行政院國家科學委員會專題研究計畫　成果報告

Serratia marcescens 表面移行相關基因功能之研究

計畫類別：個別型計畫
計畫編號：NSC93-2314-B-002-281-
執行期間：93 年 08 月 01 日至 94 年 07 月 31 日
執行單位：國立臺灣大學醫學院醫事技術學系暨研究所

計畫主持人：賴信志

報告類型：精簡報告

處理方式：本計畫可公開查詢

中　華　民　國 94 年 12 月 12 日

JOURNAL OF BACTERIOLOGY, May 2005, p. 3407–3414 Vol. 187, No. 10
0021-9193/05/$08.00+0 doi:10.1128/JB.187.10.3407–3414.2005

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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Received 30 November 2004/Accepted 8 February 2005

Serratia marcescens **swarms at 30°C but not at 37°C on a nutrient-rich (LB) agar surface. Mini-Tn*5* 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 swarms at 30°C. Analysis of this mutant revealed that the transposon was 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 the two mutants exhibited similar swarming phenotypes on LB swarming agar, in which swarming not only occurred at 37°C but also initiated at a lower cell density, on a surface with a higher agar concentration, and more rapidly than the swarming of the parent strain at 30°C. Both mutants also exhibited increased hemolysin 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 fatty acid profiles of the parent and the *rssA* and *rssB* mutants grown at 30°C or 37°C and under different nutrition conditions revealed a relationship between cellular fatty acid composition and swarming phenotypes. The cellular fatty acid profile was also observed to be affected by RssA and RssB. SFA-dependent inhibition of swarming was also observed in *Proteus mirabilis*, 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.**

When inoculated onto L broth (LB) solidified with 0.8% (wt/vol) 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 hyperflagellated swarm cells which migrate coordinately and rapidly away from the colony (10). 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 coordinated, multicellular effort involving groups of differentiated cells functioning through close cell-cell interactions (10).

Swarming is the result of the regulated expression of gene networks required to initiate the complex processes underlying the required morphological and physiological changes (10, 11, 15, 16). 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 (AHL)-dependent quorum-sensing system are global regulators of flagellar motility and cell population density, respectively (12, 18, 25, 26). Cellular differentiation is only one part of this process, which requires a lag period prior to the commencement of swarming migration, during which cellular proliferation occurs up to the required population density and a large amount of biosurfactant is produced (1, 18, 25). An additional layer of regulation of surface migration may also be exerted through the posttranscriptional regulator RsmA (4). When overexpressed, *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 *rsmA* in *Pseudomonas aeruginosa* has been reported to negatively regulate AHL synthase expression (27).

Many regulatory pathways involved in control of swarming

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remain to be characterized at the molecular level. For example, exposure of cells to a surface 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 been characterized yet. *S. marcescens* swarming is known to be a temperature-dependent behavior which occurs at 30°C but not at 37°C. Bacteria inoculated onto identical plates and incubated at 37°C do not initiate swarming even after 48 h of culture and form only small defined colonies. This may be a consequence of reduced flagellar motility (26) and/or a reduction in biosurfactant production following the temperature upshift. To begin unraveling the underlying regulatory mechanism(s), we utilized mini-Tn*5* 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 (17) which we termed RssA-RssB. Here we present evidence showing that swarming of *S. marcescens* is inhibited by the presence of saturated fatty acids (SFAs) and that this effect is mediated via the RssA-RssB two-component system. The cellular fatty acid composition, hemolysin production, and cell surface topography were also shown to be regulated by RssA-RssB.

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 (26). No AHL quorum-sensing signals were detected in CH-1 cells. *S. marcescens* CH-1ΔA and CH-1ΔB are *rssA* and *rssB* knockout mutants, respectively, that were constructed via insertion of an Ω cassette (Sm^r) from pHP45Ω (28) and subsequent homologous recombination. *Escherichia coli* strains JM109, CC118 λ*pir*, S17-1 λ*pir*, Top10 F′, and XL-1 Blue were cultured at 37°C and *S. marcescens* was cultured at 30°C in L broth (Difco) (29) unless indicated otherwise. M9 salt solution (29) was used to make minimal growth medium (MGM), into which 0.8% Eiken agar (Eiken, Japan) was incorporated to make MGM plates. In the case of mixed cultures (e.g., conjugations), incubation was at 30°C.

The pUT::mini-Tn*5*-Km1 construct (9) was used in the transposon mutagenesis assays for screening precocious-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. pSA400(P*shlBA*::*luxCDABE*), used as the bioreporter for monitoring the promoter activity of P*shlBA*, was generated by insertion of a PCR-amplified *shlBA* promoter region upstream of *luxCDABE*, derived from pSB1075 (32), into the pACYC184 (Tc^r Cm^r) vector (8). pWC200 (Tc^r Cm^r) was constructed by PCR cloning a 1,766-bp DNA fragment containing full-length *rssA* into a pACYC184 vector, the expression of which was driven by its native promoter. pWC201 (Cm^r) was constructed by cloning a 1,014-bp DNA fragment containing the complete *rssB* structural gene into pBCSK (Stratagene), which was also expressed via its native promoter. pWC202 contained the complete PCR-amplified 2.5-kb *rssB-rssA* genes. Swarming motility of *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 each agar plate. Cellular elongation and polyploidy were examined microscopically as described previously (22, 26). To determine bacterial growth rates, hourly increases in the optical densities at 600 nm of broth cultures were determined.

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 (United States) or Takara Biomedicals (Japan). Other laboratory grade chemicals were purchased from Sigma (United States), Merck (Germany), or BDH (United Kingdom).

Recombinant DNA techniques. Unless indicated otherwise, 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 (29), and conjugation (9). Southern blotting 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 using pCRII and a TA cloning kit (Invitrogen). The sequences of the primers used in this study are available from us. DNA sequencing and analysis were performed using a Perkin-Elmer model 377 Autosequencer with a *Taq* DyeDeoxy 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 and nonredundant protein sequence databases, respectively, using blastn or blastx via the National Center for Biotechnology Information internet homepage (http://www.ncbi.nlm.nih.gov/).

Screening of precocious-swarming mutants by mini-Tn*5* mutagenesis. For effective transfer of the pUT-mini-Tn*5*-Km1 recombinant plasmid from *E. coli* to *S. marcescens* CH-1 by conjugation, the protocols of de Lorenzo and Timmis (9) were used. After conjugation, bacteria were suspended in 5 ml $MgSO_4$ broth and spread onto modified LB agar plates (0.04% NaCl, 2% glycerol, 0.5% yeast extract, 1% Bacto Tryptone [Difco], 0.8% Eiken agar, 50 μg ml^{-1} kanamycin, and 13 μg ml^{-1} tetracycline), which was followed by incubation at 37°C. Transconjugants that exhibited swarming migration at 37°C were then selected. A total of 6,000 colonies were screened, from which 17 colonies were finally selected. Southern blot hybridization using a labeled kanamycin 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 (18) was designed to introduce specific HindIII sites into *rssA* and *rssB* genes for subsequent insertion of a HindIII-digested Ω (Sm^r) gene cassette (28). For gene inactivation mutagenesis by homologous recombination, the respective plasmid constructs were transferred from the permissive host strain *E. coli* S17- 49 λ*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-crossover 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 designated CH-1ΔA and CH-1ΔB, respectively.

Complementation of precocious-swarming mutants. Transfer of pWC200, pWC201, pWC202, and a control plasmid into *S. marcescens* CH-1ΔA or CH-1ΔB via electroporation was used to test 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. An Autolumat LB 953 luminometer (EG&G, Germany) with the "replicates" program was used for bioluminescence measurement. For all procedures we followed the protocols supplied by the manufacturer.

Measurement of hemolysin activity. Cell-associated hemolysin (ShlA) activity was assayed as described previously (20) and was expressed in arbitrary hemolytic units (1 U caused the release of 50 mg hemoglobin/h in the standard assay).

AFM. Bacteria were cultured overnight in LB, diluted 1:100 in fresh LB, and incubated at 37°C with vigorous shaking (225 rpm) for 2 h. Prior to imaging, bacteria were gently washed with distilled water, and final concentrations of 10^4 to 10^5 cells/ml were used for atomic force microscopy (AFM) experiments. AFM experiments were performed using SOLVER BIO atomic force microscopy (NT-MDT, Moscow, Russia). The procedures for AFM imaging have been described by Hansma et al. (14). Silicon nitrite tips were used, with a force constant of 5.5 N/m. Imaging was carried out with dry samples. AFM images were generated at line frequencies between 1 and 3 Hz, with 256 lines per image. Images were obtained using semicontact (tapping) mode AFM. The data were analyzed with the SMENA software (Advanced Technologies Center, Russia) (standard error of the mean, <5%).

Analysis of cellular fatty acids by the gas chromatography-fatty acid methyl ester method. Both swarming and nonswarming bacteria were seeded onto plates under different growth conditions. Extraction of cellular fatty acids was performed by standard procedures (MIDI, United States). Samples were prepared for analysis by the MIDI gas chromatography-fatty acid methyl ester analysis system (Microbe Inotech Laboratories, United States). Three independent experiments were performed, and the results obtained were expressed averages of three experiments (standard error of the mean, <10%).

Nucleotide sequence accession number. The 3,009-bp nucleotide sequence containing *rssA* and *rssB* has been deposited in the DDBJ/EMBL/GenBank databases under accession no. AF465237.

RESULTS

***S. marcescens* mutant defective in temperature control of swarming migration.** To characterize the genetic determinants of temperature-dependent regulation of swarming behavior in *S. marcescens* CH-1, mini-Tn*5* transposon mutagenesis experiments were performed to screen for mutants that swarmed on modified LB agar plates at 37°C. A total of 17 of 6,000 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% (wt/vol) agar or even a 1% (wt/vol) agar swarming plate and incubated at 37°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, cloning, and sequencing of a 3,009-bp DNA fragment flanking the mini-Tn*5* insertion site in the *S. marcescens* WC100 genome were performed. The mini-Tn*5* insertion in WC100 was revealed to be within a 1,407-bp open reading frame, 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 open reading frame, *orf2*, encoding a putative 219-amino-acid (24.7-kDa) polypeptide in the same direction.

Computer-assisted homology searches using BLASTP (3) of protein sequences stored in the DDBJ/EMBL/GenBank databanks indicated that Orf1 is highly homologous to members of the two-component sensor kinase family of proteins, including *E. coli* EvgS (22% identity, 41% similarity) (10). Orf2 exhibits homology with two-component response-regulator elements, including 39% identity and 57% homology throughout the alignment to the two-component response regulator PmrA of *Pectobacterium carotovorum* subsp. *carotovorum* (19). We designated *orf1 rssA* (regulation of *Serratia* swarming A), and *orf2* was designated *rssB*.

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 the WC100, CH-1ΔA, and CH-1ΔB mutants were characterized by their growth dynamics, cell elongation patterns, cell densities, and temperature-regulated swarming behaviors. No differences were observed between WC100 and *S. marcescens* CH-1ΔA in any of these analyses. While swarming of CH-1 was inhibited at 37°C on LB swarming plates, CH-1ΔA and CH-1ΔB swarming was normal (Fig. 1A).

The swarming behavior and velocity of CH-1ΔA, CH-1ΔB, and CH-1 at 30°C were monitored by using the method of Gygi et al. (13). Depending on the moisture contents of the plates, CH-1 cells typically spent 4 to 5 h in the initial lag phase, which was approximately 1 h longer than the CH-1ΔA and CH-1ΔB mutants spent. Following initiation of swarming, CH-1ΔA cells migrated at an increasingly higher velocity than CH-1 cells (Fig. 1B). The average velocity for *S. marcescens* CH-1 swarming increased from 1 mm/h (between 5 and 6 h) to 3.3 mm/h (between 7 and 8 h), whereas the average velocity for *S. marcescens* CH-1ΔA swarming increased from 2 mm/h to 5 mm/h during the same period (Fig. 1B). Due to a shorter lag phase and the higher migration velocity, CH-1ΔA mutant cells therefore translocated a greater distance in the same period than the CH-1 parental cells. Swarming of CH-1ΔA mutants was essentially the same at 37°C as at 30°C (Fig. 1A and B).

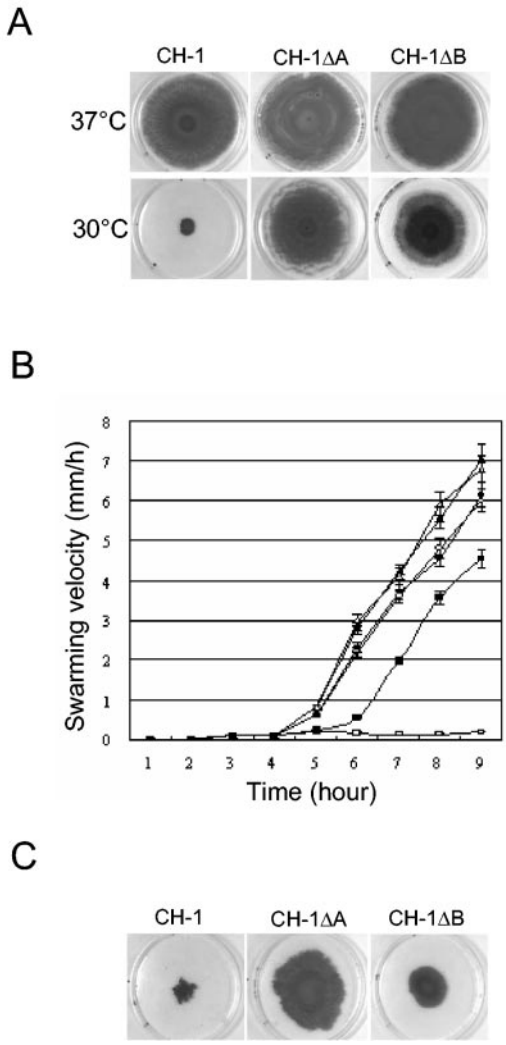

FIG. 1. Swarming assays with *S. marcescens* CH-1, CH-1ΔA, and CH-1ΔB cells. (A) Assays carried out at both 30°C and 37°C on 0.8% agar LB swarming plates. (B) Comparison of swarming velocities (13). Cells were grown overnight in LB and washed once in 1× phosphate-buffered saline, and 5 μl of inoculum was added to the center of each plate. The plates were then incubated for 12 h, during which the extent of swarming migration was assessed. Symbols: ■, CH-1, 30°C; □, CH-1, 37°C; ▲, CH-1ΔA, 30°C; △, CH-1ΔA, 37°C; ●, CH-1ΔB, 30°C; ○, CH-1ΔB, 37°C. The error bars indicate the standard errors of the means (<5%; $n = 4$). (C) Swarming results for the three *S. marcescens* strains after 10 h of incubation on LB plates containing 1.2% agar at 30°C.

CH-1ΔB exhibited a slower swarming phenotype than CH-1ΔA at both temperatures (Fig. 1A and B). Both CH-1ΔA and CH-1ΔB cells swarmed at agar concentrations of up to 1% (wt/vol) at 30°C (Fig. 1C), and on an average they looked shorter than CH-1 cells following growth on LB containing

0.8% (wt/vol) agar, as determined by light microscopy at a magnification of ×1,000.

Transformation of plasmid pWC200 (pACYC184::*rssA*) expressing the *rssA* gene restored CH-1ΔA to the nonswaming phenotype characteristic of parent strain CH-1 incubated at 37°C (data not shown), confirming that *rssA* was the gene responsible for the precocious-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 that *rssB-rssA* may form an operon and both genes are involved in swarming regulation.

RssAB negatively regulates hemolysin production in *S. marcescens*. The cell-surface-associated hemolysin ShlA has been identified as a major virulence factor in *S. marcescens* (21). To determine whether RssAB regulated hemolysin production, we assayed both hemolytic activity (Fig. 2A) and *shlA* expression (Fig. 2B). Figure 2A shows that the hemolytic activity in CH-1ΔA and CH-1ΔB cells was higher than that in CH-1 cells at both 30°C (1.7-fold and 1.4-fold increases for CH-1ΔA and CH-1ΔB, respectively) and 37°C (1.6-fold and 1.4-fold increases for CH-1ΔA and CH-1ΔB, respectively) after 5 h of growth to the late log phase. Further experiments using a plasmid-borne *shlBA*::*luxCDABE* fusion (pSA400) as a reporter for hemolysin gene (*shlA*) activity revealed four- to eightfold and three- to fourfold increases in *shlBA* promoter activity in CH-1ΔA and CH-1ΔAB, respectively, compared with the parent strain (Fig. 2B). These data indicate that RssA-RssB is a negative regulator of *shlBA* promoter activity.

RssA and RssB are involved in determination of cellular surface architecture. The detailed cellular surface topography of log-phase CH-1, CH-1ΔA, and CH-1ΔB cells was studied using AFM. A clear trend emerged, which showed that, on average, the calculated surface texture parameter root mean square values for the length and width of CH-1, CH-1ΔA, and CH-1ΔB cells were 1,981 by 734 nm, 1,518 by 555 nm, and 1,644 by 680 nm, respectively (standard error of the mean, <5%). This was consistent with our previous observation that the average cell dimensions of both the CH-1ΔA and CH-1ΔB mutants were less than those of CH-1 cells as determined by light microscopy at a magnification of ×1,000.

The cell surface topography of both CH-1ΔA and CH-1ΔB mutants changed when they were cultured in LB at 37°C (Fig. 3A), and the changes are shown in Fig. 3B. CH-1 showed a mean valley-to-peak distance of 11.900 nm, with a maximal value of 25.900 nm, and a surface roughness of 2.652 nm. CH-1ΔA and CH-1ΔB, however, showed mean valley-to-peak distances of 9.075 and 7.775 nm, respectively, with maximal values of 17.700 and 14.675 nm, respectively, and surface roughness values of 1.999 and 1.545 nm, 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. CH-1 was observed to exhibit ample migratory swarming on defined MGM plates with or without Casamino Acids (1%) added at both 30 and 37°C (Fig. 4A). 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. 4A). These observations suggested that a higher environmental temperature per se is not inhibitory for CH-1 swarming, and some components (or their metabolic derivatives) of yeast extract inhibited parental CH-1 cell swarming at 37°C. Subsequent assays showed that apart from certain fatty acids, none of the other medium components tested, such as amino acids, sugars, or iron compounds, inhibited CH-1 swarming (Fig. 4A and data not shown). Whereas unsaturated fatty acids (UFAs) exerted no significant swarming inhibition effects on CH-1, swarming was completely inhibited by the addition of lauric or myristic acid and was slightly inhibited by palmitic or stearic acid at a final concentration of 0.01% (wt/vol) (Fig. 4A). The fatty acids tested did not have significant inhibitory effects on CH-1ΔA and CH-1ΔB swarming (Fig. 4A and data not shown). The myristic acid-dependent phenome-

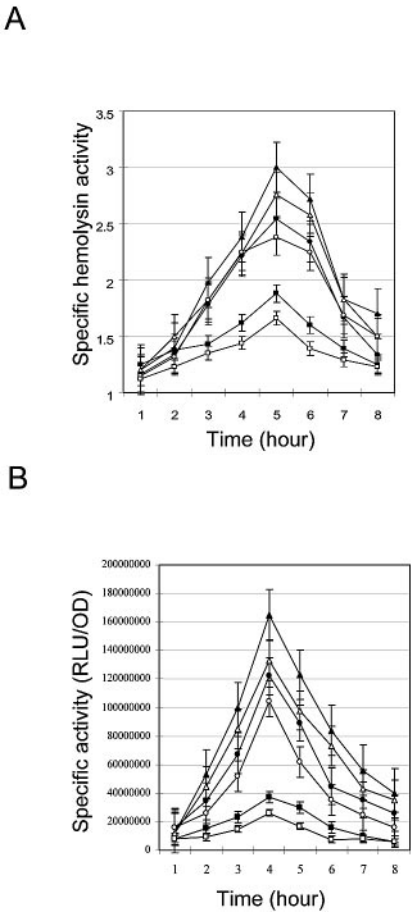

FIG. 2. *S. marcescens* CH-1ΔA and CH-1ΔB cells show increased hemolysin activity at 30°C and 37°C. The specific hemolysin activity (20) (A) and the transcriptional activity of the hemolysin gene *shlA* expressed as the specific bioluminescence activity (B) of CH-1(pSA400), CH-1ΔA(pSA400), and CH-1ΔB(pSA400) were monitored at 30°C and 37°C. (A) Symbols: ■, CH-1, 30°C; □, CH-1, 37°C; ▲, CH-1ΔA, 30°C; △, CH-1ΔA, 37°C; ●, CH-1ΔB, 30°C; ○, CH-1ΔB, 37°C. (B) Symbols: ■, CH-1(pSA400), 30°C; □, CH-1(pSA400), 37°C; ▲, CH-1ΔA(pSA400), 30°C; △, CH-1ΔA(pSA400), 37°C; ●, CH-1ΔB(pSA400), 30°C; ○, CH-1ΔB(pSA400), 37°C. RLU, relative light units; OD, optical density.

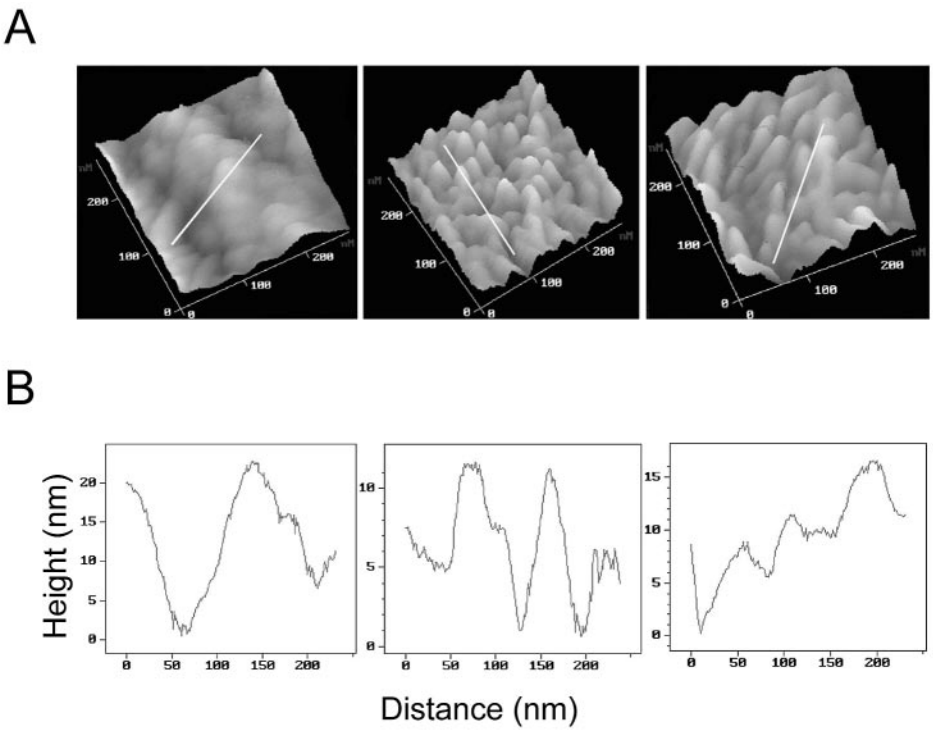

FIG. 3. Cellular surface topology changes in *S. marcescens* CH-1ΔA and CH-1ΔB cells. *S. marcescens* CH-1, CH-1ΔA, and CH-1ΔB cells were subjected to surface structure characterization by AFM. Cells were cultured overnight in LB, followed by 1:100 dilution in fresh LB for further culturing at 37°C for 2 h. Cells were harvested and treated to enable AFM observation (14). (A) Cell surface images. (B) Characterization of at least 20 cells of each strain.

non was further observed to be dose dependent at 37°C. The swarming lag times of CH-1 cells were delayed with increasing myristic acid concentrations from 0.00125% to 0.01% (wt/vol) (Fig. 4B). A similar myristic acid inhibitory effect was also observed at 30°C on both LB swarming plates and MGM-Casamino Acid plates, and lauric acid exhibited similar inhibitory effects (Fig. 4A). Taken together, these data indicate that in addition to a temperature shift, initiation of swarming is regulated by the availability of SFAs in a concentration-dependent manner and that this effect is likely to be mediated via RssA and RssB.

Swarming behavior is closely related to cellular fatty acid profiles regulated by RssA-RssB. 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 nonswarming phenotype. To examine this, the cellular fatty acid profiles expressed as percentages of the total cellular fatty acids of cells grown under swarming and nonswarming conditions were determined by extraction and analysis using gas chromatography. The culture conditions tested included LB or MGM-Casamino Acids plates supplemented with different fatty acids. The results are summarized in Table 1 and indicate that for CH-1 cells growing under nonswarming conditions, including either at 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 + 18:1w7c]) was much higher than the ratio for cells grown under swarming conditions (Table 1), with the percentage of 12:0 showing the most significant difference (Fig. 4C). In contrast, CH-1 cells grown under swarming conditions, including lower incubation temperatures or media lacking SFAs, contained higher percentages of UFAs and branched-chain fatty acids, with the 18:1w7c and 15:0 anteiso contents showing the greatest variations (Fig. 4C). The calculated average ratios were 2.7 for swarming cells and 6.2 for nonswarming cells.

Comparison of the cellular fatty acid compositions of the corresponding mutants showed that the fatty acid profile of CH-1 cells cultured on an LB agar surface at 37°C was significantly different from the profiles of both CH-1ΔA and CH-1ΔB swarming cells, which showed swarming fatty acid profiles at this temperature (Table 1). For these mutants, the ratios of saturated to nonsaturated fatty acids were 1.8 and 2.6, respectively, whereas the ratio was 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 compositions differed (Table 1). Analysis of the fatty acid profiles of the three strains also revealed differences between the strains when they were cultured on MGM media at 37°C (Table 1).

Taken together, the data showed that the *S. marcescens* CH-1 cellular fatty acid profile is affected by both temperature and the medium fatty acid content and that the profile is closely related to the swarming phenotype, with swarming cells containing relatively more UFAs and branched-chain fatty acids and nonswarming cells containing more SFAs. The data also suggested that consistent with swarming regulation, cellular fatty acid profiles are regulated via RssA-RssB.

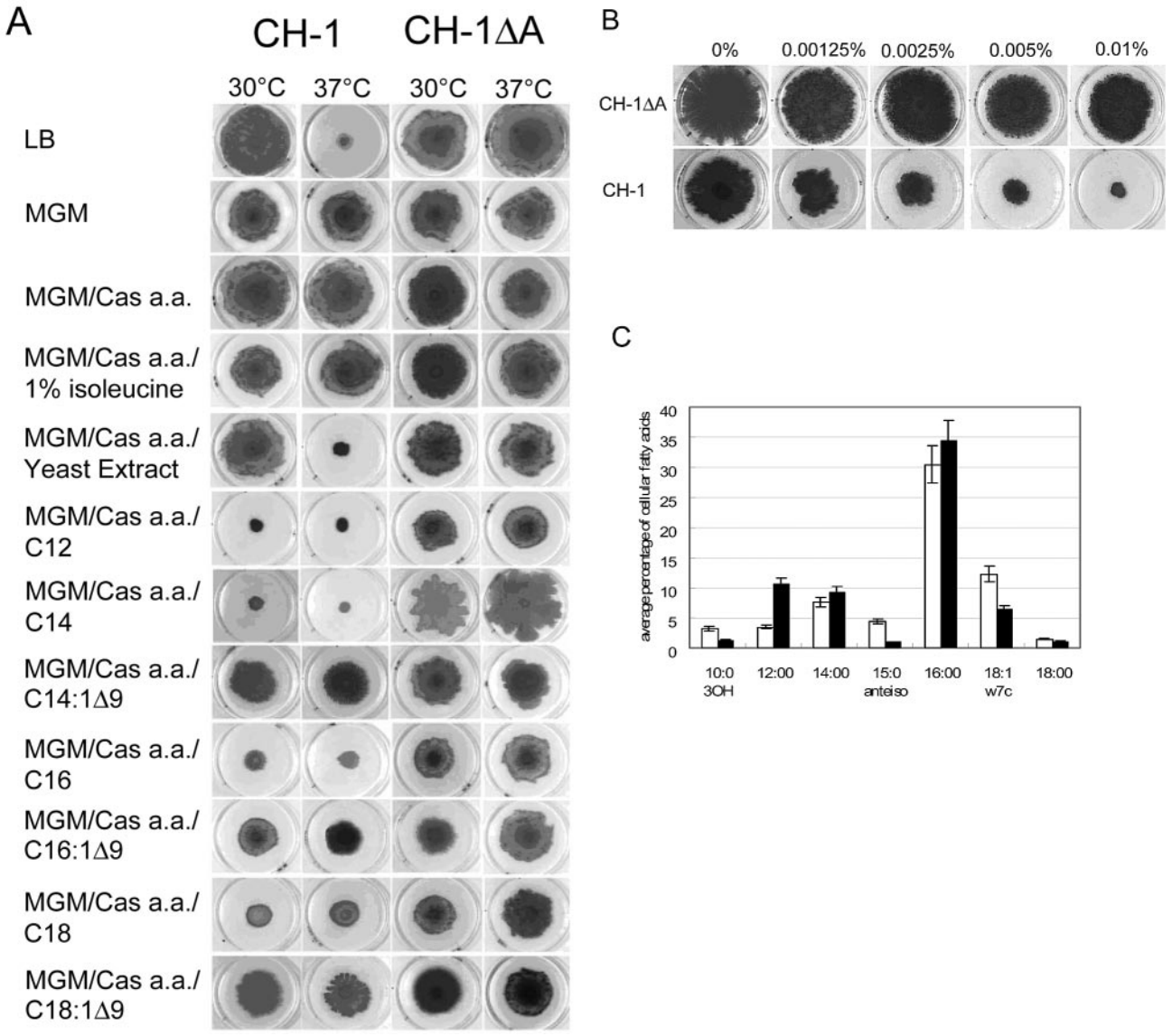

FIG. 4. Effects of fatty acids on the swarming of *S. marcescens* CH-1 and CH-1ΔA and the swarming phenotype are closely related to the cellular fatty acid profile. (A) Swarming assays with CH-1 and CH-1ΔA cells performed on MGM-0.8% agar plates either with or without additives at both 30°C and 37°C. Cas a.a., Casamino Acids. (B) Swarming of CH-1, but not swarming of CH-1ΔA, is inhibited by myristic acid in a dose-dependent manner at 37°C after 12 h of incubation. (C) Average percentages of cellular fatty acids for swarming cells (open bars) and nonswarming cells (solid bars). The cellular fatty acid profiles of *S. marcescens* cells grown under different growth conditions leading to either swarming or nonswarming phenotypes were analyzed by gas chromatography. The data are the means for three independent experiments (standard errors of the means, <10%).

DISCUSSION

Although there is extensive literature on bacterial swarming motility, the signals sensed and the underlying genetically programmed molecular mechanisms are still far from being fully understood. Here we 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 "precocious-swarming" phenotype on LB agar. This phenotype is reminiscent of that observed by Belas et al. (7) and Liaw et al. (23), who observed that precocious-swarming mutants of *Proteus 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 the homology to the sensor kinase protein family, RsbA is composed of 897 amino acids and RssA is composed of 469 amino acids, and the two proteins do not have significant homology or identity as determined by amino acid sequence comparisons, apart from a low level of identity within certain short polypeptide sequences. *P. mirabilis* BB2000 and

TABLE 1. Percentages of cellular fatty acids in both swarming and nonswarming *S. marcescens* cells[a]

Cells	Temp (°C)	Strain	Medium[b]	% of total fatty acids							Ratio[c]
				10:0 3OH	12:0	14:0	15:0 anteiso	16:0	18:1w7c	18:0	
Swarming	30	CH-1	LB		4.26	7.52	2.95	25.96	17.08	2.92	2.0
	30	CH-1	MGM		2.9	8.08	6.72	30.42	10.22	0.83	2.5
	30	CH-1	MGM-Cas		3.44	6.08	6.41	28.66	8.82		2.5
	30	CH-1	MGM-Cas-YE		3.55	7.42	3.69	29.54	17.21		1.9
	30	CH-1	MGM-Cas-C14:1 Δ9		2.42	9.2	2.81	30.82	10.89	1.99	3.2
	30	CH-1	MGM-Cas-C16:1 Δ9		3.62	8.63	3.06	31.48	14.84		2.4
	30	CH-1	MGM-Cas-C18:1 Δ9		3.13	8.18	2.47	29.52	13.64	2.17	2.7
	30	CH-1ΔA	LB		2.71	6.21	1.9	29.21	15.85		2.1
	30	CH-1ΔB	LB		3.41	6.2	2.47	27.37	17.63		1.8
	37	CH-1	MGM		2.02	9.09	5.57	38.99	6.83		4.0
	37	CH-1	MGM-Cas	3.21	1.9	7.17	1.38	33.53	7.58	1.19	3.6
	37	CH-1ΔA	LB		4.28	7.04	7.48	25.92	13.58		1.8
	37	CH-1ΔA	MGM			6.47	11.26	33.95	5.6		2.4
	37	CH-1ΔA	MGM-Cas			5.99	1.64	32.47	7.7	1.64	4.3
	37	CH-1ΔA	MGM-Cas/C14		1.12	13.14	1.49	35.42	14.96	0.85	3.1
	37	CH-1ΔB	LB		2.91	7.49	3.75	33.98	13.57		2.6
	37	CH-1ΔB	MGM		8.14	7.67	9.17	33.64	7.42		3.0
	37	CH-1ΔB	MGM-Cas	3.18	2.32	7.65	1.34	35.01	8.11	1.02	3.6
Nonswarming	30	CH-1	MGM-Cas-C12	1.76	24.75	5.97	1.16	30.01	5.66	0.9	7.2
	30	CH-1	MGM-Cas-C14	1.63	3.65	12.78	0.6	36.25	8.15	0.73	5.1
	30	CH-1	MGM-Cas-C16	1.63	11.12	7.35	0.89	35.63	6.42	0.89	6.2
	37	CH-1	LB		7.75	8.25	1.35	29.8	7.27	2.63	5.6
	37	CH-1	MGM-Cas-YE	1.08	5.48	10.28	0.6	36.33	5.82	1.12	7.1

[a] *S. marcescens* CH-1, CH-1ΔA, CH-1ΔB were inoculated under different culture conditions, leading to either swarming or nonswarming phenotypes. The composition of cellular fatty acids was analyzed by gas chromatography (MIDI, united States). The data are the means for three independent determinations (standard errors of the means, <10%).

[b] MGM, M9 minimal growth medium; YE, yeast extract; Cas, Casamino Acids.

[c] Calculated by dividing the total percentage of 12:0, 14:0, 16:0, and 18:0 by the total percentage of 10:0 3OH, 15:0 anteiso, and 18:1w7c. The average ratios for swarming and nonswarming cells were 2,7 and 6.2, respectively.

P19, in contrast to *S. marcescens* CH-1, swarm on LB plates at 37°C (7, 23), suggesting that RsbA and RssA may not be functionally equivalent at least with respect to their temperature dependence. However, since swarming migration in *P. mirabilis*, like that in *S. marcescens*, can also be inhibited by SFAs through RsbA (24) and since the research results of Armitage et al. (5, 6) showed that there are cell wall and membrane composition differences between filamentous swarming cells and short nonswarming cells of *P. mirabilis*, conserved regulatory mechanisms may still exist. In *Salmonella enterica* serovar Typhimurium, a number of transposon mutants which have defects in putative two-component regulatory systems and which exhibit aberrant swarming have been described (31). Among these, a mutant defective in the *rsbA* homologue *yojN* showed substantially reduced flagellin levels. It is thus possible that the regulation of swarming through the control of membrane fluidity via a two-component system is conserved in gram-negative bacteria.

Currently, it is not clear whether RssA senses SFAs per se or the change in membrane lipid composition in response to addition of fatty acids. It is conceivable that RssA-RssB is involved in a regulatory loop in which either SFAs or temperature upshifts influence swarming behavior through changing the cellular fatty acid profile and altering the ratio of SFAs to UFAs. This may be sensed by RssA and RssB, which also regulate cellular fatty acid composition. For example, in LB at 30°C, CH-1 has a "swarming" fatty acid profile (i.e., a ratio of saturated- to nonsaturated 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 exogenous provision of lauric acid or myristic acid. These acids change the fatty acid ratio from 2.5 (i.e., a swarming profile) to 7.2 and 5.1 (i.e., nonswarming profiles) respectively. However, myristic acid was unable to inhibit swarming by the *rssA* 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 in 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 SFA function, an *S. marcescens* mutant defective in fatty acid transport would have to be constructed and the effect of SFAs on its swarming would have to be characterized.

An important question that remains is the identity of the molecular mechanism underlying swarming regulation by RssA-RssB. In *P. mirabilis*, an acidic capsular polysaccharide (Cmf-CPS) is proposed to enhance growth medium surface fluidity by extracting water from the agar, thus reducing friction and aiding swarming migration (13). In *E. coli* K-12, capsule synthesis and swarming have been reported to be regulated by a *yojN-rcsB-rcsC* signal transduction system (30). The *rsbA-rscB-rscC* (7) locus has been found to be responsible for swarming regulation in *P. mirabilis*, in which RsbA is a homologue of YojN. These findings suggest that *P. mirabilis* and *E. coli* may use a common signal transduction pathway to regulate capsule synthesis and swarming. Given that the average cell lengths of the CH-1ΔA and CH-1ΔB mutants are

about 10% less than that of parental strain CH-1, this suggests that cell length determination, which is known to be regulated by $FlhDC_{Sm}$, is also regulated by RssA-RssB. Thus, it is speculated that in both swarming mutants the flagellar motility may be affected. Furthermore, the cell surface topography of *rssA* and *rssB* mutants is markedly different from that of CH-1, as revealed by AFM, suggesting that RssA and RssB are involved in homeostasis maintenance of the cellular surface structures and function. Further experiments to measure some physiological parameters involved in swarming regulation in *S. marcescens* are being performed. These parameters include the $flhDC_{Sm}$ flagellar motility and amounts of biosurfactant and extracellular polysaccharides synthesized. These experiments should help us understand the regulatory mechanism for swarming in *S. marcescens*.

ACKNOWLEDGMENTS

This work was supported by grants from the National Science Council (grants NSC-91-2314-B-002-258 and NSC-92-2314-B-002-356), the Technology Development Program for Academia, Ministry of Economical Affairs (grant 91-EC-17-A-10-S1-0013), and the Environmental Protection Administration (grant EPA-91-NSC-01-B003) of Taiwan and by a joint Royal Society U.K./National Science Council of Taiwan travel project grant, which is gratefully acknowledged.

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