APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1993, p. 3447–3454
0099-2240/93/103447-08$02.00/0

Vol. 59, No. 10

Invasion and Exclusion among Coexisting *Pseudomonas syringae* Strains on Leaves

LINDA L. KINKEL[1]* AND STEVEN E. LINDOW[2]

Department of Plant Pathology, University of Minnesota, St. Paul, Minnesota 55108,[1] and Department of Plant Pathology, University of California, Berkeley, California 94720[2]

Received 19 April 1993/Accepted 28 July 1993

The invasion and exclusion abilities of coexisting *Pseudomonas syringae* strains were quantified on leaves. Twenty-nine *P. syringae* strains were inoculated onto plants in 107 pairwise combinations. All pairs were duplicated so that each strain was inoculated both first as an antagonist strain (day 0) and second as a challenge strain (day 3). The population size of each strain in a mixture was quantified on day 6 following incubation under moist conditions. For *P. syringae* strains, the presence of an established population often significantly reduced the growth of subsequently arriving challenge strains on the leaf surface. Invasion and exclusion abilities, quantified by contrasting population sizes of challenge strains in the presence and in the absence of another strain, varied significantly among *P. syringae* strains and were partly a function of the particular strain pair. The population size of a strain when present alone on a leaf was not predictive of invasion or exclusion ability. Successful invaders were significantly less likely to exclude challenge populations than were nonsuccessful invaders. Population sizes of successful excluders were negatively correlated with population sizes of coexisting challenge strains, while population sizes of successful invaders were positively correlated with those of coexisting antagonist strains. The patterns of interaction among coexisting strains suggest mechanisms for successful invasion and exclusion among *P. syringae* strains on leaves.

Biological control with antagonistic microbes has been investigated for many fungal and bacterial plant diseases (e.g., 1, 4, 21, and 27). Resource competition (14), antibiosis (3, 5, 18), or a combination of both of these forms of interaction (24) has been suggested to explain disease reductions in cases in which mechanisms of control have been studied. However, despite some successes, biological control continues to be sporadic and to have low predictability in most systems. This lack of consistent control is partly a consequence of our limited understanding of the nature of the interactions of microorganisms with plants and with each other. For example, we have little quantitative data on the diversity and specificity of competitive abilities for pathogenic strains and potential biological control organisms. Successful prediction and enhancement of biological control require such information.

Strains of the bacterium *Pseudomonas syringae* van Hall cause disease on a wide range of plant hosts (10). In addition, *P. syringae* plays an important role in plant frost injury because many strains are capable of catalyzing ice formation at warm, subfreezing temperatures (2, 17). Prior work has shown that bacterial antagonists of ice nucleation-active (Ice^+) bacteria are promising as means of controlling frost injury to many plant species in the field (11, 13). Plant frost injury and population sizes of Ice^+ *P. syringae* strains are significantly reduced in the presence of large populations of non-Ice^+ (Ice^-) *P. syringae* strains (13). Furthermore, resource competition rather than antibiosis appears to be the primary mechanism of interaction that explains the reductions in Ice^+ bacteria and frost damage on plant surfaces (14). However, we know relatively little of the dynamics between Ice^+ and Ice^- *P. syringae* strains on leaves and consequently are limited in possible strategies for enhancing biological control.

* Corresponding author.

In this study, we investigated competitive interactions between pairwise mixtures of *P. syringae* strains on leaves. Specifically, we sought to quantify two distinct aspects of the competitive interactions between bacterial strains: invasion, or the ability of a strain to establish a population on leaves that have already been colonized by another strain, and exclusion, or the ability of an established strain to inhibit the development of a population of a second strain on leaves. Our objective was to provide basic information on the variability among strains in competitive ability and on the quantitative impacts of intraspecific interactions on bacterial populations.

MATERIALS AND METHODS

Bacterial strains. Twenty-nine strains of *P. syringae* were isolated from healthy pear, almond, potato, strawberry, and navel orange leaves. Leaves (ca. 20 g) were placed in Erlenmeyer flasks containing 200 ml of sterile washing buffer (0.1 M potassium phosphate buffer [pH 7.0] containing 0.1% Bacto Peptone) and sonicated in an ultrasonic cleaner (Bransonic 52) for 7 min. Tenfold serial dilutions of the wash suspension were plated onto King's medium B (KB) (8) containing 100 μg of cycloheximide per ml. Ice^+ bacteria were isolated by a replicate freezing technique from dilution platings of leaf washings and subsequently purified by several single-colony transfers in a manner similar to that described by Lindow et al. (16). Strains tentatively identified as *P. syringae* exhibited fluorescent pigment production on KB, negative arginine dihydrolase (25) and oxidase (23) reactions, and positive tobacco hypersensitivity and ice nucleation (15) reactions and were unable to rot potato. Strains were stored at −80°C in a solution of 15% (vol/vol) glycerol.

Mutant strains resistant to the antibiotic rifampin were selected as single colonies arising after a suspension (0.1 ml, 10^9 cells per ml) of each parental *P. syringae* strain was

spread onto KB containing 100 μg of rifampin per ml (KBR) and incubated at 30°C for 4 days. Individual mutant colonies were purified by streaking on KBR, retested for the biochemical and physiological characteristics listed above, and demonstrated to have growth rates similar to those of the corresponding parental strains on KB.

Ice^- deletion mutants Cit7del1b, TLP2del1, and 31R1-R1P6, derived from Ice^+ parental *P. syringae* Cit7, TLP2, and 31R1, respectively, were produced by marker exchange mutagenesis with a cloned gene that confers ice nucleation in each of these strains. The isolation, production, and characteristics of these Ice^- deletion mutants have been described elsewhere (11, 12).

The isolation and ice nucleation properties of Ice^- mutant strains 31R1-28 and Cit13R-12, identified after mutagenesis with ethyl methanesulfonate of Ice^+ *P. syringae* 31R1 and Cit13, respectively, have been described elsewhere (2, 13).

Experimental design. Logistic considerations prevented the evaluation of interactions among all possible pairwise combinations of the 29 strains. One hundred seven strain pair interactions were investigated in eight different experiments. In each experiment, between three and five different bacterial strains were evaluated, and every strain was inoculated as both an antagonist and a challenger. Antagonist strains were inoculated onto leaves first (see below). Challenge strains were inoculated onto plants 3 days later and at a lower inoculum concentration than antagonist strains. In a single experiment, antagonist strains were either all Ice^- or all Rif^s. Challenge strains were Ice^+ or Rif^r variants of the antagonist strains. Each challenge strain in an experiment was paired with all antagonist strains (both homologous and heterologous) in that experiment. Thus, in an experiment evaluating four bacterial strains, 16 different strain pair interactions were quantified (each strain as an antagonist paired with each strain as a challenger). In addition, challenge strains were inoculated onto plants that had not been inoculated with any antagonist strains as a control.

Plant inoculations. Bean (Bush Blue Lake 274) or corn (Northrup King PX74 single-cross) seedlings were grown (10 plants per pot) for about 12 days in a greenhouse. Plants were exposed to air movement by use of an oscillating fan to facilitate structural development of the plants and to minimize the duration of wetness on leaves following watering. Care was taken to avoid wetting the leaves of plants during watering operations. The population size of indigenous bacteria on the leaves of these plants was generally less than ca. 10^4 cells per g (fresh weight) at the time of inoculation, as was observed in other studies (20).

Inocula of *P. syringae* strains were grown on KB plates for 2 days at 24°C. Cells were removed from plates by scraping and suspended in sterile distilled water. Inoculum concentrations were determined by use of a spectrophotometer, and concentrations were adjusted by dilution with sterile distilled water.

The antagonist inoculum was sprayed onto plants (ca. 1.0 ml per plant) at a concentration of 10^8 cells per ml with an air-pressurized atomizer. Leaves were sprayed to runoff. Plants were incubated in a humid chamber maintained at 21°C and misted intermittently to facilitate bacterial growth and establishment. A set of misting nozzles operating at a constant air pressure delivered jets of atomized distilled water over the plants at 10-min intervals. The jets were operated for 1 min every 10 min. Thus, the plants were kept slightly moist during their entire incubation in the humid chamber. After 3 days, plants were inoculated with a homologous or heterologous challenge strain at a concentration of 10^5 cells per ml by spraying to runoff as described above. Plants were returned to the humid chamber and incubated for 3 days at 21°C with periodic misting as described above.

Measurement of bacterial populations on leaves. For estimation of bacterial population sizes, 18 to 30 leaves were harvested from each of four pots (replicates) of plants from each treatment. Leaves from each pot were weighed and immersed in 200 ml of washing buffer (16) in 500-ml Erlenmeyer flasks, and bacterial cells were removed by sonication as described above.

In experiments in which Ice^- antagonist strains and Ice^+ challenge strains were evaluated, serial dilutions of leaf washings were plated onto KBR amended with 100 μg of cycloheximide per ml (Ice^+ parental strains were rifampin resistant). Plates were incubated for 5 days at 21°C. Total bacterial colonies enumerated on KBR provided estimates for combined antagonist (Ice^-) and challenge (Ice^+) populations. Ice^+ challenge strain populations were determined by a replicate freezing technique as described previously (16), and Ice^- antagonist strain populations were estimated as the difference between total KBR populations and Ice^+ populations. Even when present as a small percentage of total *P. syringae* (less than 0.01%), Ice^+ (challenge) populations could be accurately estimated by this procedure.

In experiments in which Rif^s antagonist strains and Rif^r challenge strains were evaluated, serial dilutions of leaf washings were plated onto both KB and KBR, each amended with 100 μg of cycloheximide per ml. Fluorescent colonies characteristic of the inoculated strains were enumerated on KB plates incubated for 4 days at 24°C. These counts represented estimates for the combined antagonist and challenge populations. Nonfluorescent indigenous bacteria represented less than ca. 10% of the colonies recovered on KB. Total colonies recovered on KBR were used as estimates of Rif^r challenge populations. Antagonist populations were thus estimated as the difference between characteristic fluorescent colonies on KB and total colonies on KBR. The plating efficiencies of the Rif^r mutants were compared on KB and KBR. There were no significant differences in the plating efficiencies of the derivative strains on the two culture media. Contaminating bacteria were rarely recovered from greenhouse plants on KBR.

Analyses. (i) Quantifying strain interactions. The goals of our analysis were to quantify differences in bacterial invasion and exclusion abilities and to characterize the relationships between invasion and exclusion abilities among strains. One- and two-way analyses of variance (ANOVA) (22) of log-transformed (7) estimates of challenge strain population sizes were used to quantify the effects of different antagonist strains on challenge populations and to quantify the differences in colonization among challenge strains. Significant interaction terms in two-way ANOVA indicated specificity of interactions between antagonist and challenge strains with respect to challenge strain population sizes.

One- and two-way ANOVA of log-transformed estimates of antagonist strain population sizes were used to evaluate differences in population sizes of antagonist strains in the presence of different challenge strains. Significant interaction terms in two-way ANOVA suggested that antagonist strain population sizes are a function of both the antagonist strain and the particular coexisting challenge strain.

(ii) Characterizing strains and summarizing relationships among strain abilities. Because any single strain-strain interaction cannot distinguish challenge and antagonist strain abilities (the challenge strain invades or the antagonist strain does not exclude), characterization of a bacterial strain is

based upon its pattern of interactions with a variety of strains. Methodologically, a large single experiment in which every strain is paired as an antagonist with every strain as a challenger is not feasible. Thus, characterization is always in relation to other strains within an experiment.

Statistically, we define invaders and excluders as follows. A successful invader is a (challenge) strain that reaches a mean population size in the presence of antagonists that is not significantly different from the population size of that challenge strain in the absence of any antagonist. This definition is stringent in that strains that establish substantial non-zero populations in the presence of an antagonist will not be classified as successful invaders if the mean challenge population over all antagonists is significantly smaller than when no antagonist is present. A successful excluder is a strain that, when present as an antagonist, restricts the mean challenge population size to a significantly lower level than that present in the absence of the antagonist. Excluder strains need not completely prohibit the development of challenge strains; they need only significantly reduce challenge strain population sizes.

Therefore, strains are characterized as successful invaders on the basis of their ability to establish populations in the presence of antagonists relative to their ability to establish populations in the absence of antagonists. Similarly, antagonist strains are characterized as successful excluders when they are able to reduce challenge population sizes significantly in relation to challenge population sizes in the absence of any antagonist. Although this approach precludes an absolute ranking of bacterial strain invasion or exclusion abilities among all experiments (not the objective of our analysis), it allows the characterization of strains as successful invaders or excluders and permits an evaluation of the relationship between invasion and exclusion abilities among strains over all experiments. Chi-square analysis was used to quantify relationships among strain characteristics.

(iii) Population size relationships. Relationships between the mean population sizes of each strain as a challenger and as an antagonist provided another approach for investigating strain interactions. Specifically, the population size of a single challenge strain averaged over all antagonist strains in an experiment provided a measure of the colonization ability (incorporated both invasion ability and carrying capacity) of the challenge strain (see challenge strain column means in Table 1). A relatively high mean challenge population suggested an effective colonist or invader. In the same manner, the mean challenge population among all challenge strains in the presence of a particular antagonist provided an index of the exclusion ability of the antagonist (see challenge strain row means in Table 1). A relatively high mean challenge population indicated low exclusion ability for the antagonist.

An analysis of population sizes of antagonist strains in the presence of challenge strains (see antagonist strain populations, Table 1) provided a measure of relative challenge strain influences on antagonist populations (inhibition index; see antagonist strain column means in Table 1) and a relative index of antagonist strain abilities to maintain populations in the presence of invading strains (see antagonist strain row means in Table 1). A high antagonist column mean suggested poor inhibition of antagonist populations by the challenge strains. A high antagonist row mean suggested successful colonization and establishment (maintenance) of the antagonist population in the presence of challenge strains.

Regression and correlation analyses of invasion, exclusion, inhibition, and maintenance population data were performed with SAS release 6.04 (SAS Institute, Cary, N.C.) and were used to describe the relationships among strain abilities for all strains studied. In addition, relationships between challenge and antagonist populations for individual strain pair combinations were evaluated by the same techniques.

RESULTS

Quantifying strain interactions: an example. Table 1 illustrates the analyses used to quantify strain interactions for a single experiment. In this experiment, there were significant differences among the antagonist strains in ability to exclude challenge strain populations (Table 1, challenge strain populations; significant antagonist effect, $P = 0.0001$). In addition, the challenge strains showed significant differences in ability to colonize leaves on which antagonist strain populations were established (Table 1, challenge strain populations; significant challenge effect, $P = 0.0001$). A significant antagonist-challenge interaction term (Table 1, challenge strain populations; $P = 0.0001$) in the two-way ANOVA for the challenge populations indicated that exclusion of challengers by antagonists and invasion by challengers were functions of the particular competing strain.

Antagonist strains varied significantly in their mean population sizes in the presence of challenge strains (Table 1, antagonist strain populations; $P = 0.0001$). However, mean antagonist populations did not vary significantly in the presence of different challenge strains ($P = 0.1262$). Still, for some individual antagonist strains, population sizes did vary significantly among challenge strains (e.g., strain 468; one-way ANOVA, $P < 0.05$). There was no significant antagonist-challenger interaction ($P = 0.4786$). Therefore, the ability of each antagonist strain to maintain high population sizes in the presence of various challenge strains was fairly consistent.

Quantifying strain interactions: summary. In all experiments, there were significant differences among *P. syringae* strains in their ability to establish populations on leaves already colonized by antagonists (eight of eight experiments; Table 2, challenge populations, challenge effect). There were significant differences among antagonist strains in their exclusion ability in seven of eight experiments (Table 2, challenge populations, antagonist effect). Significant antagonist-challenge interaction terms in all experiments (Table 2, challenge populations) indicated that the colonization ability of a given challenge strain was dependent on the antagonist strain present on the plant or that the exclusion ability of an antagonist strain was dependent on the specific challenge strain. Analyses were performed to evaluate whether the ability of a challenge strain to invade a plant colonized by an antagonist was consistently greater or less in the presence of a homologous versus a heterologous antagonist strain. No patterns were observed: strains were not consistently more successful or less successful in invading plants colonized by homologous rather than heterologous antagonists (full analyses not shown; however, e.g., Table 1, challenge strain populations, isogenic ANOVA, $P = 0.3986$).

Challenge strains had a significant influence on antagonist population sizes in three of eight experiments (Table 2, antagonist populations, challenge effect). There were significant differences in population sizes of *P. syringae* antagonists in seven of eight experiments (Table 2, antagonist populations, antagonist effect). These may reflect differences in the abilities of the antagonists to withstand invasion or may reflect differences in carrying capacity among antagonists. Significant interaction terms in all experiments (Table

TABLE 1. Mean population sizes[a] of challenge strains in the presence of different antagonist strains and antagonist strains in the presence of different challenge strains

Populations and antagonist strain	Population size (log CFU/g [fresh wt]) with the following challenge strain:				
	468R	584R	563R	1199R	Mean[b]
Challenge strain populations					
468	5.11	3.98	3.80	4.99	4.47 A
584	1.02	4.36	3.82	4.48	3.42 C
563	2.73	4.00	4.09	3.73	3.64 BC
1199	4.10	4.59	3.80	3.93	4.11 AB
None	5.17	5.31	5.66	4.82	5.24 A
Mean[c]	3.63 B	4.45 A	4.23 A	4.39 A	
Antagonist strain populations					
468	5.43	6.01	5.93	5.57	5.74 B
584	6.06	6.21	6.12	5.89	6.07 A
563	6.28	6.10	6.20	6.27	6.21 A
1199	4.95	5.39	5.75	5.33	5.35 C
Mean[c]	5.68 B	5.93 AB	6.00 A	5.77 AB	

[a] Log CFU per gram of leaf tissue, averaged over four replicates for each combination and based upon dilution plating of leaf sonicates. Challenge strains were inoculated onto plants 72 h after antagonist strains. Population sizes for both antagonist and challenge strains were quantified 72 h after the challenge strains were introduced.

[b] Values in the column for challenge strain populations or for antagonist strain populations that are followed by the same letter are not significantly different ($P < 0.05$), as determined by Fisher's least-significant-difference (LSD) test. Two-way ANOVA results for both the challenge population data and the antagonist population data indicated a significant antagonist effect in each case ($P = 0.0001$).

[c] Values in the row followed by the same letter are not significantly different ($P < 0.05$), as determined by Fisher's LSD test. Two-way ANOVA results for the challenge strain population data indicated a significant challenge effect ($P = 0.0001$) and a significant antagonist-challenge interaction ($P = 0.0001$). Two-way ANOVA results for the antagonist strain population data indicated no significant challenge effect ($P = 0.1262$) (Note that these results contrast with the results of Fisher's LSD test, which controls the type I comparison-wise error rate but not the experiment-wise error rate) or challenge-antagonist interaction ($P = 0.4786$).

2, antagonist populations) indicated that, like challenge population sizes, antagonist population sizes were partly a function of a particular strain pair combination.

Characterizing strains and summarizing relationships among strain abilities. Individual bacterial strains were characterized as successful invaders or excluders on the basis of their interactions with a random collection of bacterial strains, as quantified by challenge strain population data. For example, strain 1199 was a successful invader (Table 1, challenge strain populations); the population of this strain was not significantly smaller in the presence of antagonists than in their absence. Strain 563 was not a successful invader. However, strain 563 was a successful excluder; the mean population size of challenge strains (when considered for all challenge strains) was significantly smaller in the presence of this antagonist than when no antagonist was present. Strain 1199 was not a successful excluder. The relationship between invasion and exclusion abilities among all strains in all experiments was as follows: 5 successful and 10 nonsuccessful invaders were successful excluders, and 11 successful and 3 nonsuccessful invaders were nonsuccessful excluders. Chi-square analysis of these data indicated that invasion and exclusion abilities were not independent in the *P. syringae* strains tested ($\chi^2 = 5.992$; $P = 0.014$). Strains that were successful invaders were significantly less likely to be successful excluders than were strains that were not successful invaders. There were, however, individual strains that effectively invaded and excluded as well as strains that neither invaded nor excluded successfully within these experiments.

Analysis of population sizes. The mean population size of a strain when inoculated as a challenge organism was positively correlated with the mean population size of challenge strains coexisting with that strain when it was evaluated as an antagonist ($R = 0.854$; $P < 0.0001$) (Fig. 1). Thus, the less successful invaders were apt to be more successful excluders.

The mean population size of antagonists maintained in the presence of a particular challenge strain was positively correlated with the mean population size of that challenge

TABLE 2. Summary of two-way ANOVA results for all experiments showing significance levels for antagonist effect, challenge effect, and antagonist-challenge interaction

Populations and term	*P* value in expt:							
	1	2	3	4	5	6	7	8
Challenge populations								
Challenge effect	0.0016	0.0001	0.0082	0.0001	0.0001	0.0001	0.0001	0.0001
Antagonist effect	0.2100	0.0007	0.0089	0.0001	0.0008	0.0001	0.0001	0.0001
Interaction	0.0031	0.0001	0.0458	0.0001	0.0056	0.0001	0.0001	0.0001
Antagonist populations								
Antagonist effect	0.0001	0.0187	0.0005	0.0001	0.0837	0.0001	0.0002	0.0001
Challenge effect	0.0030	0.1312	0.3295	0.1262	0.0016	0.0744	0.0924	0.0007
Interaction	0.0001	0.0375	0.1152	0.4786	0.0001	0.0130	0.0010	0.0001

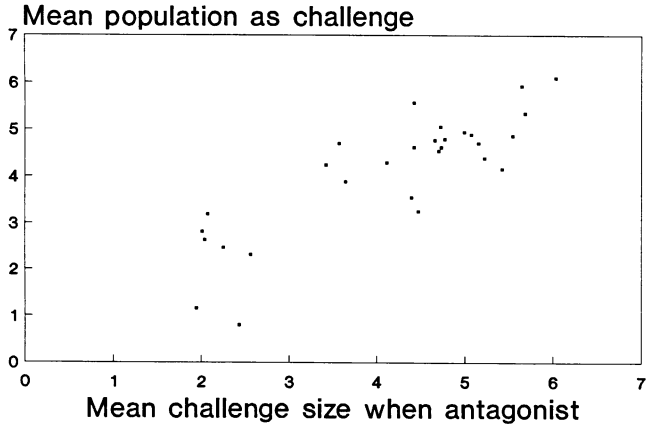

FIG. 1. Relationship for individual *P. syringae* strains between the mean population size of each strain when inoculated as a challenge strain and the mean population size of challenge strains coexisting with that strain when it was evaluated as an antagonist ($R = 0.845$; $P < 0.0001$). Relatively higher mean challenge populations coexisting with an antagonist (abscissa) suggest less antagonist exclusion ability; higher challenge populations of the indexed strain (ordinate) suggest better colonization ability (invasion ability and high carrying capacity).

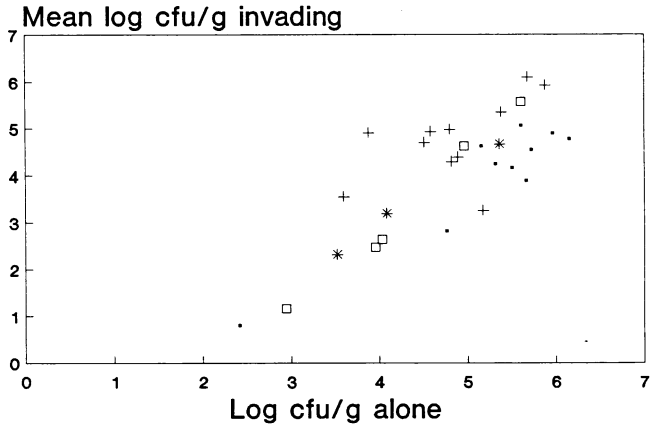

FIG. 3. Mean population size (log CFU/gram) of individual *P. syringae* strains when present alone on leaves in relation to the mean population size of each strain when present on leaves as a challenge strain (challenge populations were averaged over all antagonist combinations). Strains were distinguished on the basis of their success as invaders and excluders (see text for definitions): ■, successful excluders, nonsuccessful invaders; +, successful invaders, nonsuccessful excluders; □, successful invaders, successful excluders; *, nonsuccessful invaders, nonsuccessful excluders.

strain when it was evaluated as an antagonist ($R = 0.502$; $P = 0.006$) (Fig. 2). More effective inhibitors tended to be less successful invaders ($R = -0.587$; $P = 0.0008$) and more successful excluders ($R = -0.595$; $P = 0.0007$). Relationships between other population measures were not statistically significant.

Mean population sizes of challenge strains when existing alone on leaves were positively correlated with the mean population size of each strain when coexisting with an antagonist population ($R = 0.7929$; $P = 0.0001$) (Fig. 3). However, population size of strains when existing alone on leaves was not predictive of successful invasion (Fig. 3) or successful exclusion as formally defined here.

Population sizes of individual challenge and antagonist strains coexisting on leaves were positively correlated when all strains having coincidental populations (log CFU per gram of leaf tissue) of >3 were considered ($R = 0.2504$; $P = 0.02$) (Fig. 4). However, for successful excluders, there was a significant negative correlation between the antagonist and coexisting challenge populations ($R = -0.3699$; $P = 0.0097$) (Fig. 4). For nonsuccessful excluders, there was a significant positive correlation between the population sizes of the antagonist strains and the coexisting challenge strains ($R = 0.5045$; $P = 0.0005$). For successful invaders, there was a significant positive correlation between the antagonist and coexisting challenge populations ($R = 0.5422$; $P = 0.0002$) (Fig. 5). Antagonist and coexisting challenge populations of nonsuccessful invaders were not significantly correlated ($R = -0.1190$; $P = 0.4529$) (Fig. 5).

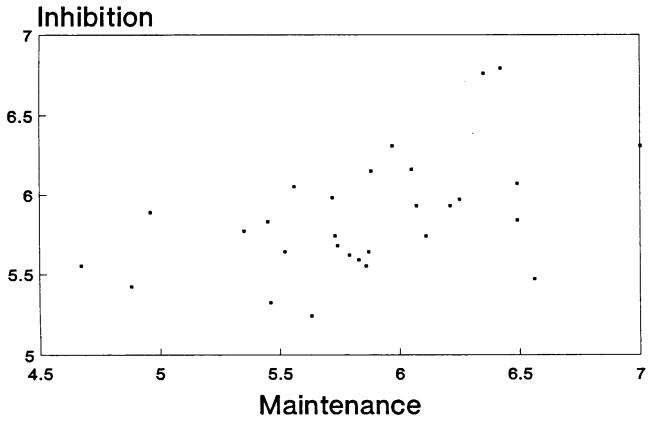

FIG. 2. Relationship for individual *P. syringae* strains between the mean population size of antagonists coexisting with a strain used as the challenge strain (inhibition) and the mean population size of that strain used as an antagonist (maintenance) ($R = 0.502$; $P = 0.006$). Relatively higher values along the abscissa suggest superior exclusion ability; higher values along the ordinate suggest inferior invasion ability.

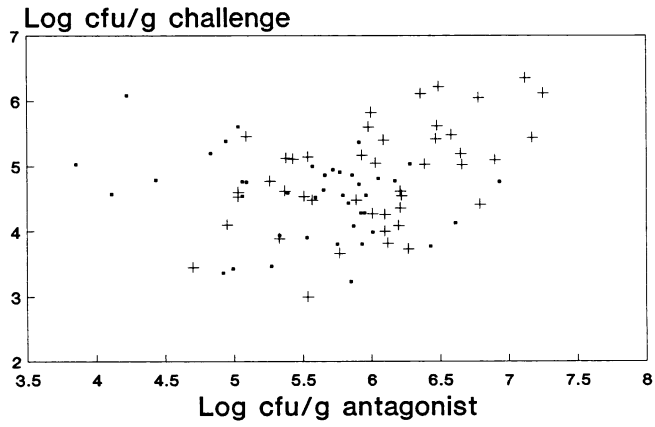

FIG. 4. Relationship between the mean population sizes (log CFU/gram) of *P. syringae* antagonist and challenge populations coexisting on the same leaves. Antagonist populations were distinguished on the basis of their success as excluders. Symbols: ■, successful excluders; +, nonsuccessful excluders.

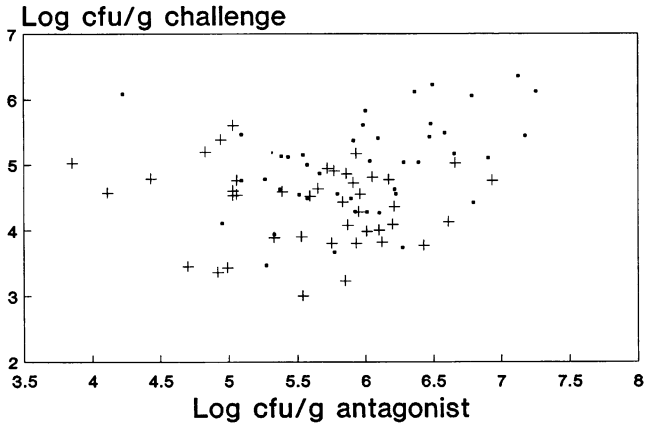

FIG. 5. Relationship between the mean population size (log CFU/gram) of *P. syringae* antagonist and challenge populations coexisting on the same leaves. Challenge populations were distinguished on the basis of their success as invaders. Symbols: ■, successful invaders; +, nonsuccessful invaders.

DISCUSSION

Competitive exclusion as a strategy for phylloplane biological control has been investigated for several diseases (11, 14, 24). However, effective implementation has been hampered by the lack of quantitative data on both the potential impacts of competition on epiphytic population dynamics and the prevalence and specificity of competitive interactions on the phylloplane. For *P. syringae* strains, the presence of an established population can have a significant quantitative influence on the ability of subsequently arriving strains to establish populations on the leaf surface. The significant variability in population sizes among *P. syringae* strains on leaves in the presence of intraspecific antagonists reflects differences in both the invasion ability and the carrying capacity of the strains. The formal definition of invasion (a successful invader is a challenge strain that reaches a population size in the presence of antagonists that is not significantly different from the population size reached by that strain in the absence of any antagonist) distinguishes invasion ability from population size alone. Thus, strains with high challenge populations in the presence of antagonists were not necessarily successful invaders; nonsuccessful invaders experienced significantly greater reductions in population size in the presence versus in the absence of antagonists than did successful invaders. In addition, population size in the absence of an antagonist was not predictive of invasion ability; successful invaders were not more likely to have high populations in the absence of antagonists than were nonsuccessful invaders. Invasion ability is not a simple function of the propensity of a strain to grow on leaves.

Exclusion ability of *P. syringae* strains also is not simply a function of population size, since strains that reached a high population size when present alone on leaves were not more likely to be successful excluders than other strains. However, among the successful excluders, antagonist population size was significantly negatively correlated with challenge population size, indicating that challenge strains were inhibited most by large antagonist populations.

Although we have characterized individual strains as successful invaders or excluders on the basis of their overall interaction with a set of strains, the significant ANOVA interaction terms in most experiments suggest a fair amount of specificity in the interactions among individual strain pairs. For example, even if an individual strain is described as a successful invader, the population size attained by that strain in the presence of an established antagonist population will depend upon the particular antagonist. This specificity among individual strain pairs suggests that it is unlikely that a single bacterial strain will be capable of either invading plants colonized by any one of all possible antagonist strains or excluding all possible challenge strains. Despite this specificity, there were distinct differences among strains in invasion and exclusion, indicating that it is possible to identify strains that are most likely to be effective competitors against particular target populations on the phylloplane. Though invasion and exclusion abilities certainly exist along a continuum, the formal definitions of invasion and exclusion used here present one possible strategy for identifying the best invaders or excluders.

Several mechanisms may mediate successful invasion or exclusion among coexisting bacteria on the phylloplane. Successful invaders may produce antibiotics that inhibit antagonist populations or may have high competitive ability relative to the antagonist strains. Alternatively, successful invasion might suggest that coexisting strains are not competing because shared resources are not limiting (the resource use patterns are nonoverlapping or the shared resources are not limiting growth). Previous work indicated that after 3 days of incubation, bacterial populations on leaves no longer increased, suggesting that some resource(s) had become limiting. However, for successful invaders, the significant positive correlation between mean antagonist and challenge population sizes suggests that most of these strains were not competing for identical resources. Successful invaders tended to coexist with already established antagonist populations. For nonsuccessful invaders, there were no significant correlations between antagonist and challenge populations.

Successful excluders presumably compete with subsequent arrivals through either direct resource competition or antibiosis. The significant negative correlation between antagonist population size and challenge population size among individual strain pairs in which the antagonist was a successfully excluding strain indicates that larger antagonist populations had larger impacts on challenge strains. However, this suggestion does not distinguish among resource competition, antibiosis, or habitat alteration as possible mechanisms of exclusion.

Antagonist strains that were nonsuccessful excluders were not simply less effective competitors than the challenge strains in these experiments. Among the nonsuccessful excluders, the significant positive correlation between paired antagonist and challenge population sizes suggests that shared resources were not limiting on leaves with high populations of both strains or that the resource needs of the strains differed (and the amounts of the distinct resources on separate leaves were positively correlated). Nonsuccessful excluders tended to coexist with challenge strains.

The nonindependent relationship between invasion and exclusion abilities for individual strains may reflect the mechanisms of interaction among strains rather than any relationship among specific traits that make a strain a superior invader or a superior excluder (6). Specifically, strains that are successful invaders but nonsuccessful excluders are likely limited by resources different from those that limit coexisting strains. Thus, these strains can readily invade leaves on which substantial antagonist populations have already been established because required resources have

not already been appropriated by the antagonist. Because these strains are not competing, the successful invader is also a nonsuccessful excluder. By the definitions used in this study, approximately 38% of the *P. syringae* strains evaluated in these experiments successfully coexisted with coinoculated strains (successful invasion, nonsuccessful exclusion), showing no significant reductions in population size as a function of the presence of a second *P. syringae* strain.

About 35% of the bacterial strains studied were categorized as successful excluders but nonsuccessful invaders. This combination suggests that these strains were competing directly for resources on the phylloplane and that the order of their arrival was important in determining their success on the leaf. Alternatively, members of this group may have produced antibiotics or altered the phylloplane habitat in such a way as to exclude subsequent arrivals. Regardless of the specific mechanism, the order of arrival on the leaf determined the outcome of the interaction for these strains.

Only about 10% of *P. syringae* strains were both nonsuccessful invaders and nonsuccessful excluders. These strains were not necessarily ineffective colonists when present alone on leaves; two of the three strains reached population sizes significantly larger than those of other strains in the same experiment. However, these strains were apparently poor competitors under the conditions of these experiments. These strains may be very sensitive to antibiosis or to habitat changes induced by competitors or may have a very narrow resource utilization pattern and require resources that many other strains share.

Five strains (17%) were successful both as invaders and as excluders. These strains were possibly the best competitors of all the strains. They may be capable of using a wide range of resources, so that if one strain has preemptively used sites or nutrients on leaves, these strains may substitute others. Likewise, they may preemptively use all resources that other strains could use. An alternative possibility is that these strains produce antibiotics or alter the phylloplane habitat during the colonization process, allowing them both to invade already occupied habitats and to exclude subsequent arrivals from these habitats. The data do not distinguish among these mechanisms of interaction.

Although not directly applicable to biological control in the field (26), these data have implications for the use of competitive exclusion as a mechanism for biological control on the phylloplane. The constant environmental conditions over the 6-day time period of each experiment were designed to heighten resource limitations and enhance possibilities for resource competition under conditions favorable for bacterial growth. Such constant physical conditions are probably rare in a field setting. However, nutrient addition has been shown to alter population sizes in the field (19), suggesting that resource competition may sometimes be important on plant surfaces in the field. These data indicate that when resource availability is limiting, presumably during extended periods of conditions favoring bacterial growth, intraspecific competitive interactions (resource competition, antibiosis, or habitat alteration) can have a significant quantitative influence on epiphytic bacterial population sizes.

In pairwise combinations, approximately 52% of strains were effective at competitively excluding subsequent arrivals. For the majority of these strains (67%), the order of arrival on the leaves was important, since they themselves were not effective at invading leaves on which large populations had already developed. Thus, in relation to biological control, the establishment of competitive excluders prior to the arrival of significant populations of other bacteria may be important in minimizing the growth of immigrant strains (9). In the search for a single potential biological control organism, regardless of the mechanism of biological control (resource competition, antibiosis, or habitat alteration), first consideration should be given to strains that are both effective excluders and effective invaders. These strains, by implication, are superior colonists for which the order of arrival on leaves is not limiting. Also, although we noted that population size itself is not predictive of invasion or exclusion ability, among the successful excluders, selection of those that reach the highest population sizes is likely to lead to the greatest level of inhibition of invading populations.

Most of the nonsuccessful competitive excluders coexisted with invading strains without any apparent negative influences on either strain, and 79% of nonsuccessful excluders were also successful invaders. This somewhat surprisingly high proportion of successful coexisting strains likely reflects the diversity of niches and resource utilization patterns among epiphytic bacteria (19).

Overall, this work highlights the diversity among *P. syringae* strains in abilities to compete and coexist with one another. The skeptic might conclude that this diversity and the observed specificity of interactions indicate that biological control of plant disease based on competition is unlikely to meet with widespread success. However, although the data suggest that it is unlikely that a single bacterial strain will be effective in excluding all possible colonists, an alternative outlook is possible. The observation that many *P. syringae* strains coexist successfully with others on the phylloplane could be used to the advantage of biological control practitioners. Complementary *P. syringae* strains that coexist well with one another could be sought for biological control. Thus, rather than attempting to exclude all possible immigrants with a single biological control strain, researchers should seek complementary strains that together exclude a wider range of strains than any single biological control agent. In addition, knowledge of the pathogen target population could be used to select particular combinations of biological control strains effective in excluding the specific target population. Towards this end, this work provides a strategy for quantifying the differences among *P. syringae* strains in abilities to invade, exclude, and coexist with others on the phylloplane and provides insight into the mechanisms of interaction that mediate these abilities.

ACKNOWLEDGMENTS

This work was partially supported by a grant from the National Science Foundation (BSR-88-06648) to Steven E. Lindow.

We thank Dorothy Okamoto and Kris Callan for excellent technical assistance and Barbara Rotz for maintenance of plants used in the study. We are also grateful to Gwyn Beattie, Mark Wilson, Andrew Ryan, and three anonymous reviewers for their suggestions for improving the manuscript.

REFERENCES

1. **Anagnostakis, S.** 1982. Biological control of chestnut blight. Science **215**:466–471.
2. **Arny, D. C., S. E. Lindow, and C. D. Upper.** 1976. Frost sensitivity of *Zea mays* increased by application of *Pseudomonas syringae*. Nature (London) **262**:282–284.
3. **Boudreau, M. A., and J. H. Andrews.** 1987. Factors influencing antagonism of *Chaetomium globosum* to *Venturia inaequalis*: a case study in failed biocontrol. Phytopathology **77**:1470–1475.
4. **Chet, I., and Y. Henis.** 1985. *Trichoderma* as a biocontrol agent against soilborne root pathogens, p. 110–112. *In* C. A. Parker, A. D. Rovira, K. J. Moore, P. T. W. Wong, and J. F. Kollmorgen (ed.), Ecology and management of soilborne plant

pathogens. American Phytopathological Society, St. Paul, Minn.
5. **Cullen, D., and J. H. Andrews.** 1984. Evidence for the role of antibiosis in the antagonism of *Chaetomium globosum* to the apple scab pathogen, *Venturia inaequalis*. Can. J. Bot. **62:**1819–1823.
6. **Grime, J. P.** 1977. Evidence for the existence of three primary strategies in plants and its relevance to ecological and evolutionary theory. Am. Nat. **111:**1169–1194.
7. **Hirano, S. S., E. V. Nordheim, D. C. Arny, and C. D. Upper.** 1982. Lognormal distribution of epiphytic bacterial populations on leaf surfaces. Appl. Environ. Microbiol. **44:**695–700.
8. **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.
9. **Kinkel, L. L., J. H. Andrews, and E. V. Nordheim.** 1989. Fungal immigration dynamics and community development on apple leaves. Microb. Ecol. **18:**45–58.
10. **Krieg, N. R., and J. G. Holt (ed.).** 1984. Bergey's manual of systematic bacteriology, vol. 1. Williams & Wilkins, Baltimore.
11. **Lindemann, J., and T. V. Suslow.** 1987. Competition between ice nucleation active wild-type and ice nucleation deficient deletion mutant strains of *Pseudomonas syringae* and *P. fluorescens* biovar I and biological control of frost injury on strawberry blossoms. Phytopathology **77:**882–886.
12. **Lindow, S. E.** 1985. Ecology of *Pseudomonas syringae* relevant to the field use of Ice^- deletion mutants constructed in vitro for plant frost control, p. 23–25. *In* H. O. Halvorson, D. Pramer, and M. Rogul (ed.), Engineered organisms in the environment: scientific issues. American Society for Microbiology, Washington, D.C.
13. **Lindow, S. E.** 1987. Competitive exclusion of epiphytic bacteria by Ice^- mutants of *Pseudomonas syringae*. Appl. Environ. Microbiol. **53:**2520–2527.
14. **Lindow, S. E.** 1988. Lack of correlation of in vitro antibiosis with antagonism of ice nucleation active bacteria on leaf surfaces by non-ice nucleation active bacteria. Phytopathology **78:**444–450.
15. **Lindow, S. E.** 1990. Bacterial ice nucleation measurements, p. 428–434. *In* D. Sands, Z. Klement, and K. Rudolf (ed.), Methods in phytobacteriology. Akademia Kiado, Budapest.
16. **Lindow, S. E., D. C. Arny, and C. D. Upper.** 1978. Distribution of ice nucleation active bacteria on plants in nature. Appl. Environ. Microbiol. **36:**831–838.
17. **Lindow, S. E., D. C. Arny, and C. D. Upper.** 1982. Bacterial ice nucleation: a factor in frost injury to plants. Plant Physiol. **70:**1084–1089.
18. **Lumsden, R. D., J. C. Locke, S. T. Adkins, J. F. Walter, and C. J. Ridout.** 1992. Isolation and localization of the antibiotic gliotoxin produced by *Gliocladium virens* from alginate prill in soil and soilless media. Phytopathology **82:**230–235.
19. **Morris, C. E., and D. I. Rouse.** 1985. Role of nutrients in regulating epiphytic bacterial populations, p. 63–82. *In* C. E. Windels and S. E. Lindow (ed.), Biological control on the phylloplane. American Phytopathological Society, St. Paul, Minn.
20. **O'Brien, R. D., and S. E. Lindow.** 1989. Effect of plant species and environmental conditions on epiphytic population sizes of *Pseudomonas syringae* and other bacteria. Phytopathology **79:**619–627.
21. **Rishbeth, J.** 1963. Stump protection against *Fomes annosus*. III. Inoculation with *Peniophora gigantea*. Ann. Appl. Biol. **52:**63–77.
22. **Snedecor, G. W., and W. G. Cochran.** 1980. Statistical methods, 7th ed. The Iowa State University Press, Ames.
23. **Steele, F. J.** 1961. The oxidase reaction as a taxonomic tool. J. Gen. Microbiol. **25:**297–301.
24. **Thomashow, L. S., and D. M. Weller.** 1988. Role of a phenazine antibiotic from *Pseudomonas fluorescens* in biological control of *Gaeumannomyces graminis* var. *tritici*. J. Bacteriol. **170:**3499–3508.
25. **Thornley, M. J.** 1960. The differentiation of *Pseudomonas* from other gram-negative bacteria on the basis of arginine metabolism. J. Appl. Bacteriol. **23:**37–52.
26. **Welden, C. W., and W. L. Slauson.** 1986. The intensity of competition versus its importance: an overlooked distinction and some implications. Q. Rev. Biol. **61:**23–44.
27. **Windels, C. E., and S. E. Lindow (ed.).** 1985. Biological control on the phylloplane. American Phytopathological Society, St. Paul, Minn.