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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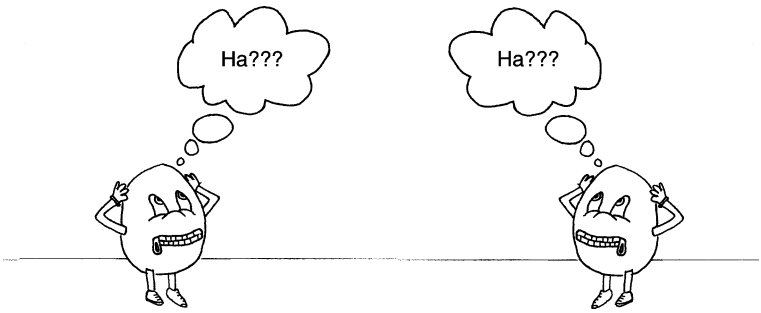

Fig. 1 Inhibition of quorum sensing as a novel mode of therapy (Illustration by Mike Beshiri, Tufts University, Cummings School of Veterinary Medicine, Department of Biomedical Sciences, Division of Infectious Diseases, North Grafton, MA, USA)

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

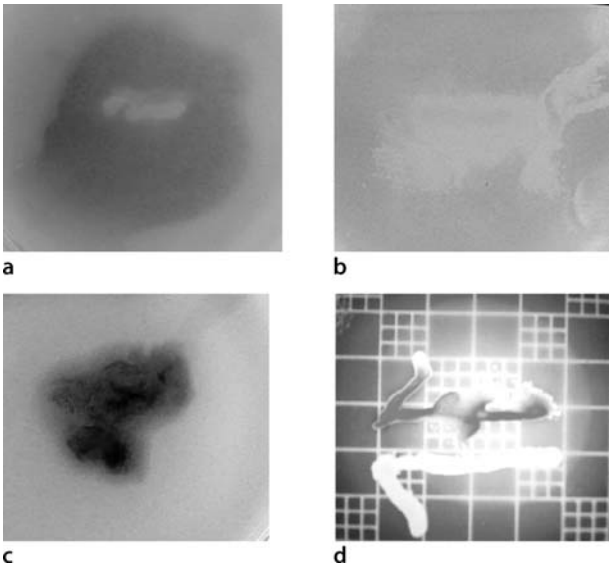

Fig. 2 A soft-agar overlay can be used for qualitative detection of *N*-acyl homoserine lactones (AHLs) using a reporter strain such as *A. tumefaciens* A136 (pCF 218) (pCF372) (Fuqua and Winans 1996). Positive control (**a**) involves testing with AHL-overproducing *A. tumefaciens* KYC6. Negative control (**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**)

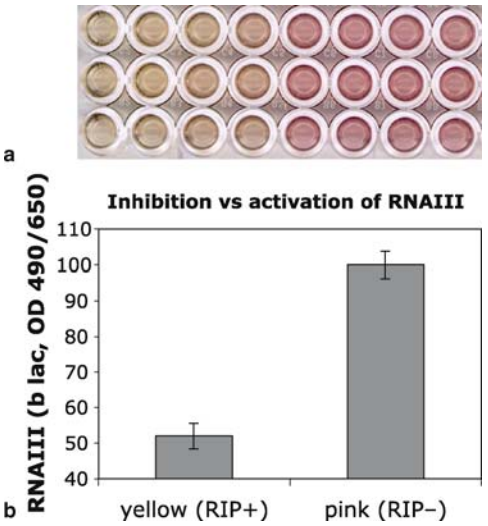

Fig. 3 a An example of beta lactamase reporter assay in *S. aureus*. *Four yellow columns* on left reflect inhibition of RNAIII production by RIP. *Four pink columns* on right reflect RNAIII production in the presence of saline. **b** Spectrophotometric analysis (absorbance at 490/650 nm × 1000) of column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

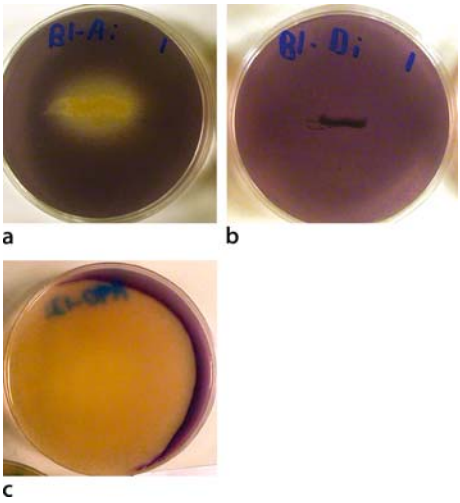

Fig. 4 Bioassay for quorum signal inhibition using *C. violaceum* ATCC 12472 (McLean et al. 2004). The cognate *N*-acyl homoserine lactone (AHL) for this strain is C6-HSL (McClean et al. 1997). Organisms such as *Pseudomonas aeruginosa* PAO1 can be used as a positive control (**a**) in that their AHLs, 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).

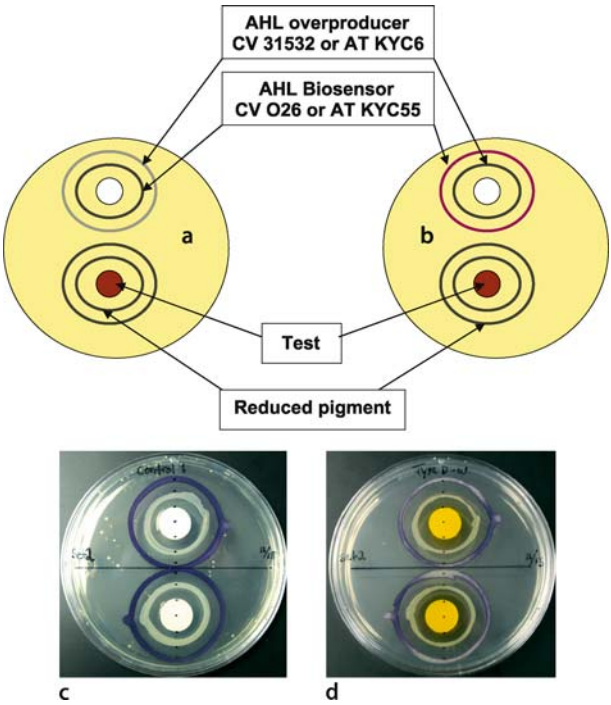

Fig. 5 Incorporation of two strains into a quorum-sensing inhibitor bioassay can distinguish whether inhibition is due to *N*-acyl homoserine lactone (AHL) response (*luxR* effect; **a**) or synthesis (*luxI* effect; **b**). Negative controls are at the top, and test samples are at the bottom. **c**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

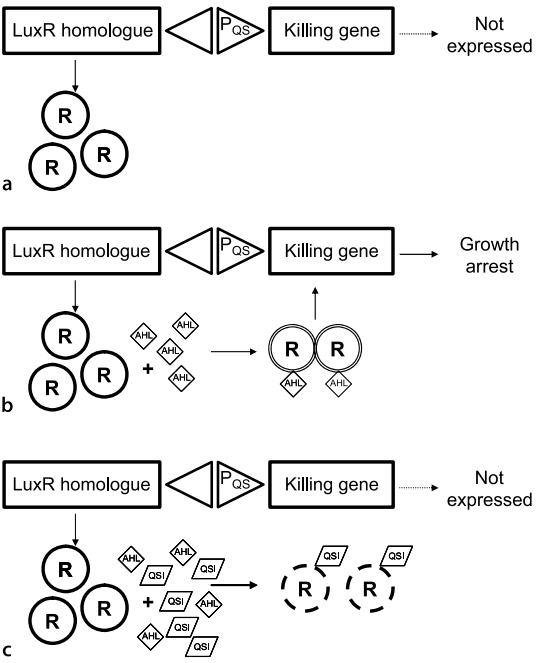

Fig. 6 General layout of the quorum-sensing inhibitor (QSI) selector systems. **a** The screening bacteria are grown without *N*-acyl homoserine lactone (AHL). The LuxR homolog is not activated, hence there is no expression from the QS-controlled promoter (P_{QS}), and, in turn, the killing is not expressed, 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)

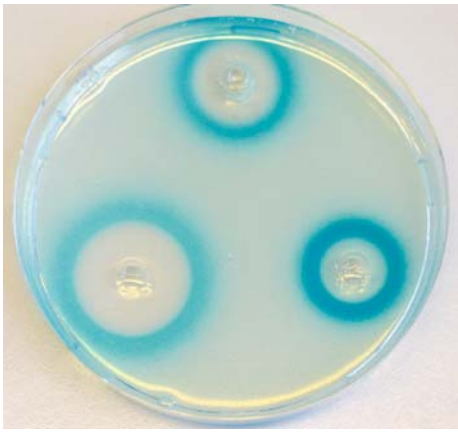

Fig. 7 The quorum-sensing inhibitor selector (QSIS) 1 screening system in action. The screening bacteria are cast into an agar plate along with *N*-acyl homoserine lactone (AHL) and other cofactors. Wells are punched in the plate, into which test compounds are added. The test compounds diffuse into the 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).

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