Membrane fluidity optimization regulates *Serratia marcescens* swarming behaviour through modulation of a temperature-dependent two-component signal transduction system

Abstract

Serratia marcescens swarming behavior is characterized by continuous populational surface migration on swarming agar at 30°C, but not at 37°C. The underlying mechanism how *S. marcescens* population starts to initiate swarming and the temperature-dependent regulation of swarming behaviour are currently uncharacterized. We identify in *S. marcescens* a genetic locus that, when mutated, results in a ”super-swarming” phenotype, which is not only defective in the temporal, but also in the temperature-dependent control of swarming behaviours. The gene mutated (*rssA*, Regulation of *Serratia* Swarming A) is homologous to membrane sensor histidine kinases of the two-component family of regulatory proteins, suggesting that RssA may function as a sensor of environmental conditions required to regulate swarming migration. Factors already identified to be involved in regulation of swarming migration are not affected in this mutant. These include amount of flagellum synthesis, activity of swimming motility, surfactant production, and synthesis of extracellular polysaccharide. Here we report that long chain fatty acids and temperature shift acting as the signal cues regulate *S. marcescens* CH-1 swarming through the RssA/RssB two component signal transduction system. We find that long chain fatty acids and temperature upshift negatively regulate the CH-1 swarming through influencing pattern of cellular fatty acid profile. The RssA-RssB two-component system mediates this response, as both partners are required to sense and transduce the signals. Fatty acid profile analysis strongly indicates that long chain fatty acid and temperature affects the signaling state of the RssA sensor protein by increasing the incorporation of the low0melting point fatty acids into membrane phospholipid. We propose that both increase in membrane fluidity at constant temperature and a temperature upshift influences fatty acid synthesis and swarming by the same mechanism. Similar phenomena of long chain fatty acid inhibition were also observed in swarming regulation of *Proteus mirabilis* and *Salmonella typhimurium*. Thus, the fatty acid profile and two component signaling pathway might provide a novel and common mechanism for regulation of swarming behaviour of not only *S. marcescens*, but also other swarming bacteria under different physiological culture conditions.

Introduction

More and more bacteria are observed to exhibit a form of cell differentiation and multicellular behaviour termed swarming migration. Swarming involves differentiation of vegetative cells into hyperflagellated swarm cells that undergo rapid and coordinated population migration across solid surfaces (Harshey 1994; Shapiro 1998, Fraser and Hughes 1999). Bacteria reported to show swarming behaviours include *Proteus* (Mobley and Belas 1995), *Vibrio* species (McCarter and Silverman, 1990), *Escherichia coli* (Harshey and Matsuyama 1994), *Salmonella typhimurium* (Harshey and Matsuyama 1994), the glucose non-fermenter *Pseudomonas aeruginosa* ( ), *Pseudomonas syringae* (Kinscherf and Willis 2002), *Pseudomonas fluorescens* (Sanchez-Contreras et al., 2002), *Burkholderia cepacia* (Huber et al., 2001), a spirochete *Treponema denticola* (Lux et al., 2002), a nitrogen-fixation bacterium *Sinorhizobium meliloti* (Soto et al., 2002), a purple photosynthetic bacterium *Rhodospirillum centenum* (Maclain et al., 2002), *Bacillus cereus* (Senesi et al., 2002), *Bacillus subtilis* (Dixit et al., 2002), also some absolute anaerobic bacteria such as *Clostridium* species (Macfarlane et al., 2001), *Serratia liquefaciens* (Givskov et al., 1998) and also *Serratia marcescens* (Alberti and Harshey 1990; Liu et al., 2000)

S. marcescens is a dimorphic, motile gram-negative bacterium associated with diseases such as pneumonia, empyema, pneumatocele, osteomyelitis and endophthalmitis (Khan et al., 1997, Parment et al., 1997, Marinella et al., 1998). *S. marcescens* secretes a large number of virulence-related proetins in the extracellular medium (Hejazi and Falkiner 1997) including proteases, nuclease and a haemolysin (ShlA) ( ). Previous works have shown that on LB medium solidified with 0.5 to 0.8% agar (LB swarming plate) *S. marcescens* shows a phenomenon of swarming where a colony of short motile vegetative rods differnetiate at the colony margin into elongated, aseptate, hyperflagellate, swarm cells which migrate rapidly and coordinately away from the colony at 30°C (Alberti and Harshey 1990; Liu et al., 2000). The process of swarmer cell differentiation and swarming migration behavior may be divided into two separate phases in *S. marcescens*: (i) the lag period prior to onset of swarming behavior and the induction of swarmer cell differentiation at the colonial edge and (ii) active motile swarming migration (or translocation) from colonial edge. Individual swarmer cells by themselves do not have the ability to swarm. Rather, swarming behavior in *S. marcescens* is continuous in nature and is the result of a coordinated, multicellular effort of groups of differentiated swarmer cells functioning through close cell-cell interactions ( ). Interestingly, Based on previous observation, swarming migration does not occur immediately upon swarmer cell differentiation on LB swarming plates, a process that occurs 1.5 to 2 h postinoculation. Rather, swarming motility is seen only after ca. **4** h (at 30°C) of incubation, long after differentiation and division have produced a visible colony of swarmer cells. Thus, the differentiated cell is only one part of the process. It requires a lag period prior to the commencement of swarming migration, during which close interaction among bacterial cells and environment occurs. *Serratia* swarming, unlike that of *Proteus* where swarming colonies exhibit regular concentric zones of consolidation (Allison and Hughes 1991), occurs at a lower agar concentration (0.8%) plate and is a continuous process in which populations of swarm cells continue in migration and result in irregular, dendritic patterns on agar surfaces (Liu et al., 2000:).

Development of a *Serratia* swarming colony requires the sensing and integration of a variety of environmental, cell-to-cell, as well as intracellular signals involving surface contact and local high population density. According to the current working hypothesis for the development of a swarming colony (; Givskov et al., 1998), exposure of the cells to surfaces with a certain viscosity is recognized by an unknown sensor, and signal transduction then progresses via the *flhDC* master operon and chemotaxis system (Burkart et al., 1998). Stimulation of *flhDC* operon initiates swarm cell differentiation that involves development of characteristic traits such as cell elongation, multinucleation, and hyperflagellation (Liu et al., 2000; Eberl et al., 1996). The population density is recognized by a homoserine lactone-dependent quorum-sensing system constituted by the *swrI* and *swrR* genes in *Serratia liquefaciens* (Eberl et al., 1996; Givskov et al., 1998), and by *spnI* and *spnR* in *S. marcescens* (Horng et al., 2002). Expansion of the colony is dependent on a functional *swrI* or *spnI* gene, both are members of the *luxI* family of autoinducer synthetase genes (Horng et al., 2002; Eberl et al., 1996). The *swrI* gene product catalyzes the formation of *N*-butanoyl-L-homoserine lactone (BHL) (C4?) and *N*-hexanoyl-L-homoserine lactone (C6?)(Eberl et al., 1996), and the *spnI* gene product mainly catalyzes the formation of C6 and 3-oxo-C6 AHLs (Horng et al., 2002). The signals produced then regulate the SwrR or SpnR activity respectively. Subsequently, production of biosurfactant was stimulated, leading to initiation of swarming or sliding (a flagellum-independent surface migration behaviour, Horng et al., 2002). The flagellar master and the quorum-sensing system are global regulators which control two separated regulons (Givskov et al., 1998).

Although the factors identified to date unravel many critical components involved in *Serratia* spp. swarming behaviour itself, many questions remain unanswered. For example, it is not explained why swarming of *S. marcescens* is observed to be strictly temperature-dependent, i.e., when inoculated on a 0.8% LB swarming plate, *S. marcescens* swarms at 30°C, but this behavior was completely inhibited at 37°C. It is also not understood why *S. marcescens* has to accumulate to a high cell density before the initiation of swarming? The potential physiological signals providing critical stimuli and the signal transduction system are also not characterized as well. We have observed that *S. marcescens* still took 4 h before intiation of swarming under the situation of over-expression of *flhDC* in the presence of biosurfactant. This phenomenon together with the fact that cell elongation and *flhDC* over-expression may be independent of solid surface contact in *S. liquefaciens* (Tolker-Nielsen et al., 2000; Lai et al., 1997) suggest that initiation of *S. marcescens* swarming may not purely be dependent on *flhDC* over-expression and formation of swarming cells. In addition, even though the importance of AHL (N-acyl homoserine lactones) quorum-sensing signals is clearly proved in many swarming bacteria including *Serratia spp.* ( ), it was found that the majority of *S. marcescens* strains do not contain detectable AHL quorum sensing system (Salmond et al., ***personal communication; Lai HC, unpublished data), including many *S. marcescens* strains that show normal swarming behaviour (Lai HC, unpublished data). These observations suggest that there must be some other uncharacterized regulatory mechanisms regulating the swarming behaviours.

In this communication, through miniTn5 mutagenesis, we have screened a group of *S. marcescens* strains that swarmed well at 37°C. One of the mutants was selected for further physiological charaterization. We describe a gene that, when mutated,

showing a “super-swarming” behaviour: decreased the length of the lag phase prior to swarming migration and swarmed not only faster, but also at higher agar concentration surface. In the process of characterization of this mutated gene and its flanking DNA sequences, we have identified a two-component regulatory system and a partial open reading frame with a protein predicted to be involved in fatty acid synthesis. Interestingly, this mutant appears to function to coordinate the initiation of swarming migration independent of *flhDC* flagellar swimming motilty, AHL quorum-sensing system, and production of biosurfactant and extracellular polysaccharide (EPS), factors that are already characterized to be involved in the regulation of populational surface migration behaviours in some bacteria species, including *S. marcescens* (Horng et al., 2002; Liu et al., 2000), *S. liquefaciens* (Lindum et al., 1998) and *P. mirabilis* (Gygi et al., 1995).

Here we show strong evidence that regulation of initiation of swarming was closely related to the homeoviscous acclimation phenomenon, i.e., acclimatizing response of cell membrane lipid in response to different environmental conditions ( ). We demonstrate that composition of cellular fatty acid profile was affected by environmental temperature and also long chain fatty acids. The pattern of fatty acid profile will subsequently lead to either swarming or non-swarming behaviour through a two-component signal transduction system. In addition, we demonstrate that cellular fatty acid profile was further regulated by this two-component signal system. Evidence also suggested that lipopolysaccharide (LPS) which mainly functions as the bacterial endotoxin, might play a role in the regulation of initiation of swarming. A similar long chain fatty acid effect on the swarming of *P. mirabilis* and *Salmonella typhimurium* was observed, suggesting that this regulation is a common phenomenon. Thus, a regulatory loop composed of the long chain fatty acids/temperature, fatty acid profile and the two-component signal transduction system provides a novel mechanism for the control of initiation of swarming in *S. marcescen.*

We also found that compared with LB swarming plate, S. marcescens swarmed immediately after it was transferred from LB broth culture tonto MGM plates.

Results

A *Serratia marcescens* mutant defective in the temperature and temporal control of swarming migration.

At 30°C, *S. marcescens* CH-1 cells exhibited typical swarming behaviour on LB medium containing 0.8% agar (LB swarming plate)(**Fig.** 1) and reached the edge of standard 9 cm diameter plates after 12h culture. In contrast, while growth dynamics of *S. marcescens* CH-1 were almost identical at 30 and 37°C, bacteria inoculated onto identical plates incubated at 37°C did not initiate swarming even after 48h culture and formed small defined colonies (**Fig.** 1).

To characterize any genetic determinants involved in the temperature-dependent regulation of swarming behaviour in *S. marcescens* CH-1, mini-Tn5 transposon mutagenesis was performed to screen for *S. marcescens* CH-1 colonies that swarmed normally at 37°C after over night culture on modified LB agar plates. A total of 17 colonies out of 6000 transconjugants were selected to show such a temperature-independent swarming behaviour. One of the mutants, named *S. marcescens* CH-1ΔA was selected for further study. When incubated on a normal 0.8% LB swarming agar plate [the same result was obtained from either Bacto agar (Difco, USA) or Eiken-agar (Eiken, Japan); Eiken agar was chosen for subsequent experiments] at 30°C, *S. marcescens* WC100 showed a more progressive translocation across the agar surface compared to its isogenic parent *S. marcescens* CH-1. This behavior resulted in a colony that moved out farther than CH-1 over a given time. Following the swarming fronts of *S. marcescens* WC100 and CH-1 cells at 30°C by the method of Gygi et al. (1995), the swarming behavior and velocity of WC100 and CH-1 were monitored, and were shown in **Fig.** 1. Conspicuous differences were observed, including the difference in time spent during the initial lag phase, the swarming velocity after initiation of swarming, and the ability of cells to swarm on higher agar concentration in the swarming plates. CH-1 cells typically spent 3 to 3 and a half hours (at 30°C) in the initial lag phase prior to the onset of swarming migration, while WC100 spent ca. 1 hour less in this phase. After initiation of swarming, *S. marcescens* WC100 swarms at a constitutively increasing velocity, which is significantly faster than that of CH-1 cells (**Fig.** 1). The average velocity of *S. marcescens* CH-1 swarming increased from 2 mm/h (between 2 to 3 hours) to 5 mm/h (between 5 to 6 hours), while *S. marcescens* WC100 increased from 3 mm/h to 6.6 mm/h at the same period (**Fig**. 1). By spending less time in the initial lag period and swarming faster than the CH-1 cells, WC100 thus translocates farther in a given time than do the CH-1 cells. Furthermore, *S. marcescens* WC100 still swarmed well at the agar concentration of up to 1% at 30°C, where CH-1 did not (**Fig.** 1).

As the incubation temperature is shifted from 30 to 37°C, CH-1 cells typically are unable to initiate swarming behavior in the LB swarming plate (**Fig.** 1). WC100 still swarmed well and behaved similarly to that at 30°C in the time of swarming initiation, average swarming velocity, and the ability of swarming at higher agar concentration (**Fig.** 1), suggesting that the temperature-dependent regulation of swarming behaviour was abolished in the mutant. We refer to this unusual swarming phenotype as "super swarming". To see whether the mutation resulting in super-swarming extends to other phenotypes, some physiological assays including growth rate and production of the red pigment prodigiosin were performed. While there was no significant difference in the growth dynamics and pattern of cell elongation during growth on plates between CH-1 and WC100 (data not shown), WC100 produced less prodigiosin than the CH-1 cells at 30 and 37°C (data not shown).

A mutation in a locus encoding RssA, a member of the two-component family of sensory proteins, leads to the superswarming phenotype

Identification of the *S. marcescens* WC100 DNA flanking the mini-Tn*5* insertion site was accomplished by conventional digestion and cloning of the DNA flanking the transposon using either *PstI*, or *KpnI* restriction endonuclease digestion of WC100 genomic DNA, followed by sequencing with primers designed from within the I end or O end of the transposon, respectively (de Lorenzo and Timmis, 1994). The result revealed that the mini-Tn*5* insertion giving rise to the super-swarming phenotype was located within a 2921-bp *Kpn*I/*Pst*I fragment of the *S. marcescens* CH-1 genome (**Fig.** 2). Sequencing revealed that mini-Tn5 had inserted in an 1185bp open reading frame (named ORF1), potentially encoding a 395-amino-acid (***KDa) polypeptide with a calculated isoelectric point of ***. Upstream of *orf1* was *orf2* identified, potentially encoding 214-amino-acid in the same direction. Upstream of *orf2* was a divergent partial *orf3* identified, potentially encoding an N-terminus of ** residues (**Fig.** 2).
The deduced protein sequences were compared with non-redundant protein sequence databases using blastn or blastx via the NCBI internet homepage. The mini-Tn*5* insertion was found to be in the *S. marcecens* genome in a region that is highly homologous to the bacterial two-component signal transduction system region (Kottayil 2002). Computer-assisted homology searches using BLASTP (**Altschul** et al., 1997) of protein sequences stored in the DDBJ/EMBL/GenBank database indicate that Orf1 is homologous to *N. meningitis* *** (29% identity, 50% similarity), *E. coli* RcsC (19% identity, 39% similarity), *Vibrio harveyi* LuxQ (22% identity, 41% similarity), and *E. coli* EvgS (22% identity, 41% similarity), which are (putative) two-component sensor elements. Orf2 showed *** % identity throughout the alignment to *** (gene) of *Pseudomonas* ( ),……., which are (putative) two-component response-regulator elements. Partial ORF3 shows high homology (**% out of the ** N-terminal amino acids) to FabG (NADPH-dependent 3-ketoacyl-ACP reductase) of *E. coli* ( ). We have chosen to call the mutated gene (*orf1*) *rssA*, for regulation of *Serratia* swarming. *orf2* was named *rssB*. The nucleotide sequence of 2921-bp encoding the three ORFs (*rssA*, *rssB* and the 5' portion of *orf3*) has been submitted to the DDBJ/EMBL/GenBank databases under accession no. AF465237. Comparison of RssA and RssB amino acid sequences with their homologues are shown in **figure 2**.

Analysis of the deduced amino acid sequence of RssA indicates that it may be localized in the inner membrane as a transmembrane protein (**Fig.** 3). The N-terminal sequence of RssA was predicted to stretch into the periplasm with only one membrane-spanning domain site predicted between residues P91 and L111. A phosphoaccepter domain was found between 172R and 239E near the middle of the protein in the cytoplasm, and a ATPase domain was observed between 284T and 395Y near the C-terminal. The hydrophobicity characteristic of RssA in which a hydrophobic region between P91 and L111 was observed was shown in **figure** 3**.** RssA has strong motif similarity to many other sensory proteins that are members of the two-component family of proteins (newer? Swanson et al., 1994; Hoch and Silhavy 1995; Alex and Simon 1994). Specifically, as shown in **figure 3**, domains, such as the H, N, G1, F and G2 blocks that are associated with histidine kinases (Hoch and Silhavy 1995) are maintained within RssA. Therefore, RssA may function as a typical sensory protein of histidine kinase in *S. marcescens* CH-1.

To confirm that the WC100 mutant phenotype was not due to artifact effect from introducing a copy of mini-Tn5 transposon, *rssA* in CH-1 was further knocked out by insertion deletion through homologous recombination to form *S. marcescens* CH-1 ΔA. The phenotypes of both *S. marcescens* CH-1 ΔA and WC100 were characterized, including growth dynamics, prodigiosin synthesis, cell density and temperature-regulated swarming behaviours and haemolysin production (see later in the text). No difference was observed between WC100 and *S. marcescens* CH-1 ΔA. Plasmid pWC*rssA* (pACYC184::*rssA*) was further transformed into both WC100 and CH-1 ΔA at 37°C. In both strains, super-swarming behaviour was restored (data not shown), confirming that *rssA* was the gene responsible for the super-swarming phenotype. *S. marcescens* CH-1 ΔA was selected for further study. The effect of over-expression of *rssA* on swarmer cell differentiation and behavior was examined by transforming pWC*rssA* into *S. marcescens* CH-1. When *rssA* is overexpressed from a multicopy pWC*rssA* at 30°C, it not only significantly delayed the initiation of CH-1 swarming for about 2 h, but also reduced its swarming velocity for about 50%. Plasmid-only controls have no significant effect on the time of swarming. The data suggest that RssA may function as a negative regulator of swarming such that increased levels of the RssA protein increases the lag phase before swarming and result in a delayed, aberrant swarming phenotype.

RssA acts as a density and temperature dependent sensor for initiation of swarming

One possible role for RssA in the initiation of swarming could be as a sensor of population cell-density. We have observed that although CH-1 does not swarm at 37°C, the onset time of *S. marcescens* CH-1 swarming behavior is dependent on the population density at 30°C (Lai et al., 1997), such that the time to swarming migration increased as the cell density decreased. It is possible that this cell-density dependent behaviour may be out of regulation in the *rssA* mutant. If RssA senses the population density or degree of cell-to-cell contact, defect in RssA should alter the ability of the cells to detect changes in the population size and may change the time of initiation of migration. To test this, we compared the timing of swarming initiation of CH-1ΔA cells to that of CH-1 at 30°C. In a series of experiments, we tested whether the initiation of *S. marcescens* CH-1ΔA swarming behavior was still correlated with population density at 30°C. As similar to many other enterobacteria ( ), the $flhDC_{Sm}$ is the master operon of flagellar regulon regulating the flagellar motility during swarming in *S. marcescens* (Liu et al., 2000), we chose to use the *flhDCsm* promoter activity as a reporter for *S. marcescens* swarming. A *S. marcescens* strain was constructed where P*flhDC*::*luxAB* (Liu et al., 2000) was inserted into the chromosome of *S. marcescens* CH-1 ΔA to form *S. marcescens* WC101. In these experiments, the initial inoculum concentration of WC101 and also the control strain *S. marcescens* F-3 (Liu et al., 2000) was varied in 10-fold increments from 1×10^8 to 1×10^5 cells (CFU) delivered in 5-µl-aliquot droplets to the LB swarming agar surface. The bioluminescence emission of WC101 which indicates the promoter activity of *flhDC* master operon (Liu et al., 2000) was monitored hourly by X-ray film exposure following the growth of bacterial colonies on LB swarming plates. The number of each bacterial colony was at the same time calculated by 10 times serial dilution and plate counts. The assay result of *S. marcescens* WC101 was shown in **figure 4**. At the earlier stage of bacterial culture where no swarming was initiated, light emission (the *flhDC* promoter activity) could be detected from the whole colony. Once the cells started to swarm, bioluminescence could only be detected at the swarming edge of the

colony, where elongated cells are actively migrating outwards. Thus the light emission pattern reflectced the real time colonial swarming behaviour. While initiation of WC101 swarming is still density dependent, it started to swarm earlier than CH-1 derived F-3 cells at a much lower cell density. WC101 only requires ca. **50 (or 100?)**-fold fewer cells to commence migration (1×10^8 F-3 cells versus 2×10^6 WC101 to begin swarming at **3 h**). Further experiments showed that *S. marcescens* WC101 also behaved similarly in a cell-density dependent swarming phenomenon at 37°C (data not shown). We further identified that the light emission pattern between F-3 and WC101 cells was identical following the growth in LB broth culture at 30 and 37°C (data not shown), suggesting that the *flhDC* promoter activity of *S. marcescens* Δ*rssA* was not affected in broth culture. These data show that although swarming initiation of *S. marcescens* Δ*rssA* is still density-dependent, it reproducibly initiates swarming at a lower cell-density, suggesting *rssA* indeed is involved in the cell-density dependent regulation of swarming behaviour, which was similar to the RsbA effect reported in *P. mirabilis* super swarming mutant (Belas et al., 1998). Furthermore, our results clearly showed that the aberrant mutant swarming behaviour was not affected by temperature-upshift (from 30 to 37°C), suggesting that RssA may also act as a temperature-dependent sensor regulating the swarming behaviour of *S. marcescen*s CH-1.

Swimming motility, flagellum synthesis, production of surfactant and extracellular polysaccharides and cell wall integrity were not affected in *S. marcescens* CH-1 ΔA **at 30 and 37**°C

Factors known to be involved in the swarming behaviours in *S. marcescens* ( ), *S. liquefaciens* (Lindum et al., 1998) and *P. mirabilis* (Gygi et al., 1995) were examined to see whether any of the known factors were upregulated, subsequently leading to the super-swarming behaviour in *S. marcescens* Δ*rssA*. These include the swimming motility ( ), amount of flagellum produced, and production of surfactant ( ) and **extracellular polysaccharides** ( ).

To see whether the swimming motility was significantly increased in CH-1ΔA, swimming assay of *S. marcescens* CH-1 ΔA and CH-1 cells at both 30 and 37°C were performed. The result in **figure** 5 shows that CH-1ΔA and CH-1 swimmed in a similar scale at both temperatures. Therefore, this mutation does not appear to affect flagellar rotation and motility. Further SDS-PAGE analysis for quantification of flagellum production also did not show significant difference between *S. marcescens* CH-1 ΔA and CH-1 at both temperatures (data not shown). To see whether solid surface contact was an important factor influencing the *S. marcescens* flagellar motility, *S. marcescens* WC101 and *S. marcescens* F-3 were cultured in either LB broth or seeding plates at both temperatures, and specific bioluminescence activity was compared between broth and plate cultures of the two strains following the growth. Still, we could not detect any significant difference between broth and plate culture conditions at 30 or 37°C (data not shown). This result was similar to what Givskov et al., ( ) has reported from a related swarming species, *Serratia liquefaciens*, indicating that solid surface contact is not the factor leading to the inhibition of CH-1 swarming at 37°C. We further supposed the CH-1ΔA super-swarming phenotype may be due to over-production of biosurfactant reducing the surface friction of swarming plates. Drop-collapsing test (Horng et al., 2002) was then performed to qualitatively assay the surface tension of over-night culture suspensions from CH-1 ΔA and CH-1 cells at both temperatures. Again, even after

repeated tests, no difference was observed between *S. marcescens* CH-1 ΔA and CH-1 (**Fig.** 5). For confirmation, TLC (Thin Layer Chromatography) (Horng et al., 2002) assay was performed to see whether there is a minor difference of biosurfactant production between the two strains, and still similar results were obtained (**Fig.** 5).

As we had used the modified LB swarming plates which contained glycerol for selecting the super-swarming mutants, and glycerol was reported to stimulate the production of polysaccharide production in *S. marcescens* strains (Aucken et al., 1997), together with the fact that extracellular polysaccharide (EPS) is shown to be essential for *P. mirabilis* swarming ( ), we hypothesized that the extracellular polysaccharide (EPS) may be over-produced in *S. marcescens* CH-1 ΔA, leading to the super swarming phenotype. Measurement of EPS (both the bound form and unbound form) ( ) were further performed to see whether they are involved in the mutant super-swarming phenotype. Again, to our surprise, no difference was observed between CH-1 and *rssA* mutant in the production of EPS at both temperatures **(Fig. 5)**.

As WC100 was screened from modified LB agar medium that contains a lower NaCl concentration [0.04% (wt/vol)] compared with the normal LB agar salt concentration [1% (wt/vol)], together with the phenomenon that the predicted products of almost all transposon–disrupted genes identified in the super-swarming mutants are closely associated with cell envelope components (data not shown), we further hypothesized that cell envelope integrity might be less compact in the *rssA* mutant. To see whether this is possible, we grow CH-1 and CH-1 ΔA for overnight in normal LB broth media before transferring the bacterial cells into normal [1% NaCl], high [1.5% NaCl] and low-osmolarity [0.04% NaCl] LB broth media at both 30 and 37°C and monitor their growth dynamics by measuring the optical density (O.D. A_{600nm}). *S. marcescens* CH-1ΔA was shown to grow with a generation time similar to that of the CH-1 cells in all the conditions assayed (data not shown). This result suggests that the RssA was not involved in the process of osmolarity regulation in *S. marcescens* CH-1. Our results suggest that some novel factors other than the factors already identified are involved in the regulation of *S. marcescens* CH-1 swarming behaviour, which, when defective, leading to the super swarming behaviour.

Hemolysin production and biofilm formation are regulated by RssA in a temperature-dependent way

As the degree of biofilm formation is reported to be closely connected to swarming ability in bacterial species including *Salmonella typhimurium* ( ), *Pseudomonas aeruginosa* ( ), Burkholderia cepacia (Huber et al., 2001) etc., we thus hypothesized that the biofilm formation was affected in *S. marcescens* CH-1 ΔA. The microtiter well assay (O'Tolle and Kolter, 1998) which monitors the ability of *S. marcescens* to attach to the wells of microtiter dishes was used to quantify the biofilm formation as described in the Materials and Methods. The assay was performed in CH-1ΔA and CH-1 strains at both 30 and 37°C. As shown in **Fig.** 6, the biofilm formation for CH-1 was better at 37°C which showed an average value of optical density at about ***, and an average absorbance value of *** was detected at 30°C under the assay condition. Biofilm formation for CH-1 ΔA is significantly defective, reaching only about 0.12 (25% of the CH-1 level) at 37°C, and **0.21 (66**% of CH-1 level) at 30°C. The results suggest that CH-1ΔA is less effective in binding to the microtiter plate surface. Further experiments by phase-contrast microscopy(confocal microscopy? Or others/) for monitoring the structures of biofilm formation showed that CH-1 ΔA at

both temperatures has different and less compact structures compared with CH-1 at both 30 and 37°C (**Fig.** 6).

The defect in biofilm formation suggested cell alignment within group rafts during swarming of *S. marcescens* CH-1 ΔA may be aberrant compared with CH-1. To confirm this, swarming assays were performed at both 30 and 37°C for *S. marcescens* CH-1 and CH-1 ΔA on LB swarming plates and the swarming edge of the bacterial colonies were observed closely under 400x and 1000x phase contrast microscopy at both temperatures. The results showed a trend that compared with *S. marcescens* Δ*rssA*, areas of cell population near the colonial swarming edge showing actively swarming motility is much less for CH-1 37°C **(Fig. 6)**, where CH-1 did not swarm and the cells near colonial edge of CH-1 looked less active and similar to the sliding edge of *S. marcescens* SS-1 (Horng et al., 2002), and the swarming fronts of *S. marcescens* Δ*rssA* was basically similar to that observed at 30°C **(Fig. 6)**. No other significant differences were observed, including the direction of bacterial axis in movement, organization of colonial texture (from short, vegetative and still cells in the colonial center, gradually outwards to the vegetative, actively turning cells in the middle, then to the medium-elongated, also actively moving cells near the swarming edge, finally to the very much elongated swarming cells in the swarming fronts) and the ratio of pigment to non-pigment producing cells under the 1000x phase contrast microscope (data not shown). Our observation showed that compared with the CH-1 parent strain whose biofilm formation and group rafts structure during swarming were under the temperature regulation, these phenotypes were aberrant in the *rssA* mutant irrespective of the temperature shift.

Expression of some virulence factors including haemolysin was co-ordinately regulated with cell differentiation during swarming ( ), and bacterial two-component system seemed to play an important role in these processes ( ). It was reported that bacterial two-component system was involved in the regulation of haemolysin production (Stibitz et al., 1989; Fournier et al., 2001), we then supposed that in the super-swarmer strain *S. marcescens* CH-1 ΔA, haemolysin production may be affected. To answer this question, we assayed whether the cell-associated haemolysin activity was affected in CH-1 ΔA. Equivalent cultures of *S. marcescens* CH-1 and CH-1 ΔA cells were harvested from LB seeding agar plates at 30 and 37°C at hourly following the growth after inoculation. The cell suspensions were subject to haemolysin activity assay ( ). The results shown in **figure 6** clearly indicated that the haemolysin activity of CH-1 ΔA was significantly higher than that of CH-1 ΔA at both 30 (320 %) and 37°C (250%) at 2 hours after inoculation. To see whether the regulation occurs at the transcriptional level, a recombinant plasmid, pSA*** [pACYC184(P*shlA*::*luxCDABE*)], was constructed to monitor the promoter activity of P*shlA*. A comparison of the light emission patterns from *S. marcescens* CH-1 ΔA (pSA**) and *S. marcescens* CH-1 (pSA***) (Fig. 6) showed an average increase of ** fold in P*shlA* activity in CH-1 ΔA, suggesting the negative regulation effect of RssA on the promoter activity of *shlA*. This result showed that in *S. marcescens* CH-1, haemolysin production was regulated by the RssA/RssB two component system at the transcriptional level, suggesting a complicated RssA/RssB regulatory network existing in *S. marcescens*, in which some uncharacterized swarming-regulatory genes and haemolysin virulence factor gene are regulated.

Long chain saturated fatty acids inhibit *S. marcescens* swarming in a

dose-dependent way

Although CH-1 does not swarm on LB swarming plate at 37°C, interestingly we found that CH-1 did swarm well on defined M9 minimal growth medium (MGM) containing 0.8% Eiken agar (MGM plate) at 37°C (figure 7). Further swarming assay using MGM plate containing 1% casamino acids/1% yeast extract showed that CH-1 swarming was completely inhibited at this temperature(figure 7). We also observed that CH-1 ΔA swarmed at the same conditions (figure 7). These observations suggested that some components contained in casamino acids/yeast extract inhibited swarming of CH-1 at 37°C, and existence of the component(s) did not affect CH-1 ΔA swarming behaviour. CH-1 and CH-1 ΔA both swarmed well at 30°C on all plates tested (Fig. 7). We hypothesized that some amino acids contents within yeast tract may inhibit the CH-1 swarming behaviour at 37°C. Using MGM-casamino acids(1%) swarming plate as the basal medium, the 20 essential(?) single amino acids including isoleucine, leucine, valine, glutamine, asparagines, ***** and **** at the concentration of 1% was separately added, followed by swarming assay at 30 and 37°C. Comparatively, a similar swarming trend was observed on the swarming scale between CH-1 and CH-1 ΔA when compared with those on MGM-casamino acids (1%) plates (data not shown), suggesting that individual amino acid in excess did not have effect on the swarming regulation of CH-1. We then continue to test whether addition of saturated or unsaturated fatty acids with different chain length into MGM-casamino acids (1%) plates would affect the swarming behaviour. Saturated fatty acids such as lauric acid (C12 carboxylic acid), myristic acid (C14 tetradecanoic acid), palmitic acid (C16 carboxylic acid) and stearic acid (C18 octadecanoic acid) and unsaturated fatty acids such as myristoleic acid (cis-9-tetradecenoic acid), palmitoleic acid (cis-9-hexadecenoic acid, 16:1 Δ9), oleic acid(cis-9-octadecenoic acid) and cis-vaccenic acid(cis-11-octadecenoic scid) at the final concentration of 0.1% were added separately into MGM-casamino acids(1%) plates followed by swarming assays for CH-1 and CH-1 ΔA. The results were shown in figure 7. While there were no significantly different swarming-regulation effect for the other fatty acids added, we found that addition of lauric acid (0.1 %) and myristic acid(0.1%), stearic acid (0.5%) and palmitic acid(0.1%) completely inhibits swarming of *S. marcescens* CH-1 while CH-1 ΔA swarming was not significantly inhibited. To further confirm whether this phenomenon was dose-dependent,

Dose-dependent response

Cellular fatty acid profile affected by long chain saturated fatty acids and growth temperature are closely related to swarming behaviour

As the expression of acyl-lipid desaturase(des) gene responsible for the maintenance of membrane fluidity in response to a decrease in growth temperature (The Des pathway) is regulated by a two-component system DesK and DesR in *Bacillus subtilis* (Larisa et al., 2002; Aguilar et al., 2001; Japan), together with the fact that *orf3* identified upstream of *rssA* and *rssB* was predicted to encode a protein with high amino acid identity to *E. coli* FabG ( ), which encodes a NADPH-dependent 3-ketoacyl-ACP-reductase involved in the fatty acid synthesis in the elongation step ( ), we thus hypothesized that the cellular fatty acid profile might be affected by environmental temperature and the long chain fatty acids added into the culture media in CH-1, which may then affect the conformation of RssA and subsequently regulate the signal transduction flow of RssA/RssB, leading to either swarming or

non-swarming phenotypes. To confirm this, cellular fatty acid profile which was expressed as percentage of fatty acid composition of *S. marcescens* CH-1 cultured at MGM plates, MGM casamino acids plates, MGM-casamino acids-long chain fatty acids and MGM-casamino acids /yeast plates at 37°C were determined by fatty acid extraction followed by fatty acid analysis by MIDI gas chromatography system ( ). The results in **Table 1** showed that fatty acid profile is indeed affected by amount of fatty acids contained in the media. Compared with the fatty acid profile pattern of CH-1 swarming cells, the non-swarming cells contained significantly less short-chain fatty acids (12:0) and branch-chain fatty acids (15:0 anteiso), but contained some 14:0 2OH, which was not observed in …… The result suggested that fatty acid composition between CH-1 swarming and non-swarming cells was indeed different.
more short chain 12:00 fatty acids and branch chain 15:0 Anteiso fatty acids were synthesized.

To see whether this is a common phenomenon between the CH-1 swarming and non-swarming cells, CH-1 swarming cells and non-swarming cells cultured at different growth conditions were harvested for fatty acid profile analysis. These include CH-1 swarming cells collected from LB swarming plates (30°C), MGM plates (30 and 37°C), MGM-casamino acids (1%) plates (30 and 37°C), and MGM-casamino acids (1%)/yeast extract(1%) plates (30°C). For CH-1 non-swarming cells, cells were collected from LB swarming plates (37°C), MGM-palmitic acid(0.1%) plate(30 and 37°C) , MGM- stearic acid(0.1%) plate(30 and 37°C), MGM-peptone(1%)/yeast extract(1%) plates (37°C). The results were shown in Table 1. We found a significant trend that…….. The more branch-chain fatty acids contained within thin cells, the less S. marcescens CH-1 will swarm.

Similar experiments were also performed in CH-1 ΔA (Table 1.). We found that for swarming CH-1 and CH-1 ΔA under different culture conditions including temperature shift and nutrients variation, while there was no significant difference in the percentage of fatty acid composition in 14:0, 16:0, 18:1 w7c, and 18:0, there was a clear difference in 12:0, 15:0 anteiso, and 14:0 2OH fatty acids……

Our results suggested that Patterns of cellular fatty acid profile is closely related to *S. marcescens* swarming behaviour. And that
Adiiton of Ile and leu …did not have effect.

We classify the fatty acid profiles into “swarming” ( ) and “non-swarming” ( ) profiles.

Expression of *fabG* is influenced by fatty acid profile in a RssA-dependent way

To further confirm that expression of *fabG* was part of the elements belonging to the temperature-dependent RssA/B regulatory network, recombinant plasmids pSA301 and pSA302 (P*fabG*::*luxCDABE*) in which the *fabG* promoter was ligated in front of the *luxCDABE* reporter were constructed. pSA301 was electroporated into CH-1 and *S. marcescens* CH-1 ΔB, and pSA302 into CH-1 ΔA, followed by monitoring the bioluminescent activity of each strain ( ) under different culturing conditions leading to either “swarming” or “non-swarming” fatty acid profiles. The result was shown in figure 8. In a “swarming” fatty acid profile, expression of *fabG* was…… and that in a “non-swarming” profile, *fabG* was…….. Expression of *fabG* was constitutively high(or low) in a *rssA* mutant background irrespective of change of amino acids contents in the culture media. These phenomena showed that expression

of *fabG* was indeed regulated by RssA/RssB two component system under different fatty acid profile condition, and such a regulation was aberrant
Northern blot hybridization using partial *fabG* (*orf3*) as the probe confirmed the regulatory phenomena (Fig. 8A), suggesting that expression of the *fabG* was regulated by *rssA* in a fatty acid profile dependent way and that more short-chain.

Similar phenomenon of shlA expression was also observed in …….(fig…?).

Under this hypothesis, together with the fact that CH-1 ΔA swarmed irrespective of environmental temperature shift and change of media nutrients (Fig. and Fig.), it might be possible that the uncontrollable CH-1 ΔA super-swarming behaviour may be due to blockage of phosphorelay signaling to RssB because of the knock out of RssA sensor kinase, even if the fatty acid profile changed under different culture conditions.

RssA acts to control swarming and *fabG* expression in response to growth temperature and lauric acid/myristic acid via RssB
In the RssA and RssB signaling pathway, we hypothesized that at 30°C, composition of membrane fatty acids affected the conformation of RssA sensor kinase, where phosphorelay reaction from RssA was not transferred to RssB. RssB was unphosphorylated, leading to swarming phenotype. When temperature was shifted to 37°C and membrane fatty acid structure changed in order to maintain fluidity, the phosphate-group was transferred from RssA to RssB, RssB was phosphorylated, leading to inhibition of swarming. To see whether regulation of swarming behaviour and expression of *fabG* by RssA was indeed through RssB, a *rssB* insertion deletion knock-out strain *S. marcescens* CH-1 named CH-1 ΔB was constructed. If the hypothesis was correct, CH-1 ΔB should behave basically like CH-1 ΔA due to the absence of phosphorylated RssB at any culture conditions. After confirmation of the mutated *rssB* genotype by Southern blot hybridization (data not shown), CH-1 ΔB was subject to swarming assay under different physiological culture conditions including LB swarming plates, MGM plates, MGM-cas amino acid(1%) plates, MGM-peptone(1%) plates, MGM-peptone(1%)/yeast extract(1%) plates, and MGM-cas amino acids plates containing a series of long chain fatty acids(C12, C14, C16, C18, C14:1 Δ9, C16:1 Δ9, C18:1 Δ9, and C18:1 Δ11) at the concentration of 0.1% at 30 and 37°C. We have found that CH-1 ΔB did behave similarly to CH-1 ΔA swarming behaviours under all conditions tested (Fig. 8B and data not shown). We then continued to test whether the expression of *fabG* was still regulated by long chain fatty acids or temperature shift in the absence of *rssB*, and whether this regulation was coordinate with swarming behaviours. To achieve this, pSA**(P*fabG*::*luxCDABE*) was electroporation transformed into *S. marcescens* CH-1 and CH-1 ΔB, followed by monitoring the intensity of light emission of both strains cultured at different conditions mentioned above.
The results were shown in figure ***. It was found that ….indeed……..
Northern blot hybridization using partial fabG DNA sequence as the probe confirmed the observation (figure 8B).

Previous work has shown that overexpression of response regulators in the absence of their cognate kinases could result in constitutive expression of the target gene(s) controlled (Powell and Kado 1990). This suggested that high concentrations of unphosphorylated response regulator could bind *in vivo* to target promoter and caused unregulated transcription. To determine whether an over-expression of *rssB*, without

the assistance of RssA, could activate transcription of fabG and stimulates swarming at 30 and 37°C, pSA***(containing rssA) was transformed into *S. marcescens* CH-1 and CH-1 ΔA followed by swarming assay and monitoring the expression of *fabG*. The results….also showed that both S. marcescens CH-1(pSA***containing rssA) and S. marcescens swarmed well not only at 30, but also at 37°C. Further experiments using pSA**(PfabG::luxCDABE) as the reporter for *fabG* expression showed that fabG was constitutively expression when rssA was over-expressed (Fig.). These experiments demonstrate that high production of RssA promotes constitutive expression of *fabG* gene and stimulates swarming without the assistance of RssB, and that the unphosphorylated RssA **alone** enhanced the expression of *fabG* and initiates swarming at 37°C and also MGM-cas amino acid medium containing long chain fatty acids. This result therefore agrees with the observation that unphosphorylated response regulators can activate transcription when they are overexpressed (Powell and Kado, 1990). Our data showed that RssB and RssA play essential roles in the regulation of swarming in response to growth temperature and long-chain saturated fatty acids.

Fatty acid profile is regulated by RssA/RssB signaling pathway

From the results we have obtained, as expression of *fabG* was regulated by RssA/RssB system, it seemed possible that pattern of fatty acid profile might be regulated by this two-component system. To confirm this, the fatty acid composition of *S. marcescens* CH-1, CH-1 ΔA and CH-1 ΔB was analysed. Cells were grown on MGM cas amioc acids(1%)/yeast extract(1%) plates at 30 and 37°C before harvesting for fatty acid analysis. The results were shown in Table 1. We found that indeed……

Long chain AHLs quorum sensing signals inhibit swarming via change of fatty acid profile and RssA/RssB signalling (test PfabG::luxAB)

Quorum sensing and regulation of populational surface migration behaviour have been identified in many bacterial species, including sliding of *S. marcescens* (Horng et al., 2002; Ang et al., 2001), and swarming of *S. liquefaciens* (Lindum et al., 1998), *Pseudomonas aeruginosa* (Fuqua et al., 2001) and *Burkholderia cepacia* (Huber et al., 2001) (any more?). To study the role of quorum sensing in the regulation of swarming motility in *S. marcescens*, we began work with identification of the quorum sensing signals produced by *S. marcescens* CH-1. Unexpectedly, we discovered that this strain was unable to produce AHLs capable of activating 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 AHL activity was detected (data not shown). As further evidence for the absence of a LuxRI/AHL-based 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).

Although *S. marcescens* CH-1 did not produce detectable AHLs, it was observed that long-chain AHLs have inhibitory effects on the sliding of *S. marcescens* SS-1 (Horng et al., 2002). We thus hypothesize that long chain AHL signals may also inhibit *S. marcescens* CH-1 swarming. To confirm this, *S. marcescens* CH-1 was used

for the swarming assay on LB swarming plates containing a range of synthetic AHLs incorporated into the medium at the final concentration of 10 μM. **Figure** 10 shows while all the short chain AHLs tested did not inhibit swarming, a clear and inhibitory effect of some specific long chain AHLs upon swarm plate motility was observed. Among the long chain AHLs tested, 3-oxo-C10, 3-oxo-C12 and especially 3-oxo-C14 inhibit the CH-1 swarming most significantly, and addition of these signals did not inhibit the swarming of CH-1 ΔA **(figure 10)**, suggesting that the long chain AHLs inhibitory effect might act through change of membrane fatty acid profile and RssA/RssB signaling system. To see whether this is true, the 3-oxo-C12 was selected and incorporated into the LB swarming media followed by monitoring the pattern of fatty acid profiles of CH-1 and CH-1 ΔA that are seedling-plate cultured at both 30 and 37°C. Results in Table 1 showed that……a significant difference was observed between********** 3-oxo-C12 on *S. marcescens* CH-1 with and without the presence of 3-oxo-C12, and ****. To see whether this effect was through RssA/RssB signaling pathway, bioluminescence activity of *S. marcescens* CH-1 (P*fabG*::*luxCDABE*) and CH-1 ΔA (P*fabG*::*luxCDABE*) seeding plated at 30 and 37°C was measured. It was observed that ……suggesting that inhibition of CH-1 swarming by long chain AHLs, especially the 3-oxo-C12 seemed to be via the fatty acid profile and RssA signaling pathway.

Fig. 10B fabG affected

Evidence suggests that production of lipopolysaccharide was regulated by RssA/RssB signalling pathway

It was reported in *Salmonella typhimurium* *** that lipopolysaccharide (LPS) was involved in the regulation of swarming, albeit the underlying mechanism was not understood (Toguchi et al., 2000). We have further found that from our another 3 super-swarming mutants characterized, genes mutated were predicted to be involved in either synthesis or modification of LPS (data not shown). It was thus possible that LPS also played some uncharacterized role in the regulation of swarming. We further hypothesized that production of LPS might be also under the control of RssB/RssA system. To test this hypothesis, LPS from *S. marcescens* CH-1, CH-1 ΔA and CH-1 ΔB were extracted and separated in ***gels. We found that …Fig. 9……

RssA/RssB was conserved among *Serratia marcescens*

To see whether rssA or rssB was conserved among S. marcescens, a total of 67 S. marcescens strains collected from National Taiwan University Hospital, 4 strains from (Paul) were subject to PCR using the primer pairs (/) and (/) for amplifying rssA and rssB, respectively. The results shown that both rssA and rssB DNA fragments were detected from 70 out of the 71 S. marcescens strains(data not shown), suggesting that the gene pair was conserved among S. marcescens strains.

By Blast comparison more others involved in membrane stress…., suggesting that ……

Long-chain saturated FA affects fatty acids profile and inhibit swarming of *P. mirabilis* and *S. typhimurium*

To see whether swarming of another two swarming bacteria was also inhibited by the long chain fatty acids, C18 was inoculated into LB media …P. mirabiluis also For S. typhi…….
Fig. 11

Discussion

It is hypothesized that bacteria might communicate among themselves by producing extracellular signal compounds that, when present during appropriate conditions and in sufficient quantities, trigger specific responses. "Quorum sensing" describes one mechanism that acts in response to population density (Swift et al., 2001). It relies on the accumulation of small extracellular signaling molecules to modulate the transcription of target genes and operons. Quorum-sensing mechanisms have been found in *S. marcescens* (Thomson et al. 2000; Ang et al., 2001, Lai et al., unpublished data), and also in *S. liquefaciens* (Eberl et al., 1996; Lindum et al., 1998), regulating the swarming and sliding behaviours.

Transduction of environmental cues such as temperature and nutrient limitation are commonly required for the transcriptional control of gene networks involved in bacterial cell differentation and virulence (Mekalanos, 1992 newer review). Although swarming differentiation has been reported in many Gram-negative and Gram-positive bacteria, the factors which induce it are poorly understood. Initiation

by surface contact was recognized in early studies of the phenomenon on agar media and the addition of viscosity agents to liquid media also induce swarming differentiation in *Vibrio parahaemolyticus*, *S. marcescens* and *P. mirabilis* (McCarter & Silverman, 1990; Alberti & Harshey, 1990; Allison et al., 1993 newer review?). Swarming of *V. parahaemolyticus* is initiated under iron limited growth conditions (McCarter & Silverman, 1989) and the amino acid glutamine is an extracellular signal initiating swarming differentiaion of *P. mirabilis* (Allison et al., 1993).

Membrane fluidity and signaling

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A fadD mutant of Sinorhizobium meliloti shows multicellular swarming migration and is impaired in nodulation efficiency on alfalfa roots.

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Using *S. marcescens* SS-1 as the study model a similar inhibitory effect of long chain AHLs upon sliding plate motility was also observed (Fig.). Similar inhibitory effect from AHL signals….

Hemolysin regulated by two-component system, also observed in Bordetella pertussis (Stibitz 1994).

V. harveyi can perceive the AI-1 and AI-2.

Bacteria that differentiate and demonstrate multicellular behaviour as part of the regulated expression of gene networks required for the complex processes underlying morphological and physiological changes are commonly observed (Shimkets 1990; Matsuyama and Matsushita 1993; Shapiro 1995; Shapiro 1998 Rice et al., 1999; Alavi and Belas 2001). Regulation of these multicellular behaviours usually involves interaction between cells to cells and/or cells to environment signals. Examples include Gram-positive bacterial antimicrobial peptide production (Kleerebezem and Quadri 2001), sporulation in *Bacillus* (Losick et al., 1989, Jelsbak and Sogaard-Andersen 2000), Gliding motility in Myxococcus xanthus (Spormann et al., 1999); myxobacteria fruiting body formation (Shimkets, 1990; Kim et al.,1992; Jelsbak and Sogaard-Andersen 2000), *Vibrio harveyi* and *Vibrio fischeri* light emission (Meighen 1999), *P. aeruginosa* biofilm formation (Costerton et al., 1999; Singh et al., 2000; Costerton 2001), production of nitrogen-fixing cells in cyanobacterium ( ), and also swarming behavior of Vibrio, *Proteus*, *Salmonella*, *Salmonella*, *E. coli*, *Clostridium* and *Serratia* species (McCarter and Silverman 1990; Harshey 1994; Fraser and Hughes 1999; Eberl et al., 1999; Macfarlane et al., 2001; Romling 2001).

The underlying interactive mechanism between bacteria and environmental cues varies. Many bacteria produce chemical signals by themselves in response to the changing of environment. Quorum sensing mediated through a N-acylhomoserine lactone (AHL) autoinducer has been described in many bacterial species (for reviews see Parsek and Greenberg 2000; Swift et al., 2001; Withers et al., 2001; Miller and Bassler 2001; Fuqua et al., 2001) include *Burkholderia cepacia* (Huber et al., 2001), *S.*

liquefaciens (Eberl et al., 1996; Eberl et al., 1999), and *S. marcescens* (Thomson et al., 2000; Horng et al., 2002?), where it has been shown to be involved in the initiation of swarming and sliding motilities through regulation of the production of secondary metabolites including biosurfactant, thus playing important roles in regulation of the swarming/sliding behaviours. In another swarming bacterium *P. mirabilis*, although no AHL signals are identified so far, initiation of swarming, swarmer cell differentiation and virulence activation grew to be concomitant and coregulated in response to extracellular glutamine and surface contact (Allison et al., 1992, 1993). Glutamine acts as a chemical signal which initiates swarming behaviour in *P. mirabilis*. The biological role of glutamine in signalling swarming of *Proteus* is unclear but the ability of *P. mirabilis* to secrete proteases with broad substrate specific may indicate a common mechanism for generation of the amino acid signal from host-derived protein substrates in vivo (Loomes et al., 1990, Hines, et al., 1988).

Bacterial two-component systems are also proved to be important for regulation of bacterial physiological behaviours under the regulation of some physical or chemical environmental signals (either identified or unidentified)(Browse and Xin 2001 more). Examples include the PhoP/PhoQ two-component system of *Salmonella typhimurium* governing transcription of some 25 loci in response to the extracellular concentration of Mg2+ (Soncini and Groisman 1996), the induction of PmrA-activated genes by hign iron concentration in *Salmonella enterica* (Wosten et al., 2000), the regulation of he *arc* two-component system of *E. coli* by redox Quinone signals (Georgellis et al., 2001), the interaction between two autoinducers (AI-1 and AI-2) and two cognate membrane-bound two-component hybrid sensor kinases called LuxN and LuxQ respectively and regulation of light emission in Vibrio harveyi (Freeman et al., 2000). Furthermore, regulation of *P. mirabilis* swarming by the putative RsbA sensor kinase (Belas et al., 1998), regulation of *P. aeruginosa* swarming, virulence factor and biofilm formation by GacA/GacS (Brinkman et al., 2001; Parkins et al., 2001 others), and the evidences of being involved in swarming behaviour by defects in putative two-component signaling components in *Salmonella enterica* serovar *typhimurium* ( ) are also reported. In the last 3 examples, the signals that interact with bacterial two-component systems remain uncharacterized.

Temperature shift is a common environmental signal directing metabolic and phenotypic changes in bacteria. Pathogenic bacteria such as *E. coli*, *Listeria*, *Shigella* and *Bordetella* frequently express virulence characteristics preferentially at 37°C as part of the adaptation required to colonize their hosts (Mekalanos, 1992 newer references). Many bacteria use the two-component signal transduction system for communication with the environmental temperature shift. These include regulation of BvgA/BvgS by low temperature, MgSO4 and nicotinic acid in *Bordetella pertussis* (Manetti et al., 1994) and *E. coli* (Utsumi et al., 1994), regulation of *des* gene expression by DesK/DesR induced by temperature shift in *B. subtilis* (Aguilar et al., 2001), cold-regulated genes under control of the cold sensor kinase Hik33 in *Synechocystis* (Suzuki et al., 2001), the temperature-dependent biosynthesis of the *Pseudomonas syringae* phytotoxin coronatine by CorS/CorP and CorS/CorR two-component regulatory systems (Ullrich et al., 1995), the increase of transcription of hrpXY up to threefold by low pH, nutrient, and temperature levels in *Erwinia amylovora* (Wei et al., 2000), and in this paper, the temperature-dependent swarming of *S. marcescens* by the RssA/RssB two-component system. Besides two-component system, many bacteria use other systems for interaction with the environment. In *Vibrio cholerae*, environmental stimuli such as temperature, pH and osmolarity

regulates expression of the ToxR virulence regulon (Skorupski and Taylor 1997). Similarly in *Yersinia pseudotuberculosis*, the *inv* gene is regulated in response to a variety of environmental signals, such as temperature, growth phase, nutrients, osmolarity and pH, and requires the product of *rovA*, a member of the SlyA/Hor transcriptional activator family (Nagel et al., 2001). In *E. coli*, the aspartate chemoreceptor (Tar) serves as a thermosensor, and its thermosensing properties are controlled by reversible methylation of the cytoplasmic signalling/adaptation domain of the protein (Nishiyama et al., 1999). In *Shigella* species, transition-related increases in virulence genes expression was governed by central regulatory loci such as virR which are in turn thought to respond to temperature change via the effects of histone-like proteins (Hromockyj et al., 1992). More examples?

Serratia species, like *Proteus*, are common opportunistic pathogens of immunocompromised and hospitalized patients but they are also frequently the cause of infections of insects and cold-blooded vertebrates (Grimont & Grimont, 1978). The ability to undergo coordinate population migration and virulence expression may therefore play a role in *Serratia* infections of these hosts as well. Our results showed that some similarities exist between *S. marcescens* and *P. mirabilis* in the regulation of swarming behaviours (**Fig.**) and expression of virulence factors (**Fig.**). Regulation of multicellular behaviour by two-component systems is an example. Similar to RsbA in *P. mirabilis*, expression of one of the virulence factors such as haemolysin ( ), and biofilm formation ( ) is under the regulation of RssA in *S. marcescens* (**Fig.**). Furthermore, like *P. mirabilis rsbA* mutant, the *rssA* mutant also shows a precocious swarming phenotype and requires only 100-fold fewer cells to start swarming than its parent strain CH-1 (Fig. 8), suggesting that the process is still density dependent despite of the fact that cell mass needed for initiation of swarming is impaired.

Even though there are similarities in the phenotypes of *P. mirabilis rsbA* and *S. marcescens rssA* super-swarming mutants, based on the current data obtained, it is still difficult to say whether there is a common mechanism involved in the swarming process between *P. mirabilis* and *S. marcescens*. First of all, RssA does not show significant similarity to RsbA either in length (393 amino acids for RssA and 898 amino acids for RsbA) or amino acid sequences (17.13% identity, and 32.4% similarity). Another example is the AHL quorum sensing system. Despite of its prevalence in many other bacterial species, AHL seems not to play a dominant role in the regulation of swarming of *P. mirabilis* strains. We have used standard autoinducer purification techniques that include organic extractions of both LB and minimal growth medium grown media with dichloromethane, and have not been able to isolate an autoinducer activity from *P. mirabilis* cultures strains from different sources (Lai et al., data not shown). On the contrary, diversity of quorum-sensing systems is observed in *S. marcescens* strains, including the *smaRI* system identified in *S. marcescens* ATCC39006 ( ), and a *S. marcescens* SS-1 *spnRI* system that regulates SS-1 sliding via regulation of surfactant production ( ). At the same time, many *S. marcescens* strains including *S. marcescens* CH-1 did not produce detectable AHL signals, even after repeated tests. It was observed that only about 30% of the clinically isolated *S. marcescens* strains produced the detectable AHL signals (Lai et al., unpublished data), suggesting that there might be complex regulatory pathways regulating the swarming of *S. marcescens*. Another regulatory factor identified in *P. mirabilis* is the *cmfA* (colony migration factor), associated with the assembly of capsular polysaccharide CPS ( ). CmfA mutants generate closely spaced terraces during cyclic swarming behavior ( ). The loss of the Cmf CPS thus results in the

loss of a lubricant, with the indirect consequence of an increase in friction of the cellular mass as it moves over the surface; this increase in friction reduces overall translocation velocity and generates the tightly clustered consolidation zones observed. For regulation of *S. marcescens* swarming, the function of capsule is still undetermined, but production of surfactant (serrawettion) is shown to play an important role for stimulation of the process ( ).

Many physiological differences are also observed in the swarming physiology of these two bacteria. For example, *S. marcescens* CH-1 spreads well at minimal growth medium, where *P. mirabilis* does not ( ). Also, glycerol used for stimulation of EPS ( ) and biosurfactant ( ) production in *S. marcescens* inhibits *P. mirabilis*, but stimulates *S. marcescens* swarming. Initiation of *Serratia* swarming is additionally thermo-regulated, occurring at 25°C and 30°C but not at 37°C, while *P. mirabilis* swarms well at both temperatures. On the other hand, it is also interesting to see that *S. marcescens* CH-1 swarms at a constitutively increasing velocity at 30°C, and does not stop and consolidate during the swarming process, which is quite different from that of *P. mirabilis* that swarms and consolidates alternatively ( ). Differences are also observed between the RssA and RsbA swarming mutants. Although both are two-component sensor kinase mutants, *S. marcescens* CH-1 ΔA swarms at a constitutively increasing velocity and significantly much faster than its parent cells (**Fig.**), and *P. mirabilis rsbA* mutant swarms at a constant velocity once started to swarm (Belas et al., 1998). These facts may suggest that both bacterial species use different mechanisms for regulation of swarming.

What may be the molecular mechanism by which RssA functions? Besides flagellar motility, the precocious mutant also does not demonstrate major defects in CPS, either cellular bound or unbound form (Fig.). CPS has been identified to be a colony migration factor in *P. mirabilis* ( ). By appearance, precocious cells are neither rough nor mucoid in appearance. Also, complementation and overexpression of RssA do not produce overt changes in the colony that can be linked to changes in CPS. The *rssA* mutant also does not demonstrate defects in production of biosurfactant that is shown to be essential for the initiation of swarming in *S. liquefaciens* ( ) and sliding in *S. marcescens* ( ). Although the direct answer to this question is not known, RssA shows high homology to many other sensor kinase proteins or sensory proteins regulated by temperature. It is possible that the temperature regulation of *rssA* might be either at the promoter transcriptional level, like that reported in *bvgAS* system in *B. pertussis* ( ), or at the protein level, such as affecting the protein conformation and thus phosphorelay process during the signal transduction process. In the temperature regulation of DesK/DesR two-component system in *B. subtilis*, it is suggested that temperature shift does not affect the transcription level of *desKR*, but might affect the conformation change of DesK through a temperature-induced change in membrane fluidity (Aguilar et al., 2001). Another example, the CorS of *Pseudomonas syringae* (Ullrich et al., 1995), using CorS::phoA translation fusion as the assay system, evidence has suggested that the membrane topology and protein conformation of the CorS changes in accordance with the change of temperature, thus affecting the availability of histidine residue for phosphorylation and the subsequent phosphorelay process during signal transduction. The temperature effect was suggested to act through modulation of membrane fatty acid profile, leading to changes in membrane fluidity and the subsequent membrane protein conformational change (Ullrich MS, unpublished data). The phenomenon of membrane fluidity affected by environmental temperature is also shown in the

cyanobacterium *Synechocystis*. Low-temperature signals are shown to induce the desaturation of fatty acids in the cell membrane, thus changing the membrane fluidity (Suzuki et al., 2000). Although the direct answer to this question is not known, as a working model, we currently hypothesize that RssA may function in a manner similar to that of *B. subtilis* DesK ( ). Evidences inclues that an ORF with high amino acid identity to fatty acid synthesis, the FabG, was identified immediately upstream of *rssA/rssB*. FabG is a fatty acid dehydrogenase that is involved in elongation process during the fatty acid synthesis step in *E. coli* ( ), but/and is involved in synthesis of biosurfactant in the bacterium *P. aeruginosa* ( ). Another evidence is that no significant difference of surfactant production is observed between *S. marcescens* CH-1 ΔA and CH-1, while a significant difference in cellular fatty acid profiles was observed between the two strains. Also, CH-1 ΔA does show significant sensitivity to hypo-osmolarity (Fig.), and aberrant cellular morphology was observed under hypo-osmolarity stress from the transmission electron microscope pictures (Fig.), suggesting that might indeed be defective in the cell wall integrity. Whether expression of the *Serratia fabG* is regulated by RssA/RssB two-component system and that whether RssA/RssB may directly or indirectly be involved in the regulation of cell wall fluidity in a temperature-dependent way remains to be determined. We are in the process of characterizing the possible genetic determinants regulated by RssA/RssB two-component system and their role in the regulation of swarming behaviour in *S. marcescens* CH-1. Using PCR and primers designed from *rssA*, we have found that *rssA* is conserved in a total of **66** clinically isolated *S. marcescens* strains (66/66 tested) (Lai et al., unpublished data), suggesting that the *rssA* two component system may play an common role in the regulation of multicellular haviuours in *S. marcescens*.

More and more genes are reported to be essential for regulation of multicellular behaviour of *S. marcescens*. Genes required for flagellum synthesis and function have been associated with aspects of swarmer cell differentiation and swarming behavior in *S. marcescens*. For example, mutation in the *flhDC* central regulator of flagellum synthesis has been described that directly affect swarmer cell differentiation and produce swarming null mutants through direct effects on the function of the flagellum (Liu et al., 2000). An additional layer of regulation may also be exerted through RsmA (an *E. coli* csrA homologue and a repressor), which functions as a global regulator and which, when over-expressed, also produces a nonspreading colony (Ang et al., 2001). The regulatory genes *flhDC* in swarming null mutants affect swarming behavior or migration mostly as a consequence of their impact on flagellar synthesis. RsmA may inhibit the *Serratia* spreading through inhibition of quorum-sensing system, which is also reported in ********** ( ). For identifying other genes involved in the regulation process, we have focused our attention on a unique group of transposon mutants that we refer to as super swarming mutants. These behavioral mutants, originally described by us as "superswarmers" because they all swarmed earlier than the wild type at 30°C, and swarmed in a similar way at 37°C as well. From the basic physiological observation, our *S. marcescens* super-swarming mutants are not necessarily defective in cell elongation and swarmer cell differentiation; however, all mutants characterized swarmed faster than their parent CH-1 cells and migrated in a constitutively increasing velocity after the initiation of swarming. From these mutants, we have observed a common phenomenon that basically no significant differences in any factors reported to be essential for swarming process are affected,

further distinguishing this group of mutant as unique, and suggesting that some other unidentified factors are involved in the regulation of swarming in *S. marcescens*.