A novel quorum-sensing system regulating the multicellular behaviour in *Serratia marcescens*

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Summary

In *Serratia marcescens* SS-1 the regulation of the production of a biosurfactant that facilitates surface translocation, the pigment prodigiosin and the NucA nuclease involves *N*-acyl homoserinelactone (acyl-HSL) dependant quorum sensing. The major *S. marcescens* SS-1 acyl-HSL signal is identified as *N*-3-oxohexanoyl homoserine lactone (3-oxo-C6-HSL). The gene for its synthase, SpnI, and the response regulator, SpnR, have been cloned and their sequences determined. SpnR over-expression and *spnR* gene deletion show that, in contrast to the two other *Serratia* quorum sensing systems investigated to date, SpnR appears to act as a repressor. SpnI over-expression, acyl-HSL supplementation and *spnI* gene deletion show that SpnR is de-repressed by 3-oxo-C6-HSL. The blockade of 3-oxo-C6-HSL signalling with long acyl chain HSLs reduces biosurfactant mediated surface translocation in both *S. marcescens* SS-1 and an acyl-HSL quorum sensing negative *S. marcescens* isolate, CH-1. Upstream of *spnI* there is a gene we have termed *spnT*. Database searches have found no homologs of SpnT and while usually reliable strategies to delete this gene from the *S. marcescens* SS-1 chromosome have failed, over-expression of this gene produces a strong inhibition of quorum sensing controlled traits.

Introduction

Serratia marcescens is noted for the production of a red pigment, prodigiosin (2-methyl-3-pentyl-6-methoxyprodigiosin) (Dauenhauer *et al.*, 1984; Hejazi and Falkiner, 1997) that is now receiving renewed interest as a consequence of its potency as a therapeutic agent (Han *et al.*, 1998; Montaner *et al.*, 2000). In addition, the β-lactam antibiotic, carbapenem (carbapen-2-em-3-carboxylic acid) has been described as a secondary metabolite produced by some strains of *Serratia* (Cox *et al.*, 1998; McGowan *et al.*, 1999). Moreover, *S. marcescens* has emerged as a nosocomial pathogen, mainly being responsible for outbreaks that affect immuno-compromised or surgical patients, and where *Serratia* multi-antibiotic resistance amplifies the problem (Arakawa *et al.*, 2000; Traub, 2000; Knowles *et al.*, 2000). Interestingly, isolates of *S. marcescens* from the clinical setting are predominantly non-pigmented (Carbonell *et al.*, 2000), however a number of other traits have been identified that may be involved in pathogenesis, including swimming and swarming motility and extracellular enzyme activities, i.e. nuclease, protease, lipase, and haemolysin (Hejazi and Falkiner, 1997).

The regulation of both prodigiosin production and carbapenem production has been demonstrated to be under the control of *N*-acylhomoserine lactone (acyl-HSL) mediated quorum sensing in *Serratia* sp. ATCC 39006 (Thomson *et al.*, 2000). Quorum sensing is a mechanism of gene regulation that requires cell-to-cell signalling and reflects the population cell density (reviewed in Dunny and Winans, 1999; Fuqua and Eberhard, 1999; Williams *et al.*, 2000; Swift *et al.*, 2001). The simple paradigm describes bacteria at low population cell density producing a small amount of a diffusible signal molecule that accumulates in the extracellular milieu. After growth to a high population cell density, the concentration of the signal reaches a threshold level and activates

transcription of the quorum sensing regulon of genes via a response regulator protein. In Gram-negative bacteria acyl-HSL signalling is predominant, although other signalling chemistries are apparent (Holden *et al.*, 2000). The LuxI family of proteins is most commonly associated with acyl-HSL signal generation. The LuxR family of proteins is most commonly associated with acyl-HSL signal response. The *luxI* and *luxR* homologous genes are often genetically linked, although they may be arranged consecutively, convergently or divergently (Salmond *et al.*, 1995).

Notably in *Serratia* sp. ATCC 39006, the quorum sensing signals produced were identified, using comparative thin-layer chromatography, as *N*-butanoylhomoserine lactone (C4-HSL; major product) and *N*-hexanoylhomoserine lactone (C6-HSL; minor product) (Thomson *et al.*, 2000). The genes encoding the signal generator and response regulator proteins were identified as a convergently transcribed pair and termed *smaI* and *smaR* (Thomson *et al.*, 2000). Protein sequence identity analyses identified SmaI and SmaR as members of the LuxI and LuxR families, sharing high levels of identity with homologues from *Erwinia chrysanthemi* (SmaR:EchR 55%), *Erwinia caratovora* (SmaR:ExpR 55%; SmaI:EcbI 53%) and *Serratia liquefaciens* (SmaR:SwrR 71%, SmaI:SwrI 55%).

The quorum sensing response in bacterial populations can favour pathogenesis, symbiosis, dissemination or dispersal, DNA transfer, microbial biofilm development, the production of antibiotics and other secondary metabolites, and the exit from dormancy (see Dunny and Winans, 1999; Swift *et al.*, 2001 for reviews). In the non-pigmented *S. liquefaciens* strain MG-1 acyl-HSL quorum sensing activates the production of a secondary metabolite with surfactant properties (Lindum *et al.*, 1998). One physiological role for this substance has been demonstrated in the facilitation of flagellar-mediated swarming motility across surfaces, e.g. that of an agar plate.

Evidence also exists for other traits forming part of a quorum sensing regulon in *S. liquefaciens*, i.e. in the quorum sensing signal generator *swrI* mutant the production of an exoprotease activity is much reduced (Eberl *et al.*, 1996). Recently this has been attributed to quorum sensing control of the LipB protein involved in the secretion of lipase, protease and S-layer monomers (Riedel *et al.*, 2001).

Surface translocation is important in colonisation and pathogenesis. For example, the swarming motility phenotype of *Proteus mirabilis* has been implicated in uropathogenesis, with mutants unable to swarm being unable to ascend to the kidney from the bladder (Allison *et al.*, 1994). Dramatic morphological and physiological changes occur in swarming cells (Allison and Hughes, 1991; Harshey, 1994). This swarmer cell differentiation involves a change from short, vegetative cells with few peritrichous flagella to elongated, multi-nucleoid swarmer cells covered with flagella that are required to produce the population movement over a surface. The regulation of swarming in *Serratia* spp., perhaps unexpectedly, also involves the FlhDC flagellar master regulator (Givskov *et al.*, 1998; Fraser and Hughes, 1999; Liu *et al.*, 2000). In addition, the *rsmA* (repressor of secondary metabolism) gene product (named after the *Erwinia* homologue; Cui *et al.*, 1995) of *Serratia* inhibits swarming motility (Ang *et al.*, 2001). The activity of *rsmA* in both *Erwinia* and *Serratia* is part of the regulatory network that also involves acyl-HSL based quorum sensing regulation (Cui *et al.*, 1995, Ang *et al.*, 2001).

In this study we have investigated the role of acyl-HSL mediated quorum sensing in two pigmented isolates of *S. marcescens* with particular emphasis upon secondary metabolism, exoenzyme production and surface translocation. The differences observed between the *Serratia* strains documented here and in previous studies are striking and may offer insight into the role of quorum sensing in bacterial ecology and the spread of

various quorum sensing loci throughout bacterial populations.

Results

Genetic structure of the quorum sensing locus in Serratia marcescens SS-1

The first reports of acyl-HSL signalling in *S. marcescens* used a pigmented, laboratory-maintained strain we have designated SS-1. In 1993, the population cell density dependent production of an acyl-HSL activating a plasmid-based biosensor was shown for *S. marcescens* SS-1 (Swift *et al.*, 1993). The biosensor technology used relies upon activation of LuxR, derived from *Vibrio fischeri*, by its cognate ligand *N*-3-oxohexanoyl homoserine lactone (3-oxo-C6-HSL), or an agonist thereof. The activated LuxR then activates transcription of the reporter gene, which in this case was *luxAB*. Swift *et al.*, (1993) also utilised the biosensor strain to screen a *S. marcescens* SS-1 DNA library for a clone that could direct the production of 3-oxo-C6-HSL, or an agonist, and a 7.5 kbp *Bam*HI DNA fragment was isolated. To extend this study, the DNA sequence of the 7.5 kbp fragment has been determined. The genes for the acyl-HSL signal synthase, *spnI*, and the response regulator, *spnR*, (*spn* stands for sliding, pigment and nuclease) were located as a convergon at one end of the *Bam*HI fragment, with 6.3 kbp cloned upstream of *spnR*. A second screening (*Nsi*I library) identified a 4 kbp DNA fragment for which DNA sequencing revealed *spnRI* and 2.5 kbp of DNA upstream of *spnI*.

The *spnRI* region sequenced comprises approximately 10 kbp and 11 complete and partial open reading frames (ORFs). The structure of the *spnRI* region upon the *S. marcescens* SS-1 genome is shown in Figure 1, and the identities of the open reading frames indicated are given in Table 1. The DNA sequence of the *spnRI* region of *S. marcescens* SS-1 has been submitted to GenBank under the accession number

AF389912. It is interesting to note here that SpnR and SpnI share most identity with the EsaR (67 %) and EsaI (57 %) proteins of *Pantoea stewartii* (von Bodman *et al.*, 1998), and not the SmaR and SmaI genes identified in *Serratia* sp. ATCC 39006 (Thomson *et al.*, 2000), with the identity of 43 % and 34 %, respectively.

C6-HSL and 3-oxo-C6-HSL are the major quorum sensing signals produced by Serratia marcescens SS-1

Cell-free supernatants from *S. marcescens* SS-1 cultures taken at various points in growth were extracted with dichloromethane and fractionated by TLC and HPLC. TLC overlays with the biosensor strain *C. violaceum* CV026 revealed major spots corresponding to C6-HSL and 3-oxo-C6-HSL in supernatants after growth to an A_{600nm} of 1.0. Allowing growth to continue to an A_{600nm} of 2.2 revealed two additional minor spots corresponding C7-HSL and C8-HSL (Figure 2a and 6b). The designations were made after comparison to synthetic acyl-HSL standards. To confirm this preliminary identification, HPLC fractionation [acetonitrile/water gradient from 20% (V/V) to 80%] and sub-fractionation of the active molecules (isocratic mobile phase) followed by mass spectrometry was performed. This analysis confirmed the two major signal molecules as C6-HSL and 3-oxo-C6-HSL and the two minor signal molecules as C7-HSL and C8-HSL (Figure 2d).

SpnR is a repressor of prodigiosin production, nuclease production and sliding motility in Serratia marcescens SS-1

To investigate the role of SpnR we examined the effects of (1) increasing the number of

SmaR molecules in the cell by introducing a plasmid copy of *spnR* and (2) deleting the *spnR* gene from the *S. marcescens* SS-1 chromosome using homologous recombination. Increasing the SpnR copy has an obvious negative effect upon the production of the red pigment, prodigiosin: colonies of *S. marcescens* SS-1 (pSC200) are white, whereas those of the control strain *S. marcescens* SS-1 (pSC201) are red. Deletion of the *spnR* gene produces colonies that are qualitatively more pigmented when compared to the parent strain.

Analysis of *S. marcescens* SS-1, *S. marcescens* SS-1ΔR, *S. marcescens* SS-1 (pSC200), and *S. marcescens* SS-1 (pSC201) in exoenzyme assays failed to demonstrate any effect upon protease or phospholipase (data not shown). As the production of exonuclease activity is partially regulated by the *flhDC* operon in *S. marcescens* (Liu *et al.*, 2000), we further investigated whether the *spnRI* system also performed a regulatory role. A microplate format DNA degradation assay (Chen *et al.*, 1992) shows that in the presence of multicopy SpnR *in trans*, exonuclease activity is reduced, but in the absence of SpnR, exonuclease activity is increased (Fig. 3a). Hence, in comparison to the parental *S. marcescens* SS-1 strain, the nuclease activity produced by SS-1ΔR is substantially higher (up to 400% of the wild-type activity; Fig. 3a). In the presence of multi-copy *spnR*, the nuclease activity of *S. marcescens* SS-1 (pSC200) is reduced (to 50% of the wild-type activity; Fig. 3a). The provision of *spnR in trans* to *S. marcescens* SS-1ΔR restores nuclease activity to give about 200% of the parental activity (Fig. 3a).

The data presented above indicates that SpnR plays a negative regulatory effect upon the production of nuclease. To determine whether this effect was due to action at the nuclease promoter or perhaps an effect at some other stage of the secretion of the nuclease, a chromosomal *nucA*::*luxAB* promoter fusion was constructed in *S.*

marcescens SS-1 (to obtain the strain *S. marcescens* N-2) and assayed in the presence/absence of pSC200. A clear negative effect of SpnR upon the expression of luciferase activity is observed (Fig. 3b). The light emission of *S. marcescens* N-2 (pSC200) is substantially lower than that of *S. marcescens* N-2 (pSC201) (35% parental activity left; Figure 3b). The inhibitory effect can be complemented by addition of 3-oxo-C6-HSL at the concentration of 10 μM (Fig. 3b). These data demonstrate that SpnR exerts a negative regulatory effect upon the exonuclease activity of *S. marcescens* SS-1 by influencing the level of *nucA* gene transcription.

S. marcescens SS-1 does not swim or swarm, a property we presume is due to the inability of this strain to produce flagellae (Fig. 4b). SDS-PAGE analysis of total cell protein and of sheared surface protein fails to show the presence of the 37 kDa flagellin subunit (data not shown). Furthermore, no flagellae are visible under the microscope after silver staining of the bacteria (Fig. 4b). Nevertheless, *S. marcescens* SS-1 is still able to spread rapidly from a point inoculation to a 0.35% agar LB plate, a phenomenon we will describe as sliding motility (see also Henrichsen, 1972 and Martinez *et al.*, 1999) (Fig. 4a). The behaviors of *S. marcescens* SS-1, *S. marcescens* SS-1 ΔR, and *S. marcescens* SS-1 (pSC200) in sliding motility assays are markedly different (Fig. 5a) and in keeping with SpnR as a negatively acting regulator, as seen with the effects upon pigment and nuclease production. Hence, in comparison to their control strains, *S. marcescens* SS-1 (pSC200) does not slide from the point of inoculation and *S. marcescens* SS-1ΔR is up-regulated for sliding motility. Moreover, the *S. marcescens* SS-1ΔR colony begins to slide much earlier after inoculation than the parent strain, and the sliding of SS-1 ΔR was inhibited in the presence of multi-copy pSC200 (Fig. 5a). A comparison of the growth rates of *S. marcescens* SS-1 with its *spnR* deletion strain and

of *S. marcescens* SS-1 (pSC200) with *S. marcescens* SS-1 (pSC201) in L-broth does not show any differences that could explain differing rates of sliding motility (data not shown).

SpnR control of sliding motility is through the regulation of the production of a biosurfactant

The role of quorum sensing in *S. liquefaciens* has been described previously to activate the production of a biosurfactant (Lindum *et al.*, 1998). One effect of biosurfactant production is the reduction of surface tension of broth cultures, which can be qualitatively assessed using the drop-collapsing test (Fig. 5a). A semi-quantitative thin layer chromatography technique has also been developed to assess biosurfactant production (Matsuyama *et al.*, 1992) (Fig. 5b). The data presented in Fig. 5 show that the extent of sliding motility can be correlated with biosurfactant production. Hence, *S. marcescens* SS-1ΔR exhibits greatest sliding motility and produces most biosurfactant, whereas *S. marcescens* SS-1 (pSC200) shows very little sliding motility and produces no detectable biosurfactant. The beginning of sliding motility is observable approximately 3 h after inoculation for *S. marcescens* SS-1. In comparison, the migration of *S. marcescens* SS-1 ΔR is reproducibly observable after only about 1.5 h (Fig. 5a). The provision of multi-copy *spnR*, *in trans*, on pSC200, represses the production of biosurfactant and increases the time of sliding initiation in *S. marcescens* SS-1 ΔR from 1.5 hours to about 5 hours (Fig. 5a).

SpnR has no effect upon acyl-HSL production but it is a positive activator of self

To determine whether SpnR has any effect upon quorum sensing signal production, acyl-HSLs were monitored through growth for *S. marcescens* SS-1, *S. marcescens* SS-1ΔR, *S. marcescens* SS-1 (pSC200) and *S. marcescens* SS-1ΔR (pSC200). The spent supernatants were collected and the amount of acyl-HSL signals was monitored using the biosensor *E. coli* JM109 (pSB401) (Winson *et al.*, 1998). The results in Fig. 6a demonstrate that the synthesis of a LuxR activating signal increases throughout the growth of *S. marcescens* SS-1. The strains tested [SS-1 (pSC201), SS-1ΔR (pSC201), SS-1 (pSC200) and SS-1ΔR (pSC200)] show no differences in their growth rates and produce similar patterns of quorum sensing dependent light emission, suggesting that synthesis of acyl-HSL signals by *S. marcescens* SS-1 is independent of the expression of SpnR. By following the pattern of acyl-HSL signal production from cultures of SS-1 and SS-1ΔR throughout growth in CV026 TLC overlay assays, we further demonstrate that SpnR has little influence upon the actual acyl-HSLs produced (Fig. 6b). In a third experiment, a recombinant plasmid pYT505 [pACYC184(P*spnI*::*luxAB*)] was constructed to determine whether any SpnR effect upon the activity of the *spnI* promoter could be detected. The intensity of light emission was monitored throughout the growth in *S. marcescens* SS-1 (pYT505) and SS-1ΔR (pYT505). The results are presented in Fig. 6c and show that a similar pattern of *spnI* promoter activity is observed in both SS-1 and SS-1ΔR strains. The promoter activity is highest when cells are in the logarithmic phase of growth, reducing as cells enter the stationary phase.

A *lux* box consensus sequence as defined in Egland and Greenberg (1999) is present in the upstream region of *spnR* (Fig. 7a) and partially overlaps with the predicted –35 promoter region, suggesting that the expression of *spnR* might be auto-regulated. To confirm this a recombinant plasmid, pSC503 [pACYC184(P*spnR*::*luxAB*)], was

constructed to monitor the promoter activity of P*spnR*. A comparison of the light emission patterns from *S. marcescens* SS-1 (pSC503) and *S. marcescens* SS-1 ΔR (pSC503) (Fig. 7b) showed an average increase of 2.4 fold in P*spnR* activity in the presence of a chromosomal copy of *spnR*, suggesting the positive auto-regulation effect of SpnR. The addition of 3-oxo-C6-HSL (10 μM) to the media further stimulated the activity of P*spnR* (to 5 times) in SS-1 (pSC503) (Fig. 7b). Taken together these data suggest that SpnR stimulates the activity of its own promoter and that this effect is enhanced by the addition of 3-oxo-C6-HSL.

3-oxo-C6-HSL and 3-oxo-C8-HSL derepress SpnR

The observation that excess SpnR represses a wide range of phenotypic characters, whereas the deletion of *spnR* resulted in the over-expression of these same traits led us to hypothesise that SpnR is acting as a repressor. To ascertain the role of the acyl-HSL signals in this process we followed three lines of investigation: (1) addition of exogenous synthetic acyl-HSLs to *S. marcescens* SS-1(pSC200); (2) increasing acyl-HSL production by *S. marcescens* strains by providing *spnI* on a multicopy plasmid; and (3) making a *spnI* deletion mutant.

The addition of *S. marcescens* SS-1 signal molecule, 3-oxo-C6-HSL and also 3-oxo-C8-HSL at 10 μM to sliding motility plates inoculated with *S. marcescens* SS-1 (pSC200) overcomes the repression by SpnR (Fig. 8a). The addition of C4-HSL, C5-HSL, C6-HSL, C8-HSL, C10-HSL, C12-HSL, 3-oxo-C4-HSL, 3-oxo-C10-HSL, 3-oxo-C12-HSL and 3-oxo-C14-HSL had no observable effect when applied at 10 μM (Fig. 8a). Further experiments showed that the de-repression effect by 3-oxo-C6-HSL and 3-oxo-C8-HSL is dose-dependent (Fig. 8b). The provision of *spnI* on the plasmid

pYT300 to strain *S. marcescens* SS-1 (pSC200) de-represses SpnR, thus allowing sliding motility (Fig. 8c). In both situations the provision of acyl-HSLs either exogenously, or via SpnI, to *S. marcescens* SS-1 (pSC200) also restores pigment and biosurfactant production (data not shown). A similar de-repressing effect from 3-oxo-C6-HSL and 3-oxo-C8-HSL on the production of nuclease was also observed (data not shown).

The deletion of the *spnI* gene results in a phenotype similar to the SpnR over-expression strain. Prodigiosin production is reduced and the colonies are essentially white (data not shown). Biosurfactant production (Fig. 5) and sliding motility (Fig. 5) are substantially reduced. In each case the *spnI* mutant can be complemented by the provision of exogenous 3-oxo-C6-HSL (Fig. 5). Interestingly, the *spnI* mutant strain retains the ability to produce reduced levels of AHLs as shown in Fig. 2c. Addition of 3 μl out of a total of 100 μl concentrated supernatant (equivalent to 30 ml of cell-free supernatant) prepared from a 1 litre overnight MGM broth culture gave faint C6-HSL and 3-oxo-C6-HSL signals from the SS1ΔI mutant in the TLC/CV026 assay (Fig. 2b). PCR and Southern blot hybridisations demonstrate that the *spnI* gene is both disrupted and has its central portion deleted. The N-terminal fragment of SpnI that remains is unable to direct the synthesis of acyl-HSLs when encoded upon a plasmid in *E. coli* (data not shown).

The nuclease activity produced by *S. marcescens* SS-1ΔI was reduced to about 25% of the parental *S. marcescens* SS-1 level. The complementation of the *spnI* mutation *in trans* with pYT200 restores nuclease activity to parental levels (Fig. 3a). The complementation of the *spnI* mutation with exogenously added synthetic acyl-HSL signals is successful for the cognate molecule 3-oxo-C6-HSL (Fig. 3a) and also 3-oxo-C8-HSL but none of the others tested [i.e. C4-HSL, C5-HSL, C6-HSL, C8-HSL,

C10-HSL, C12-HSL, 3-oxo-C4-HSL, 3-oxo-C10-HSL, 3-oxo-C12-HSL and 3-oxo-C14-HSL](data not shown). Using *S. marcescens* N-2 (which carries a chromosomal P*nucA*::*luxAB* reporter fusion), it was shown that addition of 3-oxo-C6-HSL at the concentration of 10 μM into the medium restores the repressed light emission of N-2 (pSC200) (Fig. 3b). These data show the repression of *nucA* expression by SpnR is relieved in the presence of the SpnR ligand, 3-oxo-C6-HSL.

Long chain acyl-HSLs antagonise sliding motility in Serratia marcescens SS-1, and swarming motility in S. marcescens CH-1

To study the role of quorum sensing in the regulation of swarming motility in *S. marcescens*, we began work with a flagellated *S. marcescens* strain, termed CH-1 (Fig. 4c), which has previously been characterised as motile in swimming and swarming assays (Liu *et al.*, 2000). Unexpectedly, we discovered that this strain was unable to produce any acyl-HSL able to activate biosensors based upon LuxR, CviR, AhyR or LasR in T-streak assays and in well assays after dichloromethane extraction of spent culture supernatants (data not shown). To exclude the possibility that inhibitory compounds were present in the crude dichloromethane extract we subjected the extract to fractionation by both HPLC and TLC and assayed using well assays or biosensor overlays respectively. In both cases no acyl-HSL activity was detected (data not shown). As further evidence for the absence of a LuxRI/acyl-HSL quorum sensing system in *S. marcescens* CH-1, DNA/DNA hybridisation in a Southern blot failed to identify any homologous DNA to the *spnRI* and *spnT* genes of SS-1 (data not shown).

To determine whether *S. marcescens* CH-1 could perceive and respond to acyl-HSLs, we performed swarm plate motility assays with a range of synthetic acyl-HSLs

incorporated into the medium at the concentration of 10 μM. Figure 9b shows while all the short chain acyl-HSLs tested did not inhibit swarming, a clear, inhibitory effect of some specific long chain acyl-HSLs upon swarm plate motility was observed. Among the long chain acyl-HSLs tested, 3-oxo-C10, 3-oxo-C12 and especially 3-oxo-C14 inhibit the CH-1 swarming most significantly. Using *S. marcescens* SS-1 as the study model a similar inhibitory effect of long chain acyl-HSLs upon sliding plate motility was also observed (Fig. 9a).

SpnT inhibits the sliding and pigment production in S. marcescens SS-1

Analysis of the *orf*s to either side of *spnRI* identified a number of transposon remnants. The only exception is *orf3* that is directly upstream of *spnI* and which we have designated *spnT*. Overexpression of *spnT* in *S. marcescens* SS-1 has a profound negative effect upon sliding motility and pigment production (Fig. 10) without affecting the bacterial growth rate. Homology searches with the deduced SpnT primary protein sequence provide little information regarding possible functions. To date we have been unable to disrupt the *spnT* gene using homologous recombination techniques that are routinely successful in *Serratia* in our hands, suggesting that SpnT is essential under the conditions used for selection of the *spnT* mutant.

Discussion

In this study we describe a quorum-sensing system that regulates expression of biological traits involved in the multicellular behaviors of *S. marcescens.* We demonstrate that the flagellar independent surface translocation, by a sliding motility, of *S. marcescens* SS-1 relies upon the production of a QS-regulated biosurfactant. In addition we identify two additional components of the QS-regulon in *S. marcescens* SS-1 as the genes responsible for the production of the pigment prodigiosin and the extracellular nuclease.

Quorum sensing has previously been described in two strains of *Serratia.* In the non-pigmented *S. liquefaciens* MG-1, flagellar dependent surface translocation by swarming motility has been shown to rely upon the production of a QS regulated surfactant. Moreover, the SwrRI system has recently been shown to regulate the secretion of the LipA lipase, PrtA metalloprotease and the SlaA S-layer monomer via the regulation of the Lip exporter (Riedel *et al.*, 2001). For *S. liquefaciens* MG-1, this study demonstrated that Swr signalling did not control the transcription of *lipA, prtA* or *slaA,* but that the effects seen in the QS regulation of protease and lipase activities in the supernatant was due to transcriptional control of components of the secretory apparatus. In contrast, for *S. marcescens* SS-1 we show using a chromosomal gene fusion in *nucA* that Spn signalling is regulating the transcription of *nucA* itself. NucA is translocated across the inner cytoplasmic membrane to the periplasm via the Sec pathway (Suh *et al.*, 1996) and so is independent of LipB. The mechanism by which NucA crosses the outer membrane is unclear (reviewed in Benedik and Strych, 1998). Benedik and Strych (1998) suggested that the growth phase dependent secretion of NucA was the result of QS regulation, perhaps via the activation of NucC (a *nucA* activator encoded upon a

cryptic prophage). Our study suggests that QS control is focussed directly upon *nucA*. However, given the variation in QS systems employed by *Serratia* observed in this study, the specific involvement of QS and NucC in the regulation of *nucA* should be determined for each strain tested. Perhaps most intriguing in relation to these observations is that *Serratia* employs genes encoded in the remnants of prophage and transposon inserts to regulate the expression of its nuclease. In the pigmented *Serratia* spp. ATCC 39006 the production of prodigiosin and also an antibiotic, carbapenem, is under QS control.

A significant finding of this study is that while the QS systems of *S. liquefaciens* MG-1 and *Serratia* spp. ATCC 39006 are closely related to each other, by virtue of protein primary sequence homologies and cognate acyl-HSLs, they do not exhibit as much similarity to the QS system of *S. marcescens* SS-1, which is most similar to the EsaRI system of *P. stewartii*. Hence, although the QS-regulated traits described in this study for a member of the genus *Serratia* are not novel, the QS apparatus and underlying regulatory mechanism used are. Nevertheless there are some organizational similarities as both the Sma and Spn QS systems are flanked by remnants of transposons, although different IS family homologues are identified (Tn3 and IS3 for SS-1, and IS1 for ATCC39006). These results suggest that complex QS regulatory systems exist in *S. marcescens* regulating the multicellular behaviours and secondary metabolites. The observations at the protein primary sequence level for SpnRI have a functional relevance as SpnR behaves as a repressor. De-repression occurs in the presence of the SpnI derived acyl-HSL. This is in contrast to most LuxR-like regulators, including SmaR and SwrR, which are thought to form complexes with acyl-HSL signals and then function as transcriptional activators. Other examples of repressor activity include EsaR of *P. stewartii* (von Bodman *et al*., 1998) and probably YpsR of *Yersinia*

pseudotuberculosis (Atkinson *et al.*, 1999). Nevertheless, although there are many similarities between SpnR and EsaR, we have identified one difference, i.e. that EsaR auto-represses (von Bodman and Farrand, 1995) but SpnR auto-activates at its own promoter. The possible mechanism may involve the location of the *lux* box in the promoter region, and is discussed later.

Although the phenotypic effects of the *spnI* mutation in *S. marcescens* SS-1 are obvious and complemented by the exogenous addition of the cognate acyl-HSLs, we do still detect low levels of acyl-HSLs (with TLC Rf values comparable to C6-HSL and 3-oxo-C6 HSL). We have confirmed by PCR and Southern blotting that the *spnI* deletion mutant has been constructed as described. We have tested the N-terminal remnant of SpnI as constructed in the plasmid used to direct the gene deletion by homologous recombination and have shown that this construct cannot direct the production of acyl-HSLs when expressed in *E. coli*. Indeed, all the residues shown by mutational analyses of LuxI homologues (Hanzelka *et al.*, 1997; Parsek *et al.*, 1997) are deleted. We therefore suggest that a second acyl-HSL synthase exists in *S. marcescens* SS-1. This is true for *Y. pseudotuberculosis*, where YpsRI and YtbRI systems exist (Atkinson *et al.*, 1999) and *P. aeruginosa*, where LasRI and RhlRI systems exist (Latifi *et al.*, 1995; Pesci *et al.*, 1997). A second possibility is that *Serratia* may possess an alternative acyl-HSL synthase of the LuxLM (Bassler *et al.*, 1993; Gilson *et al.*, 1995) or HdtS families (Laue *et al.*, 2000).

The most revealing studies of the activity of a member of the LuxR family to date have focussed upon TraR from *Agrobacterium tumefaciens*. An analysis of the accumulated data for TraR suggests that transcriptional activation is promoted upon the acyl-HSL induced dimerisation (Zhu and Winans, 1999; 2001; Qin *et al.*, 2000). Further evidence obtained with TraR suggests that inactive monomers reside at the

inner cell membrane, where they may interact with incoming acyl-HSL signals, undergo conformational change, form dimers that are released into the cytosolic fraction and which are capable of activating transcription (Qin *et al.*, 2000). In LuxR and TraR distinct regions containing amino acids that are involved in transcriptional activation, DNA binding, dimerisation have been localised, although there is some difference between the location of some activities between these two proteins (Luo and Farrand, 1999; Trott and Stevens, 2001). In contrast, data gathered for CarR (an acyl-HSL activated regulator of carbapenem biosynthesis in *Erwinia carotovora*) (McGowan *et al.*, 1995; Welch *et al.*, 2000) and EsaR (an acyl-HSL de-repressed regulator of exopolysaccharide biosynthesis in *P. stewartii*)(von Bodman *et al.*, 1998; Qin *et al.*, 2000) show dimerisation in the absence of ligand. The data presented here suggests that SpnR behaves in a similar fashion to its closest homolog, EsaR, and hence we hypothesise that it forms DNA binding dimers in the absence of acyl-HSL. Thus in the absence of acyl-HSL we see repression of target genes and in the presence of de-repressing levels of acyl-HSL we see de-repression and the potential for target gene expression. Nevertheless, data presented in this study also suggests that SpnR can act as an activator of *spnR* gene expression. A potential *spnR* promoter contains a 20 bp region of dyad symmetry, which overlaps 1 bp with a putative -35 region and closely resembles the canonical *lux* box sequence (Fig.7a). It is tempting to speculate that SpnR activates its own expression by interacting with this *lux* box-like element. No *lux*-box like sequence is found in the promoter region of *spnI*, so it seems reasonable that, as indicated by our data, SpnR does not regulate the expression of *spnI*. A similar situation is observed in *P. stewartii* (von Bodman *et al.*, 1998). Furthermore, EsaR represses the expression both *esaR* and *cps* operons, where the *lux*-box like elements are all located at the –10 region, blocking access for transcription (von Bodman *et al.*, 1998).

Although the *spnR* and *spnI* gene produces are key components in the regulatory network, other genes are also important. For instance, *spnT* in multi-copy causes a dramatic reduction of pigment synthesis, change of red-white colonial variation and complete inhibition of sliding motility (Fig. 10). SpnT is a novel protein and appears potentially as a pivotal regulator of SS-1 multicellular phenotypes**.** Further studies in progress should clarify the role of this unique regulator in the regulation of multicellular behaviors.

Spn-signalling may also play roles in the bacterial translocation and colonization during the process of interaction between bacteria and eukaryotic host organisms. The ability to coordinate the behaviour of a population of bacterial pathogens may contribute to establishing a successful infection. The ability to interfere with this co-ordination may be valuable in combating bacterial infection where the blockade of quorum sensing appears to be a viable target (see Finch *et al*., 1998 for a review and Givskov *et al*., 1996; Mayville *et al*., 1999; Swift *et al*., 1999 for experimental evidence). In this study we show that simple QS antagonists in the form of long chain acyl-HSLs can inhibit the surface translocation of not only *S. marcescens* SS-1, which controls biosurfactant production via acyl-HSL signalling, but also of *S. marcescens* CH-1, which has not yet been shown to use acyl-HSL signalling, despite considerable efforts to do so. An understanding of the molecular basis of how *S. marcescens* CH-1 perceives and reacts to the presence of acyl-HSLs is now required to underpin possible future investigations that may focus on controlling or manipulating quorum-sensing systems to intervene in pathogenic interactions.

Experimental procedures

Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this study are described in Table 1. *E. coli* was cultured at 37 ^{o}C and *S. marcescens* and *Chromobacterium violaceum* CV026 at 30 ^{o}C in L-broth (LB) medium (Difco, USA) (Sambrook *et al.*, 1989). In cases of mixed cultures, e.g., conjugations, incubations were at 30 ^{o}C. For extraction of acyl-HSLs, M9 minimal growth medium (M9 MGM, Sambrook *et al.*, 1989) was used for overnight bacterial culture. Swimming motility was examined on motility agar [LB solidified with 0.3% Eiken agar (Eiken, Japan)] by sterile needlepoint inoculation from an overnight culture into the centre of the agar plate. Sliding assay was performed on L-broth medium with 0.35% Eiken agar and swarming motility was examined on swarming agar plates (LB solidified with 0.8% Eiken agar) by inoculating 5 µl of an overnight broth culture onto the centre of the agar plate. Bacterial colonies were examined by light microscopy at a magnification of 1x or 30x using an Olympus SZH microscope. Images were captured using an Olympus C35AD photo system. An Olympus BH2 light microscope was used for observation of single bacterial cells at a magnification of 1000x. For determining the bacterial growth rate, hourly increases in the optical density of broth cultures at 600 nm were observed.

Analysis of Flagellum production

To prepare flagellae for SDS-PAGE analysis cells from the surface of agar plate were harvested by washing into 3 ml of LB. Flagellin from the supernatant of vortexed cells

was precipitated by 10% trichloro-acetic acid (Allison *et al.*, 1992), normalized to cell mass [A_{600nm} x (ml) cell suspension volume =5], separated by SDS-PAGE (Sambrook *et al.*, 1989) and stained with Coomassie brilliant blue. Flagellae were stained on whole cells using the silver-plating method of West *et al.*, (1977).

Enzymes and chemicals

DNA restriction and modification enzymes were purchased from Roche (Germany). *Taq* polymerase and PCR-related products were from Perkin Elmer (USA) or Takara Biomedicals (Japan). Other laboratory grade chemicals were purchased from Sigma (USA), Merck (Germany) or BDH (UK).

Recombinant DNA techniques

Unless mentioned specifically, standard protocols were used for DNA/DNA hybridization, the isolation of plasmid and chromosomal DNA, transformation, electroporation, PCR, restriction digestion, agarose gel electrophoresis, DNA recovery from agarose gels, the ligation of DNA fragments (Sambrook *et al.*, 1989), and conjugation (de Lorenzo and Timmis, 1994). DNA/DNA hybridization and washes were carried out at 68 ^{o}C. DNA sequencing and analysis was performed using a Perkin-Elmer Autosequencer model 377 with a Taq DyeDeoxy^{TM} terminator cycle sequencing kit (Applied Biosystems, USA). The DNA sequence of PCR products was confirmed by sequencing both strands from two or three independent reactions.

Analysis of DNA and protein sequences

The DNA and deduced protein sequences were compared with GenBank DNA or non-redundant protein sequence databases using blastn or blastx via the NCBI internet homepage (http://www.ncbi.nlm.nih.gov/). Protein sequence identities were obtained using the ALIGN program available on the GENESTREAM network server at Institut de Génétique Humaine, Montpellier, France (http://vega.igh.cnrs.fr/bin/align-guess.cgi).

***Construction of S. marcescens N-2* (*P*nucA::luxAB *in the chromosome*)**

To construct the *S. marcescens* N-2 (P*nucA*::*luxAB*) strain, a 170-bp putative promoter region of the *nucA* gene (Chen *et al*., 1992) was amplified by PCR using the primers, R*nucA* and F*nucA* (Table 3), and was ligated in front of the *luxAB* genes of pUT-*luxAB*-Km (de Lorenzo and Timmis, 1994) as a *Not*I/*Sfi*I fragment to form pUT-P*nucA*-*luxAB*-Km. For transfer to the *S. marcescens* chromosome, pUT-P*nucA*-*luxAB*-Km was transformed into *E. coli* S17-1 λpir (de Lorenzo and Timmis, 1994) and conjugated into wild-type *S. marcescens* SS-1 at 30 ^{o}C. A kanamycin resistant exconjugant showing a positive light signal and normal sliding was selected and designated *S. marcescens* N-2.

Detection of luxAB reporter luciferase activity

The Autolumat LB 953 luminometer (EG&G, Germany) with the program 'replicates' was used for bioluminescence measurement. All procedures followed the protocols supplied by the manufacturer.

N-*Acyl homoserine lactone bioassays*

The biosensor strains used were *E. coli* JM109 (pSB401) (Winson *et al.*, 1998) and *C. violaceum* CV026 (McClean *et al.*, 1997). The presence of acyl-HSLs produced by bacteria growing on agar plates was detected in T-streaks against biosensor organisms (see Swift *et al.*, 1999 for a review). The presence of acyl-HSLs in filter (0.25-mm pore size)-sterilized conditioned media was detected according to McClean *et al.*, (1997) and Winson *et al.*, (1998). The presence of acyl-HSLs in conditioned media after high-pressure liquid chromatography (HPLC) fractionation was detected as described in Camara *et al.*, (1998). The presence of acyl-HSLs in conditioned media after thin layer chromatography (TLC) fractionation was detected as described in Shaw *et al.*, (1997) with the modifications described by McClean *et al.*, (1997). Positive assays were judged as induction of the purple pigment violacein in the *C. violaceum* CV026 reporter (McClean *et al.*, 1997) or the induction of bioluminescence in *E. coli* JM109 carrying either plasmid pSB401 detected with a Hamamatsu Argus 100 Vim3 (Hamamatsu Photonics, UK), a Berthold LB980 (E.G. & G, Germany) photon video camera or a Autolumat LB 953 luminometer with the program 'replicates'. Pigment production by *C. violaceum* CV026 was scored after over-night incubation at 30 ^{o}C. Bioluminescence was determined after 4 h of incubation at 30 ^{o}C, and quantitative results are expressed as plots of $\log_{10}$ [luminescence per unit test culture optical density] against test culture optical density.

Recombinant *E. coli* libraries were screened for the presence of a *luxI* homolog either by toothpicking into *C. violaceum* CV026 overlays or by cross-streaking CV026 against 24-h growth on plates of *E. coli* JM109 transformants. Alternatively, plasmid-based libraries were introduced into *E. coli* JM109(pSB401) by electroporation

and the recombinant clones obtained were examined for bioluminescence as described in Swift *et al.*, (1993). In both cases, incubation was at 30 ° C.

N-*Acylhomoserine lactones*

The acyl-HSLs used in this study, *N*-butanoyl-L-homoserine lactone, (C4-HSL); *N*-hexanoyl-L-homoserine lactone, (C6-HSL); *N*-octanoyl-L-homoserine lactone, (C8-HSL); *N*-decanoyl-L-homoserine lactone, (C10-HSL); *N*-dodecanoyl-L-homoserine lactone, (C12-HSL); *N*-(3-oxobutanoyl)-L-homoserine lactone, (3-oxo-C4-HSL); *N*-(3-oxohexanoyl)-L-homoserine lactone, (3-oxo-C6-HSL); *N*-(3-oxooctanoyl)-L-homoserine lactone, (3-oxo-C8-HSL); *N*-(3-oxodecanoyl)-L-homoserine lactone, (3-oxo-C10-HSL); *N*-(3-oxododecanoyl)-L-homoserine lactone, (3-oxo-C12-HSL) and *N*-(3-oxotetradecanoyl)-L-homoserine lactone (3-oxo-C14-HSL) were synthesized as described by Chhabra *et al.*, (1993). Each compound was purified to homogeneity by semipreparative HPLC, and its structure was confirmed by MS and proton nuclear magnetic resonance spectroscopy (see Camara *et al.*, 1998 for a review). Stock solutions at 10 mM in acetonitrile (far-UV grade) were diluted into the growth medium to give the stated concentrations.

Purification and characterization of acyl-HSLs

Spent supernatants (1 liter) from stationary-phase cultures of *S. marcescens* SS-1 and *E. coli* JM109 transformed with the recombinant plasmid (both grown in M9 minimal growth medium) were extracted three times with dichloromethane (700:300

supernatant:dichloromethane). The dried extract was reconstituted in 50 µl acetonitrile, and then samples were subjected to analytical and preparative thin-layer chromatography (TLC) and preparative HPLC. Tentative identification of acyl-HSLs was made by comparing the *Rf* values of the positive sample spots with those of synthetic standards. For preparative TLC, samples were separated as described above and the silica matrix at the relevant *Rf* was collected. Acyl-HSLs were extracted from the TLC matrix three times with 2 ml of acetone and evaporated to dryness. For preparative HPLC, samples were separated by using a Kromasil KR100-5C8 (250 by 8 mm) reverse-phase column (Hichrom, England) with an isocratic mobile phase of 70% (vol/vol) acetonitrile in water at a flow rate of 2 ml per min and monitored at 210 nm. Fractions showing activity in the CV026 reporter assay were pooled and rechromatographed by using 60% (vol/vol) acetonitrile in water; the procedure was repeated, using a final chromatographic separation employing 35% (vol/vol) acetonitrile in water. Active fractions with the same retention times were pooled and analyzed by mass spectrometry (MS) on a V.G.70-SEQ instrument (Fisons Instruments, VG Analytical, England). Samples were ionized by positive-ion fast atom bombarbment (FAB), and the molecular ion (M+1 H) peaks recorded by FAB-MS were further analyzed by tandem MS (MS-MS).

Assay of nuclease and surfactant production

Nuclease activity was detected using the DNase microplate assay following the protocol of Chen *et al.*, (1992). Qualitative assay of surfactant production was performed using the drop collapsing method (Lindum *et al.*, 1998). Semi-quantitative assay of biosurfactant was performed using a TLC based assay as described by Matsuyama *et al.*,

(1992).

Construction of S. **marcescens** *SS-1* **spnI** *and* **spnR** *deletion insertion mutants*

PCR primers (Table 3) were designed to introduce specific *Hind*III sites into *spnI* and *spnR* respectively for subsequent insertion of *Hind*III digested Ω (Sm^r) gene cassette (Prentki and Krisch, 1984). Primer pairs SI-1/SI-2 (for *spnI*) and SR-7/SR-8 (for *spnR*) were used to amplify the 5'-region (approximately 400 bp) of the gene to be inactivated. PCR products were T-cloned into pCR2.1 (Stratagene, UK), sub-cloned as an *Xba*I/*BamH*I fragment into pZero-2 (Invitrogen, Nederland) and excised as a *Xba*I/*Hin*dIII fragment. A second PCR product encompassing the 3'-region of the gene to be inactivated (approximately 400 bp) was generated using primer pairs SI-3/SI-4 (*spnI*) and SR-5/SR-6 (*spnR*), T-cloned into pCR2.1 and excised as an *Hin*dIII/*Eco*RI fragment. The 2 kb Sm-resistant Ω DNA fragment was excised from pHP45Ω (Prentki and Krisch, 1984) as a *Hin*dIII fragment. The three DNA fragments were ligated together with *XbaI/EcoRI* digested suicide vector pUT-mini-Tn5-Km (de Lorenzo and Timmis 1994). The resultant pUT-*spnI*::Sm and pUT-*spnR*::Sm vectors were selected as conferring streptomycin resistance upon the permissive *E. coli* strain CC118 and verified by restriction enzyme mapping. For gene inactivation mutagenesis by homologous recombination, the respective plasmids were transferred from the permissive host strain *E. coli* S17-1 λpir to *S. marcescens* SS-1 by conjugation and the transonjugants were spread on LB plates with streptomycin (100 µg/ml) and tetracycline (13 µg/ml). The mutants with double cross over events were selected by CV026 T-streaking (*spnI*::Sm) or by selecting mutants with fast-sliding phenotype followed by PCR screening (*spnR*::Sm). Southern hybridization using the PCR

amplified *spnI* gene (SI-1 and SR-6 as primers) or *spnR* gene (SR-8 and SI-3 as primers) as the probe respectively was performed. In a *Bam*HI digestion of chromosomal DNA from the parent strain, a single band corresponding to the predicted size of 7.4 kbp hybridized to the probe. In the SS-1 ΔI mutant, a 9 kbp and a 0.4 kbp band hybridized (data not shown). In the SS-1 ΔR mutant a band of 0.8 kbp and a band of 8.6 kbp hybridized (data not shown). The data confirmed that a double-crossover event had taken place and the new strains were designated SS-1 ΔI and SS-1 ΔR strains.

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