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The RssAB Two-Component Signal Transduction System in *Serratia marcescens* Regulates Swarming Motility and Cell Envelope Architecture in Response to Exogenous Saturated Fatty Acids

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ABSTRACT

Serratia marcescens swarms at 30°C, but not at 37°C on a nutrient rich (LB) agar surface. Mini-Tn5 mutagenesis of *S. marcescens* CH-1 yielded a mutant (WC100) that swarms not only vigorously at 37°C, but also earlier and faster than the parent strain at 30°C. Analysis of this mutant revealed that the transposon had inserted into a gene (*rssA*) predicted to encode a bacterial two component signal transduction sensor kinase, upstream of which, a potential response regulator gene (*rssB*) was located. *rssA* and *rssB* insertion-deletion mutants were constructed through homologous recombination and both exhibited similar swarming phenotypes on LB swarming agar whereby swarming not only occurs at 37°C, but also initiates at a lower cell density, on a surface of higher agar concentration and more rapidly than the parent strain at 30°C. Both mutants also exhibited increased haemolysin activity and altered cell surface topologies compared with the parent CH-1 strain. Temperature and certain saturated fatty acids (SFAs) were found to negatively regulate *S. marcescens* swarming via the action of RssA-RssB. Analysis of the respective fatty acid profiles of the parent, *rssA* and *rssB* mutants grown at 30^{o}C or 37^{o}C and different nutrition conditions revealed a relationship between cellular fatty acid composition and swarming phenotypes. The cellular fatty acid profile is further observed to be affected by RssA and RssB. SFA-dependent inhibition of swarming was also observed in

Proteus mirabilis and *Salmonella typhimurium*, suggesting that either SFAs *per se* or the modulation of cellular fatty acid composition and hence homeostasis of membrane fluidity may be a conserved mechanism for regulating swarming motility in Gram negative bacteria.

INTRODUCTION

When inoculated onto LB medium solidified with 0.8% w/v agar at 30°C, *Serratia marcescens* exhibits a characteristic swarming phenotype in which short motile vegetative rods at the colony margin differentiate into elongated, aseptate and hyperflagellate swarm cells which migrate coordinately and rapidly away from the colony (11). Phenotypically, the process of swarming cell differentiation and migration in *S. marcescens* may be divided into two separate phases: (i) the lag period prior to the onset of swarming behavior and the induction of swarm cell differentiation at the colony edge and (ii) active motile swarming migration (or translocation) from the colony edge. Individual bacterial cells do not possess the intrinsic ability to swarm as swarming behavior in *Serratia* is a result of the coordinated, multicellular effort of groups of differentiated cells functioning through close cell-cell interactions (11).

Swarming is the result of the regulated expression of gene networks required to initiate the complex processes underlying the required morphological and physiological changes (11, 13, 21, 22). Development of a *Serratia* surface swarming colony requires the processing and integration of multiple environmental, cell-to-cell and intracellular signals involving surface contact and local high bacterial population densities (2). The flagellar master operon (*flhDC*) and an *N*-acylhomoserine

lactone-dependent quorum-sensing system are global regulators of flagellar motility and cell population density, respectively (14, 24, 33, 35). Cellular differentiation is only one part of this process which requires a lag period prior to the commencement of swarming migration, during which time cellular proliferation occurs up to the required population density, and a large amount of biosurfactant was produced (1, 24, 32). An additional layer of regulation of surface migration may also be exerted through the post-transcriptional regulator RsmA (5). When over-expressed, *rsmA* inhibits the formation of a spreading colony in *S. marcescens* (4) and this effect may be mediated via repression of AHL-dependent quorum-sensing, since overexpression of *rsm*A in *P. aeruginosa* has been reported negatively regulate AHL synthase expression (38).

Many regulatory pathways involved in control of swarming remain have yet to be characterized at the molecular level. For example, exposure of the cells to surfaces with a specific viscosity is likely to require recognition by an unknown sensor. Furthermore, the physiological signals that provide essential stimuli for the initiation of swarming and the underlying signal transduction pathways have also not yet been characterized. *S. marcescens* swarming is known to be a temperature-dependent behaviour, which occurs at 30°C, but not at 37°C. Bacteria inoculated onto identical plates and incubated at 37°C did not initiate swarming even after 48h culture and

formed only small defined colonies. This may be a consequence of reduced flagellar motility (35) and/or a reduction of biosurfactant production following temperature upshift. To begin unravelling the underlying regulatory mechanism(s), we have utilized mini-Tn5 mutagenesis to screen a group of *S. marcescens* mutants that demonstrated swarming proficiency at 37°C. In the process of characterizing one of these mutants, we identified a bacterial two-component signal transduction system (23) which we termed RssA-RssB. This two component system functions in the coordination of swarming migration which is independent of surface *flhDC* flagellar motility, AHL-dependent quorum-sensing, and the production of biosurfactants, factors which have previously been identified as regulators of population surface migration behavior in *Serratia* (15, 24). Here we present evidence to show that swarming of *S. marcescens* is inhibited by presence of saturated fatty acids (SFAs) and that this effect is mediated via the RssA-RssB two component system. The cellular fatty acids composition, haemolysin production and cell surface topography were also shown to be regulated by RssA-RssB. The negative regulatory effect of myristic acid on bacterial swarming was also observed in bacteria such as *Salmonella typhimurium* and *Proteus mirabilis*, suggesting existence of a common regulatory pathway in Gram negative bacterial swarming.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions. *S. marcescens* CH-1 is a clinical isolate and is functionally wild-type for swimming motility and swarming migration behavior. No AHL quorum sensing signals were detected from CH-1 cells. *S. marcescens* CH-1ΔA and CH-1ΔB are *rssA* and *rssB* knock-out mutants, respectively, via the insertion of an Ω cassette (Sm^r) from pHP45Ω (39) and subsequent homologous recombination. Bacterial strains *Salmonella typhimurium* SJW1103 and *Proteus mirabilis* P19 are both wild-type for motility and chemotaxis and were gifts from R. Macnab (Yale University) and C. Hughes (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) (40) unless otherwise indicated in the text. M9 salt solution (40) was used to make minimal growth medium (MGM), into which 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 construct (10) was used in the transposon mutagenesis assays for screening super-swarming mutants. pZErO2.1 (Km^r), pCR2.1 (Ap^r; Km^r)(Invitrogen), pBCSK+ (Cm^r) and pBluescript SKII+ (Stratagene) constructs were used as DNA fragment cloning vectors. pPC300(P*flhDC*::*luxCDABE*) and pSA400(P*shlBA*::*luxCDABE*) constructs, used as bioreporters for monitoring the promoter activity of $P\mathit{flhDC}_{Sm}$ and P*shlBA*, respectively, were generated by insertion

of a PCR amplified promoter region upstream of *luxCDABE,* derived from pSB1075 (47), into the pACYC184(Tc^r; Cm^r) vector (9). The PCR primer pairs were: 5'GATATCCAGCCTCAGGCGGAGGG3'/5'GGATCCATTCCCCATCCCGACAG ACTA3' (P*flhDC*$_{Sm}$) and 5'GCAGCAGCGCCGGTATAAGCAC3'/5'ATCGCCAGCGCAGCGGCCAGTT3' (P*shlBA)*. pWC200 (Tc^r, Cm^r) was constructed by PCR cloning of a 1766 bp DNA fragment containing full length *rssA* into a pACYC184 vector, the expression of which was driven by its native promoter. Full length *rssA* PCR was via the primer pair 5'ACCATTATTTTCCAGGTGCT3'/5'ATACAGAGTGTCGATAATTT3'. The pWC201 (Cm^r) was constructed by cloning a 1014 bp DNA fragment containing the complete *rssB* structural gene into pBCSK(Stratagene) which was also expressed via its native promoter. The *rssB* primers were: 5'TGCGGCCTGCGCGCAGGCGC3'/5'AGAATATTGGCGATGCCTGC3'. pWC202 containing the complete 2.5 Kb *rssB-rssA* genes amplified by the primer pair 5'TGCGGCCTGCGCGCAGGCGC3'/5'ATACAGAGTGTCGATAATTT3'.

Swimming motility for *S. marcescens* was examined on motility agar [LB solidified with 0.35% Eiken agar (Eiken, Japan)] by sterile needlepoint inoculation from an overnight culture into the center of the agar plate. Swarming motility for *S. marcescens* was examined on swarming agar plates (LB solidified with 0.8% Eiken agar) by inoculating 5 μl of an overnight broth culture onto the center of the agar plate, with some modifications for *S. typhimurium* SJW1103 and *P. mirabilis* P19 described in the figure legends. Swimming motility and swarming migration distances were recorded at hourly intervals to enable comparisons. Swarm cell differentiation i.e., the overproduction of flagella, cellular elongation and polyploidy, was also examined microscopically as described (29, 35). Briefly, for cell elongation assays, after

overnight culture, bacteria were diluted 1:100 and cultured in 20ml LB broth at 30^{o}C and 37^{o}C with vigorous shaking at 200 rpm. An Olympus BH2 light microscope was used for observation of individual bacterial cells at 1000 X magnification. Images were captured using an SPOT CCD camera integrated with a PC computer (Diagnostics Instruments). Bacterial colonies were examined under light microscopy at a magnification of 1X or 30X using an Olympus SZH microscope. For determining bacterial growth rates, hourly increases in the optical density of broth cultures at 600nm were measured.

Enzymes and chemicals. DNA restriction and modification enzymes were purchased from Roche (Germany). *Taq* polymerase and PCR-related products were obtained from either 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 otherwise indicated, standard protocols were used for DNA/DNA hybridization, plasmid and chromosomal DNA preparation, transformation, electroporation, PCR, restriction digestion, agarose gel electrophoresis, DNA recovery from agarose gels, DNA ligation (40), and conjugation (10). Southern blotting analysis of chromosomal DNA was performed using nylon membranes (HybondN^{+}; Amersham) and a 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 sequences of PCR products were confirmed by sequencing both strands from two or three independent reactions.

Analysis of DNA and protein sequences. Deduced DNA and protein sequences were compared with GenBank DNA or non-redundant protein sequence databases, respectively, using blastn or blastx via the NCBI internet homepage (http://www.ncbi.nlm.nih.gov/). Protein sequence identities were analyzed 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).

Screening of super-swarming mutants by Mini-Tn5 mutagenesis. For the effective transfer of the pUT-miniTn5-Km1 recombinant plasmid from *E. coli* to *S. marcescens* CH-1 by conjugation (10), the *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^{o}C in 10 ml LB broth, with the addition of streptomycin 50 μgml^{-1} and kanamycin 50 μgml^{-1} for *E. coli* cultures. Mating was achieved by mixing 100μl of each bacterial suspension together, followed by the 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^{o}C. The bacteria were then suspended in 5 ml $MgSO_4$ broth, that can be kept at 4 ^{o}C for one week, and spread onto modified LB agar plates (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 ^{o}C. Transconjugants that exhibited swarming migration at 37 ^{o}C were then selected. A total of 6000 colonies were screened, from which 17 were finally selected. Southern blot hybridization using labeled Km gene as a probe was performed to confirm insertion of only one transposon copy in the mutants.

Construction of *S. marcescens* CH-1ΔA and CH-1ΔB insertion deletion mutants. A PCR protocol was designed to introduce specific *Hind*III sites into *rssA* and *rssB* genes for subsequent insertion of a *Hind*III digested Ω (Sm^{r}) gene cassette (39). The Primer pairs 5'GGTTTACGATCGAGACAACC3'/5'GGATCCGTGCGGGCGATCTG3' (*rssA)* and 5'GCATTGGAGCTGGCCGGCTTTA3'/5'GGATCCAAAGTCGTTCGGGCTGT 3' (*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' and 5'GGATCCCATCAGGTCATGCTGGATA3'/5'CTACTCTTTCTTCAGCAAATAGC C3' for *rssA* and *rssB*, respectively, and was 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 (39) as a *Hin*dIII fragment. The three DNA fragments were ligated to the *XbaI/EcoRI* digested suicide vector pUT-mini-Tn5-Km (10). The resultant pUT-*rssA*::Sm and pUT-*rssB*::Sm vectors were selected by streptomycin resistance in 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 pir 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). Mutants with double cross over events were selected by colony PCR screening. Southern hybridization using PCR amplified *rssA* or *rssB* genes as probes was performed to confirm the mutant genotypes (data not shown). The data confirmed that a double-crossover event had

occurred and the new strains were hence designated as CH-1ΔA and CH-1ΔB strains.

Complementation of super-swarming mutants. Transfer of pWC200, pWC201, pWC202 and control plasmid into *S. marcescens* CH-1ΔA or CH-1ΔB mutant via electroporation tested the effect of overexpressing *rssA, rssB or rssB-rssA* in the mutant backgrounds for complementation assays. Transformants that were Cm^r were selected for further characterization of swarming and cell differentiation behavior.

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

Measurement of hemolysin activity, surfactant production and flagellum production. Cell-associated haemolysin (ShlA) activity was assayed as described (27) and calculated in arbitrary hemolytic units (1 unit causing the release of 50 mg hemoglobin/hr in the standard assay). A qualitative assay for surfactant production was performed using the drop collapsing method (34). A semi-quantitative assay for biosurfactant was performed using a TLC based assay as described by Matsuyama *et al*., (37). To quantify the amount of flagellin, cells from surfaces of agar plates were harvested by washing with LB and normalized to a fixed cell mass [OD(A600nm) x cell suspension volume(ml) = 3]. Whole cell lysates were separated by SDS-PAGE

(40) followed by flagellin detection using anti *Salmonella typhimurium* polyclonal antibodies (ViroStat) and ECL kit (Amersham).

Atomic force microscopy(AFM). Bacteria were cultured overnight in LB medium and diluted 1:100 in fresh LB and incubated at 37℃ with vigorous shaking (225rpm) for 2 hr. Prior to imaging, bacteria were gently washed with distilled water and final concentrations 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 distilled water was applied onto a treated slide. After adsorption for 30 minutes, distilled water was added to remove the unadsorbed cell. AFM experiments were performed using SOLVER BIO atomic force microscopy (NT-MDT, Moscow, Russia). The procedures for AFM imaging are described in Hansma *et al.* (20). Silicon nitrite tips were used, with a force constant of 5.5 N/m. Imaging was carried out on dry samples. 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. Both swarming and non-swarming bacteria were seeded onto plates under growth conditions described in the Results section. Extraction of cellular fatty acids was performed by standard procedures (MIDI, USA). Samples were prepared for analysis by the MIDI

GC-FAME analysis system (Microbe Inotech Laboratories, USA). Three independent experiments were performed and results obtained were averages of three experiments (SEM< 10%).

RESULTS

A *Serratia marcescens* mutant defective in both temperature and temporal control of swarming migration. To characterize the genetic determinants of temperature-dependent regulation of swarming behavior in *S. marcescens* CH-1, mini-Tn5 transposon mutagenesis experiments were performed to screen for mutants that swarmed on modified LB plates at 37°C. A total of 17 out of 6000 transconjugants that showed such temperature-independent swarming behavior, were obtained. One of these mutants, designated *S. marcescens* WC100, was selected for further characterization. When inoculated on a 0.8% w/v or even a 1% w/v agar swarming plate and incubated at 37^{o}C, WC100 in contrast to the parent strain CH-1 exhibited swarming motility. This suggested that the temperature-dependent regulation of swarming behavior had been abolished in this mutant.

Analysis of the locus which the transposon interrupted. Conventional digestion and cloning a 3009-bp DNA fragment of the *S. marcescens* CH-1 genome followed by sequencing with primers designed from within the I or O end of the transposon, respectively (10) were used to identify *S. marcescens* WC100 DNA flanking the mini-Tn*5* insertion site. This analysis revealed that the mini-Tn*5* insertion in WC100 was within a 1407bp open reading frame (ORF), designated *orf1*, encoding

a putative 469-amino-acid (52.8 kDa) polypeptide with a calculated isoelectric point of 5.45. Upstream of *orf1* we identified an additional ORF, *orf2*, encoding a putative 219-amino-acid (24.7 kDa) in the same direction. Upstream of *orf2* was a divergent ORF, *orf3*, encoding a putative 231-amino-acid protein (Fig. 1A).

The deduced protein sequences were compared with non-redundant protein sequence databases using blastn or blastx, via the NCBI internet homepage. Computer-assisted homology searches using BLASTP (3) of protein sequences stored in the DDBJ/EMBL/GenBank databanks indicate that ORF1 is highly homologous to members of the two-component sensor kinase family of proteins, including *E. coli* EvgS (22% identity, 41% similarity)(12). Orf2 shares homology to two-component response-regulator elements, including a 39 % identity and 57% homology throughout the alignment to a two-component response regulator PmrA of *Pectobacterium carotovorum* subsp. *carotovorum* (25).

Orf1 and Orf2 were further observed to contain the domain regions highly conserved in two-component sensor kinase and response regulator domains, respectively. Domains such as the H box (Met-242 to Val-257) (a phosphoaccepter domain) and motifs N (Leu-353 to Arg-373), D/F (Gly-385 to Gly-414) and G (Thr-415 to Lys-443) that are associated with the sensor proteins (48) are maintained within RssA (Fig. 1B and 1D) (41). The amino acid phosphorylation domain including

conserved Asp-8, 9, 51 (the putative phosphorylation site) and Lys-101 and the DNA-binding domain (42) which are typical of OmpR family proteins can be identified in RssB (Fig. 1C and 1D). The results therefore suggest that RssA and RssB may function as a typical sensory histidine protein kinase and response regulator pair in *S. marcescens* CH-1. Orf3 shows high homology to a potential oxido-reductase YgfF in *E. coli* (64% identity, 78% homology) (8). We have designated the gene encoded by *orf1*, '*rssA*', for regulation of *Serratia* swarming A. *orf2* is denoted '*rssB*' and *orf3* as $ygfF_{Sm}$. The nucleotide sequence of 3009bp encoding all three ORFs (*rssA*, *rssB* and $ygfF_{Sm}$) has been submitted to the DDBJ/EMBL/GenBank databases under the accession no. AF465237.

Analysis of the deduced amino acid sequence of RssA from the ExPASy proteomics tools at internet homepage http://tw.expasy.org identified two N-terminal hydrophobic regions (Lys5-Trp33 and Gly162-Arg189), indicating that RssA may form two membrane helixes and localize as a inner membrane transmembrane protein (Fig. 1D). A HAMP domain, which is predicted to play an active role in transmembrane signal transduction (6), was identified between 33 and 162. Additionally, the phospho-accepting domain was located near the middle of the protein in the cytoplasm, and a predicted ATPase domain is observed between 353 and 443 towards the C-terminal (Fig. 1D). The predicted N-terminal phosphorylation

domain and carboxyl terminal DNA-binding motif (residues 189–201) of RssB are shown in figure 1D.

Construction of *rssA* and *rssB* insertion-deletion mutants and characterization of swarming-related phenotypes. Mutations were separately introduced into *rssA* and *rssB* in CH-1 cells by insertion deletion via homologous recombination, to form the CH-1ΔA and CH-1ΔB mutants, respectively. The phenotypes of WC100, CH-1ΔA and CH-1ΔB mutants were characterized by their growth dynamics, cell elongation pattern, cell densities and temperature-regulated swarming behaviors. No differences were observed between WC100 and *S. marcescens* CH-1ΔA in any of these analyses. Effect of temperature on swarming of CH-1, CH-1ΔA and CH-1ΔB on LB swarming plates were shown in figure 2A.

By analysis of the swarming fronts of *S. marcescens* CH-1ΔA, CH-1ΔB and CH-1 at 30°C, using the method of Gygi *et al*. (18), the swarming behavior and velocity of CH-1ΔA, CH-1ΔB and CH-1 was monitored. Depending on the moisture content of the plates, CH-1 cells typically spent 4-5 h (at 30°C) in the initial lag phase, which was approximately one hour longer than that of the CH-1ΔA and CH-1ΔB mutants. Following the initiation of swarming, CH-1ΔA cells migrate at an increasingly higher velocity than CH-1 cells (Fig. 2B). 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), whereas *S. marcescens* CH-1ΔA increased from 2 mm/h to 5 mm/h during the same period (Fig. 2B). Due to a shorter lag phase and higher migration velocity, CH-1ΔA mutants therefore translocate a greater distance in the same period than CH-1 parental cells. Swarming of CH-1ΔA mutants was essentially the same at 37°C as that observed at 30°C (Fig. 2 A and 2B).

CH-1ΔB exhibited a slower swarming phenotype than CH-1ΔA at both temperatures (Fig. 2 A and 2B). Both CH-1ΔA and CH-1ΔB cells swarmed at an agar concentration of up to 1% w/v at 30°C (Fig. 2C), and on an average measured about 10% shorter than that of CH-1 cells following growth on 0.8% w/v LB agar surface (data not shown). When incubated at 30°C, both CH-1ΔA and CH-1ΔB cells initiated swarming earlier than CH-1 cells and required ca. 50-fold fewer cells to commence migration (3.4×10^6 /50mm^2 versus 1.7×10^8/50mm^2 cells to initiate swarming). Similar cell-population density dependent initiation of swarming for both strains was also observed at 37°C (data not shown).

Transformation of plasmid pWC200 (pACYC184::*rssA*) expressing the *rssA* gene restored CH-1ΔA to the non swaming phenotype characteristic of the parent strain CH-1 incubated at 37°C (data not shown), confirming *rssA* as the gene responsible for the super-swarming phenotype of CH-1ΔA. This observation also suggested that RssA may function as a negative regulator of swarming at 37°C. On the

other hand, *rssB-rssA* (pWC202, pACYC184::*rssB-rssA*) in trans, but not *rssB* (pWC201, pACYC184::*rssB*) alone, inhibited CH-1ΔB swarming at 37°C (data not shown), suggesting *rssB-rssA* may form an operon and both genes were involved in swarming regulation.

Factors such as swimming motility, the flagellar master operon *flhDC* and biosurfactant which are known to be essential for swarming migration in *Serratia* were examined to determine whether they are controlled by RssAB. A swimming assay at 37°C showed that the motilities of CH-1, CH-1ΔA and CH-1ΔB were similar (data not shown). Monitoring of $flhDC_{Sm}$ promoter activity by plasmid pPC300 (P$flhDC_{Sm}$::*luxCDABE*) and Western blot analysis for quantification of flagellin production on 0.8% w/v LB agar surfaces also did not identify any measurable differences between mutants and parent (data not shown). These observations indicate that, compared with the parent strain CH-1, expression of the $flhDC_{Sm}$ regulon is unaffected in either *rssA* or *rssB* mutant cells on swarming agar surfaces.

The super-swarming phenotype of CH-1ΔA or CH-1ΔB cells may be due to over-production of biosurfactant, hence reducing the surface friction on swarming plates. A drop-collapsing test (24) was performed to qualitatively assay the surface tension of stationary phase culture suspensions from CH-1ΔA, CH-1ΔB and CH-1 cells grown on LB swarming plates at 37°C. No reproducible differences were

observed, (data not shown). Similar results were obtained using thin layer chromatography (TLC) analysis(36). Taken together, the swarming behaviour of *S. marcescens* CH-1ΔA and CH-1ΔB at 37°C is not due to an increase in surface flagellar motility or biosurfactant production.

RssAB negatively regulates haemolysin production in *S. marcescens*. The cell-surface associated haemolysin, ShlA has been identified as a major virulence factor in *S. marcescens* (28). To determine whether RssAB regulated haemolysin production we assayed both haemolytic activity (Fig. 3A) and *shlA* expression (Fig. 3B). Figure 3A shows that haemolytic activity in CH-1ΔA and CH-1ΔB cells was higher than in CH-1 cells at both 30°C (1.7-fold and 1.4-fold increase for CH-1ΔA and CH-1ΔB respectively) and 37°C (1.6-fold and 1.4-fold increase for CH-1ΔA and CH-1ΔB respectively) after 5 h of growth into the late log phase. Further experiments using a plasmid-borne *shlBA::luxCDABE* fusion (pSA400) as a reporter for haemolysin gene (*shlA*) activity revealed a 4 to 8 fold and 3 to 4 fold increase in *shlBA* promoter activity in CH-1ΔA and CH-1ΔAB, respectively, compared with the parent strain (Fig. 3B). This data indicate that RssA-RssB is a negative regulator of *shlBA* promoter activity.

RssA and RssB are involved in the determination of cellular surface architecture. The detailed cellular surface topography of log phase CH-1, CH-1ΔA

and CH-1ΔB cells was studied using atomic force microscopy (AFM) by systematically targeting regions within the cellular envelope. A larger scan area (3 x 3 μm^2) was initially utilized to select suitable cells for higher-resolution imagery. In each of the CH-1, CH-1ΔA and CH-1ΔB strains, more than twenty individual vegetative cells were examined. Although some variations do exist in the calculation of exact cellular size, a clear trend emerged that, on average, the calculated surface texture parameter RMS (Root-Mean-Square**)** values for length and width of CH-1, CH-1ΔA and CH-1ΔB cells were 1981 x 734 nm, 1518 x 555 nm and 1644 x 680 nm, respectively. This was consistent with our previous observation that the average cell dimensions of both CH-1ΔA and CH-1ΔB mutants were shorter than CH-1 cells under 1000X light microscopy.

The cell surface topography of both CH-1ΔA and CH-1ΔB mutants changed when cultured in LB at 37°C (Fig. 4A), and characterization of these changes are shown in figure 4B. CH-1 showed a mean valley-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. Both CH-1ΔA and CH-1ΔB, however, 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 suggest that RssA and RssB are either directly or indirectly involved in

determination of *S. marcescens* cell surface topology.

Inhibition of *S. marcescens* swarming by saturated fatty acids via RssA. Although CH-1 cells do not swarm on 0.8% w/v LB agar plates at 37°C, we found that CH-1 did exhibit ample migratory swarming on defined MGM plates (M9 minimal growth medium containing 0.8% Eiken agar) and on MGM plates containing casamino acids (1%) at both 30 and 37°C (Fig. 5A), suggesting that a higher environmental temperature per se is not inhibitory for *S. marcescens* CH-1 swarming under these culture conditions. We also noted that CH-1 swarming began very quickly after it was transferred from LB broth cultures to either MGM or MGM-casamino acid plates at both temperatures (within 1 h and at a much lower colony cell population density). This observation is very striking when compared with the cell-population density dependent swarming behavior of CH-1 at 30°C and inhibition of swarming at 37°C on normal LB swarming plates, indicating that the absence of specific nutrients in the minimal medium promotes the early appearance of swarming. Swarming assays on MGM-casamino acids (1%)/yeast extract (1%) plates further revealed that whereas CH-1ΔA swarmed at both temperatures, CH-1 swarming was completely inhibited at 37°C (Fig. 5A). These observations suggested that some components (or their metabolic derivatives) of yeast extract inhibited parental CH-1 cell swarming at 37°C. We therefore examined the effects of amino acids, glucose,

mono- and di- saccharides, saturated (SFAs) and unsaturated (UFAs) fatty acids, iron starvation and pH on the swarming behavior of *S. marcescens* CH-1 (Fig. 5A and data not shown). These data revealed that apart from certain fatty acids, none of the other medium components or environmental conditions influenced the CH-1 swarming. We found that whereas UFAs exerted no significant swarming-inhibition effects on CH-1, swarming was completely inhibited by the addition of lauric or myristic acids and slightly inhibited by the presence of palmitic or stearic acids (Fig. 5A). None of the fatty acids tested showed significant inhibitory effects towards CH-1ΔA swarming (Fig. 5A).

To determine whether this SFA-dependent phenomenon was dose-dependent, myristic acid at concentrations of 0.01%, 0.005%, 0.0025% and 0.00125% (w/v) was added to MGM-casamino acid plates, followed by swarming assays at 37°C. While there was no effect on CH-1ΔA, the swarming lag times of CH-1 cells are delayed with increasing concentrations of myristic acid and swarming was completely inhibited by 0.01% (w/v) myristic acid (Fig. 5B). A similar myristic acid inhibitory effect was also observed at 30°C on both LB swarming plates and MGM-casamino acid plates, with lauric acid also exhibiting similar inhibitory effects (Fig. 5A). 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 and such effect is likely to be mediated via RssA.

Swarming behavior is closely related to "swarming" or "non-swarming" cellular fatty acid profiles. We postulated that the cellular fatty acid profile of CH-1 might be affected by environmental fatty acids which may in turn influence RssA-RssB signaling leading to either a swarming or a non-swarming phenotypes. To this end, the cellular fatty acid profiles (expressed as percentage of the total fatty acids of CH-1 cultured at 37°C) of cells grown under different conditions were evaluated by extraction and analysis using gas chromatography (GC). These growth conditions included swarming conditions (MGM plates and MGM casamino acids (1%) plates) and non-swarming conditions (LB plates and MGM-casamino acids (1%)/yeast extracts (1%) plates). Major observed changes in the profiles of cells grown under non-swarming conditions were increases in the content of lauric acid (12:0), myristic acid (14:0) and palmitic acid (16:00) (Fig. 5C; Table 1). For cells grown in swarming conditions, a concomitant increase in unsaturated fatty acids (especially 18:1 w7c) and branch-chain fatty acids (especially 15:0 anteiso) were observed (Fig. 5C; Table 1). These results highlight the link between cellular fatty acid composition and swarming behavior at constant temperatures in *S. marcescens*.

The observed changes in fatty acid profile under different nutrient conditions at 37°C that coincide with the swarming or non-swarming behavior of CH-1 supported

our hypothesis of a direct correlation between fatty acid profile and swarming regulation. To confirm this finding, we collected both swarming and non-swarming CH-1 cells cultured under different growth conditions for fatty acid profile analysis. CH-1 swarming cells were collected at 30°C from LB swarming plates, MGM-casamino acids (1%)/yeast extract (1%) plates, MGM-casamino acids (1%)-myristoleic acids (0.01%), MGM-casamino acids(1%)-palmitoleic acids (0.01%) and MGM-casamino acids (1%)-oleic acids (0.01%) (30°C), and at 30°C and 37°C from MGM plates and MGM-casamino acids (1%) plates. For CH-1 non-swarming cells, cells were collected at 30°C from MGM-casamino acids (1%)-lauric acid (0.01%) plate, MGM-casamino acids (1%)-myristic acid (0.01%) plate and MGM-casamino acids (1%)-palmitic acid (0.01%) plates, and at 37°C from LB swarming plates.

The results are summarized in Table 1 and indicate that for CH-1 cells growing at non-swarming conditions, including either a higher incubation temperature or in the presence of growth nutrients containing SFAs, the ratio of high melting point 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 in cells grown under swarming conditions (Table 1). The percentage of 12:0 showed the most significant difference (Fig. 5C). In contrast, CH-1 cells grown under swarming conditions, including lower incubation temperatures or media

lacking SFAs, contain higher percentages of UFAs and branch-chain fatty acids, with 18:1 w7c and 15:0 anteiso contents showing the greatest variations (Fig. 5C). The calculated average ratio was 2.7 for swarming cells and 6.2 for non-swarming cells. The fatty acid profiles of the two super-swarming mutants, CH-1ΔA and CH-1ΔB, inoculated either on LB swarming plates (30°C or 37°C), MGM, MGM-casamino acids or CH-1ΔA on MGM-casamino acids-myristic acid (0.01%) (37°C) were basically similar (Table 1).

Taken together, these results suggest that the *S. marcescens* CH-1 cellular fatty acid profile is affected by both temperature and medium fatty acid content and that the profile is closely related to swarming phenotypes, with swarming cells containing relatively more UFAs and branch-chain fatty acids and non-swarming cells containing more SFAs. Thus the two different cellular fatty acid profiles could be classified as "swarming" and "non-swarming" profiles.

RssA-RssB regulate cellular fatty acid composition. To determine whether RssA-RssB influence cellular fatty acid composition, the cellular fatty acid compositions of the corresponding mutants was determined by GC. Table 1 shows that the fatty acid profile of CH-1 cells cultured on an LB agar surface at 37°C is significantly different from both CH-1ΔA and CH-1ΔB swarming cells which showed swarming fatty acid profiles at this temperature. For both mutants, the ratio of

saturated- to non- saturated fatty acids is 1.8 and 2.6 respectively whereas it is 5.6 for the parent strain (Table 1). Furthermore, although CH-1, CH-1ΔA and CH-1ΔB all showed swarming fatty acid profiles at 30°C, their overall fatty acid composition differed (Table 1). Further experiments analyzing the fatty acid profile of the three strains also found variance between them when cultured on MGM media at 37°C (Table 1). These data indicate that, consistent with swarming regulation, cellular fatty acid profiles are regulated via RssA-RssB.

Myristic acid inhibits swarming in both *P. mirabilis* and *S. typhimurium*. To determine whether myristic acid also influenced swarming migration in other Gram negative bacteria, *P. mirabilis* P19 and *S. typhimurium* LT2 at 37°C were inoculated onto 0.5 % or 2.0 % w/v Eiken agar LB plates supplemented with myristic acid at a concentration of 0.01% (w/v). Figure 6 shows that myristic acid inhibited the swarming of *P. mirabilis* and *S. typhimurium* LT2. These results indicate that fatty acid-mediated inhibition of swarming motility is conserved among these Gram negative bacterial genera.

DISCUSSION

Although there is an extensive literature on bacterial swarming motility, the signals sensed and underlying genetically programmed molecular mechanisms are still far from being fully understood. Here we have identified a two component regulatory system, RssA-RssB which negatively controls swarming in response to SFAs in *S. marcescens* strain CH-1. Mutation of the sensor, RssA or the response regulator, RssB confers a "vigorous-swarming" phenotype on LB agar. This phenotype is reminiscent of that observed by Belas *et al.*,(7) and Liaw et al., (31) where precocious swarming mutants of *P. mirabilis* BB2000 and P19 which were defective in the temporal control of swarming migration initiated swarming some 60 min earlier than the parent. In each case, the corresponding mutation was localized to a gene termed *rsbA*, which codes for a putative sensor kinase. Despite the functional similarities and their homology to the sensor kinase protein family, RsbA is composed of 897 amino acids and RssA is composed of 469 amino acids, and both proteins do not have significant homology or identity following amino acid sequence comparisons, apart from a low percentage of identity within certain short polypeptide sequences. *P. mirabilis* BB2000 and P19, in contrast to *S. marcescens* CH-1, swarm on LB plates at 37^{o}C (7, 30), suggesting that RsbA and RssA may not be functionally equivalent at least with respect to their temperature dependency. In *E. coli* K12

swarming is regulated via a locus termed *yojN-rcsB-rcsC* (44) with the same genetic organization as that found in *P. mirabilis* i.e. *rsbA-rscB-rscC* (7) where YojN is a homologue of RsbA. This suggests that *P. mirabilis* and *E. coli* may use a common signal transduction pathway to regulate swarming.

It is currently not clear whether RssA senses SFAs *per se* or the change in membrane lipid composition in response to feeding of fatty acids, although the regulatory effect of SFAs on *Serratia* swarming is identified in this report. If, like *E. coli* and *Salmonella*, *Serratia* takes up fatty acids it is offered and incorporates these into its membranes, this will affect membrane fatty acid composition. Although not clear yet, evidences suggested that changes in cellular fatty acid profile which may either directly or indirectly be involved in altering the ratio of SFAs to UFAs may be sensed by RssA and RssB. For both mutants, the ratio of saturated- to non- saturated fatty acids is 1.8 and 2.6 respectively whereas it is 5.6 for the parent strain when cultured on LB plates at 37 ^{o}C (Table 1). These fatty acid profiles correspond to "swarming" and "non-swarming" phenotypes respectively suggesting that changes in membrane fluidity may be sensed via the RssA-RssB system, which may either directly or indirectly be involved in altering the ratio of SFAs to UFAs. Furthermore, these changes in fatty acid profiles and hence swarming phenotype can also be modified by temperature shifts or by the provision of a SFA. For example, in LB at

30°C, CH-1 has a "swarming" fatty acid profile i.e. a ratio of saturated- to non-saturated fatty acids of 2.0 whereas at 37 °C, it fails to swarm and has a fatty acid ratio of 5.6 (Table 1). On a minimal medium (MGM plus casamino acids), CH-1 swarms at both temperatures but swarming can be inhibited by the exogenous provision of lauric acid or myristic acid. These convert the fatty acid ratio from 2.5 (i.e. swarming profile) to 7.2 and 5.1 (i.e. non swarming profiles) respectively. However, myristic acid was unable to inhibit swarming by the *rss*A mutant at 37°C, which retained a swarming fatty acid profile with a ratio of 3.1. Thus as a putative sensor kinase, RssA is likely to sense changes either in membrane fluidity or the ratio of saturated to unsaturated fatty acids or even variations in the levels of specific fatty acids either exogenously or within the cell envelope. To further clarify SFAs function, a *S. marcescens* mutant defective in fatty acid transport would have to be constructed and effect of SFAs on its swarming being characterized.

Since swarming and haemolysin production are upregulated in both *rssA* and *rssB* mutants, it is possible that the response regulator RssB, when phosphorylated via RssA, functions either as a repressor or as an activator of a repressor. RssA in *S. marcescens* may sense different signaling states under varying growth temperatures and nutritional conditions which lead to changes in membrane fatty acid composition in order to maintain cellular homeostasis. This may be accomplished by regulating the

ratio of kinase to phosphatase activities of RssA, such that a kinase-dominant state is present at high growth temperatures or in the case of SFA-rich nutrient availability. RssA is predicted to possess two transmembrane domains and either the periplasmic or cytoplasmic region may function to propagate a conformational change that is sufficient to significantly alter its activity. Such a conformational change is likely to be governed by the physical state of the membrane lipid bilayer. Lipids in biological membranes are usually maintained in a fluid, liquid-crystalline state (46) and the correct physical state of membrane lipids is essential for optimal membrane structure and function. Temperature markedly affects membrane lipid composition in order to maintain an appropriate liquid crystalline state. As the growth temperature increases, the proportion of high-melting-point fatty acids in the membrane lipids increases. This change from an less ordered to an ordered membrane composition may stimulate activation of kinase activity in RssA, resulting in autophosphorylation of the conserved histidine contained within its transmitter domain followed by phosphotransfer to RssB and consequently down-regulation of down-stream genes involved in swarming regulation. While the down-stream genes involved in modulating the *S. marcescens* cellular fatty acid profile remains to be established, it is conceivable that RssA-RssB is involved in a regulatory loop where either SFAs or temperature upshifts influence swarming behavior via the action of RssA and RssB,

which also regulate cellular fatty acid composition. Since swarming migration in *P. mirabilis* and *S. typhimurium*, in common with *S. marcescens,* can be inhibited by SFAs, it is possible that the regulation of swarming through the control of membrane fluidity via a two component system is conserved in Gram negative bacteria. Indeed in *S. typhimurium*, a number of transposon mutants with defects in putative two component regulatory systems and which exhibit aberrant swarming have been described (45). Amongst these, a mutant defective in the *rsbA* homologue *yjoN* showed substantially reduced flagellin levels.

An important question that remains is the identity of the molecular mechanism underlying swarming regulation by RssA-RssB. Given that the *Serratia rssA* and *rssB* mutants do not exhibit defects in known components of the swarming pathways such as biosurfactant and *flhDC*, these findings imply the existence of additional signalling pathways involving RssAB. Indeed, given that the average cell lengths of CH-1ΔA and CH-1ΔB mutants are about 10% shorter than parental CH-1 cells and that $flhDC_{Sm}$ expression is not affected, this suggests that cell length determination is also regulated by the RssA-RssB system, independent of $flhDC_{Sm}$. Furthermore, the cell surface topography of *rssA* and *rssB* mutants is markedly different to that of the CH-1 wild type as revealed by AFM. In *P. mirabilis*, an acidic capsular polysaccharide (Cmf-CPS) facilitates swarming migration (17). Cmf-CPS is proposed to enhance

growth medium surface fluidity by extracting water from the agar so reducing friction and aiding bacterial surface translocation. Capsule synthesis under the control of two-component system is also reported to be involved in regulation of swarming in *E. coli* K12 (43). In *S. marcescens*, so far we have no evidence that the extracellular polysaccharide (EPS) has contribution to swarming motility. Further experiments will be required to determine whether RssA-RssB regulates swarming via the control of EPS synthesis.

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Table 1. Percentage of cellular fatty acids in both swarming and non-swarming *S. marcescens* cells. Strains including *S. marcescens* CH-1, CH-1ΔA, CH-1ΔB were inoculated under different culture conditions, leading to either swarming or non-swarming phenotypes. The composition of cellular fatty acids was analyzed by gas chromatography (MIDI, USA). Results are the means of three independent determinations (SEM<10%). "Ratio*" means the ratio of the 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 (anteiso)	16:00	18:1 (w7c)	18:00	Ratio*
swarming cells								2.7 (ave)
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/Cas/YE		3.55	7.42	3.69	29.54	17.21		1.9
30CH-1/MGM/Cas/ C14:1 Δ9		2.42	9.2	2.81	30.82	10.89	1.99	3.2
30CH-1/MGM/Cas/ C16:1 Δ9		3.62	8.63	3.06	31.48	14.84		2.4

30CH-1/MGM/Cas/ C18:1 Δ9		3.13	8.18	2.47	29.52	13.64	2.17	2.7
30CH-1ΔA/LB		2.71	6.21	1.9	29.21	15.85		2.1
30CH-1ΔB/LB		3.41	6.2	2.47	27.37	17.63		1.8
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-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/C14		1.12	13.14	1.49	35.42	14.96	0.85	3.1
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
non-swarming cells								6.2 (ave)
30CH-1/MGM/Cas/ C12	1.76	24.75	5.97	1.16	30.01	5.66	0.9	7.2
30CH-1/MGM/Cas/ C14	1.63	3.65	12.78	0.6	36.25	8.15	0.73	5.1
30CH-1/MGM/Cas/ C16	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

1

* 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. MGM, M9 minimal growth medium; YE, yeast extract; Cas, cas amino acids.

FIGURE LEGENDS

Fig. 1. Location of the Tn*5* insertion in the *S. marcescens* chromosome and analysis of the RssA and RssB protein sequences. The insertion point of the mini-Tn*5*-Km transposon in *rssA* region is shown in (A). Nucleotide sequences containing *rssA* and *rssB* has been submitted to the DDBJ/EMBL/GenBank databases under accession no. AF465237. Alignment of RssA and RssB domains with those of related bacterial sensor proteins (40) in which conserved sequences are highlighted are shown in B and C, respectively. Asterisks indicated identical amino acids and encircled asterisks indicate essential amino acid residues H248 in RssA and A51 in RssB for phosphorylation and phosphorelay reaction. (D) Predicted of RssA and RssB protein topology by program DAS in the ExPASy Molecular Biology Server of the Swiss Institute of Bioinformatics (SIB) (http://tw.expasy.org).

Fig. 2. Swarming assays of *S. marcescens* CH-1, CH-1ΔA and CH-1ΔB cells. Assays were carried out at both 30°C and 37°C on 0.8% LB swarming plates (A). Comparisons of swarming velocities (16) are shown in (B). Cells were grown overnight in LB broth, washed once in 1 × PBS, and 5-µl of inoculum was added to the center of the plate. Plates were then incubated for 12 h, during which time the extent of swarming migration was assessed. Swarming assays of the three *S.*

marcescens strains after 10 h incubation on 1.2% LB agar plates at 30°C is shown in (C). 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 respective means ($n = 4$).

Fig. 3. *S. marcescens* CH-1ΔA and CH-1ΔB cells show increased haemolysin activity at 30°C and 37°C. Specific haemolysin activity (26) 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°C and 37°C are shown in (A) and (B), respectively. Symbols **A**: (■)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 **B**: (■)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. 4. Cellular surface topology changes in *S. marcescens* CH-1ΔA and CH-1ΔB cells. *S. marcescens* CH-1, CH-1ΔA and CH-1ΔB cells were subjected to surface structure characterization by atomic force microscopy (AFM). Cells were cultured overnight in LB broth followed by 1:100 dilution in fresh LB for further culturing at

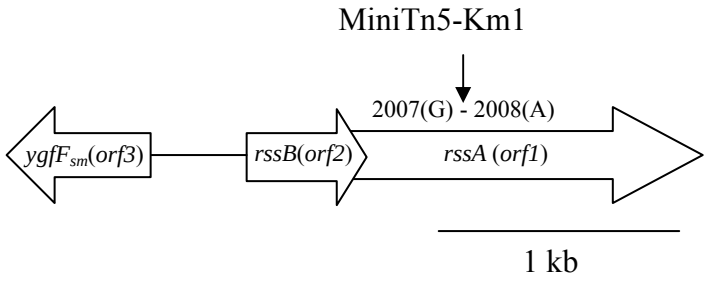
37 °C for 2 hours. Cells were harvested and treated to enable AFM observations (19). Cell surface images, and characterization of at least 20 cells from each strain, are shown in (A) and (B) respectively.

Fig. 5. Effect of fatty acids on swarming of *S. marcescens* CH-1 and CH-1ΔA, and swarming phenotype is closely related to cellular fatty acid profile. (A) Swarming assays of CH-1 and CH-1ΔA cells performed on 0.8% MGM plates either with or without additives at both 30°C and 37°C. (B) Swarming of CH-1, but not CH-1ΔA is inhibited by myristic acid in a dose-dependent way at 37°C after 12 hours of incubation. (C) Average percentage of cellular fatty acids between swarming (white bars) and non-swarming (black bars) cells. The cellular fatty acid profiles of CH-1, CH-1ΔA and CH-1ΔB cells grown under different growth conditions leading to either swarming or non-swarming phenotypes were analyzed by Gas Chromatography. Results were the mean of 3 independent experiments (SEM<10%).

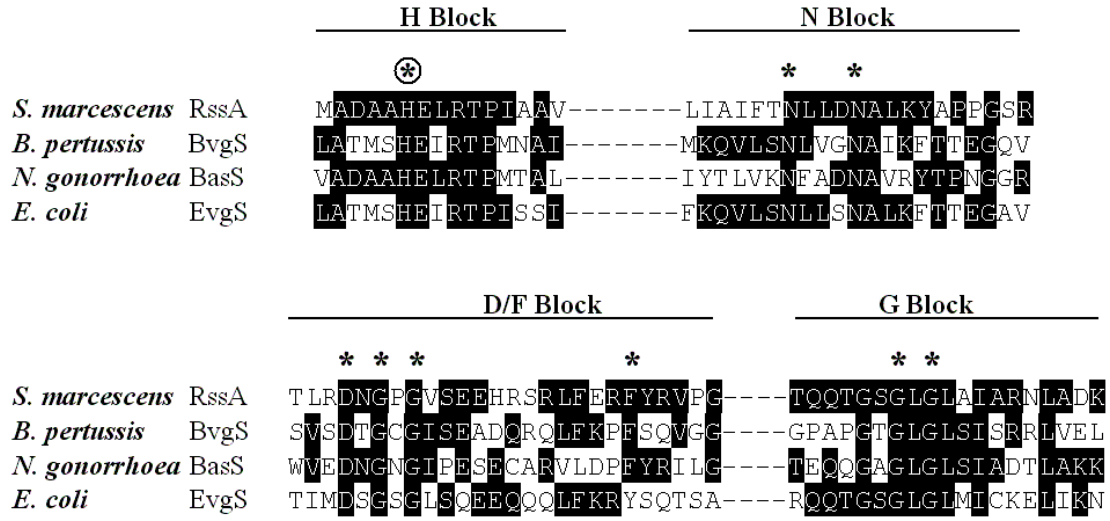
Fig. 6. Effect of myristic acid on swarming migration of *S. typhimurium* SJW1103 and *P. mirabilis* P19. *S. typhimurium* SJW1103 and *P. mirabilis* P19 were inoculated on 0.5% and 2% LB Eiken agar plates, respectively, containing myristic acid at a concentration of 0.01% (w/v). This was followed by swarming assays at 37 °C for 10 hr.

Fig.1

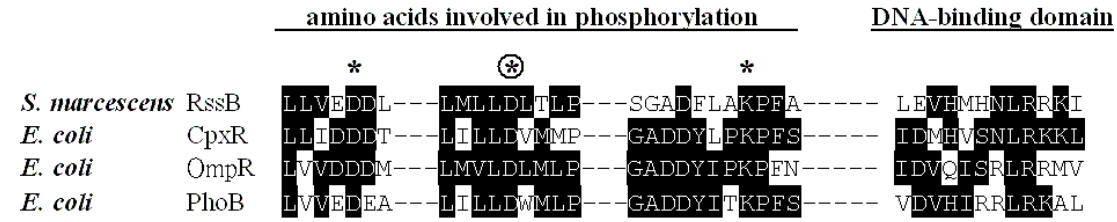
A


MiniTn5-Km1
2007(G) - 2008(A)
ygfF$_{sm}$(orf3)
rssB(orf2)
rssA (orf1)
1 kb

B


H Block
N Block
S. marcescens RssA
B. pertussis BvgS
N. gonorrhoea BasS
E. coli EvgS
D/F Block
G Block

C


amino acids involved in phosphorylation
DNA-binding domain
S. marcescens RssB
E. coli CpxR
E. coli OmpR
E. coli PhoB

D

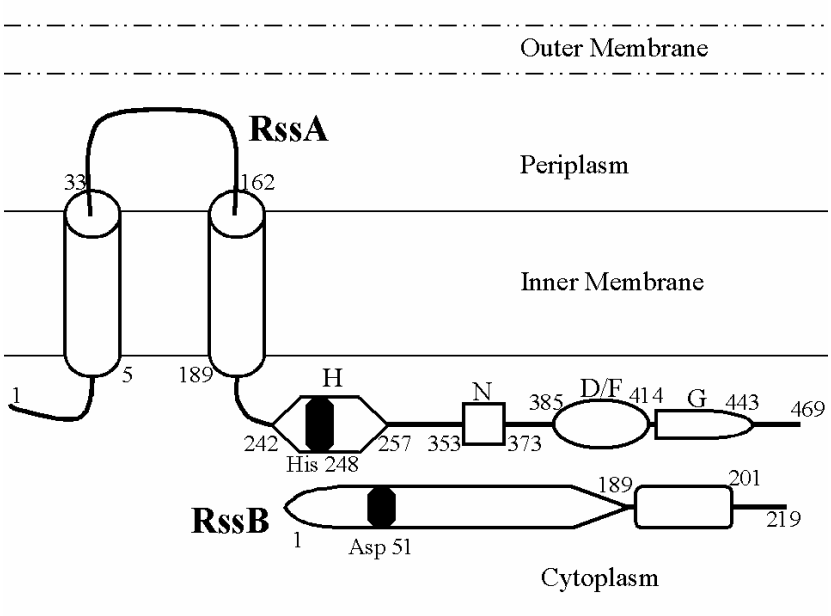
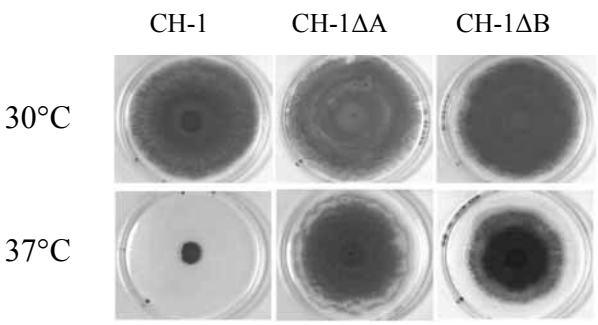
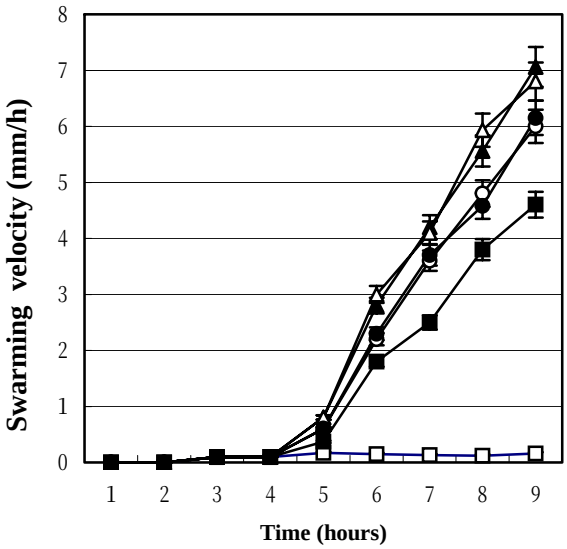

Outer Membrane
RssA
Periplasm
33
162
Inner Membrane
1
5
189
H
N
385
D/F
414
G
443
469
242
His 248
257
353
373
189
201
RssB
1
Asp 51
219
Cytoplasm

Fig. 2

A


CH-1
CH-1ΔA
CH-1ΔB
30°C
37°C

B


Swarming velocity (mm/h)
Time (hours)
0
1
2
3
4
5
6
7
8
1
2
3
4
5
6
7
8
9

C

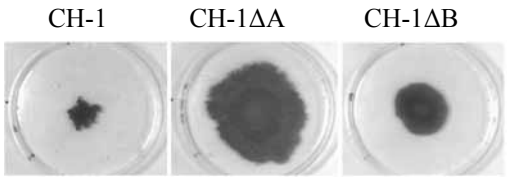
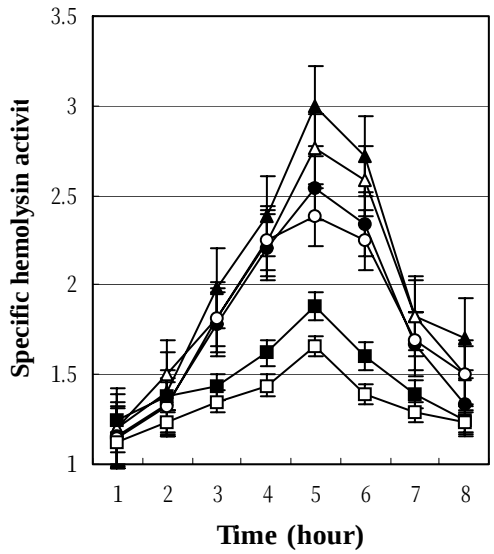

CH-1
CH-1ΔA
CH-1ΔB

Fig. 3

A


Specific hemolysin activit
3.5
3
2.5
2
1.5
1
1 2 3 4 5 6 7 8
Time (hour)

B

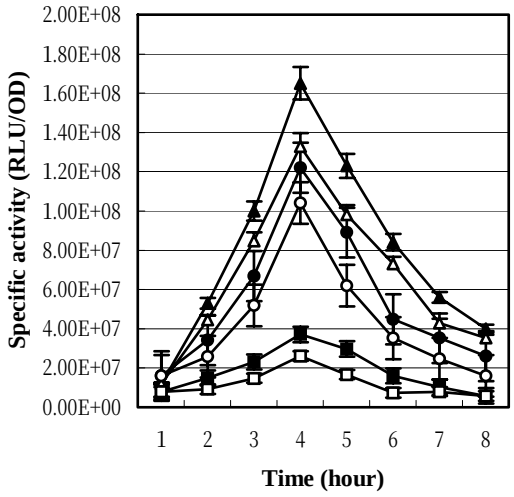

Specific activity (RLU/OD)
2.00E+08
1.80E+08
1.60E+08
1.40E+08
1.20E+08
1.00E+08
8.00E+07
6.00E+07
4.00E+07
2.00E+07
0.00E+00
1 2 3 4 5 6 7 8
Time (hour)

Fig. 4

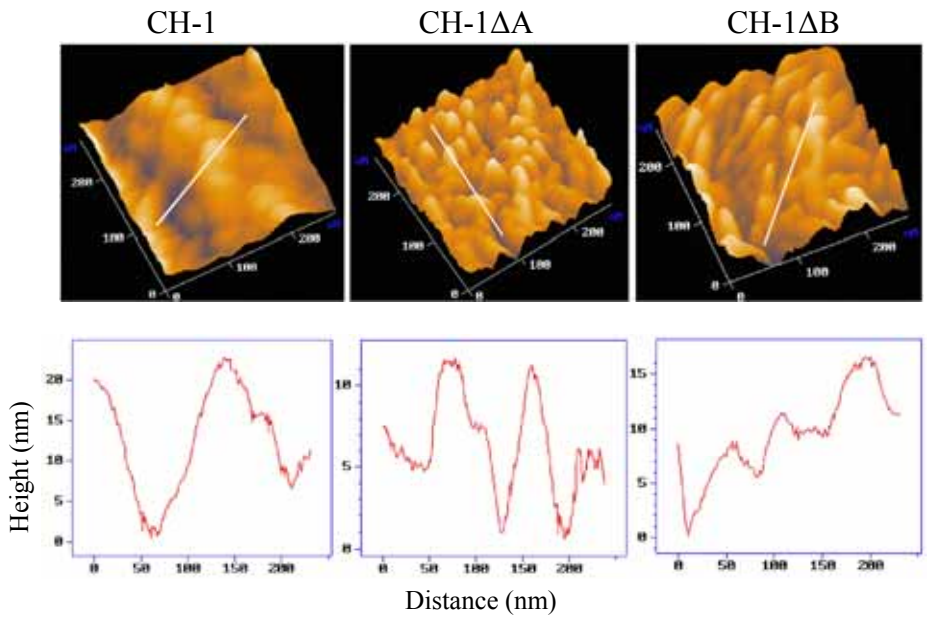
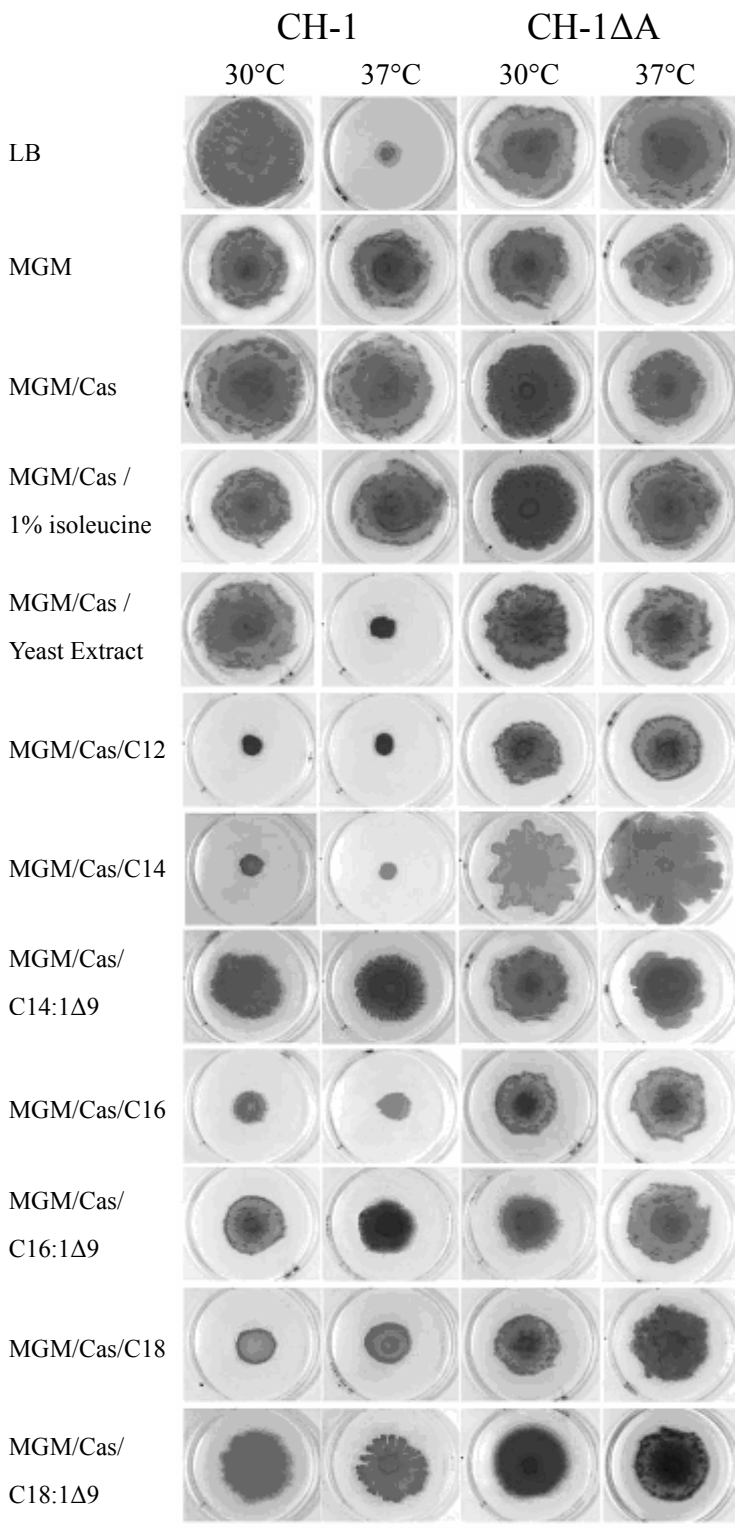
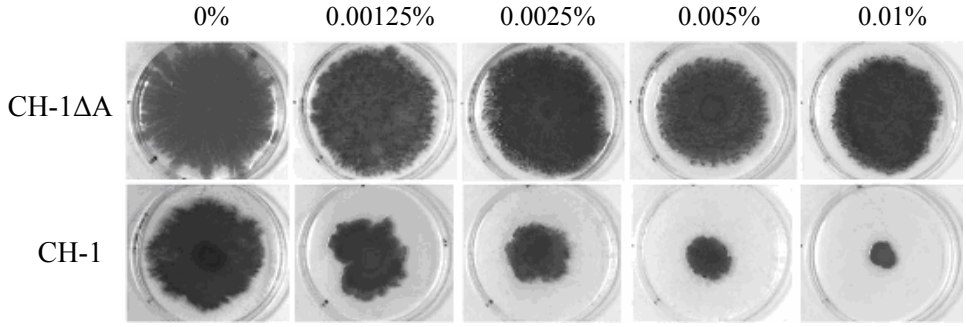


Fig. 5

A


CH-1
CH-1ΔA
30°C
37°C
30°C
37°C
LB
MGM
MGM/Cas
MGM/Cas /
1% isoleucine
MGM/Cas /
Yeast Extract
MGM/Cas/C12
MGM/Cas/C14
MGM/Cas/
C14:1Δ9
MGM/Cas/C16
MGM/Cas/
C16:1Δ9
MGM/Cas/C18
MGM/Cas/
C18:1Δ9

B


0%
0.00125%
0.0025%
0.005%
0.01%
CH-1ΔA
CH-1

C

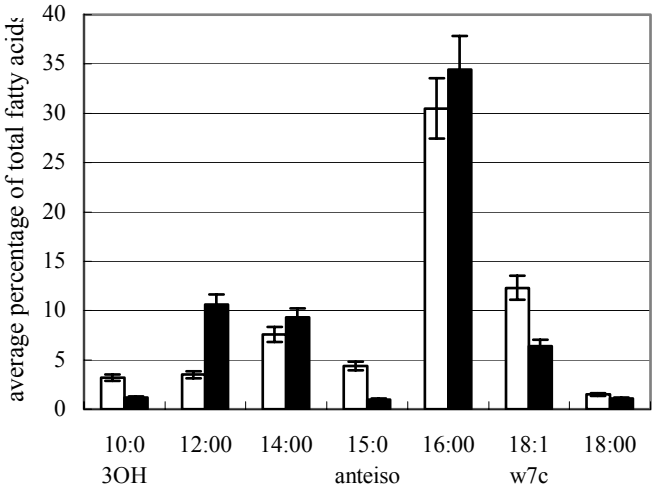

average percentage of total fatty acids
40
35
30
25
20
15
10
5
0
10:0 3OH
12:00
14:00
15:0 anteiso
16:00
18:1 w7c
18:00

Fig. 6