Characterization of the *dapA-nlpB* Genetic Locus Involved in Regulation of Swarming Motility, Cell Envelope Architecture, Hemolysin Production, and Cell Attachment Ability in *Serratia marcescens*

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Swarming migration of *Serratia marcescens* requires both flagellar motility and cellular differentiation and is a population-density-dependent behavior. While the *flhDC* and quorum-sensing systems have been characterized as important factors regulating *S. marcescens* swarming, the underlying molecular mechanisms are currently far from being understood. *Serratia* swarming is thermoregulated and is characterized by continuous surface migration on rich swarming agar surfaces at 30°C but not at 37°C. To further elucidate the mechanisms, identification of specific and conserved regulators that govern the initiation of swarming is essential. We performed transposon mutagenesis to screen for *S. marcescens* strain CH-1 mutants that swarmed at 37°C. Analysis of a “precocious-swarming” mutant revealed that the defect in a conserved *dapA_{Sm}-nlpB_{Sm}* genetic locus which is closely related to the synthesis of bacterial cell wall peptidoglycan is responsible for the aberrant swarming phenotype. Further complementation and gene knockout studies showed that *nlpB_{Sm}*, which encodes a membrane lipoprotein, NlpB_{Sm}, but not *dapA_{Sm}*, is specifically involved in swarming regulation. On the other hand, *dapA_{Sm}* but not *nlpB_{Sm}* is responsible for the determination of cell envelope architecture, regulation of hemolysin production, and cellular attachment capability. While the *nlpB_{Sm}* mutant showed similar cytotoxicity to its parent strain, the *dapA_{Sm}* mutant significantly increased in cytotoxicity. We present evidence that DapA_{Sm} is involved in the determination of cell-envelope-associated phenotypes and that NlpB_{Sm} is involved in the regulation of swarming motility.

An increasing number of bacterial strains are being shown to exhibit a form of cellular differentiation and multicellular behavior termed swarming migration. Swarming migration involves the differentiation of vegetative cells at the colony edge into elongated, aseptate, and hyperflagellated cells that undergo rapid and coordinated population migration across solid surfaces (14, 20, 38). Swarming is also the result of the regulated expression of gene networks required to initiate the complex processes underlying the required morphological and physiological changes. Individual cells do not have the ability to swarm, as swarming migration is the result of a coordinated, multicellular effort of groups of differentiated cells functioning through close cell-cell interactions (12, 19).

When inoculated onto Luria broth (LB) medium solidified with 0.8% 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 (12, 18, 19). 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 swarming-cell differentiation at the colony edge and (ii) active-motile swarming migration (or translocation) from the colony edge.

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 locally high bacterial population densities (2). The flagellar master operon and the quorum-sensing system are global regulators of flagellar motility and cell population density, respectively (16, 22, 26, 27). 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 is produced (1, 22, 26). An additional layer of regulation of surface migration may also be exerted through RsmA. When overexpressed, *rsmA* inhibits the formation of a spreading colony in *S. marcescens* (6) and the effect may be via the repression of quorum sensing, which has also been reported in *Pseudomonas aeruginosa* (31).

S. marcescens swarming is observed to be a temperature-dependent behavior which occurs at 30°C but not at 37°C. This may be explained by a decrease in flagellar motility (27) and reduction of biosurfactant production at temperature upshift.

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To unravel the underlying regulatory mechanism, we have screened a *S. marcescens* mini-Tn5 mutant library to isolate strains that demonstrated proficient swarming at 37°C. In the process of characterizing one of the mutants, we identified a *dapA-nlpB* genetic locus involved in the regulation of *Serratia* swarming. Here, we present several lines of evidence to show that while DapA_{Sm}, which acts as a dihydrodipicolinate synthase involved in the synthesis of murein *meso*-diaminopimelate (m-DAP), is involved in the determination of cellular morphogenesis, hemolysin production, and cell attachment capability, the membrane lipoprotein NlpB_{Sm} is specifically involved in swarming regulation. Compared with the parent strain and *nlpB*_{Sm} mutant, a significant increase in cytotoxicity was further observed in the *dapA*_{Sm} mutant.

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 (*N*-acylhomoserine lactone) quorum-sensing signals were detected from *S. marcescens* CH-1 cells. *Escherichia coli* strains (CC118 λ pir, S17-1 λ pir, and Top10 F') were cultured at 37°C and *S. marcescens* at 30 or 37°C in LB medium (Difco) unless otherwise indicated in the text.

Swimming motility was examined on motility agar (LB medium 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 was examined on swarming agar plates (LB medium solidified with 0.8% Eiken agar) by inoculating 5 μ l of an overnight broth culture onto the center of the agar plate. Swimming motility and swarming migration distances were recorded at hourly intervals to enable comparisons. Swarming-cell differentiation i.e., the overproduction of flagella, cellular elongation, and polyploidy, was also examined microscopically as described previously (27). For determining bacterial growth rates, hourly increases in the optical density (OD) of broth cultures at A_{600} were measured.

Recombinant DNA techniques. Unless otherwise indicated, standard protocols of DNA manipulation and related techniques were used following the protocols of Sambrook et al. (35). Southern blotting analysis of chromosomal DNA was performed using nylon membranes (HybondN⁺; Amersham) and a digoxigenin (DIG) High Prime labeling kit according to the recommendations of the manufacturer (Roche). PCR DNA amplicons were cloned by using pCRII and the TA cloning kit (Invitrogen). DNA sequencing and analysis were performed using a Perkin-Elmer Autosequencer model 377 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.

Northern blot hybridization. Total cellular RNA was prepared by the hot phenol method (35) and transferred to nylon filters and hybridized with DNA probes labeled with DIG (Roche). The *dapA*_{Sm} and *nlpB*_{Sm} probes were a 350-bp partial *dapA*_{Sm} DNA fragment and a 365-bp partial *nlpB*_{Sm} DNA fragment amplified by PCR/DIG labeling (Roche) using the primer pair 5'-CGCGCGA GCCTGAAAAAATTGA-3'/5'-GCGAAGGCACGTTATACAGG-3' for *dapA*_{Sm} probe amplification and 5'-CTTCGCCTGCAGCAGGC-3'/5'-AAGTCGACGG TAGCAAAAGTAGTG-3' for *nlpB*_{Sm} probe amplification.

Analysis of DNA and protein sequences. Deduced DNA and protein sequences were compared with GenBank DNA or nonredundant protein sequence databases, respectively, using BLASTN or BLASTX via the National Center for Biotechnology Information Internet homepage (<http://www.ncbi.nlm.nih.gov/>). Protein sequence identities were analyzed by using ExPASy proteomics tools (Dense Alignment Surface method, Tmpred, SOSUI, PredictProtein, and ProtScale) in the ExPASy Molecular Biology Server of the Swiss Institute of Bioinformatics (<http://tw.expasy.org>).

Screening of "precocious-swarming" mutants by mini-Tn5 mutagenesis. For effective transfer of the pUT-mini-Tn5-Km1 recombinant plasmid from *E. coli* to *S. marcescens* CH-1 by conjugation (11), *S. marcescens* CH-1 and *E. coli* S17-1 λ -pir carrying the pUT derivative recombinant plasmid were grown overnight with vigorous shaking at 30°C in 10 ml LB medium, and LB medium containing 50 μ g ml⁻¹ kanamycin, respectively. Mating was achieved by mixing 100 μ l of each bacterial suspension together, followed by the addition of 5 ml of 10 mM MgSO₄. The mixture was then filtered through a type HA filter membrane (Millipore) using a negative-pressure pump (Stratagene). The drained membrane was sub-

sequently placed onto the surface of a 1.2% LB agar plate and incubated for 8 to 18 h at 30°C. The bacteria were then suspended in 5 ml 10 mM MgSO₄ solution and spread onto modified LB agar plates (0.04% NaCl, 2% glycerol, 0.5% yeast extract, 1% Bacto tryptone, 0.8% Eiken agar, 50 μ g/ml kanamycin, 13 μ g/ml tetracycline) 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 were finally selected. Southern blot hybridization using the labeled Km gene as a probe was performed to confirm the insertion of only one transposon copy in the mutants.

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.

Construction of *S. marcescens* CH-1 *nlpB*_{Sm} and *dapA*_{Sm} insertion deletion mutants. A PCR protocol was designed for the specific insertion of a 2-kb Sm-resistant Ω cassette excised from pHP45 Ω (33) into the *dapA*_{Sm} and *nlpB*_{Sm} genes, respectively. For construction of the *dapA*_{Sm} mutant, the primer pair DapK1 and DapK2 (Table 1) was used to amplify the central region of *dapA*_{Sm}. PCR products were T-cloned into pCR2.1 (Stratagene), excised as an EcoRI fragment, and ligated with an Ω cassette into a *tnp*-deleted pUT vector to form pUT-*dapA*_{Sm}::Sm (pSC301). For construction of the *nlpB*_{Sm} mutant, the 5' region of *nlpB*_{Sm} was generated using primer pair NlpBk1 and NlpBk2, T-cloned into pCR2.1, and excised as a SalI-HindIII fragment. A second PCR product encompassing the 3' region of *nlpB*_{Sm} was generated using primer pair NlpBk3 and NlpBk4, T-cloned into pCR2.1, and excised as a HindIII-EcoRI fragment. The two DNA fragments together with the Ω cassette were ligated to the SalI-EcoRI-digested pUT-mini-Tn5-Km1 suicide vector (11) to form plasmid pUT-*nlpB*_{Sm}::Sm (pSC300).

For gene inactivation by homologous recombination, pUT-*dapA*_{Sm}::Sm and pUT-*nlpB*_{Sm}::Sm were transferred from *E. coli* S17-1 λ pir to *S. marcescens* CH-1 by conjugation. Transconjugants were spread on LB plates containing streptomycin (100 μ g/ml) and tetracycline (13 μ g/ml). Mutant candidates were screened by colony PCR. Southern blot hybridization using either the *dapA*_{Sm} or *nlpB*_{Sm} gene as probe was performed to confirm the mutant genotypes (data not shown). Results confirmed that a single-crossover event and a double-crossover event had occurred. The strains were designated PC101 for the *nlpB*_{Sm} mutant strain and PC102 for the *dapA*_{Sm} mutant strain, respectively.

Complementation of precocious-swarming mutants with *dapA*_{Sm} and *nlpB*_{Sm}. For complementation, pBG200, pBG201, pBG203, and control plasmid pBAD18-Cm were separately transformed into *S. marcescens* PC100 or PC101 via electroporation. Transformants that were Cm^r were selected for confirmation of the correct plasmid and for further characterization of swarming and cell differentiation behavior.

Cell attachment assay. Quantification assays were performed as previously described (30). Briefly, 10 μ l of an overnight culture was used to inoculate PVC microtiter wells containing 90 μ l of LB medium without NaCl but supplemented with 2% glucose. The covered microtiter dish was sealed with Parafilm during incubation at 30 and 37°C. Cell suspensions were removed to determine the OD (A_{600}). The wells were rinsed with distilled water and dried at room temperature for 15 min before the addition of 200 μ l of crystal violet (1%) for 20 min. The stained wells were rinsed several times with distilled water, allowed to dry at room temperature for 15 min, and extracted twice with 200 μ l of 95% ethanol. The OD (A_{630}) was estimated using a Beckman DU-640B spectrophotometer after adjusting the volume to 1 ml with distilled water.

Measurement of hemolysin activity and pattern of cell-surface-associated proteins. Cell-associated hemolysin (ShlA) activity was assayed as described previously (24) and calculated in arbitrary hemolytic units (1 U causing the release of 50 mg hemoglobin/h in the standard assay). To analyze cell-surface-associated protein patterns, cells grown on agar plate surfaces were harvested by washing with 3 ml of LB broth followed by centrifugation for concentration. Cells were vortexed for 10 min before precipitation with 10% trichloroacetic acid (4), normalized to the cell mass (OD [A_{600}] $\times$ cell suspension volume [ml] = 5), separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and stained with Coomassie brilliant blue (35).

Transmission electron microscopy. Bacteria were adsorbed to 200- μ m-pore-size mesh copper electron microscopy grids coated with carbon and Formvar. The grid was then floated on a drop of 1% (wt/vol) phosphotungstic acid for 15 s to negatively stain the sample. Cells were observed in a Hitachi H-7100 transmission electron microscope operated under standard conditions with the cold trap in place.

Scanning electron microscopy (SEM). Bacterial cells were fixed with 2% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS) buffer (pH 7.2) for 1 h, postfixed with 1% osmium tetroxide in 0.1 M PBS buffer (pH 7.2), dehydrated with serial concentrations of ethanol and acetone, and critical point dried, fol-

TABLE 1. Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Relevant characteristic(s)	Source or reference
Strains		
<i>Serratia marcescens</i>		
CH-1	Clinical isolate	27
PC100	CH-1 <i>dapA_{Sm}::Km</i> , Km ^r	This study
PC101	CH-1 <i>nlpB_{Sm}::Sm</i> , Sm ^r	This study
PC102	CH-1 <i>dapA_{Sm}::Sm</i> , Sm ^r	This study
<i>Escherichia coli</i>		
CC118 λ pir	λ -pir lysogen of CC118 [D(<i>ara-leu</i>) <i>araD</i> <i>DlacX74</i> <i>galE</i> <i>galK</i> <i>phoA20</i> <i>thi-1</i> <i>rpsE</i> <i>rpoB</i> <i>argE</i> (Am) <i>recA1</i>]; permissive host for suicide plasmids requiring the Pir protein	22
S17-1 λ pir	λ -pir lysogen of S17-1 [<i>thi pro</i> <i>hsdR</i> <i>hsdM</i> ⁺ <i>recA</i> RP4 2-Tc::Mu-Km::Tn7 (Tp ^r Sm ^r)]; permissive host able to transfer suicide plasmids requiring the Pir protein by conjugation to recipient cells	22
Plasmids		
pACYC184	Cloning vector, P15A replicon, Tc ^r , Cm ^r	22
pBCSK+	Cloning vector, ColE1 replicon, Amp ^r	Stratagene
pUT-Sm	Suicide plasmid requiring the Pir protein for replication and containing a mini-Tn5 cassette containing Sm ^r genes	22
pBAD18-Cm	Cloning vector, arabinose regulation; Cm ^r	Colin Hughes (Cambridge, United Kingdom)
pBG200	pBAD18-Cm:: <i>dapA_{Sm}</i> ; Cm ^r	This study
pBG201	pBAD18-Cm:: <i>nlpB_{Sm}</i> ; Cm ^r	This study
pBG203	pBAD18-Cm:: <i>dapA_{Sm} nlpB_{Sm}</i> ; Cm ^r	This study
pBG401	pACYC184 (<i>PshIBA_{Sm}::luxAB</i>), Cm ^r	This study
pSC300	pUT-Sm derivative carrying <i>nlpB_{Sm}::Sm</i> ^r insertion, Sm ^r	This study
pSC301	pUT-Sm derivative carrying <i>dapA_{Sm}::Sm</i> ^r insertion, Sm ^r	This study
Primers		
Dapk1	5-ATCTAGACGCGCGAGCCTGAAAA-3	
Dapk2	5-ATCTAGAGCGAAGGCACGTTATACAGG-3	
NlpBk1	5-AAGCTTCGCCTGCAGCAGGC-3	
NlpBk2	5-AAGTCGACGGTAGCAAAAGTAGTG-3	
NlpBk3	5-GATATCGCTGGTTTTGCTGAAGGC-3	
NlpBk4	5-AAGCTTCGAGAGCGGCGCCG-3	

lowed by gold palladium alloy coating. Samples were examined with a scanning image observing device equipped with an electron microscope (Autoscan Etec). The images in the figures are representative of what was observed in 10 random fields in each of three independent experiments.

Cytotoxicity assays. Approximately 5×10^4 Hep-2 cells (human larynx epithelium cells) were plated in flat-bottom well plates in RPMI 1640 medium–1% fetal bovine serum. Bacterial strains were cultivated to stationary phase in LB broth at 37°C. To each bacterial sample, 100 μ l was added to wells that contained Hep-2 cells, which were then incubated at 37°C in humidified 5% CO₂–95% air for 5 h. The wells were washed with PBS and incubated with 5 mg of 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT; Amersham Life Sciences)/ml of RPMI 1640 medium (GIBCO) for 3 to 4 h at 37°C. The Hep-2 cells were washed with PBS, and the colored formazan product was solubilized by treating cells with a lysis solution composed of 90% isopropanol containing 40.6 mM HCl and 0.5% SDS. Conversion of MTT to formazan was quantified by measuring the optical density at 570 nm with the subtraction of background absorbance at 690 nm using a Multiskan RC plate reader (Thermo Labsystems).

Globomycin assay. Bacterial cultures grown overnight in LB broth at 37°C were diluted 1:100 to fresh medium and incubated with vigorous shaking at 37°C for 2 h. Arabinose at a final concentration of 0.2% and globomycin (Sankyo, Japan) at a final concentration of 200 μ g/ml were then added. Cells were cultured for an additional 1 h before being harvested for SDS-PAGE analysis.

Hypotonic tolerance assay. Bacterial strains were cultured overnight in LB broth. A 100- μ l sample of each culture was transferred to 100 ml distilled water for hypotonic stress (final concentration of 0.17 mM NaCl). Bacterial suspensions were periodically removed and spread onto LB-ampicillin (50 μ g/ml) plates. After overnight culturing, CFU were counted for calculation of the survival rate.

Nucleotide sequence accession number. We have designated *orf1* as *dapA_{Sm}*, *orf2* as *nlpB_{Sm}* and *orf3* as *gcvR_{Sm}*, and the nucleotide sequence of the 6,500-bp region containing these three *orfs* has been submitted to the DDBJ/EMBL/GenBank databases under the accession number AY502943.

RESULTS

A *Serratia marcescens* mutant with aberrant precocious-swarming behavior. *S. marcescens* CH-1 cells exhibit swarming migration at 30°C, but this motility is inhibited at 37°C (Fig. 1A). To further characterize the genetic determinants of the temperature-dependent regulation of swarming behavior in *S. marcescens* CH-1 cells, we performed mini-Tn5 transposon mutagenesis and then screened for *S. marcescens* mutant colonies swarming at 37°C. One such mutant, designated *S. marcescens* PC100, showed the precocious-swarming phenotype (Fig. 1B) and was selected for further analysis. Southern blot hybridization using a mini-Tn5 DNA fragment as a probe confirmed that only a single insertion occurred in this clone (data not shown). To examine whether the precocious-swarming mutation results in additional phenotypes, a number of physiological assays, including growth rate and production of the red pigment prodigiosin, were performed. While there was no sig-

FIG. 1. Swarming behavior of *S. marcescens* strains. (A) Swarming of *S. marcescens* CH-1 cells on 0.8% Eiken agar plates at 30°C and 37°C. (B) Swarming of CH-1 and PC100 cells on 0.8% Eiken agar plates at 37°C (i) and CH-1 and PC101 cells on 1.0% Eiken agar plates at 30°C (ii).

nificant difference in the growth dynamics of PC100 cells during growth in LB broth, these cells did show significantly reduced prodigiosin synthesis at 30°C (data not shown).

Characterization of the locus interrupted by transposon.

Identification of the *S. marcescens* PC100 DNA flanking the mini-Tn5 insertion site was accomplished by conventional digestion and cloning, followed by sequencing with primers designed from within the I end or O end of the transposon (11). Sequencing results revealed that the mini-Tn5 insertion, giving rise to the precocious-swarming phenotype, was located within a 6,500-bp DNA fragment of the *S. marcescens* CH-1 genome. These data also revealed that the transposon had inserted into an 882-bp open reading frame (*orf1*) encoding ORF1, a putative 294-amino-acid (32.34-kDa) polypeptide. Downstream of *orf1* and in the same orientation, *orf2* was identified, which encodes a putative 350-amino-acid product. Upstream of *orf1*, we also identified a divergent *orf3*, potentially encoding a 228-amino-acid peptide (Fig. 2A).

The deduced protein sequences were compared with nonredundant protein sequences stored in the DDBJ/EMBL/GenBank databanks using BLASTP (5) via the National Center for Biotechnology Information Internet homepage. The mini-Tn5 insertion in the *S. marcescens* CH-1 genome was found to be in a region that is highly homologous to the bacterial *dapA-nlpB* region involved in the synthesis of cell wall components. ORF1 is homologous to *E. coli* dihydrodipicolinate synthase DapA (75% identity) and also to DapAs of *Haemophilus influenzae* (58% identity), *Vibrio cholerae* (57% identity), and *Pseudomonas aeruginosa* (57% identity). ORF2 was found to have homology to *E. coli* K-12 lipoprotein 34 (named NlpB_{Ec}) (66% identity) and *Yersinia pestis* lipoprotein 34 (73% identity). The

FIG. 2. Genetic organization and characterization of the *S. marcescens* CH-1 *gcvR_{Sm}*-*dapA_{Sm}*-*nlpB_{Sm}* region and results of swarming complementation analysis. (A) Physical map of the *S. marcescens* CH-1 *gcvR_{Sm}*(*orf3*)-*dapA_{Sm}*(*orf1*)-*nlpB_{Sm}*(*orf2*) region. In PC100 cells, the mini-Tn5 transposon was inserted into *dapA_{Sm}*. The black arrowhead indicates the transposon insertion site. Transcomplementation of PC100 mutant phenotypes was performed with pBG200 (pBAD18-Cm::*dapA_{Sm}*), pBG201 (pBAD18-Cm::*nlpB_{Sm}*), and pBG203 (pBAD18-Cm::*dapA_{Sm}*-*nlpB_{Sm}*), respectively. (B) Results of complementation of PC100 swarming at 37°C. (a) CH-1; (b) PC100(pBAD18-Cm); (c) PC100(pBG200); (d) PC100(pBG201); (e) PC100 (pBG203); (f) PC100 (m-DAP, 200 µg/ml); (g) PC100 (lysine, 200 µg/ml); (h) PC100 (m-DAP, 200 µg/ml, and lysine, 200 µg/ml). (C) *dapA_{Sm}*-*nlpB_{Sm}* forms an operon. Northern blot hybridization analysis using *dapA_{Sm}* and *nlpB_{Sm}* probes, respectively, against total cellular RNA prepared from *S. marcescens* CH-1 cells grown to an OD (*A*₆₀₀) of 0.3. M, DNA size standards. A 2.5-kb DNA fragment predicted to be the *dapA_{Sm}*-*nlpB_{Sm}* operon and a 1.5-kb fragment were both identified using *nlpB_{Sm}* as a probe. A single 2.5-kb fragment was identified using *dapA_{Sm}* as a probe.

structure of the *dapA_{Sm}*-*nlpB_{Sm}* operon identified in *S. marcescens* CH-1 is conserved with that in *E. coli* (9).

ORF3 was found to have a high level of homology to *E. coli* GcvR (76% identity), which is a transcriptional repressor in the glycine cleavage system, and also to GcvR homologs from

TABLE 2. Complementation and phenotypic characterization of *S. marcescens* mutant strains defective in the *dapA_{Sm}-nlpB_{Sm}* locus at 37°C^a

Strain	Phenotype at 37°C ^a			
	Swarming	Biofilm	Hemolysin	Cell wall integrity
CH-1 (wild type)	—	N	N	N
PC100	+	D	H	D
PC100(pBG200)	+	N	N	N
PC100(pBG201)	—	D	H	D
PC100(pBG203)	—	N	N	N
PC101	+	N	N	N
PC101(pBG201)	—	N	N	N
PC102	+	D	H	D
PC102(pBG200)	+	N	N	N
PC102(pBG201)	—	D	H	D
PC102(pBG203)	—	N	N	N

^a —, nonswarming; +, swarming; N, normal; D, defective; H, hemolysin overproduction.

Salmonella enterica subsp. *enterica* serovar Typhimurium (76% identity), *Shigella flexneri* (82% identity), and *Yersinia pestis* (77% identity).

***nlpB_{Sm}* is specifically involved in swarming regulation.** Transformation of the recombinant plasmid pBG203, containing the complete *dapA_{Sm}-nlpB_{Sm}* operon under the control of its native promoter, into PC100 cells inhibited precocious-swarming behavior at 37°C (Fig. 2A and B, panel e), suggesting that a defect at this locus is responsible for the PC100 phenotype and that either *dapA_{Sm}* or *nlpB_{Sm}* or both are responsible for the mutant phenotype. To clarify this, the pBG200 construct (pBAD18-Cm::*dapA_{Sm}*), containing the *dapA_{Sm}* gene under the control of the arabinose pBAD promoter, was first transformed into PC100. No inhibition of the precocious-swarming phenotype in PC100(pBG200) cells at 37°C was observed in the presence of 0.2% arabinose (Fig. 2A and B, panel c). Subsequent experiments using a series of concentrations (between 50 µg/ml and 500 µg/ml) of lysine and/or DAP for complementation assays were performed, and the precocious-swarming phenotype was still evident (Fig. 2B). PC100 was transformed with pBG201 (pBAD18-Cm::*nlpB_{Sm}*) and assayed for swarming at 37°C. Swarming of PC100 cells was clearly inhibited (Fig. 2A and B, panel d). The results of complementation assays are also summarized in Table 2.

As *dapA_{Ec}-nlpB_{Ec}* was previously shown to form an operon in *E. coli* (9), *dapA_{Sm}-nlpB_{Sm}* might also form an operon in *S. marcescens* CH-1. Northern blot hybridization using 400-bp partial *dapA_{Sm}* or *nlpB_{Sm}* DNA fragments as probes was performed. A single RNA transcript of ca. 2,500 bp was identified using either the *dapA_{Sm}* or the *nlpB_{Sm}* probe, and an additional RNA transcript of ca. 1,500 bp was further identified using *nlpB_{Sm}* as the probe (Fig. 2C). These data suggested that *dapA_{Sm}-nlpB_{Sm}* does form an operon in *S. marcescens* CH-1 and that *nlpB_{Sm}* expression is also under the control of its own promoter. Consequently, in PC100 mutant cells where *dapA_{Sm}* is disrupted by a mini-Tn5 transposon insertion, there may be reduced levels of *nlpB_{Sm}* RNA transcripts and subsequent translation products. Indeed, Western blot analysis confirmed that the amount of NlpB_{Sm} in PC100 cells was reduced to about one-fourth of the CH-1 levels (see Fig. 4A). These data suggest that insertion of the mini-Tn5 transposon into *dapA_{Sm}*

resulted in a reduction in the amount of NlpB_{Sm} synthesized and subsequently the precocious-swarming behavior.

For the confirmation of *nlpB_{Sm}* function, *nlpB_{Sm}* was further mutated in CH-1 cells by insertion deletion via homologous recombination to generate the *S. marcescens* PC101 mutant strain (Fig. 3A). Southern blot hybridization (data not shown) and Western blot analysis (Fig. 3B) confirmed that *nlpB_{Sm}* was specifically deleted in PC101 cells. No difference in growth rate was observed between PC101 and CH-1 cells. The swarming phenotype of *S. marcescens* PC101 was characterized and compared with that of CH-1 and PC100. PC101 displayed the precocious-swarming trait at 37°C (Fig. 3C, panel i). PC101 was further shown to continue to swarm as the agar concentration was increased up to 1.0% at 30°C, whereas CH-1 cells did not (Fig. 1B, panel ii). For the purpose of complementation analysis, the plasmid pBG201 (pBAD18-Cm::*nlpB_{Sm}*) was transformed into PC101 cells. Western blot analysis showed that the NlpB_{Sm} level was restored in strain PC101(pBG201) (Fig. 3B). Subsequent swarming assays revealed that the precocious-swarming motility of PC101 was also inhibited by *nlpB_{Sm}* in *trans* (Fig. 3C, panel i) and in a dose-dependent way at 37°C (Fig. 3C, panel ii). These data suggest that a defect in *nlpB_{Sm}* is the underlying mechanism leading to the precocious-swarming phenotype of PC100 and PC101.

***NlpB_{Sm}* is a constitutively expressed membrane lipoprotein.** Analysis of NlpB_{Sm} by the Dense Alignment Surface method for hydrophobicity (10) and PSORT (Prediction of Protein Localization site) (<http://psort.nibb.ac.jp/form.html>) identified an N-terminal hydrophobic region and a 26-amino-acid signal peptide containing a conserved lipobox Leu-Ala-Ala-Cys sequence [Leu-(Ala or Ser)-(Gly or Ala)-Cys at the -3 to +1 position] which was predicted to be the lipoprotein signal peptidase II recognition site and the site for lipid modification (21). In addition, an outer membrane amino acid sorting signal serine is located at the +2 position in NlpB_{Sm} so that NlpB_{Sm} was predicted to be a lipoprotein located in the outer membrane (28, 37). The globomycin assay (8), in which globomycin specifically inhibits the signal peptidase II enzyme that cleaves lipoprotein signal sequences, was performed to ascertain whether NlpB_{Sm} is a lipoprotein (23). In the presence of 200 µg/ml globomycin, a slower migratory form of NlpB_{Sm}, consistent with the presence of an unprocessed precursor, was observed as the dominant species from CH-1(pBG201) cells after Western blot analysis (Fig. 4A). These data indicated that NlpB_{Sm} is a membrane lipoprotein. To monitor the synthesis pattern of NlpB_{Sm} following the growth of CH-1 cells on LB plate cultures, cells were periodically harvested for quantification of NlpB_{Sm} with an anti-NlpB_{Sm} antibody. NlpB_{Sm} was observed to be synthesized constitutively at both 30°C and 37°C (Fig. 4B).

***dapA_{Sm}* is involved in the determination of cell wall integrity, cell attachment ability, and hemolysin production in *S. marcescens*.** The *dapA_{Sm}* function was characterized. In further experiments, the *dapA_{Sm}* gene was mutated by single cross-homologous recombination to generate the *S. marcescens* strain PC102 (Fig. 3A, panel ii). Phenotypic characterization of PC102 showed that these cells behaved similarly to PC100, including a similar growth rate and reduced protein level of NlpB_{Sm} to about one-fourth that of the CH-1 levels (data not shown). As *dapA* is a gene responsible for the synthesis of

FIG. 3. Construction of *nlpB_{Sm}* and *dapA_{Sm}* mutant strains and characterization of (complemented) PC101 swarming phenotypes. (A) Strategies used for the construction of *nlpB_{Sm}* or *dapA_{Sm}* knockout strains. (i) Construction of *nlpB_{Sm}* knockout strain PC101. (ii) Construction of *dapA_{Sm}* knockout strain PC102. Ω , a streptomycin resistant gene cassette (22). (B) Western blot analysis of NlpB_{Sm} using anti-NlpB_{Sm} polyclonal antibody. Lane 1, CH-1(pBAD18-Cm); lane 2, PC101(pBAD18-Cm); lane 3, PC101(pBG201). (i) Whole-cell crude extract stained with Coomassie brilliant blue. (ii) Result of Western blot analysis. (C) Swarming phenotypes of PC101 under different culture conditions. (i) On 0.8% Eiken agar plates (0.2% arabinose) at 37°C. pBAD18-Cm, a negative control vector; PC101(pBG201), PC101 cells containing pBAD18-Cm::*nlpB_{Sm}*. (ii) PC101(pBG201) was inoculated on 0.8% Eiken agar plates containing different arabinose (ara) concentrations ranging from 0% to 0.1% at 37°C.

m-DAP and the amino acid lysine, both of which are components of bacterial cell wall peptidoglycan (17, 34, 43, 44), the disruption of *dapA_{Sm}* might result in defects in cell wall integrity. Indeed, compared to CH-1, a significant increase of loosely bound cell surface proteins was observed after PC102 vortexing (Fig. 5A). These observations suggested that PC102

is defective in cell wall integrity. Hypotonic tolerance assay (41) showed that the survival rate of PC102 cells was found to decrease to about 38% 4 h after hypotonic shock, in contrast to CH-1 cells (85%) (Fig. 5B), suggesting that PC102 cells are more sensitive to hypotonic shock. Cell morphology observation by SEM showed an aberrant irregular elliptical shape of

FIG. 4. NlpB_{Sm} is a membrane lipoprotein produced constitutively at 30°C and 37°C. (A) Characterization of NlpB_{Sm} as a membrane lipoprotein. Whole-cell crude extracts of *S. marcescens* strains were separated by SDS-PAGE followed by bromothymol blue staining. Lane 1, CH-1; lane 2, PC100; lane 3, CH-1(pBG201) treated with 0.2% arabinose; lane 4, CH-1(pBG201) treated with 0.2% arabinose and 200 µg/ml globomycin. (a) NlpB_{Sm} precursor form; (b) mature form of NlpB_{Sm}. (B) Synthesis of NlpB_{Sm} following the growth of CH-1 seeded on 0.8% Eiken agar plates at 30 and 37°C. Cells were harvested at 2, 4, and 6 h after inoculation. (i) Whole-cell crude extract separated by SDS-12% PAGE. (ii) Western blot analysis using polyclonal antibodies against NlpB_{Sm}.

PC102 cells, with swelling in the middle and narrower ends (Fig. 5C). These data suggested that PC102 has a disrupted cell wall structure. Similar phenomena were observed in PC100 and PC100(pBG201) but not in CH-1, PC101, or PC100(pBG200) (Fig. 5C; Table 2).

The microtiter well assay (30), which monitors the ability of *S. marcescens* to attach to the wells of microtiter dishes, was used to quantify attachment ability in CH-1 and PC102 cells at 37°C. PC102 attachment is significantly defective, reaching only 60% of the parental CH-1 levels (A_{630} of 0.57 for PC102 versus 0.95 for CH-1) (Fig. 6A). These data suggested that a defect in *dapA_{Sm}* leads to reduced cell adhesion ability at 37°C.

Cell-surface-associated hemolysin has been identified as a major virulence factor in *S. marcescens* (25). The production of hemolysin was analyzed to see if it was affected in the *dapA_{Sm}* mutant. Hemolysin activity in PC102 cells was significantly higher than that in CH-1 cells at 37°C by up to fourfold (Fig. 6B). To determine whether this dysregulation occurs at the transcriptional level, a recombinant plasmid, pBG401 (*PshlBA::luxCDABE*), was constructed as a reporter for the promoter activity of *PshlBA*. A comparison of the light emission patterns from PC102(pBG401) and CH-1(pBG401) showed an average 10-fold increase in *shlBA* promoter activity in PC102 (Fig. 6C), suggesting that the hemolysin dysregula-

tion seen in these cells occurred mainly at the transcriptional level.

For complementation analysis, defect in cell wall integrity, reduced cell attachment ability, and increased hemolysin production of PC102 can all be complemented by either *dapA_{Sm}-nlpB_{Sm}* or *dapA_{Sm}* but not by *nlpB_{Sm}* (Table 2). Lysine and/or DAP complementation assays (between 50 µg/ml and 500 µg/ml) showed that while the PC102 precocious-swarming phenotype was still evident, the defect in cell wall integrity was restored (data not shown). Meanwhile, PC101 cells displayed normal cell attachment ability, hemolysin production levels, and cell wall integrity (Table 2). These data suggested that *dapA_{Sm}* but not *nlpB_{Sm}* is responsible for the aberrant phenotypes described. In summary, our findings present strong evidence that *dapA_{Sm}* is principally responsible for cell attachment ability, hemolysin production, and cell wall integrity and that *nlpB_{Sm}* is specifically involved in swarming regulation in *S. marcescens*.

PC100 and PC102 show an increase in *S. marcescens* cytotoxicity. A cytotoxicity assay using human larynx epithelial cells (Hep-2) as the study model was performed to evaluate the pathogenesis of the *S. marcescens* strains. Results in Fig. 6D showed that while PC101 showed a cytotoxicity level similar to that of the wild-type strain CH-1, PC100 and PC102 were significantly more cytotoxic to Hep-2 cells. These assays showed that *dapA_{Sm}* and not *nlpB_{Sm}* is closely related to *S. marcescens* pathogenesis.

DISCUSSION

S. marcescens CH-1 is a clinically isolated bacterial strain that exhibits swarming migration and produces prodigiosin at normal levels. Using mini-Tn5 transposon mutagenesis, we have identified a conserved genetic locus, *dapA_{Sm}-nlpB_{Sm}*, which encodes a dihydrodipicolinate synthase and a membrane lipoprotein, NlpB_{Sm}, respectively. Although NlpB_{Sm} homologs are commonly identified in the GenBank database, including those from *E. coli*, *Shigella flexneri*, *Shewanella oneidensis*, *Vibrio parahaemolyticus*, *Yersinia pestis*, *Salmonella enterica* subsp. *enterica* serovar Typhi, *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2, *Photobacterium luminescens*, *Vibrio vulnificus*, and *Vibrio cholerae*, etc. (<http://www.ncbi.nlm.nih.gov/entrez/>), the function of NlpB and the underlying mechanism of its relationship to swarming are basically not characterized. In *E. coli*, *nlpB_{Ec}* was shown to be cotranscribed with *dapA_{Ec}* and to code for a 344-amino-acid lipoprotein with a potential lipobox signal sequence (7). NlpB_{Ec} is detected in outer membrane vesicles prepared from osmotically lysed spheroplasts and also appears to be nonessential. Since a strain in which the *nlpB_{Ec}* gene is disrupted by the insertion of a chloramphenicol resistance gene is still able to grow and shows no discernible phenotype, the function of *E. coli* NlpB_{Ec} remains undetermined. In this study, complementation and knockout experiments in *S. marcescens* CH-1 showed that *nlpB_{Sm}* specifically regulates the swarming phenotype but not other physiological traits associated with cell envelope. These findings thus preliminarily clarify the functions of *nlpB_{Sm}* in *S. marcescens*.

NlpB_{Sm} is shown to be a membrane lipoprotein in *S. marcescens* CH-1. Inner and outer membrane lipoproteins in gram-

FIG. 5. Cell wall integrity assay and electron microscopy. (A) Patterns of cell-surface-associated proteins were analyzed by SDS-PAGE after vortexing. Bacteria were cultured on LB plates until the OD (A_{600}) reached 0.8 before harvesting cells for vortexing. Lane 1, CH-1; lane 2, PC100; lane 3, the *dapA_{Sm}* knockout strain PC102. (B) Determination of cell wall integrity by hypotonic tolerance assay. ▲, PC102; ■, CH-1. Data shown are means of three independent experiments (standard error of the mean < 10%). (C) Observation of cell surface morphology by SEM. (a) CH-1; (b) PC100; (c) PC100(pBG200); (d) PC100(pBG201); (e) PC101, a *nlpB_{Sm}* knockout strain; (f) PC102, a *dapA_{Sm}* knockout strain.

negative bacteria undergo processing by signal peptidase II. This is achieved after diacylglycerol transferase has transferred a diacylglycerol moiety to the sulfhydryl group of the N-terminal cysteine to be processed, forming a thioether linkage. After removal of the signal sequence, *N*-acyltransferase acylates the α amino group of the cysteine with a long-chain fatty acid to yield the mature lipoprotein (15, 32). In *E. coli*, the N terminus of a major membrane lipoprotein, Lpp, has been shown to be situated in the outer membrane, with its C terminus linked to DAP (39). In conjunction with this and other studies of *E. coli* NlpB_{Ec}, we speculated that NlpB_{Sm} might also be connected to the outer membrane through its N terminus, with its C-terminal domain extended to peptidoglycan through a direct linkage to DAP. In this situation, NlpB_{Sm} might monitor changes to extracellular/outer membrane conditions using its N terminus and to periplasmic peptidoglycan conditions using its C-terminal domains. Under these conditions, complementation of *nlpB_{Sm}* restored normal NlpB_{Sm} levels and subsequently a normal swarming phenotype.

The fact that the levels of NlpB_{Sm} synthesis did not vary following cellular growth at both temperatures, together with the fact that *S. marcescens* CH-1 swarms at 30°C but not at 37°C and that the *nlpB_{Sm}* mutant, PC101, swarms at both these

temperatures, suggests that although NlpB_{Sm} is expressed constitutively at both temperatures, it functions differently. Current evidence suggests that NlpB_{Sm} is actively functioning as a negative swarming regulator at 37°C and is inactive at 30°C. The function of NlpB_{Sm} might well be conformation dependent, with the protein acting as a membrane lipoprotein, and its conformation may be affected by the cellular membrane structure where the fatty acid composition can be influenced by temperature.

DapA_{Sm} was expected to be involved in the synthesis of cell-wall-related components. DAP is an enzyme responsible for the synthesis of m-DAP, one of the key linking units of peptidoglycan (17, 43). The biochemical synthesis of DAP in bacteria is involved mainly in a pathway for lysine biosynthesis. Such a pathway provides both lysine and m-DAP for protein synthesis and the construction of a bacterial peptidoglycan cell wall, respectively (36). Although in PC100, a defect in *dapA_{Sm}* leads to precocious-swarming and aberrant phenotypes associated with cell envelope architecture, swarming of PC100 cells was not inhibited by the addition of either m-DAP or lysine in the media at 37°C. These observations, together with the fact that PC100 precocious-swarming could not be inhibited by the complementation of *dapA_{Sm}*, suggest that *dapA_{Sm}* and

FIG. 6. Level of cell attachment, hemolysin production, and cytotoxicity in *S. marcescens* strains. (A) CH-1 and PC102 cell attachment capability in PVC wells at 37°C. The results were quantified and expressed as OD (A_{630}) values. (B) Hemolysin activity of CH-1 and PC102. Cells grown to an OD (A_{600}) of 0.8 were incubated with horse red blood cells, and the hemolysin activity was determined (24). The released hemoglobin was measured at the OD (A_{543}). (C) *shlBA* promoter activity of CH-1(pBG401) and PC102(pBG401) following the growth in LB broth at 37°C. The specific light intensity was expressed as total RLU (relative light units)/OD divided by 1×10^4 . (D) Cytotoxicity assay using human larynx epithelial cells (Hep-2) was performed to evaluate the cytotoxicity of *S. marcescens* strains. Control, no bacteria added; CH-1, *S. marcescens* wild-type strain; PC100, transposon-inserted mutant strain; PC101, *nlpB_{Sm}* knockout strain; PC102, *dapA_{Sm}* knockout strain. All data shown are means of three independent experiments (standard error of the mean < 10%).

m-DAP/lysine might not play a direct role in the regulation of *Serratia* swarming. Additionally, both abnormal cellular morphology and overproduction of hemolysin were restored by the transformation of multicopy *dapA_{Sm}* plasmids, indicating that *dapA_{Sm}* is involved mainly in bacterial-cell-wall-related morphogenesis. Our observations thus discriminate between the functions of *dapA_{Sm}* and *nlpB_{Sm}*. However, we still could not completely rule out a possible role for *dapA_{Sm}* in the regulation of *Serratia* swarming, as we have not yet been able to successfully construct a mutant containing an in-frame deletion of *dapA_{Sm}* where the expression of *nlpB_{Sm}* is not affected. Although *dapA_{Ec}* has been reported to be essential in *E. coli* (7, 9), the fact that there are three slightly different pathways for the synthesis of m-DAP (42) in eubacteria suggests the possibility of an alternative pathway for m-DAP synthesis in *Serratia*. It is also possible that although cell wall integrity in PC100 cells is defective, low levels of m-DAP may still be synthesized, and this may be linked to the remaining *NlpB_{Sm}* in PC100, which is at about 25% that of the levels in CH-1.

Expression of virulence genes and swarming-cell differentiation have been previously shown to be coordinately regulated in swarming bacteria during the process of bacterial population migration (3). In *Proteus mirabilis*, motile mutants unable to differentiate into swarming cells were comparably reduced in their hemolytic, ureolytic, proteolytic, and invasive phenotypes. These findings suggest that most phenotypes mentioned, if not

all, are under the control of the *flhDC* operon (13). In this study, increased production of the major virulence factor hemolysin and an increase in cytotoxicity were observed in PC100 and PC102, which are defective in *dapA_{Sm}* and show a reduced amount of *NlpB_{Sm}*. We further found that the *nlpB_{Sm}* mutant PC101 does not show any abnormalities in the phenotypes tested, except for the precocious-swarming behavior. These data suggest that increased hemolysin production in PC100 and PC102 may be the underlying mechanism of increase of cytotoxicity and that *NlpB_{Sm}* is specifically involved in a regulatory pathway regulating *Serratia* swarming. In this work, we have characterized the functions of the *dapA_{Sm}-nlpB_{Sm}* genes and showed that *nlpB_{Sm}*, which encodes a membrane lipoprotein, is specifically involved in the regulation of *Serratia* swarming, while *dapA_{Sm}*, which is predicted to synthesize a peptidoglycan component, m-DAP, is required for cell envelope integrity.

An important question that now remains is the elucidation of the molecular mechanism underlying swarming regulation by *NlpB_{Sm}*. Although the precise mechanism(s) remains unclear, cumulative evidence shows that *Serratia* mutants that are defective in genes involved in the synthesis of either fatty acids or lipopolysaccharide (LPS) are also defective in swarming regulation. For example, another three precocious-swarming mutants isolated in this laboratory showed a defect in the genes involved in LPS synthesis (data not shown). It is demonstrated

that LPS modification not only is involved in antimicrobial resistance but also plays a role in *P. mirabilis* swarming due to surface charge alterations (29). Soto et al. reported that a *fadD* (a gene involved in fatty acid degradation) mutant of *Sinorhizobium meliloti* shows swarming migration and that nodulation efficiency is impaired on alfalfa roots (40). Consequently, experiments will be performed to clarify the connection between NlpB_{Sm} and the effects of LPS modification.

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