (1996) Agrobacterium-plant cell DNA transport: have virulence proteins, will travel. Plant Cell 8:1699–1710

Shimizu T, Yaguchi H, Ohtani K, Banu S, Hayashi H (2002) Clostridial VirR/VirS regulon involves a regulatory RNA molecule for expression of toxins. Mol Microbiol 43:257–265

Singh PK, Schaefer AL, Parsek MR, Moninger TO, Welsh MJ, Greenberg EP (2000) Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. Nature 407:762–764

Smith RS, Iglewski BH (2003) *P. aeruginosa* quorum-sensing systems and virulence. Curr Opin Microbiol 6:56–60

Smith RS, Harris SG, Phipps R, Iglewski B (2002a) The *Pseudomonas aeruginosa* quorum-sensing molecule *N*-(3-oxododecanoyl) homoserine lactone contributes to virulence and induces inflammation in vivo. J Bacteriol 184:1132–1139

Smith RS, Kelly R, Iglewski BH, Phipps RP (2002b) The *Pseudomonas* autoinducer *N*-(3-oxododecanoyl) homoserine lactone induces cyclooxygenase-2 and prostaglandin E2 production in human lung fibroblasts:implications for inflammation. J Immunol 169:2636–2642

Sperandio V, Mellies JL, Nguyen W, Shin S, Kaper JB (1999) Quorum sensing controls expression of the type III secretion gene transcription and protein secretion in enterohemorhagic and enteropathogenic *Escherichia coli*. Proc Natl Acad Sci USA 96:15196–15201

Sperandio V, Torres AG, Jarvis B, Nataro JP, Kaper JB (2003) Bacteria-host communication: the language of hormones. Proc Natl Acad Sci USA 100:8951–8956

Telford G, Wheeler D, Williams P, Tomkins PT, Appleby P, Sewell H, Stewart GS, Bycroft BW, Pritchard DI (1998) The *Pseudomonas aeruginosa* quorum-sensing signal molecule *N*-(3-oxododecanoyl)-L-homoserine lactone has immunomodulatory activity. Infect Immun 66: 36–42

Trott AE, Stevens AM (2001) Amino acid residues in LuxR critical for its mechanism of transcriptional activation during quorum sensing in *Vibrio fischeri*. J Bacteriol 183:387–392
Tsang LH, Daily ST, Weiss EC, Smeltzer MS (2007) Mutation of traP in Staphylococcus aureus has no impact on expression of agr or biofilm formation. Infect Immun 75:4528–4533
Vannini A, Volpari C, Gargioli C, Muraglia E, Cortese R, De Francesco R, Neddermann P, Marco SD (2002) The crystal structure of the quorum sensing protein TraR bound to its autoinducer and target DNA. EMBO J 21:4393–4401
Velicer GJ (2003) Social strife in the microbial world. Trends Microbiol 11:330–337
Vendeville A, Winzer K, Heurlier K, Tang CM, Hardie KR (2005) Making "sense" of metabolism: autoinducer-2, LuxS and pathogenic bacteria. Nat Rev Microbiol 3:383–396
Vieira-da-Motta O, Damasceno Ribeiro P, Dias da Silva W, Medina-Acosta E (2001) RNAIII inhibiting peptide (RIP) inhibits *agr*-regulated toxin production. Peptides 22:1621–1627
Vuong C, Saenz HL, Gotz F, Otto M (2000) Impact of the agr quorum-sensing system on adherence to polystyrene in Staphylococcus aureus. J Infect Dis 182:1688–1693
Vuong C, Gerke C, Somerville GA, Fischer ER, Otto M (2003) Quorum-sensing control of biofilm factors in *Staphylococcus epidermidis*. J Infect Dis 188:706–718
Wagner VE, Bushnell D, Passador L, Brooks AI, Iglewski BH (2003) Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment. J Bacteriol 185:2080–2095
Walters M, Sperandio V (2006) Autoinducer 3 and epinephrine signaling in the kinetics of locus of enterocyte effacement gene expression in enterohemorrhagic *Escherichia coli*. Infect Immun 74:5445–5455
Waters CM, Bassler BL (2005) Quorum sensing: cell-to-cell communication in bacteria. Annu Rev Cell Dev Biol 21:319–346
Watson WT, Minogue TD, Val DL, von Bodman SB, Churchill ME (2002) Structural basis and specificity of acyl-homoserine lactone signal production in bacterial quorum sensing. Mol Cell 9:685–694
Whitehead NA, Byers JT, Commander P, Corbett MJ, Coulthurst SJ, Everson L, Harris AK, Pemberton CL, Simpson NJ, Slater H, Smith DS, Welch M, Williamson N, Salmond GP (2002) The regulation of virulence in phytopathogenic *Erwinia* species: quorum sensing, antibiotics and ecological considerations. Antonie Van Leeuwenhoek 81:223–231
Whiteley M, Greenberg EP (2001) Promoter specificity elements in Pseudomonas aeruginosa quorum-sensing-controlled genes. J Bacteriol 183:5529–5534
Winans SC, Bassler BL (2002) Mob psychology. J Bacteriol 184:873–883
Winzer K, Williams P (2001) Quorum sensing and the regulation of virulence gene expression in pathogenic bacteria. Int J Med Microbiol 291:131–143
Withers H, Swift S, Williams P (2001) Quorum sensing as an integral component of gene regulatory networks in Gram-negative bacteria. Curr Opin Microbiol 4:186–193
Wopperer J, Cardona ST, Huber B, Jacobi CA, Valvano MA, Eberl L (2006) A quorum-quenching approach to investigate the conservation of quorum-sensing-regulated functions within the *Burkholderia cepacia* complex. Appl Environ Microbiol 72:1579–1587
Xavier KB, Bassler BL (2005) Regulation of uptake and processing of the quorum-sensing autoinducer AI-2 in *Escherichia coli*. J Bacteriol 187:238–248
Yang G, Cheng H, Liu C, Xue Y, Gao Y, Liu N, Gao B, Wang D, Li S, Shen B, Shao N (2003) Inhibition of *Staphylococcus aureus* pathogenesis in vitro and in vivo by RAP-binding peptides. Peptides 24:1823–1828
Yang G, Gao Y, Dong J, Liu C, Xue Y, Fan M, Shen B, Shao N (2005) A novel peptide screened by phage display can mimic TRAP antigen epitope against *Staphylococcus aureus* infections. J Biol Chem 280:27431–27435
Zhang L, Ji G (2004) Identification of a staphylococcal AgrB segment(s) Responsible for group-specific processing of AgrD by gene swapping. J Bacteriol 186:6706–6713
Zhang L, Gray L, Novick RP, Ji G (2002a) Transmembrane topology of AgrB, the protein involved in the post-translational modification of AgrD in *Staphylococcus aureus*. J Biol Chem 277:34736–34742

Zhang RG, Pappas T, Brace JL, Miller PC, Oulmassov T, Molyneaux JM, Anderson JC, Bashkin JK, Winans SC, Joachimiak A (2002b) Structure of a bacterial quorum-sensing transcription factor complexed with pheromone and DNA. Nature 417:971–974

Ziebandt AK, Weber H, Rudolph J, Schmid R, Hoper D, Engelmann S, Hecker M (2001) Extracellular proteins of *Staphylococcus aureus* and the role of SarA and sigma B. Proteomics 1:480–493

Detection In Vitro of Quorum-Sensing Molecules and Their Inhibitors

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Abstract Bacterial population density signaling (quorum signaling) is now recognized as a widespread phenomenon in microorganisms. In some cases, quorum signaling is an essential regulatory component of virulence and other attributes, including biofilm formation. Several organisms compete with bacteria by virtue of disrupting quorum signal production or the signal receptor, or by degrading the signals themselves. While some have been described in the literature, many others await discovery. Here, we explore bioassay-based strategies that could be used to identify novel quorum-signal inhibitors.

Many of the quorum sensing (QS)-controlled genes, such as in *P. aeruginosa* and *S. aureus*, encode known virulence factors. These include the toxins elastase, alkaline protease, chitinases, cyanide, phenazines, lectins, and rhamnolipids by *P. aeruginosa* (Schuster et al. 2003; Wagner et al. 2003; Hentzer et al. 2003; Vasil 2003;

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N. Balaban (ed.) *Control of Biofilm Infections by Signal Manipulation*
Springer Series on Biofilms 2
doi: 10.1007/978-3-540-73853-4, © Springer 2008

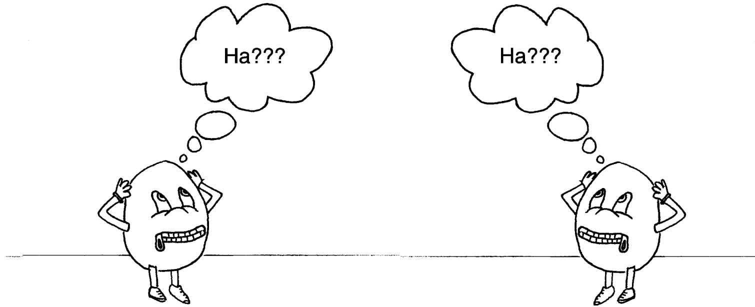

Fig. 1 Inhibition of quorum sensing as a novel mode of therapy (Illustration by Mike Beshiri, Tufts University, Cummings School of Veterinary Medicine, Department of Biomedical Sciences, Division of Infectious Diseases, North Grafton, MA, USA)

Hassett et al. 1999). In *S. aureus* QS-regulated virulence factors include alpha, beta, gamma, and delta-hemolysin, triacylglycerol lipase precursor, glycerol ester hydrolase, hyaluronate lyase precursor, staphylococcal serine protease (V8 protease), cysteine protease precursor, cysteine protease, staphopain-cysteine proteinase, 1-phosphatidylinositol phosphodiesterase, zinc metalloproteinase aureolysin precursor, holing-like proteins, and capsular polysaccharide synthesis enzymes (Lowy 1998; Korem et al. 2005). Also controlled are genes involved in iron limitation in biofilms (Hentzer et al. 2005) as well as the adaptive response that limits the deleterious effects of the reduced pH associated with anaerobic growth conditions in biofilms (Beenken et al. 2004).

Given the fact that QS systems control many different unwanted bacterial phenotypes, including toxin production and biofilm formation, and given the fact that they function by means of extracellular signal, they are promising targets for developing novel antimicrobials (Fig. 1). In the following sections, we will review such compounds.

The importance of QS-dependent gene expression for bacterial virulence has been established in several animal models, as shown in the chapter *In Vivo Studies: Inhibiting Biofilm-Associated Bacterial Infections Using SQIs*, and in clinical studies (discussed in the chapter *Clinical Wound Healing Using Signal Inhibitors*).

1 Detection of Quorum Signals

Several strains have been developed for detecting QS activators and inhibitors (Table 1), based on fusing a QS-controlled promoter to a reporter gene. A characteristic of some of these strains is that they lack the ability to produce their own QS but are able to respond to exogenous QS with a visible phenotype, such as violacein pigment production in *Chromobacterium violaceum* CV026 (McClean et al. 1997; Adonizio et al. 2006), prodigiosin pigment production in *Serratia marcescens* ATCC 39006 (Glansdorp et al. 2004), light production in *Vibrio harveyi* D1 (Cao and Meighen 1993), and swarming in *Serratia liquefaciens* MG44 (Eberl et al.

Table 1 Representative quorum-sending (QS) bacteria and plasmids

Strain	Major characteristics	Refs.
Agrobacterium tumefaciens		
A136 (pCF218)(pCF372)[a]	Detects wide range of AHLs (C6-C14 HSLs)	Fuqua and Winans 1996; Zhu et al. 1998
KYC55 (pJZ372)(pJZ384)(pJZ410)[a]	Increased detection of AHLs (C4-C18 HSLs)	Zhu et al. 2003
KYC6	3-oxo-C8-HSL overproducer	Fuqua et al. 1995
Chromobacterium violaceum		
CV026[b]	Detects C4-C8-HSLs	McClean et al. 1997
ATCC 31532	Nonpigmented overproducer of C6-HSLs	McClean et al. 1997
ATCC 12472[b]	wt pigmented strain used in QS inhibitor assay	McLean et al. 2004
Pseudomonas aureofaciens		
30–84[b]	wt pigmented strain used in QSI assay	McLean et al. 2004; Wood and Pierson 1996
Serratia liquefaciens		
MG44[c]	*swrI* mutant for C4-HSL detection	Eberl et al. 1996
Serratia marcescens		
ATCC39006[b]	*smaI* mutant for C4-HSL detection	Glansdorp et al. 2004
Sinorhizobium meliloti		
Rm41 *sinI*::*lacZ*(pJNSinR)[a]	AHL reporter for long chain (up to C16) HSLs	Llamas et al. 2004
Vibrio harveyi BB170	AI-2 detection	Bassler et al. 1994
Plasmids		
pSB406[d]	*lux*-based reporter plasmid for AHL detection	Winson et al. 1995
pSB403[d]	*lux*-based reporter plasmid for AHL detection	Winson et al. 1998
pJBA89[e]	Stable gfp-based reporter plasmid for AHL detection	Andersen et al. 2001
pJBA132[e]	Unstable gfp-based reporter plasmid for AHL detection Most sensitive for C6-HSL	Andersen et al. 2001
pAS-C8[e]	Unstable gfp-based reporter plasmid for AHL detection most sensitive for C8-HSL	Steidle et al. 2001
pKR-C12[e]	Unstable gfp-based reporter plasmid for AHL detection most sensitive for 3-oxo-C12-HSL	Steidle et al. 2001
pMHLB	*lasB-gfp*; unstable reporter for detection of 3-oxo-C12-HSL	Hentzer et al. 2002
S. aureus		
pRN6683 in lab strain RN6390[f]	RNAIII-*blaZ* transcriptional fusion	Novick et al. 1995

[a] *lacZ* reporter [b] Pigment reporter [*C. violaceum*, violacein (purple); *P. aureofaciens*, phenazine (orange); *S. marcescens*, prodigiosin (red)] [c] Swarming reporter [d] *lux* reporter [e] *gfp* reporter; unstable *gfp* constructs have a C-terminus deletion, making them more susceptible to endogenous proteolytic activity in bacteria (Andersen et al. 2001). They are used for real-time measurements of AHL levels. [f] Beta-lactamase reporter

1996). A bioluminescent strain of *V. harveyi* BB170 was developed for the bioassay of AI-2 (Bassler et al. 1994), and RNAIII-*blaZ* transcriptional fusion was developed in *S. aureus* for detecting *agr* as a marker for virulence (Novick et al. 1995).

QS-mediated gene expression does not always result in a readily visible phenotype. One example of this is in the plant pathogen *Agrobacterium tumefaciens*, in which conjugation is mediated by QS (Fuqua and Winans 1996). Two *A. tumefaciens* biosensor strains have been constructed, A136 (pCF218)(pCF372) (Fuqua and Winans 1996) and KYC55 (pJZ372)(pJZ384)(pJZ410) (Zhu et al. 2003). These two strains overexpress *traR*, which is the *luxR* homolog. Although these strains are most sensitive (often in the sub-pmol concentration) to the cognate *N*-acyl homoserine lactone (AHL) [3-oxo-octanoyl homoserine lactone (3-oxo-C8 HSL)], they are also able to detect a wide range of other AHLs at less sensitivity (μmol to nmol range; Zhu et al. 1998, 2003). A representative example is shown in Fig. 2. Other AHL biosensor strains have also been constructed using other reporter genes, including *gfp* and *lux*, that have been fused to *luxR* homologs (Andersen et al. 2001; Swift et al. 1993). Positive controls for AHL detection include WT strains of the biosensor organism with intact *luxI* genes and AHL synthetic capability. Alternatively, AHL overproducers such as *A. tumefaciens* KYC6 (Fuqua and Winans 1996) and *C. violaceum* 31532 (McClean et al. 1997) can be used for the *Agrobacterium* and *Chromobacterium* bioassays, respectively. A number of AHLs are commercially

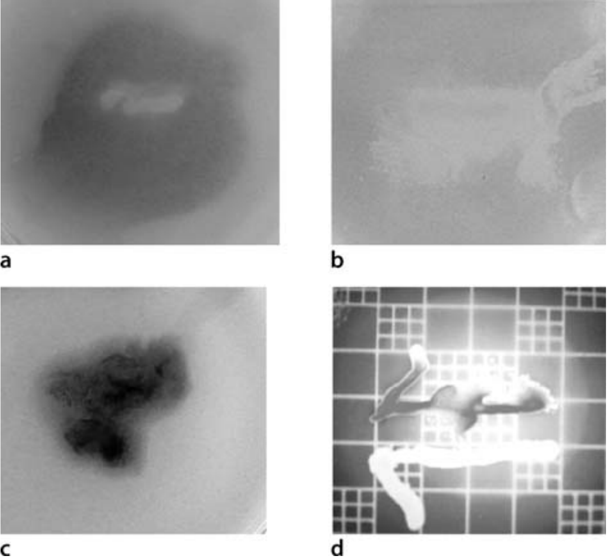

Fig. 2 A soft-agar overlay can be used for qualitative detection of *N*-acyl homoserine lactones (AHLs) using a reporter strain such as *A. tumefaciens* A136 (pCF 218) (pCF372) (Fuqua and Winans 1996). Positive control (**a**) involves testing with AHL-overproducing *A. tumefaciens* KYC6. Negative control (**b**) involves testing with non-AHL-producing A136 reporter strain. An AHL-producing environmental isolate is shown in (**c**). For comparison, a positive control cross-streaking plate is shown in (**d**)

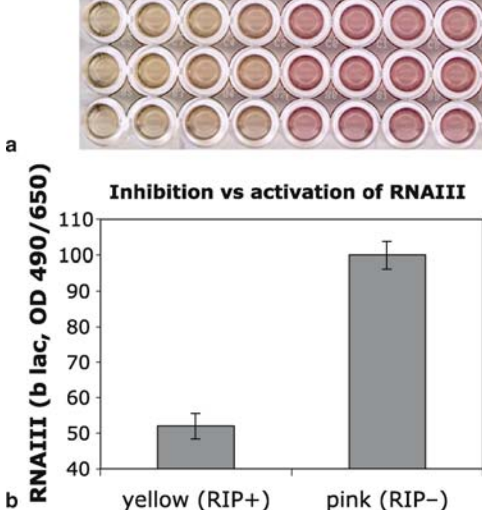

Fig. 3 a An example of beta lactamase reporter assay in *S. aureus*. *Four yellow columns* on left reflect inhibition of RNAIII production by RIP. *Four pink columns* on right reflect RNAIII production in the presence of saline. **b** Spectrophotometric analysis (absorbance at 490/650 nm × 1000) of column 1 on far left (*yellow*, RIP+) and column 8 on far right (*pink*, RIP−)

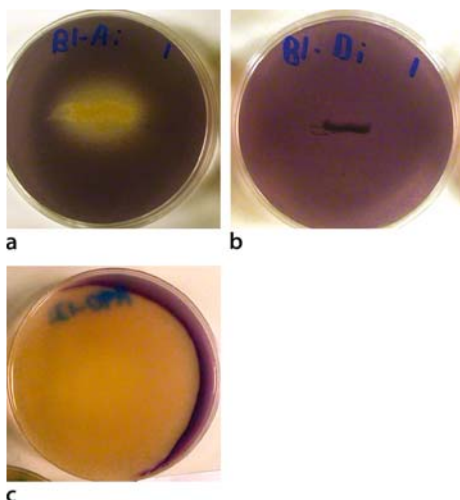

Fig. 4 Bioassay for quorum signal inhibition using *C. violaceum* ATCC 12472 (McLean et al. 2004). The cognate *N*-acyl homoserine lactone (AHL) for this strain is C6-HSL (McClean et al. 1997). Organisms such as *Pseudomonas aeruginosa* PAO1 can be used as a positive control (**a**) in that their AHLs, 3-oxo-C12-, and C4-HSL will compete with C6-HSL for binding on *cviR* (*luxR* homolog) of *C. violaceum*. The negative control, *C. violaceum* 12472, is shown in (**b**). Quorum-sensing inhibitor of aquifer isolate (McLean et al. 2005) is shown in (**c**)

available, or they can be produced via chemical synthesis (Eberhard and Schineller 2000).

For increased sensitivity or resolution of different AHLs, biosensor strains have also been incorporated along with other analytical techniques such as high-performance liquid chromatography (Moré et al. 1996; Charlton et al. 2000a,b) and thin-layer chromatography (Shaw et al. 1997). Alternatively, violacein, a QS-regulated pigment in *C. violaceum*, can be extracted with acetone or ethanol, and measured with a spectrophotometer (Blosser and Gray 2000). There is a recent report of a mass spectrometry technique for AHL detection that rivals the picomolar detection limits of the best biosensors (Makemson et al. 2006).

RNAIII-*blaZ* transcriptional fusion was developed to detect *agr* activity, using the *agr* P3-*blaZ* fusion plasmid pRN6683 in lab strain RN6390 (Novick et al. 1995). Beta-lactamase activity can be measured by the addition of nitrocefin. A red color indicates activation of *agr*, and yellow indicates inhibition of *agr*. This assay can be carried out in microtiter plates (Fig. 3).

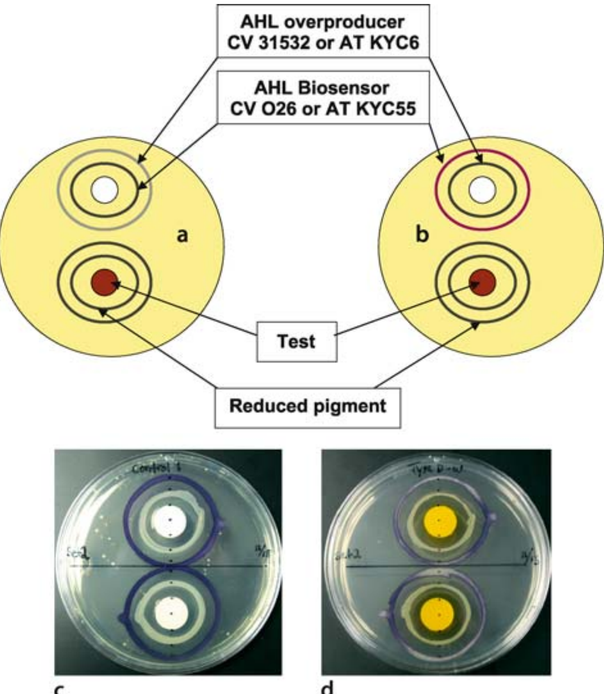

Fig. 5 Incorporation of two strains into a quorum-sensing inhibitor bioassay can distinguish whether inhibition is due to *N*-acyl homoserine lactone (AHL) response (*luxR* effect; **a**) or synthesis (*luxI* effect; **b**). Negative controls are at the top, and test samples are at the bottom. **c** Negative control for *luxI* effect. **d** Positive reaction for inhibition of AHL production due to green tea (*Camellia sinensis*) extract

2 Detection of Quorum Signal Inhibitors

As these reporter systems are fused to a QS-controlled promoter, they become activated when the bacteria encounter exogenous signal molecules. Conversely, when the bacteria are challenged with quorum signal inhibitors (QSIs), the signal from the reporter systems is reduced. Hence, there is a positive "hit" when expression of the reporter is significantly reduced.

Several strategies can be employed to identify QSIs. A general strategy can involve taking a biosensor strain (Table 1) and exposing it to test compounds that would cause a loss of signal response. Alternatively, a wild-type strain containing a QS-regulated phenotype can be used as a biosensor (Table 1). Bacteria using Gfp or beta-lactamase-based screening systems, for instance, can be grown in liquid media in microtiter dishes in which many different compounds and/or concentrations can be probed at a time. An example of inhibition of *S. aureus* QS by RIP is shown in Fig. 3.

The *Chromobacterium violaceum* AHL bioassay (McClean et al. 1997) and pigmented *Pseudomonas aureofaciens* strain 30–84 (Wood and Pierson 1996), for example, have been used for detecting potential QSIs (McLean et al. 2004). Here, biological material (typically plant components or bacterial cultures) is placed in close proximity to either WT *C. violaceum* ATCC 12472 or *P. aureofaciens* 30–84. These QSI indicator strains can be incorporated into a soft-agar overlay (Fig. 4) or, alternatively, can be streaked in close proximity to the test material. Potential QSI activity is observed via a loss of pigmentation (purple violacein production in *C. violaceum* or loss of orange phenazine in *P. aureofaciens*).

Potential antibiotics or other antimicrobial agents can also be detected by growth inhibition of the QSI strains. The AHL normally used by *C. violaceum* and *P. aureofaciens* is *N*-hexanoyl homoserine lactone (C6-HSL). Other AHLs will competitively bind to the LuxR homologs in these two organisms, CviR and PhzR (McClean et al. 1997; Chancey et al. 1999), but are not otherwise biologically active, thus inhibiting their AHL-regulated pigmentation. Although competitive inhibition by AHLs (other than C6-HSL) can be used as a positive control for this bioassay, one should also test samples with QS biosensor strains such as *A. tumefaciens* A136 or *C. violaceum* CV026. In this fashion, one can readily detect potential QSI and also determine whether it is due to AHL-mediated inhibition.

A QSI bioassay was recently modified to discern whether potential inhibitors target AHL synthesis (via LuxI) or AHL response (via LuxR) (Vattem, Bryant, and McLean, unpublished). As shown in Fig. 5, two strains are used: an AHL overproducer such as *A. tumefaciens* KYC6 or *C. violaceum* 31532, and an AHL biosensor such as *A. tumefaciens* A136 or *C. violaceum* CV026. A test compound is usually placed in an absorbent, sterile filter paper and the AHL overproducer and biosensor strains inoculated in varying proximity to the test material. To test for potential LuxI inhibition, the AHL overproducer is placed in close proximity to the test substance and the AHL biosensor placed distal. To test for LuxR inhibition, the locations of the AHL overproducer and biosensor strains are reversed. In either case, potential QSI activity results in a lowered signal from the AHL biosensor. Although the QSI

bioassay at present is a qualitative assay, we are currently developing a quantitative QSI bioassay (Vattem, Bryant, and McLean, unpublished) analogous to a previously published violacein extraction QS assay (Blosser and Gray 2000).

Usually, both growth (OD) and expression of the reporter system are monitored over time. One major drawback to this type of screening system is that compounds that either inhibit or slow growth inevitably reduce reporter expression and consequently may lead to scoring of false positives. Hence, growth of the bacterial screen has to be carefully monitored to ensure that the test compounds are not interfering with growth and thereby with general protein synthesis. To circumvent this problem, another type of screen, termed the QSI selector (QSIS), has been developed (Rasmussen et al. 2005).

In this system, the QS-controlled promoter is fused to a gene causing growth arrest when expressed. The screening bacterium does not produce any QS signals by itself, so in the absence of AHL molecules, growth is unrestrained. If the growth medium is supplemented with AHL molecules, the QS-controlled killing system becomes activated, leading to growth arrest (Fig. 6). Further addition of a QSI com-

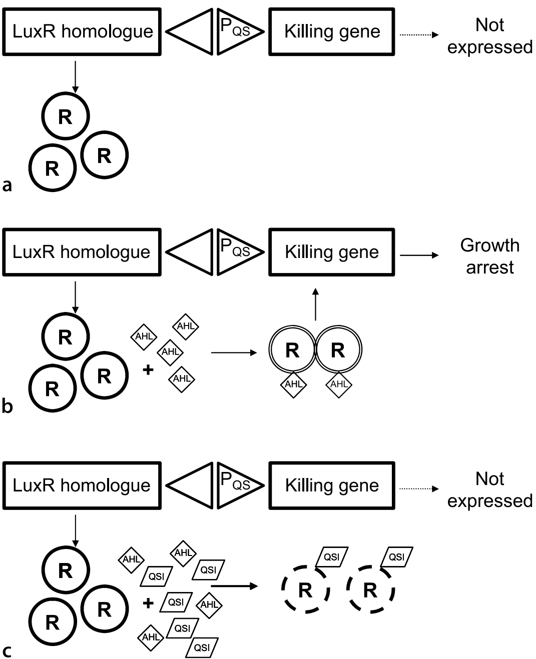

Fig. 6 General layout of the quorum-sensing inhibitor (QSI) selector systems. **a** The screening bacteria are grown without *N*-acyl homoserine lactone (AHL). The LuxR homolog is not activated, hence there is no expression from the QS-controlled promoter (P_{QS}), and, in turn, the killing is not expressed, and the cells survive. This situation is used when the bacteria are grown for purposes other than screening. **b** Exogenously added AHL molecules activate the P_{QS} promoter, and the killing gene is thereby expressed, causing growth arrest of the bacteria. **c** A QSI blocks QS, and the killing gene is not expressed, allowing growth of the bacteria (Rasmussen et al. 2005)

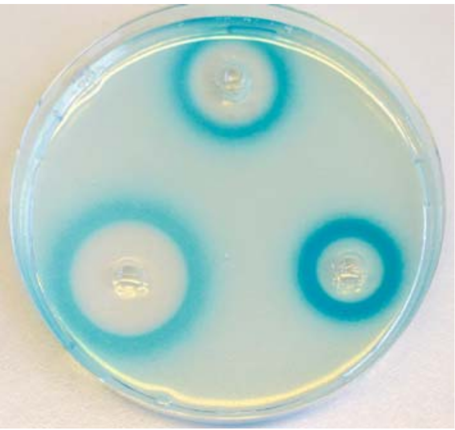

Fig. 7 The quorum-sensing inhibitor selector (QSIS) 1 screening system in action. The screening bacteria are cast into an agar plate along with *N*-acyl homoserine lactone (AHL) and other cofactors. Wells are punched in the plate, into which test compounds are added. The test compounds diffuse into the agar, and where the concentration is appropriate, quorum sensing is blocked, allowing growth of the bacteria. Growth is indicated by a *blue ring* as the bacteria produce beta-galactosidase turning over X-gal in the plate

pound inhibits expression of the QS-controlled killing cassette, and the bacteria are allowed to grow (Fig. 6). Hence, the presence of a hit is indicated by growth. Furthermore, the bacteria express phenotypes that ease identification of growth, such as beta-galactosidase and bioluminescence (Fig. 7).

Briefly, the bacteria are cast into agar along with signal molecules that will activate the killing cassette. Wells are punched in the agar, and compounds or mixtures to be tested are added to the wells. From the wells, compounds diffuse into the semisolid agar, establishing a concentration gradient with the highest concentration closest to the well. This enables the researchers to test the effect of numerous concentrations in just one assay. If the compound has no QSI activity, the killing system in the bacteria is active due to the AHLs present in the agar; hence, the bacteria are killed, no growth is observed, and a negative screen is observed. If the compound is toxic to the bacteria, no growth is observed. This is also scored as a negative outcome of the screen. Only if the test compound has nontoxic properties and exhibits QSI activity will the bacteria be rescued and a positive outcome of the screen be scored (Fig. 7) (Rasmussen et al. 2005).

References

Adonizio AL, Downum K, Bennett BC, Mathee K (2006) Anti-quorum sensing activity of medicinal plants in southern Florida. J Ethnopharmacol 105:427–435

Andersen JB, Heydorn A, Hentzer M, Eber L, Geisenberger O, Christensen BB, Molin S, Givskov M (2001) *gfp*-based *N*-acyl homoserine lactone sensor systems for detection of bacterial communication. Appl Environ Microbiol 67:575–585

Bassler BL, Wright M, Silverman MR (1994) Multiple signalling systems controlling expression of luminescence in Vibrio harveyi: sequence and function of genes encoding a second sensory pathway. Mol Microbiol 13:273–286

Beenken KE, Dunman PM, McAleese F, Macapagal D, Murphy E, Projan SJ, Blevins JS, Smeltzer MS (2004) Global gene expression in Staphylococcus aureus biofilms. J Bacteriol 186:4665–4684

Blosser RS, Gray KM (2000) Extraction of violacein from Chromobacterium violaceum provides a new quantitative bioassay for *N*-acyl homoserine lactone autoinducers. J Microbiol Methods 40:47–55

Cao JG, Meighen EA (1993) Biosynthesis and stereochemistry of the autoinducer controlling luminescence in Vibrio harveyi. J Bacteriol 175:3856–3862

Chancey ST, Wood DW, Pierson LS (1999) Two-component transcriptional regulation of *N*-acyl-homoserine lactone production in Pseudomonas aeruginosa. Appl Environ Microbiol 65:2294–2299

Charlton TS, de Nys R, Netting A, Kumar N, Hentzer M, Givskov M, Kjelleberg S (2000a) A novel and sensitive method for the quantification of *N*-3-oxoacyl homoserine lactones using gas chromatography-mass spectrometry: application to a model bacterial biofilm. Environ Microbiol 2:530–541

Charlton T, Givskov M, deNys R, Andersen JB, Hentzer M, Rice S, Kjelleberg S (2000b) Genetic and chemical tools for investigating signaling processes in biofilms. Methods Enzymol 336:108–128

Eberhard A, Schineller JB (2000) Chemical synthesis of bacterial autoinducers and analogs. Methods Enzymol 305:301–315

Eberl L, Winson MK, Sternberg C, Stewart GS, Christiansen G, Chhabra SR, Bycroft B, Williams P, Molin S, Givskov M (1996) Involvement of *N*-acyl-L-homoserine lactone autoinducers in controlling the multicellular behaviour of *Serratia liquefaciens*. Mol Microbiol 20:127–136

Fuqua C, Burbea M, Winans SC (1995) Activity of the *Agrobacterium* Ti plasmid conjugal transfer regulator TraR is inhibited by the product of the traM gene. J Bacteriol 177:1367–1373

Fuqua C, Winans SC (1996) Conserved cis-acting promoter elements are required for density-dependent transcription of *Agrobacterium tumefaciens* conjugal transfer genes. J Bacteriol 178:435–440

Glansdorp FG, Thomas GL, Lee JK, Dutton JM, Salmond GPC, Welch M, Spring DR (2004) Synthesis and stability of small molecule probes for *Pseudomonas aeruginosa* quorum sensing modulation. Org Biomol Chem 2:3329–3336

Hassett DJ, Ma JF, Elkins JG, McDermott TR, Ochsner UA, West SE, Huang CT, Fredericks J, Burnett S, Stewart PS, McFeters G, Passador L, Iglewski BH (1999) Quorum sensing in *Pseudomonas aeruginosa* controls expression of catalase and superoxide dismutase genes and mediates biofilm susceptibility to hydrogen peroxide. Mol Microbiol 34:1082–1093

Hentzer M, Riedel K, Rasmussen TB, Heydorn A, Andersen JB, Parsek MR, Rice SA, Eberl L, Molin S, Høiby N, Kjelleberg S, Givskov M (2002) Inhibition of quorum sensing in *Pseudomonas aeruginosa* biofilm bacteria by a halogenated furanone compound. Microbiology 148:87–102

Hentzer M, Wu H, Andersen JB, Riedel K, Rasmussen TB, Bagge N, Kumar N, Schembri MA, Song Z, Kristoffersen P, Manefield M, Costerton JW, Molin S, Eberl L, Steinberg P, Kjelleberg S, Høiby N, Givskov M (2003) Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. EMBO J 22:3803–3815

Hentzer M, Eberl L, Givskov M (2005) Transcriptome analysis of *Pseudomonas aeruginosa* biofilm development: anaerobic respiration and iron limitation. Biofilms 2:37–61

Hume EB, Baveja J, Muir B, Schubert TL, Kumar N, Kjelleberg S, Griesser HJ, Thissen H, Read R, Poole-Warren LA, Schindhelm K, Willcox MD (2004) The control of *Staphylococcus epidermidis* biofilm formation and in vivo infection rates by covalently bound furanones. Biomaterials 25:5023–5030

Jones MB, Jani R, Ren D, Wood TK, Blaser MJ (2005) Inhibition of *Bacillus anthracis* growth and virulence gene expression by inhibitors of quorum-sensing. J Infect Dis 191:1881–1888

Korem M, Gov Y, Kiran MD, Balaban N (2005) Transcriptional profiling of target of RNAIII-activating protein, a master regulator of staphylococcal virulence. Infect Immun 73:6220–6228

Llamas MA, Sparrius M, Kloet R, Jimenez CR, Vandenbroucke-Grauls C, Bitter W (2006) The heterologous siderophores ferrioxamine B and ferrichrome activate signaling pathways in *Pseudomonas aeruginosa*. J Bacteriol 188:1882–1891

Lowy FD (1998) *Staphylococcus aureus* infections. N Engl J Med 339:520–532

Makemson J, Eberhard A, Mathee K (2006) Simple electrospray mass spectrometry detection of acylhomoserine lactones. Luminescence 21:1–6

Manefield M, Harris L, Rice SA, de Nys R, Kjelleberg S (2000) Inhibition of luminescence and virulence in the black tiger prawn (*Penaeus monodon*) pathogen *Vibrio harveyi* by intercellular signal antagonists. Appl Environ Microbiol 66:2079–2084

Manefield M, Welch M, Givskov M, Salmond GPC, Kjelleberg S (2001) Halogenated furanones from the red alga, *Delisea pulchra*, inhibit carbapenem antibiotic synthesis and exoenzyme virulence factor production in the phytopathogen *Erwinia carotovora*. FEMS Microbiol Lett 205:131–138

Manefield M, Rasmussen TB, Hentzer M, Anderson JB, Steinberg P, Kjelleberg S, Givskov M (2002) Halogenated furanones inhibit quorum sensing through accelerated luxr turnover. Microbiology 148:1119–1127

McClean KH, Winson MK, Fish L, Taylor A, Chhabra SR, Camara M, Daykin M, Lamb JH, Swift S, Bycroft BW, Stewart GS, Williams P (1997) Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones. Microbiology 143:3703–3711

McLean RJC, Pierson LS, Fuqua C (2004) A simple screening protocol for the identification of quorum signal antagonists. J Microbiol Methods 58:351–360

McLean RJC, Barnes MB, Windham MK, Merchant MM, Forstner MRJ, Fuqua C (2005) Cell–cell influences on bacterial community development in aquatic biofilms. Appl Environ Microbiol 71:8987–8990

Moré MI, Finger LD, Stryker JL, Fuqua C, Eberhard A, Winans SC (1996) Enzymatic synthesis of a quorum-sensing autoinducer through the use of defined substrates. Science 272:1655–1658

Novick RP, Projan SJ, Kornblum J, Ross HF, Ji G, Kreiswirth B, Vandenesch F, Moghazeh S (1995) The *agr* P2 operon: an autocatalytic sensory transduction system in *Staphylococcus aureus*. Mol Gen Genet 248:446–458

Rasmussen TB, Bjarnsholt T, Skindersoe ME, Hentzer M, Kristoffersen P, Kote M, Nielsen J, Eberl L, Givskov M (2005a) Screening for quorum-sensing inhibitors (QSI) by use of a novel genetic system, the QSI selector. J Bacteriol 187:1799–1814

Ren D, Sims JJ, Wood TK (2002) Inhibition of biofilm formation and swarming of *Bacillus subtilis* by (5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(5*H*)-furanone. Lett Appl Microbiol 34:293–299

Ren D, Bedzyk LA, Thomas SM, Ye RW, Wood TK (2004b) Differential gene expression shows natural brominated furanones interfere with the autoinducer-2 bacterial signaling system of *Escherichia coli*. Biotechnol Bioeng 88:630–642

Ren D, Zuo R, Wood TK (2005) Quorum-sensing antagonist (5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(5*H*)-furanone influences siderophore biosynthesis in *Pseudomonas putida* and *Pseudomonas aeruginosa*. Appl Microbiol Biotechnol 66:689–695

Schuster M, Lostroh CP, Ogi T, Greenberg EP (2003) Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis. J Bacteriol 185:2066–2079

Steidle A, Sigl K, Schuhegger R, Ihring A, Schmid M, Gantner S, Stoffels M, Riedel K, Givskov M, Hartmann A, Langebartels C, Eberl L (2001) Visualization of *N*-acylhomoserine lactone-mediated cell-cell communication between bacteria colonizing the tomato rhizosphere. Appl Environ Microbiol 67:5761–5770

Shaw PD, Ping G, Daly SL, Cha C, Cronan JE Jr, Rinehart KL, Farrand SK (1997) Detecting and characterizing *N*-acyl-homoserine lactone signal molecules by thin layer chromatography. Proc Natl Acad Sci USA 94:6036–6041

Swift S, Winson MK, Chan PF, Bainton NJ, Birdsall M, Reeves PJ, Rees CED, Chhabra SR, Hill PJ, Throup JP, Bycroft BW, Salmond GPC, Williams P, Stewart GSAB (1993) A novel strategy for the isolation of luxI homologues: evidence for the widespread distribution of a luxR:luxI superfamily in enteric bacteria. Mol Microbiol 10:511–520

Vasil ML (2003) DNA microarrays in analysis of quorum sensing: strengths and limitations. J Bacteriol 185:2061–2065

Wagner VE, Bushnell D, Passador L, Brooks AI, Iglewski BH (2003) Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment. J Bacteriol 185:2080–2095

Winson MK, Camara M, Latifi A, Foglino M, Chhabra SR, Daykin M, Bally M, Chapon V, Salmond GPC, Bycroft B, Lazdunski A, Stewart GS, Williams P (1995) Multiple *N*-acyl-L-homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in *Pseudomonas aeruginosa*. Proc Natl Acad Sci USA 92:9427–9431

Winson MK, Swift S, Fish L, Throup JP, Jorgensen F, Chhabra SR, Bycroft B, Williams P, Stewart GS (1998) Construction and analysis of *luxCDABE*-based plasmid sensors for investigating *N*-acyl homoserine lactone-mediated quorum sensing. FEMS Microbiol Lett 163:185–192

Wood DW, Pierson LS (1996) The phzI gene of *Pseudomonas aureofaciens* 30–84 is responsible for the production of a diffusible signal required for phenazine antibiotic production. Gene 168:49–53

Zhu J, Beaber JW, Moré MI, Fuqua C, Eberhard A, Winans SC (1998) Analogs of the autoinducer 3-oxooctanoyl-homoserine lactone strongly inhibit activity of the TraR protein of *Agrobacterium tumefaciens*. J Bacteriol 180:5398–5405

Zhu J, Chai Y, Zhong Z, Li S, Winans SC (2003) Agrobacterium bioassay strain for ultrasensitive detection of *N*-acylhomoserine lactone-type quorum-sensing molecules: detection of autoinducers in *Mesorhizobium huakuii*. Appl Environ Microbiol 69:6949–6953

Quorum-Sensing Inhibitory Compounds

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Abstract Biofilm formation plays an important role in antimicrobial resistance, posing a global threat to the economy and public health. Hence, discovery of novel antagonists is critical. In this chapter, we introduce a class of compounds, brominated furanones, which have a broad spectrum of activities against bacterial multicellular behaviors, such as biofilm formation and quorum sensing. The application of these compounds and the mechanisms of inhibition are reviewed. This chapter also reviews inhibitors of quorum sensing in Gram-positive bacteria, especially those of staphylococci. The use of these inhibitors to prevent and treat biofilm diseases in humans and animals is shown in subsequent chapters.

1 Inhibitors of Quorum Sensing in Gram-Negative Bacteria[1]

The quorum-sensing (QS) systems offer three points of attack: the signal-generating LuxI homolog, the *N*-acyl homoserine lactone (AHL) molecule itself, and the signal receptor LuxR. Obviously, if the bacteria do not produce AHL molecules, the QS-controlled genes will not be expressed. Likewise, if the signal molecules are destroyed after they are produced, they will be unable to interact with the LuxR

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N. Balaban (ed.) *Control of Biofilm Infections by Signal Manipulation*
Springer Series on Biofilms 2
doi: 10.1007/978-3-540-73853-4, © Springer 2008

homolog protein, so none of the QS-controlled virulence factors will be produced. There are several methods by which the signals can be eradicated: they can be metabolized or chemically or enzymatically degraded. A third strategy to inhibit QS is by preventing the signal molecules from activating the LuxR homolog. There are two ways to achieve this goal, with both relying on small molecule chemistry. One way is to block the AHL binding pocket either by means of an AHL homolog or by introducing a conformational change in the R-protein by binding a small molecule to another part, thereby preventing the cognate signal from reaching the binding pocket. The second method relies on molecules that cause the LuxR protein to misfold, thereby making it prone to degradation by host proteases (Table 1).

1.1 Inhibition of AHL Signal Generation

The LuxI homologs use acyl-ACP and *S*-adenosylmethionine (SAM) from the central amino acid and fatty acid catabolism as precursors for AHL production. One could imagine that blocking an enzyme that uses these important building blocks would also obstruct other enzymes using the same precursors. Not only do bacteria use these molecules but eukaryotes also employ acyl-ACP and SAM in many biochemical reactions. Hence, interfering with these reactions would affect the viability of both bacteria and host. However, the LuxI homologs have a special reaction chemistry that indicates that it is indeed possible to create substrate analogs or other compounds that will specifically inhibit AHL production. Analogs of SAM have been found to be potent inhibitors of the RhlI synthetase from *P. aeruginosa*, at least in the in vitro systems. The compounds include L/D-*S*-adenosylhomocysteine, sinefungin, butyryl-SAM, and the most effective L-*S*-adenosylcysteine that was able to reduce the activity of RhlI by 97% (Parsek et al. 1999).

It should be noted that these compounds have not been tested in vivo. Some macrolide antibiotics that interfere with protein synthesis are able to inhibit AHL production by LuxI homologs. Examples include azithromycin and erythromycin. They lower production of AHL signal molecules and virulence factors when applied in sub-MIC concentrations to *P. aeruginosa* (Pechere 2001; Sofer et al. 1999; Tateda et al. 2001). The exact molecular mechanism of how this inhibition takes place has not yet been elucidated, but because the macrolides are widely used as antibiotics, resistance mechanisms directed against the compounds have already evolved. It is unknown how the resistance affects the ability of the macrolides to inhibit QS.

1.2 Destruction of the AHL Signal

The AHLs are inherently unstable at pH levels above neutral, where they undergo lactonolysis – ring opening. Some bacteria. such as *E. caratovora, P. aeruginosa*, and *Y. pseudotubercolosis*, raise the pH when they enter the stationary phase (at

Table 1 Some compounds tested for agonistic (quorum sensing, QS) and antagonistic (quorum-sensing inhibitor, QSI) effects

Compound/enzyme	Fig.	Screening system	Result	Infection model	Result	Mode of action	Refs.
2,4,5-tri-bromo-imidazole	5a	QSIS1/LuxR	QSI			Blockage of LuxR	Rasmussen et al. 2005a
3-nitrobenzene-sulfonamide	5c	QSIS1/LuxR	QSI			Blockage of LuxR	Rasmussen et al. 2005a
3-oxo homocysteine thio-lactone	3c	*V. fischeri/lux*	QSI			Blockage of LuxR	Schaefer et al. 1996
3-oxo-(2-amino-cyclo-hexanol)	4b	*P. aeruginosa/LasR*	QS				Smith et al. 2003a
3-oxo-(2-amino-cyclo-hexanone)	4c	*P. aeruginosa/LasR*	QSI				Smith et al. 2003a
3-oxo-C12-(2-aminophenol)	4d	*P. aeruginosa/LasR*	QSI				Smith et al. 2003b
3-oxo-C6 caprolactam	3f	*V. fischeri/lux*	No effect				Schaefer et al. 1996
4-nitro-pyridine-*N*-oxide	5d	*P. aeruginosa/LasR* + QSIS1/*LuxR*	QSI	*C. elegans*	QSI	Blockage of LuxR homologs	Rasmussen et al. 2005a
5-methyl-3-oxo-C6-HSL	2b	*V. fischeri/lux*	QS				Reverchon et al. 2002
AiiA (produced by *Bacillus*)		*P. aeruginosa*	QSI	Tobacco plant	QSI	Lactonolysis of AHL	Dong et al. 2000, 2002
Azithromycin		*P. aeruginosa*	QSI			Lower amounts of AHL produced	Tateda et al. 2001
Cyclohexyl-3-oxo-C6-HSL	2d	*V. fischeri/lux*	QS				Reverchon et al. 2002
Cyclopentene-C4-HSL	2g	*A. tumefaciens/TraR*	QSI			Blockage of TraR	Geske et al. 2005
Cyclopentyl-3-oxo-C6-HSL	2c	*V. fischeri/lux*	QS				Reverchon et al. 2002
Furanone #1[*]	7a	*V. fischeri/lux*	QSI				Givskov et al. 1996
Furanone #6[*]	7b	*V. fischeri/lux*	QSI	*Paneus monodon*	QSI	Blockage of LuxR homologs	Manefield et al. 2000

[*]Table 2

Table 1 (continued)

Compound/enzyme	Fig.	Screening system	Result	Infection model	Result	Mode of action	Refs.
Furanone #4*; also known as compound 30	7c	*P. aeruginosa/LasR* + *V. fischeri/LuxR*	QSI	Mice (lung infection)	QSI	Blockage of LuxR homologs	Hentzer et al. 2003
Furanone #3*; also known as compound 56	7d	*P. aeruginosa/LasR* + *V. fischeri/LuxR*	QSI	Mice (lung infection)	QSI	Blockage of LuxR homologs	Hentzer et al. 2003
Garlic extract		*P. aeruginosa/LasR* + QSIS1/*LuxR*	QSI	*C. elegans* + mice (lung infection)	QSI	Blockage of LuxR homologs	Rasmussen et al. 2005a
Hypochlorous acids			QSI			Reacts with 3-oxo group in the side chain of AHLs	Borchardt et al. 2001
Indole	5b	QSIS1/LuxR	QSI			Blockage of LuxR	Rasmussen et al. 2005a
L-S-adenosylcysteine		RhlI in vitro expression system	QSI			Blockage of AHL signal generation	Parsek et al. 1999
Para-benzoquinone		QSIS1/LuxR	QSI			Blockage of LuxR	Rasmussen et al. 2005a
Paraoxynase (produced by epithelia cells)			QSI			Hydrolysis of the ring part of AHL	Ozer et al. 2005; Chun et al. 2004; Hastings 2004
Patulin	6b	*P. aeruginosa/LasR* + QSIS1/*LuxR*	QSI	Mice (lung infection)	QSI	Blockage of LuxR homologs	Rasmussen et al. 2005b
Penicillic acid	6a	*P. aeruginosa/LasR* + QSIS1/*LuxR*	QSI	Mice (lung infection)	QSI	Blockage of LuxR homologs	Rasmussen et al. 2005b
pH > 8			QSI			Causes ring opening of the AHL molecules	Yates et al. 2002; Byers et al. 2002
phenyl-3-oxo-C6-HSL	2h	*V. fischeri/lux*	QSI			Blockage of LuxR	Reverchon et al. 2002
PvdQ and QuiP (produced by *P. aeruginosa*)		*C. violaceum*	QSI			Lactonolysis of AHL	Huang et al. 2003, 2006; Sio et al. 2006
Sulfide AHLs	2e	*P. aeruginosa/LasR*	QSI			Blockage of LasR	Persson et al. 2005
Sulfonoxide AHLs	2f	*P. aeruginosa/LasR*	QSI			Blockage of LasR	Persson et al. 2005
Variovorax paradoxus	1		QSI			Metabolism of AHLs	Leadbetter and Greenberg 2000

*Table 2

least when growing in shake flask cultures), leading to almost complete destruction of the signal molecules. Even exogenously, AHL is rapidly inactivated if added to cell-free supernatants of the above-mentioned bacteria. Increasing the temperature also influences the lactonolysis reaction. In addition, the reaction is dependent on the length of the side chain of the AHLs. Taken together, this means that AHLs with a side chain shorter than four carbon atoms cannot retain its activity at physiological conditions. Interestingly, no bacteria have been found to produce AHLs with side chains shorter than C4 (Yates et al. 2002; Byers et al. 2002).

Some plants use this pH-dependent instability against infecting bacteria. For example, when the plant pathogen *E. carotovora carotovora* attacks, the plant may respond by increasing the pH to 8.2, thereby achieving destruction of the signal molecules that direct expression of the virulence factors harming the plant (Byers et al. 2002). The marine alga *Laminaria digitata* secretes oxidized halogen compounds such as hypobromous and hypochlorous acids, which react with 3-oxo-substituted AHL compounds such as 3-oxo-C6-HSL produced by many marine *Vibrio* strains. By secreting these compounds, the alga is able to control formation of biofilm and biofouling on its leaves (Borchardt et al. 2001).

Another strategy to interfere with QS is enzymatic destruction of the signal molecule, referred to as quorum quenching. This can be achieved by the action of AiiA AHL lactonase. This enzyme and analogs thereof are produced by many *Bacillus* species such as *B. cereus, B. mycoides,* and several subspecies of *B. thurengensis* (Dong et al. 2000, 2002). The enzyme is highly specific for acylated homoserine lactones (HSLs) because it does not degrade nonacyl lactones and noncyclic esters. Conversely, the enzyme can degrade AHLs with a wide variety of side-chain lengths and different substitutions at the C3 position (Wang et al. 2004).When expressed in *P. aeruginosa*, this enzyme not only reduces the concentration of AHL in the surrounding media, but it also lowers expression of virulence factors such as elastase, rhamnolipids, hydrogen cyanide, and pyocyanin (Reimmann et al. 2002). When AiiA is expressed in *E. carotovora,* the AHL-controlled production of extracellular pectolytic enzymes is abolished. This leads to lowered virulence against potato, egg plant, carrots, and many other vegetables in which *Erwinia* causes soft rot disease (Dong et al. 2000, 2004). To prove the feasibility of the approach in vivo, the *aiiA* gene was inserted into tobacco and potato plants. These plants, which are normally prone to attack by *E. carotovora*, became resistant against the bacterium, which became attenuated by the quorum-quenching enzymes (Dong et al. 2001).

A variety of other bacteria, including *A. tumefaciens, Arthrobacter* sp., *Klebsiella pneumoniae, Commomamonas* sp., *Rhodococcus* sp., and *Streptomyces* sp., also produce enzymes that are able to interfere with rhizosphere pathogens by producing AHL-degrading enzymes (Uroz et al. 2003; Carlier et al. 2003; Park et al. 2003, 2005). The expression of AHL-degrading enzymes has probably evolved as a survival strategy of some soil bacteria to compete with the AHL-producing strains in natural ecosystems. The AHL-degrading enzymes have potential commercial interest because they are likely to be used in agriculture and food manufacturing, but for treatment of human patients they have limited use. Because of the obstacles involved in delivering proteinaceous agents systemically, AHL lactonases can, at best, be ap-

Fig. 1 Proposed pathway of C4 homoserine lactone degradation (Leadbetter and Greenberg 2000)

plied topically. One thing about the lactonolysis reaction should be kept in mind: It is reversible at acidic pH. A ring-opened AHL molecule will spontaneously undergo ring formation if the environment is not alkaline. Hence, if lactonase is applied, steps should be taken to prevent reversal of the AHL molecules to the active form.

Intriguingly, *P. aeruginosa* produces two enzymes that degrade AHLs: PvdQ and QuiP. The two enzymes have different substrate specificities: PvdQ enables the bacterium to grow on short-chain AHLs, whereas QuiP is responsible for growth on long-chain AHLs as a carbon and energy source (Huang et al. 2003, 2006; Sio et al. 2006).

Some soil bacteria such as *Variovorax paradoxus* and *P. aeruginosa* PAI-A are able to grow and proliferate using AHLs as their sole source of carbon, energy, and nitrogen. The molar growth yield of the bacteria correlates directly with the length of the side chain, indicating that this part of the signal molecule is used as a carbon source. The bacteria produce an aminoacylase that cleaves the amide bond of the signal molecular into an organic acid (from the side chain) and the HSL moiety (from the ring part). The acid undergoes beta-oxidation and is used as an energy source and for building up cell material. By action of lactonases, ammonium is released from the HSL molecule and is subsequently used as a nitrogen source by the bacteria (Fig. 1) (Huang et al. 2003; Leadbetter and Greenberg 2000).

Differentiated epithelial cells are also able to produce enzymes that degrade AHL signal molecules. They produce the enzymes paraoxynase 1, 2, and 3 (PON1–3) that are able to degrade 3-oxo-C12-HSL. PON1–3 enzymes act as lactonases degrading the AHL by hydrolysis of the ring part of the molecule. The enzymes produced by the epithelial cells are highly specific for the AHLs they can degrade. In addition to 3-oxo-C12-HSL, they can degrade C6-HSL, but, intriguingly, not 3-oxo-C6-HSL or C4-HSL molecules. This indicates that it is both the length of the side chain and its oxidation state that determine whether the AHL molecule can be targeted by the enzymes. Treatment with purified human PON1 enzyme was able to abolish biofilm formation of *P. aeruginosa*. Treatment with 0.25% serum from wild-type mice was also able to abolish biofilm formation by *P. aeruginosa*, while serum from PON1 knockout mice did not have this ability (Ozer et al. 2005; Chun et al. 2004; Hastings 2004).

1.3 Inhibition of the Signal Receptor

A highly investigated strategy to achieve QS inhibition is blocking the LuxR homolog signal receptor by means of small molecules.

Substitutions on the side chain. AHL analogs can be substituted in either the side chain or the ring moiety. Analogs of the 3-oxo-C6 HSL molecule with different substituents in the side chain (Fig. 2) are able to displace the native signal from the LuxR receptor. However, most of these compounds also exhibit agonists effects, which limit their use as QSIs (Schaefer et al. 1996). If the C-3 atom in the side chain is replaced by a sulfur atom, it will produce a potent inhibitor of both the *lux* and *las* systems (Persson et al. 2005). Likewise, if the C-1 atom is replaced by a sulfonyl group, a QSI is also generated (Castang et al. 2004).

Another strategy to modify the AHL signal molecules is to place atoms or groups at the end of the side chain. Substituting secondary alkyl groups at the C6 atom of 3-oxo-C6 HSL (Fig. 2) gives rise to agonists, whereas positioning of a secondary aryl group on that location (Fig. 2) gives rise to an antagonist. Because the size difference between the two types of molecules is negligible, the differences in activity are probably due to the ability of the aryl compounds to interact hydrophobically with aromatic amino acids in the protein. If the size of the substituents is increased to include tertiary alkyl derivatives or even larger alkyl and aryl moieties (Fig. 2),

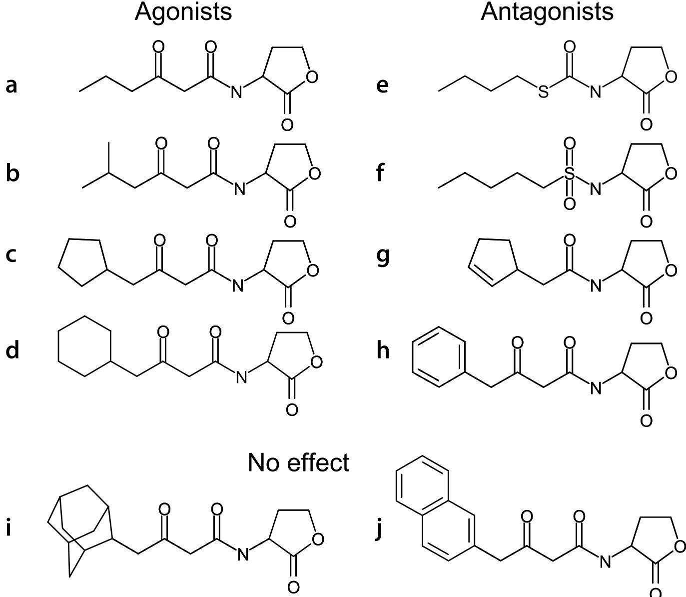

Fig. 2 *N*-acyl homoserine lactone (AHL) analogs with changes in the side chain. **a** 3-oxo-C6 HSL signal molecule. **b–d** Analogs with agonist effect. **e–h** Analogs with antagonistic effect. **i–j** Analogs without any effect (Schaefer et al. 1996; Reverchon et al. 2002; Geske et al. 2005; Castang et al. 2004; Persson et al. 2005)

agonistic activity of the molecules is lost, indicating that they are too bulky to enter the AHL binding site in the receptor protein (Reverchon et al. 2002; Geske et al. 2005).

Substitutions on the HSL ring. Most compounds with a keto-oxygen in the ring or an extra carbon expanding the ring have exhibited little or no binding to the LuxR protein (Schaefer et al. 1996). One exception to this is when the substituents are placed on the C3 carbon atom of the ring (Fig. 3). Compounds having acyl alcohols or acyl amides attached at this position are still able to function as agonists of LuxR (Fig. 3). Conversely, if the substituents are placed on the C4 atom, the compounds are not able to interact with the LuxR receptor. This indicates that there is more "free space" around the C3 atom of the ring inside the AHL binding pocket (Olsen et al. 2002).

Instead of single substitutions, the entire ring can be exchanged with another cyclic structure. In research exploring compounds able to interfere with QS in *P. aeruginosa*, the side chains of 3-oxo-C12 HSL and C4 HSL were attached to amino-cyclo-alcohol and amino-cyclo-ketone with either five or six carbons in the ring (Fig. 4). The C12 aminocyclohexanol compound was a strong activator of the LasR protein, whereas the C4 keto compounds were the most potent agonists of the RlhR protein. This indicates that the two QS receptors do not perceive the HSL moiety of AHL signal in the same manner. Another molecule, 3-oxo-C12-(2-amino-cyclohexanone) (Fig. 4), is an inhibitor of the LasR-based QS system. It is able to downregulate LasR-dependent expression of the LasI AHL synthase. When applied

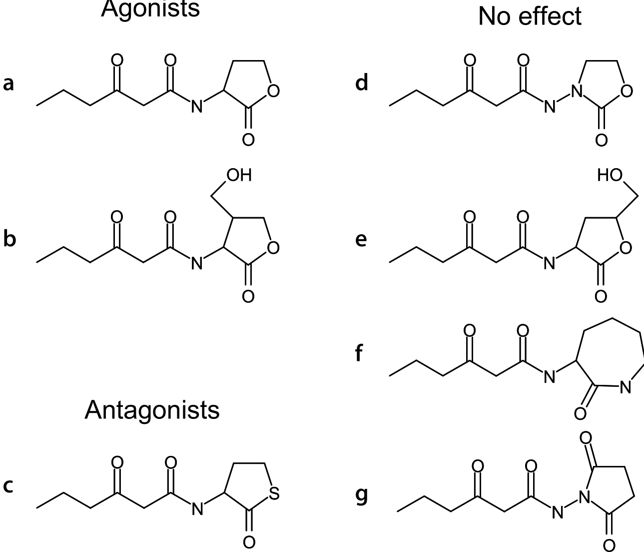

Fig. 3 *N*-acyl homoserine lactone (AHL) analogs with changes in the ring. **a** 3-oxo-C6 HSL signal molecule. **b** Analog with agonist effect. **c** Analog with antagonistic effect. **d**–**g** Analogs without any effect (Schaefer et al. 1996; Olsen et al. 2002)

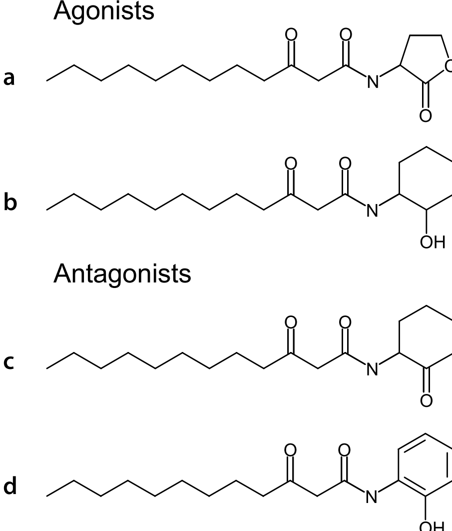

Fig. 4 *N*-acyl homoserine lactone (AHL) analogs with exchanged ring part. **a** 3-oxo-C12 HSL signal molecule. **b** Analog with agonist effect. **c**,**d** Analog with antagonistic effect (Smith et al. 2003a,b)

in a concentration of 100 μM (which is relatively high for a QSI), the compound significantly reduced the production of exoproteins (Smith et al. 2003a). An even more potent inhibitor of LasR is 3-oxo-C12-(2-aminophenol) (Fig. 4), which is able to abolish production of pyocyanin and elastase; in addition, it can disrupt normal biofilm formation by *P. aeruginosa*. Interestingly, the very similar molecule 3-oxo-C12-(2-aminocyclohexanol), with the phenol replaced by a hexane ring, is a potent agonist of the *las* system (Smith et al. 2003b). A similar situation is seen with analogs of 3-oxo-C6 HSL from the *lux* system. If the HSL ring is replaced by a hexane ring, the ability to activate LuxR is retained. On the other hand, if the HSL ring is replaced by a benzyl group, an inhibitor of LuxR is generated (Reverchon et al. 2002).

1.4 Non-AHL-Based Inhibitors

QSIs have also been identified by screening random libraries of chemical compounds. Among the identified inhibitors able to interfere with both the *lux* system from *V. fischeri* and the QS systems in *P. aeruginosa* are para-benzoquinone; 2,4,5-tri-bromo-imidazole; indole; 3-nitrobenzene-sulfonamide, and 4-nitropyridine-*N*-oxide (4-NPO), the latter being the most effective in this group (Fig. 5). Transcriptomic analysis of gene expression shows that 4-NPO mainly affects genes that are regulated by either RhlR alone or RhlR and LasR in concert. This suggests that

Fig. 5 Molecules with quorum-sensing inhibitor properties. **a** 2,4,5-tri-bromo-imidazole. **b** Indole. **c** 3-nitrobenzene-sulfonamide. **d** 4-nitro-pyridine-*N*-oxide (4-NPO) (Rasmussen et al. 2005a)

4-NPO interacts most readily with the RhlR receptor. In total, 37% of the QS-regulated genes in *P. aeruginosa* (when the planktonic cells are investigated at $OD_{600} = 2$) are significantly downregulated by treatment with 100 μM 4-NPO (Rasmussen et al. 2005a).

1.5 QSI Compounds Produced by Bacteria

Some bacteria produce compounds that are able to interfere with QS. The filamentous *Streptomyces* produces furanone compounds that are intermediates in butanolide production. Inhibitors of the QS-controlled purple pigment production in *C. violaceum* have been identified by screening a library of furanone compounds (and analogs thereof) produced by *S. antibioticus* (Martinelli et al. 2004; see below). As discussed in the next section, bacteria are by far not the only organisms that produce compounds able to interfere with QS (Fig. 6).

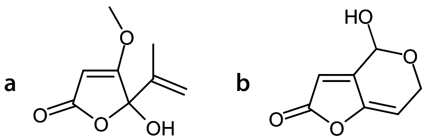

Fig. 6 The two quorum-sensing inhibitors (**a**) penicillic acid and (**b**) patulin produced by certain fungi (Rasmussen et al. 2005b)

1.6 Brominated Furanones: Novel Biofilm Inhibitors

The marine macro-alga (seaweed) *Delisea pulchra* has been found to posses some remarkable antifouling features (de Nys et al. 1993) (Fig. 7). More than 30 brominated furanones (also known as fimbrolides) are produced by *D. pulchra* as sec-

Fig. 7 Halogenated furanone compounds. **a**,**b** Natural compounds produced by the algae *D. pulchra*. **c**,**d** Synthetic furanones (Hentzer et al. 2002, 2003; Manefield et al. 1999; Givskov et al. 1996)

ondary metabolites on the surface of this seaweed and protect it from the colonization by both prokaryotes and eukaryotes (de Nys et al. 1993, Kazlauskas et al. 1977). Since this discovery, methods have been developed to synthesize the natural furanones and their synthetic derivatives in laboratory conditions (Beechan and Sims 1979; Manny et al. 1997). Table 2 summarizes the most-studied furanones and their activities on gram-negative and gram-positive (discussed in Sect. 2.4) bacteria.

1.7 Inhibition of Biofilm Formation of Gram-Negative Bacteria by Furanones

1.7.1 *E. coli* Biofilm Formation

Furanone #1 (Table 2) was reported to inhibit the biofilm formation of *E. coli* XL1-blue on mild steel (steel that contains less than 0.2% carbon) surfaces in batch experiments (Ren et al. 2001). The analysis, using scanning confocal laser microscopy and staining with the LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen), demonstrated that furanone #1 at 60 μg/ml decreased the biofilm thickness by 52% and reduced the percentage of live cells by 87%. However, the furanone showed no effect on cell growth in suspension cultures with concentrations up to 100 μg/ml, suggesting that the inhibition was not caused by repression of general metabolism. The confocal images of the vertical sections of biofilms revealed that the presence of furanone #1 led to damage of the biofilm structure (for instance, decreased water channels). Hence, the cell death may be caused by nutrient depletion and accumulation of toxic wastes. In a later study, Ren et al. (2004c) reported that furanone #1 also inhibited the air-liquid biofilm formation of *E. coli* JM109 in 96 well plates without affecting suspension growth.

Table 2 Representative natural and synthetic brominated furanones (Note: Different numbering systems have been used in the literature, attention should be paid to the structures when referring to other publications)

Number	Structure	Activities
1		Inhibits swarming of *Serratia liquefaciens* (Givskov et al. 1996), bioluminescence and virulence of *V. harveyi* (Manefield et al. 2000), quorum sensing of AI-2 (Ren et al. 2004b), and swarming and biofilm formation of *E. coli* (Ren et al. 2001). Represses siderophore synthesis of *P. putida* and stimulates that of *P. aeruginosa* (Ren et al. 2005). Accelerates the turnover of *V. fischeri* LuxR expressed in *E. coli* (Manefield et al. 2002). Inhibits swarming of *Proteus mirabilis* (Gram et al. 1996). Inhibits the growth of *B. anthracis* (Jones et al. 2005), growth, swarming, and biofilm formation of *B. subtilis* (Ren et al. 2002) and the corrosion of mild steel by *Desulfotomaculum orientis* (Ren and Wood 2004)
2		Inhibits the growth of *B. anthracis* (Jones et al. 2005)
3 (also known as compound 56)		Affects biofilm development of *P. aeruginosa* and promotes sloughing (Hentzer et al. 2002). Accelerates the turnover of *V. fischeri* LuxR expressed in *E. coli* (Manefield et al. 2002)
4 (also known as compound 30)		Represses quorum sensing (QS) and virulence factor expression of *P. aeruginosa* PAO1. Increases its susceptibility to SDS and tobramycin. Improve clearance of PAO1 from mouse lungs (Hentzer et al. 2003). Accelerates the turnover of *V. fischeri* LuxR expressed in *E. coli* (Manefield et al. 2002). Inhibits the growth of *B. anthracis* (Jones et al. 2005)

Table 2 (continued)

Number	Structure	Activities
5		Inhibits the growth of *B. anthracis* (Jones et al. 2005)
6		Inhibits QS-controlled exoenzyme virulence factor production and carbapenem antibiotic synthesis in *Erwinia carotovora* (Manefield et al. 2001) Accelerates the turnover of *V. fischeri* LuxR expressed in *E. coli* (Manefield et al. 2002)
7		Inhibits biofilm formation of *S. epidermidis* on polymer materials through surface modification by physical adsorption (Baveja et al. 2004b) or covalent binding (Hume et al. 2004)
8		Inhibits QS-regulated swarming of *S. liquefaciens* (Givskov et al. 1996)

1.7.2 *P. aeruginosa* Biofilm Formation

Among the biofilm-forming bacteria, *P. aeruginosa* has attracted particular attention among researchers because it is the most common gram-negative bacterium found in nosocomial infections, especially in immunocompromised individuals (Van Delden and Iglewski 1998). *P. aeruginosa* is also the major pathogen causing lung infections in cystic fibrosis patients (Singh et al. 2000). Biofilm and QS play a critical role in these infections, and QS has been found to control *P. aeruginosa* biofilm formation (Davies et al. 1998).

Hentzer et al. (2002) studied the effect of furanone #3 (C-56; see Table 2) on biofilm formation of *P. aeruginosa* PAO1. This strain was labeled with constitutively expressed red florescence protein to quantify the biofilm mass and study the effect of furanone on biofilm development in flow cells. Furanone #3 showed no apparent effects on day 1. By day 7, however, furanone #3 at 5 μg/ml had significantly lowered the amount of biomass (61±6 μm without furanone versus 23±4 μm with furanone). The biofilm in the presence of furanone #3 was strongly similar to that of *P. aeruginosa* PAO1 *lasI* mutant (Davis et al. 1998), supporting the mechanism that furanone treatment promotes sloughing of the biofilm and leads to defects in the maturation of *P. aeruginosa* biofilm.

Hentzer et al. (2003) also studied the effects of synthetic furanone #4 (C-30; see Table 2) on *P. aeruginosa* biofilm removal. Although this compound had no effect on general cell growth, it significantly decreased the drug resistance of *P. aeruginosa* PAO1. Compared with the control sample (no furanone treatment), furanone caused efficient removal of biofilm when treating biofilm together with 0.1% sodium dodecyl sulfate (SDS). Furthermore, furanone #4 increased the sensitivity of *P. aeruginosa* biofilm to tobramycin. The antibiotic was found to kill 85–90% of furanone-treated biofilm cells, whereas the tobramycin alone could kill only the cells on the surface of the biofilm (Fig. 8). In a mouse pulmonary model, furanone #4 was found to improve the clearance of *P. aeruginosa* infection by immune system. This result was also corroborated by another report (Wu et al. 2004), in which furanones #3 and #4 were studied for inhibiting *P. aeruginosa* infection in lungs using a mouse model. Both compounds were found to inhibit bacterial colonization, improve the clearance of bacteria from the host, and reduce the tissue damage in lungs.

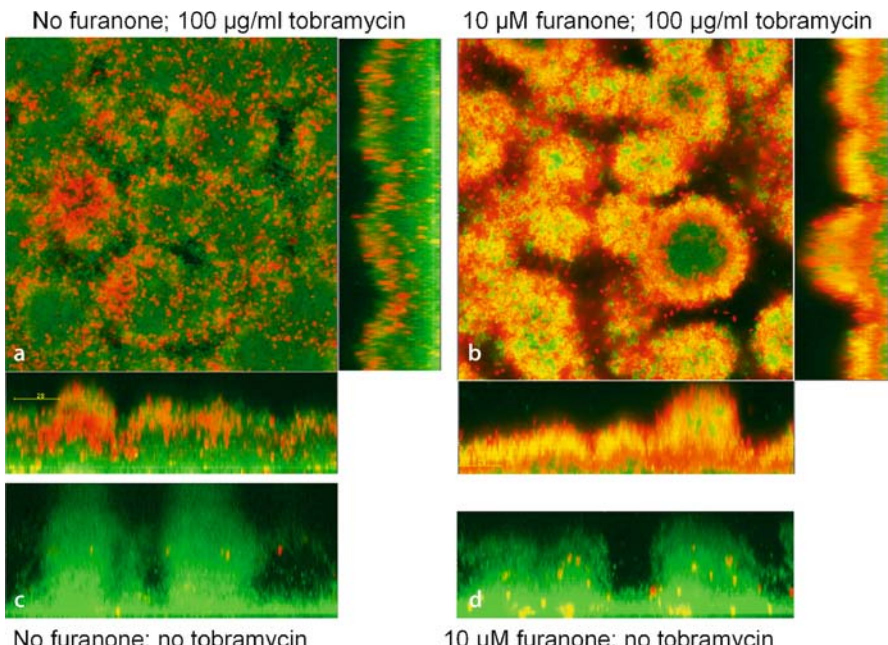

Fig. 8 Furanone #4 (C-30; refer to Table 2) enhances the sensitivity of *P. aeruginosa* biofilms to tobramycin. The biofilms were grown with (*left panel*) or without (*right panel*) 10 μM furanone #4 for 3 days. Then the biofilms were exposed to tobramycin (100 μg/ml) for 24 h. The images were obtained with scanning confocal laser microscopy after staining the biofilms with the LIVE/DEAD BacLight Bacterial Viability Kit. The *red* color represents dead cells, and the *green* color indicates that the cells are alive. The samples were exposed to (**a**) 100 μg/ml tobramycin and no furanone, (**b**) 100 μg/ml tobramycin and 10 μM furanone #4, (**c**) untreated control, or (**d**) 10 μM furanone #4 and no tobramycin. (Reprinted by permission from Macmillan Publishers, The EMBO Journal; Hentzer et al. 2003, © 2003.)

1.8 Mechanisms of Inhibition by Furanones

Unlike traditional antibiotics, furanones inhibit the biofilm formation and other phenotypes of gram-negative bacteria at concentrations noninhibitory to growth. The structural similarity between furanones and AHLs (see Fig. 2 in the chapter *Bacterial Cell-to-Cell Communication*) led to the hypothesis that furanones may interrupt the QS circuits (Givskov et al. 1996). QS is based on specific chemical (autoinducer)-protein (transcriptional activator) interactions, and changes in autoinducer structure could lead to low affinity or inhibitory effects (Schaefer et al. 1996; Koch et al. 2005). It has been shown that furanones are a class of antagonists of both AHL and AI-2 QS (Ren et al. 2001).

1.8.1 Furanones Inhibit AHL-Mediated QS

Using ^{3}H-labeled *N*-3-(oxohexanoyl)-L-homoserine lactone (OHHL) and the *V. fischeri* LuxR overexpressed in *E. coli*, Manefield et al. (1999) demonstrated that furanones displaced the [^{3}H]OHHL from *E. coli* cells overexpressing LuxR protein and that the effect was dose dependent. Furanone #6 (Table 2) was found to have the highest inhibitory activities, followed by furanone #1 and #2 (Table 2). It is worth noticing that no reaction was found between [^{3}H]OHHL and furanone #6, and furanones had no apparent effects on general metabolic activities as evidenced by comparison of protein expression profiles using two-dimensional gel electrophoresis. These data suggest that the inhibition was a result of competition between the OHHL and furanones for binding to the receptor (Manefield et al. 1999).

Because furanones disrupt the QS circuit of gram-negative bacteria with no effect on general cell growth and metabolism, it is a conceivable hypothesis that furanones may affect the fate of the transcriptional regulator R protein. To test this hypothesis, Manefield and colleagues (2002) studied the effects of two natural furanones (#1, 6; Table 2) and three synthetic homologs (#2, 3, and 4; Table 2) on the stability of LuxR of *V. fischeri* overexpressed in *E. coli*. It was found that furanones increased the turnover of LuxR in a concentration-dependent manner. Furanones #4 and #6 exhibited the strongest effects, followed by furanones #1 and #3 (Table 2) with moderate activity. Furanone #2 (Table 2) showed no apparent effect. Consistent with the inhibitory effects of furanones on QS, the QS signal OHHL was found to protect the LuxR from turnover by furanone #4 (Table 2). However, the protection could be obtained only if the OHHL was added before furanone.

Inhibition of QS was also observed in biofilms. Hentzer et al. (2002) used the synthetic furanone #3 (Table 2) to study the inhibition of QS in *P. aeruginosa* biofilms. To investigate the expression level of QS-controlled virulence gene *lasB*, a reporter was constructed by fusing the *lasB* promoter to an unstable *gfp* gene and monitoring fluorescence. The unstable version of *gfp* gene was constructed by adding a short peptide sequence (ASV) to its C-terminal, making it more susceptible to the protease activity (Andersen et al. 1998). In the suspension culture of *P. aeruginosa* PAO-JP2 (the *lasI rhlI* double mutant of the wild type *P. aeruginosa* PAO1

(Pearson et al. 1997)), it was found that furanone #3 inhibited the QS-mediated florescence in a concentration-dependent manner; for example, furanone #3 repressed the florescence by 50% in the presence of 100 nM QS signal OdDHL. The effect was reversible by the addition of pure OdDHL. Furanone #3 at 10 μg/ml affected neither the growth of *P. aeruginosa* PAO1 and PAO-JP2 nor the protein synthesis of PAO1, suggesting that the inhibition was specifically on the *las* QS system. In support of this hypothesis, the inhibition was found to be titrated with increasing concentrations of OdDHL, and intensified with increasing concentrations of furanone. However, complete inhibition was not achieved. To ensure the constant dosage of the reporter gene, the *lasB-gfp* (ASV) was also integrated into the PAO-JP2 chromosome. The PAO-JP2 cells were allowed to grow for 24 h in flow cells before the OdDHL and furanone were applied. As expected, the addition of OdDHL induced the florescence of the cells. However, furanone #3 at 2 μg/ml repressed the florescence with the presence of 40 nM OdDHL. This inhibitory effect was found to be dose dependent; for instance, the inhibition was rescued with 80 nM OdDHL and regained when furanone concentration was increased to 4 μg/ml.

Wu et al. (2000) studied QS inhibition by furanone #3 and #4 (Table 2). Using *luxR-PluxI-gfp*-labeled *E. coli* MT102 in a mouse model, they found that both furanone #3 and #4 could repress OHHL-based QS, while furanone #4 was more efficient. In addition, the inhibition by furanones was found to be titrated by increasing concentrations of the QS signal OHHL, suggesting that the furanone compounds inhibited the GFP by interrupting QS.

In addition to the above studies using traditional methods, the novel DNA microarray technology has been used to identify the genes controlled by AHL and furanones on a genome-wide scale. Hentzer et al. (2003) used DNA microarrays to study the *P. aeruginosa* genes affected by furanone #4 and compared them with the genes differentially expressed when in contact with the QS signals. It was found that furanone #4 repressed 85 genes and induced 10 genes more than fivefold. Interestingly, 80% of the genes repressed by furanone were also induced by QS, suggesting that furanone #4 specifically interrupts the QS circuit of *P. aeruginosa*.

Overall, both the in vitro and in vivo experiments (see below) suggest that furanones are specific blockers of AHL-mediated QS, where some may and others may not compete with the AHLs for binding to the transcriptional activator (Koch et al. 2005). Binding, however, disrupts the function of the receptor and consequently represses the expression of QS-regulated genes. Furanones do not kill the cells directly; however, they can disrupt the bacterial multicellular structure and ease the clearance of bacteria by other drugs and the host's immune system.

1.8.2 Furanones Inhibit AI-2-Mediated QS

In addition to the well-documented inhibition on AHL-mediated QS, furanone #1 (Table 2) was found to inhibit QS via AI-2. Using the AI-1 and AI-2 reporter systems based on *V. harveyi* (Surette and Bassler 1998), Ren et al. (2001) reported that furanone #1 at 10 μg/ml inhibited by 3300-fold the QS of *V. harveyi* via AI-1

and inhibited by 5500-fold that of *V. harveyi* via AI-2, as well as inhibited by 26 600-fold the QS of *E. coli* via AI-2. This provided the first experimental proof that furanones can affect both QS systems found in gram-negative bacteria. Furanone #1 at 100 μg/ml was also found to reduce AI-2 production in *E. coli* by 49% (Ren et al. 2004b).

The inhibition was also evidenced by a genomic study. Ren et al. (2004c) reported that furanone #1 specifically inhibited the AI-2 QS in *E. coli*. As shown in the DNA microarray results, 79% of the genes repressed by furanone #1 were also induced by AI-2, including the genes for chemotaxis, flagellar synthesis, and motility. Motility has been shown to be critical for initial attachment and maturation of *E. coli* biofilms (Pratt and Kolter 1998; Wood et al. 2006). This result may help understand the inhibition of *E. coli* biofilm formation by furanones (Ren et al. 2004b).

1.8.3 Additional Targets of Furanones

In addition to the inhibition of AHL and AI-2 QS systems, several lines of evidence indicate the existence of other targets of furanones. First, furanone #1 (Table 2) was found to inhibit the biofilm formation and swarming motility of *E. coli* XL1-blue, which has neither AHL nor AI-2 activity (Ren et al. 2001). The inhibition was obtained at concentrations without any effects on growth rate, suggesting that furanone #1 affected a specific pathway rather than the general metabolism. In addition, both furanone #1 and AHL were found to positively regulate the siderophore synthesis in *P. aeruginosa*, contradictory to the inhibitory effect of furanone #1 on AHL-mediated QS (Ren et al. 2005; Stintzi et al. 1998).

As discussed previously, the genomic studies on gram-negative bacteria showed specific interaction between furanones and QS systems. However, none of the genes for QS signal synthesis or detection was affected, suggesting that inhibition was at a posttranscriptional level (Hentzer et al. 2003; Ren et al. 2004b). Consistently, Defoirdt et al. (2007) reported that furanone #1 (Table 2) can decrease the DNA binding activity of *V. harveyi* LuxR protein.There are a number of genes affected only by furanones or QS in each microarray dataset. Further study on these genes may help identify other targets of furanones.

Since QS plays a central role in controlling bacterial physiology (Bassler 1999), and because furanones are QS blockers for gram-negative bacteria, it is not surprising that furanones control other phenotypes in addition to biofilm development. In fact, a number of studies have shown that furanones inhibit a broad spectrum of phenotypes of gram-negative bacteria, including the swarming of *S. liquefaciens* (Givskov et al. 1996), *Proteus mirabilis* (Gram et al. 1996), and *E. coli* (Ren et al. 2001); bioluminescence and virulence of *V. harveyi* (Manefield et al. 2000); exoenzyme virulence factor production; and carbapenem antibiotic synthesis in *Erwinia carotovora* (Manefield et al. 2001).

Several furanones have been found to repress the growth of gram-positive bacteria (Jones et al. 2005), a finding that cannot be explained by the mechanism of inhibiting AHL QS (see Sect. 2.4). Further studies are essential to understand the furanones' activity and to help find better antagonists.

1.9 Toxicity of Furanones

Although furanones inhibit a variety of detrimental bacterial phenotypes, the toxicity of furanones to animal cells must be addressed before they can be used for therapeutic or engineering applications. Current data suggest that some furanones, especially the synthetic ones, could repress bacterial phenotypes without inhibiting animal cells. Several furanones, including #1, #4, and #7 (Table 2), have been found to inhibit the growth of gram-positive bacteria at concentrations not inhibitory to gram-negative bacteria and mammalian cells. In addition, Baveja et al. (2004a) reported that furanone #7 is not cytotoxic as evidenced by a study using human blood. There were negligible changes in expression of the surface receptors of neutrophils (CD11b, CD18, and CD44) with and without furanone, while the positive control using lipopolysaccharide significantly changed the expression of these factors. This result suggests that this synthetic furanone will not cause significant acute inflammatory responses. In vivo studies using QSIs against gram-negative bacterial infections are presented in the chapter *In Vivo Studies: Inhibiting Biofilm-Associated Bacterial Infections Using QSIs*.

1.10 Effect of QSIs of Gram-Negative Bacteria on Components of Host Immune System

A histopathological investigation of mice lungs revealed that those receiving treatment with garlic extract were inflamed to a much higher degree on day 5 postinfection. In concert with the inflammation, a high number of PMNs and monocytes were detected in the lungs of garlic extract treated mice indicating that the immune system is actively removing the bacteria. This fits well with the finding that *P. aeruginosa* contain a QS-controlled system that paralyzes polymorphonuclear leukocytes (PMNs) and other cells of the host immune system. This can be visualized with a *P. aeruginosa* biofilm present in a flow cell. When PMNs were inoculated on top of a *P. aeruginosa* biofilm, the PMNs failed to become activated and developed oxidative burst. In contrast, when the biofilm was formed in the presence of QSIs including garlic extract, the PMNs developed oxidative burst (Bjarnsholt et al. 2005a,b). Similarly, the PMNs were unable to graze on the communicating biofilms, while the QSIs including garlic-extract-treated biofilms were readily phagocytosed by the neutrophils (Bjarnsholt et al. 2005a,b). This indicates that QSI compounds including garlic extract block the system(s) in *P. aeruginosa* that is (are) responsible for paralyzing the PMNs. If this is true in vivo, administration of a QSI drug will be expected to flip the balance between the biofilm and immune system in favor of the host's defense clearance mechanisms. (Refer to the information the chapter *In Vivo Studies: Inhibiting Biofilm-Associated Bacterial Infections Using QSIs*).

2 Inhibitors of QS in Gram-Positive Bacteria[4]

2.1 Inhibitors of QS in Staphylococci

Inhibitors to staphylococcal QS 1 (SQS 1), which inhibit the RAP/TRAP system (see section 2.1.1 in the chapter *Bacterial Cell-to-Cell Communication*), have been extensively studies in vivo and are discussed in detail in section 2 in the chapter *In Vivo Studies: Inhibiting Biofilm-Associated Bacterial Infections Using QSIs*. These include RNAIII-inhibiting peptide (RIP), RAP-binding peptides, and anti-TRAP antibodies. In the following sections, inhibitors to SQS 2 (the *agr* system) will be discussed.

2.2 Inhibitors to SQS 2

In contrast to the vast in vivo data on inhibitors to SQS 1 (see section 2 in the chapter *In Vivo Studies: Inhibiting Biofilm-Associated Bacterial Infections Using QSIs*), very little in vivo data are available on the use of inhibitors to SQS 2, and published data (Mayville et al. 1999; Wright et al. 2005) include only studies using very few mice ($n = 3$). In addition to that, the type of studies reported may have little practical relevance because they included pretreatment of a very specific type of *S. aureus* with a very specific type of inhibitor. Thus, the clinical significance of these inhibitors is not yet known.

The structure of SQS 2 inhibitors has been extensively reviewed (e. g., Abraham 2006; Chun et al. 2004) and will only be briefly described below.

The *agr*-encoded autoinducing peptide (AIPs) are short (eight or nine amino acids) peptides containing a thiolactone structure in which the carboxyl group of the C-terminal amino acid is linked to the sulfhydryl group of a cysteine, which is always the fifth amino acid from the C-terminus of the peptide (an exception is *S. intermedius*). The high-energy thiolactone linkage appears to be necessary for activity of the peptides. One unique aspect of the *agr* system is that AIPs activate the *agr* and virulence expression in one group of *S. aureus* strains also inhibit *agr* and virulence expression in other groups of *S. aureus* strains (Lyon et al. 2000). Evolutionary divergence within the *agr* locus has given rise to multiple specificity groups, of which there are four in *S. aureus* and at least 20 others in non-*aureus* staphylococci. As mentioned above, a key feature of this diversity is that heterologous AIPs competitively inhibit *agr* activation by the cognate ligand (Wright et al. 2005). Switching the receptor-histidine kinase of AIP, AgrC, between strains of different *agr* specificity types has shown that intragroup activation and intergroup inhibition are mediated by the same group-specific receptors. These results have been suggested to facilitate the development of a global inhibitor of virulence in *S. aureus*, but this has not yet been tested in vivo. This variant, a truncated AIP group II peptide lacking the

tail, is proposed to act by interfering with AIP binding to the receptor (Lyon et al. 2000).

In the two in vivo studies that have been reported using inhibitory AIPs (Mayville et al. 1999; Write et al. 2005), *S. aureus agr* type I was incubated with supernatants of *S. aureus* type II (containing AIP II) prior to injection into the mice (using the murine cellulites/abscess mouse model). The clinical significance of these studies is not yet known, not only because of the low number of animals used but also because clinical infections are caused by various *agr* types of *S. aureus*, so the use of an inhibitor of one type of *S. aureus* might actually activate another one. For example, biofilm formation and *agr* type II strains were associated with nosocomial methicillin-resistant *S. aureus* infections (Manago et al. 2006); *agr* type III strains were associated with noninvasive infections; *agr* type I strains were associated with invasive infections, especially bacteremia (Ben Ayed et al. 2006); *agr* group IV strains were associated with generalized exfoliative syndromes; and endocarditis strains mainly belonged to *agr* groups I and II (Jarraud et al. 2002). Put together, because *agr* pheromones or derivatives may have strongly varying activity against different staphylococcal strains, it will not be easy to evaluate their therapeutic use in a patient who normally carries different staphylococcal strains. Furthermore, the selection of resistant strains may quickly occur, as might have occurred already during the competition between staphylococcal strains during evolution.

2.3 Inhibition of Streptococcus Pneumoniae Virulence by Synthetic Competence-Stimulating Peptide

Two main patterns of gene expression of *S. pneumoniae* have been observed by quantitative real-time reverse transcription polymerase chain reaction during infection in the host; one was characteristic of bacteria in blood and one of bacteria in tissue, such as brain and lung. Gene expression in blood was characterized by increased expression of pneumolysin, while pneumococci in tissue infection showed increased expression of neuraminidases, metalloproteinases, oxidative stress, and competence genes. In vitro situations with similar expression patterns have been detected in liquid culture and in a pneumococcal model of biofilm, respectively. The biofilm model was dependent on the addition of synthetic competence-stimulating peptide (CSP), and no biofilm was formed by CSP receptor mutants. Induction of the competence system by the QS peptide, CSP, not only induced biofilm formation in vitro but also increased virulence in pneumonia in vivo. In contrast, a mutant for the ComD receptor, which did not form biofilm, also showed reduced virulence in pneumonia.

These results were in contrast to those found in a bacteremic sepsis model of infection, in which the competence system was downregulated. When pneumococci in the different physiological states were used directly for challenge, sessile cells grown in a biofilm were more effective in inducing meningitis and pneumonia,

while planktonic cells from liquid culture were more effective in inducing sepsis. Using in vivo gene expression and in vivo modulation of virulence data, it has been postulated that there is a distinction between two main types of disease. During bacteremic sepsis, pneumococci resemble planktonic growth, while during tissue infection, such as pneumonia or meningitis, pneumococci are in a biofilm-like state (Oggioni et al. 2006). For detail in this topic, refer to the chapter *Quorum Sensing in Streptococci.* .

2.4 Effect of Furanones on Biofilm Formation of Gram-Positive Bacteria

In addition to the phenotypes of gram-negative bacteria discussed above, furanones have also been found to control biofilm formation of gram-positive bacteria. However, furanones appear to be toxic to gram-positive cells, and the mechanism of inhibition is not yet understood.

Ren et al. (2002) reported that furanone #1 (Table 2) inhibits biofilm formation of *B. subtilis* BE1500 on mild steel surfaces in a concentration-dependent manner. The presence of 40 μg/ml of furanone led to significant cell death (64%) and decreased biofilm thickness (25%). Because this furanone was found to have bactericidal effects on *B. subtilis*, the inhibition of biofilm was likely due to the toxicity of furanone #1.

Furanone #1 (Table 2) was also tested for inhibiting biofilm formation of a gram-positive sulfate-reducing bacterium, *D. orientis*. Sulfate-reducing bacteria (SRB) are a group of anaerobic bacteria that use sulfate as the terminal electron acceptor and produce hydrogen sulfide as the reduced product (Hamilton 1985). SRB (both gram-positive and gram-negative) are a major source of microbial-induced corrosion and are estimated to be responsible for 50% of all corrosion problems (Hamilton 1985). Furanone #1 at concentrations of 20 μg/ml and 40 μg/ml inhibited 58% and 96% of the *D. orientis* growth, respectively. Consequently, biofilm formation on mild steel surfaces was inhibited as evidenced by surface analysis using scanning electron microscopy (Fig. 9), and the weight loss was reduced up to 80% (Ren and Wood 2004). These results suggest that biofilm inhibition by furanones also has engineering applications.

In addition to the experiments in free solution, furanones have been studied to develop novel coating methods. Baveja et al. (2004b) modified surfaces with furanone #7 (Table 2) through physical adsorption. Six polymer materials were tested, including poly(vinyl chloride), silicone, polyethylene, polypropylene, polyether polyurethane, and polytetrafluoroethylene. All of the modified materials were found to reduce the bacterial load and slime production of *S. epidermidis* 24 h after inoculation. No apparent changes in surface hydrophobicity or roughness were identified, suggesting that the effects were due to the inhibitory effects of furanone #7.

To further improve the surface modification, Hume et al. (2004) modified materials with furanone #7 (Table 2) through covalent binding, either by copolymerizing

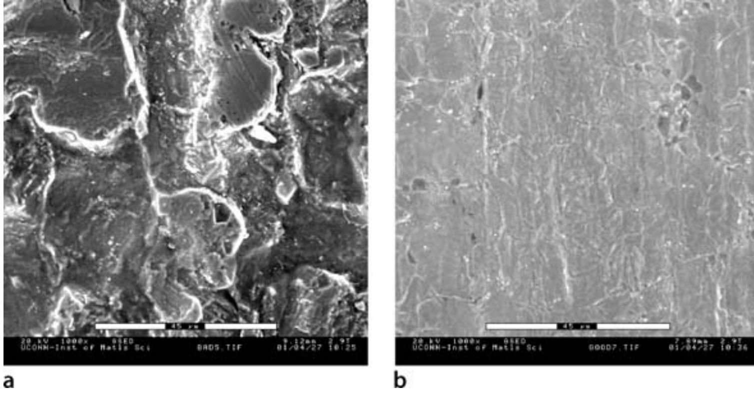

Fig. 9 Furanone #1 (refer to Table 2) at 40 μg/ml protects mild steel from corrosion of *D. orientis*. Images show the scanning electron microscope analysis of unprotected (**a**) and protected (**b**) mild steel coupons from batch experiments. The scale bar is 250 μm. (Reprinted with permission from Blackwell Publishing; Ren and Wood 2004.)

into polystyrene or by coating catheters by plasma-1-ethyl-3-(dimethylaminopropyl) carbodiimide reaction. The modified materials were found to reduce the biofilm formation of *S. epidermidis* by 89% (polystyrene-furanone disks) and 78% (furanone-coated catheters), respectively. In an in vivo study using a graft sheep model (done in a similar fashion to the rat graft model described in the chapter *Quorum Sensing in Streptococci*), furanone-coated catheters inhibited biofilm formation for 65 days. However, the furanone leached off between 65 and 84 days after implementation (Hume et al. 2004).

In addition to biofilm formation, furanones have been shown to inhibit the growth of *B. anthracis* (Jones et al. 2005) as well as the growth and swarming of *B. subtilis* (Ren et al. 2002). Compared with the well-described QS inhibition in gram-negative bacteria, the inhibitory effects of furanones on growth of gram-positive bacteria are less understood. Recent study has shown that deletion of the *B. anthracis luxS* gene (AI-2 synthesis gene) leads to apparent defects in cell growth (Jones and Blaser 2003). Hence, furanones may interfere with some signaling pathways of gram-positive bacteria. Ren et al. (2004a) used DNA microarrays to study the effects of furanone #1 on *B. subtilis* gene expression at a sublethal concentration (5 μg/ml). Consistent with the furanones' inhibition on growth, heat-shock genes were induced. This study, however, was based on a single time point and single furanone concentration. More studies with multiple conditions could be helpful for understanding the molecular basis of growth inhibition by furanones.

References

Abraham WR (2006) Controlling biofilms of gram-positive pathogenic bacteria. Curr Med Chem 13:1509–1524

Andersen JB, Sternberg C, Poulsen LK, Bjørn SP, Givskov M, Molin S (1998) New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. Appl Environ Microbiol 64:2240–2246

Bassler BL (1999) How bacteria talk to each other: regulation of gene expression by quorum sensing. Curr Opin Microbiol 2:582–587

Baveja JK, Li G, Nordon RE, Hume EBH, Kumar N, Willcox MDP, Poole-Warren LA (2004a) Biological performance of a novel synthetic furanone-based antimicrobial. Biomaterials 25:5013–5021

Baveja JK, Willcox MDP, Hume EBH, Kumar N, Odell R, Poole-Warren LA (2004b) Furanones as potential anti-bacterial coatings on biomaterials. Biomaterials 25:5003–5012

Beechan CM, Sims JJ (1979) The first synthesis of fimbrolides, a novel class of halogenated lactones naturally occurring in the red seaweed Delisea fimbriata (Bonnemaisoniaceae). Tetrahedron Lett 19:1649–1652

Ben Ayed S, Boutiba-Ben Boubaker I, Samir E, Ben Redjeb S (2006) Prevalence of agr specificity groups among methicillin-resistant *Staphylococcus aureus* circulating at Charles Nicolle Hospital of Tunis. Pathol Biol (Paris) 54:435–438

Bjarnsholt T, Jensen PØ, Burmølle M, Hentzer M, Haagensen JA, Hougen HP, Calum H, Madsen KG, Moser C, Molin S, Høiby N, Givskov M (2005a) *Pseudomonas aeruginosa* tolerance to tobramycin, hydrogen peroxide and polymorphonuclear leukocytes is quorum-sensing dependent. Microbiology 151:373–383

Bjarnsholt T, Jensen PØ, Rasmussen TB, Christophersen L, Calum H, Hentzer M, Hougen HP, Rygaard J, Moser C, Eberl L, Høiby N, Givskov M (2005b) Garlic blocks quorum sensing and promotes rapid clearing of pulmonary *Pseudomonas aeruginosa* infections. Microbiology 151:3873–3880

Borchardt SA, Allain EJ, Michels JJ, Stearns GW, Kelly RF, McCoy WF (2001) Reaction of acylated homoserine lactone bacterial signaling molecules with oxidized halogen antimicrobials. Appl Environ Microbiol 67:3174–3179

Byers JT, Lucas C, Salmond GP, Welch M (2002) Nonenzymatic turnover of an *Erwinia carotovora* quorum-sensing signaling molecule. J Bacteriol 184:1163–1171

Carlier A, Uroz S, Smadja B, Fray R, Latour X, Dessaux Y, Faure D (2003) The Ti plasmid of *Agrobacterium tumefaciens* harbors an attM-paralogous gene, *aiiB*, also encoding *N*-acyl homoserine lactonase activity. Appl Environ Microbiol 69:4989–4993

Castang S, Chantegrel B, Deshayes C, Dolmazon R, Gouet P, Haser R, Reverchon S, Nasser W, Hugouvieux-Cotte-Pattat N, Doutheau A (2004) *N*-Sulfonyl homoserine lactones as antagonists of bacterial quorum sensing. Bioorg Med Chem Lett 14:5145–5149

Chun CK, Ozer EA, Welsh MJ, Zabner J, Greenberg EP (2004) Inactivation of a *Pseudomonas aeruginosa quorum*-sensing signal by human airway epithelia. Proc Natl Acad Sci USA 101:3587–3590

Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton JW, Greenberg EP (1998) The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science 280:295–298

Defoirdt T, Miyamoto CM, Wood TK, Meighen EA, Sorgeloos P, Verstraete W, Bossier P (2007) The natural furanone (5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(5*H*)-furanone disrupts quorum sensing-regulated gene expression in *Vibrio harveyi* by decreasing the DNA-binding activity of the transcriptional regulator protein luxR. Environ Microbiol 9:2486–2495

de Nys R, Wright AD, Konig GM, Sticher O (1993) New halogenated furanone from the marine alga *Delisea pulchra* (cf. fimbriata). Tetrahedron 49:11213–11220

Dong YH, Xu JL, Li XZ, Zhang LH (2000) AiiA, an enzyme that inactivates the acylhomoserine lactone quorum- sensing signal and attenuates the virulence of *Erwinia carotovora*. Proc Natl Acad Sci USA 97:3526–3531

Dong YH, Wang LH, Xu JL, Zhang HB, Zhang XF, Zhang LH (2001) Quenching quorum-sensing-dependent bacterial infection by an *N*-acyl homoserine lactonase. Nature 411:813–817

Dong YH, Gusti AR, Zhang Q, Xu JL, Zhang LH (2002) Identification of quorum-quenching *N*-acyl homoserine lactonases from *Bacillus* species. Appl Environ Microbiol 68:1754–1759

Dong YH, Zhang XF, Xu JL, Zhang LH (2004) Insecticidal *Bacillus thuringiensis* silences *Erwinia carotovora* virulence by a new form of microbial antagonism, signal interference. Appl Environ Microbiol 70:954–960

Geske GD, Wezeman RJ, Siegel AP, Blackwell HE (2005) Small molecule inhibitors of bacterial quorum sensing and biofilm formation. J Am Chem Soc 127:12762–12763

Givskov M, de Nys R, Manefield M, Gram L, Maximilien R, Eberl L, Molin S, Steinberg PD, Kjelleberg S (1996) Eukaryotic interference with homoserine lactone-mediated prokaryotic signalling. J Bacteriol 178:6618–6622

Gram L, de Nys R, Maximilien R, Givskov M, Steinberg P, Kjelleberg S (1996) Inhibitory effects of secondary metabolites from the red alga *Delisea pulchra* on swarming motility of *Proteus mirabilis*. Appl Environ Microbiol 62:4284–4287

Hamilton WA (1985) Sulphate-reducing bacteria and anaerobic corrosion. Annu Rev Microbiol 39:195–217

Hastings JW (2004) Bacterial quorum-sensing signals are inactivated by mammalian cells. Proc Natl Acad Sci USA 101:3993–3994

Hentzer M, Riedel K, Rasmussen TB, Heydorn A, Andersen JB, Parsek MR, Rice SA, Eberl L, Molin S, Høiby N, Kjelleberg S, Givskov M (2002) Inhibition of quorum sensing in *Pseudomonas aeruginosa* biofilm bacteria by a halogenated furanone compound. Microbiology 148:87–102

Hentzer M, Wu H, Andersen JB, Riedel K, Rasmussen TB, Bagge N, Kumar N, Schembri MA, Song Z, Kristoffersen P, Manefield M, Costerton JW, Molin S, Eberl L, Steinberg P, Kjelleberg S, Høiby N, Givskov M (2003) Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. EMBO J 22:3803–3815

Huang JJ, Han JI, Zhang LH, Leadbetter JR (2003) Utilization of acyl-homoserine lactone quorum signals for growth by a soil pseudomonad and *Pseudomonas aeruginosa* PAO1. Appl Environ Microbiol 69:5941–5949

Huang JJ, Petersen A, Whiteley M, Leadbetter JR (2006) Identification of QuiP, the product of gene PA1032, as the second acyl-homoserine lactone acylase of *Pseudomonas aeruginosa* PAO1. Appl Environ Microbiol 72:1190–1197

Hume EBH, Baveja J, Muir B, Schubert TL, Kumar N, Kjelleberg S, Griesser HJ, Thissen H, Read R, Poole-Warren LA, Schindhelm K, Willcox MDP (2004) The control of *Staphylococcus epidermidis* biofilm formation and in vivo infection rates by covalently bound furanones. Biomaterials 25:5023–5030

Jarraud S, Mougel C, Thioulouse J, Lina G, Meugnier H, Forey F, Nesme X, Etienne J, Vandenesch F (2002) Relationships between *Staphylococcus aureus* genetic background, virulence factors, *agr* groups (alleles), and human disease. Infect Immun 70:631–641

Jones MB, Blaser MJ (2003) Detection of a *luxS*-signaling molecule in *Bacillus anthracis*. Infect Immun 71:3914–3919

Jones MB, Jani R, Ren D, Wood TK, Blaser MJ (2005) Inhibition of *Bacillus anthracis* growth and virulence-gene expression by inhibitors of quorum-sensing. J Infect Dis 191:1881–1888

Kazlauskas R, Murphy PT, Quinn RJ, Wells RJ (1977) A new class of halogenated lactones from the red alga *Delisea fimbriata* (bonnemaisoniaceae). Tetrahedron Lett 1:37–40

Koch B, Liljefors T, Persson T, Nielsen J, Kjelleberg S, Givskov M (2005) The LuxR receptor: the sites of interaction with quorum-sensing signals and inhibitors. Microbiology 151:3589–3602

Leadbetter JR, Greenberg EP (2000) Metabolism of acyl-homoserine lactone quorum-sensing signals by *Variovorax paradoxus*. J Bacteriol 182:6921–6926

Lyon GJ, Mayville P, Muir TW, Novick RP (2000) Rational design of a global inhibitor of the virulence response in *Staphylococcus aureus*, based in part on localization of the site of inhibition to the receptor-histidine kinase, AgrC. Proc Natl Acad Sci USA 97:13330–13335

Manago K, Nishi J, Wakimoto N, Miyanohara H, Sarantuya J, Tokuda K, Iwashita M, Yamamoto K, Yoshinaga M, Maruyama I, Kawano Y (2006) Biofilm formation by and accessory gene regulator typing of methicillin-resistant *Staphylococcus aureus* strains recovered from patients with nosocomial infections. Infect Control Hosp Epidemiol 27:188–190

Manefield M, de Nys R, Kumar N, Read R, Givskov M, Steinberg P, Kjelleberg S (1999) Evidence that halogenated furanones from *Delisea pulchra* inhibit acylated homoserine lactone (AHL)-mediated gene expression by displacing the AHL signal from its receptor protein. Microbiology 145:283–291

Manefield M, Harris L, Rice SA, de Nys R, Kjelleberg S (2000) Inhibition of luminescence and virulence in the black tiger prawn (*Penaeus monodon*) pathogen *Vibrio harveyi* by intercellular signal antagonists. Appl Environ Microbiol 66:2079–2084

Manefield M, Welch M, Givskov M, Salmond GPC, Kjelleberg S (2001) Halogenated furanones from the red alga, *Delisea pulchra*, inhibit carbapenem antibiotic synthesis and exoenzyme virulence factor production in the phytopathogen *Erwinia carotovora*. FEMS Microbiol Lett 205:131–138

Manefield M, Rasmussen TB, Hentzer M, Andersen JB, Steinberg P, Kjelleberg S, Givskov M (2002) Halogenated furanones inhibit quorum sensing through accelerated LuxR turnover. Microbiology 148:1119–1127

Manny AJ, Kjelleberg S, Kumar N, de Nys R, Read RW, Steinberg P (1997) Reinvestigation of the sulfuric acid-catalysed cyclisation of brominated 2-alkyllevulinic acids to 3-alkyl-5-methylene-2(5*H*)-furanones. Tetrahedron 53:15813–15826

Martinelli D, Grossmann G, Séquin U, Brandl H, Bachofen R (2004) Effects of natural and chemically synthesized furanones on quorum sensing in *Chromobacterium violaceum*. BMC Microbiol 4:25

Mayville P, Ji G, Beavis R, Yang H, Goger M, Novick RP, Muir TW (1999) Structure-activity analysis of synthetic autoinducing thiolactone peptides from *Staphylococcus aureus* responsible for virulence. Proc Natl Acad Sci USA 96:1218–1223

Oggioni MR, Trappetti C, Kadioglu A, Cassone M, Iannelli F, Ricci S, Andrew PW, Pozzi G (2006) Switch from planktonic to sessile life: a major event in pneumococcal pathogenesis. Mol Microbiol 61:1196–1210

Olsen JA, Severinsen RE, Rasmussen TB, Hentzer M, Givskov MC, Nielsen J (2002) Synthesis of new 3- and 4-substituted analogues of acyl homoserine lactone quorum sensing autoinducers. Bioorg Med Chem Lett 12:325–328

Ozer EA, Pezzulo A, Shih DM, Chun C, Furlong C, Lusis AJ, Greenberg EP, Zabner J (2005) Human and murine paraoxonase 1 are host modulators of *Pseudomonas aeruginosa* quorum-sensing. FEMS Microbiol Lett 253:29–37

Park SY, Lee SJ, Oh TK, Oh JW, Koo BT, Yum DY, Lee JK (2003) AhlD, an *N*-acylhomoserine lactonase in *Arthrobacter* sp, and predicted homologues in other bacteria. Microbiology 149:1541–1550

Park SY, Kang HO, Jang HS, Lee JK, Koo BT, Yum DY (2005) Identification of extracellular *N*-acylhomoserine lactone acylase from a *Streptomyces* sp. and its application to quorum quenching. Appl Environ Microbiol 71:2632–2641

Parsek MR, Val DL, Hanzelka BL, Cronan JE, Greenberg EP (1999) Acyl homoserine-lactone quorum-sensing signal generation. Proc Natl Acad Sci USA 96:4360–4365

Pearson JP, Pesci EC, Iglewski BH (1997) Roles of *Pseudomonas aeruginosa las* and *rhl* quorum-sensing systems in control of elastase and rhamnolipid biosynthesis Genes. J Bacteriol 179:5756–5767

Pèchere JC (2001) Azithromycin reduces the production of virulence factors in *Pseudomonas aeruginosa* by inhibiting quorum sensing. Jpn J Antibiot 54(suppl C):87–89

Persson T, Hansen TH, Rasmussen TB, Skindersø ME, Givskov M, Nielsen J (2005) Rational design and synthesis of new quorum-sensing inhibitors derived from acylated homoserine lactones and natural products from garlic. Org Biomol Chem 3:253–262

Pratt LA, Kolter R (1998) Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type i pili. Mol Microbiol 30:285–293

Rasmussen TB, Bjarnsholt T, Skindersoe ME, Hentzer M, Kristoffersen P, Köte M, Nielsen J, Eberl L, Givskov M (2005a) Screening for quorum-sensing inhibitors (QSI) by use of a novel genetic system, the QSI selector. J Bacteriol 187:1799–1814

Rasmussen TB, Skindersoe ME, Bjarnsholt T, Phipps RK, Christensen KB, Jensen PO, Andersen JB, Koch B, Larsen TO, Hentzer M, Eberl L, Høiby N, Givskov M (2005b) Identity and effects of quorum-sensing inhibitors produced by *Penicillium* species. Microbiology 151:1325–1340

Reimmann C, Ginet N, Michel L, Keel C, Michaux P, Krishnapillai V, Zala M, Heurlier K, Triandafillu K, Harms H, Défago G, Haas D (2002) Genetically programmed autoinducer destruction reduces virulence gene expression and swarming motility in *Pseudomonas aeruginosa* PAO1. Microbiology 148:923–932

Ren D, Wood TK (2004) (5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(5*H*)-furanone reduces corrosion from *Desulfotomaculum orientis*. Environ Microbiol 6:535–540

Ren D, Sims JJ, Wood TK (2001) Inhibition of biofilm formation and swarming of *Escherichia coli* by (5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(5*H*)-furanone. Environ Microbiol 3:731–736

Ren D, Sims JJ, Wood TK (2002) Inhibition of biofilm formation and swarming of *Bacillus subtilis* by (5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(5*H*)-furanone. Lett Appl Microbiol 34:293–299

Ren D, Bedzyk LA, Setlow P, Thomas S, Ye RW, Wood TK (2004a) Differential gene expression to investigate the effect of (5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(5*H*)-furanone on *Bacillus subtilis*. Appl Environ Microbiol 70:4941–4949

Ren D, Bedzyk LA, Thomas SM, Ye RW, Wood TK (2004b) Differential gene expression shows natural brominated furanones interfere with the autoinducer-2 bacterial signaling system of *Escherichia coli*. Biotechnol Bioeng 88:630–642

Ren D, Bedzyk LA, Ye RW, Thomas SM, Wood TK (2004c) Stationary-phase quorum-sensing signals affect autoinducer-2 and gene expression in *Escherichia coli*. Appl Environ Microbiol 70:2038–2043

Ren D, Zuo R, Wood TK (2005) Quorum-sensing antagonist (5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(5*H*)-furanone influences siderophore biosynthesis in *Pseudomonas putida* and *Pseudomonas aeruginosa*. Appl Microbiol Biotechnol 66:689–695

Reverchon S, Chantegrel B, Deshayes C, Doutheau A, Cotte-Pattat N (2002) New synthetic analogues of *N*-acyl homoserine lactones as agonists or antagonists of transcriptional regulators involved in bacterial quorum sensing. Bioorg Med Chem Lett 12:1153–1157

Schaefer AL, Hanzelka BL, Eberhard A, Greenberg EP (1996) Quorum sensing in *Vibrio fischeri*: probing autoinducer-LuxR interactions with autoinducer analogs. J Bacteriol 178:2897–2901

Singh PK, Schaefer AL, Parsek MR, Moninger TO, Welsh MJ, Greenberg EP (2000) Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. Nature 407:762–764

Sio CF, Otten LG, Cool RH, Diggle SP, Braun PG, Bos R, Daykin M, Cámara M, Williams P, Quax WJ (2006) Quorum quenching by an *N*-acyl-homoserine lactone acylase from *Pseudomonas aeruginosa* PAO1. Infect Immun 74:1673–1682

Smith KM, Bu Y, Suga H (2003a) Induction and inhibition of *Pseudomonas aeruginosa* quorum sensing by synthetic autoinducer analogs. Chem Biol 10:81–89

Smith KM, Bu Y, Suga H (2003b) Library screening for synthetic agonists and antagonists of a *Pseudomonas aeruginosa* autoinducer. Chem Biol 10:563–571

Sofer D, Gilboa-Garber N, Belz A, Garber NC (1999) "Subinhibitory" erythromycin represses production of *Pseudomonas aeruginosa* lectins, autoinducer and virulence factors. Chemotherapy 45:335–341

Stintzi A, Evans K, Meyer J-M, Poole K (1998) Quorum-sensing and siderophore biosynthesis in *Pseudomonas aeruginosa*: *lasR/lasI* mutants exhibit reduced pyoverdine biosynthesis. FEMS Microbiol Lett 166:341–345

Surette MG, Bassler BL (1998) Quorum sensing in *Escherichia coli* and *Salmonella typhinurium*. Proc Natl Acad Sci USA 95:7046–7050

Tateda K, Comte R, Pechere JC, Köhler T, Yamaguchi K, Van Delden C (2001) Azithromycin inhibits quorum sensing in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 45:1930–1933

Uroz S, D'Angelo-Picard C, Carlier A, Elasri M, Sicot C, Petit A, Oger P, Faure D, Dessaux Y (2003) Novel bacteria degrading *N*-acylhomoserine lactones and their use as quenchers of quorum-sensing-regulated functions of plant-pathogenic bacteria. Microbiology 149:1981–1989

Van Delden C, Iglewski BH (1998) Cell-to-cell signaling and *Pseudomonas aeruginosa* infections. Emerg Infect Dis 4:551–560

Wang LH, Weng LX, Dong YH, Zhang LH (2004) Specificity and enzyme kinetics of the quorum-quenching *N*-Acyl homoserine lactone lactonase (AHL-lactonase). J Biol Chem 279:13645–13651

Wood TK, González Barrios AF, Herzberg M, Lee J (2006) Motility influences biofilm architecture in *Escherichia coli*. Appl Microbiol Biotechnol 72:361–367

Wright III JS, Jin R, Novick RP (2005) Transient interference with staphylococcal quorum sensing blocks abscess formation. Proc Natl Acad Sci USA 102:1691–1696

Wu H, Song Z, Hentzer M, Andersen JB, Heidorn A, Mathee K, Moser C, Eberl L, Molin S, Høiby N, Givskov M (2000) Detection of *N*-acyl-homoserine lactones in lung tissues of mice infected with *Pseudomonas aeruginosa*. Microbiology 146:2481–2493

Wu H, Song Z, Hentzer M, Andersen JB, Molin S, Givskov M, Høiby N (2004) Synthetic furanones inhibit quorum-sensing and enhance bacterial clearance in *Pseudomonas aeruginosa* lung infection in mice. J Antimicrob Chemother 53:1054–1061

Yates EA, Philipp B, Buckley C, Atkinson S, Chhabra SR, Sockett RE, Goldner M, Dessaux Y, Cámara M, Smith H, Williams P (2002) *N*-acylhomoserine lactones undergo lactonolysis in a pH-, temperature-, and acyl chain length-dependent manner during growth of *Yersinia pseudotuberculosis* and *Pseudomonas aeruginosa*. Infect Immun 70:5635–5646

Mathematical Modeling of Quorum-Sensing Control in Biofilms

John Ward

Abstract This chapter begins with an overview of the relevant literature on theoretical approaches to modeling biofilms, quorum sensing in bacteria, and anti-quorum-sensing treatment. Following this, new mathematical models are proposed to investigate anti-quorum-sensing treatment in batch cultures and in biofilm environments. Details for the models' derivation are aimed so that readers with a nonmathematical background will have a good idea of how such models are constructed and studied. Three anti-quorum-sensing targets are investigated, and a wide variety of outcomes in terms of successful treatment are predicted depending on treatment type, strength, and timing. The many interesting conclusions that can be drawn from the presented results are discussed in detail, including ideas for new experiments, many of which would be considered routine, that will provide deeper insights into how anti-quorum-sensing treatments could be highly effective means of controlling bacterial behavior in a variety of situations and environments.

1 Introduction

The application of mathematics to biology in antiquity was probably little more than a tool to assist in the counting of sheep or cattle. The modeling of population growth seems to have had its beginnings in the Middle Ages, the most famous work being that of Fibonacci of Pisa. In *Liber Abaci*, published in 1202, he derived a formula for rabbit population growth which states that the current population is given by the sum of those of the previous two months, thereby generating his famous eponymous sequence (1, 1, 2, 3, 5, 8, 13, 21, 34, 55, etc.), which essentially amounts to exponential growth. There have, of course, been considerable advancements in the fields

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N. Balaban (ed.) *Control of Biofilm Infections by Signal Manipulation*
Springer Series on Biofilms 2
doi: 10.1007/978-3-540-73853-4, © Springer 2008

of science, mathematics, and computation since then; however, the basic modeling process is no different from that undertaken by Fibonacci. The aim of mathematical modelers is to formulate a model based on scientifically motivated mechanisms, investigating the resulting system of equations to see whether they can reproduce observations and make new predictions to motivate further experimentation. Ideally, through repeated cycles of model development, validation, and modification, significantly more insight into a problem will be gained than would be possible from experimentation alone. The modeling of quorum-sensing (QS) inhibition therapies in biofilms is in the first such cycle of model development.

Because of their importance in many industrial processes, there has been a long history in biofilm modeling by mathematicians and engineers. Broadly speaking, most mathematical modeling falls into two categories:

1. Continuum models, i.e., models that generate differential equations. See, for example, Atkinson and Davies (1974a,b), Bakke et al. (1984), Dillon et al. (1996), Dockery and Klapper (2001), Freter et al. (1983), and Rittmann and Manem (1992). More examples are discussed below. A pre-1999 review is given in Chaudhry and Beg (1998).
2. Cellular automata, i.e., computational models that use probabilistic "rules" to describe movement and growth from one time-step to the next. See, for example, Eberl et al. (2000), Gonpot et al. (2000), Kreft (2004), Kreft et al. (1998), Noguera et al. (1999), Picioreanu et al. (1998a,b, 2000, 2004), and Wimpenny and Colasanti (1997).

The first approach has the advantage of being more amenable to mathematical analysis and is usually computationally less expensive. In this chapter, a continuum model will be presented that views the biofilm as a multiphase fluid whose growth is principally governed by nutrients that diffuse into the biofilm from the surrounding fluid. This general approach has been adopted by a number of authors investigating single-species biofilm growth (Pritchett and Dockery 2001; Stewart 1994), multispecies biofilm growth (Wanner and Gujer 1986; Wanner and Reichert 1995), growth in porous media (Chen-Charpentier 1999; Tiwari and Bowers 2001), growth in conduits (Szego et al. 1993), the role of the biofilm matrix (Cogan et al. 2005), and antimicrobial resistance (Cogan and Keener 2004).

The mathematical modeling of QS has less history, starting with the near simultaneous publications from three groups, namely James et al. (2000), Dockery and Keener (2001), and Ward et al. (2001). The first two of these papers modeled QS at the molecular level, using the law of mass action to formulate a model consisting of a system of ordinary differential equations. Here, the relevant timescale is in the order of minutes, whereby the population will remain approximately constant and thus act as a parameter in their system. Within appropriate parameter regimes, the authors' analyses showed that there exists a population threshold between very low and very high QS activity. Fagerlind et al. (2003, 2005) extended the work of James et al. (2000) to model the QS system of *Pseudomonas aeruginosa* to study in detail the QS regulatory hierarchy (Fagerlind et al. 2003) and the role of an endogenously produced acyl-homoserine lactone (AHL) antagonist (Fagerlind et al.

2005). Recently, Gorychev et al. (2006) used the same modeling approach to investigate the role of AHL binding with dimeric transcriptional regulators, concluding that dimerization enhances the QS switchlike behavior. Dockery and Keener (2001) and Nilsson et al. (2001) extended these ideas to investigate QS behavior in biofilms, though biofilm growth was not considered in detail. The majority of models have focused on LuxRI homolog systems in gram-negative bacteria, Koerber et al. (2005) used a stochastic modeling approach to describe the QS system employed by the gram-positive *Staphylococcus aureus*. In particular, they studied the role of QS of individual bacteria internalized by nonprofessional phagocytes within endosomes (Hudson et al. 1995), a pathogenic trait that enables bacteria to reproduce within living cells, which is believed to play an important part in prolonging infection.

In the interest of developing a model that can be parametrized using results from routine batch culture experiments, as well as one that can be easily extended to model more complicated situations, Ward et al. (2001) proposed a simple population scale model of QS activity. The timescale of interest is in the order of hours, and the model tracks changes in total population and in subpopulations, the latter reflecting QS activity governed by AHL concentration. The model has relatively few parameters, and data fitting was made possible using results from batch cultures of several strains of *P. aeruginosa*, whereby hourly samples were taken and analyzed to obtain population density and concentrations of the AHLs 3-oxo-C12-homoserine lactone (OdDHL) and *N*-butanoyl-L-homoserine lactone (BHL). The model predicts that the switch from a principally downregulated to an upregulated population occurs very rapidly in comparison to population growth. Ward et al. (2004) extended this model to investigate a range of negative feedback mechanisms known to be involved in QS. A key result was the prediction that a population that would be considered to be QS-active may consist only of a small proportion of upregulated cells at any one time (approximately 5–20%). A simple model for QS in a wound was investigated by Koerber et al. (2002). This model couples the QS model of Ward et al. (2001) with a compartmentalized representation of a wound in which AHL molecules can be lost to the bacteria through diffusion into neighboring regions. Interestingly, the model predicts a rapid jump in QS activity just like that observed in batch cultures, indicating that the QS process is robust even when AHL loss by diffusion is an issue.

Chopp et al. (2002, 2003) and Ward et al. (2003) studied models that coupled biofilm growth and QS, with the aim of identifying key parameters (kinetics, biofilm depth) that govern QS activity. The modeling of biofilms by Chopp et al. is very similar to that described in Sect. 2.2; however, the QS aspects are handled differently. These researchers imposed a critical concentration in their models above which AHL production is massively enhanced; this is not necessary in the model proposed in the next section, as such enhancement of production occurs naturally from the dynamics.

Whereas Chopp et al. (2002, 2003) studied a depth-based model, Ward et al. (2003) modeled biofilms growing over a substratum. The biofilm growth model was not mechanistic (not governed by nutrient concentration) but was chosen so that

the number of parameters is minimal, yet growth can be "made to measure" with regard to how fast the biofilm grows up and along the substratum. This model was coupled with the QS model of Ward et al. (2001) so that the level of AHL buildup within a biofilm can be assessed. This model, and indeed the models of Chopp et al., predict that the shift from low to high QS activity occurs very rapidly throughout the biofilm.

The only publications to date regarding the mathematical modeling of QSIs (as well as antibiotic treatments) are a series of papers by Anguige et al. (2004, 2005, 2006). To explicitly model the effects of furanones (Manefield et al. 1999, 2002; Hentzer et al. 2003) and anti-AHL lactonases (Dong et al. 2002; Lee et al. 2002; Ulrich 2004; Xu et al. 2003), it was necessary to focus on the detailed biochemistry of QS, akin to the approach of James et al. (2000) and Dockery and Keener (2001). The three papers covered the scenarios of batch cultures (Anguige et al. 2004), early biofilm development (Anguige et al. 2005), and mature biofilm development and exopolysaccharide (EPS) production (Anguige et al. 2006). Because of the complexity of these models, the predicted dynamics can be very complex; however, an important result is that the amount of QSI required to be effective in biofilms increases exponentially with biofilm depth. Other key results discussed in these papers will be reproduced by the model studied in this chapter.

In the next section, simplified versions of the mathematical models of Anguige et al. (2004, 2005, 2006) are presented to describe anti-QS treatments in batch cultures and biofilms. This will involve the systematic scale-up from the molecular level to the population level, based on the assumption that the biochemical processes operate on a considerably faster timescale to that of growth. The modeling will consider three QSIs applied to *P. aeruginosa* colonies, namely anti-LasR (e.g., furanones), anti-AHL (e.g., lactonases), and a putative anti-LasI agent. Results from simulations are presented and discussed in the subsequent two sections, and the key points are summarized in the final section.

2 Mathematical Modeling

The anti-QS models developed and studied by Anguige et al. (2004, 2005, 2006) were simplified using the basic ideas of Ward et al. (2001, 2004). The key features of the former studies are maintained, but the new model has fewer parameters, many of which should be determinable using fairly routine experiments.

The model is constructed on the basis of the biochemical pathways involved in the LasRI QS system of *P. aeruginosa*, although it will be applicable to most LuxRI homolog systems. The population of cells (planktonic or sessile) is assumed to consist of two subpopulations:

Downregulated cells. Population density N_d. These cells have an empty *lux*-box and, in the case of *P. aeruginosa*, are nonvirulent and produce AHLs and biofilm matrix EPS at a very low background rate.

Upregulated cells. Population density N_u. These cells have a LasR-AHL complex-bound *lux*-box and will express virulent characteristics and produce AHLs and EPS at a significantly enhanced rate.

The total population density is thus $N_T = N_d + N_u$. It is unclear during cell division and chromosomal replication whether a LasR-AHL complex will remain bound to the *lux*-box or not; it will be assumed for simplicity that a downregulated cell will divide into two downregulated cells and that an upregulated cell will divide into one up- and one downregulated cell. The upregulation of downregulated cells is mediated by QS, and downregulation occurs spontaneously (detailed below).

The modeling for QS is based on the work of Ward et al. (2001, 2004); however, because the modeling details are important for the anti-QS therapies, they will be discussed at some length here. Figure 1 shows a schematic LasR/LasI system in *P. aeruginosa* showing the reactions relevant to the modeling. Within all cells it is assumed that LasR (concentration R) is produced at a constitutive rate R_0, binds within a reversible reaction with AHL (A) to form the LasR-AHL complex (P), and

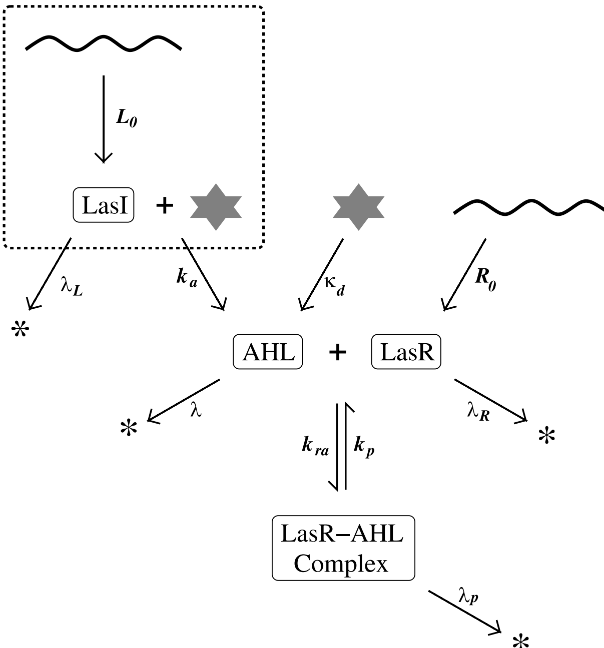


Fig. 1 Schematic of the quorum sensing system LasR/LasI system in *P. aeruginosa* used in the modeling. The figure shows the reactions that are assumed to be occurring in all bacteria, apart from those in the *dashed box* which occur only in the upregulated cells. The *grey stars* represent the reaction between the C_{12}-ACP and *S*-adenosylmethionine that produces the AHL 3-oxo-C12-homoserine lactone (Fuqua and Greenberg 2002), the *wavy lines* represent transcription of the relevant protein and the "*" represent breakdown products. The reaction rate constants for each of the chemical reactions are shown

decays naturally, hence

$$\frac{\mathrm{d}R}{\mathrm{d}t} = R_0 - k_{\mathrm{ra}}AR + k_{\mathrm{p}}P - \lambda_{\mathrm{R}}R \,. \quad (1)$$

The LasR-AHL complex equation is given by

$$\frac{\mathrm{d}P}{\mathrm{d}t} = k_{\mathrm{ra}}AR - k_{\mathrm{p}}P - \lambda_{\mathrm{p}}P \,, \quad (2)$$

in which natural decay of P is also assumed. In upregulated cells, output of LasI occurs at a constant rate and decays naturally according to

$$\frac{\mathrm{d}L}{\mathrm{d}t} = L_0 - \lambda_{\mathrm{L}}L \,. \quad (3)$$

The AHL concentration A represents the measurable AHL concentration in the fluid growth media. Pearson et al. (1999) observed for the AHL 3-oxo-C12-homoserine lactone that the equilibration between internal and external concentrations occurs quickly (in less than 5 min), with internal concentration partitioned to be about three times that of the external. This simply means that $A_{\mathrm{internal}} = \delta A$ (here $\delta \approx 3$) and the relevant parameters below have contained within them this factor δ. AHLs are produced at some background level κ_{d} and decay or become sequestered in fluid with rate constant λ; this, together with the reaction with LasR, yields for downregulated cells

$$\text{the rate of change of AHL in downregulated cells} = \kappa_{\mathrm{d}} - k_{\mathrm{ra}}AR + k_{\mathrm{p}}P - \lambda A \,, \quad (4)$$

and for upregulated cells

$$\text{the rate of change of AHL in upregulated cells} = k_{\mathrm{a}}L + \kappa_{\mathrm{d}} - k_{\mathrm{ra}}AR + k_{\mathrm{p}}P - \lambda A \,, \quad (5)$$

where $k_{\mathrm{a}}L$ describes the massive increase in production of AHLs by upregulated cells. We note that the rate of change of AHL in the external media is simply $-\lambda A$. Compared with cell division time, the timescale adopted in the modeling, these reactions occur very rapidly, and it is reasonable to assume that Eqs. 1–3 are in equilibrium (mathematically, that means $\mathrm{d}R/\mathrm{d}t = \mathrm{d}P/\mathrm{d}t = \mathrm{d}L/\mathrm{d}t = 0$). Hence,

$$L = L_\infty \,, \quad P = \frac{P_\infty}{R_\infty}RA \,, \quad R = \frac{R_\infty}{1 + \mu_{\mathrm{R}}A} \,,$$

where $L_\infty = L_0/\lambda_{\mathrm{L}}$, $R_\infty = R_0/\lambda_{\mathrm{R}}$, $P_\infty = R_\infty k_{\mathrm{ra}}/(k_{\mathrm{p}} + \lambda_{\mathrm{p}})$ and $\mu_{\mathrm{R}} = \lambda_{\mathrm{p}}P_\infty/\lambda_{\mathrm{R}}R_\infty$. Fitting the resulting model to experimental data as described in Ward et al. (2004) indicates that $\mu_{\mathrm{R}}A \approx 0$ (i.e., very small), suggesting that most of the LasR is degraded before binding with AHL. For simplicity we will assume that the LasR concentration remains roughly constant at $R = R_\infty$, from which we deduce $P = P_\infty A$. We note that dimerization of R can be modeled in the same way, and it does add a small

amount of complexity to the problem, but the reduction to $R = R_\infty$ will nevertheless result. Substituting these approximations into the AHL production rate equations gives

$$\text{the rate of change of AHL in downregulated cells} = \kappa_d - \sigma A - \lambda A \tag{6}$$

$$\text{the rate of change of AHL in upregulated cells} = \kappa_u + \kappa_d - \sigma A - \lambda A\,, \tag{7}$$

where $\kappa_u = k_a L_\infty$ and $\sigma = \lambda_p P_\infty$. We note in the earlier models (Ward et al. 2001, 2003, 2004; Koerber et al. 2002) that κ_d was absorbed into the κ_u term; however, in order to model the action of the anti-LasI agent explicitly, we shall maintain the terms in their current form. The upregulation rate of cells is assumed to be proportional to the complex concentration $P_\infty A$; letting α_a be the constant of proportionality, then

$$\text{upregulation rate} = \alpha A\,,$$

where $\alpha = \alpha_a P_\infty$. The downregulation rate of cells is governed by the decay rate of *lux*-bound complexes, which is taken to be β; it is possible that bound complexes have the same degradation properties as their free-floating counterparts, whereby $\beta = \lambda_p$. Three forms of QSI therapies will be investigated. The first two involve the action of molecules currently being investigated by a number of experimental investigators and discussed by Anguige et al. (2004–2006). The third therapy involves the action of a putative anti-LasI agent.

1. **Anti-LuxR (homolog) agents**, such as halogenated furanones. Using Q_1 to represent the concentration of this agent (to be consistent with the earlier papers), the assumed reaction with LasR can be summarized as follows:

   $$\text{LasR} + Q_1 \xrightarrow{k_1} (1-\nu_1)Q_1 + \text{by product(s)}\,,$$

   where ν_1 is the average amount of Q_1 lost by the reaction. Using a similar argument to that above, at equilibrium the LasR concentration is given by $R = R_\infty/(1+\gamma_1 Q_1)$, where $\gamma_1 = k_1/k_R$. This leads the upregulation rate and the LasR-AHL binding rate to be reduced by a factor of $(1+\gamma_1 Q_1)$; i.e., the "constants" α and σ now become $\alpha/(1+\gamma_1 Q_1)$ and $\sigma/(1+\gamma_1 Q_1)$, respectively.
2. **Anti-AHL agents**, such as lactonases that degrade/quench AHLs. Let Q_2 be the concentration of the lactonase, and then the modeling of lactonase action is based on the simple reaction

   $$\text{AHL} + Q_2 \xrightarrow{\mu_2} (1-\nu_2)Q_2 + \text{by product(s)}\,,$$

   where ν_2 is the average amount of Q_2 lost by the reaction. This results in an additional "$-\mu_2 Q_2 A$" term in Eqs. 6 and 7.

3. **Anti-LuxI (homolog) agents**, putative to the author's knowledge. Let Q_3 be the concentration of this agent, and then we assume

$$\mathrm{AHL} + Q_3 \xrightarrow{\mu_3} (1-\nu_3)Q_3 + \text{by product(s)} ,$$

where ν_3 is the average amount of Q_3 lost by the reaction. Similar to the action of Q_1 on LasR, the LasI equilibrium level L_∞ will be reduced to $L_\infty/(1+\gamma_3 Q_3)$ by the agent, where $\gamma_3 = k_3/\lambda_L$. Thus, the AHL output rate term κ_u for upregulated cells now becomes $\kappa_u/(1+\gamma_3 Q_3)$.

All the quantities involved in the modeling are assumed to evolve continuously in time and space, with random stochastic effects neglected. The solutions, therefore, are a prediction of the "average" outcome to be observed experimentally or in situ.

2.1 Anti-QS Treatment in Batch Cultures

The system of equations below is derived from the application of the assumptions given above. For the discussion in the subsequent sections, we will focus on batch culture colony during the exponential phase of growth (doubling time = $\ln(2)/r$), with the drug being introduced either at the start of an experiment or being drip-fed at a rate ϕ_i ($i = 1,2,3$ for Q_1, Q_2, Q_3, respectively). The equations are

$$\frac{dN_d}{dt} = rN_T - \frac{\alpha A}{1+\gamma_1 Q_1} N_d + \beta N_u , \tag{8}$$

$$\frac{dN_u}{dt} = \frac{\alpha A}{1+\gamma_1 Q_1} N_d - \beta N_u , \tag{9}$$

$$\frac{dA}{dt} = \frac{\kappa_u}{1+\gamma_3 Q_3} N_u + \kappa_d N_T - \frac{\sigma A}{1+\gamma_1 Q_1} N_T - \lambda A - \mu_2 Q_2 A , \tag{10}$$

$$\frac{dQ_1}{dt} = \phi_1 - \frac{\mu_1 Q_1}{1+\gamma_1 Q_1} N_T - \lambda_1 Q_1 , \tag{11}$$

$$\frac{dQ_2}{dt} = \phi_2 - \mu_2 \nu_2 A Q_2 - \lambda_2 Q_2 , \tag{12}$$

$$\frac{dQ_3}{dt} = \phi_3 - \frac{\mu_3 Q_3}{1+\gamma_3 Q_3} N_u - \lambda_3 Q_3 , \tag{13}$$

where $N_T = N_u + N_d$. Equations 8–10 in the absence of any drugs are similar to those investigated by Ward et al. (2004), the main difference being that "$\sigma A N_T$" replaces "$A(\alpha N_d + \eta N_T)$" in Eq. 10 and in the absence of the negative feedback term $g(A)$ in Eq. 10. These modifications lead to an adjustment in the best-fit parameters (notably with α and β; see Table 1), but the main results from the mathematical

Table 1 The model parameters and estimated values used in the simulations to follow. The parameters relevant to batch cultures are given above the dividing line, and the additional parameters required for the biofilm are below this line. The values labeled "CS" (current study) are obtained in a semisystematic fashion using best-fit approximation to experimental data (Ward et al. 2004). (For reasons noted in this paper, the best-fit values quoted here are order-of-magnitude approximations and should not be considered as fundamental rate constants.) Figure 2a shows the best-fit solution. L = Lewandowski et al. (1991), S = Stewart (1994), W_1 = Ward et al. (2001), W_2 = Ward et al. (2004), E = estimated value, A = assumed value

Parameter	Units	Description	Value	Source
r	h^{-1}	Cell birth rate	0.5	W_2
α	$\mu M^{-1}\ h^{-1}$	Maximal upregulation rate	9×10^4	CS
β	h^{-1}	Downregulation rate	5.8×10^4	CS
κ_u	μM ml $cell^{-1}\ h^{-1}$	AHL prod. rate by upregulated cells	2.8×10^{-8}	CS
κ_d	μM ml $cell^{-1}\ h^{-1}$	AHL prod. rate by downregulated cells	1.7×10^{-14}	CS[a]
λ	h^{-1}	AHL decay rate	1.5	W_1[b]
σ	ml $h^{-1}\ cell^{-1}$	AHL loss rate by LasR/AHL binding	3.8×10^{-8}	CS
μ_1, μ_3	ml $h^{-1}\ cell^{-1}$	Drug loss rate due to QSI action	1	A
μ_2	$\mu M^{-1}\ h^{-1}$	Drug loss rate due to QSI action	6×10^5	A
γ_1,γ_3	μM^{-1}	1/conc. when QSI is 50% effective	1.7×10^{-6}	A
ν_1	Dimensionless	Mean Q_2 loss in reaction with AHL	1	A
φ_i	$\mu M\ h^{-1}$	Drip rate of QSI i	varied	–
λ_i	h^{-1}	Decay rate of QSI i	0.06	A[c]
W_0	Dimensionless	Void fraction at maximum bacterial packing	0.3	E
H_0	cm	Initial biofilm depth	0.0002	–
θ	Dimensionless	EPS-generated pore space constant	30	E
ω	Cell/ml biofilm	Maximum density of cells in biofilms	10^{12}	A
κ_E	h^{-1}	Max. EPS prod. rate by upregulated cells	1	A
E_0	h^{-1}	Background EPS production rate	10^{-4}	A
λ_E	h^{-1}	EPS decay rate	0	A[d]
D_a	$cm^2\ h^{-1}$	Diffusion rate of AHL	9×10^{-3}	E[e]
D_i	$cm^2\ h^{-1}$	Diffusion rate of species (Q_i)	9×10^{-3}	$= D_a$
D_c	$cm^2\ h^{-1}$	Diffusion rate of oxygen	9×10^{-2}	S
Q_a	cm h^{-1}	Surface AHL mass transfer rate	90	A[f]
B_1	h^{-1}	Maximum birth rate	0.1	S
B_2	h^{-1}	Maximum death rate	0.1	E
c_{ext}	μM	Dissolved O_2 concentration	2.3	L
c_1	μM	Half max. birth rate oxygen concentration	0.47	L
c_2	μM	Half max. death rate oxygen concentration	0.47	$= c_1$
τ	Dimensionless	Sets minimum death rate ($= B_2(1-\tau)$)	1	E
ρ	μM	Oxygen consumption constant	4×10^5	L

[a] too small to be estimated reliably; [b] measurement in bovine serum; [c] 10 hour half life; [d] assumed negligible in the timescale of interest; [e] assumed 1/10th that of oxygen; [f] biofilms are assumed to readily leak AHLs into the environment, hence a large Q_a/D_a is imposed

analysis described in the earlier work are entirely relevant to the current model. The parameters yet to be defined are the rate constants $\mu_1 = \nu_1 k_1$ and $\mu_3 = \nu_3 k_3$.

Figure 2a shows that the best-fit solutions of Eqs. 8–10 are in good agreement with experimentally measured AHL concentrations; the parameter values are listed in Table 1. The data were obtained from a batch culture experiment during the expo-

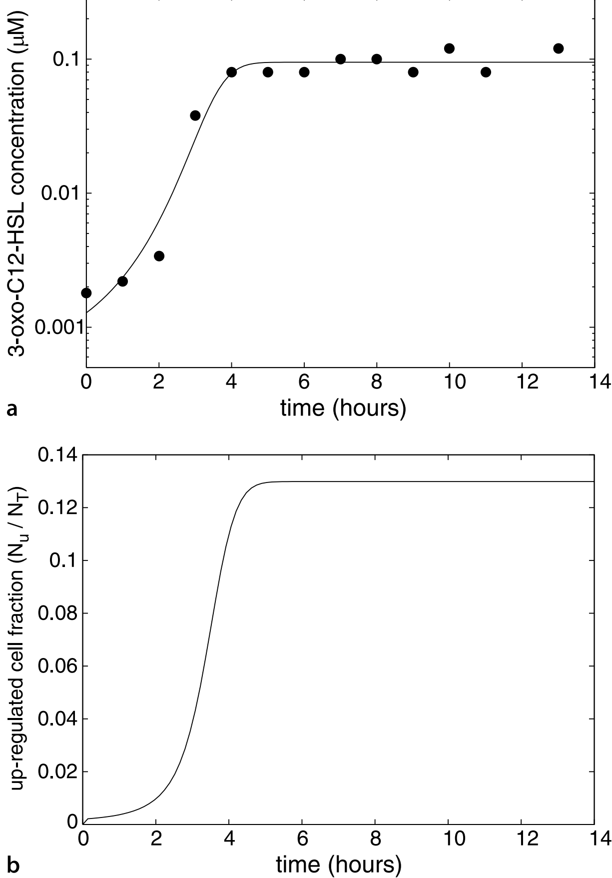


Fig. 2 Plot **a** shows the evolution of 3-oxo-C12-HSL concentration against time in an exponentially growing bacterial colony in a batch culture; the *solid line* is the solution of the model equations (8)–(10) using the parameters in Table 1, and "•" indicate the experimental values. Plot **b** depicts the fraction of upregulated cells for the same simulation

nential growing phase of *P. aeruginosa* strain PAB1 (a clinical burn wound isolate). Full details of the experimental work are given in Ward et al. (2001, 2004).

2.2 Anti-QS Treatment in Biofilms

To model biofilms it is necessary to consider bacterial cell distributions as functions of time t and space z, where z is the "perpendicular" distance from the base of the biofilm such that the biofilm surface is located at $z = H(t)$. Here, the bacterial subpopulations N_u and N_d are to be viewed as volume fractions, so that the volume

fraction of living cells is $N_u + N_d = N_T$. Through continued nutrient deprivation, cells die and occupy a volume fraction M; it is assumed that dead cells remain intact for the timescale of interest (a few days). The remaining space in the biofilm is occupied EPS (volume fraction E) and water (volume fraction W), hence $N_T + M + E + W = 1$. In the report by Anguige et al. (2006), it was further assumed that as EPS is produced, the resulting pore space increases proportionately, thus entraining more water, i.e., $W = W_0 + \theta E$ for constants W_0 and θ (in Anguige et al. 2006, α is used instead of θ). This leads to the condition

$$N_T + M + (1+\theta)E = 1 - W_0 , \tag{14}$$

where $1 - W_0$ is the maximum fraction of space that cells occupy. For example, if cells are spherical, then, according to Kepler's conjecture (or perhaps more familiarly, the "grocer's orange-stacking problem"), $W_0 \approx 0.26$ (Sloane 1998). Biofilm growth is assumed to be governed by the QS-regulated EPS production and nutrient concentration c (entering the biofilm by diffusion through the surface); although the relevant parameters regarding nutrients are based on oxygen, the modeling can be used to consider any nutrient. The local changes in volume by cell division, death, and EPS production cause movement within the biofilm; this process is called advection, and the speed of movement is described by velocity v. The growth rate of the biofilm surface is thus given by the surface value of v. The QS process is modeled in exactly the same way as that of the batch culture described above. The AHL, QSIs, and nutrient are assumed to be diffusible compounds, and all, apart from the anti-AHL agent Q_2, can enter and leave cells in such a way that the internal and external concentrations equilibrate rapidly (in a few minutes) compared to the biofilm growth timescale (hours). After a little simplification in the same manner as that described by Anguige et al. (2006), the system of equations is as follows:

$$\frac{\partial N_T}{\partial t} + \frac{\partial v N_T}{\partial z} = N_T(F_b(c) - F_d(c)) , \tag{15}$$

$$\frac{\partial M}{\partial t} + \frac{\partial v M}{\partial z} = N_T F_d(c) , \tag{16}$$

$$\frac{\partial N_u}{\partial t} + \frac{\partial v N_u}{\partial z} = \frac{\alpha A}{1+\gamma_1 Q_1} N_d - \beta N_u , \tag{17}$$

$$\frac{\partial E}{\partial t} + \frac{\partial v E}{\partial z} = (E_0 N_T + \kappa_E N_u) F_b(c) - \lambda_E E , \tag{18}$$

$$0 = D_a \frac{\partial^2 A}{\partial z^2} + \frac{\kappa_u^*}{1+\gamma_3 Q_3} N_u + \kappa_d^* N_T - \frac{\sigma^* A}{1+\gamma_1 Q_1} N_T - \lambda A - \mu_2 Q_2 A , \tag{19}$$

$$0 = D_1 \frac{\partial^2 Q_1}{\partial z^2} - \frac{\mu_1^* Q_1}{1+\gamma_1 Q_1} N_T - \lambda_1 Q_1 , \tag{20}$$

$$0 = D_2 \frac{\partial}{\partial z}\left(W \frac{\partial Q_2}{\partial z}\right) - \mu_2 \nu_2 A W Q_2 - \lambda_2 W Q_2 \,, \tag{21}$$

$$0 = D_3 \frac{\partial^2 Q_3}{\partial z^2} - \frac{\mu_3^* Q_3}{1+\gamma_3 Q_3} N_\mathrm{u} - \lambda_3 Q_3 \,, \tag{22}$$

$$0 = D_\mathrm{c} \frac{\partial^2 c}{\partial z^2} - \rho N_\mathrm{T} F_\mathrm{b}(c) \,, \tag{23}$$

$$\frac{\partial v}{\partial z} = \frac{1}{1-W_0}\left(N_\mathrm{T} F_\mathrm{b}(c) + (1+\theta)(E_0 N_\mathrm{T} + \kappa_\mathrm{E} N_\mathrm{u}) F_\mathrm{b}(c) - \lambda_\mathrm{E} E\right) \,, \tag{24}$$

$$\frac{\mathrm{d}H}{\mathrm{d}t} = v(H,t) \,. \tag{25}$$

Here, functions F_b and F_d are the birth and death rates of living cells, respectively, so the cell growth rate is simply the difference between the two functions in Eq. 15. Equation 16 states that living cells die at a rate $F_\mathrm{d} N_\mathrm{T}$, although M can be eliminated from the system using Eq. 14 since $M = 1 - W_0 - N_\mathrm{T} - (1+\theta)E$. The right-hand side of Eq. 17 is the same as Eq. 9, where the volume fraction of downregulated cells can be obtained from $N_\mathrm{d} = N_\mathrm{T} - N_\mathrm{u}$. Equation 18 states that the EPS is produced at a background level by all living cells (at rate $E_0 N_\mathrm{T} F_\mathrm{b}$), but the production rate is significantly enhanced by upregulated cells ($k_\mathrm{E} N_\mathrm{u} F_\mathrm{b}$). Equations 10–13 become Eqs. 19–22 to account for diffusion; for simplicity we have assumed the quasisteady (no time derivatives) form of these and the nutrient (Eq. 23) equations, due to as is generally the case, the fact that the chemical diffusion processes are rapid in comparison to growth. Because the anti-AHL concentration within the water is Q_2, the overall concentration is thus $W Q_2$ (recalling $W = W_0 + \theta E$), hence Eq. 21 results. The constants labeled with * are modified versions of the batch culture case due to the change of definition from cell density to cell fraction, so that $\kappa_\mathrm{u}^* = \omega \kappa_\mathrm{u}$, where ω is the maximum number of cells per millileter of biofilm (approximately 10^{12} cells/ml^{-1}); constants κ_d^*, σ^*, μ_1^* and μ_3^* are similarly redefined. Equation 24 is derived by summing Eqs. 15, 26, and $\theta \times$ Eq. 18 and applying Eq. 14. The last equation states that the speed of the biofilm surface moves at the same velocity as the cells on the surface. The functions $F_\mathrm{b}(c)$ and $F_\mathrm{d}(c)$ are bacterial cell birth and death rates, respectively, as functions of c; typically, the birth rate increases and the death rate decreases as the nutrient increases. In the simulations to follow, Michaelis-Menten (or monod)-type kinetics are used, namely

$$F_\mathrm{b}(c) = B_1 \frac{c}{c_1 + c} \,, \quad F_\mathrm{d}(c) = B_2 \left(1 - \tau \frac{c}{c_2 + c}\right) \,.$$

The rate of cell birth is assumed to reflect the amount of general activity a cell is undertaking; hence, EPS production and nutrient consumption are assumed to be proportional to $F_\mathrm{b}(c)$. Equations 15–25 require a set of initial boundary conditions to be fully specified. The simulations are assumed to start shortly after the first few bacteria have settled onto a substrate. There are no upregulated cells or EPS. The

following are imposed:

$$t = 0 \qquad N_{\mathrm{T}} = 1 - W_0 \,, \quad N_{\mathrm{u}} = 0 \,, \quad E = 0 \,, \quad H = H_0$$

$$z = 0 \qquad \frac{\partial A}{\partial z} = 0 \,, \quad \frac{\partial c}{\partial z} = 0 \,, \quad \frac{\partial Q_1}{\partial z} = 0 \,, \quad \frac{\partial Q_2}{\partial z} = 0 \,, \quad \frac{\partial Q_3}{\partial z} = 0 \,, \quad v = 0$$

$$z = H(t) \quad c = c_{\mathrm{ext}} \,, \quad D_{\mathrm{a}} \frac{\partial A}{\partial z} = -Q_{\mathrm{a}} A \,.$$

The conditions at $x = 0$ are no flux conditions; that is, no material is allowed to cross $x = 0$. The nutrient concentration is assumed to be of fixed concentration c_{ext} (oxygen dissolved in water at saturated levels is imposed), and the condition on AHL means that the flux of AHL out of the biofilm is proportional to the difference of internal A and external (assumed zero) concentrations.

We note that the model described here is one-dimensional, with growth occurring perpendicular to a solid surface. We therefore do not intend to describe the elaborate spatial structures (water channels, mushrooms) that are often observed in experimental studies (Hentzer et al. 2001). Describing these features requires a significant development of the modeling specifically, the fluid-dynamic aspects, which are beyond the scope of the current study. Furthermore, no mechanisms are included in the model that will lead to growth limitations, such as planktonic cell escape or sloughing of biofilm material due to the effect of shear forces on its surface from the fluid media. However, the current model should describe fairly well the first few days of development, during which time most experimental studies are undertaken.

In the next two sections, simulations of the models described above are presented to investigate the effect of the three types of QSIs on the QS process. In the absence of specific data, particularly with regard to drug kinetics, the simulations are aimed at presenting the key qualitative results that might be expected in experiments, along with their implications for effective treatment for biofilms. We will first consider the batch culture case and how QSI potency and dosage affect upregulation. For biofilms, this too will be considered together with the key issues of treatment timing and drug delivery via diffusion.

3 Anti-QS Therapies in Batch Cultures

Figure 2 shows a typical time evolution of AHL concentration and upregulated cell fraction ($N_{\mathrm{u}}^{\mathrm{frac}}$, given by $N_{\mathrm{u}}^{\mathrm{frac}} = N_{\mathrm{u}}/N_{\mathrm{T}}$) for an exponentially growing population in an untreated batch culture. Figure 2b demonstrates the key characteristic feature of QS. After an initial period in which very little upregulation occurs, there is very rapid upregulation (here, at around 3 h), whereby a maximally upregulated population is reached. It is worth noting that the concept of "critical AHL concentration" is not included in the model, and such a phenotypic shift is in fact due to the basic dynamics of the QS system at a molecular level. However, a fascinating prediction by the model is that only a portion of cells in an "upregulated" population will be

upregulated (here, about 12–13%) at any time. The model predicts this because there is a balance between AHL molecule production and upregulation rates against AHL loss by unused LasR-AHL complex formation and by spontaneous downregulation by upregulated cells.

The key mechanisms involved in upregulating bacteria in batch cultures are dependent on the growth phase. Using the mathematical methods described by Ward et al. (2001, 2004), it can be shown that to get substantial upregulation in a LuxR-LuxI homolog QS system, the parameter values must satisfy the following:

$$\text{exponential growth phase:} \quad \Theta = \alpha\kappa_\mathrm{u} - \sigma(\beta + r) > 0 \tag{26}$$

$$\text{stationary phase:} \quad \Psi = \alpha\kappa_\mathrm{u} K - \beta(\sigma K + \lambda) > 0\,, \tag{27}$$

where K is the total population density at the stationary phase. The parameters to the left of the "–" sign are concerned with the mechanisms that promote QS, while those to the right are inhibiting mechanisms. Thus, Θ and Ψ are formulations representing the balance between the positive and negative QS mechanisms for each of the two growth phases. Using the data in Table 1, we have $\Theta \approx 3 \times 10^{-3}$ and $\Psi \approx 3 \times 10^{6}$ (assuming a stationary phase density of 10^{10} cells/ml), both being positive, meaning that substantial upregulation is expected. Using the same mathematical methods, when $\Theta > 0$ and $\Psi > 0$, the fraction of upregulated cells $N_\mathrm{u}^\mathrm{frac}$ in a quorate population can be calculated:

$$\text{exponential growth phase:} \quad N_\mathrm{u}^\mathrm{frac} = 1 - \frac{\sigma(\beta + r)}{\alpha\kappa_\mathrm{u}}\,,$$

$$\text{stationary phase:} \quad N_\mathrm{u}^\mathrm{frac} = 1 - \frac{\beta(\sigma K + \lambda)}{\alpha\kappa_\mathrm{u} K}\,.$$

Given appropriate data, we can use these formulas to estimate how virulent a bacterial colony can be. Furthermore, they make explicit how targeting a parameter, using a putative QSI, will restrict QS and perhaps prevent expression of virulence characteristics.

In the next two subsections, solutions to the model equations are discussed for the cases in which the the QSI is administered by drip-feeding or by pretreatment of the growth media. The former case is perhaps more relevant to anti-QS treatment of biofilms discussed in Sect. 4, in which diffusion acts as a "feeding" mechanism to cells deep within the colony.

3.1 Drip-Feed Administration of QSI

Figure 3 shows the steady-state AHL concentration and upregulated cell fraction against population size K for an untreated culture 3a and for cultures drip-fed with QSIs 3b–d. Because very little data are currently available regarding the dynamics of the QSIs, the parameters were chosen so that the rapid jump in Fig. 3b and the

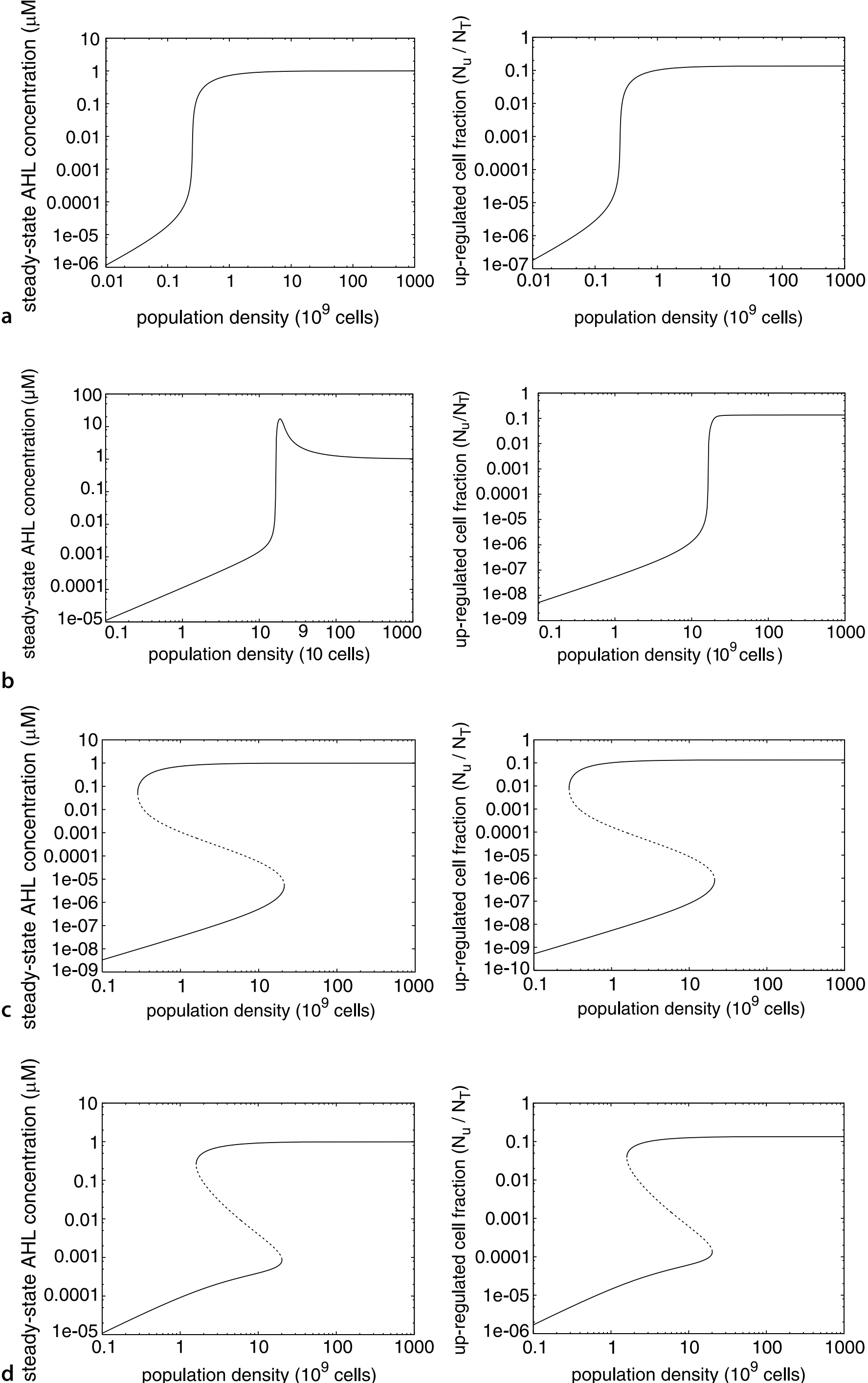
right-most fold in Fig. 3c and d occur at about the same population density (approximately 2×10^{10} cells/ml). In the absence of treatment, Fig. 3a shows that increasing K leads to a smooth increase in upregulated cell fraction, rising very sharply (at $K = K_{\text{jump}} \approx 2\times10^{8}$ cells/ml), beyond which a maximal level is reached (approximately 12–13% of cells). In contrast to the predictions of the modeling of Dockery and Keener (2000), the current model does not predict a hysteresis response; this is due to the assumption here that LasR production is not upregulated by QS (see Anguige et al. 2004).

Figure 3b–d demonstrates the range of results that can occur with the anti-AHL treatments. Anti-LasR agents lead to results that are qualitatively similar to those of the no-treatment case, while AHL and LasI blocking agents can lead to a hysteresis response. The rather unexpected hump around 10^{10} cells/ml in the left graph of Fig. 3b is due to there being less LasR present to soak up the AHLs, leading to greater accumulation; with increasing population, the anti-LasR agents become less effective, so AHL levels decrease to those of the no-treatment case. Two rather bold observations can be made from these figures with regard to effectiveness of QSI:

1. Direct comparison of the potency of the QSI can be meaningfully made only between the anti-LasR and anti-LasI, due to these having similar kinetic terms. Of particular interest, given that the rate constants are same, is that the drip-feed rate of the anti-LasI agent is 100 times less than that of the anti-LasR agent, suggesting that an anti-LasI agent will, by a considerable margin, be the more effective of the two treatments.
2. In practice, application of QSIs will be combined with antibiotics, and hysteresis is an unfavorable property for effective treatment. For the cases shown in Fig. 3c and d, an established quorate population will require more antibiotic drug to force the population density to levels below the left-hand folds in the curves. In this respect, anti-LasR treatment seems likely to be the most effective QSI.

Of course, these observations are based on a particular parameter set, and any conclusions must be treated with some caution. However, they highlight the need for experimental data to determine the parameters involved so that assertions such as these can be made with more confidence.

The evolution of upregulated cell fraction is shown in Fig. 4, in which the effects of each QSI can be compared with the untreated case. Here the population density is growing exponentially, starting with a density of 10^7 cells/ml. As expected, each treatment delays the onset of substantial upregulation, but in these simulations they will not prevent substantial upregulation as the exponentially growing population will eventually exceed the critical densities depicted in Fig. 3. However, as would be expected in practice, the limitations on attainable population density mean that sufficient drug will totally inhibit QS in batch cultures. The contrast in qualitative behaviors between the agents is interesting. For both the anti-LasR and anti-AHL cases, there is a rapid jump in cell fraction at about $t = 18$ h, roughly corresponding to the time when the population density reaches the critical level; the hump in the anti-LasR case is due to a brief overshoot in AHL concentration resulting from


steady-state AHL concentration (μM)
up-regulated cell fraction (N_u / N_T)
population density (10^9 cells)
population density (10 cells)
9
a
b
c
d
10
1
0.1
0.01
0.001
0.0001
1e-05
1e-06
1e-07
1e-08
1e-09
1e-10
100
1000

◀ **Fig. 3** Log-log plots showing the steady-state 3-oxo-C12-HSL concentration (*left*) and upregulated cell fraction (*right*) as functions of the population density for **a** untreated cultures, **b** with anti-LuxR agent (with $\varphi_1 = 3.3 \times 10^{-3}$ μM/h), **c** anti-AHL agent ($\varphi_2 = 5 \times 10^{-3}$ μM/h) and **d** anti-LuxI agent ($\varphi_3 = 3.3 \times 10^{-5}$ μM/h). Figures **c** and **d** exhibit hysteresis, the *solid* and *dashed curves* indicating the stable and unstable solutions respectively. The remaining parameters are given in Table 1

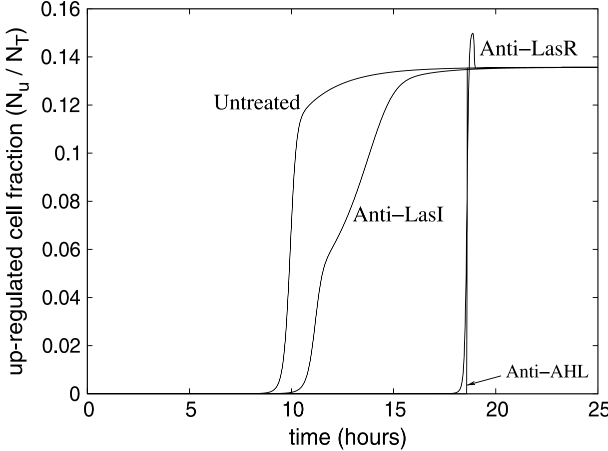


Fig. 4 Evolution of the upregulated cell fraction against time for exponentially growing bacterial colonies for untreated and drip-fed treated cultures (drip-feed rates were the same as those used in Fig. 3). In this simulation, the anti-AHL and anti-LasR cases are superimposed. The remaining parameters are given in Table 1

less available LasR at this point to soak up the AHLs. The gradual rise of upregulation in the anti-LasI case is a result of the relatively low drip-feed rate, leading to considerably less agent being present at the critical time when the population becomes quorate. However, this is very sensitive to the drip-feed rate; for example, doubling the rate to $\phi_3 = 6.6 \times 10^{-5}$ requires the population density to reach unphysical levels (approximately 2×10^{13}) before a jump to quorate levels can occur (not shown).

3.2 *Quorum Sensing in Pretreated Media*

Probably the simplest way to investigate the effects of QSIs on QS is to grow batch cultures in pretreated growth media. Figure 5 shows a simulation of such an experiment, with the initial agent concentration chosen so that the colony becomes quorate at around the same time (note that the drip-feed component of the model has been "switched off", i.e., $\phi_i = 0$). The time interval required to sufficiently consume the QSI as the population grows leads to a delay in the onset of extensive upregulation, as expected. The anti-LasI agent compares well against the anti-LasR agent in that

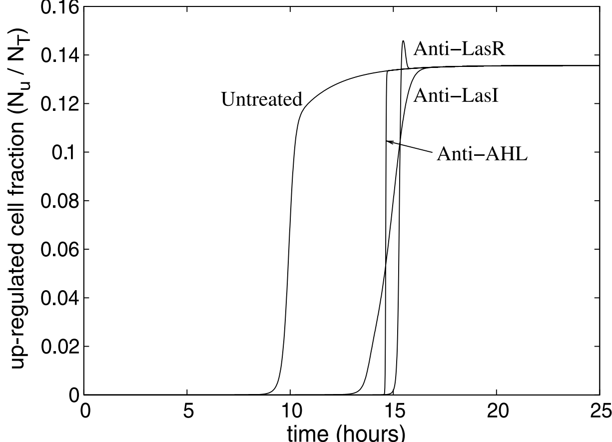


Fig. 5 Evolution of the upregulated cell fraction against time for exponentially growing bacterial colonies for an untreated culture and cultures grown in pretreated media. The initial concentrations of the anti-LasR, anti-AHL, and anti-LasI agents are $1.7 \times 10^{-2}\,\mu$M, $1.7 \times 10^{-2}\,\mu$M, and $5 \times 10^{-5}\,\mu$M, respectively. The remaining parameters are given in Table 1

significantly less agent was initially required to produce similar results (recall that the equivalent kinetic rate constants are the same for each).

Figure 6 shows the effects of the initial QSI concentration on the timescale for substantial upregulation. As expected, low concentrations of the agent have minimal impact, and the concentration needs to be above a certain threshold before the effects are noticeable. Interestingly, the curves for anti-LasR and anti-AHL are

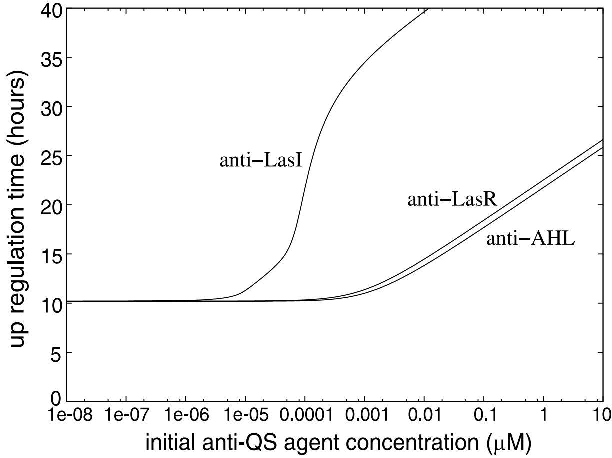


Fig. 6 The effects of the initial QSI concentration on the timescale for substantial upregulation (taken to be when $N_u/N_T = 10\%$) for batch culture colonies grown in pretreated media. The remaining parameters are given in Table 1

qualitatively similar; we note that the quantitative similarity is an accident of parameter choice. Noting that the horizontal scale is logged, the model predicts that beyond the concentration threshold of anti-LasR or anti-AHL agent, the timescale increases in a logarithmic fashion; this means that a substantial increase in agent is required to effect a noticeable delay in upregulation. Once again, the anti-LasI agent has apparently outperformed the other two agents by inducing considerable delays in upregulation at much lower concentrations. We note that in these simulations the population density is increasing exponentially and the stationary phase of growth has not been accounted for; if the agent succeeds in suppressing QS upregulation into the stationary phase, then the timescales shown in Fig. 6 will be pessimistic lower bound.

4 Anti-QS Therapies in Biofilms

Results from a typical simulation of growth of an untreated biofilm are shown in Figs. 7 and 8. The vast majority of experimental results discussed in the literature tend to be qualitative rather than quantitative in terms of biofilm growth, so accurate estimation of the parameters concerning growth is not possible. The parameters for the simulations were chosen so that the biofilm grows at a rate of approximately 6 μm/h, with a thickness of around 450 μm after 100 h, which seems reasonable given various experimental data. The model predicts that after an initial acceleration of growth, because of all bacterial cells being adequately nourished and undergoing cell division, the biofilm growth rate will slow down, eventually growing linearly in time without limitation (Fig. 7a,b). Of course, it is not possible for biofilms to grow indefinitely, but, as stated above, the aim is to have a good description of growth in the first few days of development. The linear growth phase results from nutrient diffusion limitations, in which only cells within a certain, eventually fixed, distance from the surface will have adequate nutrients to divide and produce EPS; cells deeper in the biofilm will be nonreproductive or dead. The assumption that EPS production is regulated by QS leads, as expected, to the significantly enhanced growth rate of the simulated wild-type over the QS-ve strain, in qualitative agreement with the experimental observations of Davies et al. (1998). This will have a significant effect on the delivery of the QSIs (discussed below) and, indeed, antibiotics (see Anguige et al. 2005, 2006). Plots of growth rate against time give a good indication of the timescale and extent of upregulation within the biofilms, and they will be used to illustrate the efficacy of the QSIs.

The advance toward high levels of QS activity is illustrated in Fig. 7c and d. For the first 26 h, AHL levels increase but remain relatively low [(see panels (a) and (b)]; thus, the growth rates between wild-type and QS-ve strains are indistinguishable. However, within a time interval of 1 h, there is a substantial rise in AHL levels (approximately 10000-fold increase), leading to rapid upregulation (see Fig. 6d) and divergence between the growth rates as the EPS production rate by wild-type cells is greatly enhanced. Here, the total upregulated cell fraction $U(t)$ is defined

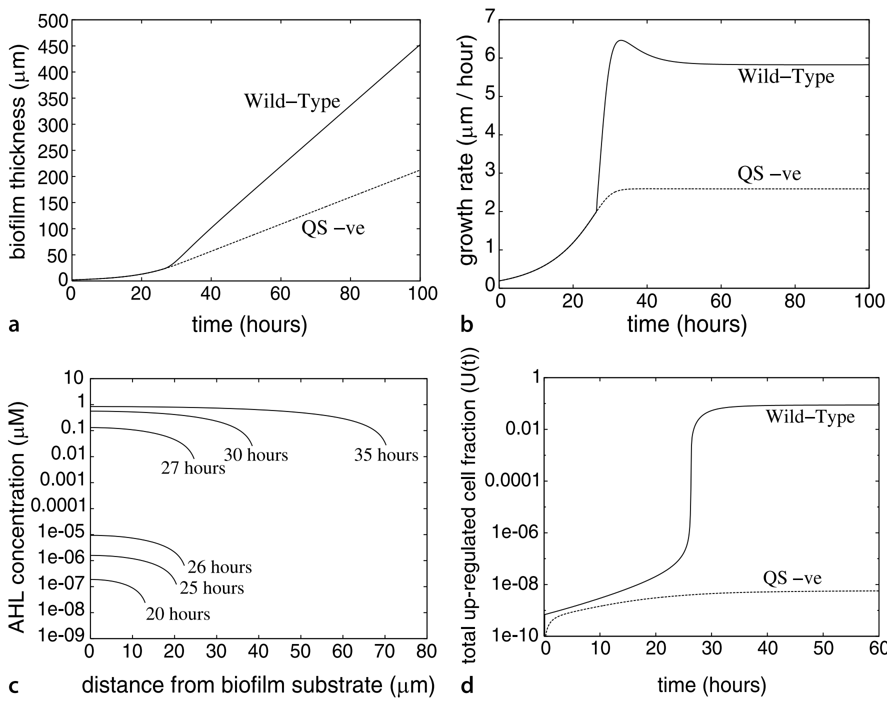


Fig. 7 Selected plots of simulated growth of an untreated biofilm. Comparison of the evolution of biofilm depth (**a**) and biofilm growth rate (**b**) for a wild-type and a QS-ve strain. Quorum sensing activity of the wild-type strain is demonstrated using AHL distribution at various time intervals up to 35 h (**c**) and the total upregulated cell fraction against time (**d**). Parameter values are given in Table 1

mathematically to be

$$U(t) = \frac{\int_0^{\mathrm{H}} N_{\mathrm{u}}(z,t)\mathrm{d}z}{\int_0^{\mathrm{H}} N_{\mathrm{T}}(z,t)\mathrm{d}z},$$

which can be viewed as the mean upregulated fraction divided by the mean live cell fraction over the entire the biofilm. Soon after, the spatial structure of the biofilm settles to the profiles indicated in Fig. 8, which move along as the biofilm grows. The overshoot in growth rate of the wild-type cells around $t = 30$ is due to the slow death rate of anoxic cells. In time, these cells die, reducing the depth at which EPS is produced and leading to a marginal slowing down of growth.

The distributions of living cells, upregulated cells, EPS, and water after 100 h are shown in Fig. 8c. The living cells are located in a region near the surface, corresponding to an area where there is a nonnegligible concentration of nutrients. The biofilm structure mainly consists of water (about 70%), and EPS occupies a volume percentage of 3%, which is in broad agreement with observations in fairly mature biofilms (Boyle et al. 1999). Because the dynamics are such that in optimum con-

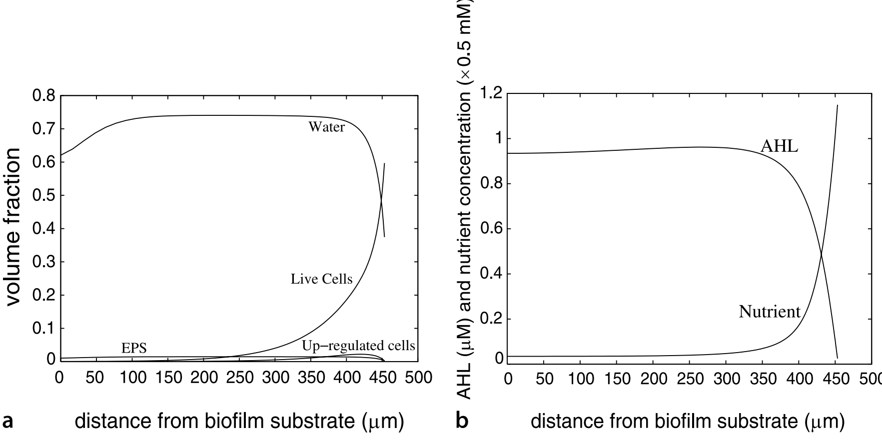


Fig. 8 Spatial distribution of live cell, EPS, water, and upregulated cell fractions (**a**) and AHL and nutrient concentration (**b**) at $t = 100$ h for an untreated wild-type biofilm. Parameter values are given in Table 1

ditions only 10% of cells will be upregulated, the upregulated cell fraction remains fairly low; however, this may still represent a population that is significantly virulent. The AHL distribution shown in Fig. 8b is elevated deep in the biofilm, dropping off sharply toward the edge because of the relatively high mass transfer rate compared with diffusion at the surface. The drop in live cell fraction moving deeper into the biofilm is paralleled by the drop in nutrient concentration (Fig. 8b), whereby much of the biofilm is predicted to be anoxic within a few days.

In the simulations to follow, the effectiveness of QSI will mostly be assessed by comparing the growth rate (i.e., $\mathrm{d}H/\mathrm{d}t$) of a treated biofilm with those of the wild-type and QS-ve strains, since EPS production and the enhanced growth it generates are markers for QS activity. The treatment will be presumed effective if the growth rate is reduced to that of the QS-ve strain, reflecting very low levels of QS activity, virulence, and EPS production.

4.1 Biofilm Growth in Media Pretreated with QSIs

Figure 9 shows the evolution of the growth rates and total upregulated cell fraction for biofilms grown in media containing a fixed concentration of QSI. Interestingly, for all treatments the shift in growth rates from the maximal to the minimal levels occur within an order of magnitude (on the μM scale) of drug concentration and is particularly sharp in the anti-AHL case.

Qualitatively, the results of the anti-LasR and anti-LasI agents are similar, there being a smooth transition down to the minimal growth rate as the drug concentration increases. For intermediate agent levels, the outer cells mostly remain downregu-

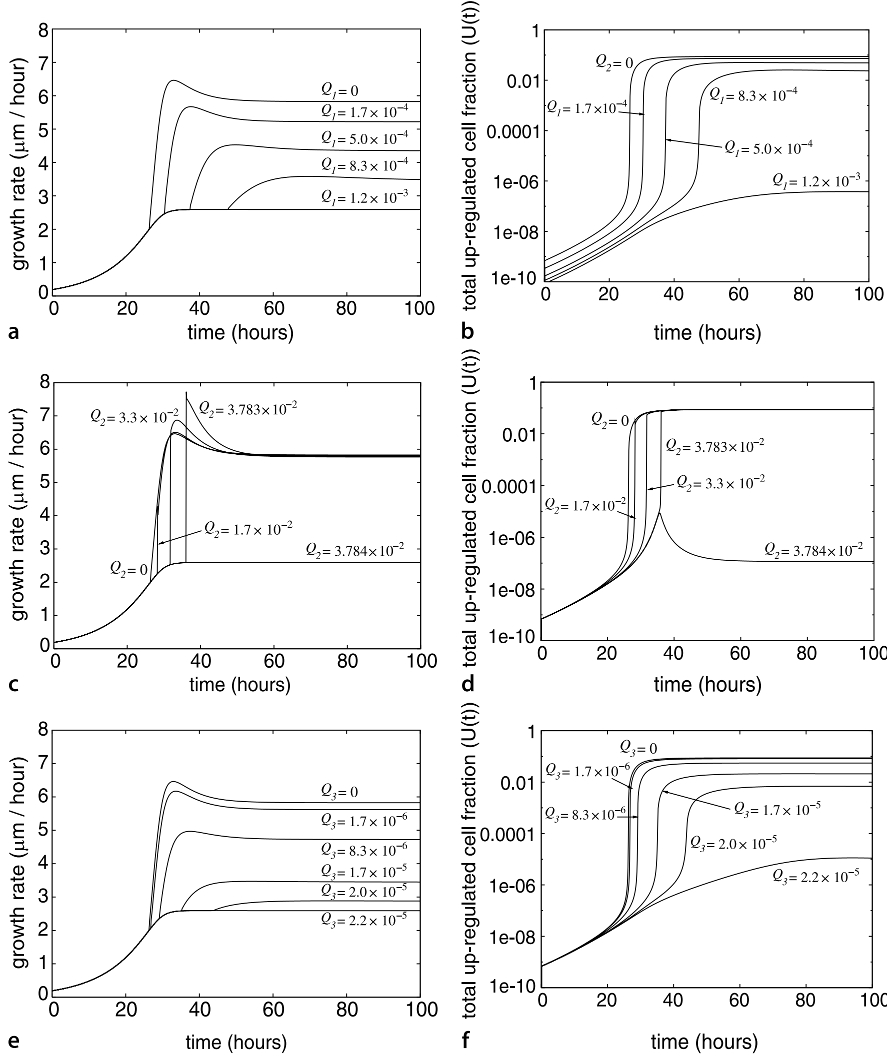


Fig. 9 The effects of applied concentrations (values in μM) of anti-LuxR (**a** and **b**), anti-AHL (**c** and **d**), and anti-LasI (**e** and **f**) agents on biofilm growth. Figures **a**, **c**, and **e** show the biofilm growth rate, and **b**, **d**, and **f** show the upregulated cell fraction (N_u/N_T) against time up to $t = 100$. Parameter values are given in Table 1

lated but prevent sufficient agent penetration to those cells deeper in the biofilm. As observed before, significantly less anti-LasI agent is required in the media to produce the desired effect. In terms of virulence (Fig. 9b,f), it can be observed that even when the agent has significantly reduced the speed of growth, the fraction of upregulated cells is considerably higher than the minimal levels (Fig. 7d), so a number of cells express virulent characteristics that could be significant in a clinical setting.

In contrast, increasing anti-AHL agent concentration makes little difference in long-term biofilm development until a threshold is reached (here, about 3.783×10^{-2} μM). The existence of the sharp transition, typical of systems with underlying hysteresis in the dynamics as described in Sect. 3, is due to the nature of the agent's target. Anti-AHL agents do not prevent production of AHLs, as these will always be produced at a background level and diffuse throughout the biofilm. The penetration of the anti-AHL agent, however, is limited by both diffusion and AHL reaction in the living cell region of the biofilm. Consequently, if insufficient anti-AHL agents reach the regions deep within the biofilm, the AHLs will accumulate there, forming a reservoir that will supply the living cells, eventually reaching a critical level that will induce mass upregulation of cells. The resulting AHL output will be sufficient to swamp the incoming anti-AHLs so that their effect will be negligible. We note that the concept of "critical concentration" of AHLs is not straightforward; the critical level of AHLs in biofilms will also depend on the bacterial growth rate, nutrient diffusion, and consumption rate, i.e., a host of parameters not directly related to QS dynamics. It is also clear from Fig. 9d that the anti-AHL agent must be above the threshold to have any effect on the eventual level of upregulation and, hence, on virulent behavior.

4.2 Growth Response to Delayed Application of QSIs

To investigate whether a more mature biofilm will have greater resistance to anti-AHLs than a nascent one, the biofilm was simulated to grow untreated for 36 h before the QSI was applied. Figure 10 illustrates the main results.

As before, for the anti-LasR and anti-LasI agents (Fig. 10a,c), the outcomes are similar; here, the concentrations used are exactly those that *just* brought about minimal growth in the pretreated media case. Both agents induced a rapid decline in growth, eventually settling to the minimal growth rate (the fact that the curves appear identical is coincidental). The sharp jump is due to the assumed fast dynamics of the biochemical processes. In the anti-LasR case (Fig. 10a), the agent will diffuse in rapidly, resulting in a near immediate shutdown of upregulation throughout, so the upregulated cells that spontaneously downregulated are not replaced. For the set of parameters used, the concentration $Q_1(H,t) = 1.2\times10^{-3}$ will always reduce growth to the minimal level. The results for the anti-LasI case (Fig. 10c) are surprising because it would be expected that when the agent is applied, the LasI present in the upregulated cells near the surface would, for a period, be sufficient to limit the agent's effect deeper into the biofilm. Although this is probably true in many situations, here the parameters are such that the processes of LasI removal, spontaneous downregulation of upregulated cells, and downturn in AHL production happen very rapidly, leading to the dramatic drop in biofilm growth rate. The results suggest that these drugs can be applied at any time and that the growth rate will be reduced accordingly. Of course, the biofilm with the delayed treatment has had a head start and will be thicker than a pretreated biofilm (Fig. 10d). This may be significant with

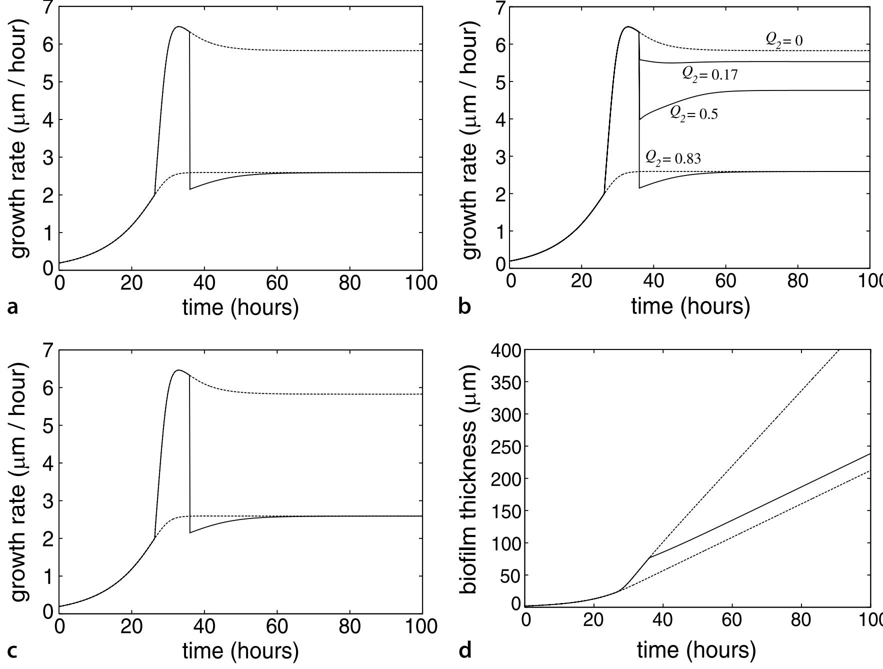


Fig. 10 The effects of a delayed application (36 h) of QSIs on the biofilm growth rate (Figs. **a**–**c**) and biofilm depth (Fig. **d**); the *dashed lines* are the corresponding curves for untreated wild-type and QS-ve bacteria (same as those shown in Fig. 7a and b). Figure **a** shows the effects of the anti-LasR agent ($Q_1(H,t) = 1.2 \times 10^{-3}$ μM), **b** anti-AHL (external concentrations as shown in μM) and **c** and **d** are the results for the anti-LasI agent ($Q_3(H,t) = 2.2 \times 10^{-5}$ μM). The *dashed curves* in **d** shows the growth rate of the wild-type (*upper*) and QS-ve strain (*lower*) and the treated biofilm is shown by the *solid line*. Parameter values are given in Table 1

regard to antibiotic penetration when a combined therapy is used; however, at the very least, the bacteria should not be virulent.

For the anti-AHL agent, a thicker biofilm has enormous implications (Fig. 10b). In these simulations, the concentration needed to induce a notable effect on the growth rate is over 10 times that for the pretreated case. This is to be expected, as after 36 h the level of upregulation is such that AHL production is at near-full capacity; consequently, there is ample AHL present to soak up the drug near the surface before it penetrates deep into the biofilm. Unlike the pretreated case, there is no longer a threshold concentration, the growth rate declining steadily as the agent increases.

4.3 Role of QSI Diffusion

One of the key issues in delivering a drug to a site of action involves its diffusive properties. Broadly speaking, larger molecules diffuse more slowly, so more of the

drug will need to be administered for it to reach the target areas. The effects of the QSI diffusion coefficient on biofilm growth rate and total upregulated cell fraction are shown in Fig. 11; only anti-LasR and anti-AHL simulations are shown because the anti-LasI produced results similar to the former agent. In the simulations up to now, the diffusion coefficients of AHL and QSIs have been assumed to be the same, which would be expected if the molecular masses are roughly the same (the molecular mass of 3-oxo-C12-HSL being 297).

Figure 11a and b shows the results of biofilm development in pretreated media containing an anti-LasR concentration of 1.7×10^{-3} μM, well within the range that would restrict biofilm growth to minimal levels given a drug diffusion of $D_1 = 9\times10^{-3}\,\mathrm{cm^2\,h^{-1}}$. As expected, the reduced drug penetration that results from decreasing the diffusion coefficient enhances biofilm growth and upregulation.

Interestingly, within the physical ranges of the diffusion coefficient D_2, the critical concentrations of anti-AHL agent in pretreated media varied very little (results not shown); for example, reducing the diffusion coefficient 100 times to

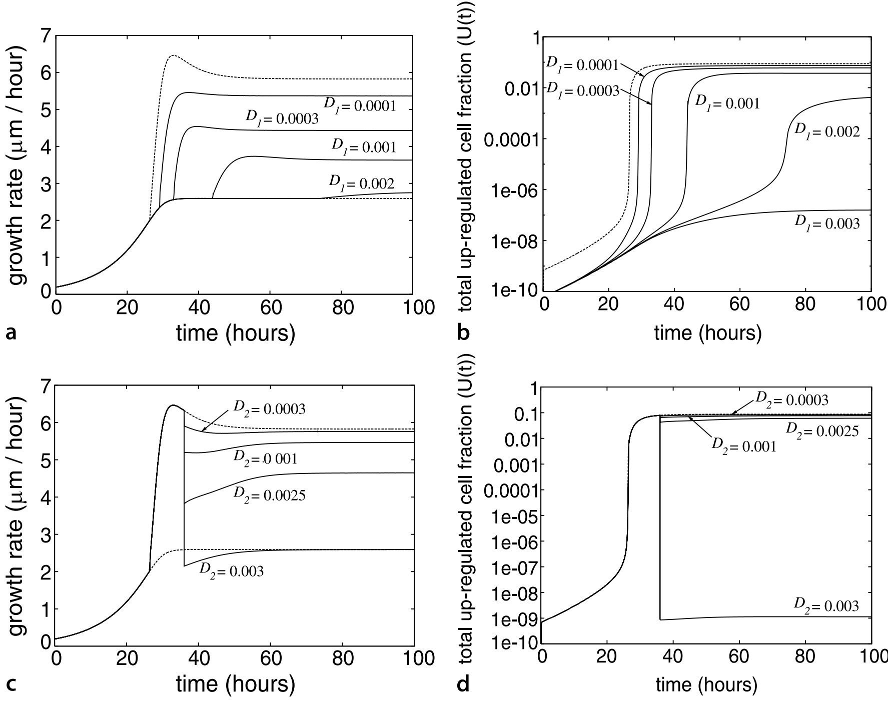


Fig. 11 The effects of the QSI diffusion coefficient (values shown have units cm^2 h^{-1}) on the evolution of the biofilm growth rate and upregulated cell fraction. Parts **a** and **b** show growth in media containing a fixed anti-LasR concentration $Q_1(H,t) = 1.7 \times 10^{-3}$ μM, and **c** and **d** shows the response to a 36-h delayed application of anti-AHL agent, at concentration $Q_2(H,t) = 1.7$ μM. The *dashed curves* are the results from the untreated wild-type and QS-mutant. Parameter values are given in Table 1

$D_2 = 9 \times 10^{-5}\,\mathrm{cm}^2\,\mathrm{h}^{-1}$ increases the critical concentration by less than 10%. This is mainly due to the anti-AHLs being present in the biofilm from the start of growth, so the transport properties of the agent are not crucial in preventing AHL accumulation. However, it is a different story when the agent is administered to a mature biofilm. Figure 11c and d shows the results of a very high anti-AHL dose (1.7 μM) administered at 36 h. Here, relatively small changes to the diffusion coefficient can have a significant effect on the outcome of biofilm development and in particular the level of upregulation that occurs (Fig. 11d). This again illustrates the underlying hysteresis in the dynamics of anti-AHL agents.

5 Concluding Remarks and Scope for Further Experimentation

The mathematical modeling described here and by Anguige et al. (2004, 2005, 2006) are the first attempts at modeling the effects of anti-QS therapies on bacteria growth, biofilm development, and virulence. These models incorporate biologically and physically relevant mechanisms and, in the absence of data, produce results that seem reasonable. Despite the current paucity of quantitative data regarding QSIs, through simulation and mathematical analysis a number of qualitative predictions and assertions can be made from the modeling:

1. In both batch cultures and biofilms, early application of a sufficient amount of QSI will delay or prevent the onset of mass upregulation. In a clinical setting, delaying QS and virulence is perhaps all that is needed for an agent to be useful, as this could buy sufficient time for the immune system to respond effectively and for an infected wound to heal normally.
2. During the exponential phases of growth in both batch cultures and biofilms, the amount of QSI required to suppress QS increases exponentially. This is illustrated for the anti-AHL and anti-LasR agents in Fig. 6 and has been shown (mathematically) by Anguige (2005) to be the case for biofilms. If dosing levels of an anti-AHL drug are an issue, then early application would be essential.
3. The dynamics of anti-AHL and anti-LasI agents have an underlying hysteresis. It is possible that once a population is upregulated, a considerable amount of agent will be needed to force downregulation. This again stresses the need for early application of QSIs, ideally prior to the onset of substantial upregulation. If hysteresis dynamics are observed experimentally for the anti-LasR agent, then it is likely that LasR is upregulated by QS (Anguige 2004); the models will need to be modified accordingly.
4. For biofilms, the range of concentrations of applied QSI that separate minimal and maximal QS suppression seems to be quite narrow, particularly in the anti-AHL case. Furthermore,when the growth rate is even slightly above the minimal level, the fraction of upregulated cells present is many times greater than that for a fully QS-suppressed colony; virulence expression in the biofilm could well be significant in such circumstances.

5. The simulations consistently suggest that the putative anti-LasI treatment seems to be the most potent one for suppressing QS. Of course, this is very much dependent on the parameter values, but its effectiveness is due largely to the fact that when few upregulated cells are present, there is very little LasI present to soak up the agent.
   Given the lack of relevant experimental data regarding anti-AHL and anti-LasR, it is difficult to make bold conclusions as to their relative merits based on results from the mathematical models. Typically, there will be considerably less LasR in the system than AHL, so less anti-LasR would be required. However, the anti-LasR must be able to get inside the cell, which may be a significant restriction in a practical setting.

Some of these qualitative predictions should be experimentally verifiable, the results of which would provide a means of validating the models and continuing the mathematical modeling cycle referred to in the introduction of this chapter.

To make quantitative, rather than qualitative, predictions and conclusions from the mathematical models, good quantitative experimental data are needed. Even with the addition of QSIs in the current model, there are only a handful of parameters to be fitted. Some of these parameters, such as the diffusion and decay rates of QSI, should be determinable from nonculture experiments. The remaining parameters can be obtained using data from batch cultures grown in media that are treated and untreated with QSIs; hourly measurements of population density, AHL concentration, and QSI concentration can be fitted to the model in the manner described by Ward et al. (2001). Such experiments will assist enormously in the development of the current mathematical model so that they can be more predictive and capable of offering further insights into the action of anti-QS treatments.

References

Anguige K, King JR, Ward JP, Williams P (2004) Mathematical modeling of therapies targeted at bacterial quorum sensing. Math Biosci 192:39–83

Anguige K, King JR, Ward JP, Williams P (2005) Modeling antibiotic- and anti-quorum sensing treatment of a *Pseudomonas aeruginosa* biofilm. J Math Biol 51:557–594

Anguige K, King JR, Ward JP (2006) A multi-phase mathematical model of quorum sensing in a maturing *Pseudomonas aeruginosa* biofilm. Math Biosci 203:240–276

Atkinson B, Davies IJ (1974a) The overall rate of substrate uptake (reaction) by microbial film. Part I – a biological rate equation. Trans Inst Chem Eng 52:260–268

Atkinson B, Davies IJ (1974b) The overall rate of substrate uptake (reaction) by microbial film. Part II – effect of concentration and thickness with mixed microbial films. Trans Inst Chem Eng 52:248–259

Bakke R, Trulear MG, Robinson JA, Characklis WG (1984) Activity of *Pseudomonas aeruginosa* in biofilms: steady state. Biotech and Bioeng 26:1418–1424

Boyle JD, Dodds I, Lappin-Scott H, Stoodley P (1999) Limits to growth and what keeps a biofilm finite. Anitmicrobial Agents Chemo 38:303–315

Chaudhry MAS, Beg SA (1998) A review on the mathematical modeling of biofilm processes: advances in fundamentals of biofilm modeling. Chem Eng Technol 21:701–710

Chopp DL, Kirisits MJ, Moran B, Parsek MR (2002) A mathematical model of quorum sensing in a growing bacterial biofilm. J Indust Microbiol Biotech 29:339–346

Chopp DL, Kirisits MJ, Moran B, Parsek MR (2003) The dependence of quorum sensing on the depth of a growing biofilm. Bull Math Biol 65:1053–1079

Cogan NG, Cortez R, Fauci L (2005) Modeling physiological resistance in bacterial biofilms. Bull Math Biol 67:831–853

Cogan NG, Keener JP (2004) The role of the biofilm matrix in structural development. Math Med Biol 21:147–166

Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton JW, Greenberg EP (1998) The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science 280:295–297

Dockery JD, Keener JP (2001) A mathematical model for quorum sensing in *Pseudomonas aeruginosa*. Bull Math Biol 63:95–116

Dockery J, Klapper I (2001) Finger formation in biofilms layers. SIAM J Appl Math 62:853–869

Dillon R, Fauci L, Fogelson A, Gaver D III (1996) Modeling biofilm processes using the immersed boundary method. J Comp Phys 129:57–73

Dong YH, Gusti AR, Zhang Q, Xu JL, Zhang LH (2002) Identification of quorum-quenching *N*-acyl homoserine lactonases from *Bacillus* species. Appl Environ Microbiol 68:1754–1759

Eberl HJ, Picioreanu C, Heijnen JJ, van Loosdrecht MCM (2000) A three-dimensional numerical study on the correlation of spatial structure, hydrodynamic conditions, and mass transfer and conversion in biofilms. Chem Eng Sci 55:6209–6222

Fagerlind MG, Nilsson P, Harlén M, Karlsson S, Rice SA, Kjelleberg S (2005) Modeling the effect of acylated homoserine lactone antagonists in *Pseudomonas aeruginosa*. Biosystems 80:201–213

Fagerlind MG, Rice SA, Nilsson P, Harlén M, James S, Charlton T, Kjelleberg S (2003) The Role of Regulators in the Expression of Quorum-Sensing Signals in *Pseudomonas aeruginosa*. J Mol Microbiol Biotechnol 6:88–100

Freter R, Brickner H, Fekete J, Vickerman M, Carey K (1983) Survival and implantation of *Escherichia coli* in the intestinal tract. Infect Immun 39:686–703

Fuqua C, Greenberg EP (2002) Listening in on bacteria: acyl-homosering lactone signalling. Mol Cell Biol 3:685–695

Gonpot P, Smith R, Richter A (2000) Diffusion limited biofilm growth. Mod Simul Mater Sci Eng 8:707–726

Goryachev AB, Toh DJ, Lee T (2006) Systems analysis of a quorum sensing network: design constraints imposed by the functional requirements, network topology and kinetic constants. Biosystems 83:178–187

Hentzer M, Teitzel GM, Balzer GJ, Heydorn A, Molin S, Givskov M, Parsek MR (2001) Alginate overproduction affects *Pseudomonas aeruginosa* biofilm structure and function. J Bacteriol 183:5395–5401

Hentzer M, Wu H, Andersen JB, Riedel KB, Rasmussen T, Bagge N, Kumar N, Schembri MA, Song Z, Kristoffersen P, Manefield M, Costerton JW, Molin S, Eberl L, Steinberg P, Kjelleberg S, Hoiby N, Givskov M (2003) Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. EMBO J 22:3803–3815

Hudson MC, Ramp WK, Nicholson NC, Williams AS, Nousiainen MT (1995) Internalisation of *Staphylococcus aureus* by cultured osteoblasts. Microb Pathog 19:409–419

James S, Nilsson P, James G, Kjelleberg S, Fagerstrøm T (2000) Luminescence control in the marine bacterium *Vibrio fischeri*: an analysis of the dynamics of *lux* regulation. J Mol Biol 296:1127–1137

Koerber AJ, King JR, Ward JP, Williams P, Croft JM, Sockett RE (2002) A mathematical model of partial-thickness burn-wound infection by *Pseudomonas aeruginosa*: quorum sensing and the build-up to invasion. Bull Math Biol 64:239–259

Koerber AJ, King JR, Williams P (2005) Deterministic and stochastic modeling of endosome escape by *Staphylococcus aureus*: “quorum” sensing by a sungle bacterium. J Math Biol 50:440–488

Kreft JU (2004) Biofilms promote altruism. Microbiology 150:2751–2760

Kreft JU, Booth G, Wimpenny JWT (1998) BacSim, a simulator for individual-based modeling of bacterial colony growth. Microbiology 144:3275–3287

Lee S, Park S, Lee J, Yum D, Koo B, Lee J-K (2002) Genes encoding the *N*-Acyl Homoserine Lactone-Degrading Enzyme Are Widespread in Many Subspecies of *Bacillus thuringiensis*. Appl Environ Microbiol 68:3919–3924

Lewandowski Z, Walser G, Characklis WG (1991) Reaction Kinetics in Biofilms. Biotech and Bioeng 38:877–882

Manefield M, De Nys R, Kumar N, Read R, Givskov M, Steinberg P, Kjelleberg S (1999) Evidence that halogenated furanones from *Dlisea pulchra* inhibit acylated homoserine lactone (AHL)-mediated gene expression by displacing the AHL signal from its receptor protein. Microbiology 145:283–291

Manefield M, Rasmussen TB, Hentzer M, Andersen JB, Steinberg P, Kjelleberg S, Givskov M (2002) Halogenated furanones inhibit quorum sensing through accelerated LuxR turnover. Microbiology 148:1119–1127

Nilsson P, Olofsson A, Fagerlind MG, Fagerstrøm T, Rice S, Kjelleberg S, Steinberg P (2001) Kinetics of the AHL regulatory system in a model biofilm system: how many bacteria constitute a "quorum". J Mol Biol 309:631–640

Noguera DR, Pizarro G, Stahl DA, Rittmann BE (1999) Simulation of multispecies biofilm development in three dimensions. Wat Sci Tech 39:123–130

Pearson JP, van Dalden C, Iglewski BH (1999) Active efflux and diffusion are involved in transport of *Pseudomonas aeruginosa* cell-to-cell signals. J Bacteriol 181:1203–1210

Picioreanu C, Kreft JU, van Loosdrecht MCM (2004) Particle-based multidimensional multispecies biofilm model. Appl Environ Microbiol 70:3024–3040

Picioreanu C, van Loosdrecht MCM, Heijnen JJ (1998a) A new combined differential-discrete cellular automaton approach for biofilm modeling: application for growth in gel beads. Biotech and Bioeng 57:718–731

Picioreanu C, van Loosdrecht MCM, Heijnen JJ (1998b) Mathematical modeling of biofilm structure with a hybrid differential-discrete cellular automaton approach. Biotech and Bioeng 58:101–116

Picioreanu C, van Loosdrecht MCM, Heijnen JJ (2000) Effect of diffusive and convective substrate transport on biofilm structure formation: a two-dimensional modeling study. Biotech and Bioeng 69:504–515

Pritchett LA, Dockery J (2001) Steady state solutions of a one-dimensional biofilm model. Math Comput Model 33:255–263

Rittmann BE, Manem JA (1992) Development and experimental evaluation of a steady-state, multispecies biofilm model. Biotech Bioeng 39:914–922

Sloane NJA (1998) Kepler's conjecture confirmed. Nature 395:435–436

Stewart PS (1994) Biofilm accumulation model that predicts antibiotic resistance of *Pseudomonas aeruginosa* biofilms. Anitmicrobial Agents Chemo 38:1052–1058

Szego S, Cinnella P, Cunningham AB (1993) Numerical simulation of biofilm growth in closed conduits. J Comp Phys 108:246–263

Tiwari SK, Bowers KL (2001) Modeling biofilm growth and porous media applications. Math Comput Model 33:299–319

Ulrich RL (2004) Quorum quenching:enzymatic disruption of *N*-acylhomoserine lactone-mediated bacterial communication in *Burkholderia thailandensis*. Appl Environ Microbiol 70:6173–6180

Viretta AU, Fusseneggar M (2004) Modeling the quorum sensing regulatory network of human-pathogenic *Pseudomonas aeruginosa*. Biotechnol Prog 20:670–678

Wanner O, Gujer W (1986) A Multispecies Biofilm Model. Biotech and Bioeng 28:314–328

Wanner O, Reichert P (1995) Mathematical modeling of mixed culture biofilms. Biotech and Bioeng 49:172–184

Ward JP, King JR, Koerber AJ, Croft JM, Sockett RE, Williams P (2003) Early development and quorum sensing in bacterial biofilms. J Math Biol 47:23–55

Ward JP, King JR, Koerber AJ, Williams P, Croft JM, Sockett RE (2004) Cell-signalling repression in bacterial quorum sensing. Math Med Biol 21:169–204

Ward JP, King JR, Koerber AJ, Williams P, Croft JM, Sockett RE (2001) Mathematical modeling of quorum sensing in bacteria. IMA J Math Appl Med Biol 18:263–292

Wimpenny JWT, Colasanti R (1997) A unifying hypothesis for the structure of microbial biofilms based on cellular automaton models. FEMS Microbiol Ecol 22:1–16

Xu F, Byun T, Dussen HJ, Duke KR (2003) Degradation of *N*-acylhomoserine lactones, the bacterial quorum-sensing molecules, by acylase. Biotechnology 101:89–96

Animal Models Commonly Used to Study Quorum-Sensing Inhibitors

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Andrea Giacometti[4], Oscar Cirioni[5]

Abstract Multiple animal models exist for the study of biofilm infections and their inhibitors in vivo. The infection models described in this chapter range from the simple nematode-killing and amoeba-plate-killing assays, to models with more relevance to human disease like the pulmonary and cellulitis infection models in mice, the graft prosthesis, and the central venous catheter infection models in rats, and the endocarditis and osteomyelitis infection models in rabbits.

Multiple animal models exist for the study of biofilm and the effect of quorum sensing inhibitors (QSIs) in vivo. Below are a few.

1 Nematode *Caenorhabditis Elegans*

One of the simplest infectious models is in the nematode *Caenorhabditis elegans*. These 1-mm-long worms feed on bacteria from its surroundings. In a laboratory setting they feed well, for example, on a nonpathogenic *E. coli* lawn on top of an agar plate. If the *E. coli* strain is replaced by a pathogenic bacterium such as *P. aerug-*

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N. Balaban (ed.) *Control of Biofilm Infections by Signal Manipulation*
Springer Series on Biofilms 2
doi: 10.1007/978-3-540-73853-4, © Springer 2008

inosa or *S. aureus*, the worms are killed by virulence factors regulated by quorum sensing (QS). *P. aeruginosa* QS-deficient mutant, for instance, kills only 10% of the worms as compared to 100% when the worms feed on the wild-type strain. This indicates that QS is indeed important for the infectious process in *C. elegans* (Rasmussen et al. 2005a; Darby et al. 1999; Mahajan-Miklos et al. 1999; Tan et al. 1999; Sifri et al. 2003; Bae et al. 2004).

1.1 Nematode Killing Assay

Bacteria are grown overnight at 37 °C in tryptone soya (TS) broth supplemented with selective antibiotics as needed. A 1:10 dilution of the saturated culture is made in culture broth, and 10 μl of the diluted culture is spread on 3.5-cm-diameter plates containing culture broth agar supplemented with 5 μg of nalidixic acid/ml. The plates are incubated at 37 °C for 4–6 h and are then allowed to equilibrate to room temperature for 30–60 min before being seeded with worms. Next, 30–40 L4-stage nematodes are added per plate (in triplicate). The plates are incubated at 25 °C and scored for live and dead worms at least every 24 h. A worm is considered dead when it fails to respond to plate tapping or gentle touch with a platinum wire (Sifri et al. 2003).

2 Amoeba *Dicyostelium Discoideum*

The haploid social soil amoeba *Dictyostelium discoideum* has been established as a host model for several pathogens, including *P. aeruginosa*, *Cryptococcus neoformans*, *Mycobacterium* spp., and *Legionella pneumophila*. A functional RhlIR QS system has been found to be important for establishing *P. aeruginosa* infections in the amoeba, where RhlR controls the production of rhamnolipid, which lyses the amoeba (Cosson et al. 2002; Pukatzki et al. 2002; Steinert and Heumer 2005).

2.1 Plate Killing Assay

Bacteria are grown in culture broth for 16 h, pelleted by centrifugation, washed once, and resuspended in SorC (16.7 mM Na_2H/KH_2PO_4/50 μM $CaCl_2$, pH 6.0) at a final optical density of 5.5 at 600 nm. *D. discoideum* cells from midlogarithmic cultures are collected by centrifugation ($1000\times g$; 4 min), washed once with SorC, and added to the bacterial suspensions at a final concentration of 5×10^2 cells/ml suspension; 0.2 ml of this mixture is plated out on SM/5 plates and allowed to dry under a sterile flow of air. Plates are incubated for 3–5 days and examined for plaques formed by *Dictyostelium* amoebae (Pukatzki et al. 2002).

3 Mouse Pulmonary Infection Model[2,3]

A model with more relevance for humans is the pulmonary infection model in mice. The initial stages of a chronic lung infection can be mimicked by casting *P. aeruginosa* into seaweed alginate beads and surgically installing them through the trachea into the mouse lung. Under normal circumstances, the activity of the mucociliary escalator clears the lungs of foreign particulate matter such as dust and bacteria. The alginate beads partly impair the function of the escalator, and neutrophils are then recruited to the sites of infection (Pedersen et al. 1990). For a short period of time, this is reminiscent of the situation in the cystic fibrosis lung. When the mice are infected with a QS mutant, both mortality of the mice as well as horizontal spread and dissemination are significantly reduced compared to the situation with the wild type (Rumbaugh et al. 1999; Bjarnsholt et al. 2005a). If rodents are infected with a QS-deficient mutant, the immune response is faster, the polymorphonuclear leukocytes (PMNs) respond with a stronger oxidative burst, and antibodies accumulate faster in the infected lungs (Smith et al. 2002a; Wu et al. 2001). Recent research points to the fact that the wild type *P. aeruginosa* contains a QS-controlled defense system that suppresses the powerful cellular immune response by paralyzing the PMNs (Bjarnsholt et al. 2005a). For more details, see section 1 in the chapter *In Vivo Studies: Inhibiting Biofilm-Associated Bacterial Infections Using QSIs*

4 Mouse Cellulitis Model

This model is based on the fact that staphylococci have an affinity to dextran beads with positive-charged DEAE groups throughout matrix (Cytodex microcar-

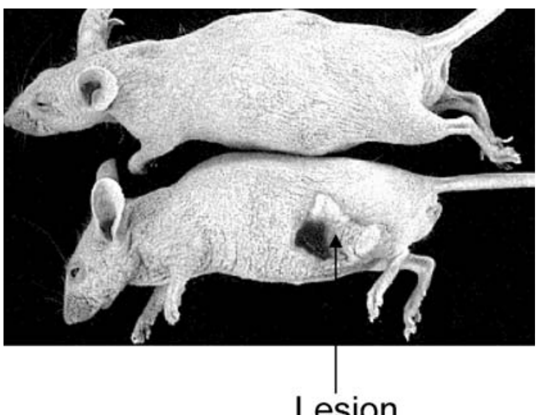

Fig. 1 Mouse cellulitis/abscess model. Mice (outbred, immunocompetent hairless male mice, strain Crl : SKH1(hrhr)Br], ($n = 10$) were injected subcutaneously with *S. aureus* and Cytodex together with the quorum-sensing inhibitor RIP (*top*) or without RIP (*bottom*). The lesion can be measured after 2 days

rier beads, GE Healthcare). When the bacteria are mixed with the beads and injected subcutaneously, a biofilm is formed, leading to a lesion or abscess that can be measured (Fig. 1). Such infections are highly dependent on the ability of the bacteria to produce toxins, and because toxin production is regulated by QS, this model is very useful in testing QSIs (Balaban et al. 1998, 2000; Gov et al. 2004).

5 Rat Graft Prosthesis Model[4,5]

The rat graft model allows for the direct quantification of bacteria on the graft itself. In the case of staphylococci, a biofilm is formed by the second day after bacterial challenge, and the graft can be removed up to 7 days after that for quantification of the bacteria on the graft. This model allows testing of compounds aimed at preventing an infection in addition to testing compounds aimed at treating an infection once a biofilm is formed.

In this model (Fig. 2), sterile collagen-sealed double velour knitted polyethylene terephthalate (Dacron) grafts are utilized as medical devices. Adult male Wistar rats ($n = 5–15$) are used, and experimental groups include control groups (no graft contamination), contaminated groups that do not receive any antibiotic or QSI prophylaxis, and treated groups that receive QSI alone or combined with other antimicrobial agents. Rats are anesthetized, the hair on the back is shaved, and the skin

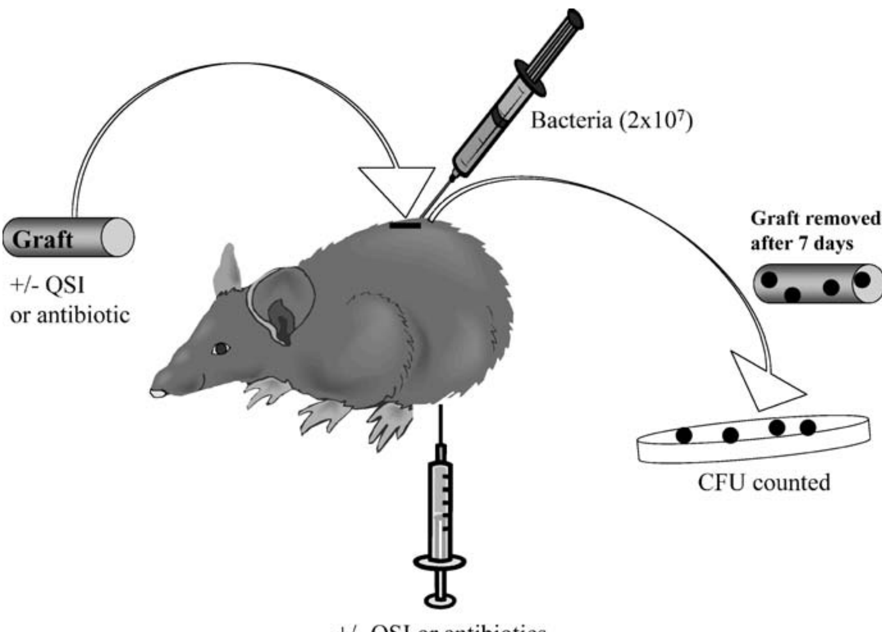

Fig. 2 Diagram of the rat graft model

is cleansed with 10% povidone-iodine solution. Subcutaneous pockets are made on each side of the median line by a 1.5-cm incision. Aseptically, 1-cm^2 sterile Dacron grafts are implanted into the pockets. Before implantation, the Dacron graft segments are impregnated (or soaked) with different concentrations of each compound. The pockets are closed by skin clips, and a physiological solution (1 ml) containing the bacteria (e. g., staphylococcal strains at a concentration of 2×10^7 CFU/ml; Balaban et al. 2005) is inoculated onto the graft surface using a tuberculin syringe to create a subcutaneous fluid-filled pocket. The animals are returned to individual cages and thoroughly examined daily. All grafts are explanted at 7–10 days following implantation. The explanted grafts are placed in sterile tubes, washed in sterile saline solution, and placed in tubes containing 10 ml of phosphate-buffered saline and sonicated (Fisher Scientific 300) at 20 000 Hz for 5 min to remove adherent bacteria. Quantitation of viable bacteria is obtained by performing serial dilutions and culturing each dilution on blood agar plates. The organisms are quantitated by counting the number of colony-forming units (CFUs) per plate. In the case of staphylococci, a biofilm will have already formed by the second day. Therefore, soaking the graft in QSI before bacterial challenge allows for testing QSIs for prevention of infection, while injecting QSI intraperitoneally for 2 days postimplantation, for example, allows for testing the reagent as a therapeutic (Balaban et al. 2007).

6 Central Venous Catheter-associated Infection Rat Model[1,4,5]

This model is very relevant clinically because more than 2 500 000 central venous catheters (CVCs) are annually implanted in the United States alone (statistics obtained from the U.S. National Center for Health Statistics, 2004), and 5% of them are associated with infectious complications leading to morbidity and mortality. The CVC may become colonized by two main routes: from the skin along the outside of the catheter or via the catheter lumen (Raad 1998; Donlan et al. 2002; Atela et al. 1997). Initial colonization is followed by development of a biofilm structure, usually developing within 3 days of catheterization (Raad 1998; Hall-Stoodly et al. 2004; Costerton et al. 1999). This model was used to evaluate the efficacy of the staphylococcal QSI RIP in preventing bacterial contamination of the CVC (Cirioni et al. 2006).

As in the graft model, these studies include control groups (no CVC infection), contaminated groups that do not receive any antibiotic or QSI prophylaxis, and contaminated groups that receive QSI-treated CVC and several schemes of antibiotic lock technique therapy ($n = 12$). Animals are anesthetized. Silastic catheters are inserted into the jugular vein and advanced into the superior vena cava. The proximal portions of the catheters are tunnelled subcutaneously to exit in the midscapular space. The catheters are then flushed with a heparin solution. A rodent restraint jacket is used to protect the catheters and to allow access to them. Twenty-four hours after CVC placement, blood cultures are obtained from the catheters to verify sterility. When established (24 h after implantation), the catheters

are filled with QSI. After 30 min the rats are challenged via the CVC, such as with 1.0×10^6 CFU staphylococci in a volume of 0.1 ml sterile saline. Several animals are sacrificed 24 h after bacterial challenge to verify, by quantitative cultures, the presence of infection. At the same time, the antibiotic lock technique can be initiated for additional delivery of QSIs or antibiotics. The drugs are allowed to dwell for 1 h, and the catheters are then flushed with a heparin solution.

Quantitative peripheral blood cultures and quantitative cultures of the catheters and surrounding venous tissues can be performed as follows: On day 9 the animals are sacrificed. For quantitative peripheral blood cultures, peripheral blood is obtained by aseptic percutaneous transthoracic cardiac puncture and cultured on sheep-blood agar plates. Plates are incubated at 37 °C for 24–48 h, and the number of CFUs per plate is determined. The isolates are identified by morphological and cultural characteristics, gram stain, etc. For quantitative cultures of the catheters and surrounding venous tissues, the location of the distal tip of the CVC in the superior vena cava is confirmed visually, and the catheters and surrounding venous tissue are removed aseptically. The explanted catheters/venous tissues are placed in tubes containing 50 ml of saline solution and are sonicated (Fisher Scientific 300 at 20000 Hz for 5 min) to remove adherent bacteria. The solution is then cultured by performing serial dilutions (0.1 ml) of the bacterial suspension and by culturing each dilution and determining CFU.

7 Rabbit Endocarditis Model

Another clinically relevant model is the rabbit endocarditis model (Garrison and Friedman 1970). Bacterial endocarditis is a lethal infection that requires the administration of high levels of bactericidal antibiotics for prolonged periods of time for cure. A significant percentage of patients with endocarditis fail therapy or suffer relapse, either because resistance develops or because not all of the infection was cleared (Oramas-Shirey et al. 2001). Native valve acute endocarditis usually has an aggressive course and is often caused by *S. aureus* or group B streptococci. Alpha-hemolytic streptococci or enterococci often cause subacute endocarditis that usually has a more indolent course. Staphylococci, gram-negative bacilli, and *Candida* species often cause early prosthetic valve endocarditis, which occurs within 60 days of valve implantation. Late prosthetic valve endocarditis occurs 60 days or more after valve implantation and is often caused by alpha-hemolytic streptococci, enterococci, or staphylococci. *S. aureus* is the most common cause of endocarditis related to intravenous drug use, which commonly involves the tricuspid valve (Baddour et al. 2005).

As reported by Oramas-Shirey et al. (2001), experiments are performed on male, specific pathogen-free (SPF) New Zealand White rabbits weighing-2.0–2.5 kg ($n = 5$–15). With the use of sterile surgical technique under anesthesia, a polyethylene catheter (PE-50) is inserted into the right carotid artery and advanced

across the aortic valve into the left ventricle. The catheter is sutured in place for the duration of the study. Of note is that only data from animals with correct catheter placement upon autopsy are included. Rabbits are infected 24 h after surgery, such as with 3.5×10^6 CFU of *S. aureus* with or without QSI and/or antibiotics. Forty-eight hours after infection, control rabbits are sacrificed, and treatment with QSIs and/or antibiotics is given. Rabbits are sacrificed 8–12 h after the final dose of material in question. Aortic valve vegetations, blood, and ventricular myocardium are removed and homogenized, and quantitative bacterial counts are determined by serial dilution and expressed as CFU/g of tissue (Oramas-Shirey et al. 2001).

8 Rabbit Osteomyelitis Model

Direct observations of the surfaces of orthopedic prostheses that have failed and of bone affected by osteomyelitis with and without the presence of prosthesis have shown that the bacteria that cause these infections live in well-developed biofilms (Costerton 2005). While the following model of osteomyelitis (Brady et al. 2006; Mader 1985) requires a large organism inoculation, it produces clinical manifestations like those seen in cases of human chronic osteomyelitis, including disruption of the normal bone architecture and periosteal elevation. Also produced is the hallmark of chronic osteomyelitis, the involucrum, which is live, encasing bone that surrounds infected dead bone within a compromised soft tissue envelope (Pesanti and Lorenzo 1998; Ehrlich et al. 2004). In addition, the recalcitrance to clearance by antimicrobial agents and the host immune system that is mediated by a biofilm mode of growth is evident after 28 days of infection (Brady et al. 2006).

In this model, 8-week-old New Zealand White female rabbits ($n = 3$) are used. Rabbits are anesthetized, and an 18-gauge needle is inserted percutaneously through the lateral aspect of the left tibial metaphysis into the intramedullary cavity. Sodium

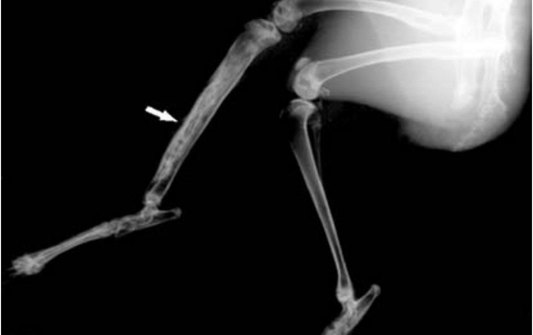

Fig. 3 Rabbit osteomyelitis model. A localized *S. aureus* osteomyelitis was surgically induced in the rabbit left lateral tibial metaphysis into the intramedullary cavity (*arrow*). Infection was allowed to progress, and the severity of osteomyelitis was determined radiographically. (Image kindly provided by Dr. Mark Shirtliff.)

morrhuate solution; 0.1 ml of *S. aureus* (10^6 CFU), for example; and sterile saline are injected sequentially. The needle is removed and the rabbits returned to their cages. The infection is allowed to progress for 42 days, with sera being drawn at days 0, 14, 28, and 42. QSI inhibitors and/or antibiotics can be administered together with the bacteria or by systemic administration at that time or after challenge. The severity of osteomyelitis can be determined radiographically at 10, 20, and 30 days following infection (Balaban et al. 2000; Fig. 3). At the conclusion of the study, rabbits are sacrificed, and both tibias are removed (one to be used as a pathogen-free control), dissected free of all soft tissue, and processed for bacterial cultures (Brady et al. 2006).

References

Atela I, Coll P, Rello J, Quintana E, Barrio J, March F, Sanchez F, Barraquer P, Ballus J, Cotura A, Prats G (1997) Serial surveillance cultures of skin and catheter hub specimens from critically ill patients with central venous catheters: molecular epidemiology of infection and implications for clinical management and research. J Clin Microbiol 35:1784–1790

Baddour LM, Wilson WR, Bayer AS (2005) Infective endocarditis: diagnosis, antimicrobial therapy, and management of complications – executive summary. Circulation 111:3167–3184

Bae T, Banger AK, Wallace A, Glass EM, Aslund F, Schneewind O, Missiakas DM (2004) *Staphylococcus aureus* virulence genes identified by bursa aurealis mutagenesis and nematode killing. Proc Natl Acad Sci USA 101:12312–12317

Balaban N, Goldkorn T, Nhan RT, Dang LB, Scott S, Ridgley RM, Rasooly A, Wright SC, Larrick JW, Rasooly R, Carlson JR (1998) Autoinducer of virulence as a target for vaccine and therapy against *Staphylococcus aureus*. Science 280:438–440

Balaban N, Collins LV, Cullor JS, Hume EB, Medina-Acosta E, Vieira-da-Motta O, O'Callaghan R, Rossitto PV, Shirtliff ME, Serafim da Silveira L, Tarkowski A, Torres JV (2000) Prevention of diseases caused by *Staphylococcus aureus* using the peptide RIP. Peptides 21:1301–1311

Balaban N, Stoodley P, Fux CA, Wilson S, Costerton JW, Dell'Acqua G (2005) Prevention of staphylococcal biofilm-associated infections by the quorum sensing inhibitor RIP. Clin Orthop Relat Res 437:48–54

Balaban N, Cirioni O, Giacometti A, Ghiselli R, Braunstein J, Silvestri C, Mocchegiani F, Saba V, Scalise G (2007) Treatment of *Staphylococcus aureus* biofilm infection by the quorum sensing inhibitor RIP. Antimicrob Agents Chemother 51:2226–2229

Bjarnsholt T, Jensen PO, Burmolle M, Hentzer M, Haagensen JA, Hougen HP, Calum H, Madsen KG, Moser C, Molin S, Hoiby N, Givskov M (2005a) *Pseudomonas aeruginosa* tolerance to tobramycin, hydrogen peroxide and polymorphonuclear leukocytes is quorum-sensing dependent. Microbiology 151:373–383

Brady RA, Leid JG, Camper AK, Costerton JW, Shirtliff ME (2006) Identification of *Staphylococcus aureus* proteins recognized by the antibody-mediated immune response to a biofilm infection. Infect Immun 74:3415–3426

Cirioni O, Giacometti A, Ghiselli R, Dell'acqua G, Orlando F, Mocchegiani F, Silvestri C, Licci A, Saba V, Scalise G, Balaban N (2006) RNAIII-inhibiting peptide significantly reduces bacterial load and enhances the effect of antibiotics in the treatment of central venous catheter-associated *Staphylococcus aureus* infections. J Infect Dis 193:180–186

Cosson P, Zulianello L, Join-Lambert O, Faurisson F, Gebbie L, Benghezal M, Van Delden C, Curty LK, Kohler T (2002) *Pseudomonas aeruginosa* virulence analyzed in a *Dictyostelium discoideum* host system. J Bacteriol 184:3027–3033

Costerton JW (2005) Biofilm theory can guide the treatment of device-related orthopaedic infections. Clin Orthop Relat Res 437:7–11

Costerton JW, Stewart PS, Greenberg EP (1999) Bacterial biofilms: a common cause of persistent infections. Science 284:1318–1322

Darby C, Cosma CL, Thomas JH, Manoil C (1999) Lethal paralysis of *Caenorhabditis elegans* by *Pseudomonas aeruginosa*. Proc Natl Acad Sci USA 96:15202–15207

Donlan RM (2002) Biofilms: microbial life on surfaces. Emerg Infect Dis 8:881–890

Ehrlich GD, Hu FE, Lin Q, Costerton JW, Post JC (2004) Intelligent implants to battle biofilms. ASM News 70:127–133

Garrison PK, Freedman LR (1970) Experimental endocarditis 1. Staphylococcal endocarditis in rabbits resulting from placement of a polyethylene catheter in the right side of the heart. Yale J Biol Med 42:394–410

Gov Y, Borovok I, Korem M, Singh VK, Jayaswal RK, Wilkinson BJ, Rich SM, Balaban N (2004) Quorum sensing in Staphylococci is regulated via phosphorylation of three conserved histidine residues. J Biol Chem 279:14665–14672

Hall-Stoodley L, Costerton JW, Stoodley P (2004) Bacterial biofilms: from the natural environment to infectious diseases. Nat Rev Microbiol 2:95–108

Mader JT (1985) Animal models of osteomyelitis. Am J Med 78:213–217

Mahajan-Miklos S, Tan MW, Rahme LG, Ausubel FM (1999) Molecular mechanisms of bacterial virulence elucidated using a *Pseudomonas aeruginosa–Caenorhabditis elegans* pathogenesis model. Cell 96:47–56

Oramas-Shirey MP, Buchanan LV, Dileto-Fang CL, Dailey CF, Ford CW, Batts DH, Gibson JK (2001) Efficacy of linezolid in a staphylococcal endocarditis rabbit model. J Antimicrob Chemother 47:349–352

Pedersen SS, Shand GH, Hansen BL, Hansen GN (1990) Induction of experimental chronic Pseudomonas aeruginosa lung infection with *P. aeruginosa* entrapped in alginate microspheres. APMI 98:203–211

Pesanti EL, Lorenzo JA (1998) Osteoclasts and effects of interleukin 4 in development of chronic osteomyelitis. Clin Orthop Relat Res 355:290–299

Pukatzki S, Kessin RH, Mekalanos JJ (2002) The human pathogen *Pseudomonas aeruginosa* utilizes conserved virulence pathways to infect the social amoeba *Dictyostelium discoideum*. Proc Natl Acad Sci USA 99:3159–3164

Raad I (1998) Intravascular-catheter-related infection. Lancet 351:893–898

Rasmussen TB, Bjarnsholt T, Skindersoe ME, Hentzer M, Kristoffersen P, Kote M, Nielsen J, Eberl L, Givskov M (2005a) Screening for quorum-sensing inhibitors (QSI) by use of a novel genetic system, the QSI selector. J Bacteriol 187:1799–1814

Rumbaugh KP, Griswold JA, Iglewski BH, Hamood AN (1999) Contribution of quorum sensing to the virulence of *Pseudomonas aeruginosa* in burn wound infections. Infect Immun 67:5854–5862

Sifri CD, Begun J, Ausubel FM, Calderwood SB (2003) *Caenorhabditis elegans* as a model host for *Staphylococcus aureus* pathogenesis. Infect Immun 71:2208–2217

Smith RS, Harris SG, Phipps R, Iglewski B (2002a) The *Pseudomonas aeruginosa* quorum-sensing molecule *N*-(3-oxododecanoyl) homoserine lactone contributes to virulence and induces inflammation in vivo. J Bacteriol 184:1132–1139

Steinert M, Heuner K (2005) Dictyostelium as host model for pathogenesis. Cell Microbiol 7:307–314

Tan MW, Mahajan-Miklos S, Ausubel FM (1999) Killing of *Caenorhabditis elegans* by *Pseudomonas aeruginosa* used to model mammalian bacterial pathogenesis. Proc Natl Acad Sci USA 96:715–720

Wu H, Song Z, Givskov M, Doring G, Worlitzsch D, Mathee K, Rygaard J, Hoiby N (2001) *Pseudomonas aeruginosa* mutations in *lasI* and *rhlI* quorum sensing systems result in milder chronic lung infection. Microbiology 147:1105–1113

In Vivo Studies: Inhibiting Biofilm-Associated Bacterial Infections Using QSIs

Naomi Balaban[1], Michael Givskov[2], Thomas Bovbjerg Rasmussen[3]

Abstract This chapter reviews animal studies carried out using quorum-sensing inhibitors of Gram-negative and Gram-positive bacterial biofilm infections. Animal studies include the use of biofilm inhibitors to suppress *Pseudomonas aeruginosa* lung infections in mice, and the use of biofilm inhibitors to suppress antibiotic-resistant staphylococcal infections like methicillin-resistant *Staphylococcus aureus* in rat graft and central venous catheter models and mouse cellulitis models. The advantages of using biofilm inhibitors over those of conventional antibiotics, and the choice of inhibitor to be used are discussed.

The use of quorum-sensing inhibitors (QSIs) to treat bacterial biofilm infections as opposed to conventional therapies such as antibiotics offers many advantages. Because QS blockers usually act as suppressors of virulence without directly killing bacterial cells, the development of resistant strains by natural selection is likely minimized. However, in bacteria that harbor more than one autoinducer or QS system, it may be necessary to disarm each system in order to attenuate virulence. If bacteria contain multiple QS systems that act in a hierarchical manner, it would be necessary to inhibit the one that is most upstream. Also, inhibitors that prevent QS in planktonic cells might not necessarily have optimal efficiency in biofilms. Therefore, it is important to test the effectiveness of a QS inhibitor in biofilms grown in vivo. Because attenuating QS in a specific bacterial species may have unpredictable synergistic effects that could lead to an unfavorable ecological imbalance, testing

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N. Balaban (ed.) *Control of Biofilm Infections by Signal Manipulation*
Springer Series on Biofilms 2
doi: 10.1007/978-3-540-73853-4, © Springer 2008

these cell-signaling blockers in vivo is a critical step in such studies. The following is a description of studies carried out using QSIs in animal models.

1 Inhibition of Gram-Negative Bacterial Infections Using QSIs[2,3]

Only a few of many QSI compounds have been reported to have been tested in various infectious in vivo models. The halogenated furanone #1 (see Table 2 in the chapter *Quorum-Sensing Inhibitory Compounds*) is able to interfere with QS-dependent virulence of the marine bioluminescent bacterium *V. harveyi*. The bacterium is a primary pathogen in many aquaculture systems in which invertebrates are raised. *V. harveyi* uses QS to control both bioluminescence and production of a 100 kDa toxin termed T1. With *V. harveyi* grown in the presence of furanone #1, both bioluminescence and T1 production were reduced in a concentration-dependent manner. If specimens of *Panaeus monodon* (black tiger prawn) were injected with cell-free supernatant of cultures grown with and without the furanone compound, a significant reduction in morality was recorded in the group of prawns injected with the treated culture compared with the untreated group. Likewise, if supernatant from the untreated culture was injected into mice, the survival rate was 20%, whereas it was 90% in the case of supernatant from the furanone-treated culture. This indicates that the furanone compound indeed interferes with the virulence of *V. harveyi* (Manefield et al. 2000).

Furanone compounds are able to attenuate *P. aeruginosa* in a mouse model. Encapsulating *P. aeruginosa* harboring a *lasB-gfp* fusion into alginate beads and installing these beads in the lungs of mice (see the chapter *Animal Models Commonly Used To Study Quorum-Sensing Inhibitors*) allows for the study of QSI efficacy in vivo. When the QS systems are activated, the infecting bacteria express green fluorescence. About 5 h after injection of furanone compounds #3 and #4 (see Table 2 in the chapter *Quorum-Sensing Inhibitory Compounds*) into the tail veins of the mice, the Gfp signal was reduced to the level of the noninduced state, indicating that the QS compounds had traveled through the blood stream, entered the lungs, and blocked QS in the infecting bacteria (Hentzer et al. 2003; Hentzer and Givskov 2003). Five hours is the approximate turnover time of the unstable Gfp reporter used. Because the bacteria were also equipped with constitutive red fluorescent tags, it was possible to determine that the bacteria were not killed or cleared. At 8–10 h after the injection, de novo Gfp expression commenced, indicating that the furanone compounds had been turned over by the host organism. This system therefore offers a rough pharmacokinetic estimate of the test compound. The model also offers an estimate of the effect on bacterial attenuation (Fig. 1). If the bacterial load of the infected lungs was followed over a period of time, mice treated with 0.7 μg/g body weight (BW) of furanone #4 (C-30) (three injections a day at 8-h intervals) had a bacterial content 1000-fold lower than an untreated group on day 5 postinfection. Also, the mortality of infected mice could be reduced by treatment with the furanone compounds. In the untreated

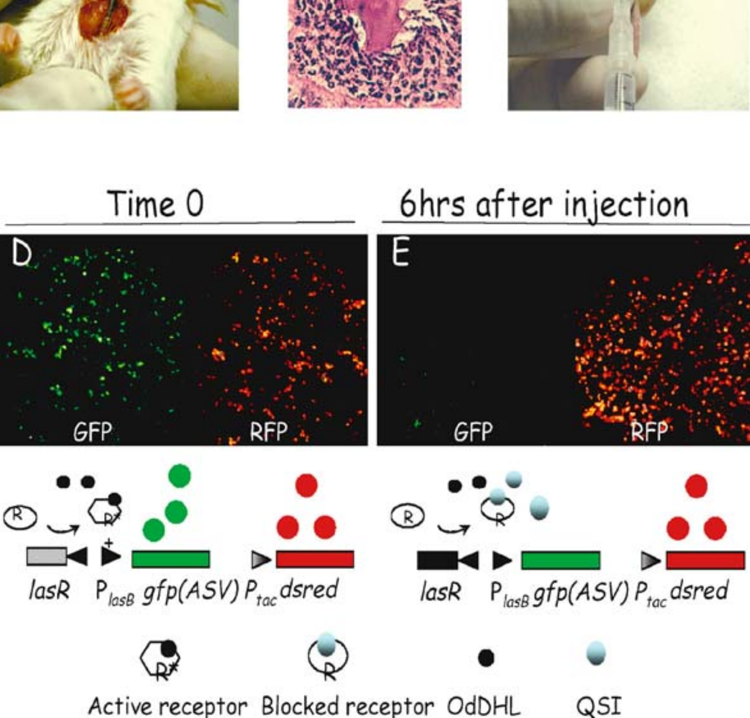

Fig. 1 The pulmonary dose-response model. **A** Mice were challenged intratracheally with alginate beads containing *P. aeruginosa*. **B** Photomicrographs of mouse lung tissue infected with *P. aeruginosa*. The *arrow* points at an alginate bead surrounded by numerous polymorphonuclear leukocytes. **C** Quorum-sensing drugs (quorum-sensing inhibitors) can be injected intravenously in the tail vein. **D**, **E** Mouse lung tissue infected with *P. aeruginosa* carrying the LasR-based monitor *PlasB-gfp* for detection of cell–cell signaling (*green fluorescence*) and a tag for simple identification in tissue samples (*red fluorescence*) examined by scanning confocal laser microscopy (SCLM). **D** Mice were administered furanone #4 (C-30) via intravenous injection at time zero. **E** Infected animals were sacrificed in groups of three at the time point indicated, and the lung tissue samples were examined by SCLM according to Hentzer et al. (2003)

half, 88% of the mice died, whereas only 55% in the treated group died (Hentzer et al. 2002, 2003). Taken together, these results indicate that the halogenated furanone compounds are able to attenuate *P. aeruginosa* in vivo.

In the nematode worm *C. elegans* model, 100% of the worms are killed when feeding on wild-type *P. aeruginosa*. If the growth medium is supplemented with 100 μM 4-nitro-pyridine-*N*-oxide (4-NPO) or 2% garlic extract, the mortality of the worms is reduced to 5% and 40%, respectively (Rasmussen et al. 2005a). In a similar fashion in the chronic lung infection model in mice, garlic extract reduces mortality

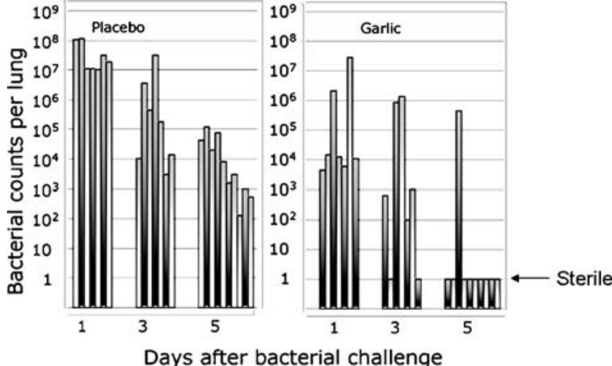

Fig. 2 Two different groups of BalB/c mice were treated either with quorum-sensing inhibitory garlic extract or placebo (saline). The treatment and placebo were given as subcutaneous injections every 24 h and continued for 8 days. The treatment concentration given was calculated to be 2% of the total mouse volume, which was estimated to be approximately 20 ml. After 3 days of prophylactic treatment, the mice were intratracheally challenged (at day 0) with alginate beads containing approximately 10^7 CFU/lung *P. aeruginosa*. In the placebo group, the bacteria grew to an average of 5^7 CFU/lung (day 1). Mice were sacrificed on days 1, 3, and 5 postinfection, and the bacterial content in the lungs was determined. At the time of sacrifice, each group contained a minimum of seven mice. The statistical significance of difference in clearance was tested by a Kruskal–Wallis test (analysis of nonparametric data), and *P*-values for the difference at days 1, 3, and 5 were 0.0636, 0.0087, and 0.0015, respectively. Data obtained from Bjarnsholt et al. 2005b

from 72% when untreated to 32% when treated with 1.5% of the extract. The clearance of bacteria is also enhanced when the mice are treated with garlic. With subcutaneous treatment of 2% garlic extract (v/w) the mice had a bacterial load in the lungs of three orders of magnitude lower than their untreated counterparts already at the second day after start of the infection and treatment. On day 5 postinfection, the treated mice had cleared their lungs whereas the mice in the untreated group harbored 10^5 colony-forming units (CFUs)/lung (Fig. 2) (Bjarnsholt et al. 2005b).

The QSI compound patulin isolated from fungi was also able to affect virulence of *P. aeruginosa* in the described infection mouse model. When treated with 2.5 μg/g BW once a day, the mice in the treated group showed a 20-fold lower bacterial load in the lungs on day 3 postinfection. Also, mortality was less than half in the patulin-treated group compared with the untreated group (Rasmussen et al. 2005b).

2 Inhibition of Gram-Positive (Staphylococcal) Bacterial Infections Using QSIs[1]

As reviewed in section 2 of the chapter *Bacterial Cell-to-Cell Communication*, *S. aureus* pathogenesis is regulated by two QS systems, termed SQS 1 and SQS 2. SQS 1 consists of the autoinducer RNAIII-activating protein (RAP) and its target

molecule TRAP (Balaban et al. 1998, 2001; Gov et al. 2004; Korem et al. 2003, 2005). SQS 1 induces the synthesis of SQS 2, which consists of the components of the *agr* system, including autoinducing peptide (AIP), its sensor AgrC, and the effector molecule RNAIII (Lyon et al. 2000). Extensive data exists on the successful use of inhibitors to SQS 1 in vivo, whereas very little data are available on the use of inhibitors to SQS 2, and those studies that are published included very few mice ($n = 3$; Mayville et al. 1999; Wright et al. 2005). The reason for that may be due to the fact that SQS 1 is highly conserved among staphylococci whereas SQS 2 is highly divergent (Ji et al. 1997; Qiu et al. 2005). In addition, inhibition of SQS 1 may be more successful in terms of treatment because SQS 1 regulates not only SQS 2-regulated virulence factors but also additional factors important for virulence and biofilm formation (see section 2 of the chapter *Bacterial Cell-to-Cell Communication* as well as Korem et al. 2005). Below is a summary of some of the animal studies using inhibitors to SQS 1.

2.1 RNAIII-Inhibiting Peptide

Staphylococcal pathogenesis can be inhibited by the linear heptapeptide RNAIII-inhibiting peptide (RIP; Gov et al. 2001). RIP interferes with SQS 1 by competing with RAP, thus inhibiting TRAP phosphorylation and *agr* expression (Balaban et al. 2001). RIP was originally discovered in the supernatants of a coagulase-negative staphylococci assumed to be *S. xylosus* or *S. warnerii* (Balaban and Novick 1995b; Balaban et al. 1998), but it is now synthesized commercially. The sequence of RIP (YSPWTNF-NH_2) is similar to the sequence of residues 4–9 of RAP (YKPITN), and several RIP derivatives designed according to the 4–9 AA sequence of RAP

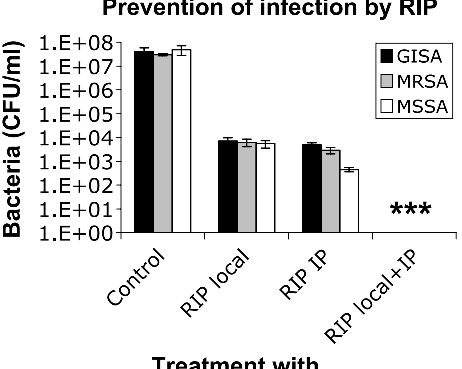

Fig. 3 RIP/RIP combination therapy (local/systemic) in the rat graft model. Grafts were soaked in RIP (20 min, 20 mg/l) combined with parenteral administration of RIP (intraperitoneal injection of 10 mg/kg). Strains tested: MSSA, MRSA, GISA MSSE, MRSE, GISE. *Asterisk* (*) indicates that no bacteria were detected (Dell'Acqua et al. 2004)

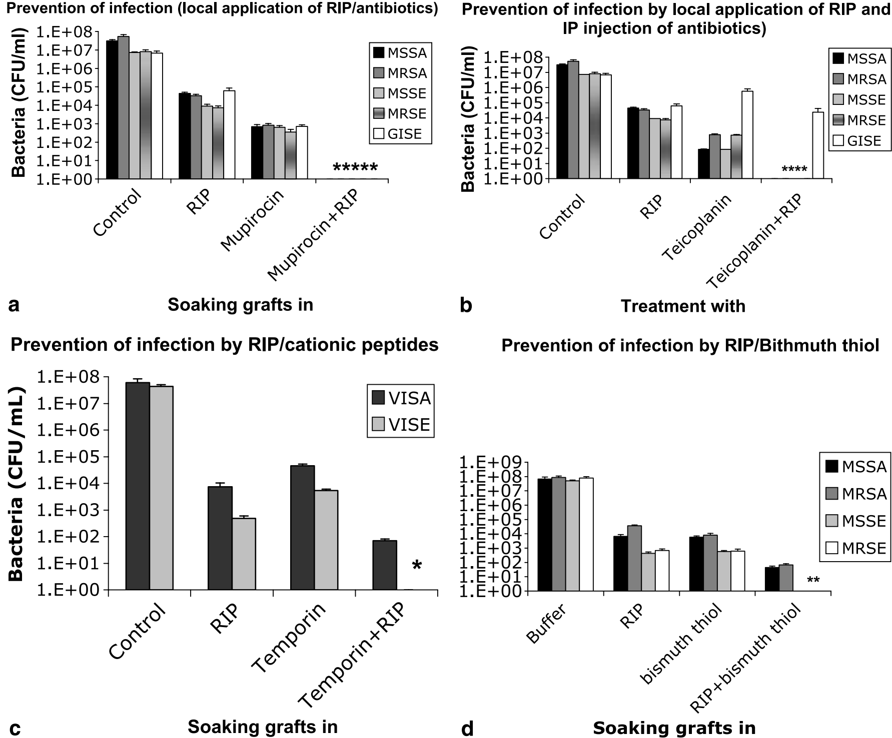

Fig. 4 RIP is synergistic to antibiotics as tested in the rat graft model. *Asterisk* (*) indicates that no bacteria were detected. **A** Local treatment: Grafts were soaked with RIP (20 min, 10 mg/l) and/or mupirocin (100 mg/l) and tested on MSSA, MRSA, MSSE, MRSE, and GISE. **B** Local/parenteral treatment: Grafts were soaked in RIP (20 min, 10 mg/l), and animals were injected with teicoplanin (intraperitoneal injection of 10 mg/kg). Strains tested: MSSA, MRSA, MSSE, MRSE, GISE (Balaban et al. 2003a; Giacometti et al. 2003). **C** Local treatment: Grafts were soaked with RIP and/or temporin (20 min, 10 mg/l) and tested on VISA and VISE (Cirioni et al. 2003). **D** Local treatment: Grafts were soaked for 20 min with RIP (10 mg/l) and/or bismuth thiol (0.1 mg/l) and tested on MSSA, MRSA, MSSE, and MRSE (Domenico et al. 2004)

compete with RAP and repress toxin production (Abraham 2006; Vieira-da-Motta 2001; Gov et al. 2001). This suggests that RIP is structurally similar to a segment of RAP and that RAP probably acts as an agonist and RIP as an antagonist to the same target protein (TRAP). Synthetic linear RIP has already been shown to prevent numerous types of *S. aureus* and *S. epidermidis* infections in vivo, including medical-device-associated infections (tested against methicillin-resistant *S. aureus*, methicillin-resistant *S. epidermidis*, vancomycin-intermediate *S. aureus*, and vancomycin-intermediate *S. epidermidis*; Dell'Acqua et al. 2004; Balaban et al. 2000, 2003a, 2004, 2005, 2007; Cirioni et al. 2003, 2006; Domenico et al. 2004; Giacometti et al. 2003, 2005; Anguita-Alonso et al. 2006) (see Figs. 3–6). These findings indicate that RIP can suppress virulence of any staphylococcal strain, which is not surprising considering that the signaling molecule it targets (TRAP) is highly conserved across strains and staphylococcal species (Gov et al. 2004).

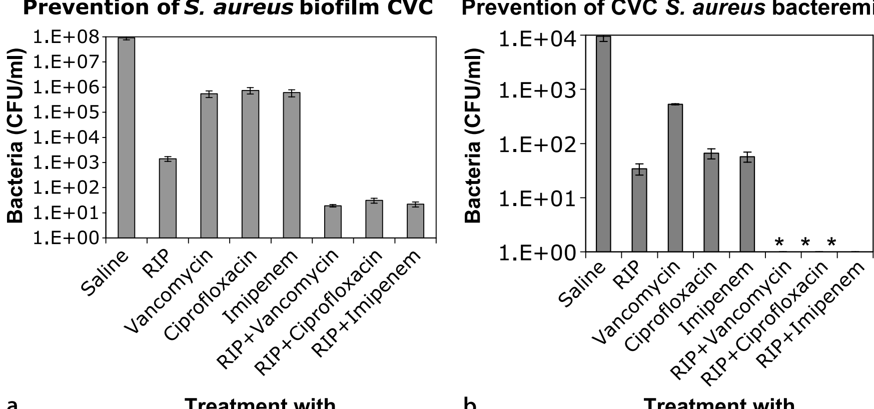

Fig. 5 Suppression of central venous catheter *S. aureus* biofilm infection (**a**) and bacteremia (**b**) by RIP and/or antibiotics. *Asterisk* (*) indicates that no bacteria were detected (Cirioni et al. 2006)

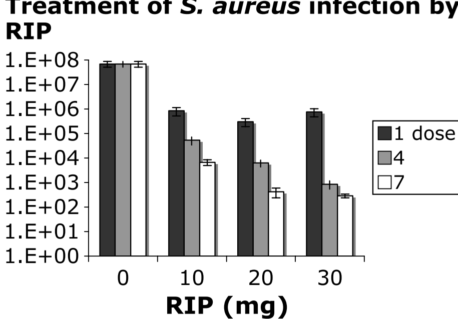

Fig. 6 Treatment of *S. aureus* biofilm infection with RIP: Using the rat graft model, rats were challenged with *S. aureus* strain Smith diffuse and injected parenterally 2 days later with multiple doses of RIP. Grafts were removed on day 10, and the bacterial load on grafts was determined and expressed as CFU/ml (Balaban et al. 2007)

2.2 Anti-TRAP Approach

A peptide TA21 (SWFDNFLYPTHD) was identified by screening a 12-mer phage-displayed peptide library using anti-TRAP antibodies. Mice vaccinated with *Escherichia coli* engineered to express TA21 on their surface (FTA21) were protected from *S. aureus* infections using sepsis and cellulitis mice models (Table 1, Fig. 7). By sequence analysis, it was found that the TA21 is highly homologous to the C-terminal sequence of TRAP (AA 156–167), which is conserved among staphylococci, suggesting that peptide TA21 may be a useful broad vaccine to protect from infection caused by various staphylococcal strains (Yang et al. 2005).

Table 1 Mice immunized with FTA21 are protected from *S. aureus* sepsis

Mice immunized with	Number of mice that died on days			Mice survival
	1–3 days	3–5 days	5–7 days	
FTA6	5	2	1	0
FTA21	0	0	0	8
Control	3	5	0	0

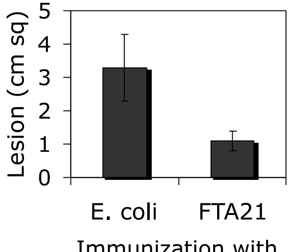

Fig. 7 Anti-TRAP approach, mouse cellulitis model. Mice were preimmunized with *E. coli* without fusion peptide or with *E. coli* expressing the TRAP-like epitope (FTA21). Mice were challenged with *S. aureus* mixed with Cytodex. The lesions were measured 72 h after injection (Yang et al. 2005)

2.3 RAP-Binding Peptides

RAP (native and recombinant) was used to select RAP-binding peptides (RBPs) from a random 12-mer phage-displayed peptide library. Two RBPs were shown to

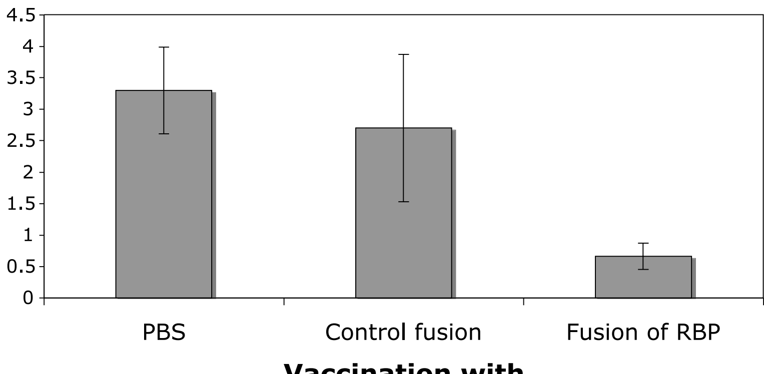

Fig. 8 Anti-RAP approach, mouse cellulitis model. *S. aureus* was preincubated with RAP-binding peptide (RBP) or controls for 90 min, mixed with Cytodex, and injected subcutaneously into immunocompetent hairless mice ($n = 8$). The lesions were measured 72 h later. Bacteria were treated with PBS control, nonrelated GST-fusion peptide, and GST fusion of RBP15 (RAP-binding peptide)

inhibit RNAIII production in vitro (RNAIII was used as a marker for pathogenesis). The peptide WPFAHWPWQYPR, which had the strongest inhibitory activity, was chemically synthesized and also expressed in *E. coli* as a GST fusion. Both synthetic peptide and GST-fusion peptide decreased RNAIII levels in a dose-dependent manner. The GST-fusion peptide was also shown to protect mice from *S. aureus* infection in vivo (tested in a murine cutaneous *S. aureus* infection model; Fig. 8). These results suggest the potential use of RAP-binding proteins in treating clinical *S. aureus* infections (Yang et al. 2003, 2006).

In summary, the RAP/TRAP QS system in staphylococci (SQS 1) is a very attractive target site for therapy due to its essential role in pathogenesis and its conservation among staphylococcal strains. RIP is now under clinical development to be used both systemically and for coating medical devices (Centegen).

References

Abraham WR (2006) Controlling biofilms of gram-positive pathogenic bacteria. Curr Med Chem 13:1509–1524

Anguita-Alonso P, Giacometti A, Cirioni O, Ghiselli R, Orlando F, Saba V, Scalise G, Sevo M, Tuzova M, Patel R, Balaban N (2006) RNAIII-inhibiting peptide-loaded polymethylmethacrylate prevents in vivo *Staphylococcus aureus* biofilm formation. Antimicrob Agents Chemother 51:2594–2596

Balaban N, Novick RP (1995b) Autocrine regulation of toxin synthesis by *Staphylococcus aureus*. Proc Natl Acad Sci USA 92:1619–1623

Balaban N, Goldkorn T, Nhan RT, Dang LB, Scott S, Ridgley RM, Rasooly A, Wright SC, Larrick JW, Rasooly R, Carlson JR (1998) Autoinducer of virulence as a target for vaccine and therapy against *Staphylococcus aureus*. Science 280:438–440

Balaban N, Collins LV, Cullor JS, Hume EB, Medina-Acosta E, Vieira-da-Motta O, O'Callaghan R, Rossitto PV, Shirtliff ME, Serafim da Silveira L, Tarkowski A, Torres JV (2000) Prevention of diseases caused by *Staphylococcus aureus* using the peptide RIP. Peptides 21:1301–1311

Balaban N, Goldkorn T, Gov Y, Hirshberg M, Koyfman N, Matthews HR, Nhan RT, Singh B, Uziel O (2001) Regulation of *Staphylococcus aureus* pathogenesis via target of RNAIII- activating protein (TRAP). J Biol Chem 276:2658–2667

Balaban N, Giacometti A, Cirioni O, Gov Y, Ghiselli R, Mocchegiani F, Viticchi C, Del Prete MS, Saba V, Scalise G, Dell'Acqua G (2003a) Use of the quorum-sensing inhibitor RNAIII-inhibiting peptide to prevent biofilm formation in vivo by drug-resistant *Staphylococcus epidermidis*. J Infect Dis 187:625–630

Balaban N, Gov Y, Giacometti A, Cirioni O, Ghiselli R, Mocchegiani F, Orlando F, D'Amato G, Saba V, Scalise G, Bernes S, Mor A (2004) A chimeric peptide composed of a dermaseptin derivative and an RNA III-inhibiting peptide prevents graft-associated infections by antibiotic-resistant staphylococci. Antimicrob Agents Chemother 48:2544–2550

Balaban N, Stoodley P, Fux CA, Wilson S, Costerton JW, Dell'Acqua G (2005) Prevention of staphylococcal biofilm-associated infections by the quorum sensing inhibitor RIP. Clin Orthop Relat Res 437:48–54

Balaban N, Cirioni O, Giacometti A, Ghiselli R, Braunstein J, Silvestri C, Mocchegiani F, Saba V, Scalise G (2007) Treatment of *Staphylococcus aureus* biofilm infection by the quorum sensing inhibitor RIP. Antimicrob Agents Chemother 51:2226–2229

Bjarnsholt T, Jensen PO, Rasmussen TB, Christophersen L, Calum H, Hentzer M, Hougen HP, Rygaard J, Moser C, Eberl L, Hoiby N, Givskov M (2005b) Garlic blocks quorum sensing

and promotes rapid clearing of pulmonary *Pseudomonas aeruginosa* infections. Microbiology 151:3873–3880

Cirioni O, Giacometti A, Ghiselli R, Dell'Acqua G, Gov Y, Kamysz W, Lukasiak J, Mocchegiani F, Orlando F, D'Amato G, Balaban N, Saba V, Scalise G (2003) Prophylactic efficacy of topical temporin A and RNAIII-inhibiting peptide in a subcutaneous rat Pouch model of graft infection attributable to staphylococci with intermediate resistance to glycopeptides. Circulation 108:767–771

Cirioni O, Giacometti A, Ghiselli R, Dell'acqua G, Orlando F, Mocchegiani F, Silvestri C, Licci A, Saba V, Scalise G, Balaban N (2006) RNAIII-inhibiting peptide significantly reduces bacterial load and enhances the effect of antibiotics in the treatment of central venous catheter-associated *Staphylococcus aureus* infections. J Infect Dis 193:180–186

Dell'Acqua G, Giacometti A, Cirioni O, Ghiselli R, Saba V, Scalise G, Gov Y, Balaban N (2004) Suppression of drug-resistant staphylococcal infections by the quorum-sensing inhibitor RNAIII-inhibiting peptide. J Infect Dis 190:318–320

Domenico P, Gurzenda E, Giacometti A, Cirioni O, Ghiselli R, Orlando F, Korem M, Saba V, Scalise G, Balaban N (2004) BisEDT and RIP act in synergy to prevent graft infections by resistant staphylococci. Peptides 25:2047–2053

Giacometti A, Cirioni O, Gov Y, Ghiselli R, Del Prete DS, Mocchegiani F, Saba V, Orlando F, Scalise G, Balaban N, Dell'Acqua G (2003) RNAIII inhibiting peptide (RIP) inhibits in vivo biofilm formation of drug-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 47:1979–1983

Giacometti A, Cirioni O, Ghiselli R, Dell'Acqua G, Orlando F, D'Amato G, Mocchegiani F, Silvestri C, Del Prete MS, Rocchi M, Balaban N, Saba V, Scalise G (2005) RNAIII-inhibiting peptide improves efficacy of clinically used antibiotics in a murine model of staphylococcal sepsis. Peptides 26:169–175

Gov Y, Bitler A, Dell'Acqua G, Torres JV, Balaban N (2001) RNAIII inhibiting peptide (RIP), a global inhibitor of *Staphylococcus aureus*: structure and function analysis. Peptides 22:1609–1620

Gov Y, Borovok I, Korem M, Singh VK, Jayaswal RK, Wilkinson BJ, Rich SM, Balaban N (2004) Quorum sensing in Staphylococci is regulated via phosphorylation of three conserved histidine residues. J Biol Chem 279:14665–14672

Hentzer M, Givskov M (2003) Pharmacological inhibition of quorum sensing for the treatment of chronic bacterial infections. J Clin Invest 112:1300–1307

Hentzer M, Riedel K, Rasmussen TB, Heydorn A, Andersen JB, Parsek MR, Rice SA, Eberl L, Molin S, Hoiby N, Kjelleberg S, Givskov M (2002) Inhibition of quorum sensing in *Pseudomonas aeruginosa* biofilm bacteria by a halogenated furanone compound. Microbiology 148:87–102

Hentzer M, Wu H, Andersen JB, Riedel K, Rasmussen TB, Bagge N, Kumar N, Schembri MA, Song Z, Kristoffersen P, Manefield M, Costerton JW, Molin S, Eberl L, Steinberg P, Kjelleberg S, Høiby N, Givskov M (2003) Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. EMBO J 22:3803–3815

Ji G, Beavis R, Novick RP (1997) Bacterial interference caused by autoinducing peptide variants. Science 276:2027–2030

Korem M, Sheoran A, Gov Y, Borovok I, Tzipori S, Balaban N (2003) Characterization of RAP, a quorum sensing activator in *Staphylococcus aureus*. FEMS Microbiol Lett 223:167–175

Korem M, Gov Y, Kiran MD, Balaban N (2005) Transcriptional profiling of target of RNAIII-activating protein, a master regulator of staphylococcal virulence. Infect Immun 73:6220–6228

Lyon GJ, Mayville P, Muir TW, Novick RP (2000) Rational design of a global inhibitor of the virulence response in *Staphylococcus aureus*, based in part on localization of the site of inhibition to the receptor-histidine kinase, AgrC. Proc Natl Acad Sci USA 97:13330–13335

Manefield M, Harris L, Rice SA, de Nys R, Kjelleberg S (2000) Inhibition of luminescence and virulence in the black tiger prawn (*Penaeus monodon*) pathogen *Vibrio harveyi* by intercellular signal antagonists. Appl Environ Microbiol 66:2079–2084

Mayville P, Ji G, Beavis R, Yang H, Goger M, Novick RP, Muir TW (1999) Structure-activity analysis of synthetic autoinducing thiolactone peptides from *Staphylococcus aureus* responsible for virulence. Proc Natl Acad Sci USA 96:1218–1223

Qiu R, Pei W, Zhang L, Lin J, Ji G (2005) Identification of the putative staphylococcal AgrB catalytic residues involving the proteolytic cleavage of AgrD to generate autoinducing peptide. J Biol Chem 280:16695–16704

Rasmussen TB, Bjarnsholt T, Skindersoe ME, Hentzer M, Kristoffersen P, Kote M, Nielsen J, Eberl L, Givskov M (2005a) Screening for quorum-sensing inhibitors (QSI) by use of a novel genetic system, the QSI selector. J Bacteriol 187:1799–1814

Rasmussen TB, Skindersoe ME, Bjarnsholt T, Phipps RK, Christensen KB, Jensen PO, Andersen JB, Koch B, Larsen TO, Hentzer M, Eberl L, Hoiby N, Givskov M (2005b) Identity and effects of quorum-sensing inhibitors produced by *Penicillium* species. Microbiology 151:1325–1340

Vieira-da-Motta O, Damasceno Ribeiro P, Dias da Silva W, Medina-Acosta E (2001) RNAIII inhibiting peptide (RIP) inhibits *agr*-regulated toxin production. Peptides 22:1621–1627

Wright JS III, Jin R, Novick RP (2005) Transient interference with staphylococcal quorum sensing blocks abscess formation. Proc Natl Acad Sci USA 102:1691–1696

Yang G, Cheng H, Liu C, Xue Y, Gao Y, Liu N, Gao B, Wang D, Li S, Shen B, Shao N (2003) Inhibition of *Staphylococcus aureus* pathogenesis in vitro and in vivo by RAP-binding peptides. Peptides 24:1823–1828

Yang G, Gao Y, Dong J, Liu C, Xue Y, Fan M, Shen B, Shao N (2005) A novel peptide screened by phage display can mimic TRAP antigen epitope against *Staphylococcus aureus* infections. J Biol Chem 280:27431–27435

Yang G, Gao Y, Dong J, Xue Y, Fan M, Shen B, Liu C, Shao N (2006) A novel peptide isolated from phage library to substitute a complex system for a vaccine against staphylococci infection. Vaccine 24:1117–1123

Quorum Sensing in Streptococci

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Abstract Emerging studies aimed at understanding the molecular underpinnings of streptococcal infections highlight the importance of quorum sensing (QS) for biofilm formation and virulence in many streptococcal species. Among streptococci, the best characterized quorum sensing system belongs to *Streptococcus pneumoniae*. Although initially QS was believed to act mainly to acquire and incorporate foreign DNA into the host chromosome, studies implicating its involvement in biofilm formation, fratricide as well as virulence suggested a broader and more varied role for this system in *S. pneumoniae*. In this chapter, we will discuss the QS system of pneumococci, as well as other streptococci including *Streptococcus mutans* and *Streptococcus pyogenes*. Furthermore, we will also discuss some interesting studies that have been conducted recently to target QS as a tool to combat or modulate infections associated with streptococcal biofilms.

1 Streptococcal Biofilms

In humans, most members of the genus *Streptococcus* belong to the normal commensal flora that inhabit the mouth, skin, intestine, and upper respiratory tract. Infections such as strep throat, dental caries, meningitis, bacterial pneumonia, endocarditis, and necrotizing fasciitis (infamously known as the "flesh-eating" disease) are caused by pathogenic streptococcal strains whose disease etiology has been studied extensively. In contrast to the original view that most of these bacteria act as individual unicellular organisms, the discovery of quorum sensing (QS) and how this

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N. Balaban (ed.) *Control of Biofilm Infections by Signal Manipulation*
Springer Series on Biofilms 2
doi: 10.1007/978-3-540-73853-4, © Springer 2008

elegant mechanism can synchronize the production of virulence actors concomitant to cell population density has shed new light on understanding and conquering infectious diseases caused by these bacteria. Moreover, the finding that streptococcal QS plays a major role in maintaining the structural and functional integrity of the biofilm phenotype makes QS an attractive and rather promising target to attenuate virulence caused by these strains. In this chapter we will discuss some of the recent findings pertaining to QS-based virulence modulation strategies that are being developed for use in *Streptococcus pneumoniae*, *Streptococcus mutans*, and *Streptococcus pyogenes* infections as potential chemotherapeutic agents to control or prevent streptococcal biofilm-mediated infections.

2 Peptide-Based Quorum Sensing in Streptococci

There is a growing body of literature strongly implicating the switch from planktonic to sessile growth as a major phenotypic shift required for streptococcal virulence (Cvitkovitch et al. 2003). One of the most well-known and best-studied pathogenic biofilms predominated by streptococci is human dental plaque, which harbors hundreds of species of bacteria (Kroes et al. 1999). This chapter will focus on the QS systems of streptococci that reside permanently or transiently in the human oral cavity and the ways that biofilms are linked to infection and signaling processes. The primary components and mechanisms utilized for QS by *S. mutans*, *Streptococcus gordonii*, and other streptococci are essentially the same as for *S. pneumoniae*. The phenotype originally associated with density-dependent signaling was the induction of genetic competence, the transient physiological state that bacteria enter to facilitate uptake and incorporation of exogenous DNA (Morrison and Lee 2000).

The signal molecule associated with the QS phenomenon is called competence-stimulating peptide, or CSP (Håvarstein et al. 1995a). CSP in *S. pneumoniae* and *S. mutans* and competence factor in *S. gordonii* are derived from their precursor propeptide molecules that contain a typical Gly–Gly cleavage site and an N-terminal leader sequence that is removed via proteolysis by the ComAB transporter during export to produce the biologically active autoinducer peptide (Håvarstein et al. 1995b). The *S. pneumoniae* CSP is a 17-amino-acid (aa) peptide processed from a 41-aa precursor (Håvarstein et al. 1995a), whereas the *S. gordonii* competence factor comprises 19 aa obtained from a 50-aa precursor (Lunsford and London 1996). The leader sequence of the *S. pneumoniae* CSP was found to belong to a family of double-glycine-type leader peptides that contained the consensus LSX_2ELX_2IXGG with hydrophobic residues at positions −4, −7, −12, and −15 relative to the Gly–Gly cleavage site (Håvarstein et al. 1994, 1995b). Sequence analysis of the *S. mutans* genome database allowed deduction of its CSP to consist of a 21-aa CSP derived from a 46-aa precursor, and the addition of synthetic CSP to growing cultures confirmed its identity (Li et al. 2001b).

In pneumococci, the QS system is encoded by two distinct genetic loci, *comCDE* and *comAB* (Håvarstein et al. 1995a). The *comC*, *comD*, and *comE* genes encode the CSP peptide-precursor, the histidine kinase (HK), and response regulator (RR), respectively (Cheng et al. 1997; Håvarstein et al. 1995a, 1996; Pestova et al. 1996). The secretion apparatus necessary for CSP maturation and export is encoded by the *comA* and *comB* genes. Based on the model proposed in *Streptococcus pneumoniae*, when the mature CSP peptide reaches a threshold concentration, it binds to the membrane-bound HK sensor ComD, causing its autophosphorylation. Phosphorylated ComD then transfers the phosphate group to its cognate intracellular RR ComE, which then activates the transcription of *comX* encoding an alternative sigma factor. ComX (Luo et al. 2003) together with the ComW protein (Luo et al. 2004; Sung and Morrison 2005) enable transcription of several late competence-related genes involved in DNA uptake and integration.

The first evidence that QS was involved in streptococcal biofilm formation came from a study of *S. gordonii*, a commensal bacterium in human dental plaque (Loo et al. 2000). These investigators recovered a biofilm-defective mutant following transposon mutagenesis that had an inactivated *comD* gene encoding the HK receptor that detects the QS autoinducer peptide. More recently, Gilmore et al. used real-time polymerase chain reaction to study the expression of *S. gordonii* genes known or assumed to be involved in biofilm formation. They demonstrated that both *comD* and *comE* – which comprise the two-component signal transduction system in its QS – were both upregulated in the biofilm phase (Gilmore et al. 2003).

Following the pioneering work in *S. gordonii*, a link between biofilm formation and QS was solidly established in *Streptococcus mutans* (Li et al. 2001b, 2002). This bacterium, which resides in the oral cavity, is strongly regarded as a principal etiologic agent of dental caries, which is one of the most prevalent chronic childhood ailments (more common than asthma). In the mouth, *S. mutans* relies on a biofilm mode of growth for its survival by anchoring itself to hard surfaces and evading the hostility of the salivary flow.

The linkage between QS and biofilm formation in *S. mutans* was established by demonstrating that mutants defective in any of the *comC*, *comD*, *comE*, or *comX* genes formed abnormal biofilms (Li et al. 2001b, 2002), and the addition of exogenous CSP to a *comC* mutant restored the normal biofilm architecture (Li et al. 2002). Other phenotypes activated by CSP were an increased resistance to acid (Li et al. 2001a) and modulation of bacteriocin production (Kreth et al. 2006).

In a recent study, Oggionni et al. (2004, 2006) established a link between the CSP QS system and biofilm formation in *S. pneumoniae*. CSP was able to induce biofilm formation *in vitro*, and a mutant defective in the ComD receptor, which did not form biofilms, also showed reduced virulence. These results were in contrast to those found in a bacteremic sepsis model of infection, in which the competence system was downregulated. These researchers also found that when the infective bacteria from different physiological states were used to infect mice, biofilm-grown cells were more effective in inducing meningitis and pneumonia whereas liquid-grown planktonic cells were more effective at inducing sepsis.

One can now argue that the simple transient phase of competence was merely the first discovery of the multiple roles of CSP-mediated QS in most streptococci, which we propose is the "master switch" between planktonic and biofilm lifestyles.

3 Population-Density-Dependent Cell Death

Although natural genetic transformation of pneumococci was extensively investigated for over eight decades following its initial discovery by Frederick Griffith in the 1920s, the source of donor DNA for transformation was traditionally regarded as DNA that originated from dead cells that fell apart from natural causes. Despite this belief, accumulating evidence from recent experiments shows that in *S. pneumoniae*, recipient or competent cells are capable of ensuring the availability of donor DNA by coordinating competence with the lysis of noncompetent cells of the same strain in the population (Steinmoen et al. 2002, 2003). Moreover, in *S. mutans* it was observed that the addition of exogenous CSP in excess of the amount necessary for competence inhibited cell growth and that a further increase of CSP led to cell death (Qi et al. 2005). Clearly, during a time in which there is a great need to develop novel strategies to combat virulence, these studies come as positive news in getting us one step closer to developing successful drugs against biofilm-mediated infections. In the following sections we will discuss competence-induced cell death in *S. pneumoniae* and *S. mutans*, as well as how we can possibly exploit QS mechanisms of these bacteria to design novel therapies against bacterial infections.

3.1 QS-Induced Allolysis in S. Pneumoniae

Steinmoen et al. (2002) recently demonstrated that during cocultivation, competent streptococci grown in liquid culture were able to actively acquire transforming DNA by killing 5–20% of their noncompetent siblings when exposed to CSP. These researchers showed that lysis and DNA release were initiated with the induction of the competence state and that the efficiency of this process was influenced by cell density (Steinmoen et al. 2003). The discovery of this fratricide phenomenon has enormous significance from an evolutionary perspective in enhancing the genetic plasticity of *S. pneumoniae*. Although identification of the molecular underpinnings of this cell-lysis mechanism is still in the early stages, a two-peptide bacteriocin called CibAB is believed to be required for the allolysis. Moreover, competence-induced allolysis also requires the production of the major autolysin LytA and the lysozyme LytC, which can be supplied by the competent cells or the targeted cells (Guiral et al. 2005; Knutsen et al. 2004; Moscoso and Claverys 2004; Steinmoen et al. 2002, 2003). In addition, it was also observed that a QS-mutant deficient in the *comE* gene was incapable of cell lysis, thereby linking the ComDE signal transduction system with DNA release and uptake (Steinmoen et al. 2002).

In their reports, Steinmoen et al. (2002, 2003) claimed that the fratricide phenomenon was present in *S. pneumoniae* to ensure the presence of sufficient homologous DNA during genetic transformation. However, a more recent investigation by Moscoso and Claverys (2004) showed that although competence decreased after 20 min following the addition of CSP, the amount of liberated DNA continued to increase and reached a maximum in the stationary phase, when cells were no longer capable of DNA uptake (Moscoso and Claverys 2004). Hence, they argued against the role of fratricide as a means to acquire DNA for maximized genetic exchange and suggested a different role for this observation, including a possible role in nutrient acquisition, biofilm formation, or the release of toxins (such as pneumolysin, teichoic, and lipoteichoic acids).

3.2 CSP-Induced Cell Death in S. Mutans

In *S. mutans*, the ComCDE QS system regulates several physiological properties, including competence development, biofilm formation, acid tolerance, and bacteriocin production. The induction of the latter three phenotypes especially suggests that the CSP-induced QS system likely responds to environmental stress; therefore, it is also likely that an overreaction to stress may cause a detrimental effect on the cells. Recently this idea was tested by Qi et al. by overdosing *S. mutans* with CSP at a concentration higher than that normally used to induce competence (Qi et al. 2005). It was demonstrated that at a slightly higher than normal (0.65 versus 0.5 μM) concentration of CSP, growth of *S. mutans* was inhibited in planktonic and biofilm cells. Further analyses revealed that CSP exerted this effect by inhibiting cell division, ultimately leading to cell death, while mutational analyses suggested that the ComDE QS pathway mediated CSP-induced cell death in a ComX-independent manner. A preliminary examination of the inhibitory effect of CSP on other *S. mutans* strains suggested that the effect was highly variable among strains. Even within the sensitive strain, only about 20% of the cell population progressed to cell death after CSP treatment. Whether this partial inhibition in a cell population would give commensal streptococci a head start to become dominant awaits further experimentation. It remains to be determined whether the higher-than-normal doses of CSP used in the various studies have relevance in the context of a biofilm in which the local concentration of peptide is likely to be high.

3.3 Competence-Induced Bacteriocin Production by S. Mutans

The involvement of CSP in multiple cellular functions has prompted scientists to search for its ecological functions in a multispecies environment such as dental biofilm. In a recent study (Kreth et al. 2005), it was demonstrated that CSP was required to activate a group of bacteriocin (mutacin) genes in *S. mutans*. One of the

mutacins, mutacin IV, was shown to have activities specifically against a group of oral streptococci, the "mitis" group streptococci, which comprise the pioneer colonizers during dental biofilm development and which are known to have antagonistic activities toward *S. mutans*. Further studies demonstrated that mutacin production was required to cause DNA release from neighboring streptococcal species when *S. mutans* became competent (Kreth et al. 2006). More interestingly, there was a programmed time delay for competence development after the addition of CSP. Although mutacin production responds to CSP within 30 min after CSP addition, competence does not develop until 2–2.5 h after the addition of CSP. This time delay was suggested as a mechanism to ensure DNA availability when the cells become competent. Recently, a lantibiotic mutacin, Smb, was also found to be regulated by CSP in strain GS-5. Whether this regulation is also related to DNA release from other streptococcal species has yet to be determined.

4 Targeting Streptococcal Quorum Sensing

4.1 CSP as a Potential Therapeutic Agent

An important study (Oggioni et al. 2004) used an *in vivo* sepsis mouse model to assay the effect of exogenous CSP addition on virulence. This group found that CSP-treated infected mice had a significant increase in survival rates, reduced pneumococcal blood counts, and increased length of survival relative to control mice. *In vitro* CSP addition at the same concentration used in the mice elicited a transient growth inhibition. A receptor HK mutant did not have a bacteriostatic phenotype *in vitro* and was not responsive to CSP *in vivo*. This work shows that CSP induces a temporary growth arrest that alters the outcome of the infection. Interestingly, this therapeutic effect was somewhat unexpected because the positive effect was obtained by activating the target molecule rather than inhibiting it.

In addition to being used as a pheromone to disrupt a cell's normal metabolic activity, the target specificity of CSP was also used recently as a homing device to deliver antimicrobial peptide to the target bacterium (Eckert et al. 2006). Eckert et al. demonstrated that by fusing the C-terminal 16 aa of the *S. mutans* CSP (the full-length CSP is 21 aa) to a wide spectrum, defensin-derived antimicrobial peptide G2, they could specifically eliminate *S. mutans* from mixed cell cultures with other closely related streptococcal species. Furthermore, the species specificity of the C16 peptide could be further narrowed down to 8 aa (C8). Interestingly, the target specificity of C16 and C8 is independent of ComD, suggesting that CSP may have a secondary binding site on the cell surface. It is worth noting that a similar process was also attempted in *S. aureus*, where the CIP pheromone fused to colicin was shown to be able to kill *S. aureus*.

Two studies have recently revealed structure-function relationships between *S. mutans* CSP and its competence- and bacteriocin-inducing abilities. The first of these studies showed that a peptide similar to CSP but lacking the three

C-terminal residues was more potent than CSP at inducing competence, biofilm formation, and bacteriocin production (Petersen et al. 2006). A subsequent study examined the ability of synthetic CSP analogs to activate or inhibit competence and modulate expression from the *comC* promotor (Syvitski et al. 2007). The use of truncated CSP peptides in conjunction with circular dichroism and nuclear magnetic resonance revealed that the *S. mutans* CSP has at least two functional domains. The C-terminal end is required for activating the signal transduction pathway, while the alpha-helical core is required for binding to the ComD receptor. If three or more peptides were deleted from the C-terminus, genetic competence was not induced, but QS could be competitively inhibited in the presence of wild-type CSP. These data suggest that at least in vitro, modulation of the CSP regulon can be manipulated by CSP analogs. Further research into their effectiveness in animal and human models may demonstrate chemotherapeutic potential of these or other QS analogs.

4.2 QS Modulation of Infection in Group A Streptococcus

S. pyogenes, or group A streptococcus (GAS), is responsible for causing a number of infections in humans, from the most common form of bacterial pharyngitis (strep throat) to the highly invasive and often fatal necrotizing fasciitis (Cunningham 2000). It has been recently suggested that a functional QS peptide modulates the pathogenic potential of this bacterium (Hidalgo-Grass et al. 2004). The SilCR peptide, or SilC, has features typical of QS signal peptides in gram-positive bacteria (Hidalgo-Grass et al. 2004; Miller and Bassler 2001). The *sil* locus was originally described in an M14 serotype strain of *S. pyogene*s demonstrated to be involved in invasive disease and DNA transfer (Hidalgo-Grass et al. 2002). This five-gene locus encodes a putative two-component system (*silA* and *silB*), a region similar to an ATP-binding cassette transporter (*silD* and *silE*), and a small open reading frame preceded by a combox-like promoter (*silC*). The SilCR region encodes a predicted 41-aa propeptide with a typical glycine-glycine sequence motif that facilitates cleavage, producing a 17-aa mature peptide. SilCR is proposed to be a QS signal peptide that modulates the expression of several unidentified genes in *S. pyogenes* (Hidalgo-Grass et al. 2004).

The exogenous application of SilCR to infection wound sites was shown to affect the outcome of infection in mice inoculated with invasive isolates of *S. pyogenes*. In this study, decreased lesion formation and size were observed in mice infected with *S. pyogenes* in the presence of exogenously added SilCR (Hidalgo-Grass et al. 2004).

Subsequent genomic analyses of several M serotypes of *S. pyogenes* indicated that either the *silCR* gene or other genes in the operon were mutated or absent in the genomes of invasive isolates. It was suggested that SilCR has a regulatory effect on the invasive potential of *S. pyogenes*, somehow reducing invasiveness when present since strains with an intact expressed operon were considered noninvasive.

The therapeutic effect of SilCR was dose dependent and was shown to reduce lesion size with the addition of as little as 3.0 μg of peptide.

The mechanism of SilCR is believed to involve the downregulation of GAS chemokine trypsin-like protease activity responsible for an increase in the migration of neutrophils to the infection site, thus limiting bacterial spread (Hidalgo-Grass et al. 2006). Three serine proteases of *S. pyogenes* were believed to be modulated: X-propyl-dipeptidyl aminopeptidase (PepXP), HtrA/DegP, and PrtS/CspA, enzymes capable of degrading interleukin 8, thus inhibiting neutrophil recruitment to the site of infection.

Further study of SilCR will likely focus on PepXP and PrtS/CspA and other GAS genes and will determine whether SilCR directly affects the expression of host factors.

5 Conclusions and Future Perspectives

It is becoming apparent that QS is nearly always involved in streptococcal biofilm infections. It is therefore logical to study these processes and manipulate these signals to alter disease outcome. The three modes of action of signal perturbation discussed in this article involve application of native signal to alter the colonization stage of infection or addition of CSP analogs to either overstimulate or inhibit CSP action. These works are in their infancy, and further testing of these concepts in animal and human subjects will ultimately determine whether they are feasible therapeutic agents.

References

Cunningham MW (2000) Pathogenesis of group A streptococcal infections. Clin Microbiol Rev 13:470–511

Cvitkovitch DG, Li YH, Ellen RP (2003) Quorum sensing and biofilm formation in streptococcal infections. J Clin Invest 112:1626–1632

Eckert R, He J, Yarbrough DK, Qi F, Anderson MH, Shi W (2006) Targeted killing of Streptococcus mutans by a pheromone-guided “smart” antimicrobial peptide. Antimicrob Agents Chemother 50:3651–3657

Gilmore KS, Srinivas P, Akins DR, Hatter KL, Gilmore MS (2003) Growth, development, and gene expression in a persistent *Streptococcus gordonii* biofilm. Infect Immun 71:4759–4766

Guiral S, Mitchell TJ, Martin B, Claverys JP (2005) Competence-programmed predation of noncompetent cells in the human pathogen *Streptococcus pneumoniae*: genetic requirements. Proc Natl Acad Sci USA 102:8710–8715

Håvarstein LS, Holo H, Nes IF (1994) The leader peptide of colicin V shares consensus sequences with leader peptides that are common among peptide bacteriocins produced by gram-positive bacteria. Microbiology 140:2383–2389

Håvarstein LS, Coomaraswamy G, Morrison DA (1995a) An unmodified heptadecapeptide pheromone induces competence for genetic transformation in *Streptococcus pneumoniae*. Proc Natl Acad Sci USA 92:11140–11144

Håvarstein LS, Diep DB, Nes IF (1995b) A family of bacteriocin ABC transporters carry out proteolytic processing of their substrates concomitant with export. Mol Microbiol 16:229–240

Håvarstein LS, Gaustad P, Nes IF, Morrison DA (1996) Identification of the streptococcal competence-pheromone receptor. Mol Microbiol 21:863–869

Hidalgo-Grass C, Ravins M, Dan-Goor M, Jaffe J, Moses AE, Hanski E (2002) A locus of group A Streptococcus involved in invasive disease and DNA transfer. Mol Microbiol 46:87–99

Hidalgo-Grass C, Dan-Goor M, Maly A, Eran Y, Kwinn LA, Nizet V, Ravins M, Jaffe J, Peyser A, Moses AE, Hanski E (2004) Effect of a bacterial pheromone peptide on host chemokine degradation in group A streptococcal necrotising soft-tissue infections. Lancet 363:696–703

Hidalgo-Grass C, Mishalian I, Dan-Goor M, Belotserkovsky I, Eran Y, Nizet V, Peled A, Hanski E (2006) A streptococcal protease that degrades CXC chemokines and impairs bacterial clearance from infected tissues. EMBO J 25:4628–4637

Knutsen E, Ween O, Håvarstein LS (2004) Two separate quorum-sensing systems upregulate transcription of the same ABC transporter in *Streptococcus pneumoniae*. J Bacteriol 186:3078–3085

Kreth J, Merritt J, Shi W, Qi F (2005) Co-ordinated bacteriocin production and competence development: a possible mechanism for taking up DNA from neighbouring species. Mol Microbiol 57:392–404

Kreth J, Merritt J, Zhu L, Shi W, Qi F (2006) Cell density- and ComE-dependent expression of a group of mutacin and mutacin-like genes in *Streptococcus mutans*. FEMS Microbiol Lett 265:11–17

Kroes I, Lepp PW, Relman DA (1999) Bacterial diversity within the human subgingival crevice. Proc Natl Acad Sci USA 96:14547–14552

Li YH, Hanna MN, Svensater G, Ellen RP, Cvitkovitch DG (2001a) Cell density modulates acid adaptation in *Streptococcus mutans*: implications for survival in biofilms. J Bacteriol 183:6875–6884

Li YH, Lau PC, Lee JH, Ellen RP, Cvitkovitch DG (2001b) Natural genetic transformation of *Streptococcus mutans* growing in biofilms. J Bacteriol 183:897–908

Li YH, Tang N, Aspiras MB, Lau PC, Lee JH, Ellen RP, Cvitkovitch DG (2002) A quorum-sensing signaling system essential for genetic competence in *Streptococcus mutans* is involved in biofilm formation. J Bacteriol 184:2699–2708

Loo CY, Corliss DA, Ganeshkumar N (2000) *Streptococcus gordonii* biofilm formation: identification of genes that code for biofilm phenotypes. J Bacteriol 182:1374–1382

Lunsford RD, London J (1996) Natural genetic transformation in *Streptococcus gordonii*: comX imparts spontaneous competence on strain wicky. J Bacteriol 178:5831–5835

Luo P, Li H, Morrison DA (2003) ComX is a unique link between multiple quorum sensing outputs and competence in *Streptococcus pneumoniae*. Mol Microbiol 50:623–633

Luo P, Li H, Morrison DA (2004) Identification of ComW as a new component in the regulation of genetic transformation in *Streptococcus pneumoniae*. Mol Microbiol 54:172–183

Miller MB, Bassler BL (2001) Quorum sensing in bacteria. Annu Rev Microbiol 55:165–199

Morrison DA, Lee MS (2000) Regulation of competence for genetic transformation in *Streptococcus pneumoniae*: a link between quorum sensing and DNA processing genes. Res Microbiol 151:445–451

Moscoso M, Claverys JP (2004) Release of DNA into the medium by competent *Streptococcus pneumoniae*: kinetics, mechanism and stability of the liberated DNA. Mol Microbiol 54:783–794

Oggioni MR, Iannelli F, Ricci S, Chiavolini D, Parigi R, Trappetti C, Claverys JP, Pozzi G (2004) Antibacterial activity of a competence-stimulating peptide in experimental sepsis caused by *Streptococcus pneumoniae*. Antimicrob Agents Chemother 48:4725–4732

Oggioni MR, Trappetti C, Kadioglu A, Cassone M, Iannelli F, Ricci S, Andrew PW, Pozzi G (2006) Switch from planktonic to sessile life: a major event in pneumococcal pathogenesis. Mol Microbiol 61:1196–1210

Pestova EV, Håvarstein LS, Morrison DA (1996) Regulation of competence for genetic transformation in *Streptococcus pneumoniae* by an auto-induced peptide pheromone and a two-component regulatory system. Mol Microbiol 21:853–862

Petersen FC, Fimland G, Scheie AA (2006) Purification and functional studies of a potent modified quorum-sensing peptide and a two-peptide bacteriocin in *Streptococcus mutans*. Mol Microbiol 61:1322–1334

Qi F, Kreth J, Levesque CM, Kay O, Mair RW, Shi W, Cvitkovitch DG, Goodman SD (2005) Peptide pheromone induced cell death of *Streptococcus mutans*. FEMS Microbiol Lett 251:321–326

Steinmoen H, Knutsen E, Håvarstein LS (2002) Induction of natural competence in *Streptococcus pneumoniae* triggers lysis and DNA release from a subfraction of the cell population. Proc Natl Acad Sci USA 99:7681–7686

Steinmoen H, Teigen A, Håvarstein LS (2003) Competence-induced cells of *Streptococcus pneumoniae* lyse competence-deficient cells of the same strain during cocultivation. J Bacteriol 185:7176–7183

Sung CK, Morrison DA (2005) Two distinct functions of ComW in stabilization and activation of the alternative sigma factor ComX in *Streptococcus pneumoniae*. J Bacteriol 187:3052–3061

Syvitski RT, Tian XL, Sampara K, Salman A, Lee ST, Jakeman PL, Li YH (2007) Structure-activity analysis of quorum-sensing signaling peptides from *Streptococcus mutans*. J Bacteriol 189:1441–1450

Ironing Out the Biofilm Problem: The Role of Iron in Biofilm Formation

E. Peter Greenberg, Ehud Banin

Abstract The opportunistic pathogen *Pseudomonas aeruginosa* causes chronic biofilm-associated infections in the lungs of cystic fibrosis patients that cannot be eradicated by antibiotics. Like most other pathogens, *P. aeruginosa* is under intense competition with the host for iron. Recent studies show that even when there is sufficient iron for growth, this element serves as a signal for biofilm development. Here, we summarize our knowledge of the role iron plays in *P. aeruginosa* biofilm development. Novel therapeutic approaches that target iron homeostasis as an antibiofilm target are also presented.

1 Introduction to the Problem

P. aeruginosa exhibits considerable nutritional and metabolic versatility, which allows it to colonize a wide variety of environments. It can be isolated from soil, water, plants, and animals. Like most organisms, *P. aeruginosa* requires iron for growth, as iron serves as a cofactor for enzymes that are involved in many basic cellular functions and metabolic pathways (Andrews et al. 2003). Recent reports show that iron also serves as an environmental signal for biofilm development (Banin et al. 2005; Singh et al. 2002).

P. aeruginosa causes chronic biofilm-associated infections in the lungs of CF patients (Costerton et al. 1999; Lyczak et al. 2000; Parsek and Singh 2003; Singh et al. 2000). CF is an autosomal recessive disorder resulting from a mutation in a gene coding for a chloride ion channel, the CF transmembrane conductance regulator

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N. Balaban (ed.) *Control of Biofilm Infections by Signal Manipulation*
Springer Series on Biofilms 2
doi: 10.1007/978-3-540-73853-4, © Springer 2008

(reviewed in Rowe et al. 2005). This single genetic defect has pleiotropic effects on the development and function of several tissues and organs. The mutation results in a hypersusceptibility of CF patients to bacterial infection and to *P. aeruginosa* in particular. Chronic *P. aeruginosa* infection of the lungs currently accounts for the majority of the morbidity and mortality seen in CF (Chernish and Aaron 2003; Davies 2003).

Although the battle to treat and cure CF is far from over, in the last decade a substantial increase in the life span of CF patients has been achieved, mainly due to improvements in antimicrobial therapy and treatment regimes. In fact, in its 2006 patient registry data report, the American Cystic Fibrosis Foundation announced that in the last 4 years alone, more than 5 years have been added to the median survival age of CF patients, which currently stands at 36.8 years. Unfortunately, one must remember that despite this encouraging improvement, half of the patients suffering from CF will not reach this age, reemphasizing the need for novel antimicrobial therapeutic approaches that can effectively eradicate *P. aeruginosa* chronic infection in the lungs.

As an opportunistic pathogen, one of the biggest challenges for *P. aeruginosa* is the acquisition of iron in the host. While iron was readily available and soluble at the beginning of life on earth, the increase of oxygen in the atmosphere as our planet matured dramatically reduced iron solubility and exposed the toxic effects associated with this element due to its ability to mediate the production of oxygen radicals (Touati 2000). Bacteria have developed sophisticated mechanisms to scavenge iron from dilute environments and to maintain iron homeostasis (reviewed in Andrews et al. 2003). It is interesting that as life on earth continued to evolve, the role of iron as an essential element was maintained. In fact, it is so crucial for life that a central part of the mammalian and human host defense against invading pathogens is based on the ability to maintain free iron concentrations at extremely low levels ($< 10^{-18}$ M) (reviewed in Schaible and Kaufmann 2004). Proteins such as lactoferrin and transferrin that tightly bind iron provide an efficient first line of defense against bacterial infections. Thus, there is intense competition for iron with the host, and bacterial acquisition of iron is recognized as one of the key steps in the establishment of an infection (Ratledge and Dover 2000; Schaible and Kaufmann 2004).

Although the role of iron in supporting microbial growth has been known for many years, only recently has it been reported that this metal also serves as a signal for biofilm development. In this chapter we review the current knowledge of how iron mediates biofilm formation, and we discuss the potential of targeting iron uptake and homeostasis as a novel antibiofilm therapeutic approach.

2 Iron Acquisition and Regulation in *P. aeruginosa*

2.1 Iron Uptake

The ability of *P. aeruginosa* to colonize a wide range of environments is, not surprisingly, accompanied by a very sophisticated and versatile iron acquisition sys-

tem. Under iron-limiting conditions, *P. aeruginosa* synthesizes two bacterial ferric ion chelating molecules (i.e., siderophores), pyoverdine and pyochelin (reviewed in Poole and McKay 2003). These siderophores bind ferric ions and transport them into cells via specific outer membrane receptors in a process that is driven by the cytosolic membrane potential and is mediated by the energy-transducing TonB-ExbB-ExdD system (reviewed in Moeck and Coulton 1998). Of the two siderophores, pyoverdine has the higher affinity and can successfully compete for iron with transferrin and lactoferrin (Wolz et al. 1994; Xiao and Kisaalita 1997). Furthermore, mutants defective in pyoverdine synthesis and transport are avirulent in several animal infection models (Meyer et al. 1996; Takase et al. 2000). Interestingly, pyoverdine can also act as a signaling molecule. The binding of ferripyoverdine to the outer membrane receptor FpvA induces, via a cell surface signaling pathway consisting of two extracytoplasmic function (ECF) sigma factors, transcription of its receptor, genes required for the production of pyoverdine, and genes encoding several virulence factors (Beare et al. 2003; Lamont et al. 2002).

Beside its endogenous siderophores, *P. aeruginosa* is also able to utilize a variety of heterologous iron-binding molecules such as heme, enterobactin, ferric citrate, and ferrioxamine to acquire iron (reviewed in Poole and McKay 2003). Two receptors, PhuR and HasR, are involved in the utilization of heme (Ochsner et al. 2002), and two, PfeA and PirA, mediate the uptake of enterobactin (Dean and Poole 1993; Ghysels et al. 2005). Our laboratory has recently shown that *P. aeruginosa* can utilize ferric citrate via a FecI/R-like system (PA3899-3901) and ferrioxamine via two receptors, FoxA (PA2466) and FiuA (PA0470) (Banin et al. 2005). FiuA has also been shown to promote ferrichrome transport (Llamas et al. 2006). It is interesting to note that these receptors probably represent only a small sample of the real iron-acquisition potential of *P. aeruginosa*. An in silico analysis of the *P. aeruginosa* genome revealed an impressive total of 34 genes encoding putative TonB-dependent outer membrane receptors (Cornelis and Matthijs 2002). Combined with the endogenous siderophores, these may give *P. aeruginosa* the ability to "steal" iron from other microorganisms, providing a competitive advantage in the environment.

2.2 Iron Regulation

Because bacteria require iron, which in itself is toxic, cellular iron levels must be tightly regulated. Two independent microarray analyses have revealed that over 200 *P. aeruginosa* genes are differently regulated by iron (Ochsner et al. 2002; Palma et al. 2003). Like many other *Proteobacteria*, iron regulation in *P. aeruginosa* is orchestrated by the ferric uptake regulator (Fur), which appears to be essential for the viability of *P. aeruginosa* (Ochsner et al. 1995; Vasil and Ochsner 1999). Fur binds to specific DNA sequences (Fur boxes) and represses iron-responsive genes. Fur is dependent on intracellular iron because it requires Fe^{2+} as a cofactor for its DNA-binding activity. Although Fur is a repressor, it can also activate expression of specific genes (such as genes coding for iron storage proteins) through its control of a pair of small regulatory RNAs, PrrF1 and PrrF2 (Wilderman et al. 2004).

Fur is not the only regulator that governs the iron response of *P. aeruginosa*. Another group of regulators consists of the iron starvation ECF sigma factors (reviewed in Visca et al. 2002). These environmentally responsive transcription factors enable bacteria to respond to the presence of specific siderophores in the environment as well as to levels of intracellular iron. Visca et al. (2002) utilized the availability of the *P. aeruginosa* genome and identified 14 gene clusters that may encode iron-responsive ECF sigma factor-regulated systems. The best-studied member of this group in *P. aeruginosa* is the ECF sigma factor PvdS, which is known to regulate the synthesis of pyoverdine genes and genes coding for several virulence factors (Beare et al. 2003; Lamont et al. 2002). The combination of Fur, sRNAs, and iron starvation ECF sigma factors provides *P. aeruginosa* a highly regulated system that can efficiently respond to changes in extracellular and intracellular iron concentrations.

3 Iron and *P. aeruginosa* Biofilm Development

3.1 Low Iron – The Lactoferrin Effect

In 2002 Singh and colleagues reported that lactoferrin blocked *P. aeruginosa* microcolony formation on glass surfaces by virtue of its ability to sequester iron (Singh et al. 2002). These authors were intrigued by the ability of healthy hosts to prevent bacterial biofilm formation. They speculated that antimicrobial peptides and proteins produced by mammals might be able to interfere with biofilm development. Mucosal secretions are known to be rich in such antimicrobial factors as lactoferrin, lysozyme, and defensins. The authors' initial study focused on lactoferrin because it is abundant in mucosal secretions. Lactoferrin is a mammalian iron chelator that can limit bacterial growth by sequestering iron. It also has bactericidal activity involving its ability to bind lipopolysaccharides and disrupt membranes (reviewed in Orsi 2004). The effect of subgrowth inhibitory concentrations of lactoferrin on biofilm development was examined (Singh et al. 2002). Using a continuous flow-cell biofilm system as a model to study biofilm development, the authors grew *P. aeruginosa* PAO1 carrying a green-fluorescent-protein-containing plasmid in the presence and absence of lactoferrin. In the absence of lactoferrin, *P. aeruginosa* cells attached and within 48 h formed microcolonies that developed to characteristic mushroom-like structures by 96 h (Fig. 1). In the presence of a subinhibitory concentration of lactoferrin, cells attached and multiplied but failed to form microcolonies and structured biofilms. Rather, they remained as a flat thin layer of cells attached to the glass surface (Fig. 1). Iron-saturated lactoferrin did not cause this inhibition, suggesting that the antibiofilm activity was dependent on lactoferrin's iron chelation activity.

To further investigate the antibiofilm activity of lactoferrin, Singh et al. (Singh et al. 2002; Singh 2004) carried out time-lapse microscopy experiments. They observed that in the absence of lactoferrin, cells attached to the surface and multiplied, and the daughter cells in most cases remained near the point of division.

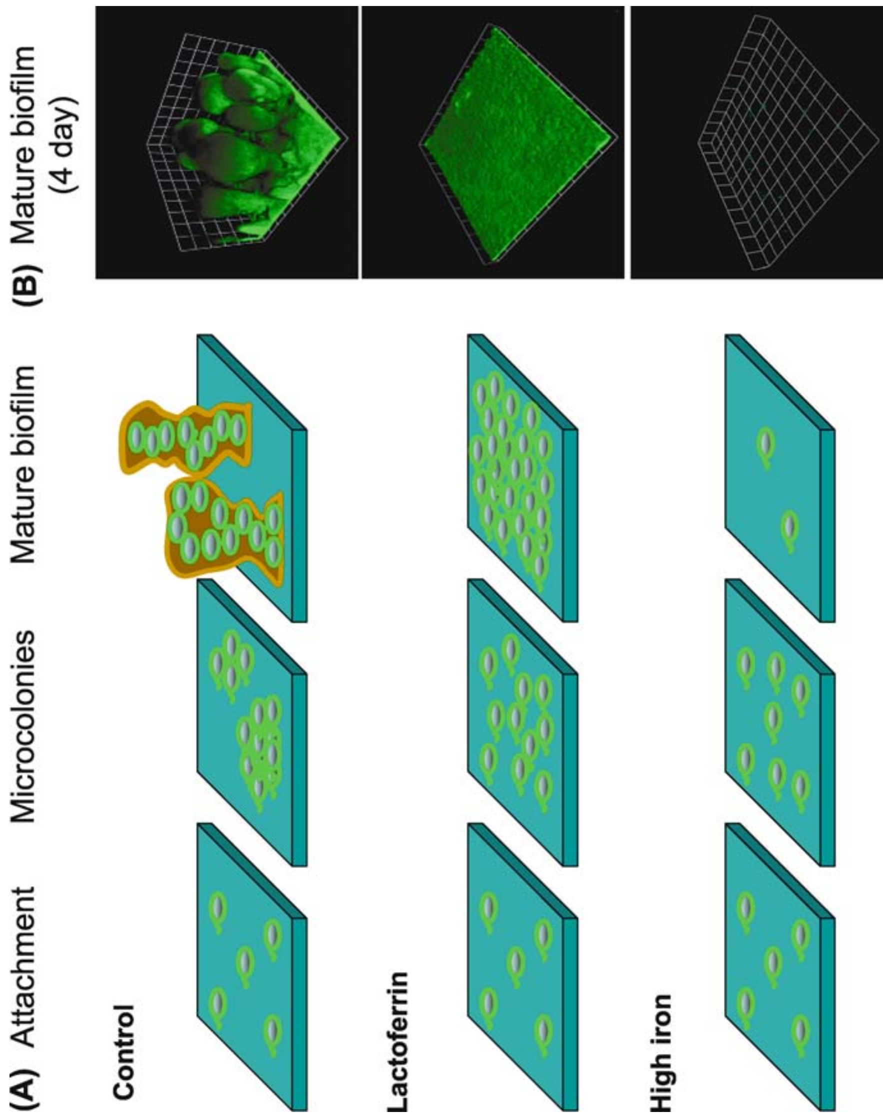

Fig. 1 Role of iron in *P. aeruginosa* biofilm development. **A** Schematic representation of the effects lactoferrin and high iron have on development of *P. aeruginosa* biofilms. Normally, bacteria attach, multiply, and develop into microcolonies that mature into structured biofilms. In the presence of subinhibitory concentrations of lactoferrin, the cells show increased surface motility; they attach and multiply, but daughter cells move away from the point of replication and thus do not form microcolonies or structured biofilms. Later, a thin, flat cell layer develops on the surface. In the presence of high iron, cells attach normally and multiply but do not remain attached to the glass surface, and biofilms do not form. **B** Effects of lactoferrin and high iron on *P. aeruginosa* biofilm development. GFP-labeled *P. aeruginosa* was grown for 4 days in continuous culture flow cells. The growth medium was 1% TSB (control) with subinhibitory concentrations of lactoferrin (20 μg/ml) or $FeCl_3$ (200 μM). Reconstructed three-dimensional images are presented; squares are 23 μm/side

This behavior was maintained as cells continued to divide and led to the formation of microcolonies. In the presence of lactoferrin, however, daughter cells used surface-associated motility, presumably twitching motility, to move away from the point of division. This constant movement on the surface interfered with the formation of sessile structures. The authors hypothesized that by chelating iron, lactoferrin induced twitching motility, which caused the cells to wander across the surface and thus disrupted their ability to settle and form structured communities. As support for this hypothesis, Singh et al. (2002) showed, by using a standard assay for twitching motility, that low iron induced *P. aeruginosa* twitching. Furthermore, they studied a twitching mutant in the flow-cell. Although not exactly resembling the wild-type biofilms in the absence of lactoferrin, the mutant was able to form microcolonies and structured biofilms even in the presence of lactoferrin.

The importance of iron for bacterial growth has been known for many years. The study by Singh et al. provided evidence that even when there is sufficient iron for planktonic growth, this element serves as a signal for biofilm development. In a recent paper, Berlutti et al. (2005) provided further evidence for the role of iron in cell aggregation and biofilm formation. They reported that in the liquid phase, iron limitation (1 μM) induced motility and transition to the planktonic mode of growth, while high iron concentrations (100 μM) facilitated cell aggregation and biofilm formation.

To further investigate the role of iron in biofilm development, we utilized a genetic approach and systematically screened mutants defective in iron acquisition and regulation for biofilm formation in the presence and absence of lactoferrin (Banin et al. 2005). Initially we examined the role of the two endogenous siderophores (pyoverdine and pyochelin) in biofilm formation. While biofilms formed by a pyochelin mutant were indistinguishable from the wild type, a pyoverdine mutant formed flat biofilms even in the absence of lactoferrin. Complementing the mutations or adding a conditioned medium containing pyoverdine restored normal biofilm development to the pyoverdine mutant. Because pyoverdine serves as an iron chelator and a signaling molecule (Lamont et al. 2002), we wanted to distinguish between these activities. We reasoned that if the activity is mediated through iron uptake, then the addition of iron should complement the mutation; however, if the activity is due to signaling, then the addition of iron would not influence biofilm development in a pyoverdine mutant. The addition of 50 μM iron was sufficient to restore biofilm formation in a pyoverdine mutant. This result suggested that the pyoverdine iron uptake activity, and not the signaling activity, is important for biofilm development. We also asked whether additional heterologous siderophores can restore biofilm formation in a pyoverdine mutant. We demonstrated that ferric citrate and ferrioxamine can both be utilized by *P. aeruginosa* pyoverdine mutant and that the pyoverdine mutant formed normal biofilms when these iron chelators were added to the culture medium. A *P. aeruginosa* genome sequence analysis revealed homologs of the ferric citrate and ferrioxamine uptake systems, and we showed that these systems are involved in the uptake of citrate or ferrioxamine chelated iron.

Our genetic analysis (Banin et al. 2005) indicated that *P. aeruginosa* requires an active iron uptake system to support biofilm development in vitro. It can utilize its endogenous siderophores pyoverdine (at low iron concentrations) and pyochelin (at high iron concentrations), or it can utilize other chelators for which it has an uptake system. Furthermore, by systemically screening mutants with defects in putative iron starvation ECF-sigma factors, we concluded that these systems are most likely not directly involved in signaling of biofilm development, but rather facilitate iron uptake of heterologous iron sources. Based on these results, we speculated that internal iron concentration is critical for normal biofilm development. Consistent with this hypothesis, mutants with abnormally functional Fur organized into structured biofilms under low iron, a condition in which the parent formed flat biofilms. The two known Fur-controlled small regulatory RNAs (PrrF1 and F2; Wilderman et al. 2004) did not appear to mediate iron control of biofilm development.

Besides being an essential nutrient for growth and a signal for biofilm development, iron stabilizes the extracellular polymeric matrix of *P. aeruginosa* biofilms (Chen and Stewart 2002). The viscosity of a mixed *P. aeruginosa–Klebsiella pneumoniae* biofilm suspension is affected by various cations. The addition of iron salts significantly increases biofilm viscosity. Electrostatic interactions contribute to biofilm cohesion, and iron cations are potent cross-linkers of the biofilm matrix. In support of these findings, we have demonstrated that chelation of iron, mediated by the addition of ethylenediamine tetraacetic acid (50 mM), initiated the detachment of cells from mature *P. aeruginosa* biofilms (Banin et al. 2006). Taken together, these results demonstrate that iron is important both as a signal for biofilm development and as a stabilizing cation of the biofilm matrix.

3.2 High Iron

The role of iron in biofilm development became even more intriguing with the work of Musk et al. (2005). Using a high throughput biofilm screen, the authors tested a library of over 4500 compounds for nonantibiotic biofilm inhibitors against *P. aeruginosa* PA14. One of the compounds that inhibited biofilm formation was ferric ammonium citrate. Neither ammonium nor citrate ion was responsible for this activity. Conversely, when other iron salts (ferric chloride, ferric sulfate, and ferrous sulfate) were examined, they showed biofilm inhibition similar to that of ammonium ferric citrate inhibition. The biofilm inhibition was significant at iron concentrations $>100\ \mu M$, but this iron level did not affect *P. aeruginosa* growth. The authors utilized a flow-cell system to further characterize the inhibitory effect of iron on biofilm formation. Growing *P. aeruginosa* in flow cells in the presence of $200\ \mu M$ ferric ammonium citrate completely inhibited biofilm formation, while in the absence of added iron, the cells formed robust structured biofilms (Fig. 1). The inhibition of biofilm formation was not due to reduced adhesion of cells to the surface, and initial biofilm formation at 10 h was similar under both conditions. Rather,

iron seemed to disrupt the later stages of biofilm development, leaving very few cells adhering to the surface by 48 h. Fourteen of the 17 clinical isolates that could form biofilms (82%) displayed >40% inhibition in biofilm formation when grown in the presence of 250 μM ferric ammonium citrate, and 41% had >60% inhibition. Interestingly, three of the strains were resistant to the treatment but were not further characterized.

Exposing mature biofilms to ferric ammonium citrate (200 μM) caused biofilms to disperse, resulting in complete clearing of the biofilms after 5 days (Musk et al. 2005). Thus, excess iron not only blocked biofilm formation but also disrupted existing biofilms. It is important to note that the addition of nutrients (such as carbon sources) to existing biofilms has been shown to induce biofilm dispersal, and this could be a more general response to nutrient excess rather than a specific response to iron (Sauer et al. 2004). The work by Musk et al. suggests that in vitro there is actually an optimal range of iron for biofilm growth and maintenance: approximately 1–100 μM) (Musk et al. 2005). When the iron concentration is too low (<1 μM) or too high (>100 μM), cells will abort normal biofilm development. It remains unclear by which mechanism high iron inhibits biofilm development and triggers dispersal.

4 Iron and Biofilm Formation In Vivo

The studies demonstrating an involvement of iron (both high and low) in biofilm development and persistence were carried out in vitro using biofilms grown on abiotic surfaces. An important question asks how relevant these findings are to the situation in vivo. The literature contains several interesting observations regarding the iron content in the CF lung that may shed light on the role iron plays in the ability to form biofilms in vivo.

4.1 Lactoferrin Activity in the Cystic Fibrosis Lung

An important piece of evidence supporting the in vitro findings comes from studies evaluating the activity of lactoferrin. Britigan et al. (1993) initially reported that lactoferrin underwent proteolytic cleavage in the lungs of CF patients, having detected lactoferrin cleavage products in bronchoalveolar lavage (BAL) samples from patients with CF. Such cleavage products were not detected in BAL samples from healthy controls. The authors also demonstrated partial cleavage of lactoferrin when lactoferrin was incubated with high concentrations of *Pseudomonas* elastase (an alkaline protease) and neutrophil elastase for prolonged periods. The authors speculated that this in vivo cleavage of lactoferrin and transferrin by neutrophil- and/or bacterial-derived proteases may have an important role in the acquisition of iron in the lungs by *P. aeruginosa*. In a more recent study, Jesaitis et al. (2003), investigating neutrophil interactions with *Pseudomonas* biofilms, postulated that a signif-

icant fraction of lactoferrin released during neutrophil degranulation was probably degraded by neutrophil-derived proteases. Rogan et al. (2004) demonstrated that cathepsins (lysosomal proteases) in CF secretions are responsible for complete and rapid cleavage of lactoferrin and that this cleavage results in the loss of lactoferrin's microbicidal and antibiofilm activity. Furthermore, lactoferrin levels and activity in sputum obtained from *P. aeruginosa*-infected and noninfected CF patients were compared. *P. aeruginosa*-positive sputum showed significantly higher cathepsin activity and lower levels of lactoferrin than *P. aeruginosa*-negative sputum. There was also reduced antimicrobial and antibiofilm lactoferrin activity in *P. aeruginosa*-positive compared with *P. aeruginosa*-negative sputum samples. Taken together, these studies suggest that lactoferrin in the CF lung is highly susceptible to cleavage and has substantial lower antimicrobial and antibiofilm activity compared with lactoferrin in healthy individuals.

4.2 Iron in the Cystic Fibrosis Lung

In light of the lactoferrin results, what is the iron content in the CF lung? It is estimated that the levels of free iron in human secretions are maintained at extremely low concentrations ($>10^{-18}$ M), which limits the growth of invading pathogens. Apparently, a high percentage of CF patients suffer from iron deficiency, which is attributed to a combination of factors such as poor dietary intake, inflammation, and gastrointestinal problems (Ehrhardt et al. 1987; Pond et al. 1996). In this condition the patient has a much lower iron pool than a healthy individual, and this implies that an invading bacterial pathogen might encounter even lower iron concentrations. However, work examining the iron content in the lungs suggests that despite the iron deficiency (which is measured as the amount of iron in the serum), sputum from CF patients is actually replete with iron. Stites et al. (1998, 1999) have examined the iron and ferritin content in the sputum of CF patients, and their results demonstrate that the concentrations of extracellular iron and ferritin are substantially higher in CF patients compared with healthy individuals. In addition, Reid et al. (2002) examined the correlation between iron deficiency, lung disease, and *P. aeruginosa* chronic infection. They demonstrated that iron and ferritin levels in the sputum of CF patients are much higher than in normal controls. These studies imply that the iron content in the lungs of CF patients is higher, which is advantageous for biofilm formation. However, we note that the confounding factor in these investigations is that the patients were already colonized with bacteria, which probably changed the environmental iron concentration.

4.3 *P. aeruginosa* Response to Iron In Vivo

It is well known that invading pathogens are in intense competition for iron with the host. However, iron content in the CF lungs might be substantially different from

that in healthy individuals, providing favorable conditions for infection and biofilm formation. Therefore, evaluating the iron response of *P. aeruginosa* in the lungs may serve as a marker for the type of environment the bacteria actually face. Haas et al. (1991) initially reported the identification of pyoverdine in sputum isolated from CF patients. This led to the conclusion that *P. aeruginosa* in the lungs most likely experiences a low iron environment and in turn secretes siderophores to scavenge iron. Wang et al. (1996) used an in vivo selection system (IVET) to identify *P. aeruginosa* genes that are specifically induced by respiratory mucus derived from CF patients. One of the genes they identified was FptA (the receptor for pyochelin); the induction was suppressed by the addition of exogenous iron, further demonstrating that the mucus is iron depleted.

It is now recognized that *P. aeruginosa* isolates from CF patients appear to originate from the environment but adapt to the milieu of the patient's airway. Beckmann et al. (2005) attempted to characterize the antigen response in early-infected CF patients during their first year of infection. By screening serum from these patients against a phage display peptide library, the authors were able to identify over 150 reactive clones. When these were sequenced and compared with the *P. aeruginosa* published genome, 76 genes encoding outer membrane and secreted proteins were identified. The majority of these were proteins involved in small-molecule transport, membrane structural proteins, and secreted factors. Interestingly, several of the genes identified by this technique were involved in iron acquisition (e.g., FptA and several siderophore receptor proteins). Furthermore, the transcriptome responses of *P. aeruginosa* exposed to differentiated human epithelia has revealed a significant overlap with the iron-responsive regulon (Chugani and Greenberg 2007). Of the set of 97 genes identified in a previous study as induced upon growth in iron-deficient conditions, 80% were induced when *P. aeruginosa* was exposed to epithelia. Taken together, these studies suggest that the bacteria are challenged by the deficiency of available iron in the epithelial milieu during the initial phase of infection.

If one continues to follow the adaptation of *P. aeruginosa* as a marker for the iron content in the airways, there is evidence that this condition may change as the infection progresses. A study examining genomic variation in *P. aeruginosa* strains isolated from children with CF (6 months to 8 years of age) revealed that several strains had a deletion in the pyoverdine locus, which eliminated biosynthesis and uptake of pyoverdine (Ernst et al. 2003). It actually appears that the pyoverdine locus is highly divergent and that some of the *pvd* genes may be under positive selective pressure (Smith et al. 2005). De Vos et al. have also reported that pyoverdine synthesis mutants seemed to accumulate as a function of the lung colonization time (De Vos et al. 2001). Although this is highly speculative, the accumulation of mutants that lose their major and most powerful siderophore (i.e., pyoverdine) may imply that the conditions in the lungs (as the disease progresses) change during colonization from iron-limiting to iron-sufficient.

5 Iron as a Signal for Biofilm Formation in Other Bacteria

Is iron involved in signaling biofilm development of other bacterial species? And if so, what is the mechanism? We found several reports in the literature that have addressed these questions (summarized in Table 1). O'Toole and Kolter (1998b) reported that, similar to our observation of *P. aeruginosa*, the addition of iron could restore biofilm development by several *Pseudomonas fluorescens* attachment mutants. *Vibrio cholerae* also requires iron for normal biofilm development. A wild-type *V. cholera* strain formed reduced biofilms in an iron-deficient medium compared with iron-replete conditions (Mey et al. 2005). This iron response is mediated in part by Fur through the sRNA *ryhB*. A *ryhB* mutant showed reduced chemotaxis in low iron and was unable to form wild-type biofilms. The biofilm defect was suppressed by the addition of iron (Mey et al. 2005).

Interestingly, we also found several reports on the negative regulation of iron on biofilm formation. In the gram-negative human pathogen *Acinetobacter baumannii*, biofilm formation is significantly enhanced under low iron conditions (Tomaras et al. 2003). The authors characterized a novel chaperone/usher pilus system that is involved in biofilm development and identified a potential Fur box upstream of the first gene in the operon. The authors speculated that this Fur-mediated pilus synthesis results in reduced biofilm formation under high iron conditions. Two gram-positive bacteria (*S. mutans* and *S. aureus*) also demonstrated improved biofilm formation under iron-depleted conditions. Berlutti et al. (2004) reported that iron-deprived saliva ($<0.1\ \mu M\ Fe^{3+}$) increases *S. mutans* aggregation and biofilm formation, whereas iron-loaded saliva ($>1\ \mu M\ Fe^{3+}$) inhibits both phenomena. These results suggest that aggregation and biofilm formation are negatively controlled by iron. The authors hypothesized that lactoferrin that is readily found in saliva may mediate this response. In agreement with this hypothesis, subinhibitory concentrations of lactoferrin stimulated biofilm formation, while iron-saturated lactoferrin decreased aggregation and biofilm formation (Berlutti et al. 2004). In *Staphylococcus aureus*, iron depletion was shown to promote biofilm formation, and this response was partially regulated by Fur (Johnson et al. 2005).

Table 1 Iron and biofilm development in diverse bacteria

Strain	Biofilm development in low iron	Motile organism	Mode of regulation	Refs.
P. aeruginosa	Reduced	Yes	Fur	Banin et al. 2005; Singh et al. 2002
P. fluorescens	Reduced	Yes	Unknown	O'Toole and Kolter 1998b
V. cholera	Reduced	Yes	Fur, RyhB	Mey et al. 2005
A. baumannii	Increased	No	Fur (?)	Tomaras et al. 2003
S. mutans	Increased	No	Unknown	Berlutti et al. 2004
S. aureus	Increased	No	Fur (partially)	Johnson et al. 2005

One common theme that arises when comparing the role iron plays in mediating biofilm development in all these bacterial species is motility (see Table 1). The three bacterial species that showed reduced biofilm formation under low iron conditions are motile bacteria (*P. aeruginosa*, *P. fluorescens*, and *V. cholera*). All of the non-motile bacteria (*A. baumannii*, *S. mutans*, and *S. aureus*) had induced biofilm formation under iron-depleted conditions. Motility is crucial to biofilm development in several motile species (O'Toole and Kolter 1998a; O'Toole et al. 2000). Furthermore, in the case of *P. aeruginosa*, induction of surface motility has provided an explanation for impaired biofilm formation in the presence of lactoferrin (Singh et al. 2002; Singh 2004). An important question is whether surface motility is directly controlled by iron directly or whether iron controls cell–cell adhesion, which in turn affects motility and biofilm formation. Further work will be needed to characterize the role motility plays in mediating the dependence of biofilm development on iron and how it is regulated.

6 Iron Metabolism as an Antibiofilm Target

The absolute requirement of iron for growth in most bacteria makes iron an appealing target for antimicrobial therapy. An initial concept was to replace Fe^{3+} with a nonmetabolizable metal ion such as scandium or indium in an effort to block iron uptake by competitive inhibition of the transport proteins (Rogers et al. 1982). Although initial results were encouraging for Sc^{3+} treatment of *Escherichia coli* and *Klebsiellla pneumoniae* mouse infections (Rogers et al. 1980), Sc^{3+} proved toxic to the animals (Plaha and Rogers 1983). A similar approach was taken in *Mycobacterium tuberculosis* using molybdenum, cobalt, and scandium, but with no major effect (Barclay and Ratledge 1986). A promising development came with the use of gallium-portoporphyrin, which had antimicrobial activity against *Yersinia enterocolitica*, methicillin-resistant *S. aureus*, and *Mycobacterium smegmatis* (Stojiljkovic et al. 1999). The gallium-porphyrin complex enters bacteria via heme transport systems and was assumed to interfere with respiration, generating reactive oxygen radicals that presumably killed the cells (Stojiljkovic et al. 1999). The gallium complexes had no toxic effect on human fibroblasts or other animal cell lines, further highlighting the potential for clinical use.

Another strategy to target bacterial homeostasis was the use of siderophore-antibiotic conjugates. This "Trojan horse" approach is based on molecules found in nature, such as albomycins, which contain an iron-binding domain (similar to ferrichrome) and a toxic molecule. Several microorganisms can introduce albomycin through the ferrichrome uptake system. Once internalized, the toxic molecule is cleaved and damages the cell (Benz et al. 1982). Imitating this strategy, several groups have chemically synthesized siderophore-antibiotic complexes and tested them for their antimicrobial activity. The most common approach was to conjugate beta-lactam antibiotics with a siderophore moiety (Ghosh et al. 1996; Ghosh and Miller 1995). These complexes show excellent anti-*Pseudomonas* activity in vitro

and in several animal infection models (Basker et al. 1984, 1989; Budzikiewicz 2001). The emerging data on the role of iron as a *P. aeruginosa* biofilm signal suggests that these Trojan-horse approaches might be particularly well suited for treating chronic *P. aeruginosa* infections.

References

Andrews SC, Robinson AK, Rodriguez-Quinones F (2003) Bacterial iron homeostasis. FEMS Microbiol Rev 27:215–237

Banin E, Vasil ML, Greenberg EP (2005) Iron and *Pseudomonas aeruginosa* biofilm formation. Proc Natl Acad Sci USA 102:11076–11081

Banin E, Brady KM, Greenberg EP (2006) Chelator-induced dispersal and killing of *Pseudomonas aeruginosa* cells in a biofilm. Appl Environ Microbiol 72:2064–2069

Barclay R, Ratledge C (1986) Metal analogues of mycobactin and exochelin fail to act as effective antimycobacterial agents. Zentralbl Bakteriol Mikrobiol Hyg [A] 262:203–207

Basker MJ, Edmondson RA, Knott SJ, Ponsford RJ, Slocombe B, White SJ (1984) In vitro antibacterial properties of BRL 36650, a novel 6 alpha-substituted penicillin. Antimicrob Agents Chemother 26:734–740

Basker MJ, Frydrych CH, Harrington FP, Milner PH (1989) Antibacterial activity of catecholic piperacillin analogues. J Antibiot (Tokyo) 42:1328–1330

Beare PA, For RJ, Martin LW, Lamont IL (2003) Siderophore-mediated cell signalling in *Pseudomonas aeruginosa*: divergent pathways regulate virulence factor production and siderophore receptor synthesis. Mol Microbiol 47:195–207

Beckmann C, Brittnacher M, Ernst R, Mayer-Hamblett N, Miller SI, Burns JL (2005) Use of phage display to identify potential *Pseudomonas aeruginosa* gene products relevant to early cystic fibrosis airway infections. Infect Immun 73:444–452

Benz G, Schroder T, Kurz J, Winsche CKW, Steffens G (1982) Constitution of the desferriform of the albomycins d1, d2. Angew. Chem Int 21:527–528

Berlutti F, Ajello M, Bosso P, Morea C, Petrucca A, Antonini G, Valenti P (2004) Both lactoferrin and iron influence aggregation and biofilm formation in *Streptococcus mutans*. Biometals 17:271–278

Berlutti F, Morea C, Battistoni A, Sarli S, Cipriani P, Superti F, Ammendolia MG, Valenti P (2005) Iron availability influences aggregation, biofilm, adhesion and invasion of *Pseudomonas aeruginosa* and *Burkholderia cenocepacia*. Int J Immunopathol Pharmacol 18:661–670

Britigan BE, Hayek MB, Doebbeling BN, Fick RB Jr (1993) Transferrin and lactoferrin undergo proteolytic cleavage in the *Pseudomonas aeruginosa*-infected lungs of patients with cystic fibrosis. Infect Immun 61:5049–5055

Budzikiewicz H (2001) Siderophore-antibiotic conjugates used as trojan horses against *Pseudomonas aeruginosa*. Curr Top Med Chem 1:73–82

Chen X, Stewart PS (2002) Role of electrostatic interactions in cohesion of bacterial biofilms. Appl Microbiol Biotechnol 59:718–720

Chernish RN, Aaron SD (2003) Approach to resistant gram-negative bacterial pulmonary infections in patients with cystic fibrosis. Curr Opin Pulm Med 9:509–515

Chugani S, Greenberg EP (2007) The influence of human respiratory epithelia on *Pseudomonas aeruginosa* gene expression. Microb Pathog 42:29–35

Cornelis P, Matthijs S (2002) Diversity of siderophore-mediated iron uptake systems in fluorescent pseudomonads: not only pyoverdines. Environ Microbiol 4:787–798

Costerton JW, Stewart PS, Greenberg EP (1999) Bacterial biofilms: a common cause of persistent infections. Science 284:1318–1322

Davies D (2003) Understanding biofilm resistance to antibacterial Agents. Nat Rev Drug Discov 2:114–122

De Vos D, De Chial M, Cochez C, Jansen S, Tummler B, Meyer JM, Cornelis P (2001) Study of pyoverdine type and production by *Pseudomonas aeruginosa* isolated from cystic fibrosis patients: prevalence of type II pyoverdine isolates and accumulation of pyoverdine-negative mutations. Arch Microbiol 175:384–388

Dean CR, Poole K (1993) Cloning and characterization of the ferric enterobactin receptor gene (*pfeA*) of *Pseudomonas aeruginosa*. J Bacteriol 175:317–324

Ehrhardt P, Miller MG, Littlewood JM (1987) Iron deficiency in cystic fibrosis. Arch Dis Child 62:185–187

Ernst RK, D'Argenio DA, Ichikawa JK, Bangera MG, Selgrade S, Burns JL, Hiatt P, McCoy K, Brittnacher M, Kas A, Spencer DH, Olson MV, Ramsey BW, Lory S, Miller SI (2003) Genome mosaicism is conserved but not unique in *Pseudomonas aeruginosa* isolates from the airways of young children with cystic fibrosis. Environ Microbiol 5:1341–1349

Ghosh M, Miller MJ (1995) Design, synthesis, and biological evaluation of isocyanurate-based antifungal and macrolide antibiotic conjugates: iron transport-mediated drug delivery. Bioorg Med Chem 3:1519–1525

Ghosh A, Ghosh M, Niu C, Malouin F, Moellmann U, Miller MJ (1996) Iron transport-mediated drug delivery using mixed-ligand siderophore-beta-lactam conjugates. Chem Biol 3:1011–1019

Ghysels B, Ochsner U, Mollman U, Heinisch L, Vasil M, Cornelis P, Matthijs S (2005) The *Pseudomonas aeruginosa pirA* gene encodes a second receptor for ferrienterobactin and synthetic catecholate analogues. FEMS Microbiol Lett 246:167–174

Haas B, Kraut J, Marks J, Zanker SC, Castignetti D (1991) Siderophore presence in sputa of cystic fibrosis patients. Infect Immun 59:3997–4000

Jesaitis AJ, Franklin MJ, Berglund D, Sasaki M, Lord CI, Bleazard JB, Duffy JE, Beyenal H, Lewandowski Z (2003) Compromised host defense on *Pseudomonas aeruginosa* biofilms: characterization of neutrophil and biofilm interactions. J Immunol 171:4329–4339

Johnson M, Cockayne A, Williams PH, Morrissey JA (2005) Iron-responsive regulation of biofilm formation in *Staphylococcus aureus* involves fur-dependent and fur-independent mechanisms. J Bacteriol 187:8211–8215

Lamont IL, Beare PA, Ochsner U, Vasil AI, Vasil ML (2002) Siderophore-mediated signaling regulates virulence factor production in *Pseudomonas aeruginosa*. Proc Natl Acad Sci USA 99:7072–7077

Llamas MA, Sparrius M, Kloet R, Jimenez CR, Vandenbroucke-Grauls C, Bitter W (2006) The heterologous siderophores ferrioxamine B and ferrichrome activate signaling pathways in *Pseudomonas aeruginosa*. J Bacteriol 188:1882–1891

Lyczak JB, Cannon CL, Pier GB (2000) Establishment of *Pseudomonas aeruginosa* infection: lessons from a versatile opportunist. Microbes Infect 2:1051–1060

Mey AR, Craig SA, Payne SM (2005) Characterization of *Vibrio cholerae* RyhB: the RyhB regulon and role of *ryhB* in biofilm formation. Infect Immun 73:5706–5719

Moeck GS, Coulton JW (1998) TonB-dependent iron acquisition: mechanisms of siderophore-mediated active transport. Mol Microbiol 28:675–681

Musk DJ, Banko DA, Hergenrother PJ (2005) Iron salts perturb biofilm formation and disrupt existing biofilms of *Pseudomonas aeruginosa*. Chem Biol 12:789–796

Ochsner UA, Vasil AI, Vasil ML (1995) Role of the ferric uptake regulator of *Pseudomonas aeruginosa* in the regulation of siderophores and exotoxin A expression: purification and activity on iron-regulated promoters. J Bacteriol 177:7194–7201

Ochsner UA, Wilderman PJ, Vasil AI, Vasil ML (2002) GeneChip expression analysis of the iron starvation response in *Pseudomonas aeruginosa*: identification of novel pyoverdine biosynthesis genes. Mol Microbiol 45:1277–1287

Orsi N (2004) The antimicrobial activity of lactoferrin: current status and perspectives. Biometals 17:189–196

O'Toole GA, Kolter R (1998a) Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. Mol Microbiol 30:295–304

O'Toole GA, Kolter R (1998b) Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signaling pathways: a genetic analysis. Mol Microbiol 28:449–461

O'Toole GA, Gibbs KA, Hager PW, Phibbs PV Jr, Kolter R (2000) The global carbon metabolism regulator Crc is a component of a signal transduction pathway required for biofilm development by *Pseudomonas aeruginosa*. J Bacteriol 182:425–431

Palma M, Worgall S, Quadri LE (2003) Transcriptome analysis of the *Pseudomonas aeruginosa* response to iron. Arch Microbiol 180:374–379

Parsek MR, Singh PK (2003) Bacterial biofilms: an emerging link to disease pathogenesis. Annu Rev Microbiol 57:677–701

Plaha DS, Rogers HJ (1983) Antibacterial effect of the scandium complex of enterochelin. Studies of the mechanism of action. Biochim Biophys Acta 760:246–255

Pond MN, Morton AM, Conway SP (1996) Functional iron deficiency in adults with cystic fibrosis. Respir Med 90:409–413

Poole K, McKay GA (2003) Iron acquisition and its control in *Pseudomonas aeruginosa*: many roads lead to Rome. Front Biosci 8:d661–686

Ratledge C, Dover LG (2000) Iron metabolism in pathogenic bacteria. Annu Rev Microbiol 54:881–941

Reid DW, Withers NJ, Francis L, Wilson JW, Kotsimbos TC (2002) Iron deficiency in cystic fibrosis: relationship to lung disease severity and chronic *Pseudomonas aeruginosa* infection. Chest 121:48–54

Rogan MP, Taggart CC, Greene CM, Murphy PG, O'Neill SJ, McElvaney NG (2004) Loss of microbicidal activity and increased formation of biofilm due to decreased lactoferrin activity in patients with cystic fibrosis. J Infect Dis 190:1245–1253

Rogers HJ, Synge C, Woods VE (1980) Antibacterial effect of scandium and indium complexes of enterochelin on *Klebsiella pneumoniae*. Antimicrob Agents Chemother 18:63–68

Rogers HJ, Woods VE, Synge C (1982) Antibacterial effect of the scandium and indium complexes of enterochelin on *Escherichia coli*. J Gen Microbiol 128:2389–2394

Rowe SM, Miller S, Sorscher EJ (2005) Cystic fibrosis. N Engl J Med 352:1992–2001

Sauer K, Cullen MC, Rickard AH, Zeef LA, Davies DG, Gilbert P (2004) Characterization of nutrient-induced dispersion in *Pseudomonas aeruginosa* PAO1 biofilm. J Bacteriol 186:7312–7326

Schaible UE, Kaufmann SH (2004) Iron and microbial infection. Nat Rev Microbiol 2:946–953

Singh PK (2004) Iron sequestration by human lactoferrin stimulates *P. aeruginosa* surface motility and blocks biofilm formation. Biometals 17:267–270

Singh PK, Schaefer AL, Parsek MR, Moninger TO, Welsh MJ, Greenberg EP (2000) Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. Nature 407:762–764

Singh PK, Parsek MR, Greenberg EP, Welsh MJ (2002) A component of innate immunity prevents bacterial biofilm development. Nature 417:552–555

Smith EE, Sims EH, Spencer DH, Kaul R, Olson MV (2005) Evidence for diversifying selection at the pyoverdine locus of *Pseudomonas aeruginosa*. J Bacteriol 187:2138–2147

Stites SW, Walters B, O'Brien-Ladner AR, Bailey K, Wesselius LJ (1998) Increased iron and ferritin content of sputum from patients with cystic fibrosis or chronic bronchitis. Chest 114:814–819

Stites SW, Plautz MW, Bailey K, O'Brien-Ladner AR, Wesselius LJ (1999) Increased concentrations of iron and isoferritins in the lower respiratory tract of patients with stable cystic fibrosis. Am J Respir Crit Care Med 160:796–801

Stojiljkovic I, Kumar V, Srinivasan N (1999) Non-iron metalloporphyrins: potent antibacterial compounds that exploit haem/Hb uptake systems of pathogenic bacteria. Mol Microbiol 31:429–442

Takase H, Nitanai H, Hoshino K, Otani T (2000) Impact of siderophore production on *Pseudomonas aeruginosa* infections in immunosuppressed mice. Infect Immun 68:1834–1839
Tomaras AP, Dorsey CW, Edelmann RE, Actis LA (2003) Attachment to and biofilm formation on abiotic surfaces by *Acinetobacter baumannii*: involvement of a novel chaperone-usher pili assembly system. Microbiology 149:3473–3484
Touati D (2000) Iron and oxidative stress in bacteria. Arch Biochem Biophys 373:1–6
Vasil ML, Ochsner UA (1999) The response of *Pseudomonas aeruginosa* to iron: genetics, biochemistry and virulence. Mol Microbiol 34:399–413
Visca P, Leoni L, Wilson MJ, Lamont IL (2002) Iron transport and regulation, cell signaling and genomics: lessons from *Escherichia coli* and *Pseudomonas*. Mol Microbiol 45:1177–1190
Wang J, Lory S, Ramphal R, Jin S (1996) Isolation and characterization of *Pseudomonas aeruginosa* genes inducible by respiratory mucus derived from cystic fibrosis patients. Mol Microbiol 22:1005–1012
Wilderman PJ, Sowa NA, FitzGerald DJ, FitzGerald PC, Gottesman S, Ochsner UA, Vasil ML (2004) Identification of tandem duplicate regulatory small RNAs in *Pseudomonas aeruginosa* involved in iron homeostasis. Proc Natl Acad Sci USA 101:9792–9797
Wolz C, Hohloch K, Ocaktan A, Poole K, Evans RW, Rochel N, Albrecht-Gary AM, Abdallah MA, Doring G (1994) Iron release from transferrin by pyoverdin and elastase from *Pseudomonas aeruginosa*. Infect Immun 62:4021–4027
Xiao R, Kisaalita WS (1997) Iron acquisition from transferrin and lactoferrin by *Pseudomonas aeruginosa* pyoverdin. Microbiology 143:2509–2515

Clinical Wound Healing Using Signal Inhibitors

Randall D. Wolcott

Abstract The cases presented in this chapter demonstrate the amazing improvement in wound-healing trajectory that is best explained by the suppression of biofilms. The individual patient with a chronic wound is not much better served today than a decade ago despite all of the advances achieved. The rate of major limb amputation and mortality is not decreasing, and the quality of life of these patients is not improving. Chronic wounds have a much higher prevalence rate of biofilms than acute ones, suggesting that presence of a biofilm is an important factor in the lack of healing of chronic wounds. As shown in this chapter, the implementation of the quorum-sensing inhibitor RNAIII-inhibiting peptide in managing complex wounds, along with other anti-biofilm strategies like lactoferrin, has dramatically changed the positive outcomes for many of the most desperate wounds.

1 Biofilm and Chronic Wound Infection

There has been an unprecedented technological explosion in science over the last decade, allowing for rapid expansion of our knowledge at the molecular and cellular levels of wound healing. Despite all our newfound knowledge, the general state of wound care is best summed up by Falanga, who stated, “Undoubtedly, we still have some way to go in the way we provide optimal care for our patients. A major stumbling block is the poor understanding of how chronic wounds fail to heal and what is their intrinsic pathogenesis that needs to be corrected” (Falanga 2001). Chronic wounds, and indeed most chronic human infections, continue to evade solid scientific explanation.

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N. Balaban (ed.) *Control of Biofilm Infections by Signal Manipulation*
Springer Series on Biofilms 2
doi: 10.1007/978-3-540-73853-4, © Springer 2008

Chronic human infections, including chronic wounds, constitute up to 80% of all human infectious disease (Costerton et al. 1995). The cost of these chronic infections is a major portion of the healthcare budget and continues to grow at exponential rates (Smith 2004). Although the big picture of chronic infections is dismal, the devastation of this problem is best understood by looking at the individual patient.

The individual patient with a chronic wound is not much better served today than a decade ago despite all the advances in our understanding. The outcomes for a patient with a diabetic foot ulcer remain bleak. The rate of major limb amputation is not decreasing, the mortality associated with a major limb amputation is not declining, and the quality of life reported by these patients is not improving (Buzato et al. 2002; Peters et al. 2001). Based on the human suffering and mounting costs, innovative explanations and management of chronic human infections must be sought.

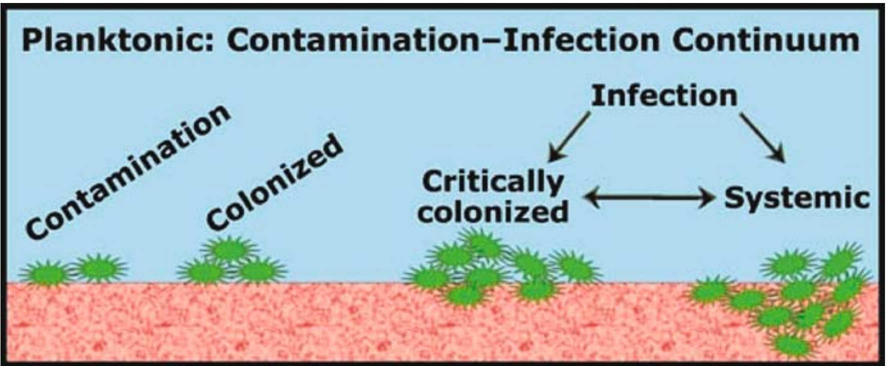
The dominant view of most human infections is based on a "planktonic" concept. For chronic wounds it is very common to see the bacterial burden on the surface of the wound explained as a "contamination-infection continuum" (Fig. 1).

This continuum suggests that bacteria randomly land on the surface of the wound, and if they can divide, they can then "colonize" the surface. These individual bacteria can then further divide, invading a short distance into the host. This is termed "critical colonization" and is identified clinically by local increased erythema, heat, pain, and swelling. If the individual bacteria can invade the host, broader involvement (infection) results. The only problem with the contamination-infection continuum is that there is no scientific validation for it, and it is a very minor pathway regarding how bacteria behave on a surface.

Endocarditis, prostatitis, sinusitis, otitis media, osteomyelitis, and other chronic biofilm infections all occur on surfaces. When a bacterium encounters a surface to which it can attach, it quickly forms a biofilm. A biofilm offers bacteria much more survivability, and therefore is the preferred phenotype in which bacteria choose to exist. Biofilm may be the best explanation for many of the clinical observations associated with chronic infections (Table 1).

As noted in other chapters, a biofilm is a colony of bacteria expressing different proteins (phenotypes) to fulfill the needs of the colony (Sauer et al. 2002). A biofilm should be considered a true multicellular organism with each individual under molecular control by the colony. The bacteria that constitute a biofilm usually arise from a number of bacterial species that work in synergy to ensure the colony's survival.

Chronic infections are very resistant to conventional treatments such as antibiotics because of the defenses that the biofilm colonies possess. Biofilms possess an extracellular polymeric substance (EPS) that protects the bacteria from environmental attacks (Costerton et al. 1999). Biofilms are also very resistant to biocides such as hydrogen peroxide, bleach, acetic acid, and others (Costerton and Stewart 2001). The biofilm phenotype, along with the physical barrier and structure provided by the EPS, makes biofilm almost impervious to host defenses (Stoodley et al. 2002). In fact, a strategy of suppression rather than eradication has yielded the best outcomes in chronic wounds (see below).



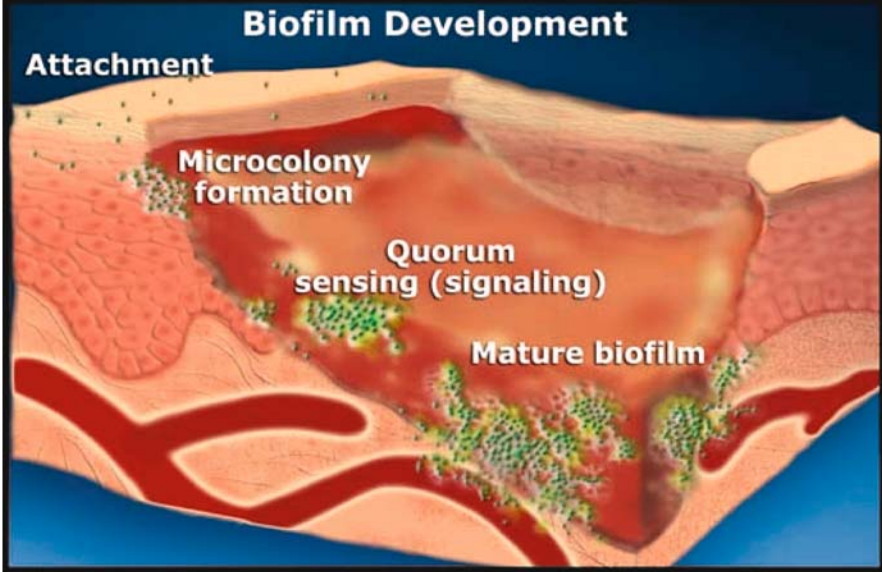


Fig. 1 A planktonic explanation for surface bacteria involved in wounds is not scientifically valid. Planktonic bacteria quickly form into microcolonies and then mature into formidable wound biofilm that evades host defenses and impairs wound healing

The process that may take place in the genesis of a chronic wound or other biofilm-based infection is as follows (Costerton et al. 1999): A planktonic bacterium finds its way through the first lines of defense, such as through a skin break, mucous membrane, or alveoli, and attaches to the underlying tissue (Fig. 2A). Once the bacterium attaches to exposed extracellular matrix components, it rapidly begins biofilm behavior to express new proteins and EPS. If the host immune system, mainly the innate system, fails to clear the bacteria before it is protected within its EPS fortress, then the immune system becomes irrelevant. Once the microcolony (early biofilm) is firmly entrenched on the tissue surface, it rapidly matures into a biofilm, which is protected from the host immune response and antibiotics (Fig. 2B,C).

Table 1 Observations in chronic wounds explained by the presence of a biofilm

Observation	Planktonic concept	Biofilm concept
In vitro	Planktonic bacterium (seed) expresses proteins and structures for motility and attachment (flagella, fimbria). Its function is to spread the colony to a different location.	Biofilm (vegetation) is a complex colony of bacteria that can express different proteins (phenotype) to fulfill different roles to help the community survive. Biofilm is stationary, protecting its location with multiple defenses.
In vivo	Planktonic bacteria are susceptible to antibiotics, biocides, and the immune system.	Biofilms are resistant to antibiotics and biocides. Once biofilm is established, it cannot be eradicated by the immune system.
Acute wounds heal in 2–4 weeks; chronic wounds in the same area heal in 4–6 months	Acute wounds have intact defenses and clear planktonic bacteria not allowing biofilm formation.	Random planktonic bacteria evade host defenses and set up biofilm. Biofilm possesses defenses against the host immune system and strategies to keep the wound open.
Antibiotics not effective in chronic wounds but work in acute wounds	Sensitive planktonic bacteria show a rapid 4- to 5-log reduction.	Sensitive biofilm phenotype bacteria show only a grudging 1- to 2-log reduction and quick adaptation.
Biocides not effective in chronic wounds	Nonspecific biocides eradicate planktonic bacteria.	Nonspecific biocides eradicate host healing cells and host defenses, doing little damage to the biofilm.
Drying the wound (open to air, heat lamp, etc.) is ineffective in chronic wounds	Planktonic bacteria are very sensitive to the environment.	Biofilms are very resistant to drying and other environmental changes.
Closing a traumatic wound after 4–12 h leads to increased dehiscence	Planktonic phenotypes dominate the surface early on (1–2 h) and are sensitive to biocides and amenable to closure.	Biofilms form in 1–2 h and are resistant to surgical scrubs. Closure of the wound puts two surfaces in contact with the biofilm and leads to dehiscence.
Increased surgical site infections in patients with chronic wounds	Surgical preps and prophylactic antibiotics are highly effective planktonic bacteria.	Chronic wounds shed biofilm fragments continuously. These fragments have all the defenses of biofilm and are resistant to surgical preps and prophylactic antibiotics.
Wet-to-dry dressings are detrimental to chronic wounds	Planktonic bacteria quickly (6 h) seed gauze and form biofilm on gauze.	Detachment fragments foul the gauze dressing, forming biofilm that produces fragments, toxins, etc., that are detrimental to the wound.

The biochemical impairment on the surface of a chronic wound is well documented. There are very high levels of MMP8, which is the major matrix metalloprotease of neutrophils. Yeager and Nwomeh have stated, “Virtually all the available

Table 1 (continued)

Observation	Planktonic concept	Biofilm concept
Autograft or allograft failure on wounds	Planktonic cells are easily cleared by neutrophils, antibodies, and common wound-bed preparations.	Laying unprotected cells over biofilm just adds a second surface and a food source, leading to the rapid deterioration of the graft, with increased exudate, inflammation, and odor.
Negative wound cultures	Most clinical bacteria in planktonic phenotype are easily cultured.	Wounds have bacteria on their surface yet culture negative when only biofilm phenotype (i.e., viable but not culturable) is present.
Wounds "stuck" in chronic inflammatory state	Planktonic bacteria are easily cleared by host's inflammatory response and try to avoid causing inflammation.	Biofilms are impervious to the host's inflammatory response and can even feed off the exudate produced by inflammation. Biofilms try to cause inflammation.
Corticosteroids known to slow the host healing response help heal some chronic wounds	Steroids decrease the host immune response, making planktonic infections worse.	Steroids rob biofilms of nutrition by decreasing the host inflammatory response.
No blood clot formation on chronic wound	Planktonic bacteria are overwhelmed by host defenses after injury, and clot will form on wound surface.	Biofilms prevent clot formation on the surface of a wound post-debridement by direct competition and an increased proteolytic environment.

evidence supports a role for neutrophils in the pathophysiology of chronic wounds" (Yager and Nwomeh 1999). Diegelman concurred, stating, "In contrast, the ulcer microenvironment appears to be an area of massive destruction due to the invasion of tremendous numbers of neutrophils and their destructive enzymes" (Diegelmann 2003).

There have been tremendous advances in imaging over the last decade, yet very few studies have examined the wound bed itself. Diegelman's study looking at tissue gram stains to demonstrate the presence of excessive neutrophils inadvertently demonstrated the presence of wound biofilm in the same area (Diegelmann 2003). The presence of wound biofilm can explain the elevated proinflammatory cytokines, elevated matrix metalloproteases, and excessive neutrophils seen in all chronic wounds.

There is now good evidence that chronic wounds are different from acute wounds because the former possess biofilm on their surface. Electron microscopy studies along with tissue gram stains conducted at the Center for Biofilm Engineering found that chronic wounds had a high prevalence of biofilm on the surface of the wound (Fig. 3a), whereas acute wounds rarely exhibited any biofilm (Fig. 3b) (James et al., unpublished). This would indicate that the difference between an acute and a chronic wound is the presence of biofilm on the chronic wound (Fig. 4).

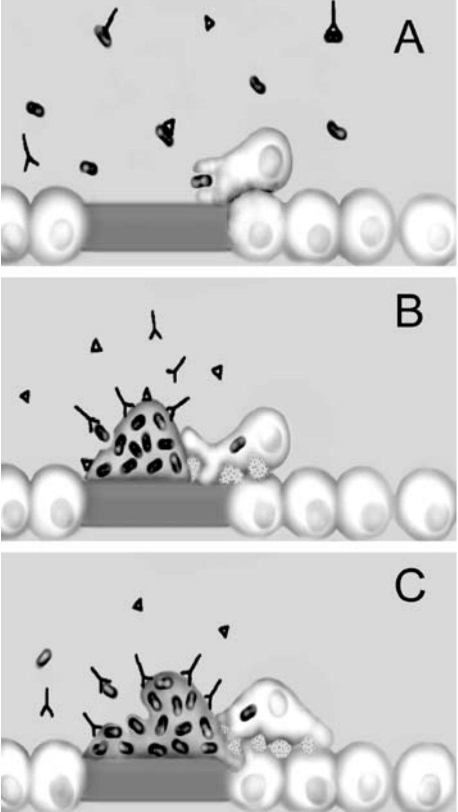


Fig. 2 Diagram of implant infection. **A** Planktonic bacteria are susceptible to the host's immune response and to antibiotics. **B** Bacteria form biofilms, such as on medical devices, where they are resistant to immune response and to antibiotics. **C** Phagocytes attach to the biofilm. They are unable to phagocytose the bacteria, but degrading enzymes are released, destroying surrounding tissue. Some bacteria are released, leading to dissemination of infection to neighboring tissue. (*Y* antibodies, Δ-antibiotics) (Illustration by Mike Beshiri, Tufts University, Cummings School of Veterinary Medicine, Department of Biomedical Sciences, Division of Infectious Diseases, North Grafton, MA, USA)

Chronic wounds, and indeed all biofilm-based human infections, are locked in a persistent chronic inflammatory state in a high proteolytic environment. As noted above, chronic wounds seem to have significant biofilm, whereas acute wounds (which do not exhibit a chronic inflammatory state) have very little biofilm on their surface. Intuitively, it makes sense for the presence of a colony of bacteria to produce a vigorous immune response from the host. But is it biofilm on the wound's surface that is causing the chronic inflammation and thus impairing healing? If wound healing improves with the use of agents that only target the biofilm behavior of bacteria, then the biofilm must be contributing to the impairment in healing. This provides indirect evidence of the role of biofilm in nonhealing chronic wounds.

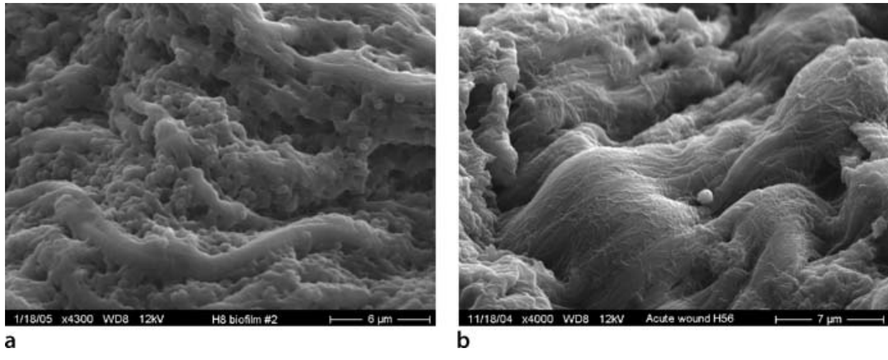


Fig. 3 a Wound biofilm as seen in this electron micrograph covers most of the surface of chronic wounds regardless of the etiology. **b** Acute wounds show expanses of extracellular matrix with occasional planktonic bacteria. Acute wounds have little biofilm on their surface

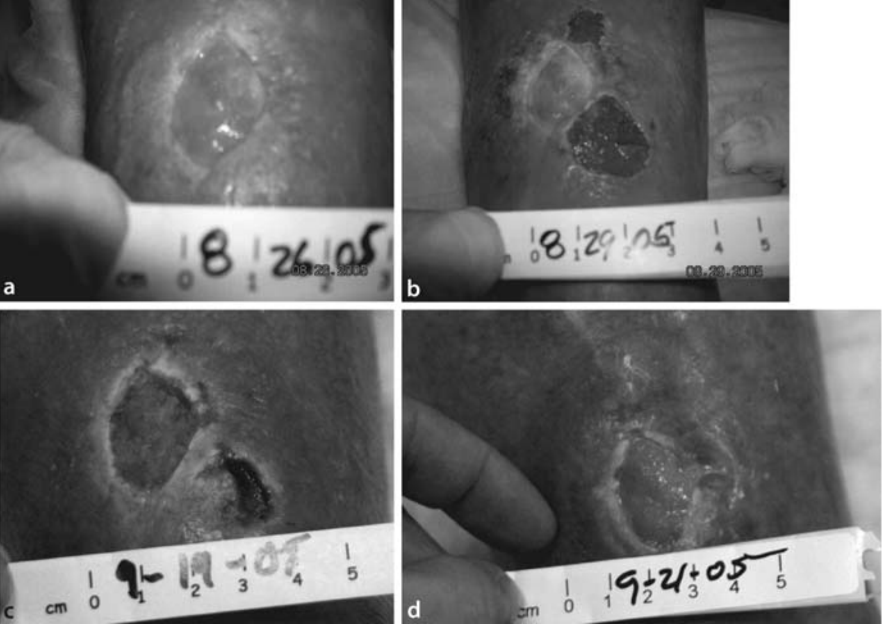


Fig. 4 This series of photographs captures the development of a full-thickness acute wound in the same region as a chronic wound. The acute wound healed in 3 weeks, but the chronic wound did not. The difference may be biofilm

Quorum-sensing inhibitors (QSIs) have the unique property of affecting only the biofilm behavior of bacteria. As noted in other chapters, quorum-sensing molecules are specific signaling agents that control the behavior of biofilms by regulating the gene expression of each of the colony bacteria. The original name given to these signaling molecules was quorum-sensing molecules because a critical density of individual bacteria (a quorum) is required to trigger biofilm gene expression. In other

words, a certain number of these molecules need to be present to cause upregulation or downregulation of suites of genes (operons and regulons) responsible for biofilm formation. It was clear from the beginning that if this communication language could be understood, certain behaviors could be manipulated (inhibited), thus averting the impenetrable defenses of biofilm.

As noted in other chapters, RIP is a staphylococcal QSI that blocks biofilm behavior and toxin production. Animal studies using rat graft or central venous catheter models show that animals treated with RIP were able to clear *S. aureus* and *S. epidermidis* biofilms and that RIP is synergistic with commonly used antibiotics. RIP suppressed any type of staphylococcal infection, including drug-resistant ones such as methicillin-resistant *S. aureus* (MRSA), methicillin-resistant *S. epidermidis*, vancomycin-intermediate *S. aureus*, and vancomycin-intermediate *S. epidermidis* (e.g., Balaban et al. 2005). This is a very exciting step toward specific, well-understood agents that may be useful in a clinical setting.

2 Use of a Quorum-Sensing Inhibitor to Treat Chronic Wound Infections

Two cases demonstrate the potential power of a QSI (RIP) in clinical application for controlling biofilm-based infectious diseases. RIP was used in these two cases of chronic wounds with osteomyelitis in patients who had exhausted all other options. Although the methods of using RIP are still in development, these two cases demonstrate the potential of QSIs for future clinical use.

2.1 Case History 1

A man with severe peripheral vascular disease and diabetes mellitus sustained minor trauma to his right little toe. In his minor diabetic foot ulcer he was found to have MRSA, *Enterobacter cloacae*, *Pseudomonas* species, *Citrobacter* species, *E. coli*, and group D enterococci. Over the next 6 months, the patient had aggressive conservative wound care. This included topical products such as Iodosorb, Hydrofera Blue, Acticoat, silver gels, enzymes, Promogran, and many other topical preparations. The wound underwent debridement frequently. Despite these measures, there was a continual dying back into the forefoot. Perfusion and oxygenation studies showed that there was adequate blood flow and oxygenation of the tissues for healing. Yet the wound relentlessly worsened. There was extensive osteomyelitis across the forefoot within 4 months. The patient was offered major limb amputation on several occasions but refused. After 7 months (Fig. 5a), the patient had just completed 2 weeks of intravenous Cubicin at 6 mg/kg along with topical application of Hydrofera Blue on a daily basis, offloading, and nutritional support. The foot was clearly worsening. After 8 months, a gel preparation of RIP at 1.7 mcg/ml was ap-

plied topically to the wound on a daily basis along with continued use of Cubicin 4 mg/kg for 3 weeks. By that time, the wound had shown significant improvement. The Cubicin was stopped, but the RIP gel was continued with steady improvement noted throughout the next few months (Fig. 5b). Three months after that, Apligraf grafting was instituted every 2–4 weeks for the next 8 months to completed healing (Fig. 5c). In summary, RIP, as a topically applied agent used adjunctively with intravenous antibiotics, cleared the biofilm that was slowly eroding through the foot. This treatment produced a spectacular salvage of this patient's limb.

2.2 Case History 2

A man with severe insulin-dependent diabetes mellitus presented with a hot, swollen left foot with blood sugars greater than 600. He was febrile and hypotensive and was immediately admitted to the hospital for intravenous antibiotics and surgical management of his left foot. The surgical decision was made to amputate the left lower extremity, which the patient refused. Local surgery was done on the foot, which had significant pus in the forefoot with osteomyelitis involving the tarsal and metatarsals. The organisms cultured were MRSA and group D enterococci. The patient was placed on Cubicin 6 mg/kg and continued to do poorly (Fig. 6a). He was dismissed from orthopedic and medical care because he continued to refuse amputation of his left lower extremity. He was placed in outpatient therapy with daily Cubicin 6 mg/kg and was injected with RIP in the tarsal area (25 mg/5 ml Marcaine). This injection was done on a daily basis for three visits. The local wound care consisted of daily ultrasonic debridement followed by sharp debridement to open up any undermining and tunneling and to remove any necrotic material. Applied topical preparations included lactoferrin 10 mg/ml, xylitol 5% by weight, and RIP 1.7 μg/ml. The compounded gel was placed on the foot on a daily basis. After 1 week, the patient's blood sugars had normalized, his malaise had cleared, and the wound was much improved. The foot edema and erythema completely resolved within 10 days (Fig. 6b). The injection of RIP in the area of obvious *S. aureus* biofilm (osteomyelitis) markedly changed the patient's clinical course and led to resolution of the osteomyelitis as well as healing of the diabetic foot ulcer (Fig. 6c).

QSIs are not the only signal-inhibiting agents that have been shown to improve wound healing by suppressing biofilm. Lactoferrin applied topically to a chronic wound has shown good ability to block reaccumulation of biofilm after debridement (see case history 3, below). Xylitol and other alcohol sugars have demonstrated an ability to weaken EPS, and therefore can be the first line of defense to improve wound outcomes. In fact, a number of excellent antibiofilm agents are well documented in the literature from other fields (Domenico et al. 2004; Katsuyama et al. 2005; Tapiainen et al. 2004; Veloira et al. 2003). The fact that these agents produce better outcomes in chronic biofilm infections demonstrates that the approach of direct biofilm suppression may be a valuable medical tool.

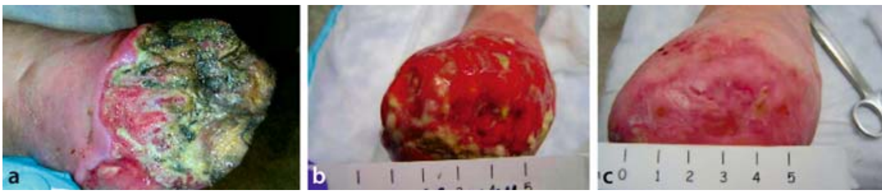


Fig. 5 Case history 1. The patient had continued dying back of the foot (**a**) secondary to infection caused by methicillin-resistant *S. aureus*, *Enterobacter cloacae*, *Pseudomonas* spp, *Citrobacter* species, *E. coli*, and group D enterococci. Once RIP was added to the regimen, there was a dramatic turnaround (**b**), and the patient went on to healing (**c**)

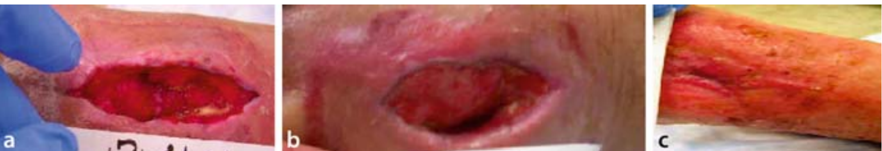


Fig. 6 Case history 2. The patient presented with overwhelming infection of the foot with osteomyelitis (**a**). The use of RIP injected in the tarsal and metatarsal region in conjunction with appropriate antibiotics (**b**) allowed for limb salvage (**c**)

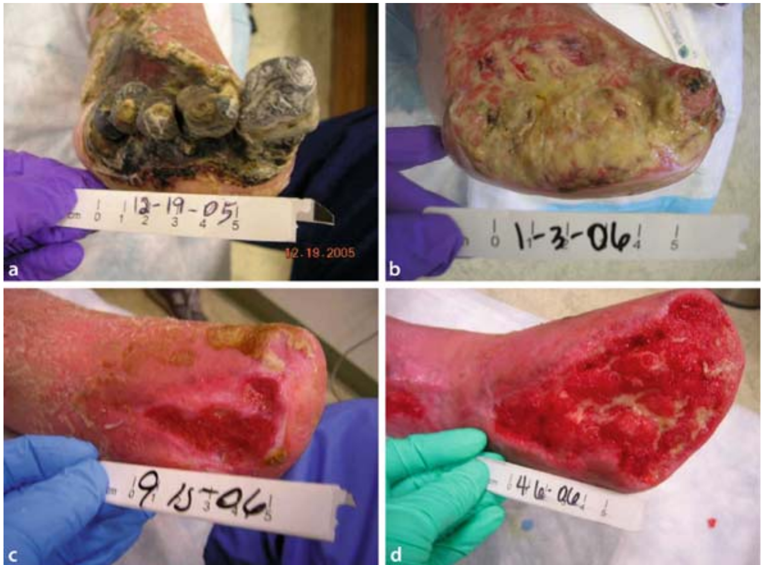


Fig. 7 Case history 3. The patient presented with overwhelming infection of the foot with osteomyelitis (**a**). The initial culture showed methicillin-resistant *S. aureus*, with subsequent culture showing *Enterococcus* and *E. coli*. Debridement was carried out at 1-week intervals. The patient was placed on Daptomycin 6 mg/kg for over 6 weeks (**b**). The topical regimen was lactoferrin 33 mg/cc, Xylitol 5%, and silver (Acticoat) (**c**), and later Apligraf and Oasis, allowing for limb salvage (**d**)

3 The Use of Lactoferrin in Wound Care

As noted in other chapters, lactoferrin is used in many products, as well as in meat packing plants, to treat meat surfaces to prevent biofilm formation and as a dietary supplement. It has been a popular nutritional supplement for a number of decades, a situation that may have contributed to a lack of rigorous study of this very powerful component of the innate immune system. Copious amounts of lactoferrin are secreted in human external secretions (tears, saliva, mucous, milk, etc.). Because lactoferrin is a main component of the innate immune system and is ubiquitous in surface secretions, it seems logical that lactoferrin would play a significant role in antimicrobial surface protection of the host. Exactly what roles it plays and how it plays these roles is just now coming to light.

The initial work of looking into the antimicrobial activity of lactoferrin suggested that the protein's affinity for iron (transferrin) was its main mechanism of action. It was demonstrated that lactoferrin sequesters iron, depleting this essential bacterial nutrient and causing a bacteriostatic action against bacteria (Bullen 1975). However, this research and several subsequent studies focused on planktonic bacteria, not biofilms (Weinberg 1993). It was only recently that the biofilm-lactoferrin connection was made (Weinberg 2004).

By 1977 there was some suggestion that the role of lactoferrin was more complex than just its ability to bind iron (Arnold et al. 1977). In planktonic bacteria, lactoferrin was found to have a direct bactericidal effect. Lactoferrin was found to bind to the lipopolysaccharide portion in the outer membrane of gram-negative bacteria. This binding to the phosphate group in the lipid A region of the membrane causes rigidification of acyl chains of the lipopolysaccharides, which causes rapid release of lipopolysaccharides and increased membrane permeability. It is increased membrane permeability that causes planktonic bacterial cell death (Brandenburg et al. 2001; Appelmelk et al. 1994; Ellison III et al. 1988).

Recently, a number of different functions have been identified for this versatile protein. For example, a definite protease activity has been identified. Lactoferrin acts as a serine protease that can cleave arginine-rich sequences. Independent from its iron binding capacity, it has been shown to induce degradation of bacterial secreted proteins necessary for attachment (Ochoa et al. 2003).

An important new finding is that lactoferrin works synergistically with polymorphonuclear cells to produce bactericidal activity. Lactoferrin acts as a reservoir for iron which is required for catalyzing hydroxyl radical production, one of the main weapons in the polymorphonuclear cells' armamentarium. It was found that lactoferrin remains stable and continues to bind iron even at a very low pH. The activated neutrophil binds lactoferrin-containing granules with the acidic phagolysosome, resulting in the needed iron source for its bactericidal activity (Ward et al. 2002).

Lactoferrin has been shown to prevent free-floating (planktonic) *P. aeruginosa* from attaching to a surface. If the attachment is prevented, then biofilm formation is prevented (Singh et al. 2002). It seems that as a planktonic bacterium adheres to a surface in the presence of lactoferrin, the movement of the individual bacteria (referred to as twitching) is increased. If twitching is increased for the parent cell

and all its progeny, they fail to group into microcolonies, which is the first step in expressing biofilm behavior. In that study, *P. aeruginosa* cultures without lactoferrin demonstrated thick biofilm formation. When lactoferrin was added, the planktonic bacteria were able to divide but not attach, so no biofilm structures were identified (Singh et al. 2002). Lactoferrin worked purely to prevent the attachment of planktonic bacteria, thus preventing biofilm formation.

To determine whether lactoferrin is effective in managing wounds, our group conducted an 8-week prospective randomized controlled study of 50 patients. Twenty-five patients with a total of 37 wounds were randomly placed in the lactoferrin gel treatment group. The control group consisted of 25 patients with 30 wounds, and they received the same gel without lactoferrin. The study demonstrated that patients receiving the lactoferrin gel had a higher percentage of complete healing in the 8 weeks. Overall, lactoferrin was an effective agent for improving the healing of chronic wounds (see case history 3).

3.1 Case History 3

A man had acute onset of necrosis of all the toes of his right foot, with critical limb ischemia. A stent was placed in the superficial femoral artery. The patient did reperfuse the foot but was still recommended to have a below-knee amputation (Fig. 7a). At the initial visit, the patient's $TcPO_2$ in the right foot was 23 mmHg. Over the course of treatment it rose slightly to 25 mmHg. Initial culture showed MRSA, with subsequent culture showing *Enterococcus* and *E. coli*. Biofilm-based wound management was initiated. Initially, debridement was targeted on removing all necrotic material and shaping the wound to benefit the host (Fig. 7b). Debridement was carried out at 1-week intervals. The patient was placed on daptomycin 6 mg/kg for over 6 weeks because he had osteomyelitis of the metatarsals; daptomycin has shown efficacy against biofilm phenotype bacteria. The topical regimen used was a mixture of lactoferrin 33 mg/cc, xylitol 5%, and Acticoat; lactoferrin, xylitol, and silver have shown good synergy in preventing reaccumulation of biofilm after removal by debridement (Fig. 7c). Surface management was continued on a weekly basis using ultrasonic debridement. By the 6th month, the biofilm was suppressed sufficiently to allow proactive agents such as Apligraf and Oasis to be applied to the wound to promote wound healing. These agents have worked effectively, and although the limb remained hypoxic, healing was almost complete by the 9th month (Fig. 7d).

Healing of a difficult, hypoxic wound in an elderly patient is difficult, and this case illustrates the relative contribution of wound biofilm in preventing wound healing. With wound biofilm suppressed, even difficult wounds demonstrate the ability to heal. It appears that wound biofilm is a major barrier to wound healing; therefore, specific targeting of wound biofilm should result in better wound healing outcomes.

4 Concluding Remarks

From a medical perspective, biofilm needs rigorous scientific attention. Wounds and other biofilm-based diseases account for a major portion of current healthcare expenditures. Biofilm diseases cause significant suffering, major disability, loss of limb, and loss of life. Molecular and cellular evaluation of biofilm and the host biofilm interaction is an important first step to mitigate this desperate situation.

The answer to biofilm infections will probably not be found in a single agent such as a single QSI. More likely, the answer will be found in a coordinated approach using multiple simultaneous strategies such as frequent repetitive removal of the biofilm, specific biocides, antibiofilm antibiotics (Cubicin) and multiple antibiofilm agents (Fux et al. 2005). Antibiofilm agents include QSIs that can act at different pathways to manipulate biofilm behavior. Using QSIs to attack biofilms simultaneously at attachment, differentiation, EPS production, and so on, may be a more robust approach. This gives much hope for eventually controlling biofilm to the point where it no longer produces devastating disease.

References

Appelmelk BJ, An YQ, Geerts M, Thijs BG, de Boer HA, MacLaren DM, de Graaff J, Nuijens JH (1994) Lactoferrin is a lipid A-binding protein. Infect Immun 62:2628–2632

Arnold RR, Cole MF, McGhee JR (1977) A bactericidal effect for human lactoferrin. Science 197:263–265

Balaban N, Stoodley P, Fux CA, Wilson S, Costerton JW, Dell'Acqua G (2005) Prevention of staphylococcal biofilm-associated infections by the quorum sensing inhibitor RIP. Clin Orthop Relat Res 437:48–54

Brandenburg K, Jurgens G, Muller M, Fukuoka S, Koch MH (2001) Biophysical characterization of lipopolysaccharide and lipid A inactivation by lactoferrin. Biol Chem 382:1215–1225

Bullen JJ (1975) Iron-binding proteins in milk and resistance to *Escherichia coli* infection in infants. Postgrad Med J 51(suppl 3):67–70

Buzato MA, Tribulatto EC, Costa SM, Zorn WG, van Bellen B (2002) Major amputations of the lower leg. The patients two years later. Acta Chir Belg 102:248–252

Costerton JW (2005) Biofilm theory can guide the treatment of device-related orthopaedic infections. Clin Orthop Relat Res 437:7–11

Costerton JW, Stewart PS (2001) Battling biofilms. Sci Am 285:74–81

Costerton JW, Stewart PS, Greenberg EP (1999) Bacterial biofilms: a common cause of persistent infections. Science 284:1318–1322

Diegelmann RF (2003) Excessive neutrophils characterize chronic pressure ulcers. Wound Repair Regen 11:490–495

Domenico P, Gurzenda E, Giacometti A, Cirioni O, Ghiselli R, Orlando F, Korem M, Saba V, Scalise G, Balaban N (2004) BisEDT and RIP act in synergy to prevent graft infections by resistant staphylococci. Peptides 25:2047–2053

Ellison RT III, Giehl TJ, LaForce FM (1988) Damage of the outer membrane of enteric gram-negative bacteria by lactoferrin and transferrin. Infect Immun 56:2774–2781

Falanga V (2001) Introduction, overview. In: Falanga V (ed) Cutaneous wound healing. Martin Dunitz, London, p 2

Fux CA, Costerton JW, Stewart PS, Stoodley P (2005) Survival strategies of infectious biofilms. Trends Microbiol 13:34–40

Katsuyama M, Kobayashi Y, Ichikawa H, Mizuno A, Miyachi Y, Matsunaga K, Kawashima M (2005) A novel method to control the balance of skin microflora. Part 2. A study to assess the effect of a cream containing farnesol and xylitol on atopic dry skin. J Dermatol Sci 38:207–213

Ochoa TJ, Noguera-Obenza M, Ebel F, Guzman CA, Gomez HF, Cleary TG (2003) Lactoferrin impairs type III secretory system function in enteropathogenic *Escherichia coli*. Infect Immun 71:5149–5155

Peters EJ, Childs MR, Wunderlich RP, Harkless LB, Armstrong DG, Lavery LA (2001) Functional status of persons with diabetes-related lower-extremity amputations. Diabetes Care 24:1799–1804

Sauer K, Camper AK, Ehrlich GD, Costerton JW, Davies DG (2002) *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. J Bacteriol 184:1140–1154

Singh PK, Parsek MR, Greenberg EP, Welsh MJ (2002) A component of innate immunity prevents bacterial biofilm development. Nature 417:552–555

Smith A (2004) Etiology of the problem wound. In: Sheffield PJ, Fife CE, Smith A (eds) Wound care practice. Best, Flagstaff, pp 3–48

Stoodley P, Sauer K, Davies DG, Costerton JW (2002) Biofilms as complex differentiated communities. Annu Rev Microbiol 56:187–209

Tapiainen T, Sormunen R, Kaijalainen T, Kontiokari T, Ikaheimo I, Uhari M (2004) Ultrastructure of *Streptococcus pneumoniae* after exposure to xylitol. J Antimicrob Chemother 54:225–228

Veloira WG, Domenico P, LiPuma JJ, Davis JM, Gurzenda E, Kazzaz JA (2003) *In vitro* activity and synergy of bismuth thiols and tobramycin against *Burkholderia cepacia* complex. J Antimicrob Chemother 52:915–919

Ward PP, Uribe-Luna S, Conneely OM (2002) Lactoferrin and host defense. Biochem Cell Biol 80:95–102

Weinberg ED (1993) The development of awareness of iron-withholding defense. Perspect Biol Med 36:215–221

Weinberg ED (2004) Suppression of bacterial biofilm formation by iron limitation. Med Hypotheses 63:863–865

Yager DR, Nwomeh BC (1999) The proteolytic environment of chronic wounds. Wound Repair Regen 7:433–441

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