

Spreading, biosurfactant production, and nuclease synthesis are inhibited by SmaR in *Serratia marcescens*

Su-Chen Deng/Yu-Tze Horng, Mavin Daykin, Po-Chi Soo, Hsin-Chih Lai, Shen-Wu Ho, Kwen-Tay Luh, and Simon Swift

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Abstract

We have identified and characterized a pair of *luxI/R* homologues termed *smaI/R* from *Serratia marcescens* SS-1. From cell-free culture supernatants, two N-acyl homoserine lactone (AHL) molecules, N-(3-oxohexanoyl) homoserine lactone (C6-HSL) and N-(3-oxooctanoyl) homoserine lactone (C8-HSL), were chemically characterized. Unlike the *swrI/R* system of *S. liquefaciens*, the presence of multicopy, plasmid-encoded *smaR* inhibits the spreading of *S. marcescens* SS-1. Further study showed that production of biosurfactants and expression of one of the virulence factors, nuclease gene *nucA*, were also repressed. These repressed phenotypes were specifically de-repressed by AHL signals produced by *smaI*. Different modifications of the AHL signals had either without or inhibitory effect on *S. marcescens* SS-1 spreading. Long chain AHLs show a trend of competition with the native AHL signals synthesized by *SmaI*. Mutation of *S. marcescens* chromosomal *smaR* shortens the time before spreading motility is initiated for 1 hour. On the contrary, mutation of *smaI* significantly delays initiation of spreading. Plasmid-encoded *P_{smaI}::luxAB* and *P_{smaR}::luxAB* reporter of *smaI* and *smaR* transcription, respectively, are constructed. Using this reporter system, the expression patterns of *smaI* and *smaR* following the growth in liquid broth culture were monitored. Further studies showed the evidence of *smaR* positive autoregulation, and that AHL signals were constitutively produced in the log phase and its production was turned off as the cells entered into stationary phase. Our findings indicate that *smaI/R* quorum-sensing system is a component of the complex regulatory network that controls multicellular behaviour of *S. marcescens*.

Introduction

Serratia marcescens is a dimorphic enteric bacterium capable of undergoing dramatic morphological and physiological changes following the swarming cycles on 0.8% LB swarming plates (Harshey, 1994; Liu et al., 2000). These changes, referred to as swarmer cell differentiation (from short, vegetative cells with few peritrichous flagella to elongated, multi-nucleoid swarmer cells covered with over-produced flagella), are required to produce the motile behavior which is characterized by flagella-assisted population movement over a surface and known as swarming (population surface migration) (Matsuyama and Matsushida; 1993; Allison and Hughes 1992).

While flagellated *S. marcescens* cells display both swimming and swarming motility (Alberti and Harahey 1990), nonflagellated (Fla-) bacteria can neither swim or swarm. However, they display another mode of surface translocation, in which the bacterial population spreads without assistance of flagella (O'Rear et al., 1992; Matsuyama et al., 1992; 1995). Sliding is one of six forms (including swimming, swarming, gliding, twitching, darting and sliding) of bacterial translocation described by Henrichsen et al., (1972). It is a way of bacterial populational migration occurring on the surface of low-agar media without the assistance of flagella. The phenomenon that *Bacillus anthracis* slides and covers the agar surface rapidly is described as early as in 1910 by Graham-Smith (1910). Formation of colonies with a medusa-head appearance by *Kurthia zopfii* after sliding provides another example of sliding (Gardner 1969). Currently, the underlying mechanism of sliding is poorly understood, but it was reported that the biosurfactants play important roles in this process (Martinez et al., 1999), as was also observed in other non-flagellated bacteria such as *Mycobacterium smegmatis* and *Mycobacterium avium* (Martinez et al., 1999).

Intercellular communication for regulation of bacterial multicellular behaviour has been appreciated for several years in *Vibrio fischeri*, *Myxococcus xanthus*, *Bacillus subtilis*, *Streptomyces spp.*, the eukaryotic slime mold *Dictyostelium discoideum*, and other species (Fuqua et al., 1994). How the intercellular communication is achieved among these bacteria is being continuously studied and auto-induction systems of *luxI/R* family were presented to be one of the controlling circuits.

Effect of a *swrI/R* quorum-sensing system on the swarming behaviour in *S.*

liquefaciens has been studied. In that system, it was reported that the Ahl signals (mainly C4 AHLs) synthesized by SwrI plays an important role in stimulating serrawettin production which is synthesized by SwrA, and the subsequent *S. liquefaciens* swarming. As *S. liquefaciens* is mainly a plant pathogen (isolated from cucumber), and there was actually no characterization of SwrR, it would be worthwhile to initiate a project on understanding the roles that quorum-sensing system might play in the population surface translocation and expression of virulence factors in *S. marcescens*. While we are finishing the study, a novel *S. marcescens* QS system was reported by Thomson et al., (2000). In that system, the *smaI* and *smaR* are homologues of *S. liquefaciens swrI* and *swrR*, respectively. Production of carbapenem antibiotic and prodigiosin pigment in *S. marcescens* ATCC39006 is controlled by this quorum-sensing system.

Using the *E. coli* JM109/pSB401 bioluminescence reporter system, we have cloned and sequenced a *luxI/R* homologue named *smaI/R* from *S. marcescens* SS-1. The role that *smaI/R* played in the process of cell differentiation, population spreading, and regulation of secondary metabolite production (such as biosurfactants and one of the virulence factors, nuclease) was characterized. Our data showed that *smaI/R* are different from both *swrI/R* (*S. liquefaciens*) and *smaI/R* (*S. marcescens* ATCC39006) quorum-sensing systems genetically and functionally.

Results

S. marcescens SS-1 shows a sliding behaviour

S. marcescens SS-1 spreads well on LB solidified with 0.3% agar, with an average speed of about 5cm per hour, but does not swarm on 0.8% LB agar. This strain did not show motility in 0.1-0.3% LB motility agar, and flagella silver stain did not show evidence of flagella production in this strain. Except the spreading motility, other phenotypes were not changed, including growth rate, prodigiosin and biosurfactant synthesis, production of virulence factors such as haemolysin, protease, nuclease, and phospholipase, and cell elongation LB broth or seeding plate cultures (data not shown).

Microscopic observation of *S. marcescens* SS-1 spreading did not reveal forward and reverse movement, such as those seen in the swarm cells; instead, the bacteria appear to push outwards continuously as a single layer. Their cell axes are often perpendicular to the direction of cell movement. The spreading behaviour is optimal at 30 °C, and inhibited at 37 °C, which is consistent with production of a transparent serrawettin (biosurfactants) zone. Based on the phenomena observed, the spreading behaviour of *S. marcescens* SS-1 was defined as “sliding”.

Cloning, sequencing and characterization of a *luxI/R* homologue from *S. marcescens*

To clone the possible *luxI/R* homologues from *S. marcescens* SS-1, *Escherichia coli* JM109/pSB401 was used. A recombinant plasmid pSS100 (pT7T3/7.5Kb *Bam*HI fragment), which gave a highlighted *E. coli* JM109/pSB401 colony, was screened. pSS100 was restriction mapped and functionally assayed to define the shortest DNA fragment complementing the light emission of *E. coli* JM109/pSB401. The 1kb *Sph*I/*Bam*HI DNA fragment that gave strong light signal was first subcloned and sequenced. A putative LuxI homologue with high amino acid sequence identity to *EsaI* of *Erwinia stewartii* was identified and named *SmaI*. Downstream of *smaI*, a complete open reading frame (ORF) with high amino acid sequence identity to *esaR* of *Erwinia stewartii* was found and was named *smaR* hereafter. and with the transcriptional direction going towards *smaI*. *SmaR* amino acid sequence overlaps with *SmaI* at the C-terminal region for 40 amino acids, and both genes are transcribed convergently. A *lux* box homologue was found in the –35 promoter region of *smaR*,

suggesting that expression of *smaR* might be auto-regulated. No *lux* box homologue was observed upstream of *smaI*, which suggested that *smaI* might not be regulated by SmaR.

As only about 70 bp upstream of *smaI* ATG translation initiation codon was contained in plasmid pSS100, which might mean that the complete promoter region of *smaI* was not obtained, an extra 2kb upstream of *smaI* ATG was cloned by chromosomal walking using complete *smaI* as the probe. Further sequencing analysis showed that a DNA region containing a complete ORF (named *smaT*) with no significant nucleotide or amino acid sequence homology to any genes available from the GenBank was found at the 97bp upstream region of *smaI* ATG codon. Transcriptional direction of *smaT* was predicted to be the same as that of *smaI*. Upstream of *smaT*, a complete ORF with high amino acid sequence homology to the resolvase of transposon Tn2501 (Michiels et al., 1987) was found.

The predicted ORF polypeptide of SmaI and SmaR were compared to the SWISSPROT Database by FASTA (GCG, Version 8) . SmaI shared 65% identity throughout the alignment with the 134-residue EsaI of *E. stewartii*, and 60% identity with the 132-residue, as well as 64% identity with the 144-residue. SmaR had 67% identify with the EsaR of *E. stewartii*, and 40% identity with the 55-residue, as well as 56% identity with the 139-residue. SmaI/R of *S. marcescens* ATCC39006 showed high identity to *S. marcescens* SS-1 SmaI/R, respectively. The *swrI* of *S. liquefaciens* showed only 39% identity (59% homology) to SS-1 *smaI/R* after the comparison. As there is no *smaR* nucleotide and SmaR amino acid sequences available from the GenBank, we could not make comparisons between these two proteins. The phylogenic relationship within the LuxI/R family was shown in Fig. 3. Our data suggested that that *smaI/R* might be a novel genetic determinant involved in the regulation of *S. marcescens* populational sliding behaviour.

Using the *smaI/R* DNA sequence as the probe, we could not identify, by Southern blot hybridization and PCR (data not shown), any similar DNA fragments from some other *S. marcescens* strains including CH-1 that does not produce detectable AHL signals by *E. coli* JM109/pSB401 biosensing system], and BG-1 that produces BHL/HHLsignals, suggesting that *smaI/R* might be rather unique for *S. marcescens* SS-1.

Analysis of AHLs produced by *S. marcescens* SmaI

AHL signals synthesized by SmaI was analyzed. The over-night culture supernatant of *E. coli* JM109/ pYT200(pCR2.1/*smaI*) was extracted, concentrated, separated by TLC plates, and detected by the CV026 assay. The results showed that 3-oxoC6HSL and 3-oxoC8HSL at a ratio of about 10:1 are the main AHL signals synthesized.

To further confirm the results, the AHL signals existing in the spent cultured supernatants of *S. marcescens* strains SS-1 and CH-1 were also analysed. The results showed that 3-oxo-C6HSL and 3-oxo-C8HSL.

To verify the structure of AHL signals produced by *S. marcescens* SS-1, extraction/HPLC/Mass Spectrometry. The data showed that AHL signals produced by *S. marcescens* SS-1 are different from the AHLs synthesized by *S. marcescens* ATCC39006 SmaI and *S. liquefaciens* SwrI.

Cell differentiation of *S. marcescens* CH-1 is not affected by quorum-sensing signals

As in *Y. pseudotuberculosis*, the quorum-sensing system was observed to regulate the swimming motility which is under the control of *flhDC* system, we would like to see whether similar phenomenon was observed in *S. marcescens* CH-1. To achieve this, another *S. marcescens* strain CH-1 which shows normal swim and swarm ability was transformed with plasmids pPC1, pPC2 and pPC4. Cell differentiation markers [expression pattern of the flagellar structural gene *fliC*, flagella over synthesis, and cell elongation without septum formation] (Lai et al., 1997; Liu et al., 2000) and motility assay were monitored. The results show that no significant difference was observed in the presence of *smaI* (data not shown), suggesting that similar to *S. liquefaciens*, cell differentiation and motility were not affected in *S. marcescens* SS-1.

Effect of *smaI/R* on the sliding of *S. marcescens* SS-1

To see whether *smaR* had any effect on the sliding behaviour of *S. marcescens* SS-1, the plasmid pSC100(pCR2.1::*smaR*) was transformed into *S. marcescens* SS-1 followed by observation of sliding behaviour. While the growth rate was not affected, SS-1 sliding was completely inhibited by multi-copy *smaR* (pSC100) after O/N incubation at 30 °C. *S. marcescens* SS-1(control vector) started to slide at the time of 3

hours after incubation, and *S. marcescens* SS-1(pSC100) showed no sign of sliding even after 48 hours of incubation. Besides sliding, it was also shown that production of the red pigment prodigiosin was nearly completely inhibited.

Further experiments showed that the swarming behaviour of *S. marcescens* CH-1 was also inhibited by pSC100, although there is no *smaI/R* homologues detected in this strain (data not shown), This suggested that the *smaR* effect might be common in the *S. marcescens* strains.

To confirm the function *smaR*, a *smaR* knock-out strain named *S. marcescens* ΔR (SM ΔR hereafter) was constructed from *S. marcescens* SS-1 (see Materials and Methods for detail). Compared with SS-1 that started to slide at the time of 3 hours after incubation, SM ΔR showed a significant earlier initiation of sliding at the time of about 1.5 hours. The multi-copy *smaR* in-trans restored the time of sliding initiation in SM ΔR from 1.5 hours to about 5 hours.

Besides sliding, similar phenomenon in the regulation of prodigiosin production was also observed (data not shown), suggesting that production of prodigiosin was also co-ordinately regulated by SmaR. These data showed that SmaR acts as a repressor for regulation of *S. marcescens* SS-1 sliding and production of prodigiosin.

To see the effect of AHL signals produced by *smaI* on the control of *S. marcescens* SS-1(pSC100) sliding and prodigiosin synthesis, 3-oxo-C6HSL or 3-oxo-C8HSL were added into the sliding plates followed by observing the sliding behaviour of *S. marcescens* SS-1(pSC100). At the concentration of 10nM, effect of 3-oxo-C6HSL and 3-oxo-C8HSL AHLs on the restoration of SS-1(pSC100) sliding was started to be observed. As the AHL concentration was increased to 10 μ M, complete restoration of SS-1(pSC100) sliding was observed. These results suggested that effect of *smaR* was derepressed by the presence of AHL signals produced by *smaI*. To further confirm the result, a *smaI* knock-out mutant named *S. marcescens* ΔI (SM ΔI hereafter) was constructed. Compared with its parent strain *S. marcescens* SS-1, SM ΔI did not show any sign of sliding until after incubation for at least 7 hours. When 3-oxo-C6HSL at the concentration of 10 μ M was added into the LB sliding plates, the time of initiation of SM ΔI sliding was restored and started to spread at the time of 3 hours after incubation. Similar inhibitory and restoration effects were observed on the production of prodigiosin. These results showed that SmaR inhibits the sliding and prodigiosin production of *S. marcescens* SS-1, and SmaI AHL signals alleviate the *smaR* effect.

Effect of *SmaR* was specifically de-repressed by AHL signals produced from SmaI

To see whether there is quorum-sensing specificity between the interaction of

AHL signals and SmaR, a series of artificially synthesized AHLs ranging from 100nM to 1mM (1mM, 100uM, 10uM, 1uM, 100nM) listed in Fig. 4. were added into the LB spreading media respectively to see whether they have any effect on derepressing the *smaR* activity on spreading of *S. marcescens* SS-1. Structures of these AHL signals vary with different carbon side chain length (from C4 to C12) and oxo-modifications (such as the position and number) were tested. Effect of the AHL signals on the spreading behaviour of *S. marcescens* SS-1/*smaR* was shown in Fig. 5. For the *S. marcescens* SS-1/*smaR* incubated on the LB plate without OHHL, usually it took at least about 48h for the bacterial colony to start to spread. With the addition of 3-oxo-C6HSL or 3-oxo-C8HSL at the concentration of 10µM, SS-1/*smaR* covers the whole 9cm LB spreading plate after O/N culture. While 3-oxo-C6HSL and 3-oxo-C8HSL showed a significant de-repressing effect, the other signals including C6HSL and C8HSL did not show any observable de-repressing effect, suggesting that there is specificity between the interaction of AHL signals and SmaR.

A significant competitive effect of AHLs with long side chains against the AHLs with short side chains was observed when the signals interact with their respective LuxR homologues, as reported in *Chromobacterium violaceum* CV026, and other examples. It was also interesting to see whether such a phenomenon occurred in *S. marcescens* SS-1 spreading. To achieve this, the AHLs used above were added into the LB spreading plates followed by observation of *S. marcescens* SS-1 spreading. After over-night culture, while there was no significant effect from other AHLs, it was observed that 3-oxo-12, 3-oxo-14, C10, and C12 AHLs showed a significant inhibitory effect on the SS-1 spreading. These results suggested that long chain AHLs might have a competitive effect on the interaction of 3-oxo-C6HSL/3-oxo-C8HSL signals with SmaR.

To see whether the de-repressing effect could also be observed in the presence of multi-copy *smaI*, the plasmid pYT300 was transformed into *S. marcescens* SS-1/pYT200. pYT300 significantly derepressed the inhibited spreading phenotype caused by multi-copy *smaR* after over-night culture. These results suggested that effect of *smaR* was specifically de-repressed by AHL signals produced from SmaI, and that long chain AHLs shows a tendency to compete with the effect of AHL signals synthesized by SmaI.

Biosurfactant production was inhibited by *smaR* and derepressed by *smaI*

As biosurfactant production was essential for the population migration behavior of many bacterial species including *S. marcescens* (Matsuyama et al., 1989, 1995; O'Rear et al., 1992; Harris et al., 1998; and *S. liquefaciens*, we then would like to see whether the effect of *smaI/R* system on the spreading behaviour of *S. marcescens* SS-1 was through regulation of the biosurfactant (serrawettin) production. To answer this assumption, *S. marcescens* SS-1/control vector, SS-1/pSC200, SMΔR, SMΔR/pSC200, SMΔI, and SMΔI(pYT100) were over-night cultured followed by drop-collapsing test to semi-quantitatively determine the amount of biosurfactants produced (Matsuyama et al., 1985; Bar-ness et al., 1988; Jain et al., 1991). In the presence of multi-copy *smaR*, the surface tension of bacterial suspension was significantly increased, suggesting the production of serrawettin was inhibited. The surface tension of SMΔR was significantly reduced and was restored by *smaR* in trans (Fig.). When *smaI* was knocked-out, the surface tension was significantly increased, and this phenomenon was reversed in the presence of *smaI* in trans. There was no significant difference of surface tension change between *S. marcescens* SS-1/control plasmid and SS-1/(pYT100). The effect of multi-copy *smaR* was derepressed by complementation of 3-oxo-AHL signals. These data showed that biosurfactant production was inhibited by *smaR*, and was derepressed by *smaI*.

To further confirm this phenomenon, a quantitative TLC assay was performed. The surfactants synthesized by strains *S. marcescens* SS-1 containing plasmids pYT200, pSC100, and control plasmid, respectively, were prepared before TLC analysis. Production of biosurfactant (serrawettin W1) was clearly reduced in the presence of multi-copy *smaR*, and was de-repressed by the presence of multi-copy *smaI* in trans.

Nuclease production (and prodigiosin synthesis) were inhibited by *smaR*, and derepressed by *smaI*

As the production of some virulence factors was regulated by quorum-sensing systems in many other bacteria, we then would like to see whether production of one of the virulence factors, nuclease, was regulated by *smaI/R* system in *S. marcescens* SS-1. To achieve this, DNA microplate degradation assay (Chen et al., 1998) was performed, and *S. marcescens* strains including SS-1/control vector, SS-1/pSC200, SMΔR, SMΔR/pSC200, SMΔI, and SMΔI(pYT100) were assayed for comparison of

production of nuclease activity. Compared with the wild-type strain SS-1/control vector, the nuclease activity produced by SM Δ R was much higher. In the presence of multi-copy *smaR* in trans, the nuclease activity of SS-1/pSC200 was significantly reduced in SM Δ R. The *smaR* in trans restored the SM Δ R nuclease activity to the wild-type level. Compared with that of its parent strain *S. marcescens* SS-1, SM Δ I nuclease activity was reduced, and was restored when it was complemented in trans with pYT100. When AHL were added for complementation, signals other than oxo-C6HSL/oxo-C8HSL did not show any “de-repressing effect” on SM Δ I (data not shown). These data suggested that production of nuclease was inhibited by *smaR*, and was specifically de-repressed by *smaI* or addition of oxo-C6HSL/oxo-C8HSL signals synthesized by SmaI AHL signals.

To see whether regulation of nuclease activity by *smaR* was at the promoter transcriptional level, a strain *S. marcescens* SS-1(P*nucA*::*luxAB* in the chromosome) was constructed. Patterns of light emission were used to monitor the promoter transcriptional level of the nuclease gene *nucA*. The light emission of SS-1(P*nucA*::*luxAB*/pSC100) was significantly lower than that of SS-1(P*nucA*::*luxAB*/control vector) for about 8 folds. Addition of 3-oxo-C6HSL into the media increased the light emission of SS-1(P*nucA*::*luxAB*/pSC100). The data showed that expression of *nucA* was repressed by *smaR* at the promoter level, and was de-repressed by addition of 3-oxo-C6HSL.

SmaR shows an evidence of positive auto-regulation

A *lux* box sequence homologue was observed in the -35 promoter region of *smaR*, which suggests that expression of *smaR* might be auto-regulated. To confirm this, a recombinant plasmid pACYC184-P*smaR*::*luxAB* was constructed. The light emission patterns of *E. coli* Top10/pACYC184-P*smaR*::*luxAB*/pSC100 and *E. coli* Top10/PACYC184-P*smaR*::*luxAB*/control vector was compared. The data showed that the intensity of light emission was much higher in *E. coli* Top10/PACYC184-P*smaR*::*luxAB*/control vector than that of *E. coli* Top10/PACYC184-P*smaR*::*luxAB*/pSC100. Addition of oxoC6HSL (10 μ M) in the media further stimulated the intensity of light emission. These data showed that *smaR* in multi-copy

stimulated the activity of its own promoter and this effect was enhanced by addition of 3-oxo-C6HSL signals.

The synthesis pattern of SmaI AHL signals

To monitor the pattern of AHL produced by SmaI following the growth, the spent supernatants harvested from *S. marcescens* SS-1, SMΔR and SS-1/pSC100 were collected. The amount of AHL signals was monitored by *E. coli* JM109/pSB401 biosensing system. The results showed that the AHL signal was constitutively synthesized following the growth in SS-1. While there was no difference in the growth rate, similar patterns of light emission were also observed from strains SMΔR and SS-1/pSC100, suggesting that synthesis of AHL signals by SmaI was independent from expression of *smaR*.

To quantitatively monitor the expression of *smaI* at the promoter level, a recombinant plasmid pACYC184/ *P_{smaI}::luxAB* was constructed. Intensity of light emission was monitored following the growth in LB broth. The results showed that transcription of *smaI* promoter is constitutive when cells were grown in the log phase. As cells enter the stationary, *smaI* promoter activity was rapidly turned off.

Discussion:

By analogy to previously characterized LuxI/R-type regulatory circuits it can be anticipated that *S. marcescens* also contains at least one autoinducer protein homologous to LuxR. Members of the LuxR family of transcriptional activators share only moderate overall similarities but 2 clusters with higher similarities have been identified: an N-terminal region that has been implicated in regulation of protein function and autoinducer binding, and a C-terminal region containing a helix-turn-helix DNA binding region. The C-terminal half of this group of proteins also shares significant homology with a much higher family of proteins (the LuxR superfamily), all of which are members of two component regulatory systems. It is, therefore, tempting to speculate that autoinduction cascades could be integrate into, or at least interact with, the signal transduction network of two component regulatory systems. The autoinducer-triggered amplification step, different from the signal transduction system, is dependent on the accumulation of autoinducer to a certain threshold concentration and thus only occurs when a critical cell density has been obtained.

Such a concept may find support in the evidence presented in this study demonstrating that initiation of swarming of *S. marcescens* is not controlled by a single event or signal, but rather requires the sensing and integrating of a variety of signals including surface recognition signal and culture density.

SmaI (*S. marcescens* SS-1) synthesizes different AHL signals than the main C4HSL signal synthesized by *S. liquefaciens* SwrI or by *S. marcescens* ATCC39006 SmaI. Further experiments show that phenotypes such as the populational spreading behaviour, and production of serrawettin and nuclease were observed to be inhibited by multi-copy *smaR*. These phenotypes were restored to normal by AHL signals specifically synthesized by SmaI. While most of the other LuxR homologues act, in combination with their AHL signals, as transcriptional activators, our results showed that SmaR acts as a repressor and is “derepressed” by AHL signals. SmaR activates the transcriptional level of its own promoter. This is different from EsaR, where it represses polysaccharide synthesis and also the transcription of its own promoter.

In this study we have unravelled a novel quorum-sensing system which regulates expression of biological traits involved in controlling the multicellular behavior of *S. marcescens*. For the first time, the sliding (instead of swarming) behaviour of *S. marcescens* is also presented, and we also show that production of biosurfactant plays

an essential role for this process.

The most significant finding of this study is that SmaR behaves as a repressor and SmaI plays a role in the process of de-repressing. This is quite unique in that all other LuxR-like regulators [EsaR as the only exception reported], when form complexes with the AHL signals produced by their corresponding LuxI homologues, function as transcriptional activators. EsaR of *Pantoea stewartii* is the first example to be reported to act as a repressor for synthesis of extracellular polysaccharide (EPS) (von Bodman et al., 1998). Synthesis of EPS is inhibited at low cell density, and is de-repressed until cells are grown to a higher cell density, when sufficient AI becomes available for de-repression to occur. The fact that derepressed *esaR* strains, which synthesize EPS constitutively at low cell densities, are significantly less virulent than the wild-type parents suggests that quorum sensing in *P. stewartii. subsp. stewartii* may be a mechanism to delay the expression of EPS during the early stages of infection so that it does not interfere with other mechanisms of pathogenesis (von Bodman et al., 1998). SmaI/R(SS-1) may be the second example of negative transcriptional regulator within the QS family published. However, even though there are similarities between SmaR and EsaR, there is difference identified. i.e. EsaR auto-represses and SmaR auto-activates its own promoter. The possible mechanism may involve the location of the *lux* box in the promoter region, and is discussed later.

It is also interesting to see that the *smaI/R* QS system of *S. marcescens* SS-1 shows many characteristics different to those of *smaI/R* of *S. marcescens* ATCC39006. For example, in SS-1, the predicted length of SmaR/SmaI is 247 and 208 a.a., respectively. For ATCC39006, they are 247 and 234 a.a., respectively. Although both systems are transcribed convergently, different AHL signals are synthesized (3-oxo-C8-HSL/ 3-oxo-C6-HSL for SS-1 and C4-HSL/ C6-HSL for ATCC39006). While SmaR(SS-1) is shown to work as a repressor, and is derepressed by AHLs, SmaR (ATCC39006) is predicted to be an activator after binding to AHLs. Production of prodigiosin is reported to be regulated by SmaI/R (ATCC39006), and besides prodigiosin, sliding, serrawettin and nuclease production are regulated by SmaI/R (SS-1). What may be common are that both QS systems are flanked by remnants of transposons, although different IS family homologues are identified (Tn3 and IS3 for SS-1, and IS1 and FecD, E for ATCC39006). These results suggest that complex QS regulatory systems exist in *S. marcescens* regulating the multicellular behaviours and secondary metabolites.

Although some phenotypes are significantly affected in the train SMΔI, some trivial signals are still detectable via *luxR*-based *E. coli* JM109/pSB401 and *C. violaceum* CV026 biosensing systems, indicating that this mutant strain still produces AHLs.

Three possible mechanisms are presented to explain this phenomenon. **First**, a second LuxR/I pair might exist in *S. marcescens* SS-1. This possibility is supported by many examples. For example, the *Y. pseudotuberculosis* YpsR/I and YtbR/I systems and *P. aeruginosa* LasR/I and RhIR/I systems (Atkinson et al., 1999). On the other hand, this second genetic locus might also be related to the *ainS* system of *V. fischeri* or the *luxLM* locus of *V. harveyi*, both of which direct acyl-HSL synthesis but display no homology with *luxI* (Bassler et al., 1993; Gilson et al., 1995). **Secondly**, It is possible that another QS signal molecules, such as the cyclic dipeptides (DKP) are produced in *S. marcescens* SS-1. By using a LuxR-based AHL biosensor, it is indicated that the DKPs [cyclo(DeltaAla-L-Val), cyclo(L-Pro-L-Tyr) and cyclo(L-Phe-L-Pro)] activates the biosensor in a concentration-dependent manner, albeit at much higher concentrations than the natural activator (such as the 3-oxo-C6-HSL from *P. aeruginosa* and other gram-negative bacteria; Holden et al., 1999). These compounds are also found in cell-free supernatants from *Proteus mirabilis*, *Citrobacter freundii* and *Enterobacter agglomerans* (Holden et al., 1999). To determine whether DKPs are produced by *S. marcescens*, the cell-free *S. marcescens* culture supernatants will further be analysed together with mass spectrometry and NMR spectroscopy to reveal the structure of the possible compound.

Finally, As a clearer understanding of the mechanism by which LuxI directs the synthesis of autoinducer signal has emerged (Hanzelka and Greenberg, 1996; Schaefer et al., 1996; Hanzelka et al., 1997), it is possible that SmaI in S Δ I strain is not completely knocked out. The results of studies from mutated LuxI and mutant forms of a related protein from *P. aeruginosa*, RhlI (Parsek et al., 1997) have indicated that the active site in which amide bond formation is catalysed is roughly in the region of residues 25 to 104, and a region in C-terminus may be involved in selection of appropriate acyl-ACP from those existing in the cellular pools. SmaI in S Δ I still retains about N-terminal 134 amino-acids and thus probably still has the functional domain. However, compared with the results from many other LuxI knock-out experiments, *S. marcescens* SS-1 SmaI should have been deleted as well. More experiments have to be performed to answer these questions.

Analysis of the functional domains among the LuxR homologues may help unravel the underlying mechanism of LuxR function. As described above, the fact that SmaR is a negative regulator is particularly intriguing given the structural similarity between SmaR and LuxR-type activators. Comparison of the amino acid sequence of SmaR with several LuxR-class proteins does not reveal any salient structural differences that could account for these gross mechanistic differences. In fact, the amino acid sequence within the putative N-terminal AHL-binding domain and the predicted C-terminal helix-turn-helix structure are remarkably conserved among all these proteins

(von Bodman et al., 1995; Fuqua et al., 1994, 1996). Because SmaR functions as a repressor, directly controlling the target genes, then it must assume a DNA-binding conformation in the absence of the AHL coinducer and a conformation unsuited for DNA binding in the presence of AHL. This situation is just opposite to the typical LuxR-type activators (Slock et al., 1990; Stevens et al., 1994). It could also be argued that in the absence of AHL, SmaR may function both as a transcriptional repressor for its targeted genes, and an activator for its own promoter.

For further study, it will be essential to elucidate how autoinducers alter the properties of SmaR and how these complexes interact with RNA polymerase to affect the transcriptional efficiency. Also, it is reported that many LuxR-type regulators appear to interact with other proteins, including chaperonins, specific antagonists, and other transcriptional regulators. To dissect the SmaR protein into functional domains will be essential, and this will help us know more about the interaction between SmaR and other macromolecules.

Model for the regulation of multicellular behavior in *S. marcescens* SS-1

In *S. marcescens* SS-1, many populational biological characteristics such as sliding, virulence factor synthesis, and prodigiosin production are regulated by SmaR and SmaI QS system. The data presented here allow me to formulate a model for the regulation of the multicellular behavior. I theorize that at low cell density, SmaR inhibits expression of the target genes by binding to its controlled promoter. Similar to the *luxI/R* system, de-repression of SmaR regulated target genes requires an increase in the pool size of AHLs.

Although the *smaR* and *smaI* gene products are key components in the regulatory network, other genes are also important. For instance, *smaT* in multi-copy causes a dramatic reduction of pigment synthesis, change of red-white colonial variation and complete inhibition of sliding motility. The phenomenon that when *smaT* was transformed to a *E. coli* strain, the bacterial cells can not grow well, indicating *smaT* may play some uncharacterized role in the host cell. SmaT is a novel protein and appears 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 behavior.