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# Chemotaxis of *Pseudomonas syringae* subsp. *savastanoi* Virulence Mutants

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**Several mutants of *Pseudomonas syringae* subsp. *savastanoi* were tested for their ability to sense and respond to a chemotactic gradient in low concentrations of yeast extract. The mutants were deficient in one or both of the genes coding for the synthesis of the plant hormones indole-3-acetic acid (IAA) and isopentenyl adenosine. Mutations which resulted in the loss of IAA production were due to the loss of the entire plasmid containing the *iaa* operon or to an 18-kb deletion of the *iaa* region. Additional mutants tested were deficient in their ability to produce isopentenyl adenosine as a result of the loss of the *ptz*-bearing plasmid. In all cases, strains which had lost the ability to produce IAA exhibited enhanced motility of up to 2.5 times that of the wild type ($IAA^+$) in medium containing 0.01% yeast extract. No differences in motility were observed on medium containing lower concentrations of yeast extract. The presence or absence of the cytokinin plasmid and the presence or absence of inorganic nitrogen in the medium had no effect on the relative mobility of the strains.**

*Pseudomonas syringae* subsp. *savastanoi* causes the production of olive and oleander galls by secreting large amounts of the plant hormones indole-3-acetic acid (IAA), zeatin, and *trans*-zeatin riboside (8) into the intercellular spaces of the host plant (3, 15). IAA synthesis genes are found on the chromosome in olive isolates and on a 72-kb plasmid (pIAA2) in oleander isolates (9). Oleander-infecting strains of *P. syringae* subsp. *savastanoi* also possess several cryptic plasmids and a 27-kb plasmid (pCYT) that contains cytokinin synthesis genes (11). The bacterium enters through fresh wounds in the plant surface, usually leaf scars or frost cracks. Sources of inoculum are epiphytic populations (2, 4) or a bacterial ooze released from overwintering galls by free moisture (16).

Many bacteria, including plant pathogens, have the ability to sense chemicals in their environment and direct their movement toward or away from them. Motility and chemotaxis are widespread and highly conserved throughout the eubacteria, indicating a necessary function for these activities and the genes which encode them. Detection of a chemical gradient is required for chemotaxis to occur, whether the chemical serves as an attractant or a repellent or whether or not the chemical is metabolized (5). Attractant concentrations must fall within a specific range to activate the chemotactic system. Too low a concentration results in the absence of stimulation of the chemosensing system; too high a concentration saturates the cellular receptors, preventing the gradient-sensing system from functioning and inhibiting the chemotactic response (6, 7). Although it has been shown that *Agrobacterium* species sense and are attracted toward molecules which activate their virulence genes (13), the role of chemotaxis in allowing bacterial pathogens to efficiently enter wound tissue has not been directly investigated. Here we describe the in vitro chemotactic response of wild-type and mutant strains of *P. syringae* subsp. *savastanoi* oleander isolates which have lost the ability to produce IAA, a virulence determinant of the organism (1).

## MATERIALS AND METHODS

*P. syringae* subsp. *savastanoi* PB213, a wild-type Italian oleander isolate, and four derivative mutant strains were used. The strains and relevant phenotypes are listed in Table 1. Swarm plate media consisted of a minimal salts solution supplemented with yeast extract (YE) and 0.3% agar (0.01 or 0.001% YE, 0.75 g of $K_2HPO_4$ per liter, 0.95 g of $KH_2PO_4$ per liter, 0.2 g of $MgSO_4 \cdot H_2O$ per liter, 20 mM $NH_4NO_3$). For some experiments the media were prepared without the addition of $NH_4NO_3$.

The motility of a strain was quantified by inoculating the center of a swarm plate with cells from a fresh single colony by using a toothpick. Swarm plates were then incubated at room temperature (25°C) or (in one experiment) at 28°C. The distance from the inoculation point to the edge of the bacterial halo was measured on a dark-field light box over a 3-day period. Experiments were repeated three times, and the measure of motility was expressed as an average of the distance (millimeters) moved per hour after 72 h.

## RESULTS

The formation of bacterial halos in the agar media provided a reproducible assay of the swarming behavior of the strains tested. Growth of the colony at the center of the plate results in the formation of a gradient as the nutrients are metabolized. Chemotactically competent cells then respond to the gradient by moving outward from the point of inoculation.

Swarm rates of the strains and media tested are presented in Table 1. In all experiments with 0.01% YE, strains that were deficient in IAA production moved away from the inoculation point faster than IAA-producing strains did. Figure 1 is a dark-field photograph of a typical experimental result, comparing the rate of movement of the wild-type strain with that of $IAA^-$ mutants and strains which either did or did not carry pCYT, the plasmid which contains the genes for cytokinin synthesis. When the concentration of YE was reduced to 0.001%, no difference was observed between the

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TABLE 1. Phenotypes and swarm rates of *P. syringae* subsp. *savastanoi* strains

| Strain | Relevant phenotype | Mean swarm rate (mm/h) on[a]: | | Source or reference |
|---|---|---|---|---|
| | | 0.01% YE | 0.001% YE | |
| PB213 | IAA$^+$, wild type | 0.18 | 0.44 | 15 |
| 213-3 | IAA$^-$ (pIAA2$^-$) | 0.45 | 0.50 | This study |
| 213-6 | IAA$^-$ (pIAA2Δ)[b] | 0.41 | 0.44 | This study |
| 213-14 | IAA$^-$ (pIAA2$^-$), pCYT$^-$ | 0.49 | 0.51 | This study |
| 213-15 | IAA$^+$, pCYT$^-$ | 0.19 | 0.34 | This study |

[a] The mean swarm rates of *P. syringae* subsp. *savastanoi* strains were determined by measuring the distance that the outer margin of the colony moved on 0.3% agar medium over time (in millimeters per hour). Plates were inoculated with a toothpick in the center and incubated at 25 or 28°C for 72 h. Swarm rate values represent the mean of three experiments in which each strain was tested at least six times.

[b] Strain 213-6 contains an 18-kb deletion of pIAA2 which includes the *iaa* synthesis operon.

swarm rates of IAA$^+$ and IAA$^-$ strains. Swarm rates of all strains tested at 25°C were identical to those obtained at 28°C.

Strains 213-3 and 213-14, which lack pIAA2, and strain 213-6, which has an 18-kb deletion that includes the biosynthetic region of pIAA2, swarm at approximately the same rate (0.43 mm/h). The swarm rate for the wild-type PB213 was 0.18 mm/h, a rate less than half that for the IAA$^-$ mutants. The rate of movement from the inoculation point was consistent over the duration of the experiment for all tested strains (data not shown). Strains that were otherwise isogenic with the wild-type and IAA$^-$ strains, but which had also lost pCYT, the plasmid which contains the cytokinin synthesis genes, were also compared. The IAA$^-$ and cytokinin-deficient (Cyt$^-$) strain (strain 213-14) had a swarm rate similar to that of the IAA$^-$ strains, whereas the IAA$^+$ Cyt$^-$ strain (strain 213-15) had a swarm rate similar to that of the wild-type strain (Table 1). The mutant and wild-type strains were extensively tested for interstrain differences in growth rates in laboratory media, and no differences were found (14).

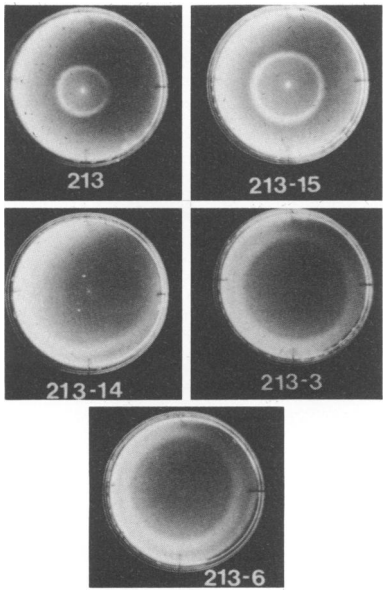


FIG. 1. Dark-field photographs of swarm agar medium plates inoculated with strains of *P. syringae* subsp. *savastanoi*. Plates were inoculated at the center and incubated at 25°C for 72 h. PB213 is the wild-type strain; 213-3, 213-6, and 213-14 are IAA$^-$ mutants. 213-14 and 213-15 are cytokinin-deficient mutants (pCYT$^-$). Strain 213-15 is IAA$^+$ and contains wild-type IAA plasmid (pIAA2).

## DISCUSSION

IAA$^-$ strains were able to move more quickly in semisolid culture medium containing 0.01% YE than IAA$^+$ (wild-type) strains were. Whether the increase in mobility of the IAA$^-$ strains of *P. syringae* subsp. *savastanoi* is due to the loss of IAA production or to the loss of some other function encoded on a neighboring region of pIAA is unknown. When the concentration of YE was reduced 10-fold, there was no apparent difference between the swarm rates of the wild-type and mutant strains. This observation suggests that the increased swarm rate of the IAA$^-$ mutants was not due to genetic changes that affected the ability of the bacterium to move (e.g., motility).

IAA synthesis is dependent on tryptophan, an IAA precursor. No difference was observed in the swarm rates of these wild-type or mutant strains tested on minimal medium or on 0.001% YE supplemented with 0.01 or 0.001% tryptophan (data not shown). However, tryptophan synthesis is influenced by the nitrogen status of the cell; therefore, the loss of a tryptophan sink such as the IAA pathway may somehow alter nitrogen levels in the cell and possibly affect the chemotactic or motile responses of the cell. The hypothesis that wild-type chemotactic responses are inhibited at higher nutrient levels is supported by the fact that swarm rates of IAA$^-$ mutants are the same as those of wild-type strains in 0.001% YE. How these metabolic changes affect the cell is unknown, but they may act upon a two-component regulatory system similar to those which have been shown to control expression of chemotaxis genes in *E. coli* (13).

Alternatively, some other gene(s) involved in carbon or nitrogen metabolism may have been lost upon deletion of the IAA region or loss of the entire pIAA plasmid. Loss of these putative genes may require the cell to use an alternate carbon or nitrogen pathway. In this model, the metabolism of an alternate substrate could reduce the substrate level in the medium to a level that is compatible with the chemotactic gradient-sensing system of the cell. In one experiment, the wild type and the pIAA2$^-$ strain 213-3 were inoculated into swarm plates which did not contain an inorganic nitrogen source (20 mM $NH_4NO_3$). Although both strains swarmed better in the presence of added nitrogen, the increase in the swarm rates of the IAA$^-$ strain was not greater than in medium unsupplemented with nitrogen.

The difference observed in IAA$^+$ and IAA$^-$ strain swarm rates appears to be unaffected by the presence or absence of the cytokinin gene-bearing plasmid. This suggests that whatever influence the IAA genes have on *P. syringae* subsp. *savastanoi* motility in this experimental system, it is independent of the cytokinin genes. Cytokinin genes are known to be expressed late in culture, and therefore they may not have been expressed under these experimental conditions.

Previous work with a closely related *P. syringae* pathovar indicates that the ability to detect and move to an infection site is an important component of the pathogenic potential of the organism (10). For *P. syringae* subsp. *savastanoi*, the number of cells surviving as epiphytes may be very low at the end of summer (4). Thus, the probability of fortuitous

infections based on the density of epiphytic populations would be low. An organism that can direct its movement toward a wound site that is secreting chemoattractant substances would have an increased chance of survival. Movement within the wound site toward conductive tissues or other microniches, where the environment is more conducive to growth of the parasite, may also affect the efficiency of disease initiation.

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