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Effect of Salicylate on Expression of Flagella by *Escherichia coli* and *Proteus*, *Providencia*, and *Pseudomonas* spp.

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Osmotic stress, salicylate, and Mar (multiple antibiotic resistance) mutation are known to block the expression of the OmpF porin. Since these conditions have also been shown to inhibit the expression of P and CFA fimbriae in *Escherichia coli*, we speculated that they might affect the expression of flagella as well. Hyperosmotic conditions have been shown to block the synthesis of flagellin and expression of flagella in *E. coli* (C. Li, C. J. Louise, W. Shi, and J. Adler, J. Bacteriol. 175:2229–2235, 1993). In the current study, sodium salicylate was found to inhibit the motility of *E. coli*, *Proteus mirabilis*, *Proteus vulgaris*, *Providencia rettgeri*, and *Providencia stuartii* in a reversible, concentration-dependent manner. Swarming did not occur at 20 mM sodium salicylate. Salicylate also blocked the synthesis of flagellin in *E. coli*. Phenotypic Mar mutants of *E. coli* derived from motile strains were amotile. Flagella were markedly reduced as determined by scanning electron microscopy when *P. mirabilis* was grown in broth containing 20 mM salicylate. Salicylate had no apparent effect, however, on expression of a 40-kDa porin protein in *P. mirabilis*. This finding suggests that the noted effect of salicylate on *Proteus* spp. may be mediated through a mechanism other than porin production or that the *Proteus* porin may not be analogous to OmpF in *E. coli*. Salicylate decreased the motility of *Pseudomonas cepacia* but had no effect on *Pseudomonas aeruginosa*. The exact mechanism by which salicylate exerts its effect is not known, but it appears to be related to osmoregulation.

Li et al. recently demonstrated that hyperosmotic conditions block the synthesis of flagellin and expression of flagella in *Escherichia coli* (17, 25). Osmotic stress also inhibits the expression of OmpF porin (2, 27) and type 1, P, S, and CFA fimbriae in *E. coli* (13). Salicylate blocks the expression of OmpF independent of osmolarity and decreases the permeability of the outer membrane of *E. coli* to beta-lactam antibiotics and other drugs (5, 11, 21, 23). Salicylate also has been shown to block the expression of P and CFA fimbriae (13). Mar (multiple-antibiotic-resistant) mutants of *E. coli* derived from P-fimbriated strains do not express OmpF and are no longer able to agglutinate Gal-Gal beads or human erythrocytes (13). In view of these observations, we wondered whether there might be a link between the expression of OmpF and the production of other outer membrane-associated organelles. To explore this possibility, we examined the effect of salicylate and Mar mutation, both of which are associated with reduced OmpF, on the expression of flagella in *E. coli*.

The ability of salicylate to inhibit swarming by *Proteus* species and other members of the family *Enterobacteriaceae* was also examined to determine whether this compound might have an effect on related bacteria. *Pseudomonas aeruginosa* was included because its porins are distinctly different from those of *E. coli* (28).

MATERIALS AND METHODS

Bacterial strains. The *E. coli*, *Proteus*, *Providencia*, and *Pseudomonas* strains used in this study are listed in Table 1. *E. coli* 1177 was isolated from a child with a first known episode of acute pyelonephritis (26). Strain U4 (O2:H7) is a clinical isolate obtained from a patient with acute cystitis (13). Tetracycline-resistant variants of *E. coli* 1177 and U4 were isolated as previously described (13). These strains had the phenotypic characteristics of Mar mutants (5). They differed from the parent strains in that OmpF was no longer apparent on polyacrylamide gel electrophoresis (PAGE); they exhibited a two- to eightfold increase in MICs of the beta-lactam antibiotics ampicillin, ampicillin/sulbactam, and cephalexin; there was a two- to fourfold increase in the MIC of ofloxacin, but no change in the MICs of the aminoglycoside antibiotics gentamicin, tobramycin, trimethoprim sulfamethoxazole, and nitrofurantoin (data not shown). *E. coli* K10 was provided by Linda Tombras Smith, University of California, Davis (16). *P. aeruginosa* PAO1 was provided by Neil Baker, The Ohio State University (28). All the other strains were obtained from the American Type Culture Collection or the Clinical Microbiology Laboratory of The Ohio State University.

Media and growth conditions. Swarming was determined as described by Li et al. (17). A single colony was isolated and grown overnight at 37°C in 1% Bacto Tryptone broth (Difco) plus 0.5% NaCl (BTB-NaCl). Approximately 10^8 CFU was inoculated at the center of petri plates containing 30 ml of BTB-NaCl in 0.25% Bacto Agar (Difco). The plates were incubated for up to 72 h at room temperature (20°C) or for 24 h at 37°C. The zone diameter of the swarm was measured at timed intervals.

Scanning electron microscopy. *Proteus mirabilis* (ATCC 7002) was incubated for 24 h at 37°C in BTB-NaCl alone or supplemented with 10 or 20 mM sodium salicylate (Sigma Chemical Company, St. Louis, Mo.). Bacterial cells were pelleted and washed twice with sterile saline. A droplet of the washed bacterial cell suspension was fixed onto a glass slide by overnight incubation at room temperature in 2.5% glutaraldehyde, washed twice for 15 min with 0.1 M cacodylate buffer (pH 7.2), incubated in 1% (wt/vol) thiocarbohydrazide for 30 min, washed five times with double-distilled H_2O, fixed with 1.33% osmium tetroxide for 2 h, washed five times with double-distilled H_2O, and dehydrated by sequential 15- or 30-min incubations in a graded series of ethanol (35 to 100%). Specimens were then critical point dried with CO_2 for 2 h, coated with gold, and viewed blindly by the same operator, using a Hitachi S800 scanning electron microscope. Two stubs were prepared for each specimen, and a minimum of 20 fields at a magnification of ×8,000 were scanned per stub for expression of flagella by individual bacterial cells. Fields were rated qualitatively as abundant (3+), moderate (2+), minimal (1+), or no (0) flagella. Representative fields were photographed at a magnification of approximately ×15,000.

Preparation of cell extracts for OMPs. Outer membrane proteins (OMPs) were prepared by differential centrifugation of crude sonic extracts obtained from cultures grown for 24 h with or without addition of sodium salicylate and solubilized with sodium-*N*-lauryl sarcosinate (14). OMPs were resolved by vertical slab gel electrophoresis (12% polyacrylamide) and stained with Coomassie brilliant blue.

Immunoblotting for flagellin. The amount of total flagellin (intracellular flagellin plus intact flagella) was determined by the method of Li et al. (17). The cells were grown in BTB-NaCl alone or supplemented with sodium salicylate at 5 or 10 mM. Cells were harvested and resuspended in Laemmli buffer (15)

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TABLE 1. Effect of sodium salicylate on the swarming diameter of bacteria incubated for 24 h at 37°C on 0.25% agar tryptone plates

Strain	Source or reference	Swarming diam (cm) at sodium salicylate concn (mM) of:				
		0	5	10	15	20
Escherichia coli						
25922	ATCC[a]	8.5	8.5	4.0	3.0	1.0
1177 (TS)[b]	26	8.5	8.5	7.2	5.8	0.5
1177 (TR)[c]	13	0.0	0.0	0.0	0.0	0.0
U4 (TS)	13	4.8	0.0	0.0	0.0	0.0
U4 (TR)	13	0.0	0.0	0.0	0.0	0.0
K10	16	8.5	2.0	0.0	0.0	0.0
Proteus mirabilis						
7002	ATCC	8.5	8.5	8.5	7.0	0.0
02495	Clinical	8.5	8.5	8.5	6.0	0.0
T68111	Clinical	8.5	8.5	3.8	0.0	0.0
T66508	Clinical	8.5	8.5	8.0	1.5	0.0
Proteus vulgaris 33420	ATCC	8.5	8.5	1.0	0.0	0.0
Providencia rettgeri T67738[d]	Clinical	8.5	6.5	2.5	0.0	0.0
Providencia stuartii 35031	ATCC	8.5	8.5	1.5	0.0	0.0
Pseudomonas aeruginosa						
27853	ATCC	8.0	8.0	8.0	8.0	8.0
PAO1	28	8.0	8.0	8.0	8.0	8.0
Pseudomonas cepacia	Clinical	2.9	2.2	1.8	1.8	1.0

[a] ATCC, American Type Culture Collection.
[b] TS, tetracycline sensitive (tetracycline MIC, 1.25 μg/ml).
[c] TR, tetracycline resistant (tetracycline MIC, >10 μg/ml).
[d] Growth at 48 h.

containing 0.1% β-mercaptoethanol and boiled for 5 min. Flagellin was isolated as described by Edelmann and Gallant (9). Protein content was determined by the modified Lowry method (19). A total of 5 μg of whole cell protein or 0.5 μg of purified flagellin was loaded into each well of a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel. The proteins were resolved by SDS-PAGE and transferred to a nitrocellulose membrane (pore size, 0.2 μm; Bio-Rad, Hercules, Calif.). Flagellin was detected with polyclonal rabbit antiflagellum antibody specific for *E. coli* (kindly provided by Robert Macnab, Yale University, New Haven, Conn.), diluted 1:1,000, and developed with alkaline phosphatase-conjugated goat anti-rabbit antibody (Bio-Rad).

RESULTS

Effect of sodium salicylate on swarming by motile bacteria. The swarming diameter was measured after incubation for 24 h at 37°C on Bacto Tryptone plates alone or containing various concentrations of sodium salicylate (Table 1). Swarming of *E. coli*, *Proteus mirabilis*, *Proteus vulgaris*, *Providencia stuartii*, *Providencia rettgeri*, and *Pseudomonas cepacia* was inhibited by sodium salicylate in a concentration-dependent manner. Swarming was completely blocked for most strains by 20 mM sodium salicylate. The effect of sodium salicylate on swarming was reversed when the bacteria were transferred to a salicylate-free medium. Sodium salicylate at 20 mM had no effect on *P. aeruginosa*. A slight decrease in the swarming diameter was noted for the *P. aeruginosa* strains at concentrations of 100 mM. Tetracycline-resistant strains of *E. coli*, which lacked OmpF, lost the swarming ability of their parent strains. The effect noted with sodium salicylate was shown to be independent of the known effects of osmolarity on OmpF expression since sodium *p*-hydroxybenzoic acid (Sigma), which has the same molecular weight, had no effect on swarming by any of the bacterial strains when tested at the same concentrations (data not shown).

Some experiments were conducted at room temperature (20°C) to slow the rate of swarming. This improved our ability to determine the effect of time and graded concentrations of sodium salicylate on the swarming diameter. Sodium salicylate delayed swarming of *E. coli* ATCC 25922 and *Proteus mirabilis* ATCC 7002 in a concentration-dependent manner (Fig. 1). Swarming progressed over time in the presence of 2.5 to 15.0 mM sodium salicylate. Swarming was completely blocked by 20 mM sodium salicylate even after incubation for up to 72 h (data not shown). This was not due to a lethal effect since growth of the microorganisms in broth supplemented with 20 mM salicylate was only slightly retarded in the presence of 20 mM salicylate and was unaffected by growth in lower concentrations (not shown). Sodium salicylate at 20 mM had no effect on the ability of *P. aeruginosa* 27853 to swarm (data not shown).

Effect of sodium salicylate on production of *E. coli* flagellin. *E. coli* K10 was grown in BTB-NaCl alone or with addition of 5 or 10 mM sodium salicylate. A flagellin band was noted on the immunoblot of whole cells grown in media alone but was absent in the immunoblot of cells grown broth supplemented with salicylate (Fig. 2).

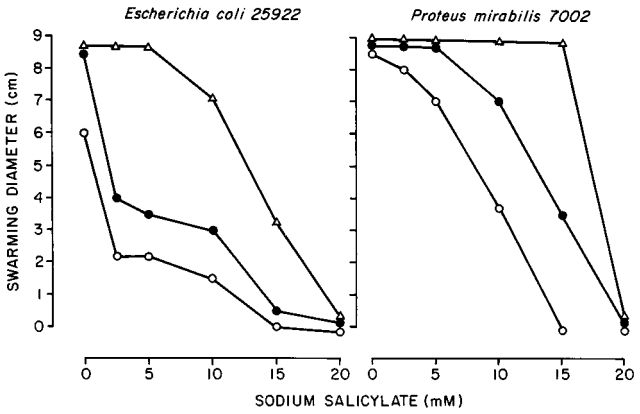

FIG. 1. Effect of sodium salicylate on the swarming diameter of *E. coli* and *Proteus mirabilis* incubated at room temperature (20°C) for 16 (○), 24 (●), and 48 (△) h.

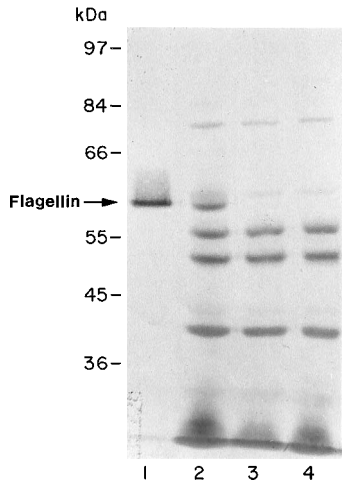

FIG. 2. Immunoblot of flagellin protein extracted from *E. coli* K10 (lane 1) and from whole cells grown in BTB-NaCl alone (lane 2), with 5 mM sodium salicylate (lane 3), and with 10 mM sodium salicylate (lane 4). Molecular mass markers are shown on the left.

Effect of sodium salicylate on OMP profiles in *Proteus mirabilis*. Salicylate has been shown to block the expression of OmpF in *E. coli* (5, 11, 21, 23). OMP profiles were obtained for *Proteus mirabilis* ATCC 7002 and T66508 grown in Trypticase soy broth alone or in the presence of 20 mM sodium salicylate. There was no apparent change in the density of the band observed at 40 kDa (not shown). This band has been reported to correspond to an outer membrane porin protein in *Proteus mirabilis* (24) but may not be analogous to OmpF in *E. coli*.

Scanning electron microscopy of *Proteus mirabilis*. No discernible difference in gross expression of flagella was noted between cells of *Proteus mirabilis* ATCC 7002 grown for 24 h at 37°C in BTB-NaCl alone and in BTB-NaCl supplemented with 10 mM sodium salicylate (Fig. 3A and B). All fields viewed for both growth conditions were rated as 2+ or 3+. However, flagella were rarely seen on cells grown in 20 mM sodium salicylate-supplemented media (Fig. 3C); all fields were rated as 0 or 1+. Specimens grown separately under identical culture conditions, were also prepared for transmission electron microscopy (data not shown). Transmission electron microscopy data corroborated the scanning electron microscopy findings.

DISCUSSION

The signals that control the process of ordered export of flagella and the mechanisms that shut off their synthesis under adverse conditions are unknown (18, 25). The observation that hyperosmolar conditions reduce the synthesis of flagellin and expression of flagella (17) as well as fimbria (13) in *E. coli* offers a potentially important clue. Salicylate and Mar mutation are known to be associated with decreased expression of the porin protein OmpF (5, 11, 13, 20, 21, 23, 27) and of P and CFA fimbriae in *E. coli* (13). We now show that salicylate reversibly blocks the expression of flagella in *E. coli*. We also found that Mar mutants lose the motility of the parent strains. These observations raise the possibility that a common mechanism controls the expression of several cell wall-associated organelles at an early stage in their synthesis or assembly. Salicylate severely depresses the translation of *ompF* and enhances the transcription of *micF* and *ompC* independent of the osmolarity of the medium (21). Salicylate can additionally induce transcription of the *mar* operon, increase *mar*-specific RNA, and decrease expression of OmpF (4). Hyperosmolar conditions appear to be linked to these mechanisms through the *micF* gene (20). *micF* RNA is reported to have a major role in osmoregulation of OmpF in *E. coli* (20). Thus, although the exact site at which control of expression of cell wall-associated organelles occurs remains unknown, it appears to be associated with osmoregulatory mechanisms in *E. coli*.

Salicylate also produces a variety of effects on several other members of the family *Enterobacteriaceae* in addition to *E. coli*. It inhibits the expression of porins in *Klebsiella pneumoniae* and *Serratia marcescens* (23) and the production of capsular polysaccharide in *K. pneumoniae* and potentiates the activity of cationic aminoglycoside antibiotics against these bacteria (6–8). It also reduces the adherence of *E. coli* to silastic catheters

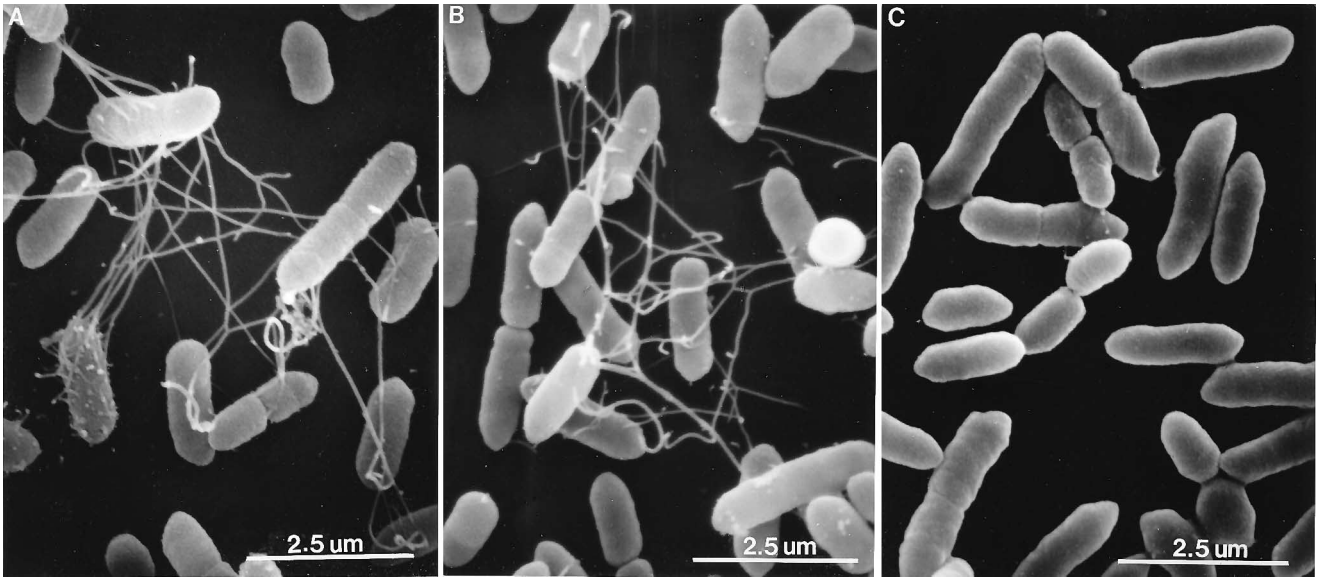

FIG. 3. Scanning electron micrographs of *Proteus mirabilis* (ATCC 7002) grown in BTB-NaCl supplemented with 0 mM sodium salicylate (A), 10 mM sodium salicylate (B), or 20 mM sodium salicylate (C). Note the relative expression of flagella in panels A and B versus panel C.

(10). In the current experiments, we found that salicylate inhibits the motility of *Proteus* and *Providencia* strains as well.

An alternate explanation is that hyperosmolarity, salicylate, and Mar mutation independently effect the expression of fimbria and flagella. The lack of an effect of salicylate on a 40-kDa porin protein in *Proteus mirabilis* suggests that it may act independently of synthesis of this porin. The single porin found in *Proteus mirabilis* may not be analogous to OmpF in *E. coli* (24). The inability of salicylate to effect the motility of *P. aeruginosa* may be due to differences in structure, function, or control mechanisms among the porins of *P. aeruginosa* and members of the family *Enterobacteriaceae*. The porins of *P. aeruginosa* are monomers and are less permeable to small molecules compared with the OmpF trimers in *E. coli* (28). The effect of salicylate on the motility of *P. cepacia* may be related to its ability to decrease expression of a pore-forming outer membrane protein in this microorganism (3).

The observations reported in this study, taken together with those of other investigators, suggest that salicylate may be the prototype of a new group of compounds that might be used to alter the expression of OmpF, fimbria and flagella, and capsular polysaccharides of enteric bacteria. Motility is not known to be a virulence factor for *E. coli* (12), but the ability of *Proteus mirabilis* to invade human urothelial cells has been shown to be coupled to motility (1). It would be expected that compounds that inhibit the expression of OmpF could also potentially decrease susceptibility to many antibiotics (3–4, 5, 11).

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