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Enhanced Epiphytic Coexistence of Near-Isogenic Salicylate-Catabolizing and Non-Salicylate-Catabolizing *Pseudomonas putida* Strains after Exogenous Salicylate Application

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The hypothesis that epiphytic bacterial populations can coexist through nutritional resource partitioning was tested with the near-isogenic bacterial strain pair *Pseudomonas putida* R20 and R20(pNAH7). The plasmid pNAH7 conferred upon R20 the ability to catabolize salicylate as a sole carbon source in vitro. *P. putida* R20(pNAH7) also catabolized exogenously applied salicylate in planta and reached a significantly larger epiphytic population size than the near-isogenic parental strain R20 under the same conditions. This supports previous observations that epiphytic populations on plants grown under nitrogen-sufficient conditions are limited by carbon availability. In the absence of exogenous salicylate, R20 and R20(pNAH7) competed for and partitioned endogenous carbon according to their inoculum proportion in replacement series experiments, exhibiting a low level of coexistence. In the presence of exogenous salicylate, however, R20(pNAH7) was solely able to catabolize the additional carbon and achieved a higher level of coexistence with R20 than was possible in the absence of exogenous carbon.

It was recently demonstrated that epiphytic populations of *Pseudomonas syringae* growing on moist leaves under constant environmental conditions in a growth chamber were limited by the availability of carbon (19, 20). Therefore, in vitro carbon source utilization profiles provided an estimate of the breadth of the limiting resource dimension of the ecological niche of these epiphytic *P. syringae* strains (20). Comparison of the in vitro carbon source utilization profiles of *P. syringae* with those of several other epiphytic species permitted quantification of the overlap of the ecological niche of *P. syringae* with the niche of each of the other species in the limiting resource dimension (20). Niche overlap indices were inversely correlated with the level of coexistence of each epiphyte with *P. syringae* in replacement series experiments (20). The epiphytic species exhibiting the lowest levels of ecological similarity to *P. syringae* (lowest niche overlap index) exhibited the highest level of coexistence (20). Coexistence between epiphytic bacteria mediated through ecological differentiation in carbon source utilization has been termed nutritional resource partitioning (17, 18). The extent of ecological niche differentiation required to achieve a measurable increase in coexistence between epiphytic populations is dependent upon the abundance of the uniquely utilized carbon sources in the phyllosphere. In theory, niche differentiation of a single carbon source could significantly alter the level of coexistence if that carbon source comprised a significant proportion of the pool of carbon present in leaf exudates.

The carbon source salicylate has been used to selectively enhance the population size of the salicylate-catabolizing strain *Pseudomonas putida* PpG7 both in soil and in the rhizosphere of tomatoes (3, 4). The plasmid pNAH7, which contains the genes for naphthalene degradation, was transferred from *P. putida* PpG7 to *P. putida* R20, conferring upon the transconjugant the ability to catabolize salicylate as a sole carbon source (3, 4). Population sizes of *P. putida* R20(pNAH7) were enhanced in salicylate-amended soil (3, 4) but were not increased in the carbon-rich spermosphere of sugar beet seed grown in soil amended with salicylate (9).

The hypothesis that epiphytic coexistence can be achieved through nutritional resource partitioning and the utilization of a unique carbon source in the carbon-limited phyllosphere was tested with the near-isogenic strain pair *P. putida* R20 and R20(pNAH7) and the exogenously applied carbon source sodium salicylate.

MATERIALS AND METHODS

Bacterial strains and inoculum preparation. The origin and phenotypic characteristics of *P. putida* R20 and the near-isogenic strain R20(pNAH7) have been described previously (3, 4). *P. putida* R20 and R20(pNAH7) were cultured on King's medium B (KB) (13) for 18 h at 28°C. Bacterial cells were scraped from the plate and suspended in phosphate buffer (0.01 M [pH 7.0]). The cell suspensions were adjusted turbidimetrically to the appropriate concentration with a standard curve relating optical density (λ = 600 nm) to culturable cells per milliliter. For coinoculations, cell suspensions were combined in equal proportions immediately prior to plant inoculation. For replacement series experiments, cell suspensions were combined in six different proportions [R20/R20(pNAH7) ratios of 0:1, 0.2:0.8, 0.4:0.6, 0.6:0.4, 0.8:0.2, and 1:0] at a constant total concentration of 10^6 CFU/ml. Where appropriate, sodium salicylate (sodium salt of 2-hydroxybenzoic acid [Sigma, St. Louis, Mo.]) was added to the inoculum immediately prior to plant inoculation.

Plant inoculation. Five replicate pots, each containing 10 bean plants (*Phaseolus vulgaris* cv. Bush Blue Lake 274), were sprayed with inoculum containing either each strain individually or both strains. The pots were covered with plastic bags to maintain a high level of relative humidity, fully randomized within the growth chamber, and incubated for 72 h at 26°C. All experiments were performed at least twice.

Enumeration of bacterial populations. Twenty leaves were collected randomly from each treatment, and individual leaves were placed in 20 ml of sterile phosphate buffer (0.01 M potassium phosphate buffer [pH 7.0]) in a glass tube. The tubes were sonicated in an ultrasonic cleaning bath for 7 min to dislodge the epiphytic microbial populations and vortexed briefly to suspend the cells. Serial dilutions of leaf washings were plated on KB amended with 100 μg of cycloheximide per ml, 50 μg of benomyl per ml (Benlate; Du Pont), and 100 μg of rifampin per ml. *P. putida* R20 and R20(pNAH7) were distinguished on plates with colonies of both strains by replica plating onto minimal medium A (15) containing sodium salicylate as a sole carbon source. The mean $\log_{10}$-transformed population size was estimated from 20 individual leaves.

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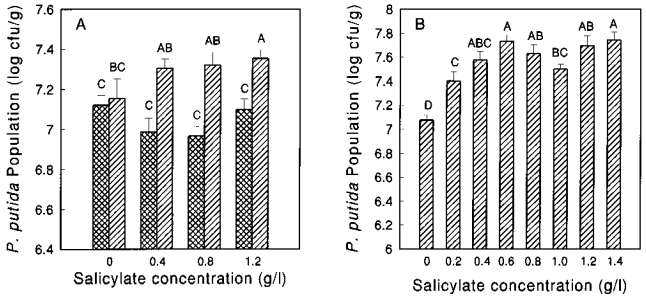

FIG. 1. Colonization of primary leaves of bean (*P. vulgaris* cv. Bush Blue Lake 274) by the near-isogenic bacterial strains *P. putida* R20 and R20(pNAH7) after amendment of the inoculum with different concentrations of sodium salicylate. Leaves were spray inoculated with each strain at an inoculum concentration of 10^6 CFU/ml and incubated for 72 h at 26°C. (A) Final population sizes of the salicylate-catabolizing strain R20(pNAH7) (hatched bars) and the noncatabolizing strain R20 (crosshatched bars). (B) Concentration response curve of *P. putida* R20(pNAH7). Bars represent 1 standard error of the mean. Population sizes followed by the same letter are not significantly different ($P = 0.05$).

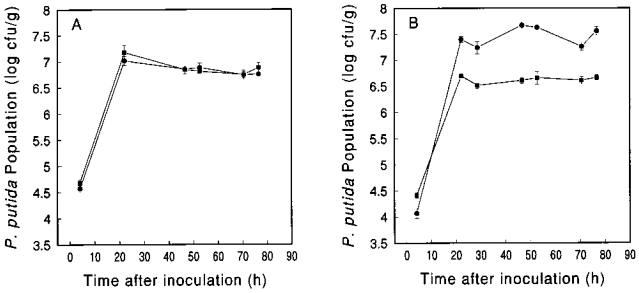

FIG. 2. Colonization of leaves of bean (*P. vulgaris* cv. Bush Blue Lake 274) by the near-isogenic bacterial strains *P. putida* R20 and R20(pNAH7). (A and B) R20 and R20(pNAH7), respectively, in the presence (circles) and absence (squares) of exogenous salicylate (1.2 g/liter). Bars represent 1 standard error of the mean.

In the replacement series experiments, the arithmetic back-transformed mean $\log_{10}$ population size of each strain and the arithmetic back-transformed mean $\log_{10}$ total population size were plotted against the inoculum proportion. In a replacement series between two strains which compete equally for all of the same limiting resources, there is a linear relationship between population size and inoculum proportion for each strain and the total population size is constant and is equal to the sum of the expected population sizes of the two strains (10). An increased level of coexistence of one strain with respect to the other is indicated by (i) a significant positive deviation from linearity in the relationship between population size and inoculum proportion for the strain showing increased coexistence and (ii) a total population size which is not constant over all proportions but which is greater in the coinoculations than when a single strain is inoculated alone (10). To test the linearity of the relationship between population size and inoculum proportion, the following model was used:

$$\log_{10}(\text{population size}_{ij}) - \log_{10}(\text{inoculum proportion}_i) = \text{mean}_i + \text{normal error}_{ij}$$

where i is the inoculum proportion and j is the leaf replicate. In this model, the population size is lognormally distributed and the relationship is linear only if all the means are equal. With analysis of variance (Proc GLM in SAS Rel. 6.08; SAS Institute, Cary, N.C.), equality of the means was determined with an F test (19).

The level of coexistence in replacement series was additionally quantified by calculation of the relative yield (RY) for each strain (i.e., the ratio of population size when coinoculated to population size when inoculated alone) and the relative yield total (RYT) for the near-isogenic strain pair [i.e., $\text{RYT} = \text{RY}_{\text{R20}} + \text{RY}_{\text{R20(pNAH7)}}$] in the presence and absence of exogenously applied salicylate. An RYT of 1.0 indicates a low level of coexistence, an RYT of greater than 1.0 indicates an increased level of coexistence, and an RYT of 2.0 indicates complete coexistence (i.e., both strains achieved the same population size when coinoculated as when inoculated alone) (10).

RESULTS AND DISCUSSION

Growth responses of the near-isogenic strains to exogenous application of sodium salicylate. Addition of the carbon source salicylate to the inoculum of the near-isogenic *P. putida* strains immediately prior to spray application onto bean plants significantly increased the final epiphytic population size of the salicylate-catabolizing strain R20(pNAH7) ($P = 0.05$) but did not significantly affect the population size of the non-salicylate-catabolizing strain R20 ($P = 0.05$) (Fig. 1A). Addition of salicylate at concentrations higher than 0.4 g/liter resulted in small but nonsignificant increases in epiphytic population size, and concentrations greater than 1.2 g/liter resulted in phytotoxicity (Fig. 1B). Hence, to achieve maximum response, a concentration of 1.2 g/liter was used in subsequent experiments.

The apparent growth rate and the final epiphytic population size of *P. putida* R20 were not significantly affected by the exogenous application of salicylate (Fig. 2A). While the growth rate of *P. putida* R20(pNAH7) was apparently not increased by the exogenous application of salicylate, the final epiphytic population size was significantly greater when the carbon source was provided than when it was not ($P = 0.05$) (Fig. 2B). The same results were observed in a separate experiment.

These data are consistent with the observations of Wilson and Lindow (19, 20) that epiphytic bacterial populations on leaves of plants grown under nitrogen-sufficient conditions and incubated under conducive environmental conditions in the growth chamber are carbon limited. The use of the near-isogenic strain pair demonstrated that the response was due to a direct effect of the carbon substrate, because only *P. putida* R20(pNAH7), which was able to catabolize salicylate in vitro as a sole carbon source, responded to the addition of salicylate. Furthermore, the magnitude of the response increased with increasing concentrations of the carbon source. These data suggest that the resource limitation of the epiphytic population of *P. putida* was alleviated by the exogenous application of carbon and that, beyond a concentration of approximately 0.4 g/liter, no further significant response was observed because the bacterial population size was then limited by a different resource, probably nitrogen. Indeed, Wilson and Lindow (19) observed that epiphytic populations of *P. syringae* were limited more by carbon than by nitrogen and that, after carbon addition, only nitrogen applications caused a further population increase. Colbert et al. (3, 4) also observed significant increases in population size of *P. putida* PpG7 and R20(pNAH7) in soil after application of sodium salicylate, and bulk soil is generally considered to be an oligotrophic, carbon-limited environment. However, no increase in population size was observed in the spermosphere of sugar beet seed, presumably because the bacterial populations growing in this environment, unlike the phyllosphere, were not carbon limited (9).

Effect of exogenous carbon addition on coexistence. In replacement series experiments with no exogenously applied salicylate, *P. putida* R20 and R20(pNAH7) coexisted at a low level [i.e., the plot of population size against inoculum proportion did not deviate significantly from linearity for strain R20(pNAH7) (Fig. 3A); the plot of population size against inoculum proportion deviated significantly from linearity for R20, but the deviation was small and was not consistent in direction (Fig. 3A); and the RYT approximated 1.0 (Fig. 3C)]. These data indicate that, in the absence of exogenous carbon, the near-isogenic strains R20 and R20(pNAH7) competed equally for limiting resources in the phyllosphere and partitioned those resources equally. When exogenous salicylate was provided, however, the level of coexistence of R20(pNAH7) with R20 was increased [i.e., the plot of population size against

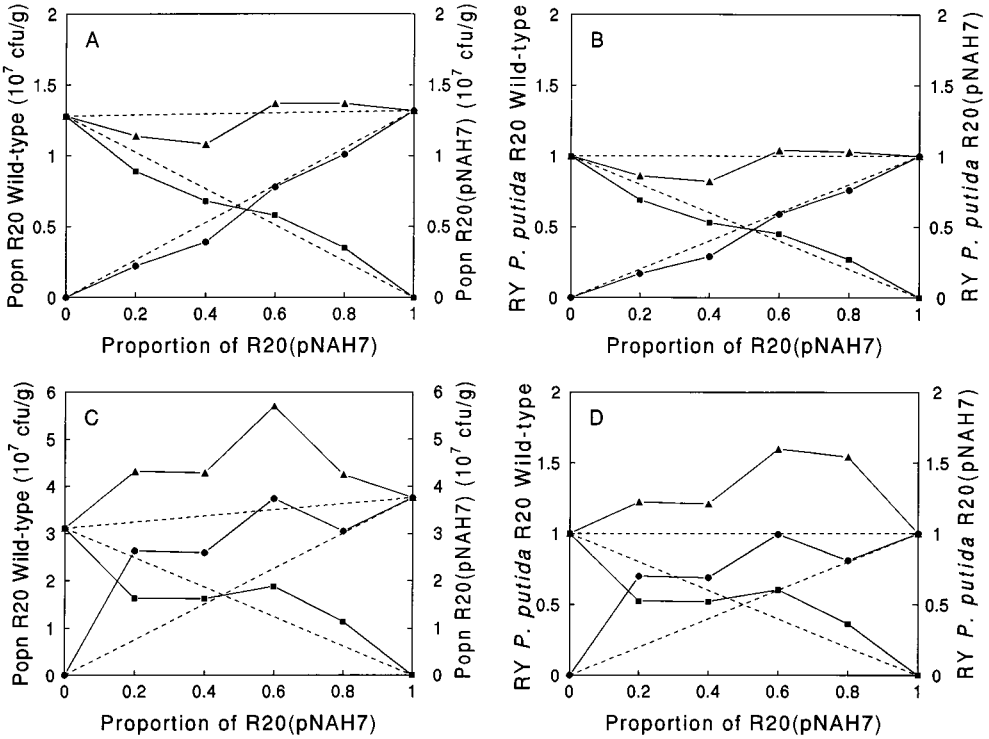

FIG. 3. Competition between the near-isogenic bacterial strain pair *P. putida* R20 and R20(pNAH7) in replacement series experiments. (A) Population sizes of R20 (squares) ($F = 3.90$, $P = 0.012$) and R20(pNAH7) (circles) ($F = 1.45$, $P = 0.243$) and total population size (triangles) in the absence of exogenous sali-cylate, plotted against inoculum proportion. (B) RY_{R20} (squares), $RY_{R20(pNAH7)}$ (circles), and RYT (triangles) in the absence of exogenous salicylate, plotted against inoculum proportion. (C) Population sizes of R20 (squares) ($F = 40.48$, $P = 0.0001$) and R20(pNAH7) (circles) ($F = 55.80$, $P = 0.0001$) and total population size (triangles) in the presence of exogenous salicylate, plotted against inoculum proportion. (D) RY_{R20} (squares), $RY_{R20(pNAH7)}$ (circles), and RYT (triangles) in the presence of exogenous salicylate, plotted against inoculum proportion. The dashed lines represent the results expected on the basis of two populations competing equally for all resources.

inoculum proportion deviated significantly from linearity in a positive direction (Fig. 3B), the $RY_{R20(pNAH7)}$ was consistently greater than expected, and the RYT was greater than 1.0 (Fig. 3D)]. In contrast, the level of coexistence of R20 with R20 (pNAH7) was not increased (i.e., while the plot of population size against inoculum proportion deviated significantly from linearity, the deviation was neither consistently positive or negative [Fig. 3B]). Similar results were observed in two other experiments, although in these experiments there was some enhancement of the population size of strain R20 when exogenous salicylate was provided.

Exogenous addition of the carbon source salicylate significantly increased the level of coexistence between the salicylate-catabolizing strain *P. putida* R20(pNAH7) and the noncatabolizing parental strain R20. In both the presence and absence of exogenous carbon, the near-isogenic strain pair competed for and partitioned the carbon compounds available in the leaf exudates and leachates. In the presence of exogenous carbon, however, *P. putida* R20(pNAH7) was apparently able to utilize the portion of the endogenous carbon that was acquired by competition with R20, plus the exogenous salicylate, and hence R20(pNAH7) achieved a population size greater than that of R20 at each inoculum proportion. The enhancement of the R20 population size observed in some of the replacement series experiments in which exogenous salicylate was applied may indicate either that the population of R20(pNAH7) utilized salicylate preferentially, thereby releasing R20 from some competition for the endogenous carbon sources, or that the population of R20(pNAH7) released a substrate that R20 was able to catabolize. While the plasmid pNAH7 conjugates at a very low frequency in vitro, even if the frequency was higher in planta, plasmid transfer from R20(pNAH7) to R20 could not account for these results.

This use of near-isogenic bacterial strains, in which one member of the pair has been engineered to catabolize an additional carbon source which is abundant in the phyllosphere because it has been added exogenously, provides a good model of species coexistence in epiphytic bacterial communities. The model employed here demonstrates that niche differentiation of one carbon source is sufficient to permit coexistence, if that carbon source is sufficiently abundant in the phyllosphere. Carbohydrate concentrations in leachates from leaf surfaces have been estimated to be approximately 0.1 $\mu g/cm^2$ in *Zea mays* (7, 8) and approximately 0.7 $\mu g/cm^2$ in *Antirrhinum nanum* (5). On the basis of the average primary leaf area, the volume of solution retained after spraying, and the substrate concentration, the applied salicylate concentration on the leaf surface was approximately 10 $\mu g/cm^2$. While this is significantly higher than the carbohydrate concentrations observed, carbon that is leaked or exuded from the leaf is likely localized at discrete sites of high concentration, where colonization subsequently occurs. To demonstrate that niche differentiation of just one or two carbon sources present at the locations and in the quantities typical of the phyllosphere is sufficient to alter the level of coexistence between epiphytic populations, more recent studies have employed transgenic plants producing a substrate that is catabolized by only one member of a near-isogenic strain pair.

Wilson and Lindow (20) hypothesized that coexistence between *P. syringae* and other epiphytic species, such as *Stenotrophomonas maltophilia* and *Methylobacterium organophilum* was

due to nutritional resource partitioning in a carbon-limited phyllosphere. On the basis of the number of carbon sources catabolized in vitro, the niche overlap between *P. syringae* and *S. maltophilia* or *M. organophilum* in the limiting resource dimension was relatively low (20); however, the niche overlap between the near-isogenic strains R20 and R20(pNAH7) would be extremely high. Wilson and Lindow (20) observed a significant inverse correlation between niche overlap and level of epiphytic coexistence, which appears to be contradicted by the high level of coexistence observed between the ecologically similar strains R20 and R20(pNAH7) in the presence of exogenous salicylate. This model, however, reinforces the recommendation of Wilson and Lindow (20) that niche overlap indices ideally should be weighted for the relative abundance of each carbon source in the phyllosphere under examination.

The ability to selectively enhance the epiphytic population size of an organism in the phyllosphere through creation of a novel catabolic niche as done here may have important implications for the biocontrol of foliar pathogens and pests. There have been several attempts to use nutritional amendments to manipulate the composition of microbial communities in favor of colonization by a biocontrol organism (1, 2, 6, 11–12, 14, 16); however, to date none has provided the selectivity that is possible with a strain engineered to catabolize a carbon source that is not catabolized by the majority of bacterial epiphytes. Hence, the plasmid pNAH7 has been mobilized into the foliar biocontrol agent *Pseudomonas fluorescens* A506, permitting future studies of improved colonization, establishment, and efficacy by the salicylate-catabolizing biocontrol agent in the presence of exogenous salicylate.

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REFERENCES

1. **Backman, P. A., R. Rodriguez-Kabana, and N. M. Kokalis.** February 1994. Methods of controlling foliar microorganism populations. U.S. patent 5,288,488.
2. **Brown, E. W., T. Van der Zwet, R. H. Bors, and W. Janisiewicz.** 1992. Identification of a carbohydrate which enhances the growth of a bacterial antagonist against *Erwinia amylovora*. Phytopathology **82:**718. (Abstract.)
3. **Colbert, S. F.** 1992. Effect of selective substrates on population dynamics of wild-type and genetically engineered rhizobacteria. Ph.D. thesis. University of California, Berkeley.
4. **Colbert, S. F., M. Hendson, M. Ferri, and M. N. Schroth.** 1993. Enhanced growth and activity of a biocontrol bacterium genetically engineered to utilize salicylate. Appl. Environ. Microbiol. **59:**2071–2076.
5. **Collins, M. A.** 1976. Colonization of leaves by phylloplane saprophytes and their interactions in this environment, p. 401–418. *In* C. H. Dickinson and T. F. Preece (ed.), Microbiology of aerial plant surfaces, Academic Press, London.
6. **Davis, R. F., P. A. Backman, R. Rodriguez-Kabana, and N. Kokalis-Burelle.** 1992. Biological control of apple fruit diseases by *Chaetomium globosum* formulations containing cellulose. Biol. Control **2:**118–123.
7. **Derridj, S., V. Gregoire, J. P. Boutin, and V. Fiala.** 1989. Plant growth stages in the interspecific oviposition preference of the European corn borer and relations with chemicals present on the leaf surfaces. Entomol. Exp. Appl. **53:**267–276.
8. **Fiala, V., C. Glad, M. Martin, E. Jolivet, and S. Derridj.** 1990. Occurrence of soluble carbohydrates on the phylloplane of maize (*Zea mays* L.): variations in relation to leaf heterogeneity and position on the plant. New Phytol. **115:**609–615.
9. **Fukui, R., M. N. Schroth, M. Hendson, and J. G. Hancock.** 1994. Interaction between strains of pseudomonads in sugar beet spermospheres and their relationship to pericarp colonization by *Pythium ultimum* in soil. Phytopathology **84:**1322–1330.
10. **Harper, J. L.** 1977. The population biology of plants. Academic Press, London.
11. **Janisiewicz, W.** 1991. Nutritional enhancement of biocontrol of postharvest diseases of pome fruits. Phytopathology **31:**1175–1176.
12. **Janisiewicz, W. J., J. Usall, and B. Bors.** 1992. Nutritional enhancement of biocontrol of blue mold on apples. Phytopathology **82:**1364–1370. (Abstract.)
13. **King, E. O., M. K. Ward, and D. E. Raney.** 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. **44:**301–307.
14. **Kokalis-Burelle, N., P. A. Backman, R. Rodriguez-Kabana, and L. D. Ploper.** 1992. Potential for biological control of early leafspot of peanut using *Bacillus cereus* and chitin as foliar amendments. Biol. Control **2:**321–328.
15. **Miller, J. H.** 1972. Experiments in bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Van der Zwet, T.** 1993. Manipulation of the epiphytic microbial community to promote biological control of *Erwinia amylovora* on pear and apple. Acta Hortic. (Wageningen) **338:**351. (Abstract.)
17. **Wilson, M., and S. E. Lindow.** 1991. Resource partitioning among bacterial epiphytes in the phyllosphere. Phytopathology **81:**1170–1171. (Abstract.)
18. **Wilson, M., and S. E. Lindow.** 1993. Coexistence among epiphytic bacterial populations resulting from nutritional resource partitioning. Phytopathology **83:**1342. (Abstract.)
19. **Wilson, M., and S. E. Lindow.** 1994. Ecological similarity and coexistence of epiphytic ice-nucleating (Ice^+) *Pseudomonas syringae* strains and a non-ice-nucleating (Ice^-) biological control agent. Appl. Environ. Microbiol. **60:** 3128–3137.
20. **Wilson, M., and S. E. Lindow.** 1994. Coexistence among epiphytic bacterial populations mediated through nutritional resource partitioning. Appl. Environ. Microbiol. **60:**4468–4477.