Modulation of Swarming and Virulence by Fatty Acids through the RsbA Protein in *Proteus mirabilis*

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After sensing external signals, *Proteus mirabilis* undergoes a multicellular behavior called swarming which is coordinately regulated with the expression of virulence factors. Here we report that exogenously added fatty acids could act as signals to regulate swarming in *P. mirabilis*. Specifically, while oleic acid enhanced swarming, some saturated fatty acids, such as lauric acid, myristic acid, palmitic acid, and stearic acid, inhibited swarming. We also found that expression of hemolysin, which has been shown to be coordinately regulated with swarming, was also inhibited by the above saturated fatty acids. Previously we identified a gene, *rsbA*, which may encode a histidine-containing phosphotransmitter of the bacterial two-component signaling system and act as a repressor of swarming and virulence factor expression in *P. mirabilis*. We found that while myristic acid, lauric acid, and palmitic acid exerted their inhibitory effect on swarming and hemolysin expression through an RsbA-dependent pathway, the inhibition by stearic acid was mediated through an RsbA-independent pathway. Biofilm formation and extracellular polysaccharide (EPS) production play an important role in *P. mirabilis* infection. We found that RsbA may act as a positive regulator of biofilm formation and EPS production. Myristic acid was found to slightly stimulate biofilm formation and EPS production, and this stimulation was mediated through an RsbA-dependent pathway. Together, these data suggest that fatty acids may act as environmental cues to regulate swarming and virulence in *P. mirabilis* and that RsbA may play an important role in this process.

*Proteus mirabilis*, a motile gram-negative bacterium, is an important pathogen of the urinary tract and is the primary infectious agent in patients with indwelling urinary catheters (42). The ability of *Proteus mirabilis* to colonize the surfaces of catheters and the urinary tract is believed to be aided by its characteristic known as swarming differentiation and migration (2). Swarming involves differentiation of vegetative cells into hyperflagellated swarm cells that undergo rapid, coordinated population migration across solid surfaces (17, 21). In *P. mirabilis*, swarming migration involves the coordinate differentiation of short, motile, vegetative cells with a few peritrichous flagella into multinucleate, aseptate swarm cells of up to 40 times the vegetative cell length and with more than 50-fold greater surface density of flagella. *P. mirabilis* swarm cells migrate rapidly away from the colony as multicellular rafts until they pause (consolidation) and undergo some dedifferentiation (4, 34). Regular cycles of migration and consolidation generate a colony on the agar surface with a characteristic pattern of concentric rings (4, 34).

The ability of *P. mirabilis* to express virulence factors, including urease, protease, and hemolysin, and to invade human urothelial cells is coordinately regulated with swarming differentiation (3, 6, 26, 27). Characterization of swarming-defective *Proteus* transposon mutants has indicated that a substantial number of proteins are involved in regulation of swarming (13, 17, 19, 22). These include FlhA, a protein involved in flagellum assembly and swarm cell differentiation (19); FlhD2C2 (het- erotetramers of FlhD and FlhC), a transcriptional activator that regulates the expression of the flagellar regulon (17); Umo proteins, which upregulate the *flhDC* operon during swarming differentiation (13); and Lrp, a global transcriptional regulator that links the physiological signals to swarming differentiation (22). Among them, FlhDC, Lrp, and the Umo proteins probably function as part of a broader regulatory network that may include bacterial two-component systems and the chemotaxis phosphoryl (17).

Previously we identified two genes, *rsbA* and *rsmA*, whose products negatively regulate swarming and virulence factor expression in uropathogenic *P. mirabilis* (27, 28). *rsbA* is a gene which may encode a histidine-containing phosphotransmitter of the bacterial two-component signaling system (7, 27, 39), and *rsmA* is a global regulatory gene widely distributed among many bacteria (28). How the RsbA- and RsmA-dependent pathways are integrated with other signal pathways to regulate swarming and virulence factor expression is currently not known. *P. mirabilis* swarming requires the sensing and integration of a variety of environmental, cell-to-cell, and intracellular signals. These signals may include those transmitted by high population density, surface contact, peptides and amino acids, and intracellular cations (3, 5, 17, 21, 25). Although the mechanisms of signal sensing and transduction are still poorly understood, it is generally believed that signals may be sensed and transmitted by two-component regulatory systems and then cytosolic regulators, leading to a complex regulatory network in which the *flhDC* master operon may be the primary site for integration of signals (17). Stimulation of the *flhDC* operon initiates swarm cell differentiation, which involves the develop-
opment of characteristic traits such as cell elongation, multi-nucleation, and hyperflagellation (14).

Fatty acids or their derivatives have been shown to be involved in regulation of swarming differentiation and virulence factor expression (29, 37, 41). It was reported that an fadD (a gene whose product is a long-chain fatty acyl-coenzyme A synthetase and involved in fatty acid degradation) mutant of Sinorhizobium melliloti shows multicellular swarming migration and is impaired in nodulation efficiency on alfalfa roots (37). In Xanthomonas campestris pv. campestris, the rpf gene cluster acts to regulate the synthesis of extracellular enzymes and virulence (36). Two members of this gene cluster, rpfB and rpfF, are involved in a novel type of regulation mediated by a small diffusible molecule called diffusible signal factor, which is probably a fatty acid derivative (41). A possible role for peptide or fatty acid signals in regulation of Proteus mirabilis swarming is also indicated by the finding that differentiation and migration are impaired by mutations in a locus that encodes components required for both the nonribosomal synthesis of peptides and also polyketide/fatty acid synthase function (18).

To further investigate the role of fatty acids or derivatives in regulation of swarming and virulence of Proteus mirabilis, in this study, we added various fatty acids to the culture medium and analyzed their effects on swarming, hemolysin activity, biofilm formation, and extracellular polysaccharide (EPS) production. We found that some saturated fatty acids (SFAs), such as lauric acid, myristic acid, and palmitic acid, could inhibit swarming and hemolysin activity. We also found that the inhibitory effect of these fatty acids was mediated through RsbA, a protein with high homology to two-component sensor proteins.

MATERIALS AND METHODS

Chromatography. All chemicals including fatty acids were purchased from Sigma. Bacterial strains and culture conditions. The bacterial strains used in this study are the wild-type P. mirabilis strain P19, the P. mirabilis P19 rbsD-defective mutant P1100, and the RsbA-complemented strain Pc (27). Bacteria were routinely cultured at 37°C in Luria-Bertani (LB) medium.

Swarming behavior assays. The swarming migration distance assay was performed as described previously (20, 27). Briefly, an overnight bacterial culture (5 μl) was inoculated centrally onto the surface of dry LB swarming agar (2%, wt/vol) plates without or with appropriate fatty acids, which were then incubated at 37°C. The swarming migration distance was assayed by following the swarm fronts of the bacterial cells and recording progress at 30- or 60-min intervals.

Measurement of cell length. Measurement of cell length was performed as described previously (27). Briefly, 150 μl of stationary-phase LB cultures were spread onto LB agar plates without or with appropriate fatty acids and incubated at 37°C for various times. After incubation, cells from the entire surface were harvested by washing into 5 ml of LB. Bacterial cells were then fixed in 4% paraformaldehyde and examined by light microscopy at a magnification of 1,000× under oil immersion with an Olympus BH2 microscope equipped with a graticule. The lengths of 100 cells in each sample were determined, and the average was calculated.

Measurement of hemolysin activity. Preparation of cells for the hemolysin assay was performed as described previously (27). Briefly, 150 μl of stationary-phase LB cultures were spread onto LB agar plates without or with appropriate fatty acids and incubated at 37°C for various lengths of time. After incubation, cells from the entire surface were harvested by washing into 5 ml of LB. Cell membrane-associated hemolysin activity was assayed as described (24).

Biofilm formation assays. Biofilm formation was assayed by measuring the ability of cells to adhere to the wells of 96-well microtiter dishes made of polystyrene (Becton Dickinson) as described (33). Briefly, 10 μl of overnight LB cultures was diluted to 1 ml with LB broth, and 100 μl was transferred to the polystyrene microtiter well. The microtiter dishes were sealed with paraffin and incubated at 37°C for 10 h. After incubation, the wells were rinsed with distilled water and air dried at room temperature for 15 min. Two hundred microliters of crystal violet (1%) solution was added to each well, and the dishes were incubated for 20 min. The crystal violet-stained biofilms were rinsed several times with distilled water, air dried at room temperature for 15 min, and then extracted twice with 200 μl of 95% ethanol. The extract containing solubilized crystal violet was adjusted to a total volume of 1 ml with distilled water, and the absorbance at 540 nm was determined with a Beckman DU-640B spectrophotometer.

Quantitative measurement of EPS production. Quantitative measurement of EPS production was performed as described previously (40). Briefly, cultures of P. mirabilis were grown overnight in LB broth. The cells were then washed twice with 0.9% NaCl solution, resuspended in equal volumes of 0.9% NaCl solution, and inoculated at 1:100 dilution into 15 ml of CPG broth (1% Casamino Acid; 1% glucose; 1% peptone). EPS was determined at 3 h postinoculation, at which time the optical density at 600 nm was about 0.6. Fixed amounts of cells were collected by centrifugation at 8,000 × g for 30 min. The unbond EPS present in the culture supernatant was precipitated with 2.5 volumes of absolute ethanol. To recover the capsular EPS fraction bound to the bacterial cells, the cell pellets were resuspended in 5 ml of high-salt buffer (10 mM K2HPO4/KH2PO4, pH 7.0; 15 mM NaCl; 1 mM MgSO4) and vortexed for 1 h. Cells were removed by centrifugation at 12,000 × g for 30 min and then resuspended in 1.5 ml of sterile H2O. The amount of total carbohydrates contained in each sample was determined by the phenol-sulfuric acid method. Briefly, 500 μl of sample solution was mixed with 500 μl of phenol solution in a glass tube, and then 2.5 ml of sulfuric acid reagent was rapidly added and vortexed. After incubation in the dark for 1 h, spectrophotometric analysis at wavelength 490 nm was performed. The amount of EPS produced was derived from a standard curve prepared with known concentrations (10 to 100 μg/ml) of t-glucose.

RESULTS

Regulation of P. mirabilis swarming by fatty acids. Besides cell density and glutamine, fatty acids have been implied as environmental signals for swarming of P. mirabilis (18). We therefore tested the effect of a series of fatty acids, including lauric acid, myristic acid, palmitic acid, stearic acid, and oleic acid, on swarming of wild-type P. mirabilis on LB swarming agar (2%) plates at 37°C. The concentration of these exog-enously added fatty acids was 0.01% (wt/vol) (lauric acid, 499 μM; myristic acid, 437 μM; palmitic acid, 389 μM; oleic acid, 354 μM) with the exception of stearic acid, which was added at 0.005% (wt/vol) (175 μM). We found that while oleic acid enhanced swarming, lauric acid, myristic acid, palmitic acid, and stearic acid inhibited swarming significantly (Fig. 1).

To test whether this inhibitory effect was dose dependent, myristic acid at 0.01%, 0.005%, 0.0025%, or 0.00125% (wt/vol) was added to LB swarming agar plates, followed by the swarming assay at 37°C. As shown in Fig. 2, swelling of P. mirabilis was inhibited by myristic acid in a dose-dependent manner. A similar dose-dependent inhibitory effect on swarming was also observed for lauric acid, palmitic acid, and stearic acid (data not shown).

The inhibitory effect of these SFAs on swarming might arise from a toxic effect on the bacteria. We therefore tested whether these SFAs would affect the growth rate of P. mirabilis. Our data showed that these SFAs did not affect the growth rate of the bacteria at the concentration used (data not shown), indicating that inhibition of swarming by SFA was unlikely to be due to its inhibitory effect on cell growth. Addition of SFA to the swarming agar plates may affect the viscosity of the agar and thereby interfere with the rotation of flagella and cause inhibition of swarming. We measured the viscosity of the agar media with and without SFA. No significant difference in viscosity was found (data not shown). Taken together, these data indicate that swelling of P. mirabilis was inhibited by some...
SFAs and that fatty acids may act as signals to regulate swarming.

Previously we identified several *P. mirabilis* superswarming mutants that contain a Tn5 insertion in the *rsbA* gene and have a higher ability to express virulence factors and to invade host cells (27). Characterization of these *rsbA* mutants led us to conclude that RsbA may act as a repressor of swarming and virulence factor expression (27). To investigate whether RsbA plays a role in SFA-mediated swarming inhibition, one of these *rsbA*-defective mutants, P1100, was tested for its response to SFAs. Consistent with our previous report (27), the *rsbA*-defective mutant migrated further in a given time than did the wild-type cells. Whereas swarming of wild-type *P. mirabilis* was inhibited by myristic acid, lauric acid, and palmitic acid, that of the *rsbA*-defective mutant was not (Fig. 3A and B). These data indicate that myristic acid, lauric acid, and palmitic acid exerted their inhibitory effect through an RsbA-dependent pathway. In contrast, when stearic acid was present in the LB swarming agar plates, swarming of wild-type and *rsbA*-defective mutant cells was inhibited to comparable levels (Fig. 3C), indicating that stearic acid inhibited *P. mirabilis* swarming through an RsbA-independent pathway.

**FIG. 1.** Effect of fatty acids on the swarming of wild-type *P. mirabilis*. (A) Histogram showing the swarming migration distance of *P. mirabilis* in the presence of different fatty acids. Aliquots (5 μl) of bacterial culture were inoculated onto the center of LB swarming agar plates without or with the indicated fatty acid. The concentration of fatty acids added was 0.01% (wt/vol) with the exception of stearic acid, which was added at 0.005% (wt/vol). The plates were incubated at 37°C, and the migration distance was measured after 7 h of incubation. The data represent the averages of four independent experiments with standard deviations. nil, no fatty acid; L, lauric acid; M, myristic acid; P, palmitic acid; S, stearic acid; O, oleic acid. (B) Halo images of swarming plates containing different fatty acids. The representative plates in A were photographed. a, palmitic acid; b, stearic acid; c, oleic acid; d, lauric acid; e, no fatty acid; f, myristic acid.

**FIG. 2.** Swarming of *P. mirabilis* is inhibited by myristic acid in a dose-dependent manner. (A) Histogram showing the swarming migration distance of *P. mirabilis* in the presence of different concentrations of myristic acid. Aliquots (5 μl) of bacterial culture were inoculated onto the center of LB swarming agar plates containing various concentrations of myristic acid. The plates were incubated at 37°C, and the migration distance was measured after 6 h of incubation. The data represent the averages of four independent experiments with standard deviations. (B) Halo images of swarming plates containing different concentrations of myristic acid. The representative plates in A were photographed. a, 0.01% (437 μM); b, 0.005% (218 μM); c, 0.0025% (109 μM); d, 0.00125% (55 μM); e, no fatty acid.
Regulation of swarming differentiation of *P. mirabilis* by SFAs. Knowing that the swarming behavior of *P. mirabilis* was inhibited by some SFAs, tests were performed to determine whether swarming differentiation of *P. mirabilis* was also affected by fatty acids. Wild-type and *rsbA*-defective mutant cells were spread on LB swarming agar plates either in the presence or in the absence of SFAs. Cell length, a marker of cell differentiation, was determined 2 h after seeding and hourly there-
after. As shown in Fig. 4, in the absence of fatty acids, the rsbA-defective mutant formed longer cells than the wild-type strain during the 7-h incubation period and became longest about 1 h earlier than wild-type cells. Moreover the rsbA-defective mutant retained its elongated cell shape for longer than the wild-type strain. These data, consistent with our previous report (27), indicate that the rsbA-defective mutant differentiated earlier and maintained a differentiated state for longer than the wild-type strain.

When lauric acid, myristic acid, or palmitic acid was added to the LB swarming agar plates, the length of the wild-type cells was reduced but that of the rsbA-defective mutant cells was not. These data indicate that lauric acid, myristic acid, and palmitic acid inhibit swarming differentiation of *P. mirabilis* and that this inhibition was mediated through an RsbA-dependent pathway. In contrast, when stearic acid was present in the LB swarming agar plates, the lengths of wild-type and rsbA-defective mutant cells were reduced to comparable levels, indicating that stearic acid inhibited swarming differentiation of *P. mirabilis* through an RsbA-independent pathway. To further investigate whether swarming differentiation of *P. mirabilis* was affected by SFAs, we tested the effect of myristic acid on the expression of the *flhDC* master operon by quantitative real-time reverse transcription-PCR. We found that myristic acid could inhibit the expression of *flhDC* by 2- and 2.5-fold at 4 and 5 h, respectively, after spot inoculation on swarming agar plates (data not shown).

**Regulation of virulence factor expression in *P. mirabilis* by SFAs.** Expression of virulence factors, including urease, protease, and hemolysin, is regulated coordinately with swarming differentiation (3, 6, 26, 27), and both are regulated by RsbA in *P. mirabilis* (27). Because swarming differentiation was inhibited by some SFAs, it was of interest to determine whether expression of virulence factors was also affected by fatty acids in *P. mirabilis*. Experiments similar to those described for measurement of cell length (see above) were performed, except that this time cell membrane-associated hemolysin activity, a dominant virulence factor in *P. mirabilis* (31), was assayed instead.

As shown in Fig. 5, in the absence of fatty acids, the rsbA-defective mutant expressed higher levels of hemolysin activity than did the wild-type strain during the 7-h incubation period, consistent with our previous report (27). Lauric acid, myristic acid, and palmitic acid inhibited the hemolysin activity of wild-type *P. mirabilis* but not the rsbA-defective mutant, indicating that these SFAs could inhibit the expression of hemolysin ac-
tivity in *P. mirabilis* through an RsbA-dependent pathway. In contrast, stearic acid inhibited the hemolysin activity of the wild-type strain and the rsbA-defective mutant to comparable levels, indicating that stearic acid, as in the case of swarming regulation, negatively regulated hemolysin expression in *P. mirabilis* through an RsbA-independent pathway.

**Regulation of biofilm formation in *P. mirabilis* by SFAs.**

Previous studies have shown that biofilm formation is closely related to swarming in many bacterial species, including *Salmonella enterica* (30), *Pseudomonas aeruginosa* (10), and *Burkholderia cepacia* (23). Since RsbA is involved in swarming regulation in *P. mirabilis*, we figured that biofilm formation might also be regulated by RsbA. To investigate this, the microtiter well assay (33), which monitors the ability of *P. mirabilis* to attach to the wells of microtiter plates, was used to quantify biofilm formation by the wild-type strain and the rsbA-defective mutant at 37°C. We found that the biofilm-forming ability of the wild-type strain was significantly higher than that of the rsbA-defective mutant (Fig. 6). These data indicate that RsbA is a positive regulator of biofilm formation in *P. mirabilis*. Addition of myristic acid to the culture medium increased biofilm formation in the wild-type strain but not in the rsbA-de-
fective mutant, suggesting that biofilm formation in *P. mirabilis* is also regulated by environmental cues such as fatty acids and that this regulation is mediated through an RsbA-dependent pathway.

**Regulation of EPS production in *P. mirabilis* by SFAs.** EPS may account for 50 to 90% of the total organic carbon of biofilms and is the primary matrix material of the biofilm (16). Knowing that biofilm formation in *P. mirabilis* was regulated by RsbA, EPS assays were performed to determine whether the production of EPS was also regulated by RsbA. Both bound and unbound forms of EPS produced by the wild-type strain and the *rsbA*-defective mutant were measured. As shown in Fig. 7, the *rsbA*-defective mutant produced less bound-form and unbound-form EPS than did the wild-type strain, indicating that RsbA was a positive regulator of EPS production in *P. mirabilis*. When myristic acid was included in the growth medium (CPG broth), production of EPS was increased in the wild-type strain but not in the *rsbA*-defective mutant. These data suggest that production of EPS, similar to biofilm formation, is also regulated by fatty acids and that this regulation involves RsbA-regulated pathways.

**Complementation of the *rsbA*-defective mutant by the wild-type *rsbA* gene restores wild-type responsiveness to fatty acids.** To further confirm that loss of responsiveness to some SFAs in the *rsbA*-defective mutant is indeed due to the defectiveness of the *rsbA* gene, an RsbA-complemented strain (Pc), which was generated by transforming the wild-type *rsbA* gene into the *rsbA*-defective mutant (27), was tested for its responsiveness to myristic acid. As shown in Fig. 8A, in the absence of myristic acid, the Pc strain was more responsive to myristic acid than the *rsbA*-defective mutant, indicating that RsbA was a positive regulator of EPS production in *P. mirabilis*. When myristic acid was included in the growth medium (CPG broth), production of EPS was increased in the wild-type strain but not in the *rsbA*-defective mutant. These data suggest that production of EPS, similar to biofilm formation, is also regulated by fatty acids and that this regulation involves RsbA-regulated pathways.

**FIG. 7.** Effect of SFAs on EPS production by the wild-type strain and *rsbA*-defective mutant of *P. mirabilis*. Bound-form, unbound-form, and total EPS produced by the wild-type strain and *rsbA*-defective mutant of *P. mirabilis* in the absence or presence of myristic acid (0.01%, wt/vol) were determined as described in Materials and Methods. The data represent the averages of 12 independent experiments with standard deviations. A significant enhancing effect of myristic acid on EPS production by the wild-type strain was observed by Student’s *t* test analysis (*P* < 0.05). w, wild type; m, mutant; nil, no fatty acid; M, myristic acid.

**FIG. 8.** Complementation of the *rsbA*-defective mutant of *P. mirabilis* by the wild-type *rsbA* gene restored wild-type responsiveness to fatty acids. The swarming migration distance (A) and the hemolysin activity (B) of the wild-type strain, the *rsbA*-defective mutant, and the RsbA-complemented strain in the absence or presence of myristic acid (0.01%, wt/vol) at various times after inoculation were determined as described in Materials and Methods. For hemolysin activity measurements, the value obtained with the wild-type cells in the absence of myristic acid at 4 h postseeding was set at 100%, and all other values were expressed relative to this value. The data represent the averages of three independent experiments. w, wild type; m, *rsbA*-defective mutant; c, RsbA-complemented strain; nil: no fatty acid; M, myristic acid.
acid, while the rsbA-defective mutant exhibited the super-swarming phenotype, the RsbA-complemented strain exhibited swarming behavior similar to that of wild-type P. mirabilis. In the presence of myristic acid, while swarming of the rsbA-defective mutant was not inhibited, that of the RsbA-complemented strain was inhibited to a level similar to that of the wild-type strain. Similar phenomena were also observed when lauric acid and palmitic acid were used (data not shown). Moreover, we also found that while swarming differentiation (as measured by cell length) of the rsbA-defective mutant was not inhibited by myristic acid, that of the RsbA-complemented strain was inhibited to a level similar to that of the wild-type strain (data not shown). Taken together, these data indicate that expression of RsbA in the rsbA-defective mutant led to the restoration of responsiveness to myristic acid, lauric acid, and palmitic acid and suggest that regulation of swarming by these SFAs is mediated through RsbA and not through other proteins in P. mirabilis.

Since some SFAs could inhibit hemolysin expression in P. mirabilis (see above), we also tested the ability of myristic acid to inhibit hemolysin expression in the RsbA-complemented strain, the rsbA-defective mutant, and the wild-type strain. While the hemolysin activity of the rsbA-defective mutant was not inhibited by myristic acid, that of the RsbA-complemented strain was inhibited to a level similar to that of the wild-type strain (Fig. 8B). These data further confirm that complementation of the rsbA-defective mutant with the rsbA gene restored its responsiveness to myristic acid and that regulation of hemolysin expression by myristic acid was mediated mainly through RsbA in P. mirabilis.

DISCUSSION

Swarm cell differentiation and swarming behavior are the results of complex sensory transduction and global control mechanisms. P. mirabilis swarming requires the sensing and integration of a variety of environmental, cell-to-cell, and intracellular signals and involves regulated expression of gene networks leading to morphological and physiological changes (3, 5, 17, 21, 25). Although a large body of information concerning swarming mechanisms in P. mirabilis has been accumulated, the signals regulating swarming and the pathways for signal transduction are still poorly understood. In this paper, we present evidence that fatty acids serve as environmental cues to regulate P. mirabilis swarming. Specifically, while oleic acid enhanced swarming, some SFAs, such as myristic acid, lauric acid, palmitic acid, and stearic acid, inhibited swarming in P. mirabilis (Fig. 1). We also found that expression of hemolysin, which is a dominant virulence factor and has been shown to be coordinately regulated with swarming (3, 6, 26, 27), was also inhibited by myristic acid, lauric acid, palmitic acid, and stearic acid in P. mirabilis (Fig. 5).

Previously we demonstrated that RsbA, which may be a His-containing phosphotransmitter of the bacterial two-component signaling system, can act as a repressor of swarming differentiation and virulence factor expression in P. mirabilis (27, 39). We found that while myristic acid, lauric acid, and palmitic acid exerted their inhibitory effect on swarming and hemolysin expression through an RsbA-dependent pathway, the inhibition by stearic acid was mediated through an RsbA-independent pathway (Fig. 3, 4, and 5). Together, these data demonstrate that fatty acids could act as extracellular signals to regulate swarming and virulence factor expression in P. mirabilis and reveal a new mechanism through which fatty acid exerted its regulatory effect.

RsbA has been shown to be able to regulate swarming differentiation, expression of virulence factors including hemolysin, protease, and urease, and cell invasion ability in P. mirabilis (27). In this paper we demonstrate that RsbA was also involved in regulation of biofilm formation and EPS production in P. mirabilis (Fig. 6 and 7). The rsbA-defective mutant produced significantly lower levels of biofilm and EPS than did the wild-type strain, suggesting that RsbA may act as a positive regulator of biofilm formation and EPS production. This is in sharp contrast to the regulation of swarming and expression of virulence factors such as hemolysin, protease, and urease, in which RsbA acts as a repressor. The mechanism by which RsbA differentially regulates swarming and hemolysin expression and biofilm and EPS production in P. mirabilis is not known.

RsbA has been suggested to be a His-containing phosphotransmitter of the bacterial two-component signaling system (7, 27, 39). It is highly homologous to the YojN protein of Escherichia coli (31% identity and 50% similarity), which was recently shown to be a His→Asp phosphorelay component of the RscC-RcsB two-component signaling system (39). In E. coli, the RscC-YojN-RcsB phosphorelay signaling pathway positively regulates capsular EPS production but negatively regulates swarming (39). A unique model has been formulated for the RscC-YojN-RcsB signaling system in E. coli. In this system, RscC, a membrane sensor histidine kinase, senses environmental stimuli, YojN serves as an intermediate for the phosphorelay, and finally RcsB, a response regulator, functions as a DNA-binding transcriptional regulator upon acquiring a phosphoryl group from YojN (39).

We propose that a similar RcsC-RsbA-RcsB signaling pathway may exist in P. mirabilis based on the following evidence. First, P. mirabilis has an rcsB-rscC locus very similar to that of E. coli (7, 39). Second, in E. coli, both rcsC- and yojN-defective mutants exhibit the superswarming phenotype (39). Interestingly, P. mirabilis rcsC and rsbA mutants resulting from Tn5 insertions also show the superswarming phenotype (7, 27). Third, rcsC- and yojN-defective mutants of E. coli lose their ability to produce capsular EPS (39). In this study, we also demonstrate that the rsbA-defective mutant of P. mirabilis had much lower ability to produce EPS (Fig. 7). Fourth, the P. mirabilis RsbA protein has a His-containing phosphotransmitter domain at its C-terminal end, as does its E. coli counterpart YojN (39).

Though not proven, we propose that RsbA, like YojN, functions as a His-containing phosphotransmitter in the RscC-RsbA-RcsB two-component signaling transduction system of P. mirabilis. Upon sensing certain environmental stimuli, RscC and RsbA may function together to phosphorylate the response regulator RcsB, which in turn regulates the expression of the response genes. The observation that the E. coli RscC-YojN-RcsB signaling pathway inversely regulates EPS production and swarming (39) is consistent with our data that RsbA upregulated EPS production but repressed swarming. That swarming and biofilm and EPS production are differentially regulated by a two-component system may be a common phe-
nommenon in bacteria. Recently, we identified a new two-component signal transduction system, RssA-RssB, which regulated swarming in *Serratia marcescens*. RssA, a sensor protein, was also found to downregulate swarming and hemolysin expression but upregulate biofilm and EPS production in *Serratia marcescens* (data not shown).

There is much evidence indicating that fatty acids or their derivatives can be involved in regulation of gene expression. In *Sinorhizobium melloti*, a mutation in fadD (a gene encoding a long-chain fatty acyl-coenzyme A synthetase) results in multicellular swarming migration and defects in nodulation on alfalfa plants, suggesting that fatty acid derivatives may act as intracellular signals controlling motility and symbiosis (37). In *E. coli*, long-chain fatty acyl-coenzyme A, the product of fadD, modulates the activity of the transcriptional regulator FadR, which regulates the expression of fatty acid biosynthesis and degradation genes (8). In *Salmonella enterica* serovar Typhimurium, loss of acyl-coenzyme A synthetase (FadD) represses the expression of a gene, *hilA*, whose product is involved in activating the expression of invasion genes, suggesting that fatty acyl-coenzyme A or its derivatives may act as intracellular signals regulating *hilA* and invasion gene expression (29).

In this paper, we demonstrate that addition of some SFAs to the culture medium can lead to the inhibition of swarming and virulence factor expression in *P. mirabilis*. It is possible that, upon uptake, these fatty acids or their derivatives may act as intracellular signals to regulate swarming and virulence factor expression through either an RsbA-dependent or RsbA-independent pathway.

Fatty acids and their derivatives have also been shown to act as intercellular communication signals in many bacteria. For instance, two groups of well-known cell-cell communication signals, acyl homoserine lactones, produced by many gram-negative bacteria (32, 35), and the 3-hydroxyacyl-CoA methyl ester from *Ralstonia solanacearum* (15), are derived from fatty acids. In *Myxococcus xanthus*, it was suggested that branched-chain fatty acids, synthesized during growth, are released from cellular phospholipids by a developmentally regulated phospholipase during fruiting-body formation. One or more of these released branched-chain fatty acids constitutes an essential signal (E-signal) which must be transmitted between cells to regulate the expression of many developmental genes (12). In *Bacillus subtilis*, KinA, a sensor kinase, is an important enzyme in a phosphorelay system that controls initiation of sporulation by regulating the phosphorylation state of the Spo0A transcriptional regulator. The activity of KinA has been shown to be regulated by fatty acids that were liberated from *B. subtilis* phospholipids and by certain purified fatty acids (38).

In *Xanthomonas campestris* pv. *campestris*, a diffusible signal factor released from the bacterial cells is perceived by RpfC, a hybrid two-component regulator containing both sensor kinase and response regulator domains, which then undergoes autophosphorylation and sets up a phosphorelay to the response regulator RpfG. Phosphorylation of RpfG leads to the activation of transcription of pathogenicity genes, such as those encoding extracellular enzymes and the biosynthetic enzymes for EPS. The diffusible signal factor was recently identified as α,β-unsaturated fatty acids which are produced by a variety of pathogenic bacteria (36, 41). Here we show that some exogenously added fatty acids can regulate swarming and virulence factor expression in *P. mirabilis* through either an RsbA-dependent or RsbA-independent pathway. It is possible that fatty acids may serve as cell-cell communication signals that interact with RcsC-RsbA or other membrane sensor proteins, leading to regulation of swarming and virulence factor expression in *P. mirabilis*.

Another intriguing hypothesis that explains fatty acid regulation of swarming and virulence factor expression is illuminated by the Des pathway of *Bacillus subtilis*. In *B. subtilis*, the Des pathway regulates the expression of the acyl-lipid desaturase Des, thereby controlling the synthesis of unsaturated fatty acids from saturated phospholipid precursors (9). The master switch for the Des pathway is a two-component regulatory system composed of a membrane-associated kinase, DesK, and a soluble transcriptional regulator, DesR, which stringently controls transcription of the *des* gene (1). When cells are shifted to the low growth temperature, membrane fluidity is decreased, which in turn activates the sensor kinase, DesK, leading to the activation of transcription of the *des* gene. In contrast, when cells are exposed to unsaturated fatty acids or some branched-chain amino acids, precursors of branched-chain fatty acids, membrane fluidity is increased, which leads to inactivation of the DesK kinase and inhibition of *des* gene expression. Therefore, the physical state of the membrane lipid bilayer may regulate the activity of membrane sensor kinases.

Here we found that some SFAs could inhibit swarming and virulence factor expression through RsbA in *P. mirabilis*. It is possible that these fatty acids may affect membrane fluidity, which in turn regulates the activity of RcsC-RsbA, leading to inhibition of swarming and virulence factor expression. In line with this proposition, we found that exogenously added lauric acid, myristic acid, palmitic acid, and stearic acid decreased membrane fluidity, while exogenously added oleic acid increased membrane fluidity. This change in membrane fluidity was found to be closely related to swarming phenotypes in *P. mirabilis* (data not shown).

It has been shown recently that cell-cell communication signals, which play important roles in functional coordination among family members in a range of biological activities, including swarming and expression of virulence genes, are promising molecular targets for the control of bacterial infection (11, 43). We have previously shown that *p*-nitrophenylglycerol can inhibit swarming and virulence factor expression in *P. mirabilis* (26, 27). Here we show that some SFAs can also inhibit swarming and virulence factor expression through RsbA in *P. mirabilis*. Moreover, in a related study, we also found that some SFAs had similar inhibitory effects on swarming of *Serratia marcescens* and *Salmonella enterica* (data not shown). These data indicate that these SFAs or their derivatives may act as negative signals for regulation of bacterial virulence. In this respect, it is important to elucidate the molecular mechanisms underlying fatty acid inhibition of swarming and virulence factor expression. Identification of the roles of fatty acids in controlling the virulence of *P. mirabilis*, *Serratia marcescens*, and *Salmonella enterica* may lead to the development of drugs that slow down bacterial infection, allowing the host to gain valuable time to activate defense mechanisms and to stop and eliminate pathogenic invaders.
ERRATUM

Modulation of Swarming and Virulence by Fatty Acids through the RsbA Protein in \textit{Proteus mirabilis}

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