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Mutational Analysis of Flagellum-Independent Surface Spreading of *Serratia marcescens* 274 on a Low-Agar Medium

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In a previous study (J. O'Rear, L. Alberti, and R. M. Harshey, J. Bacteriol. 174:6125–6137, 1992) we reported the isolation of several transposon mutants of *Serratia marcescens* 274 that were defective either in swarming alone or in both swimming and swarming motility. All the nonflagellate (Fla$^-$) mutants, while defective in both types of motility, were able to spread rapidly on the surface of low-agar (0.35%) media. We show here that some of the swarming-defective mutants are defective in the production of serrawettin W1, an extracellular cyclic lipopeptide produced by *S. marcescens* 274. When combined with a Fla defect, the serrawettin (Swt) mutants are deficient in spreading on low-agar media. The spreading deficiency can be overcome by serrawettin supplied extracellularly. Introduction of Fla defects into chemotaxis mutants does not affect this mode of surface translocation. These results suggest that spreading may be a passive form of translocation. We also report that swarming defects in all mutants showing a Dps phenotype (able to swarm within the inoculated area but unable to move outward) in the earlier study can be overcome by changing the commercial source of agar.

Serratia marcescens is a pigmented enteric bacterium found in variety of niches, which include soil, water, air, plants, and animals (9). It is also an opportunistic human pathogen, causing many nosocomial infections (7). *S. marcescens* is unique among enteric bacteria in many respects. It secretes extracellular chitinases, several proteases, a nuclease, and a lipase (13) and produces a wetting agent or surfactant called serrawettin, which helps in the colonization of surfaces (14, 15, 17). In keeping with its varied habitat, *S. marcescens* produces alternate forms of differentially flagellated cells which display different types of motility depending on whether the growth medium is liquid or solid (2). Nonflagellated cells of *S. marcescens* can also translocate (spread) efficiently over the surface of low-agar media (20).

Surface colonization via swarming requires the flagellar apparatus as well the chemotaxis system (20). A large-scale transposon mutagenesis study of *S. marcescens* identified several additional functions that were needed for swarming (20). A sizable subset of the mutants showed a Dps phenotype (cells were capable of swarming but remained confined to the inoculated area, i.e., were unable to move outwards). A distinct group of the Dps mutants (DpsI) appeared not to produce the extracellular slime layer generally visible around the bacterial colony. We show here that some of the DpsI mutants as well as some Dis (completely defective in swarming) mutants are defective in the production of serrawettin W1, an extracellular cyclic lipopeptide (14, 24). Flagellum-independent spreading of *S. marcescens* 274 on the surface of low-agar media also requires serrawettin. Defects in chemotaxis functions, however, had no effect on this mode of surface colonization. Our results reveal the importance of bacterial surface layers (serrawettins, slimes, and lipopolysaccharides [LPS]) as well as that of some physicochemical property of the growth surface for effective bacterial colonization.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids are listed in Table 1.

Plasmid pGA1 was constructed as follows. Plasmid pSM7 (1.25 kb of the flagellin-encoding gene *hag* cloned in a multiple cloning site vector [20]) was digested with *Pst*I and *Eco*RV restriction endonucleases, which cut within the coding region of *hag* (11). An oligonucleotide with recognition sites for *Sal*I and *Bam*HI was cloned into this region. The resulting plasmid was cut with *Sal*I and ligated to a 1.14-kb *Xho*I DNA fragment encoding Cm^r from pACYC184 (4). This plasmid was called pAB1. A 2.4-kb *hag*::Cm fragment obtained by digestion with *Xba*I and *Sac*I (which cut in the multiple cloning sites) of pAB1 was cloned into *Xba*I and *Sac*I sites on the broad-host-range conjugable suicide vector plasmid pGP704 (a derivative of R6K [19]) and transformed into strain SM10 λ*pir* (Table 1) to generate pGA1. pGP704 replicates only if the *pir* gene product π protein of plasmid R6K is supplied in *trans*. Therefore, efficient plasmid suicide results upon transfer to bacteria not harboring *pir*.

All motility mutants were derivatives of *S. marcescens* 274 and are listed in Table 2.

Swarming motility was observed on Luria-Bertani (LB) medium solidified with 0.75% agar. The agar was obtained either from Difco or from Eiken Chemical Co., Tokyo, Japan, and will be referred to below simply as Difco or Eiken agar. Spreading was observed on peptone-glycerol (PG) medium (2) solidified with 0.35% agar. The antibiotics and concentrations used were kanamycin, 50 μg/ml; ampicillin, 100 μg/ml; and chloramphenicol, 50 μg/ml.

Gene replacement: construction of chromosomal *hag* gene disruption. pGA1 was conjugated from SM10 λ*pir* into all of the SMu (chemotaxis-defective [Che$^-$], Dps, and Dis) strains of *S. marcescens* 274 listed in Table 2 as follows. Conjugating pairs of cells were spotted together on LB agar plates, incubated overnight at 37°C, streaked on LB plates with 50 μg of chloramphenicol per ml, and incubated again at 30°C overnight. Cm^r red (*S. marcescens*) exconjugants were isolated and tested for Ap^r. Cm^r Ap^s colonies were tested for motility on LB-swim (0.35%) agar plates. Replacement of the chromosomal *hag* gene with the *hag*::Cm disruption was confirmed by Southern analysis.

Chemical analysis of serrawettins. Methods for the identification and preparation of serrawettins were described previously (17). Fast atom bombardment mass spectrometry (FAB-MS) was carried out by using a JEOL SX 102. As matrices, triethanolamine and *p*-nitrobenzyl alcohol were used for positive and negative ion analyses, respectively.

Screening for defects in serrawettin production. Bacterial wetting activity, which is one of the phenotypes of production of surface-active exolipids, was examined by the glass slide method (14) as follows. A small amount of wet bacterial mass was suspended in a drop (8 μl) of distilled water on the surface of a clean glass slide (Pre-Cleaned Microslide Glass; Matsunami, Tokyo, Japan). Wetting activity was scored as a prompt dendritic spreading of the bacterial suspension. Specific spots of serrawettin W1 or W2 produced by *S. marcescens* were visualized by direct colony thin-layer chromatography as described previously (18). Briefly, the bacterial mass to be examined was directly placed on a thin-layer chromatography plate, and extraction development was carried out for 15 min in chloroform-methanol (2:1 [vol/vol]). After being dried, the plate was

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
S. marcescens 274	Wild type	20
S. marcescens NS 38	Wild type	17
S. marcescens NS 25-23	Serrawettin W2 producer, Fla$^-$	15
S. marcescens 274 AB-1	Serrawettin W1 producer, Fla$^-$	This study
E. coli SM10λ*pir*	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km λ*pir*R6K	19
Plasmids		
pSM7	*hag* Ap	20
pGP704	*ori* R6K *mob* RP4 Ap	19
pGA1	*hag ori* R6K *mob* RP4 Cm Ap	This study

developed in chloroform–methanol–5 M ammonia (80:25:4 [vol/vol/vol]) and examined for the presence of specific spots as reported previously (18).

Extracellular complementation of serrawettins. Paper disks containing 50 μg of one of the bacterial surface-active exolipids serrawettin W1, serrawettin W2, rhamnolipid from *Pseudomonas aeruginosa* (a gift from Y. Ishigami, National Chemical Laboratory for Industry, Tsukuba, Japan), and surfactin from *Bacillus subtilis* (Wako, Tokyo, Japan) were placed over sites point inoculated with mutant bacteria on low-agar media. After appropriate incubation times, the effects of these exolipids on the spreading of bacteria were recorded. Control disks contained no wetting agent. Disks containing non-wetting agents such as lactose (a nonmetabolizable sugar for *S. marcescens*) elicited no spreading, similarly to control disks with no wetting agent.

RESULTS

Flagellum-independent spreading of *S. marcescens* 274. Flagellated *S. marcescens* cells display both swimming and swarming motility (2). Swimming can be observed in liquid media or in low-agar (0.2 to 0.4%) media. In media of the latter type, the bacteria occupy a subsurface space, apparently swimming through water channels inside the agar (Fig. 1A). Higher agar concentrations inhibit swimming motility (2). Upon growth on media with 0.5 to 0.8% agar, the bacteria differentiate into hyperflagellated swarmer cells which swarm over the surface (Fig. 1B). Nonflagellated (Fla$^-$) bacteria can neither swim nor swarm (Fig. 1C). However, they display another mode of surface translocation, termed spreading, on the surface of low-agar media (Fig. 1D). Microscopic observation of spreading bacteria does not reveal forward and reverse movement, such as that seen in swarmer cells (20). Instead, the bacteria appear to be pushed outward continuously, their cell axes often perpendicular to the direction of cell movement. Spreading is best observed on PG medium. All three forms of movement (swimming, swarming, and spreading) are optimal at 30°C and inhibited above 37°C, a temperature permissive for growth.

Consequence of introduction of a Fla mutation into motility mutants. A large class of *S. marcescens* mutants are defective specifically in swarming (20). The Dps mutants swarm within the inoculated area but are unable to migrate beyond this area, while the Dis mutants (which include chemotaxis mutants) display no swarming. Spreading on low-agar medium is only displayed by Fla$^-$ or Mot$^-$ (absence of motor function) mutants.

In order to determine if functions that affect swarming also affect the spreading movement displayed by nonflagellate bacteria, a large number of the Dps and Dis mutants were made nonflagellate (Table 2). This was done by replacement of the flagellin-encoding chromosomal *hag* gene in the mutants with a *hag*::Cm allele carried on a suicide vector (see Materials and Methods). All 14 of the chemotaxis-defective mutants, which are completely defective in swarming (Dis), were made Fla$^-$. We found that none of the Che$^-$ Fla$^-$ double mutants thus constructed were defective in spreading (Fig. 2A). These results suggest that chemotaxis functions most likely do not play a role in spreading.

TABLE 2. *S. marcescens* 274 mutant properties with respect to spreading behavior and serrawettin production

Strain	Phenotype(s)			
	Swarming[a]	Spreading[b]	Wetting activity[c]	Serrawettin W1[d]
SMu 11a	Dis (Che$^-$)	+	+	+
SMu 13b	Dis (Che$^-$)	+	+	+
SMu 25a	Dis (Che$^-$)	+	+	+
SMu 30a.1	Dis (Che$^-$)	+	+	+
SMu 30c	Dis (Che$^-$)	+	+	+
SMu 37a	Dis (Che$^-$)	+	+	+
SMu 48c	Dis (Che$^-$)	+	+	+
SMu 1450	Dis (Che$^-$)	+	+	+
SMu 1811	Dis (Che$^-$)	+	+	+
SMu 2087	Dis (Che$^-$)	+	+	+
SMu 2461	Dis (Che$^-$)	+	+	+
SMu 3262	Dis (Che$^-$)	+	+	+
SMu 3401	Dis (Che$^-$)	+	+	+
SMu 4401	Dis (Che$^-$)	+	+	+
SMu 3202	Dis	−	−	−
SMu 3354	Dis	NT	+	+
SMu 3451	Dis	NT	+	+
SMu 3551	Dis	−	−	−
SMu 4201	Dis	NT	+	+
SMu 4802	Dis	+	+	+
SMu 4851	Dis	+	+	+
SMu 4855	Dis	NT	+	+
SMu 1a	DpsI	−	−	−
SMu 2b	DpsI	+	+	+
SMu 4e	DpsI	−	−	−
SMu 13a	DpsI	−	−	−
SMu 27a	DpsI	+	+	+
SMu 4502	DpsI	+	+	+
SMu 4803	DpsI	+	+	+
SMu 4804	DpsI	+	+	+
SMu 4853b	DpsI	−	−	−
SMu 4854	DpsI	+	+	+
SMu 30a	DpsII	NT	+	+
SMu 2473	DpsII	+	+	+
SMu 3502	DpsII	NT	+	+
SMu 3452	DpsII	+	+	+
SMu 3653	DpsII	+	+	+
SMu 4101	DpsII	NT	+	+
SMu 4102	DpsII	NT	+	+
SMu 4202	DpsII	NT	+	+
SMu 4304	DpsII	NT	+	+
SMu 4353	DpsII	+	+	+
SMu 4402	DpsII	NT	+	+
SMu 4454	DpsII	NT	+	+
SMu 4455	DpsII	+	+	+
SMu 4501	DpsII	+	+	+
SMu 4503	DpsII	NT	+	+
SMu 4552B	DpsII	NT	+	+
SMu 15002	DpsII	NT	+	+

[a] Swarming phenotype on 0.75% Difco agar swarming media. Che$^-$, chemotaxis defective; Dps, swarming within inoculated area only; Dis, defective in swarming.

[b] After disruption of the chromosomal *hag* gene, each mutant was examined for spreading behavior on 0.35% Difco agar PG media. NT, not tested.

[c] Examined in the single mutants (Fla$^+$) by the glass-slide method (14).

[d] Serrawettin W1 production was examined by direct colony thin-layer chromatography.

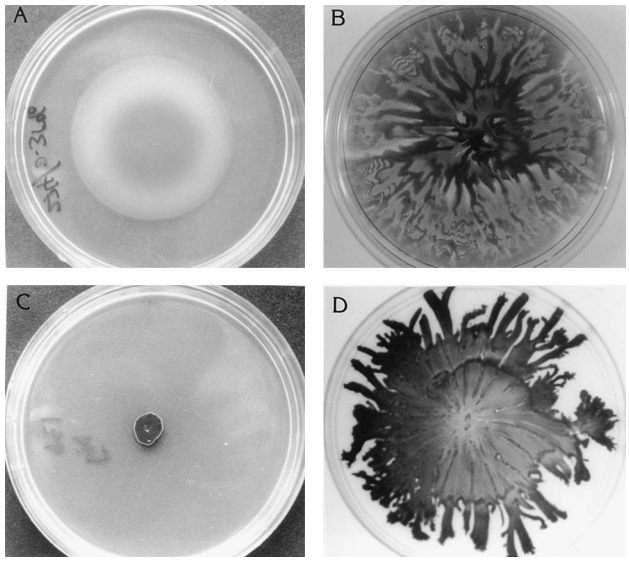

FIG. 1. Colony morphology of *S. marcescens* 274 during various modes of translocation. (A) Swimming motility (observed in flagellated cells) on 0.35% LB agar plates 8 h after inoculation. Under the microscope, the bacteria are seen to occupy a subsurface space. (B) Swarming motility (observed in flagellated cells) on the surface of 0.75% LB agar plates. (C) Defective swarming of a Fla$^-$ mutant (274 AB-1) on 0.75% LB agar plates. (D) Spreading movement of nonflagellated cells (274 AB-1) of the same line as that shown in panel C on the surface of 0.35% PG agar plates. Cells were inoculated in the center of the petri dish and incubated at 30°C overnight, unless otherwise indicated.

The Dps mutants show two different morphologies (20). The presence of the extracellular slime layer could be visually discerned in DpsII mutants but not in DpsI mutants. The Fla defect was introduced into all of the DpsI mutants and into a subset of the DpsII as well the Dis mutants. Six (four DpsI and two Dis) of the double mutants thus obtained were defective in spreading (Fig. 2B and C), while all the DpsII Fla$^-$ double mutants tested were proficient in spreading (Fig. 2D). As described below, there was a total correlation between the ability of these double mutants to spread and their ability to produce serrawettin.

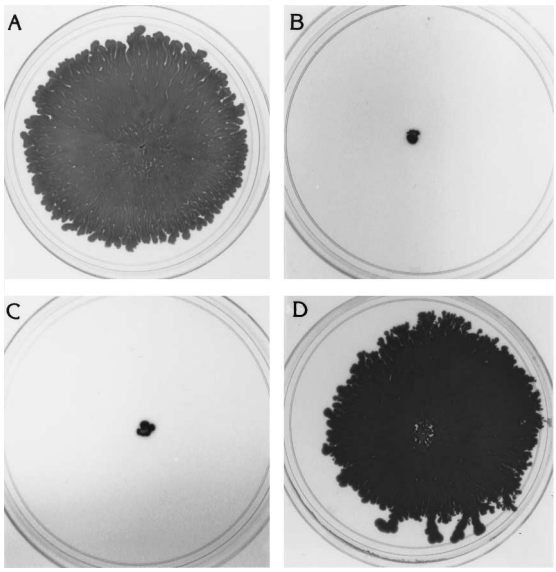

FIG. 2. Spreading in nonflagellated swarming-defective mutants of *S. marcescens* 274. Che, Dps, and Dis mutants of *S. marcescens* were made Fla$^-$ (see the text) and tested on 0.35% agar PG plates for spreading. (A) SMu 11a (Che$^-$); (B) SMu 3202 (Dis); (C) SMu 1a (DpsI); (D) SMu 2473 (DpsII).

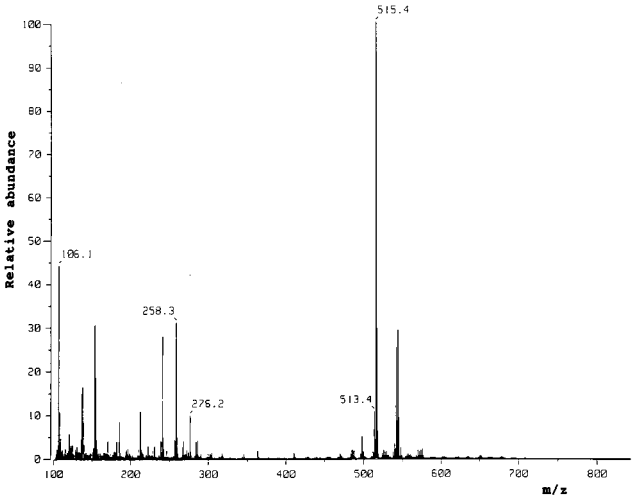

FIG. 3. Positive FAB-MS spectrum of surface-active exolipid from *S. marcescens* 274.

Characterization of serrawettin defects in motility mutants. *S. marcescens* 274 grown at 30°C demonstrated wetting activity when the bacterial mass was mixed with water on a glass slide, suggesting production of a wetting agent by the bacteria. After observation of the presence of a characteristic large spot having the same R_f value as that of serrawettin W1 (from strain NS 38) in a thin-layer chromatogram of lipids extracted from the strain, the exolipid was purified by preparative thin-layer chromatography and its wetting activity was confirmed. Positive and negative FAB-MS analyses of the exolipid gave spectra fairly identical to those of serrawettin W1. The mass spectrum shown in Fig. 3 was obtained by positive FAB-MS and clearly demonstrates an MH^+ main peak at *m/z* 515.4 and a small peak of its molecular isomer at *m/z* 515.4 + 28 (composed of 3-hydroxydodecanoic acid instead of 3-hydroxydecanoic acid). Thus, we have identified the surface-active exolipid produced by strain 274 as serrawettin W1. This is consistent with our previous observation that pigmented *S. marcescens* strains are serrawettin W1 producers (17).

Next, *S. marcescens* mutants showing characteristic defects in swarming behavior were examined for their wetting activity and production of serrawettin W1. The results, summarized in Table 2, clearly indicate that Fla$^-$ mutants defective in spreading were defective in serrawettin production (Swt$^-$). Direct colony thin-layer chromatograms of some of the Swt$^-$ Fla$^-$ DpsI mutants which were defective in spreading are shown in Fig. 4. In contrast to the chromatograms of reference strains NS 25-23 (a serrawettin W2 producer) and NS 38 (a serrawettin W1 producer) and to those of parent strains 274 and 274 AB-1 (a Fla$^-$ mutant of 274), absence of the W1 spot in the chromatograms of these DpsI mutants is evident.

Extracellular complementation. The effects of external supply of serrawettin on bacterial spreading were examined. After point inoculation of Swt$^-$ Fla$^-$ mutants, a paper disk containing serrawettin W1 or W2 was placed over the inoculated site. In contrast to Fla$^-$ Swt$^-$ mutants covered with a paper disk containing no exolipids, all Fla$^-$ Swt$^-$ mutants covered with a paper disk with the serrawettins demonstrated spreading behavior. In Fig. 5A, effects of serrawettin W1 and W2 on one of

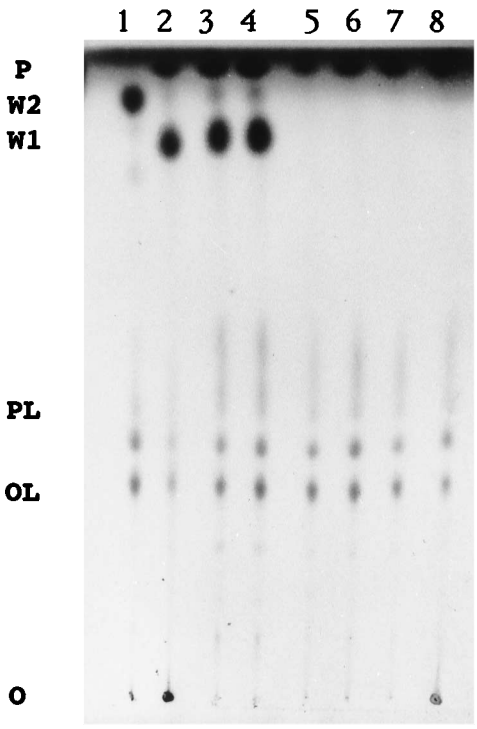

FIG. 4. Thin-layer chromatograms (direct colony) of *S. marcescens* NS 25-23 (lane 1), NS 38 (lane 2), 274 (lane 3), 274 AB-1 (lane 4), SMu 1a (Fla^-) (lane 5), SMu 4e (Fla^-) (lane 6), SMu 13a (Fla^-) (lane 7), and SMu 4853b (Fla^-) (lane 8). P, prodigiosin; W1 and W2, serrawettin; PL, phospholipids; OL, ornithine-containing lipid; O, origin. Bacteria were grown on PG agar medium for 2 days at 30°C. After development, the plate was sprayed with 50% (vol/vol) H_2SO_4 and heated at 200°C for 20 min.

Fla^- Swt^- mutants are shown. Surface-active exolipids produced by other species of bacteria, surfactin from *B. subtilis* and rhamnolipid from *P. aeruginosa*, were also seen to be effective on the spreading of Fla^- Swt^- *S. marcescens* (Fig. 5B). These exolipids also promote spreading of nonmotile bacteria such as *Klebsiella pneumoniae*, albeit to a much lesser extent than that seen with *S. marcescens* (not shown). An artificial surface-active lipid (Tween 80) was also effective for bacterial spreading on a low-agar medium (data not shown).

The commercial source of swarm agar influences surface colonization by many swarming-defective mutants. We have recently shown that agar obtained from a Japanese manufacturing company (Eiken) is effective in promoting swarming in organisms not observed to swarm before (10, 12). We therefore tested Swt^- Fla^- mutants on low-agar medium solidified with Eiken agar. As seen in Fig. 6A, compared with complete absence of translocation on PG medium with Difco agar upon overnight incubation, the Swt^- mutants showed a small zone (0.5 to 1 cm) of spreading.

We next tested all the swarming-defective transposon mutants of *S. marcescens* (20) for swarming on media prepared with Eiken agar. Of the two mutant categories (Dis and Dps), we found that the Dps phenotype is a conditional one, in that if the source of the agar was Eiken, all of these mutants showed normal swarming behavior (Fig. 6B). These results suggest that some surface property of the Eiken agar overrides the swarming defect in these mutants. Since Swt^- mutants constitute only a fraction of the Dps mutants, other components besides serrawettin (perhaps those affecting cell surface properties) must also play an important role in surface colonization.

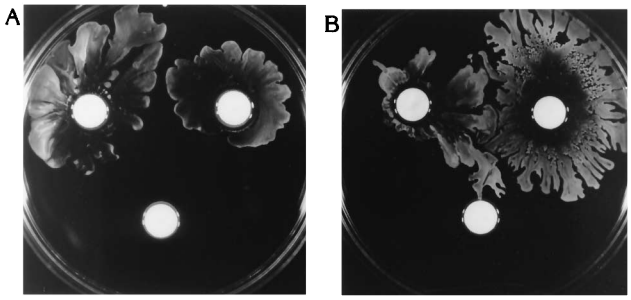

FIG. 5. Effect of purified serrawettins and bacterial exolipids on spreading of an *S. marcescens* Fla^- Swt^- mutant. (A) A sterile paper disk containing 50 μg of serrawettin W1 (upper right), serrawettin W2 (upper left), or no serrawettin (lower center) was placed at the site point inoculated with a Swt^- Fla^- mutant, 2 h after inoculation. The 0.35% agar PG plate was incubated at 30°C for 24 h. (B) A sterile paper disk containing 50 μg of rhamnolipid (upper right) or surfactin (upper left) or neither (lower center) was placed at the site point inoculated with an Swt^- Fla^- mutant, as described for panel A.

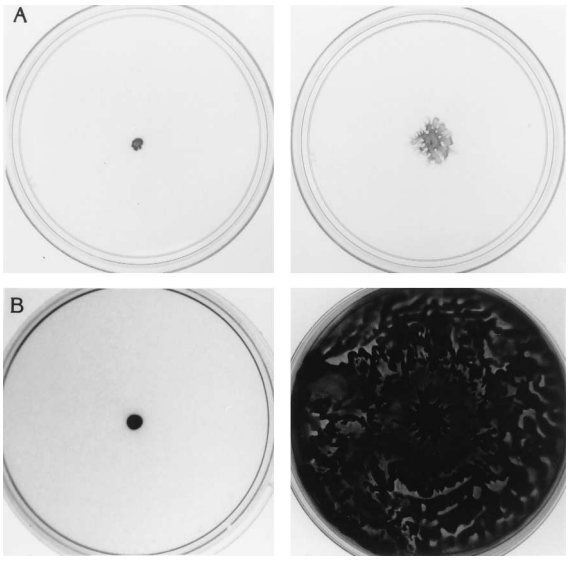

FIG. 6. Effects of commercial source of agar on surface translocation of *S. marcescens* 274. (A) Spreading behavior of an Swt^- Fla^- mutant on 0.35% agar PG medium solidified with either Difco (left) or Eiken (right) agar and incubated at 30°C overnight. (B) Swarming behavior of a Dps mutant on 0.75% LB medium solidified with Difco (left) or Eiken (right) agar and incubated as for panel A.

DISCUSSION

Serrawettin W1, produced by many pigmented *S. marcescens* strains, is a surface-active cyclodepsipeptide [cyclo-(D-3-hydroxydecanoyl-L-seryl)$_2$] identical to serratamolide, which was discovered by Wasserman et al. as an antibiotic (23, 24). Serrawettin gets its name by virtue of its wetting activity on various hydrophobic and hydrophilic surfaces (14). The extracellular amount of serrawettin constitutes 15 to 17% of the dry weight of bacterial mass (14). Isolated serrawettin lowers the surface tension of saline (17). In earlier studies, serrawettins were demonstrated to promote a highly branched two-dimensional population expansion (termed fractal morphogenesis) of colonies of *S. marcescens* (flagellated or nonflagellated), during several days (3 to 30) of growth on minimal hard (1.5%) agar (16). Fractal morphogenesis can also be observed in *B. subtilis* and *P. aeruginosa*, organisms known to produce surface-active compounds, as well as in *Escherichia coli*, *Salmonella typhimurium*, and *K. pneumoniae*, provided that 0.4% glucose is

added to the minimal medium (see reference 16). Serrawettin does not, however, affect swimming of *S. marcescens* in the subsurface space observed in low-agar media (15). Defects in serrawettin production, as well as growth at 37°C (which markedly reduces serrawettin production), interfere with fractal morphogenesis, and purified serrawettins can complement this behavior when supplied extracellularly at the higher temperature. Thus, extracellular lipids appear to contribute specifically to bacterial colonization of surfaces by exhibiting surfactant activity.

We have shown in this study that the spreading movement of nonflagellated *S. marcescens* 274 on low-agar media is also dependent on the presence of serrawettin W1. Spreading is independent of the presence of chemotaxis functions as well as other functions important for swarming. Chemotaxis functions have been proposed to play a role in gliding and in other social behaviors in the nonflagellated gliding bacterium *Myxococcus xanthus* (21, 22). Our results imply that the spreading movement analyzed here is most likely a passive form of surface translocation.

Bacteria can have a variety of surface layers external to the cell wall (3). These include capsules, external slimes, sheaths, and S layers. These external layers must play an important role in fostering both cell-cell contact and cell-substratum contact, thereby promoting the effective bacterial colonization of surfaces. We have shown that flagellate but nonswarming Dps mutants of *S. marcescens* with defects in serrawettin production do not swarm on media solidified with Difco agar. Wetting agents are therefore important in controlling swarming on certain surfaces. However, the existence of a large number of Dps mutants of *S. marcescens* (with defects other than in serrawettin production) whose ability to colonize the surface by swarming depends on the source of the agar (Difco or Eiken) implicates several bacterial functions besides serrawettin, possibly operating at the cell surface, in colonization of surfaces. Mutants of *S. typhimurium* LT2 lacking various regions of the outer membrane LPS layer, for example, show a similar conditional ability to swarm on media prepared with Difco versus Eiken agar (O-polysaccharide [Ra] mutants can swarm on Difco agar but core oligosaccharide mutants [Rb_1, Rc, Rd_1, Rd_2, and Re] can swarm only on Eiken agar [10a]). The integrity of the LPS layer, as well as the secretion of extracellular polysaccharides, is important for the gliding motility of myxobacteria (22). Cell surface polysaccharides and sulfonolipids have also been implicated in the colonization of surfaces by other nonflagellate gliding bacteria (1, 8).

In view of the above discussion, the dramatic effect of Eiken versus Difco agar on the colonization of the agar surface by swarming motility is not surprising. Eiken agar either may induce the synthesis of bacterial compounds that aid surface colonization or may provide some physical or chemical component that helps this process. (The presence of an active wetting agent in Eiken agar, however, could not be demonstrated in an examination by the glass slide method [13a, 14]). These observations are in keeping with our recent demonstration of swarming on Eiken agar in organisms such as *E. coli* and *S. typhimurium* and a number of other gram-negative bacteria (10, 12). In another recent study using epifluorescence, scanning confocal laser, and on-line visualization microscopy, major differences were found in the morphologies of a marine bacterium when it was grown on hydrophobic surfaces and when it was grown on hydrophilic surfaces (6). Thus, both the surface layers of microorganisms (LPS, slimes, and surfactants) and the surface properties of the growth substrata are important in bacterial colonization. Since many pathogenic bacteria colonize the surfaces of tissues as well as those of prosthetic devices introduced into the human body (5), altering surface layers of bacteria and altering surface properties of the colonized substrates should be important strategies for intervention in pathogenesis.

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