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Engineering of new prodigiosin-based biosensors of *Serratia* for facile detection of short-chain *N*-acyl homoserine lactone quorum-sensing molecules

Simon Poulter,[1] Timothy M. Carlton,[1] Xianbin Su,[2] David R. Spring[2] and George P. C. Salmond[1]*

Departments of [1]Biochemistry and [2]Chemistry, University of Cambridge, Cambridge, UK.

Summary

Many Gram-negative bacteria use quorum sensing (QS) to regulate expression of multiple genes, by utilizing small diffusible signalling molecules called *N*-acyl homoserine lactones (acyl-HSLs). *Serratia* sp. ATCC 39006 produces the red pigment prodigiosin under QS control, in response to the short-chain signal C4-HSL. In this study, we have demonstrated that an acyl-HSL-deficient mutant can be used as a visual biosensor to detect short-chain acyl-HSLs. We have quantified the acyl-HSL sensitivity spectrum of the *Serratia* 39006 prodigiosin QS system, and have demonstrated a strong specificity for the natural ligand C4-HSL. Mutations in the *pigX* and *pigZ* genes in *Serratia* 39006 resulted in an overproduction of prodigiosin, caused by increased transcription of the prodigiosin biosynthetic operon. A new biosensor (SP19) with enhanced prodigiosin production was created by addition of *pigX* and *pigZ* mutations to the existing biosensor. We have demonstrated that SP19 is superior to biosensor strains CV026 and *Agrobacterium* NTL4 (pZLR4) for the detection of short-chain acyl-HSLs present in spent culture supernatants. Researchers working with QS bacteria that produce short-chain acyl-HSLs can use strain SP19 as a simple visual acyl-HSL biosensor with no requirement for expensive detection equipment.

Introduction

Many Gram-negative bacterial species regulate gene expression in response to population cell density, by utilizing intercellular signalling molecules called *N*-acyl homoserine lactones (acyl-HSLs) in a cell-to-cell communication mechanism termed quorum sensing (QS) (Whitehead *et al.*, 2001). Typical Gram-negative QS systems consist of a LuxI family acyl-HSL synthase and a partner LuxR family transcriptional regulator, whose activity is modulated by binding to the acyl-HSL ligand. Acyl-HSLs produced by LuxI proteins consist of an invariant lactone ring coupled to an acyl chain, which ranges in length in different bacterial species from four to 16 carbons. The oxidation status of the acyl chain C3 carbon is also variable, sometimes containing a carbonyl (3-oxo) or hydroxyl group (Whitehead *et al.*, 2001).

The development of several bacterial biosensor strains for the detection of acyl-HSL molecules has aided the identification and analysis of bacterial species that participate in QS (Steindler and Venturi, 2007). Two of the most commonly used acyl-HSL biosensors are *Chromobacterium violaceum* CV026, and the broad range *Agrobacterium tumefaciens* NTL4 (pZLR4). CV026 produces the purple pigment violacein in response to detected acyl-HSLs, making it a simple visual assay that requires no expensive photon camera or luminometer equipment (McClean *et al.*, 1997; Steindler and Venturi, 2007). The *A. tumefaciens* strain NTL4 (pZLR4) is a β-galactosidase-based biosensor with a very high sensitivity level, and has the broadest acyl-HSL specificity of any biosensor, especially for longer-chain acyl-HSLs ranging from C_8 to C_{12} (Shaw *et al.*, 1997; Cha *et al.*, 1998; Farrand *et al.*, 2002; Zhu *et al.*, 2003). Importantly however, both the CV026 and NTL4 (pZLR4) biosensors respond poorly to the short-chain C4-HSL and 3-oxo-C4-HSL, relative to their cognate acyl-HSLs, thus limiting the utility of these strains for detection of short-chain acyl-HSL signals produced by several important QS microorganisms, e.g. various *Aeromonas*, *Pseudomonas* and *Serratia* species.

Quorum sensing regulates multiple phenotypes in the Gram-negative bacterium *Serratia* sp. ATCC 39006, including the biosynthesis of a bright red tripyrrole pigment, prodigiosin (2-methyl-3-pentyl-6-methoxyprodigiosin) (Harris *et al.*, 2004). The QS circuit in *Serratia* ATCC 39006 consists of the SmaI protein, which synthesizes C4-HSL, and the transcriptional repressor SmaR. The SmaR protein mediates QS control of the prodigiosin biosynthetic operon (*pigA–O*) via a 'derepression' mechanism, by inhibiting transcription in the

Received 25 September, 2009; accepted 18 December, 2009. *For correspondence. E-mail gpcs@mole.bio.cam.ac.uk; Tel. (+44) 1223 333650; Fax: (+44) 1223 766108.

absence of the C4-HSL ligand (i.e. at low cell density), an effect which is relieved by the interaction of SmaR with C4-HSL as cell-density increases (Thomson *et al*., 2000).

In addition to regulation by QS, recent studies have uncovered a complex regulatory network which integrates a number of environmental cues into control of prodigiosin production (Harris *et al*., 2004; Fineran *et al*., 2005a,b). Two such regulators are PigX (a GGDEF/EAL domain protein) and PigZ (a TetR family repressor) (Fineran *et al*., 2007; Gristwood *et al*., 2008). Mutation of these prodigiosin regulators causes an increase in transcription of the *pigA–O* biosynthetic operon, resulting in elevated prodigiosin production and a characteristic 'hyper-pigmented' phenotype.

The aim of this study was to utilize current knowledge of prodigiosin regulation in *Serratia* ATCC 39006 to construct a simple visual biosensor which is specific for short-chain acyl-HSL signals. This was achieved by significantly improving the sensitivity of an existing *Serratia* 39006 biosensor strain by the addition of two mutations (*pigX* and *pigZ*) that cause hyperproduction of prodigiosin, resulting in a new biosensor, SP19.

Results and discussion

Sensitivity of the standard LIS biosensor to acyl-HSLs

A previous study isolated a *smaI* mutant of *Serratia* 39006, called LIS (*smaI*::miniTn*5* Sm/Sp). LIS produces no acyl-HSL molecule, and therefore no prodigiosin. Addition of the cognate C4-HSL signal to strain LIS restored wild type levels of prodigiosin production, suggesting its potential use as a biosensor for short-chain acyl-HSLs (Thomson *et al*., 2000). In this study, the sensitivity of the LIS biosensor to acyl-HSLs was tested qualitatively on an agar plate assay, using chemically synthesized acyl-HSL molecules with straight-chain and 3-oxo acyl chains that ranged in length from C_4 to C_{12} (Fig. 1A). At the acyl-HSL concentrations tested, LIS produced prodigiosin in response to C4-HSL and C6-HSL, with a weaker response to 3-oxo-C4-HSL and C8-HSL.

To assess the sensitivity spectrum of strain LIS in a quantitative fashion, prodigiosin production by LIS was measured in liquid cultures, in response to synthetic acyl-HSLs (Table 1). The natural *Serratia* 39006 ligand, C4-HSL, was the strongest agonist in this assay, causing half-maximal prodigiosin production at a concentration of

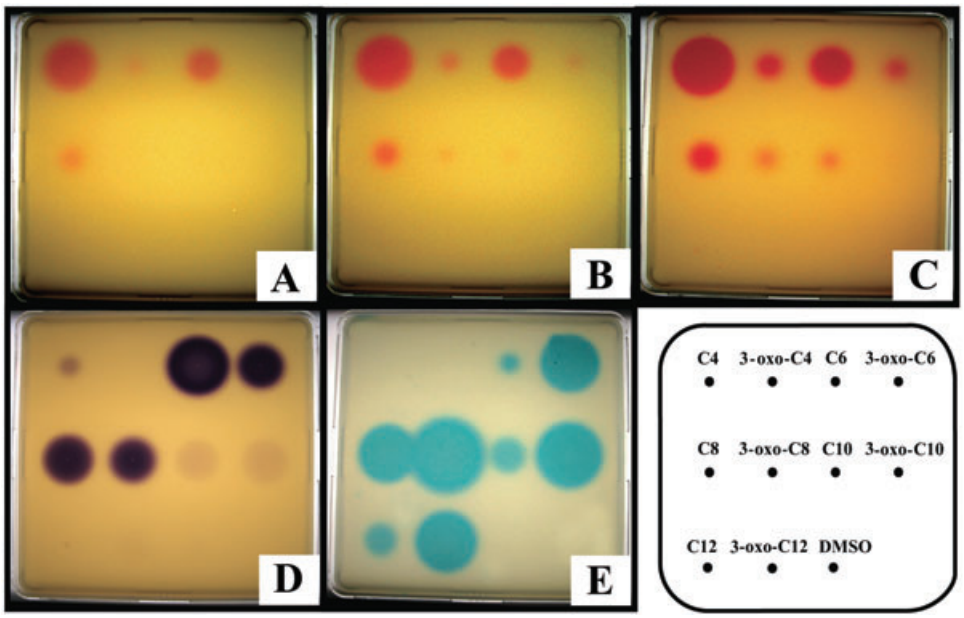

Fig. 1. Detection of acyl-HSL signals by bacterial biosensor strains. Prodigiosin production in response to exogenously applied acyl-HSL molecules by *Serratia* ATCC 39006 biosensor strains (A) LIS (*smaI*), (B) SP16 (*smaI*, *pigZ*) and (C) SP19 (*smaI*, *pigZ*, *pigX*). (D) Violacein production by *Chromobacterium violaceum* biosensor CV026. (E) β-Galactosidase activity of *Agrobacterium tumefaciens* NTL4 (pZLR4). *Serratia* 39006 and CV026 bioassay plates contained 400 ml of solidified 1.5% (w/v) Luria–Bertani agar (LBA) (Miller, 1972) overlaid with 100 ml 0.7% LBA seeded with 1 ml of an overnight culture of biosensor. *Agrobacterium tumefaciens* NTL4 (pZLR4) plates contained 400 ml of solidified 1.5% AB glucose minimal agar (Chilton *et al*., 1974) containing 40 μg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) overlaid with 100 ml of 0.7% AB minimal agar containing 40 μg ml^{-1} X-Gal and 1 ml of an overnight culture of biosensor. Prior to 24 h incubation at 30°C, 2.5 nmol (*Serratia* strains, CV026) or 0.1 nmol (*Agrobacterium* NTL4) of each acyl-HSL was spotted onto the plates and dried. Acyl-HSLs were synthesized as described in Glansdorp and colleagues (2004), and dissolved in dimethyl sulfoxide (DMSO).

Table 1. Concentrations of acyl-HSLs required to induce half-maximal prodigiosin production and prodigiosin biosynthetic operon transcription in biosensor strain LIS (*smaI*).

	$[AHL]_{50}$ (nM)[a]		Relative $[AHL]_{50}$[b]	
Acyl-HSL	Prodigiosin	*pigA* expression	Prodigiosin	*pigA* expression
C4-HSL	70	70	1	1
C6-HSL	870	920	12	13
C8-HSL	7 000	6 000	100	86
C10-HSL	76 000	70 000	1086	1000
C12-HSL	> 100 000	> 100 000	–	–
3O-C4-HSL	6 400	4 000	91	57
3O-C6-HSL	30 000	20 000	429	286
3O-C8-HSL	70 000	70 000	1000	1000
3O-C10-HSL	> 100 000	> 100 000	–	–
3O-C12-HSL	> 100 000	> 100 000	–	–

a. $[AHL]_{50}$ is the concentration of each acyl-HSL required to elicit half-maximal prodigiosin production or β-galactosidase activity from a *pigA*::*lacZ* transcriptional fusion. Samples were taken from early stationary phase (10 h) cultures of LIS (prodigiosin) or MCPI-1 (*pigA* expression) grown in LB, in the presence of acyl-HSLs (0, 10, 100, 1000, 10 000 and 100 000 nM) added at the start of growth. β-Galactosidase enzyme activity was assayed using *o*-nitrophenol-β-galactoside (ONPG) as a substrate as described previously (Miller, 1972). β-Galactosidase activity was expressed as the initial rate of reaction per ml of sample per unit OD_{600} of bacterial culture (ΔA_{420} min^{-1} ml^{-1} OD_{600}^{-1}). Data shown are the means of three independent experiments.
b. Relative $[AHL]_{50}$ corresponds to the ratio of concentration of each acyl-HSL required to elicit half-maximal prodigiosin production, versus the cognate C4-HSL ligand.

70 nM. Response to straight-chain acyl-HSL ligands dropped by an order of magnitude with each addition of a further two carbon atoms onto the acyl-chain of the signalling molecule, reflecting the high degree of specificity of the SmaR transcriptional regulator for its cognate ligand (Table 1). In contrast to C4-HSL, the 3-oxo derivative 3-oxo-C4-HSL was 91-fold less active, requiring 6.4 μM for half-maximal pigment production. This result reflects a general trend in which the *Serratia* 39006 QS system responds much more strongly to straight-chain acyl-HSLs than to 3-oxo derivatives of the same acyl-chain length. The transcription of the *pigA* gene (representative of transcription of the entire prodigiosin operon) in response to each acyl-HSL very closely matched the production of prodigiosin in each case, confirming that derepression of the *pigA* promoter in response to the applied acyl-HSLs was responsible for the increase in prodigiosin production (Table 1).

Combination of a pigZ *and* pigX *mutation causes a 'hyperpigmented' prodigiosin phenotype, caused by increased expression of the prodigiosin biosynthetic operon*

To increase the pigmentation, and therefore the sensitivity, of the LIS biosensor, mutations which cause an overproduction of prodigiosin were exploited. Two significant regulators of prodigiosin biosynthesis are the products of the *pigX* and *pigZ* genes (Fineran *et al.*, 2007; Gristwood *et al.*, 2008). Prodigiosin production by a *pigX*, *pigZ* double mutant was approximately ninefold higher than the wild type strain, and at least two- to threefold higher than either the *pigX* or *pigZ* single mutant, an effect that is mediated at the level of transcription of the prodigiosin biosynthetic operon (Fig. S1).

Construction of new biosensor strains

The hyperpigmented phenotype caused by the *pigX* and *pigZ* mutations was exploited to improve the LIS acyl-HSL biosensor strain. The *Serratia* 39006 transducing phage ΦOT8 (Evans *et al.*, 2009) was used to create two double mutant biosensors, SP16 (*smaI*, *pigZ*) and SP17 (*smaI*, *pigX*), which were then combined to create the triple mutant biosensor strain SP19 (*smaI*, *pigX*, *pigZ*). Due to the *smaI* mutation, no detectable prodigiosin production was observed in the three new biosensor strains (Fig. 2). This indicates that, in spite of the presence of mutations that derange *pigA* expression, prodigiosin production in these *smaI* biosensor mutants remained under tight QS control. Addition of 1 μM C4-HSL restored prodigiosin production in these strains to the level of their hyperpigmented progenitor mutant, indicating their potential as improved biosensors for short-chain acyl-HSLs (Fig. 2).

Use of strain SP19 versus existing acyl-HSL biosensors

A plate bioassay was performed to test the utility of the newly constructed *Serratia* strains as simple visual biosensors for acyl-HSLs (Fig. 1A–C). As expected from liquid prodigiosin assays, strain SP19 produced more prodigiosin than LIS or SP16 in response to short-chain acyl-HSLs (Fig. 1C). Increased prodigiosin production by the SP19 biosensor strain also enabled detection of 3-oxo-C6-HSL, 3-oxo-C8-HSL and C10-HSL, all of which were

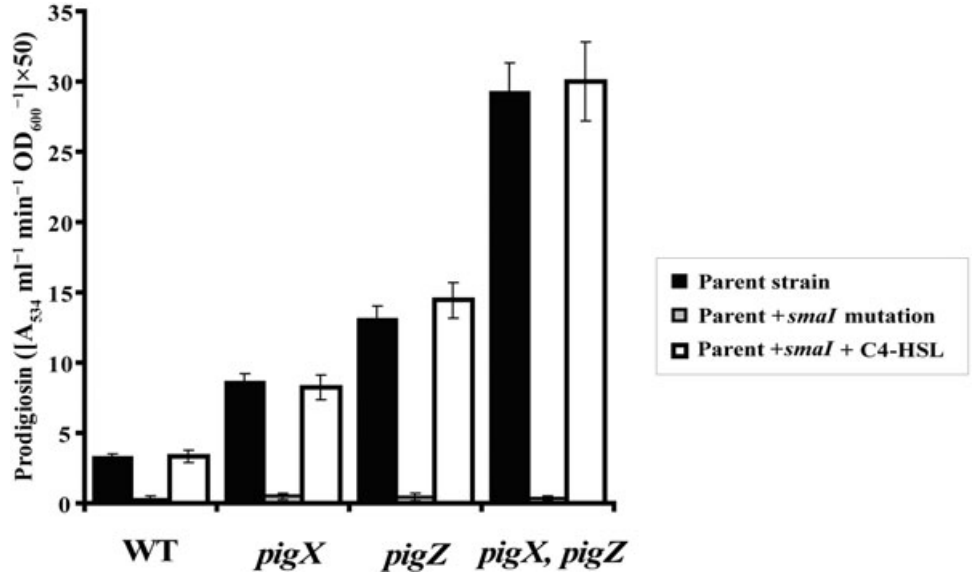

Fig. 2. Loss of pigmentation in *smaI* biosensor mutants is restored by C4-HSL. Prodigiosin production was measured from *pigX* and *pigZ* single and double mutant parent strains (black bars), following the addition of a *smaI* mutation (grey bars), and with supplementation with 1 μM C4-HSL (open bars). Samples were taken from early stationary-phase (10 h) cultures grown at 30°C in Luria–Bertani (LB) medium, at 300 r.p.m. Prodigiosin was measured as absorbance at 534 nm as described previously (Fineran *et al*., 2005b), and expressed as [(A_{534} ml^{-1} OD_{600}^{-1}) × 50]. Data shown are the means ± SD of three independent experiments.

undetectable using the original LIS biosensor at the acyl-HSL concentrations tested.

The response of SP19 to non-cognate acyl-HSLs was then quantified in liquid culture, and compared with the progenitor strain LIS. Although the absolute quantity of prodigiosin produced by SP19 was higher than LIS in each case, the concentration of each acyl-HSL required to induce half-maximal prodigiosin production remained identical (data not shown), indicating that addition of *pigX* and *pigZ* mutations did not alter the acyl-HSL specificity of the biosensor.

SP19 was then compared with two other acyl-HSL biosensors, *C. violaceum* CV026 (Fig. 1D) and *A. tumefaciens* NTL4 (pZLR4) (Fig. 1E). Both the CV026 and NTL4 (pZLR4) biosensors were able to respond with higher sensitivity than SP19 to acyl-HSL molecules with chain lengths between C_6 and C_8, and the longer chained C_{10} and C_{12} acyl-HSLs elicited a strong response from NTL4 (pZLR4). However, strain SP19 gave the strongest signal in response to the short-chain C4-HSL and 3-oxo-C4-HSL molecules, to which the CV026 and NTL4 (pZLR4) respond poorly.

Utility of SP19 as a biosensor using spent supernatant samples

The most appropriate use of the CV026 biosensor is for the detection of acyl-HSLs in spent culture supernatants (McClean *et al*., 1997). To prove that strain SP19 could also detect these physiologically relevant concentrations of acyl-HSLs, filter-sterilized supernatants extracted from various QS species were added to holes punched in an SP19 bioassay plate (Fig. 3A). SP19 responded strongly to supernatant samples from the C4-HSL-producing species *Serratia* ATCC 39006 (Thomson *et al*., 2000) and *Pseudomonas aeruginosa* PA01 (Stover *et al*., 2000). Strain SP19 also produced a weaker response to supernatants from *C. violaceum* ATCC 31532 (C6-HSL producer) (McClean *et al*., 1997) and *Erwinia carotovora* ssp. *carotovora* ATCC 39048 (3-oxo-C6-HSL producer) (McGowan *et al*., 1996), but was unable to respond to supernatant from *E. carotovora* SCC3193, which synthesizes 3-oxo-C8-HSL (Koiv and Mae, 2001).

Biosensor strain SP19 can also be used to detect acyl-HSL production throughout growth in culture. Samples from a culture of wild-type *Serratia* 39006 were taken at hourly intervals throughout growth and the cell-free supernatant was applied to the SP19 biosensor, allowing semi-quantification of the temporal dynamics of acyl-HSL production by providing a 'snap-shot' of acyl-HSL production at each time point (Fig. 3B and C). Strain SP19 is also suitable for large-scale screens for C4-HSL production, or for the identification of acyl-HSL mutants following mutagenesis screens, by patching or spotting colonies or cell suspensions onto the bioassay plate.

Conclusions

Despite the development of various *lux*- and β-galactosidase-based acyl-HSL biosensor strains with

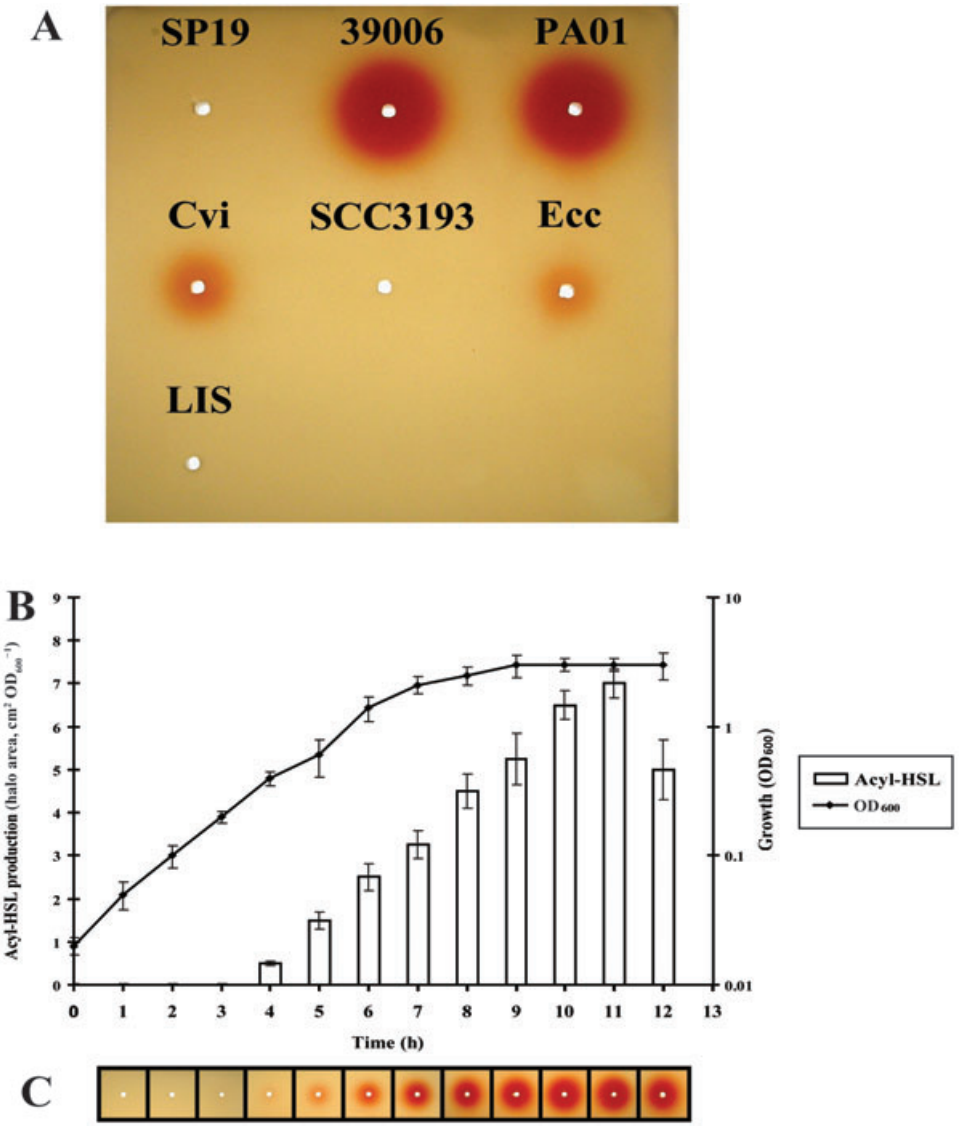

Fig. 3. A. Detection of acyl-HSLs from spent culture supernatants by SP19. Cut-well agar plate bioassay with strain SP19, following 24 h incubation with sterile-filtered supernatants from overnight cultures of: 39006, *Serratia* ATCC 39006; PA01, *Pseudomonas aeruginosa* PA01; Cvi, *Chromobacterium violaceum* ATCC 31532; SCC3193, *Erwinia carotovora* SCC3193; Ecc, *Erwinia carotovora* ssp. *carotovora* ATCC 39048. Acyl-HSL-negative strains LIS (*smaI*) and SP19 (*smaI*, *pigX*, *pigZ*) were used as negative controls. Holes cut with a sterile cork borer were filled with 200 µl of sterile-filtered culture supernatant, prior to incubation.
B. Detection of wild type *Serratia* 39006 acyl-HSL production throughout growth by strain SP19. Solid symbols and lines represent the growth curve (OD_{600}), and open bars represent acyl-HSL production, plotted as prodigiosin halo area (cm^2 OD_{600}^{-1}). Data shown are the means ± SD of three independent experiments.
C. Pigmented halos from the cut-well agar plate assay at each time point.

apparently higher levels of sensitivity, the simple CV026 assay remains extremely popular among QS researchers (Steindler and Venturi, 2007). The popularity of CV026 is due to its low cost, its simplicity and its appealing visual phenotype, characteristics all shared by the SP19 biosensor presented here. However, as shown in this study, the CV026 assay is limited by its relatively weak response to short-chain C_4 acyl-HSLs. Therefore, for researchers working with C4-HSL-producing microorganisms such as species of *Aeromonas*, *Pseudomonas* and *Serratia*, strain SP19 can be used as a powerful tool for the facile detection of short-chain acyl-HSL molecules, and can replace CV026 as a simple visual short-chain acyl-HSL biosensor with no requirement for expensive technical detection equipment.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Effect of *pigX* and *pigZ* mutations on *Serratia* ATCC 39006 prodigiosin production (A) and *pigB* gene expression (B). Samples (1 ml) for prodigiosin assays and *pigB* qRT-PCR analysis were taken from early stationary phase (10 h) cultures of a *pigX* mutant (CH9), a *pigZ* mutant (TG39) and a *pigX*, *pigZ* double mutant (SP15), grown in LB broth. Prodigiosin was measured as absorbance at 534 nm, as described previously (Fineran *et al*., 2005b). Quantitative PCR was performed on the *pigB* gene, as representative of the entire prodigiosin operon, as described previously (Williamson *et al*., 2008). A 5 μl aliquot of template DNA was used in a 25 μl reaction containing 1× SYBR Green PCR Master Mix (Applied Biosystems) and 10 pmol of primers for *pigB* (Table S1). PCR amplification was performed using an Applied Biosystems Prism 7300 sequence detection system,

using reaction conditions as described previously (Williamson *et al.*, 2008). Fluorescence data were processed using SDS software (ABI) to produce threshold cycle (Ct) values for each sample. Relative gene expression was obtained using 16S rRNA as the control with mRNA/16S rRNA = 1 in the wild-type control. Data shown are the means ± SD of three independent experiments.

Table S1. Bacterial strains, phages and primers used in this study.

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