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Membrane fluidity optimization regulates swarming through modulation of a two-component signal transduction thermosensor in *Serratia marcescens*

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Abstract

Serratia marcescens swarming behavior is thermoregulated, which is characterized by continuous populational surface migration on rich swarming agar surface at 30°C, but not at 37°C. Here we report that environmental temperature shift and fatty acids (especially the saturated fatty acids, SFAs) act as the signal cues regulating *S. marcescens* swarming through an RssA-RssB two component signal transduction system. SFAs or temperature upshift specifically and negatively regulate *S. marcescens* swarming, but not swimming motility or the identified factors known to be involved in *Serratia* swarming, possibly through influencing pattern of cellular fatty acid profile. Fatty acid profile analysis strongly indicates that addition of SFAs at a constant temperature and temperature upshift may either directly or indirectly affect the signaling state of the RssA sensor protein by increasing the incorporation of the high-melting point fatty acids into membrane phospholipid, which subsequently inhibits *S. marcescens* swarming. The RssA-RssB two-component system mediates this response, as both partners are required to sense and transduce the signals. Expression of a fatty acid synthesis gene *fabG* and a hemolysin gene *shlA* is coordinately regulated with swarming by RssA-RssB. Biofilm formation, cell surface structure and extracellular polysaccharide are also affected in the *rssA* or *rssB* mutant. Similar phenomena of SFA inhibition are also observed in swarming regulation of *Proteus mirabilis* and *Salmonella typhimurium*. Thus, evidence suggests it may be common that membrane fluidity optimization through modulation of cellular fatty acid profile under different physiological culture conditions may subsequently affect two-component signaling pathway, which regulates the composition of cellular fatty acids, expression of virulence factor and multicellular swarming behaviours. This may provide an unidentified and common regulatory circuit for controlling swarming behaviour of not only *S. marcescens,* but also other swarming bacteria.

Introduction

More and more bacteria are identified 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). Bacterial species reported to show swarming behaviours include *Serratia* (Alberti and Harshey 1990; Givskov et al., 1998; Liu et al., 2000), *Proteus* (Mobley and Belas 1995), *Vibrio* (McCarter and Silverman, 1990), *Aeromonas* (Kirov et al., 2002), *Escherichia coli* (Harshey and Matsuyama 1994), *Salmonella enterica serovar typhimurium* (Harshey and Matsuyama 1994), *Pseudomonas* (Kohler et al., 2000; Kinscherf and Willis 2002; Sanchez-Contreras et al., 2002), *Burkholderia cepacia* (Huber et al., 2001), *Myxococcus xanthus* (Jelsbak and Sogaard-Andersen 2000), a spirochete *Treponema denticola* (Lux et al., 2002), a nitrogen-fixation bacterium *Sinorhizobium meliloti* (Soto et al., 2002), a purple photosynthetic bacterium *Rhodospirillum centenum* (McClain et al., 2002), *Bacillus* (Senesi et al., 2002; Dixit et al., 2002; Ghelardi et al., 2002), and also some absolute anaerobic bacteria such as *Clostridium* species (Macfarlane et al., 2001).

We are interested in unraveling the underlying mechanism of *S. marcescens* swarming. Previous works have shown that when inoculated on LB medium solidified with 0.8% agar (LB swarming plate) at 30°C, *S. marcescens* shows a swarming phenomenon where a colony of short motile vegetative rods differentiate at the colony margin into elongated, aseptate and hyperflagellate swarm cells which migrate rapidly and coordinately away from the colony (Alberti and Harshey 1990; Liu et al., 2000). Phenotypically, 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 *Serratia* is the result of a coordinated, multicellular effort of groups of differentiated swarmer cells functioning through close cell-cell interactions (Eberl et al., 1999). 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. 4-5 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 propagation and close interaction among bacterial cells and environment occur. *Serratia* swarming, unlike that of *Proteus* where swarming colonies exhibit regular concentric zones of consolidation and swarm at a higher agar concentration (2.0%) surface (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 (Givskov et al., 1998; Liu et al., 2000).

Development of a *Serratia* surface expansion 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 (Liu et al., 2000) and chemotaxis system (Burkart et al., 1998). Stimulation of *flhDC* operon initiates swarmer cell differentiation that involves development of characteristic traits such as cell elongation and multinucleation, and hyperflagellation (Eberl et al., 1996; Liu et al., 2000). The population density is recognized by a N-acyl-homoserine lactone(AHL)-dependent quorum-sensing system constituted by the *swrI* and *swrR* genes in *Serratia liquefaciens* (Eberl et al., 1996; Givskov et al., 1998) and *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 (Eberl et al., 1996; Horng et al., 2002). 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 operon and the quorum-sensing system are global regulators which control two separated regulons involved in swarming regulation (Givskov et al., 1998). An additional layer of regulation of surface migration may also be exerted through *S. marcescens* RsmA (an *E. coli* CsrA homologue and a repressor), which functions as a global regulator and which, when over-expressed, also produces a non-spreading colony (Ang et al., 2001). RsmA may inhibit the *Serratia* spreading through inhibition of quorum-sensing system, which is also reported in *P. aeruginosa* (Pessi et al., 2001).

Although previous works have unraveled many critical components involved in *Serratia* spp. swarming behaviour itself, many questions remain elusive. For example, it is not explained why swarming of *S. marcescens* is 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. Furthermore, the potential physiological signals providing critical stimuli for initiation of swarming and the signal transduction system are also not characterized. We observed that *S. marcescens* under the situation of artificial over-expression of *flhDC* in the presence of excess amount of biosurfactant still took 4-5 h before initiation of swarming at 30°C and still did not initiate swarming at 37°C(data not shown). This observation together with the fact that cell elongation and *flhDC* over-expression may be independent of solid surface contact in *S. liquefaciens* and *S. marcescens* (Tolker-Nielsen et al., 2000; Lai et al., 1997) suggest that initiation of *S. marcescens* swarming may not purely be dependent on accumulation of surfactant, *flhDC* over-expression and formation of

swarmer cells. In addition, even though the importance of AHL signals is clearly identified in many swarming/sliding bacteria including *Serratia spp.* (Givskov et al., 1998; Horng et al., 2002), we and other colleagues have found that many *S. marcescens* strains, especially strains that show normal swarming behaviour, do not contain detectable *spnRI*, *swrRI* or *smaRI* AHL quorum-sensing systems (G. Salmond, personal communication; HC Lai, unpublished data). Furthermore, study from another swarming bacterium *Salmonella typhimurium* (Toguchi et al., 2000) failing to identify any specific swarming signals such as amino acids, pH changes, oxygen, iron starvation, increased viscosity, flagellar rotation, or quorum-sensing autoinducers leads us to consider that there must be some uncharacterized regulatory pathway controlling the behaviour of bacterial swarming in response to the versatile environmental conditions.

In this communication, through mini-Tn5 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 characterization. We describe a gene that, when mutated, lead to defect in haemolysin production and biofilm formation, and showed 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 motility, AHL quorum-sensing system, and production of biosurfactant-- factors already characterized to be involved in the regulation of population surface migration behaviours in many bacterial species including *S. marcescens* (Horng et al., 2002; Liu et al., 2000) and *S. liquefaciens* (Lindum et al., 1998).

Here we present several lines of evidences to show that regulation of initiation of swarming is closely correlated to the homeoviscous acclimation of bacterial cell membrane, i.e., acclimatizing response of cell membrane lipid in response to different environmental conditions (Browse and Xin 2001). We present evidences that pattern of cellular fatty acid profile is affected by environmental temperature and also saturated fatty acids (SFAs) through a common mechanism. To swarm or not-to-swarm is determined by pattern of fatty acid profile which acts through a two-component signal transduction system. In addition, we demonstrate that expression of $fabG_{Sm}$, a NADPH-dependent 3-ketoacyl-ACP reductase, haemolysin production and composition of cellular fatty acid was further regulated by this two-component signal system. Evidence also suggested that cell surface topology and

extracellular polysaccharide (EPS) production which originally functions as a lubricant during swarming of many bacteria including *S. typhimurium* (Mireles et al., 2001) and *P. mirabilis* (Gygi et al., 1995) might also play a role in the regulation of *Serratia* swarming. A similar SFA effect on the swarming of *P. mirabilis* and *S. typhimurium* was also observed, suggesting that this regulation is a common phenomenon. Thus, a regulatory loop composed of the nutrients condition (especially the SFAs), environmental temperature, patterns of fatty acid profile, the two-component signal transduction system, fatty acid synthesis gene and cellular fatty acid synthesis, haemolysin production and amount of EPS produced provides a novel mechanism for the control of swarming behaviour in *S. marcescens*.

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)(Figure 1A) 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 (Figure 1A).

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 at 37°C on modified LB swarming 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* WC100 was selected for further characterization. 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 with 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 Figure1. 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. Depending on the wetness of plates, CH-1 cells typically spent 4-5 h (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 (Figure1C). The average velocity of *S. marcescens* CH-1 swarming increased from 1 mm/h (between 5 to 6 h) to 3.3 mm/h (between 7 to 8 h), while *S. marcescens* WC100 increased from 2 mm/h to 5 mm/h at the same period (Figure1C). 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. *S. marcescens* WC100 was further observed to swarm even if the agar concentration was increased up to 1% at 30°C, where CH-1 did not (Figure1B).

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 (Figure 1A). WC100 still swarmed well and behaved similarly to that at 30°C in the time of swarming initiation, average swarming velocity (Figure 1C), and the ability of swarming at 1% agar concentration (data not shown), 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 during growth on plates, WC100 cells were about 10% shorter under 1000x microscopy and produced less prodigiosin than 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 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 3003-bp DNA fragment of the *S. marcescens* CH-1 genome (Figure 2). Sequencing revealed that mini-Tn5 had inserted in an 1179bp open reading frame (named ORF1), potentially encoding a 393-amino-acid (43.94 KDa) polypeptide with a calculated isoelectric point of 5.45. 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 109 residues (Figure 2A).

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 databanks indicate that Orf1 is homologous to elements in the two-component sensor family, including *Vibrio harveyi* LuxQ (22% identity, 41% similarity), *E. coli* EvgS (22% identity, 41% similarity) and *E. coli* RcsC (19% identity, 39% similarity). Orf2 is also homologous to two-component response-regulator elements, including a 38 % identity and 58% similarity throughout the alignment to a putative two-component response regulator of

Ralstonia solanacearum (Salanoubat et al., 2002). Partial ORF3 shows high homology to members of the short-chain dehydrogenase/reductase family, including 62% identity out of the 109 N-terminal amino acids to YgfF of *E. coli* (Blattner et al., 1997). We have chosen to call the mutated gene (*orf1*) *rssA*, for regulation of *Serratia* swarming. *orf2* was named *rssB*, and *orf3* named $fabG_{Sm}$. The nucleotide sequence of 3003-bp encoding the three ORFs (*rssA*, *rssB* and the 5' portion of $fabG_{Sm}$) has been submitted to the DDBJ/EMBL/GenBank databases under accession no. AF465237.

Analysis of the deduced amino acid sequence of RssA, together with the domain search by MotifScan (Falquet et al., 2002) and hydrophobicity characteristic analysis by DAS (Cserzo et al., 1997) (Figure 2B), TMpred (Hofmann and Stoffel 1993), SOSUI (Mitaku and Hirokawa 1999) and PredictProtein (Columbia University, USA) from the ExPASy proteomics tools internet homepage (http://tw.expasy.org) indentified a hydrophobic region between P90 and L112**,** which indicates that it may form a membrane helix and localized in the inner membrane as a transmembrane protein (Figure 2C). The N-terminal sequence of RssA was predicted to stretch into the periplasm and was not predicted to contain signal peptide (Figure 2C). A HAMP domain which plays an active role in transmembrane signal transduction (Appleman and Stewart 2003) was identified between 90(D) and 158(A), a phosphoaccepter domain was found between 162(R) and 227(E) near the middle of the protein in the cytoplasm, and an ATPase domain was observed between 272(T) and 382(S) near the C-terminal (Figure 2C). RssA has strong motif similarity to many other sensory proteins that are members of the two-component family of proteins (Hoch and Silhavy 1995). Specifically, as shown in Figure 2D, domains, such as the H, N, D/F and G 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 *S. marcescens* WC100 mutant phenotype was not due to artifact effect from introducing a copy of mini-Tn5 transposon, *rssA* in CH-1 was further mutated by insertion deletion through homologous recombination to form *S. marcescens* CH-1ΔA. The phenotypes of both *S. marcescens* WC100 and CH-1ΔA 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-1ΔA. Plasmid pWC200(pACYC184::*rssA*) was further transformed into both WC100 and CH-1ΔA 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-1ΔA was selected for further study. The effect of over-expression of *rssA* on swarmer cell differentiation and behavior was examined

by transforming pWC200 into *S. marcescens* CH-1. When *rssA* is overexpressed from a multicopy pWC200 at 30°C, it delayed the initiation of CH-1 swarming for about 1 h, and also reduced its swarming velocity for about 40%. Plasmid-only controls have no significant effect on the time of swarming (data not shown). 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 senses populational density and temperature for controlling initiation of swarming

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. One possible role for RssA in the regulation of initiation of swarming could be as a sensor of population cell-density, which, when defective, leads to super-swarming behaviour. If RssA senses the population density or degree of cell-to-cell contact, defect in RssA should alter the ability of 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 CH-1ΔA swarming behavior was still correlated with population density at 30°C. As similar to many other enterobacteria (Fraser and Hughes 1999), 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. In these experiments, the initial inoculum's concentration of CH-1ΔA(pPC300) and also the control strain *S. marcescens* CH-1(pPC300) was varied in 10-fold increments from 1×10^6 to 1×10^2 cells (CFU) delivered in 5-µl-aliquot droplets to the LB swarming agar surface. The light emission of swarming bacteria which indicates the promoter activity of $flhDC_{Sm}$ master operon (Liu et al., 2000) was monitored hourly by X-ray film exposure following the growth of bacterial colonies on LB swarming plates and CH-1ΔA(pPC300) swarming activity was shown in figure 3A. The number of each bacterial colony expressed as colony forming units (CFUs) was at the same time calculated by 10 times serial dilution and plate counts. Similar to CH-1(pPC300), at the earlier stage of CH-1ΔA(pPC300) culture where no swarming was initiated, light emission could be detected from the whole colony. Once the cells started to swarm, light emission could only be detected at the swarming edge of the colony, where elongated cells are actively migrating outwards (Figure 3). Thus the light emission pattern reflected the real time colonial

swarming behavior. Although initiation of CH-1ΔA(pPC300) swarming is still density dependent, it started to swarm earlier than CH-1(pPC300) cells at a much lower cell density. CH-1ΔA(pPC300) only requires ca. 50-fold fewer cells to commence migration (3.4×10^6 versus 1.7×10^8 cells to initiate swarming) (Figure 3B). Further experiments showed that CH-1ΔA(pPC300) also behaved similarly in a cell-density dependent swarming phenomenon at 37°C (data not shown). In summary, although initiation of *S. marcescens* CH-1ΔA swarming 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. The phenomenon that the *rssA* mutant swarming behaviour was not affected by temperature-upshift (from 30 to 37°C) further suggested that RssA also acts as a temperature-dependent sensor regulating the swarming behaviour of *S. marcescen*s CH-1.

Swimming motility and biosurfactant production are not affected in *rssA* mutant

Factors already identified to be essential for or involved in the regulation of swarming process in *Serratia* (Liu et al., 2000; Horng et al., 2002; Givskov et al., 1998; Harshey 1994) were examined to see whether expression of any of these factors were aberrant, leading to the super-swarming behaviour in *S. marcescens* CH-1ΔA. These include the swimming motility, amount of flagellum produced and production of surfactant.

We first checked whether the swimming motility was significantly activated in CH-1ΔA. Swimming assay and monitoring of average swimming velocity using CH-1 and CH-1ΔA cells at both 30 and 37°C were performed. The result in Figure 4A and 4B shows that CH-1 and CH-1ΔA swimmed in a similar scale at both 30 and 37°C. Therefore, this mutation does not appear to affect flagellar rotation and motility. Further monitor of $flhDC_{Sm}$ promoter activity by pPC300 (Figure 4C) and SDS-PAGE analysis for quantification of flagellum production (data not shown) also failed to show difference between CH-1 and CH-1ΔA at 37°C, suggesting that the $flhDC_{Sm}$ regulon is not affected by *rssA* mutation.

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 performed to qualitatively assay the surface tension of stationary phase culture suspensions from CH-1ΔA and CH-1 cells at both temperatures. Again, even after repeated tests, no difference was observed (Figure 4D). For confirmation of the assay, TLC (Thin Layer Chromatography) (Horng et al., 2002) was performed to quantify the biosurfactant production to see whether there is a minor difference between the two strains. Still, no difference was observed (Figure 4E), suggesting that biosurfactant production is not affected in

CH-1ΔA.

As CH-1ΔA was screened from modified LB agar medium that contains a lower NaCl concentration [0.04% (w/v)] compared with the normal LB agar salt concentration [1% (w/v)], together with the fact that the predicted gene products of almost all transposon–disrupted mutant genes from the screened super-swarming mutants are closely associated with cell envelope components (data not shown), we then hypothesized that cell envelope integrity might be less compact in the *rssA* mutant. To see whether this is possible, we grew CH-1 and CH-1ΔA for overnight in normal LB broth media before transferring the bacterial cells into normal [1% NaCl], high [1.5% NaCl] and low [0.04% NaCl]-osmolarity LB broth media at both 30 and 37°C and monitored their growth dynamics by measuring the optical density (A_{600nm}). *S. marcescens* CH-1ΔA was shown to grow with a generation time similar to that of the CH-1 cells under all the conditions assayed (data not shown). This result suggests that the RssA was not involved in osmolarity regulation in CH-1. Taken together, 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 phenotype.

Biofilm formation and haemolysin production are regulated by RssA in a temperature-dependent way

Previous works have shown that biofilm formation is closely related to swarming in many bacterial species including *Salmonella typhimurium* (Mireles et al., 2001), *P. aeruginosa* (Deziel et al., 2001) and *B. cepacia* (Huber et al., 2001). We reasoned that biofilm formation might also be aberrant in the CH-1ΔA super-swarming mutant. To validate this, 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 of CH-1 and CH-1ΔA at both 30 and 37°C. We found that biofilm-forming ability of CH-1 was higher at 30°C(Figure 5A), with an average absorbance value of 0.34 was detected, and was reduced at 37°C(Figure 5A), where an average value of optical density at 0.28 was detected (Figure 5B). Biofilm formation for CH-1ΔA is significantly defective (figure 5A), reaching only about 0.20 (59% of CH-1 level) at 30°C and 0.12 (43% of the CH-1 level) at 37°C (Figure 5B). The data suggest that biofilm-forming ability of *S. marcescens* CH-1 is altered under different environmental temperatures; with 30°C being more proficient than 37°C, and CH-1ΔA is less proficient in binding to the microtiter plate surface at both temperatures, confirming that biofilm formation is related to swarming behaviour under the control of RssA.

The defect in biofilm formation suggested cell alignment within group rafts

during swarming of CH-1ΔA may be aberrant compared with CH-1. To confirm this, swarming assays were performed at 30 and 37°C for CH-1 and CH-1ΔA 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. Compared with CH-1ΔA, CH-1 did not swarm at 37°C and the cells looked significantly non-active near the colonial edge, where the way of cell alignment was similar to the sliding edge of *S. marcescens* SS-1 (Horng et al., 2002). Similar cell alignment and motility were observed near the swarming edge of CH-1 and CH-1ΔA at 30°C (data not shown). No other significant differences were observed between the two strains, including colonial texture organization (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 400x phase contrast microscopy (data not shown).

The cell-surface associated haemolysin is identified to be a dominant virulence factor in *S. marcescens* (Kurz et al., 2003). Previous works have shown that expression of some virulence factors including haemolysin is closely coupled to swarming in *P. mirabilis* (Fraser et al., 2002). It is also shown that production of hemolysin is under the control of bacterial two-component systems in *Bordetella pertussis* (Stibitz 1994) and *Staphylococcus aureus* (Fournier et al., 2001). We decided to determine whether production of haemolysin was affected in CH-1ΔA. To this end, haemolysin activity was assayed in CH-1 and CH-1ΔA. Equivalent number of CH-1 and CH-1ΔA cells were harvested from LB seeding agar plates at 30 and 37°C at an hourly interval following the growth. It was clearly observed that the haemolysin activity of CH-1ΔA was significantly higher than that of CH-1 at both 30 (320 %) and 37°C (250%) (Figure 5C). To see whether the regulation occurs at the transcriptional level, a recombinant plasmid, pSA400 (P*shlA*::*luxCDABE*) was constructed as a reporter for the promoter activity of P*shlA*. A comparison of the light emission patterns from CH-1ΔA(pSA400) and CH-1(pSA400) showed an average 4 to 8 fold increase in *shlA* promoter activity in CH-1ΔA(Figure 5D), indicating the negative regulation effect of RssA on the promoter activity of *shlA*. Together, these data indicate that in *S. marcescens* CH-1, a complicated two-component regulatory network existing in *S. marcescens*, in which biofilm formation and haemolysin virulence factor gene, together with some uncharacterized swarming-related genes are components.

S. marcescens stops swarming in the presence of saturated fatty acids 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 MGM plate (M9 minimal growth medium containing 0.8% Eiken agar) and MGM plate containing casamino acids(1%), not only at 30, but also 37°C (Figure 6A), suggesting that higher environmental temperature is not a must condition for inhibition of *S. marcescens* CH-1 swarming. We further observed that CH-1 started to swarm almost immediately (within 1 hour, at a much lower colonial cell density) after it was transferred from saturated LB broth culture to MGM plate or MGM-casamino acids (1%) plate at both temperatures. This observation is striking when compared with the cell-density dependent CH-1 swarming behaviour on LB swarming plate, indicating that absence of some contents in the minimal media lead to the immediate CH-1 swarming. Further swarming assays on MGM-casamino acids (1%)/yeast extract (1%) plate showed that while CH-1ΔA swarmed, CH-1 swarming was completely inhibited at 37°C (Figure 6A). These observations suggested that some components (or the metabolic molecules derived from the components) contained in yeast extract inhibited CH-1 swarming at 37°C, and existence of the component(s) did not inhibit CH-1ΔA swarming. We hypothesized that some amino acids contents in excess might inhibit the CH-1 swarming at 37°C. Using MGM-casamino acids (1%) swarming plate as the basal medium, the 20 single amino acids including the branch chain amino acids isoleucine, leucine and valine, and glutamine, asparagines, histidine, lysine, phenylanaline, threonine, tryptophan, valine, tyrosine, serine, proline, glycine, glutamine, glutamate, cysteine, aspartate and alanine at the final concentration of 1% was separately incorporated, followed by swarming assay at 30 and 37°C. Comparatively, a similar swarming pattern was observed on the swarming scale between CH-1 and CH-1ΔA 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 inhibition of swarming of CH-1. While changes of other culture conditions including the concentration of glucose, addition of other mono- or di-saccharides, iron starvation, medium pH…etc. did not lead to change of CH-1 swarming behaviour (data not shown), we continued to determine whether exogenous saturated or unsaturated fatty acids with different chain length have some effect on the CH-1 swarming. Saturated fatty acids (SFAs) such as lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0) and stearic acid (C18:0) and unsaturated fatty acids (UFAs) such as myristoleic acid (14:1 Δ9), palmitoleic acid (16:1 Δ9) and oleic acid (C18:1 Δ9) at a final concentration of 0.01% (w/v) were added separately into MGM-casamino acids (1%) plates followed by swarming assays for CH-1 and CH-1ΔA at 37°C. We have found that while there were not so significantly different swarming-inhibition effect

from the UFAs tested, *S. marcescens* CH-1 swarming was completely inhibited by addition of lauric acid and myristic acid, and slightly inhibited by the presence of palmitic acid and stearic acid (Figure 6A). All fatty acids tested did not show significant inhibitory effect on CH-1ΔA swarming (Figure 6A). To further confirm whether this regulatory phenomenon was dose-dependent, myristic acid at the concentration of 0.01%, 0.005%, 0.0025% and 0.00125% (w/v) was added into MGM-casamino acids plates, followed by swarming assays at 37°C. The experiments showed that the time spent before initiation of swarming of CH-1 was delayed as the concentration of myristic acid was increased and that swarming was completely inhibited by myristic acid at the concentration of 0.01% (w/v) (Figure 6B). A similar myristic acid inhibitory effect was also observed at 30°C on LB swarming plates or MGM-casamino acids plates, and lauric acid also showed a similar inhibitory trend (Figure 6A). Taken together, these data indicate that, in addition to temperature shift, initiation of swarming is regulated by the availability of SFAs in a concentration-dependent manner under a constant temperature.

Swarming behaviours are closely related to "swarming" and "non-swarming" cellular fatty acid profiles

The findings that SFAs inhibit CH-1 swarming and *fabG*$_{Sm}$ identified upstream of *rssA* and *rssB* was predicted to encode a protein with high amino acid identity to members of short-chain dehydrogenase/reductase family, which basically were NADPH-dependent 3-ketoacyl-ACP-reductases involved in the fatty acid synthesis during the elongation step (Magnuson et al., 1993), together with the fact that the membrane fluidity determined by composition of membrane fatty acids is optimized by a DesK-DesR two component pathway in *B. subtilis* (Aguilar et al., 2001), we reasoned that the cellular fatty acid profile of CH-1 might be affected by environmental nutrients contained in the culture media, which may then affect the conformation of RssA and subsequently regulate the signal transduction of RssA-RssB two-component pair, leading to either swarming or non-swarming phenotypes. To this end, cellular fatty acid profile expressed as percentage of total fatty acids of CH-1 cultured at 37°C on MGM plates, MGM casamino acids(1%) plates, MGM-peptone(1%) plates (swarming initiated), LB plates, MGM-casamino acids(1%)/yeast extracts (1%) plates, and MGM-peptone(1%)/yeast extracts (1%) plates (swarming inhibited) was determined by fatty acid extraction followed by fatty acid analysis by MIDI gas chromatography system. The major changes observed in fatty acid profile from cells grown on non-swarming conditions were the significant increase in the composition of lauric acid (12:0) and then myristic acid (14:0) and palmitic acid (16:00) (Figure 6C; Table 1). For cells grown in the swarming condition,

a concomitant increase in unsaturated fatty acids (especially 18:1 w7c) and branch-chain fatty acids (especially 15:0 anteiso) were observed (Figure 6C; Table 1). The results highlight the importance of exogenous fatty acids influcing the composition of cellular fatty acids and swarming behaviour at a constant temperature in *S. marcescens*.

The changes in fatty acid profile under different nutrient conditions at 37°C that coincide with the swarming or non-swarming behaviour of CH-1 reinforced our hypothesis of a direct correlation between fatty acid profile and swarming regulation. To this end, we collected CH-1 swarming cells and non-swarming cells cultured under different growth conditions for fatty acid profile analysis. CH-1 swarming cells were collected from LB swarming plates (30°C), MGM-casamino acids (1%)/yeast extract(1%) plates (30°C), MGM-casamino acids(1%)-myristoleic acids (0.01%) (30°C), MGM-casamino acids(1%)-oleic acids (0.01%)(30°C), MGM-casamino acids(1%)-palmitoleic acids (0.01%)(30°C), MGM plates and MGM-casamino acids (1%) plates (30 and 37°C). For CH-1 non-swarming cells, cells were collected from MGM-casamino acids(1%)-lauric acid (0.01%) plate (30°C), MGM-casamino acids(1%)-myristic acid (0.01%) plates(30°C), MGM-casamino acids(1%)-palmitic acid (0.01%) plates(30°C), LB swarming plates(37°C), and MGM- casamino acids(1%)/myristic acid(0.01%) plates(37°C). The results were summarized in Table 1. We found a significant trend that for CH-1 cells growing at non-swarming conditions including either higher incubation temperature or nutrients containing SFAs, the ratio of high melting point fatty acids to low melting point fatty acids (12:0+14:0+16:0+18:0/10:0 3OH+15:0 anteiso+18:1 w7c) is much higher than that from cells growing at swarming conditions (Table 1), with the percentage of 12:0 showing the most significant difference **(Figure 6C)**. On the contrast, CH-1 cells growing at swarming conditions, including lower incubation temperature or media that do not contain SFAs, contain higher percentage of UFAs and branch-chain fatty acids, with 18:1 w7c and 15:0 anteiso contents showing the most significant difference **(Figure 6C)**.

The fatty acid profiles of the two super-swarming mutants CH-1ΔA and CH-1ΔB (see later in the text) inoculated at either on LB swarming plates (30 or 37°C), MGM, MGM-casamino acids, or MGM-casamino acids-myristic acid(0.01%) (37°C), similar to some other super-swarming mutants including no. 3, 6, and O3 on LB swarming plates at 30 or 37°C, also contained a similar swarming fatty acid profile (Table 1). In conclusion, we have presented a clear evidence that *S. marcescens* CH-1 cellular fatty acid profile is affected by environmental temperature and fatty acids, and that the cellular fatty acid profile is closely related to swarming phenotypes, where swarming and non-swarming cells show a different pattern, with swarming cells containing

relatively more UFAs and branch-chain fatty acids and non-swarming cells containing more SFAs. We classify the two fatty acid patterns into “swarming” and “non-swarming” profiles for subsequent studies.

Expression of *fabGsm* is activated by myristic acid

Effect of myristic acid on *fabG*$_{Sm}$ promoter activity was evaluated. A recombinant plasmid pSA401 (P*fabG*$_{Sm}$::*luxCDABE*) in which the *fabG*$_{Sm}$ promoter ligated in front of the *luxCDABE* reporter was constructed. pSA401 was electroporated into CH-1 followed by monitoring the bioluminescent activity in LB broth culture with and without 0.01% (w/v) myristic acid at 30°C. While grown in LB broth, *fabG*$_{Sm}$ promoter activity in CH-1 reached its peak soon at about 30 minutes while cells were still growing at lag phase after it was transferred from LB over-night broth culture into fresh LB broth containing myristic acid 0.01% (w/v), decreased rapidly thereafter as cells are growing into log phase and maintained at a very low level as cells were grown into stationary phase (Figure 7A). Addition of myristic acid activates *fabG*$_{Sm}$ promoter activity up to 2-fold, followed by a similar decrease pattern as cultured in LB broth (Figure 7A). The data suggested that *fabG*$_{Sm}$ promoter responds very sensitively to myristic acid in LB medium.

RssA and RssB down-regulate *fabG*$_{Sm}$ expression

To see whether *fabG*$_{Sm}$ expression was regulated by RssA, a recombinant plasmid pSA401 (P*fabG*$_{Sm}$::*luxCDABE*) in which the *fabG*$_{Sm}$ promoter ligated in front of the *luxCDABE* reporter was constructed. pSA401 was electroporated into CH-1 and CH-1ΔA followed by monitoring the bioluminescent activity in LB broth culture containing 0.01% (w/v) myristic acid at 30°C. Compared with CH-1 *fabG*$_{Sm}$ promoter activity which decreased rapidly, *fabG*$_{Sm}$ promoter in CH-1ΔA was maintained at a much higher level for longer time for 2 hours followed by slower reduction (figure 7A). Northern blot hybridization using partial *fabG*$_{Sm}$ as the probe further confirmed this phenomenon (Figure 7B). Briefly, a clear trend was observed that when growing in a culture condition containing myristic acid, the *fabG*$_{Sm}$ promoter activity in CH-1 was activated and was rapidly down-regulated. Such an effect was not observed when *rssA* was mutated, suggesting that expression of *fabG*$_{Sm}$ was inhibited by RssA in response to effect of myristic acid.

We hypothesized that the reason why CH-1ΔA swarmed under all the culture conditions tested may be due to blockage of phosphorelay transfer to the transcriptional regulator RssB, albeit the fatty acid profile varied under different culture conditions. To test whether *rssB* was involved in regulation of swarming behaviour and *fabG*$_{Sm}$ expression, we constructed a strain *S. marcescens* CH-1ΔB, which contains a streptomycin resistance gene (Sm^{R}) cassette interrupting the *rssB*

gene. After confirmation of the mutated *rssB* genotype by Southern blot hybridization (data not shown), CH-1ΔB was subject to swimming assay and the swimming velocity and *flhDC*$_{Sm}$ promoter activity following the growth in LB broth culture were measured. While there is no significant difference observed compared with *S. marcescens* CH-1(Figure 4B; 4C), CH-1ΔB was further assayed for surfactant production, where still no difference was observed compared with that of CH-1 (data not shown). CH-1ΔB was tested for swarming motiltiy under different physiological culture conditions including LB swarming plates, MGM plates, MGM-casamino acid(1%) plates, MGM-gelatin(1%) plates, MGM-gelatin(1%)/yeast extract(1%) plates, and MGM-casamino acids plates containing a series of 0.01% (w/v) fatty acids (C12, C14, C16, C18, C14:1 Δ9, C16:1 Δ9, and C18:1 Δ9) at both 30 and 37°C. CH-1ΔB showed an unique "super-swarming" phenotype under all conditions tested, i.e., similar to CH-1ΔA, CH-1ΔB swarmed on all plates tested at both temperatures. CH-1ΔB swarming behaviour on LB swarming plates was shown in Figure 1A. Although CH-1ΔB only showed slight decrease in biofilm formation (Figure 5A), it was observed to swarm much faster than CH-1 (when swarmed) but slower than CH-1ΔA. The result of comparison of average swarming velocity among CH-1, CH-1ΔA and CH-1ΔB on LB swarming plates was shown in Figure 1C. On 1% LB swarming plates at 30°C, CH-1ΔB migrated farther than CH-1, but less than CH-1ΔA within a given time of 9 hr incubation (Figure 1B). The data suggest that RssB may act as a negative regulator involved in the regulation of CH-1 swarming.

Expression of *fabG*$_{Sm}$ may also be regulated RssB. To confirm this, myristic acid at the concentration of 0.01% (w/v) were added into LB broth and the intensity of light emission from *S. marcescens* CH-1(pSA401) and CH-1ΔB(pSA401) was monitored following the growth at 30°C. Similar to CH-1ΔA, effect of myristic acid on the *fabG*$_{Sm}$ expression was aborted when *rssB* was mutated (Figure 7A). Northern blot analysis using partial *fabG*$_{Sm}$ as the probe further confirmed the observation (Figure 7B). In conclusion, we have shown a strong evidence that RssA and RssB are both involved in a signal transduction regulatory pathway negatively regulating swarming and *fabG*$_{Sm}$ expression in response to environmental nutrients, especially SFAs.

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. While we reasoned that phosphorylated RssB may act as an negative regulator, an over-expression of *rssB* without the assistance of RssA might rapidly down-regulate transcription of *fabG*$_{Sm}$ and inhibit CH-1ΔA

swarming at 30°C, pSA402(pBCSK::*rssB*) was transformed into *S. marcescens* CH-1ΔA followed by swarming assay and monitoring the expression of $fabG_{Sm}$. Initiation of swarming in CH-1ΔA(pSA402) was significantly delayed for about 2h compared with CH-1ΔA containing control plasmid (data not shown). Further experiments using pSA401 as the reporter for $fabG_{Sm}$ expression showed that $fabG_{Sm}$ promoter activity was decreased up to 2-fold when *rssB* was over-expressed. These experiments demonstrate that high production of RssB down-regulates $fabG_{Sm}$ expression and inhibits swarming without the assistance of RssA at 30°C. This result therefore agrees with the observation that unphosphorylated response regulators can still regulates transcription when they are overexpressed (Powell and Kado, 1990).

RssA-RssB regulate cellular fatty acid composition

As expression of $fabG_{Sm}$ was regulated by RssA-RssB in response to different environmental culture conditions, and $FabG_{Sm}$ was predicted to be involved in the cellular fatty acid synthesis, we reasoned that pattern of cellular fatty acid profile is regulated by the RssA-RssB two-component system. The fatty acid composition of CH-1, CH-1ΔA and CH-1ΔB grown on LB plates at 30 and 37°C was analysed by gas chromatography and results were shown in Table 1. Although CH-1, CH-1ΔA and CH-1ΔB all show a swarming fatty acid profile at 30°C, the fatty acid composition of these 3 strains was different from each other (Table 1). The fatty acid profile of CH-1 cultured at 37°C, which showed a typical non-swarming profile, is also significantly different from those of CH-1ΔA and CH-1ΔB at 37°C, which showed a swarming profile (Table 1). Further experiments analyzing the fatty acid profile of the three strains also showed vary from each other when cultured on MGM media at 37°C (Table 1). These data indicate that in accordance with swarming, cellular fatty acid profile is regulated by RssA-RssB two component system.

3-oxo-C12 AHL signal inhibits swarming and modulates fatty acid profile

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 *spnTIR* genes of *S. marcescens* SS-1 (Horng et al., 2002).

Previously we have found that long-chain AHLs have inhibitory effect on the sliding behaviour of *S. marcescens* SS-1 (Horng et al., 2002). Although *S. marcescens* CH-1 did not produce detectable AHLs, we hypothesized that long chain AHL signals produced from other sources may inhibit CH-1 swarming. To confirm this, CH-1 was subject to swarming assay on LB swarming plates containing a range of synthetic AHLs incorporated into the medium at the final concentration of 10 μM at 30°C. Figure 8A 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, and especially 3-oxo-C12 and 3-oxo-C14 inhibit the CH-1 swarming most significantly, and addition of these signals did not significantly inhibit the swarming of CH-1ΔA (data not shown), suggesting that the inhibitory effect of the long chain AHLs might act through change of membrane fatty acid profile and RssA-RssB signaling system. To see whether this is possible, the long chain AHL signal 3-oxo-C12 at the concentration of 10 μM was incorporated into the LB swarming media followed by analyzing fatty acid profiles of CH-1 and CH-1ΔA that are seeding-plate cultured at 30°C. Results in Table 1 showed that a clear difference was observed on the fatty acid profile between the two strains, which was concordant with swarming behaviours. While CH-1 showed a swarming fatty acid profile in the absence, and a non-swarming fatty acid profile in the presence of 3-oxo-C12 at 30°C, CH-1ΔA showed a similar swarming fatty acid profile irrespective the presence of 3-oxo-C12. This observation suggests that although CH-1 does not bear AHL quorum sensing system, 3-oxo-C12 does regulate CH-1 swarming and such a regulation might act through affecting the cellular fatty acid profile.

We continue to see whether expression of $fabG_{Sm}$ was affected by 3-oxo-C12. The light emission assay using pSA401 as a reporter was performed in LB broth culture containing 10 μM 3-oxo-C12 at 30°C. $fabG_{Sm}$ promoter activity in CH-1 was only slightly affected by the presence of 3-oxo-C12 (Figure 8B; C). Compared with CH-1, CH-1ΔA and CH-1ΔB, we also did not observe a significant effect of 3-oxo-C12 on $fabG_{Sm}$ promoter activity in mutant strains (Figure 8B; C). A similar result was obtained using pSA400 as a reporter for monitoring the effect of 3-oxo-C12 on *shlA* expression (data not shown). In brief, although 3-oxo-C12 does inhibit CH-1 swarming and affect cellular fatty acid profile, it seems not to regulate $fabG_{Sm}$ and *shlA* through RssA-RssB signaling pathway.

RssA and RssB are involved in determination of cellular surface structure

The detailed cellular surface topography of CH-1, CH-1ΔA and CH-1ΔB growing in the log phase was studied using atomic force microscopy (AFM) by systematically zooming in on target cells and regions within the cell envelope. A larger scan area (3 x 3 μm^2) was first made in order to select the desired cells for higher-resolution images. For each CH-1, CH-1ΔA and CH-1ΔB strain, over twenty individual vegetative cells were examined. Although some variations exist in the calculation of exact cellular size, a clear trend was observed that on an average, the calculated RMS values of length and width of CH-1, CH-1ΔA and CH-1ΔB were 1981 x 734 nm, 1518 x 555 nm and 1644 x 680 nm, respectively, confirming our previous observation that average cell length and width of both CH-1ΔA and CH-1ΔB was shorter than that of CH-1 cells under 1000x light microscopy. The cellular surface topography of both CH-1ΔA and CH-1ΔB changed when cultured in LB broth at 37°C (Figure 9A), and characterization of these strains were shown in Figure 9B. CH-1 showed a mean valey-to-peak distance (Rmean) of 11.900nm, with a maximal value (Rmax) of 25.900nM, a surface roughness (Ra) of 2.652nm, and a root-mean-square (Rq) 3.344nm. CH-1ΔA and CH-1ΔB showed an Rmean of 9.075 and 7.775nm, an Rmax of 17.700 and 14.675nm, an Ra of 1.999 and 1.545 nm, and an Rq of 2.240nm and 1.948nm, respectively. These data showed that Rssa and RssB are either directly or indirectly involved in determination of *S. marcescens* surface topology.

Evidence suggests that extracellular polysaccharide is reduced in *rssA* mutant

As we used modified LB swarming plates which contained glycerol for selecting the super-swarming mutants, and glycerol was reported to stimulate extracellular polysaccharide (EPS) production in *S. marcescens* strains (Aucken et al., 1997), together with the fact that although not yet been identified in *S. marcescens*, EPS is shown to play an important role in stimulating swarming in many other bacterial species: (i) a colony migration factor for *P. mirabilis* swarming (Gygi et al., 1995); (ii) involved in *E. coli* swarming under the control of a two-component signaling system (Takeda et al., 2001), and (iii) important for *V. parahaemolyticus* swarming by a functionally uncharacterized three-gene-operon *scrABC* (Boles and MaCarter, 2002), we reasoned that production of EPS may be altered in CH-1ΔA or CH-1ΔB, leading to the super swarming phenotype. Measurement of EPS production (both the bound form and unbound form) (von Bodman et al., 1998) was performed under the bacterial growth conditions of CPG (Dolph et al., 1988) or LB broth, and seeding plate cultures. Unexpectedly but consistently from all culture conditions tested, while there was no significant difference in the amount of bound-form EPS produced among the 3 strains, we found that CH-1ΔA produced less (about 40%) un-bound form EPS than CH-1 and CH-1ΔB at 3 hr after inoculation at both temperatures, and the results from LB

seeding plates at 30°C were shown in Figure 10, suggesting that production of EPS is, instead of increased, reduced in CH-1ΔA and CH-1ΔB.

rssA-rssB are conserved among _Serratia marcescens_

To see whether *rssA* and *rssB* was conserved among *S. marcescens* strains, a total of 67 *S. marcescens* strains collected from National Taiwan University Hospital, one strain (*S. marcescens* NewCDC) isolated from Germany, and three strains (*S. marcescens* 1324E, S1220 and 4444) isolated from U.K. were subject to PCR amplification using the primer pairs (5'CCATCATCGTCACCTTGCTGTTTACC3'/GAGCGACAGTTCCACATCCTTTTCCA3')and(5'TGCTGGATCTCACGCTGCCG3'/5'CCGGTTGACAGCCTTGACGC3') designed from within the open reading frame region of *rssA* and *rssB* for amplifying *rssA* and *rssB*, respectively. We have detected both *rssA* and *rssB* DNA fragments from 68 out of the 71 *S. marcescens* strains tested (data not shown), suggesting that the *rssA-rssB* gene pair was conserved among *S. marcescens* strains.

Long chain SFAs regulate swarming of _P. mirabilis_ and _S. typhimurium_

To see whether swarming of another two bacterial strains, *P. mirabilis* P19 and *S. typhimurium* LT2 was also inhibited by SFAs, SFAs and UFAs at the concentration of 0.01%(w/v) was incorporated into 2% Eiken agar LB plates and 0.5% Eiken agar LB plates followed by swarming assay of *P. mirabilis* and *S. typhimurium*, respectively, at 37°C. We found that a similar inhibitory effect of SFAs on swarming of both bacteria was observed and that UFAs showed either stimulate or inhibit the swarming of both bacteria (data not shown). The effect of myristic acid and myristoleic acid on swarming of both bacteria was shown in Figure 11. Both myristic acid and myristoleic acid significantly inhibited swarming of both bacteria. The results suggested a common regulatory effect of fatty acids on swarming bacterial species.

Discussion

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 and/or cells to environment signals. Examples include Gram-positive bacterial antimicrobial peptide production (Kleerebezem and Quadri 2001), sporulation in *Bacillus* (Ryan and Shapiro 2003), light emission in *Vibrio* (Meighen 1999), biofilm formation in *Pseudomonas* (Costerton et al., 1999; Costerton 2001), production of nitrogen-fixing cells in cyanobacterium (Adams 2000), and also populational surface migration of many bacterial species (Velicer and Yu 2003; Horng et al., 2002; Romling 2001; Macfarlane et al., 2001; Fraser and Hughes 1999; Eberl et al., 1999; Harshey 1994; Shapiro 1998; McCarter and Silverman 1990).

Although a large body of information concerning the swarming mechanisms has been accumulated in *Serratia* and related bacterial species, the molecular mechanism(s) of swarming is far from understood. This is because the wide spectrum effects of physiology in swarming bacteria make it difficult to study the mechanisms directly. Furthermore, a potential specific and conserved regulator governing initiation of swarming has not been clearly identified, albeit a regulator of swarming behaviour of *P. mirabilis*, RsbA, has been reported by Belas et al., (1998). The identification of such a swarming regulator gene is thus an essential step in understanding the underlying mechanism of swarming. In this paper we present strong evidence that determination of *S. marcescens* swarming is controlled by acclimation of membrane fluidity, which is affected by environmental factors including temperature and nutrients, especially the provision of SFA substrates. We propose that the change of membrane fluidity is sensed by a pair of two-component regulatory proteins, RssA and RssB, which subsequently govern the expression of hemolysin gene *shlA* and $fabG_{Sm}$ gene coding for the NADPH-dependent 3-ketoacyl-ACP reductase, an enzyme involved in fatty acid synthesis (Rawlings and Cronan 1992). In *P. aeruginosa*, FabG is also reported to be involved in rhamnolipid synthesis and 3-oxo-homoserine lactone acyl chain length determination (Campos-Garcia et al., 1998; Hoang et al., 2002). Although kinase-response regulator pairs of this type were frequently reported as governors of a wide variety of pathways in response to a myriad of environmental signals (Dutta et al., 1999; Hoch et al., 2000), we have for the first time shown the requirement for temperature- and SFAs- dependent regulation of swarming via these genes. The genetic studies shown in this work indicate that RssA-RssB may form a signal transduction pathway controlling the fatty acid

profile-dependent regulation of *fabG*$_{Sm}$ gene expression and subsequently fatty acid profile, *shlA* hemolysin gene expression and initiation of swarming.

We hypothesized that when *S. marcescens* cells are growing into stationary phase at 30°C, no phosphorelay signaling is transferred between the RssA-RssB pair so RssB is mostly in a unphosphorylated form (inactive repressor) and at 37°C, the phosphorelay occurs and RssB is mostly phosphorylated (active repressor):(i) at 30°C, RssB in stationary phase cells is potentially in a unphosphorylated form in the presence or absence of RssA, expression of *fabG* $_{Sm}$ and *shlA* is not down-regulated, and bacteria swarm; when *rssB* is mutated, CH-1ΔB thus showed a swarming, albeit "slow-swarming" phenotype. (ii) at 37°C, compared with CH-1 whose RssB is potentially phosphorylated to become an active repressor, *fabG*$_{Sm}$ expression is inhibited and cells do not swarm, both CH-1ΔA and CH-1ΔB show a decrease in *fabG*$_{Sm}$ expression and super-swarming phenotype, although CH-1ΔA swarms faster and CH-1ΔB slower. On the basis that no response regulator has yet been identified to be active in the unphosphorylated form (Hoch, 2000), we propose that RssB may act as an active repressor binding to the promoter of *fabG*$_{Sm}$ when phosphorylated at 37°C, and binds to nearby promoter DNA region at 30°C (Lai et al., unpublished data). The role of RssA would then be to act as a phosphatase that selectively dephosphorylates RssB at 30°C. However, after a temperature upshift RssA would function as a specific kinase phosphorylating RssB, the cognate response regulator, which inhibits *fabG*$_{Sm}$ expression and also swarming initiation on LB swarming plate. The results in this report strongly suggest that the sensor protein RssA is a bifunctional enzyme having both kinase and phosphatase activities. These two opposite activities of the sensor protein have been demonstrated in different two-component systems (for a review see Dutta et a., 1999). We have further shown that the transcriptional activity of the *fabG*$_{Sm}$ promoter and subsequently the "swarming" or "non-swarming" fatty acid profile can be regulated by not only temperature shift, but also SFAs at a constant temperature. Temperature downshift or SFA deficiency may mediate activation of RssA phosphatase activity, leading to dissociation of unphosphorylated RssB, and subsequently RssB binding to another *fabG*$_{Sm}$ promoter DNA region (Lai et al., unpublished data). Currently experiments are being performed to confirm these questions.

A provisional model accounting for our results is shown in Figure 12. We envisage that RssA could assume different signaling states under varying growth temperatures and nutrient conditions which lead to different membrane fatty acid composition for the sake of homeostasis. This could be accomplished by regulating the ratio of kinase to phosphatase activities, such that a kinase-dominant state is present at high growth temperature or SFA-rich nutrients. RssA possesses a single

transmembrane domain and either the periplasmic domain or cytoplasmic domain would function to propagate a conformational change that is sufficient to significantly alter its activity. This conformational change could be governed by the physical state of the membrane lipid bilayer. Lipids in biological membranes are usually maintained in the fluid, liquid-crystalline state (Vigh et al., 1998). The correct physical state of membrane lipids is required for optimal membrane structure and function. Temperature markedly affects membrane lipid composition, and changes in lipid composition are thought to occur in order to maintain an appropriate liquid crystalline state. The major way in which bacteria, generally lacking cholesterol, maintain this functional membrane physical state is by changing their fatty acid composition (Vigh et al., 1998). As the growth temperature decreases, the proportion of low-melting-point fatty acids in the membrane lipids increases. The phenomenon of membrane fluidity affected by environmental temperature is also shown in the cyanobacterium *Synechocystis*, where low-temperature signals are shown to induce the desaturation of fatty acids in the cell membrane, thus changing the membrane fluidity (Sakamoto and Murata 2002). Membranes at 30°C are normally in a less-liquid crystalline form and will undergo a transition to a more-fluid phase state when the temperature increases (Cronan and Rock 1996; Vigh et al., 1998). This change from a non-fluid (ordered) to a liquid state (less ordered) might cause activation of the kinase activity, resulting in autophosphorylation of a conserved histidine (His 188) contained in the transmitter domain of RssA. The phosphoryl group of His188 could be directly transferred to RssB, which down-regulates transcription of $fabG_{Sm}$ or *shlA*. Activation of $fabG_{Sm}$ results in a synthesis of "non-swarming fatty acid profile". This metabolic pathway, therefore, generates a regulatory loop where SFAs or temperature upshift stimulates $fabG_{Sm}$ transcription by favouring RssB phosphorylation and promotes phosphorylated RssB binding to its binding site. In *B. subtilis*, cells respond to a decrease in ambient growth temperature by desaturating the fatty acids of their membrane lipids and by increasing the proportion of anteiso-branched fatty acids (Aguilar et al., 2001). This pathway, termed the Des pathway, responds to a decrease in growth temperature by enhancing the expression of the *des* gene coding for an acyl-lipid desaturase (Aguilar et al., 2001). The Des pathway is also uniquely and stringently regulated by a two-component system composed of a membrane-associated kinase, DesK, and a soluble transcriptional activator, DesR. It is suggested that activation of this transduction pathway might be mediated by a decrease in membrane fluidity provoked by a temperature downshift (Aguilar et al., 2001). It is also found in *B. subtilis* that a decrease in the content of isoleucine-derived fatty acids in the membrane at a constant temperature could mimic a drop in growth temperature and

that both stimuli could induce UFAs synthesis (Cybulski et al., 2002). Although so far based on our experimental results there is no evidence the *S. marcescens* uses branch-chain amino acids for maintaining the membrane fluidity, changes of fatty acid profile in response to environmental temperature, nutrients or chemical signals is a common phenomenon. Therefore, this adaptive response could be important in providing an appropriate degree of membrane fluidity for bacterial survival under ever-changing physiological conditions. Here so far as we know, we have for the first time connecting the synthesis of fatty acids to be regulated by the two-component system and phenomenon of membrane fluid optimization to the regulation of swarming.

It is a common phenomenon that bacterial two-component systems are proved to be important for regulation of bacterial physiological behaviours under the regulation of physical or chemical environmental signals. Examples include the PhoP/PhoQ system of *S. enterica* governing transcription of some 25 loci in response to the extracellular concentration of Mg^{2+} (Soncini and Groisman 1996), the regulation of he *arc* 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, mostly regulation of swarming, although the signals that interact with bacterial two-component systems and thus the mechanism remain uncharacterized, regulation of *P. mirabilis* swarming by the putative RsbA sensor kinase (Belas et al., 1998), *P. aeruginosa* swarming, virulence factor expression and biofilm formation by GacA-GacS (Brinkman et al., 2001; Parkins et al., 2001), *E. coli* swarming and capsule synthesis by RcsC-YojN-RcsB (Takeda et al., 2001), and the *S. enterica* swarming by the putative two-component signaling components are also reported (Toguchi et al., 2000).

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). Many bacteria use the two-component signal transduction system for adaptation of the environmental temperature shift. These include regulation of BvgA/BvgS by temperature, $MgSO_4$ 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 *P. syringae* phytotoxin coronatine by

CorS/CorP and CorS/CorR regulatory systems (Smirnova et al., 2002), 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 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).

Besides regulation of *S. marcescens* swarming, we also demonstrate that swarming of *P. mirabilis* and *Salmonella enterica* is also inhibited by fatty acids. Similar to *S. marcescens* CH-1, *P. mirabilis* and *S. enterica* do not produce AI-1 AHL signals. The AI-2 signal produced in *P. mirabilis* is not involved in the regulation of swarming or swimming (Scheider et al., 2002) and so far as we know, there is also no report on the regulation of *Salmonella* swarming by AI-2, although it is reported that some normal swimming but aberrant swarming *S. typhimurium* transposon mutants have defects in putative two-component signaling components (Toguchi et al., 2000), and that a *P. mirabilis rsbA* (a putative sensor kinase) mutant also shows a "super-swarming" behaviour (Belas et al., 1998). It may be possible that swarming of the two bacterial species is also regulated by the membrane fluid optimization through two-component system.

What may be the molecular mechanism of swarming regulation by which RssA-RssB functions? Although we have established clear and conclusive evidence that *S. marcescens* swarming is closely related to culture conditions (especially to the existence of SFAs), membrane fatty acid profile, RssA-RssB signal transduction, hemolysin production and fatty acid synthesis, due to the fact that the *rssA* mutant does not change in any identified swarming-related factors in *Serratia* spp., our finding may open a novel research direction that focuses on understanding the underlying mechanism of cell-cell and cell-environment communication. The fact that average cell length of CH-1ΔA and CH-1ΔB is about 10% shorter that that of CH-1

although the expression of *flhDC*$_{Sm}$ is not affected suggested that cell length determination is also partially regulated by RssA-RssB system independent of *flhDC*$_{Sm}$. Although the actual mechanism remains not totally clear, more and more evidences gathered indicate that bacterial mutants defective in genes involved in synthesis of either fatty acids or LPS are defective in swarming regulation, recognition function or regulation of virulence factors. Although the main function of LPS for bacterium is probably to provide an impermeable barrier against entry of harmful substances, it also plays various roles in interaction with other bacteria or eukaryotic cells (Raetz et al, 1991). In animals, LPS fractions are powerful endotoxins and contribute to disease symptoms. Recognition of LPS, which is an indispensable component of the Gram–negative bacterial cell surface, can thus sensitize surrounding bacterial cells to the presence of high or low density bacteria. For example, besides the genes identified from another 3 "super-swarming" mutants isolated in this laboratory (one is predicted to be involved in the modification of LPS, and the other 2 are predicted to play a role in LPS synthesis), McCoy et al., (2001) demonstrate that LPS modifications is not only involved in the antimicrobial resistance, but also play a role in *P. mirabilis* swaming due to surface charge alterations (McCoy et al., 2001). Using transposon mutagenesis, Toguchi et al., (2000) indicated a critical role for swarming regulation in *S. typhimurium*. It is also reported that a novel highly unsaturated fatty acid moiety of lipo-oligosaccharide signals determines host specificity of *Rhizobium* (Spaink 1994). Soto et al., (2002) report that a *fadD* (a gene whose product is involved in fatty acid degradation) mutant of *Sinorhizobium meliloti* shows multicellular swarming migration and is impaired in nodulation efficiency on alfalfa roots. In *Xanthomonas campestris*, from a cluster of genes, the *rpf* cluster, which comprise eight genes (*rpf*A-H), mutation of any of which leads to a reduction of synthesis of all extracellular enzymes and xanthan and loss of pathogenicity. Two members of this gene cluster, *rpfB* and *rpfF*, are involved in a novel type of regulation mediated by a small diffusible molecule. The factor responsible for these effects has been named DSF (for Diffusible Signal Factor), which is probably a fatty acid derivative. It is speculated that *rpfF* and *rpfB* are involved in diverting intermediates of lipid metabolism to DSF production. Production of DSF is suggested to be under control of two–component system including RpfC, RpfG and RpfH network. Indeed, from one of the other *S. marcescens* super-swarming mutants identified in our laboratory, evidence has shown that some unidentified volatile chemical signals can stimulate CH-1 swarming at 37°C across the air (data not shown), suggesting that *S. marcescens* may also use chemicals similar to DSF for regulation of the swarming behaviour. Currently, experiments are performed trying to characterize these molecules.

Another potential factor identified to be involved in swarming regulation is the EPS. In *P. mirabilis*, *cmfA* (colony migration factor), associated with the assembly of capsular polysaccharide CPS is identified to be important for swarming (Gygi et al., 1995). 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, although the function of capsule is still undetermined, in this report we have shown that while production of surfactant (serrawettin) is unchanged in *S. marcescens* CH-1ΔA, production of EPS is significantly reduced in the mutant, which basically agrees with our AFM observation (Figure 9). Although the EPS is thought to aid bacterial coordinated motility, it is interesting to see that extracellular addition of purified EPS delays the swarming of *S. marcescens* CH-1 (data not shown). A similar effect was also observed in *P. mirabilis* (data not shown). One of the reasons may be that LPS supposed to be involved in cell-cell interaction is easily exposed in the mutant strains, so that cell-to-cell communication becomes easier. More experiments are currently being performed to unravel the biochemical and physiological function of EPS on *S. marcescens* swarming.

Using *S. marcescens* SS-1 as the study model a similar inhibitory effect of long chain AHLs upon sliding plate motility is also observed (Horng et al., 2002). These results suggest that long chain AHLs not only inhibit sliding, but also swarming of *S. marcescens*. Although we did not demonstrate that presence of the long chain AHLs can be perceived by RssA-RssB two-component system, *S. marcescens* CH-1 did show a non-swarming fatty acid profile in the presence of 10 μM 3-oxo-C12 AHL signal **(Table 1)**. The results suggest that effect of 3-oxo-C12 on the swarming inhibition of CH-1 may act through some other unidentified pathway.

In summary, we provide evidence for the first time that a transcriptional factor that responds directly to membrane fluidity optimization regulated by temperature and nutrients (especially SFAs) is regulated by a histidine sensor kinase and acts as a molecular switch for the regulated transcription of a fatty acid synthesis gene, a haemolysin gene and some other unidentified swarming-related genes, where EPS synthesis genes might be the candidates. Future studies aim to understand the bacterial cell-to-cell and cell-to-environment interaction. These include to unravel the role(s) of the EPS/LPS components, to establish the chemical nature of *S. marcescens* EPS/LPS both in CH-1 and in those “super-swarming” mutants cultured in different physiological condition, and to evaluate the role of change of EPS/LPS structure in the sensing of environment and the subsequent regulation of initiation of *S. marcescens* swarming. The biochemical functions of the potential EPS/LPS-related

genes mutated in the other 3 super-swarming strains will be identified. Also, for the group of transposon mutants that we refer to as “super-swarming” mutants, products of the mutants are mostly predicted to be located in cell wall (data not shown), and all mutants swarmed in a similar way to the *rssA* mutant. Preliminary data gathered from these mutants show that like *S. marcescens* CH-1ΔA, their cellular fatty acid profiles all show the characteristics of the “swarming” profile, further distinguishing this group of mutants as unique, and suggesting that some other unidentified factors are involved in the regulation of swarming in *S. marcescens*. Such research may help us further understand the molecular mechanism of swarming.

Materials and Methods

Bacterial strains, plasmids, and culture conditions

S. marcescens CH-1 is a clinical isolate and is wild-type for swimming and swarming behaviors. No AHL quorum sensing signals was detected from CH-1. *S. marcescens* strains NewCDC (isolated from Germany), 1324E, S1220 and 4444 (isolated from U.K) are clinical strains provided by Peter ***(University). *S. marcescens* CH-1ΔA and CH-1ΔB were *rssA* and *rssB* knock-out mutants through insertion of an Ω cassette (Sm^r) from pHP45Ω (Prentki and Krisch, 1984) and homologous recombination. *S. marcescens* no.3, no.6 and O3 were three of the 17 super-swarming mutants screened in this study. *Salmonella typhimurium* SJW1103 and *Proteus mirabilis* P19 are both wild-type for motility and chemotaxis and are gifts from Macnab R (Yale University) and Hughes C (Cambridge University), respectively. *E. coli* strains (JM109; CC118 λ*pir* ; S17-1 λ*pir*; Top10 F' and XL-1 Blue) were cultured at 37 ^oC and *S. marcescens* at 30 ^oC in L-broth (LB) medium (Difco) (Sambrook *et al*., 1989) unless under conditions specifically described in the text. M9 salt solution (Sambrook et al., 1989) was used to make minimal growth medium (MGM), where 0.8% Eiken agar was incorporated to make MGM plates. In cases of mixed cultures, e.g., conjugations, incubations were at 30^oC. The pUT::mimi-Tn5-Km1 (de Lorenzo et *et al*., 1994) was used for transposon mutagenesis assay for screening super-swarming mutants. pZErO2.1 (Km^r), pCR2.1 (Ap^r; Km^r)(Invitrogen), pBCSK+ (Cm^r) and pBluescript SKII+ (Stratagene) were used as cloning vectors for cloning DNA fragments. pPC300(P*flhDC*::*luxCDABE*), pSA400(P*shlA*::*luxCDABE*) and pSA401(P$fabG_{Sm}$::*luxCDABE*) used as bioreporters for monitoring the promoter activity of P$flhDC_{Sm}$, P*shlA*, and P$fabG_{Sm}$ respectively were constructed by insertion of a PCR amplified promoter region ligated in front of *luxCDABE* derived from pSB1075 (Winson et al., 1998) into pACYC184(Tc^r; Cm^r) (Chang and Cohen, 1978) and primer pairs used were (5'GATATCCAGCCTCAGGCGGAGGG3'/5'GGATCCATTCCCCATCCCGACA GACTA3') for P$flhDC_{Sm}$, (5'GCAGCAGCGCCGGTATAAGCAC3'/5'ATCGCCAGCGCAGCGGCCAGTT3') for P*shlA*, and (5'GCCGGTTACCAATGCCACTTT3'/5'GGGGGAGCGAAACATGCAG5') for P$fabG_{Sm}$. pWC200 (Tc^r, Cm^r) was constructed by PCR cloning an 1460 bp DNA fragment containing complete *rssA* into pACYC184 and expressed from its native promoter by using primer pair (5'ACCATTATTTTCCAGGTGCT3'/5'ATACAGAGTGTCGATAATTT3').
pSA402 (Cm^r) was constructed by cloning a 1320 bp DNA fragment containing complete *rssB* structural gene into pBCSK(Stratagene) and expressed from its native promoter by using primer pair (5'TGCGGCCTGCGCGCAGGCGC3'/5'AGAATATTGGCGATGCCTGC3').
Swimming motility was examined on motility agar [LB solidified with 0.35% Eiken agar (Eiken, Japan)] by sterile needlepoint inoculation from an overnight culture into the centre of the agar plate. Swarming motility was examined on swarming agar plates (LB solidified with 0.8% Eiken agar) by inoculating 5 μl of an overnight broth culture onto the centre of the agar plate. The swimming and swarming distances were recorded hourly for comparison. Swarmer cell differentiation, i.e., the overproduction of flagella, cellular elongation, and polyploidy, was also examined microscopically as described (Lai et al., 1997; Liu et al., 2000). Basically, for cell elongation assay, after overnight broth culture, bacteria were 1:100 diluted and cultured in 20ml LB broth at

30°C and 37°C with vigorous shaking at 200 rpm. An Olympus BH2 light microscope was used for observation of single bacterial cells at a magnification of 1000x. Images were captured using an SPOT CCD camera integrated with a PC computer (Diagnostics Instruments). Bacterial colonies were examined by light microscopy at a magnification of 1x or 30x using an Olympus SZH microscope. For determining the bacterial growth rate, hourly increases in the optical density of broth cultures at 600nm were observed.

Enzymes and chemicals

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

Recombinant DNA techniques

Unless mentioned specifically, standard protocols were used for DNA/DNA hybridization, the isolation of plasmid and chromosomal DNA, transformation, electroporation, PCR, restriction digestion, agarose gel electrophoresis, DNA recovery from agarose gels, the ligation of DNA fragments (Sambrook *et al*., 1989), and conjugation (de Lorenzo and Timmis, 1994). Southern blot analysis of chromosomal DNA bound to a nylon membrane (HybondN^{+}; Amersham) was hybridized and washed at 68 ^{o}C and was performed by using the DIG High Prime labeling kit according to the recommendations of the manufacturer (Roche). PCR DNA amplicons were cloned by pCR^{TM}II$^{®}$ and the TA Cloning Kit (Invitrogen). DNA sequencing and analysis was performed using a Perkin-Elmer Autosequencer model 377 with a Taq DyeDeoxyTM terminator cycle sequencing kit (Applied Biosystems). The DNA sequence of the PCR was confirmed by sequencing both strands from two or three independent reactions.

Northern Blot hybridization

Total cellular RNA prepared by the hot phenol method (Koronakis and Hughes, 1988) was transferred to nylon filters and hybridized with DNA probes labelled with DIG (Roche). The *fabG*$_{Sm}$ gene probe was a 327 bp partial *fabG*$_{Sm}$ DNA fragment amplified by PCR/ DIG-labelling (Roche) using primer pair 5'-ATGACGAAAGTGGCATTGGTAACC-3' and 5'-GAGGACCTGATTGATGCGGTC-3'.

Analysis of DNA and protein sequences

The DNA and deduced protein sequences were compared with GenBank DNA or non-redundant protein sequence databases using blastn or blastx via the NCBI internet

homepage (http://www.ncbi.nlm.nih.gov/). Protein sequence identities were analysed by ExPASy proteomics tools (DAS, Tmpred, SOSUI, PredictProtein and ProtScale) in the ExPASy Molecular Biology Server of the Swiss Institute of Bioinformatics (SIB) (http://tw.expasy.org).

Mini-Tn5 mutagenesis for screening super-swarmer mutants

For transfer of pUT-miniTn5-Km1 recombinant plasmid from *E. coli* to *S. marcescens* CH-1 by conjugation (de Lorenzo et al., 1994), *S. marcescens* CH-1 recipient strain and *E. coli* S17-1 *λ-pir* donor strain carrying the pUT derivative recombinant plasmid were grown overnight with vigorous shaking at 30℃ in 10 ml LB broth, with addition of streptomycin 50 μgml^{-1} and kanamycin 50 μgml^{-1} for *E. coli* culture. Mating was achieved by mixing 100µl of each bacterial suspension followed by addition of 5 ml of 10 mM $MgSO_4$. The mixture was then filtered through a Type HA Filter membrane (Millipore) using a negative-pressure pump (Stratagene). The drained membrane was subsequently placed onto the agar surface of a normal 1.2% LB plate and incubated for 8-18 hour at 30℃. The bacteria were then suspended in 5 ml $MgSO_4$ broth that can be kept at 4℃ for one week, and spread onto the modified LB agar plate (0.04% NaCl, 2% glycerol, 0.5% yeast extract, 1% Bacto-tryptone, 0.8% Eiken agar, 50μgml^{-1} kanamycin and 13μgml^{-1} tetracycline) followed by incubation at 37℃. Transconjugants that swarmed at 37℃ were selected. A total of 6000 colonies were screened, among which 17 were selected. Southern blot hybridization using labelled Km gene as the probe was performed to confirm that only one transposon copy was inserted in to the chromosome of the mutants.

Detection of bioluminescence luciferase activity

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

Construction of S. marcescens* CH-1ΔA and CH-1ΔB *deletion insertion mutants

PCR were designed to introduce specific *Hind*III sites into *rssA* and *rssB* respectively for subsequent insertion of *Hind*III digested Ω (Sm^r) gene cassette (Prentki and Krisch, 1984). Primer pairs (5'GGTTTACGATCGAGACAACC3'/5'GGATCCGTGCGGGCGATCTG3')for *rssA* and (5'GCATTGGAGCTGGCCGGCTTTA3'/5'GGATCCAAAGTCGTTCGGGCTGT3') for *rssB* were used to amplify the 5'-region of the gene to be inactivated. PCR products were T-cloned into pCR2.1 (Stratagene), sub-cloned as an *Xba*I/*BamH*I

fragment into pZero2.1 (Invitrogen) and excised as a *Xba*I/*Hin*dIII fragment. A second PCR product encompassing the 3'-region of the gene to be inactivated was generated using primer pairs (5'GGATCCGCTCGACGGCAGCGAGGA3'/5'TCCGGCAAATCGATGATGAAGC C3') for *rssA* and (5'GGATCCCATCAGGTCATGCTGGATA3'/5'CTACTCTTTCTTCAGCAAATAG CC3') for *rssB*, T-cloned into pCR2.1 and excised as an *Hin*dIII/*Eco*RI fragment. The 2 kb Sm-resistant Ω DNA fragment was excised from pHP45Ω (Prentki and Krisch, 1984) as a *Hin*dIII fragment. The three DNA fragments were ligated together with *XbaI/EcoRI* digested suicide vector pUT-mini-Tn5-Km (de Lorenzo and Timmis 1994). The resultant pUT-*rssA*::Sm and pUT-*rssB*::Sm vectors were selected as conferring streptomycin resistance upon the permissive *E. coli* strain CC118 and verified by restriction enzyme mapping. For gene inactivation mutagenesis by homologous recombination, the respective plasmids were transferred from the permissive host strain *E. coli* S17-1 λpir to *S. marcescens* CH-1 by conjugation and the transconjugants were spread on LB plates with streptomycin (100 μg/ml) and tetracycline (13 μg/ml). The mutants with double cross over events were selected by colony PCR screening. Southern hybridization using the PCR amplified *rssA* gene or *rssB* gene as the probe respectively was performed, which confirmed the mutant genotype (data not shown). The data confirmed that a double-crossover event had taken place and the new strains were designated CH-1ΔA and CH-1ΔB strains.

Overexpression of rssA and complementation of super swarming mutant

Transferring pWC200 and control plasmid into *S. marcescens* CH-1 via electroporation tested the effect of overexpressing *rssA* in a wild-type background. The resulting Cm^r Km^r colonies were then assayed for swarming and other phenotypic characteristics as previously described. Plasmid pWC200 was also electroporated into the super-swarming mutant *S. marcescens* WC100 or CH-1ΔA for complementation assay. The transformants that were Cm^r were selected for characterization of swarming and cell differentiation behaviors.

Biofilm assay

The quantification assay was performed as previously described (O'Tolle and Kolter 1998). Basically, 10μl of an overnight culture was used to inoculate PVC microtiter wells containing 90μl of LB without NaCl but with 2% glucose. The covered microtiter dish was sealed with parafilm during incubation at 30°C. Cultures were removed to determine the optical density at 630nm (A_{630nm}), and the wells were rinsed with distilled water. After drying at room temperature for 15min, 200μl of crystal

violet (1%) was added to the wells for 20min. The stained biofilms were rinsed several times with distilled water, allowed to dry at room temperature for 15min, and extracted twice with 200µl of 95% ethanol. The OD(A_{550nm}) was estimated using a Beckman DU-640B spectrophotometer after adjusting the volume to 1ml with distilled water.

Quantitative measurement of extrapolysaccharide (EPS) Production

The procedures were basically followed by the protocols from von Bodman et al., (1998). Briefly, cultures of *S. marcescens* CH-1 and WC100 strains were grown overnight in LB broth. Cells were then washed twice in equal volumes of 0.9% NaCl and suspended in 0.9% NaCl. Then cell suspensions were 1:100 inoculated into 15 ml CPG broth (Dolph et al., 1988). EPS was determined at 3 hr where OD(A_{600nm}) reached 0.6. Fixed amount of cells were collected by centrifugation at 8,000×*g* for 30min. The unbound EPS present in the culture supernatant were precipitated with 2.5 volumes of absolute ethanol. To recover the capsular EPS fraction bound to the bacterial cells, the cell pellets were resuspended in 5ml of high-salt buffer (10mM K_2HP0_4/KH_2P0_4, pH 7.0/15 mM NaCl/1 mM $MgS0_4$) and vortex for 1 hr. Cells were removed by centrifugation at 12,000 ×*g* for 30min. Dislodged EPS was precipitated from the supernatant with 2.5 volumes of ethanol. The EPS precipitates were collected by centrifugation at 12,000×*g* for 30min and then resuspended in 1.5ml of sterile H_2O. The amount of total carbohydrates contained in each sample was determined by the phenol/sulfuric acid method (Hanson and Philips 1981). Briefly, 500 µl sample solution is mixed with 500 µl of phenol solution in a glass tube, and then 2.5ml of sulfuric acid reagent is rapidly added and vortexes. The tubes were incubated in the dark for 1 hour, when the absorbance at OD(A_{490nm}) is measured. Glucose standards (10 to 100 µg/ml) were used to construct the standard curve.

Measurement of hemolysin activity, surfactant production and flagellum production

Cell-associated haemolysin (ShlA) activity was assayed as described (Koronakis et al., 1987) and calculated in arbitrary haemolytic units (1 unit causing the release of 50 mg haemoglobin/hr in the standard assay). A qualitative assay for surfactant production was performed using the drop collapsing method (Lindum *et al*., 1998). A semi-quantitative assay for biosurfactant was performed using a TLC based assay as described by Matsuyama *et al*., (1992).

To prepare flagellae for SDS-PAGE analysis cells from the surface of agar plate were harvested by washing into 3 ml of LB. Flagellin from the supernatant of vortexed cells was precipitated by 10% trichloro-acetic acid (Allison *et al*., 1992), normalized to cell mass [OD(A_{600nm}) x cell suspension volume(ml) = 5], separated by SDS-PAGE

(Sambrook *et al*., 1989) and stained with Coomassie brilliant blue. Flagella were stained on whole cells using the silver-plating method of West *et al*., (1977).

N-*Acylhomoserine lactones*

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

Atomic force microscopy(AFM)

Bacteria were cultured overnight on LB medium. The bacterial culture was diluted 1:100 in fresh LB broth and incubated at 37°C with vigorous shaking (225rpm) for 2 hr. Prior to imaging, bacteria were gently washed with distilled water and final concentration of 10^4-10^5 cells/ml was used for AFM experiments. Pre-cleaned slides were treated with poly-L-lysine 0.01% and left to dry. A 20µl drop of bacterial suspension in distillated water was applied onto treated slide. After adsorption for 30 minutes, distilled water was added to remove the unadsorbed cell. AFM experiments were performed using a SOLVER BIO atomic force microscopy (NT-MDT, Moscow, Russia). The procedures for AFM imaging are described in Hansma *et al.* (1994). Silicon nitrite tips were used, with a force constant of 5.5 N/m. Imaging was carried out on dry sample. AFM images were generated at line frequencies of between 1 and 3 Hz, with 256 lines per image. Images were obtained using semi-contact (tapping) mode AFM. The data were analyzed with SMENA software (Advanced Technologies Center, Russia).

Analysis of cellular fatty acids by GC-FAME

Bacteria either swarming or non-swarming were seeded on plate conditions mentioned in the text. Extraction of cellular fatty acids was performed followed the standard procedure (MIDI, USA). Samples were prepared for analysis in MIDI GC-FAME analysis system (Microbe Inotech Laboratories, USA).

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DOEA project…..

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Figure Legends:

Fig. 1. Swarming of *S. marcescens* CH-1, *S. marcescens* CH-1ΔA and *S. marcescens* CH-1ΔB. Swarming assay of the three strains on 0.8% LB swarming plates and comparison of swarming velocity (Gygi et al., 1995) were performed at both 30 and 37°C and were shown in (A) and (C) respectively. Cells were grown overnight in LB broth, washed once in 1 × PBS, and a 5-µl spot of inoculum was added to the center of the agar. The plates were incubated and observed after 12 h. Swarming assay of the three strains on 1.2% LB agar plates at 30°C after 10 h incubation was shown in (B). Symbols: (■)CH-1, 30°C; ( )CH-1, 37°C; (▲)CH-1ΔA, 30°C; (Δ)CH-1ΔA, 37°C; (●)CH-1ΔB, 30°C; (o)CH-1ΔB, 37°C. Error bars, standard errors of the means (n = 4).

Fig. 2. Location of the Tn*5* insertion on the *S. marcescens* chromosome and analysis of the RssA protein sequence. The insertion point of the mini-Tn*5*-Km transposon in 5'region of *rssA* was shown in (A). The nucleotide sequence containing *rssA* and *rssB* has been submitted to the DDBJ/EMBL/GenBank databases under accession no. AF465237. A hydropathy profile of RssA constructed by DAS (Cserzo et al., 1997) was shown in (B). The solid and dashed lines represent the strict and loose cutoffs, respectively. Prediction of membrane topology (Mitaku and Hirokawa 1999) was shown in (C). Alignment of RssA domains with those of related bacterial sensor proteins (Hoch and Silhavy 1995) was shown in (D). Conserved sequences are highlighted.

Fig. 3 *S. marcescens* CH-1ΔA initiates swarming at a lower cell density.
The density-dependent initiation of swarming behavior of CH-1ΔA(pPC300) was assayed on 0.8% LB swarming plates followed by hourly light microscopy observation (Ai) and X-ray film exposure (Aii) for monitoring the light emission pattern which is a reporter for $flhDC_{Sm}$ expression (Liu et al., 2000) and bacterial swarming. Number means the time (hr) of incubation. The density-dependent initiation of swarming behavior in CH-1 and CH-1ΔA cells was compared. The initial inoculum concentration was varied in 10-fold increments from 1×10^6 to 1×10^2 cells (CFU) delivered in 5-µl-aliquot droplets to the LB swarming plates. The onset time of swarming behavior and the lowest cell number (CFU) needed for swarming initiation were then recorded (B). Bacterial cell number shown was the mean of 4 independent experiments.

Fig. 4 The flagellar swimming motility and production of surfactant were not affected in CH-1ΔA and CH-1ΔB mutants. Swimming assay of CH-1, CH-1ΔA and CH-1ΔB

was performed on 0.35% LB plates at both 30 and 37°C for over night (A). The swimming velocity measured at 30 and 37°C (B) and *flhDC*$_{Sm}$ promoter activity assayed at 37°C (C) for the 3 strains were recorded. Drop collapsing assay (Horng et al., 2002) (D) and TLC assay (Horng et al., 2002) (E) were performed to qualitatively and semi-quantitatively determine serrawettin production in CH-1 and CH-1ΔA at 30 and 37°C. f, TLC front; p, prodigiosin pigment; w, serrawettin; o, TLC origin. Symbols **B**: (■)CH-1, 30°C; ( )CH-1, 37°C; (▲)CH-1ΔA, 30°C; (Δ)CH-1ΔA, 37°C; (•)CH-1ΔB, 30°C; (o)CH-1ΔB, 37°C. Symbols **C**: (■)CH-1(pPC300); (▲)CH-1ΔA(pPC300) and (•)CH-1ΔB(pPC300).

Fig. 5 *S. marcescens* CH-1ΔA and CH-1ΔB are defective in biofilm formation and show increased haemolysin activity at 30 and 37°C. Microtiter well assay (O'Tolle and Kolter 1998) was performed to monitor the biofilm formation activity of CH-1, CH-1ΔA and CH-1ΔB (A). Intensity of crystal violet measured at 550nm (OD_{550}) was shown in (B), where blank column means test performed at 30°C and black column means at 37°C. The specific haemolysin activity (Koronakis et al., 1987) and transcriptional activity of the haemolysin gene *shlA* expressed as specific bioluminescence activity of CH-1(pSA400), CH-1ΔA(pSA400) and CH-1ΔB(pSA400) at 30 and 37°C were shown in (C) and (D), respectively. Symbols **C**: (■)CH-1, 30°C; ( )CH-1, 37°C; (▲)CH-1ΔA, 30°C; (Δ)CH-1ΔA, 37°C; (•)CH-1ΔB, 30°C; (o)CH-1ΔB, 37°C. Symbols **D:** (■)CH-1(pSA400), 30°C; ( )CH-1(pSA400), 37°C; (▲)CH-1ΔA(pSA400), 30°C; (Δ)CH-1ΔA(pSA400), 37°C; (•)CH-1ΔB(pSA400), 30°C; (o)CH-1ΔB(pSA400), 37°C.

Fig. 6 *S. marcescens* CH-1 swarming phenotype is inhibited by saturated fatty acid in a dose-dependent way, and shows close correlation to cellular fatty acid profiles. Swarming assay of CH-1 and CH-1ΔA was performed on 0.8% MGM plates either without or with additives at 30 and 37°C (A)**.** Effect of myristic acid (C14) on swarming was further assayed on 0.8% MGM plates containing 2x serially diluted myristic acid from 0.01% to 0.00125% at 37°C for 12 hr (B). The cellular fatty acid profiles of CH-1, CH-1ΔA and CH-1ΔB inoculated under different growth conditions leading to either swarming or non-swarming phenotypes were analysed by Gas Chromatography. The average percentage of fatty acids between swarming (white bars) and non-swarming cells (black bars) was shown in (C). Results were the mean of 3 independent experiments.

Fig. 7 Effect of myristic acid on the activation of *fabG*$_{Sm}$ in *S. marcescens* CH-1 was down-regulated by RssA-RssB. CH-1(pSA401), CH-1ΔA(pSA401) and

CH-1ΔB(pSA401) grown in LB broth containing myristic acid at the concentration of 0.01% (w/v)were tested for *fabG*$_{Sm}$ promoter activity using *luxCDABE* as the reporter following the growth at 30°C and was shown in (A). Symbols: (▲)CH-1(pSA401); (◆)CH-1ΔA(pSA401); (■)CH-1ΔB(pSA401). Northern blot hybridization using partial *fabG*$_{Sm}$ as the probe detecting *fabG*$_{Sm}$ transcriptional level in CH-1, CH-1ΔA and CH-1ΔB was shown in (B).

Fig. 8 Effect of AHLs on the swarming of *S. marcescens* CH-1 and 3-oxo-C12 inhibits swarming independent of RssA-RssB two-component system. The extent of CH-1 swarming on LB swarming plates supplemented with 10μM synthetic AHLs C4-HSL, C5-HSL, C6-HSL, C8-HSL, C10-HSL, C12-HSL, 3-oxo-C4-HSL, 3-oxo-C6-HSL, 3-oxo-C8-HSL, 3-oxo-C10-HSL, 3-oxo-C12-HSL and 3-oxo-C14-HSL is shown after incubation at 30^{o}C for 12 hours (A). Effect of 3-oxo-C12 on *fabG*$_{Sm}$ expression (P*fabG*$_{Sm}$::*luxCDABE*) was assayed in CH-1(■), CH-1ΔA(▲) and CH-1ΔB(●) containing pSA401 at 30°**C**. Cells were inoculated in LB broth with (B) or without (C) addition of 10 μM 3-oxo-C12 followed by culture under 200 rpm shaking. Cells were harvested hourly for detecting the light emission intensity.

Fig. 9 Cellular surface topology is changed in CH-1ΔA and CH-1ΔB. CH-1, CH-1ΔA and CH-1ΔB were subject to surface structure characterization by atomic force microscopy (AFM). Cells were over-night cultured in LB broth followed by 1in 100 dilution in fresh LB broth for further culture at 37 ^{o}C for 2 hours. Cells were harvested and treated for AFM observation (Hansma et al., 1994), where cellular surface image and characterization of at least 20 cells from each strain were shown in (A) and (B) respectively.

Fig. 10 Production of EPS is changed in CH-1ΔA and addition of EPS inhibits CH-1 swarming at 30 ^{o}C. CH-1 and CH-1ΔA were seeding plated on 1.2% LB plates and incubated at 30 ^{o}C for 3 hr before harvesting for EPS preparation (von Bodman et al., 1998). Bound form (grey column), unbound form (black column) and total amount of EPS (white column) were shown.

Fig. 11 Effect of myristic acid and myristoleic acid on swarming of *S. typhimurium* SJW1103 and *P. mirabilis* P19. *S. typhimurium* SJW1103 and *P. mirabilis* P19 were respectively inoculated on 0.5% and 2% LB Eiken agar plates containing either myristic acid or myristoleic acid under the concentration of 0.01% (w/v), followed by swarming assay at 37 ^{o}C for 10 hr.

Fig. 12 Model of swarming control of *S. marcescens* by two-component temperature signal transduction proteins. It is proposed that RssA assumes different signalling states in response to a temperature- or SFAs-induced change in membrane fatty acid composition, which is due to bacterial maintenance of membrane fluidity for homeostasis. This is accomplished by regulating the ratio of kinase to phosphatase activity such that a phosphatase-dominant state is present at either 30°C or absence of SFAs, when membrane lipids are ordered, whereas a kinase-dominant state predominates upon an increase in the proportion of ordered membrane lipids after a temperature upshift to 37°C or presence of SFAs. RssA-mediated phosphorylation of RssB results in transcriptional inhibition of both $fabG_{Sm}$ of *shlA*, and also *S. marcescens* swarming, and vice versa. Composition of membrane fatty acid profile is further affected by RssA-RssB and subsequently $fabG_{Sm}$ expression, forming a regulatory circuit. Change of membrane fatty acid composition from rigid (more SFAs) to less rigid (more UFAs) leads to RssA dephosphorylation of RssB-P or by causing binding of DesR-P to another promoter binding site (see text for further details).

Table 1. Percentage of cellular fatty acids of swarming and non-swarming cells in *S. marcescens*. Cells including *S. marcescens* CH-1, CH-1ΔA, CH-1ΔB, no.3, no.6 and O3 were inoculated on different culture conditions leading to either swarming or non-swarming phenotypes. The composition of cellular fatty acids was analysed by gas chromatography (MIDI, USA). Each assay was performed at lease for 3 times and the average of each fatty acid was shown. "Ratio*" means ratio of calculated fatty acid composition (12:0+14:0+16:0+18:0/10:03OH+15:0 anteiso+18:1 w7c).

	Percentage of total fatty acids							
	10:0 3OH	**12:00**	**14:00**	**15:0 anteisc**	**16:00**	**18:1 w7c**	**18:00**	**Ratio***
swarming cells								(ave)2.6
30CH-1/LB		4.26	7.52	2.95	25.96	17.08	2.92	2.0
30CH-1/MGM		2.9	8.08	6.72	30.42	10.22	0.83	2.5
30CH-1/MGM/Cas		3.44	6.08	6.41	28.66	8.82		2.5
30CH-1/MGM/peptone		3.16	5.54	3.5	26.86	17.65		1.7
30CH-1/MGM/Cas/yeast extract		3.55	7.42	3.69	29.54	17.21		1.9
30CH-1/MGM/Cas/ myristoleic		2.42	9.2	2.81	30.82	10.89	1.99	3.2
30CH-1/MGM/Cas/palmitoleic		3.62	8.63	3.06	31.48	14.84		2.4
30CH-1/MGM/Cas/oleic		3.13	8.18	2.47	29.52	13.64	2.17	2.7
37CH-1/MGM		2.02	9.09	5.57	38.99	6.83		4.0
37CH-1/MGM/Cas	3.21	1.9	7.17	1.38	33.53	7.58	1.19	3.6
37CH-1/MGM/peptone		3.08	8.3	1.91	30.63	13.22	1.18	2.9
30CH-1ΔA/LB		2.71	6.21	1.9	29.21	15.85		2.1
30CH-1ΔA/LB/OC12		2.56	5.92	1.64	29.38	16.15		2.1
37CH-1ΔA/LB		4.28	7.04	7.48	25.92	13.58		1.8
37CH-1ΔA/MGM			6.47	11.26	33.95	5.6		2.4
37CH-1ΔA/MGM/Cas			5.99	1.64	32.47	7.7	1.64	4.3
37CH-1ΔA/MGM/Cas/myristic		1.12	13.14	1.49	35.42	14.96	0.85	3.1
30CH-1ΔB/LB		3.41	6.2	2.47	27.37	17.63		1.8
37CH-1ΔB/LB		2.91	7.49	3.75	33.98	13.57		2.6
37CH-1ΔB/MGM		8.14	7.67	9.17	33.64	7.42		3.0
37CH-1ΔB/MGM/Cas	3.18	2.32	7.65	1.34	35.01	8.11	1.02	3.6
37CH-1Oaf/LB		6.21	8	8.04	23.82	11.41	1.17	2.0
30CH-1Oaf/LB		5.03	6.41	9.73	26.63	12.81		1.7
37CH-1Ssp/LB		5.13	7.72	3.93	27.57	12.87	1.1	2.5
37CH-1No8/LB		2.43	7.86	2.75	30.15	12.79		2.6
37CH-1Dap/LB			9.52	6.43	31.73	12.17		2.2
non-swarming cells								(ave) 6.9
30CH-1/OC12/LB		5.63	7.28	1.58	29.16	8.92	1.5	4.2
30CH-1/MGM/Cas/lauric	1.76	24.75	5.97	1.16	30.01	5.66	0.9	7.2
30CH-1/MGM/Cas/myristi	1.63	3.65	12.78	0.6	36.25	8.15	0.73	5.1
30CH-1/MGM/Cas/palmiti	1.63	11.12	7.35	0.89	35.63	6.42	0.89	6.2
37CH-1/LB		7.75	8.25	1.35	29.8	7.27	2.63	5.6
37CH-1/MGM/Cas/YE	1.08	5.48	10.28	0.6	36.33	5.82	1.12	7.1
37CH-1/MGM/Cas/pepto	1.31	7.93	12.11	0.8	34.21	4.79	0.9	8.0
37CH-1/MGM/peptone/Y	0.98	6.82	13.24	0.79	38.12	6.02	1.03	7.6

* Calculated as the total percentage of 12:00, 14:00, 16:00 and 18:00 divided by total percentage of 10:0 3OH, 15:0 anteiso and 18:1 w7c