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Utility of Microcosm Studies for Predicting Phylloplane Bacterium Population Sizes in the Field

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Population sizes of two ice nucleation-active strains of *Pseudomonas syringae* were compared on leaves in controlled environments and in the field to determine the ability of microcosm studies to predict plant habitat preferences in the field. The *P. syringae* strains investigated were the parental strains of recombinant deletion mutant strains deficient in ice nucleation activity that had been field tested for their ability to control plant frost injury. The population size of the *P. syringae* strains was measured after inoculation at three field locations on up to 40 of the same plant species that were studied in the growth chamber. There was seldom a significant relationship between the mean population size of a given *P. syringae* strain incubated under either wet or dry conditions in microcosms and the mean population size which could be recovered from the same species when inoculated in the field. Specifically, on some plant species, the population size recovered from leaves in the field was substantially greater than from that species in a controlled environment, while for other plant species field populations were significantly smaller than those observed under controlled conditions. Population sizes of inoculated *P. syringae* strains, however, were frequently highly positively correlated with the indigenous bacterial population size on the same plant species in the field, suggesting that the ability of a particular plant species to support introduced bacterial strains is correlated with its ability to support large bacterial populations or that indigenous bacteria enhance the survival of introduced strains. Microcosm studies therefore seem most effective at assessing possible differences between parental and recombinant strains under a given environmental regime but are limited in their ability to predict the specific population sizes or plant habitat preferences of bacteria on leaves under field conditions.

Genetically engineered microorganisms may prove useful in a wide variety of agricultural and environmental contexts. Many naturally occurring microorganisms already have been used to control diseases on both the aerial and subterranean surfaces of plants (3, 8, 10, 46, 48). When successful, the alterations in the microbial community on plants achieved by the application of antagonistic microorganisms can reduce or eliminate the need for chemical pesticides for disease control. As the phenotypes determining the interactions between antagonistic microorganisms and their target pathogens or host plants are better elucidated, it becomes increasingly likely that novel strains will be modified in ways that optimize biological control in a particular agricultural context. Likewise, entomopathogenic bacteria and fungi have become increasingly attractive as alternatives to chemicals for the control of insect pests (12, 13). The molecular determinants of virulence in some insect pathogens, such as the characterization of genes determining the production of beta-endotoxins in strains of *Bacillus thuringiensis*, have enabled the creation of novel microbial strains with unique or enhanced insecticidal properties (12). Such genetically modified insect pathogens may prove to be important in future insect control strategies (12). Additionally, as the genetic determinants of virulence in plant pathogenic microorganisms are better understood, it may be possible to more reproducibly utilize such organisms for the biological control of weedy plants (42). A knowledge of the molecular determinants of symbiotic nitrogen fixation in *Rhizobium* and *Bradyrhizobium* species has also enabled the production of recombinant strains of these species which are capable of more efficient reduction of molecular nitrogen (1, 11, 37). The bioremediation of environmental pollutants such as petroleum hydrocarbons or recalcitrant synthetics may also become an important application of recombinant bacteria (6, 9, 28). Most uses of these organisms, by definition, will require their introduction into the open environment.

Considerable attention has been given to the risks associated with the release of genetically engineered microorganisms (14, 15, 41, 44, 48). The abundance of a released microbial strain in a given location and its ability to disperse from a release site are important factors that could contribute to the risks associated with the release of a recombinant microorganism. Both of these parameters are relatively easy to measure, and for this reason, several studies have addressed the population dynamics of bacteria introduced into a contained environment in an attempt to predict the behavior of such strains in the field (4, 5, 16, 31, 40, 43, 47). Unfortunately, few experiments have been conducted in both a contained environment and the field. Thus, we lack knowledge as to how relevant data from contained environments may be in predicting the behavior of microorganisms in noncontained environments.

Tests designed to evaluate the efficacy of recombinant bacteria for the control of plant frost damage necessitated predictions of the risks associated with their release into the open environment (31, 32, 35). Certain ice nucleation-active (Ice^+) bacterial species are common on plants and are responsible for inciting frost damage to many frost-sensitive agricultural plants by limiting their supercooling ability (29, 30, 33, 34). The surface of healthy plants is the primary habitat for most species of Ice^+ bacteria such as *Pseudomonas syringae* and *Erwinia her-*

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bicola (33). Recombinant Ice$^-$ mutants of *P. syringae* were produced by replacement of the chromosomal *ice* gene with a deletion-containing gene produced in vitro (31, 36). Recombinant Ice$^-$ *P. syringae* strains are capable of preemptive exclusion of Ice$^+$ bacteria under controlled environmental conditions (31). The population dynamics of isogenic Ice$^+$ and Ice$^-$ *P. syringae* strains were determined on 65 plant species under wet and, subsequently, dry greenhouse conditions to predict the relative habitat preference of these strains under field conditions; this information was used in support of an experimental use permit from the U.S. Environmental Protection Agency for field tests of the recombinant Ice$^-$ strains (32). The similar population sizes of isogenic Ice$^+$ and Ice$^-$ strains on the plants in the greenhouse under a given set of environmental conditions indicated that the Ice$^-$ strains would be expected to exhibit population dynamics similar to those of the Ice$^+$ strains in the field (31, 32). Since Ice$^+$ *P. syringae* strains are indigenous on most plant species, including plants in the region in which these recombinant strains were to be released (29), the relative population sizes of the Ice$^+$ and Ice$^-$ strains should be similar (31).

While some knowledge of the relative habitat preference of Ice$^+$ and Ice$^-$ *P. syringae* strains on plants in the greenhouse has been available, no predictions of the specific population sizes of Ice$^-$ strains under field conditions have been made. In this study, we evaluated the population dynamics, under field conditions, of the two Ice$^+$ *P. syringae* strains which were the parental strains for recombinant Ice$^-$ deletion mutants that have been field tested for biological frost control (32, 35). To determine if the controlled environment microcosm data were predictive of the relative population sizes of these strains under field conditions, *P. syringae* Cit7 and TLP2 were inoculated onto 40 plant species in a controlled environment and in the field and bacterial population dynamics were monitored in both settings. Because the physical environment is known to play a substantial role in determining epiphytic population dynamics, experiments were conducted under a variety of physical conditions. Specifically, the microcosm experiments were conducted under both wet and dry conditions, and the field investigations of bacterial population sizes were performed at three different times of year. Thus, these data address two questions: (i) what is the value of microcosm studies in predicting specific bacterial population sizes on leaves in the field, and (ii) what is the value of microcosm studies in predicting the relative ability of different plant species to support bacterial populations in the open environment?

MATERIALS AND METHODS

Bacterial strains. *P. syringae* TLP2 was isolated from an asymptomatic potato leaflet collected near Tulelake, Calif. *P. syringae* Cit7 was isolated from an asymptomatic navel orange leaf from an untreated tree located near Exeter, Calif. Both strains are Ice$^+$ and were nonpathogenic on 74 plant species tested (31). Characteristics of both strains have been described previously (31). Both strains are resistant to 100 μg of rifampin per ml. Laboratory cultures were stored at −80°C in sterile 15% (vol/vol) glycerol in 10 mM potassium phosphate buffer (pH 7.0). Strains were cultured on King's medium B (24) containing 100 μg of cycloheximide per milliliter (KB) at 24°C for 24 h for inoculum production. Cells were harvested from plates with a sterile loop and suspended in sterile distilled water. The cell concentration in each suspension was determined turbidimetrically, and the suspensions were diluted with sterile distilled water to the appropriate concentration.

Plant growth and bacterial inoculations. The following plant species were grown under controlled environmental conditions that minimized leaf wetting and therefore epiphytic bacterial populations: 1, potato (*Solanum tuberosum* cv. Russett Burbank); 2, black nightshade (*Solanum nigrum*); 3, onion (*Allium cepa*); 4, orchard grass (*Dactylis glomerata* cv. Able); 5, pea (*Pisum sativum* cv. Tinga); 6, perennial ryegrass (*Lolium perenne*); 7, pigweed (*Chenopodium album*); 8, purslane (*Portulaca oleracea*); 9, red clover (*Trifolium pratense* cv. Redman); 10, bean (*Phaseolus vulgaris* cv. Red Mexican); 11, beet (*Beta vulgaris* cv. Ruby Queen); 12, safflower (*Carthamus tinctorius*); 13, soybean (*Glycine max* cv. Lindarin); 14 and 15, alfalfa (*Medicago sativa* cv. Apollo and cv. Vernal, respectively; 16, zinnia (*Zinnia elegans* cv. Burpeeana Giant Redman); 17, cucumber (*Cucumis sativus* cv. Ashley); 18, dandelion (*Taraxacum officinale*); 19, sudan grass (*Sorghum sudanense* cv. Piper); 20, tomato (*Lycopersicum esculentum* cv. Big Boy); 21, wild oats (*Avena fatua*); 22, oats (*Avena sativa* cv. Cayuce); 23, horseradish (*Armoracia rusticana*); 24, wheat (*Triticum aestivum* cv. Modoc); 25 and 26, barley (*Hordeum vulgare* cv. Steptoe and cv. Larker), respectively; 27, bean (*P. vulgaris* cv. Eagle); 28, corn (*Zea mays* cv. PX744); 29, johnson grass (*Sorghum halapense*); 30, strawberry (*Fragaria* × *Ananassa* cv. Chandler); 31, mallow (*Malva rotundifolia*); 32, ponderosa pine (*Pinus ponderosa*); 33, jimson weed (*Datura stramonium*); 34, petunia (*Petunia* × *hybrida* cv. Purple Cascade); 35, pepper (*Capsicum annuum* cv. Early Cal Wonder); 36, quackgrass (*Agropyron repens*); 37, Italian ryegrass (*Lolium multiflorum*); 38, morning glory (*Ipomoea purpurea*); 39, cheatgrass (*Bromus tectorum*); and 40, an *Atriplex* sp. Plants were approximately 20 to 30 cm in height at the time of inoculation. Plants were spray inoculated with an aqueous suspension of each bacterium at a concentration of about 10^5 cells per ml and then placed in a mist chamber maintained at 24°C for 2 days. These conditions were chosen to approximate conditions optimal for epiphytic growth and survival. Leaves were then harvested from half of the plants to enumerate bacterial populations, and the remaining plants were placed in a growth chamber and held at 30°C and 40% relative humidity with a 16-h photoperiod for two additional days. These conditions were chosen to approximate conditions that are not conducive to epiphytic growth. After 2 days, bacterial population sizes were again measured. Population sizes of *P. syringae* TLP2 and Cit7 on some of these plants under these conditions have been reported previously (31). Four replicate samples, each consisting of from 3 to 20 leaves (total leaf mass, approximately 25 g) from a single pot, were collected from four pots of a plant species at every sampling time.

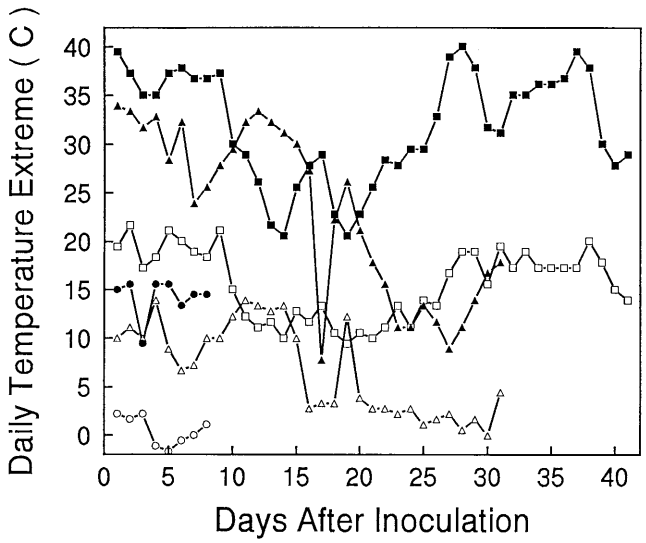

FIG. 1. Daily temperature extremes at three field sites following inoculation with *P. syringae* strains. Maximum daily high temperatures (filled symbols) and daily low temperatures (open symbols) are shown for the experimental site near Fresno from 8 May until 16 June (squares), the Fresno site from 23 to 30 November (circles), and the Tulelake site from 25 August until 24 September (triangles).

The plant species listed above were established in a field site at the University of California Kearney Agricultural Research and Extension Center, located near Fresno, Calif., on 1 March 1987 (early) and 2 April 1987 (late). All plants except for potato, horseradish, and Ponderosa pine were established from seeds. Potato and horseradish were established from non-surface-sterilized tuber pieces, while 1-year-old Ponderosa pine seedlings (approximately 30 cm in height) were transplanted from nursery plantings. Each replicate of each treatment consisted of two adjacent rows (1 m apart) that were 3 m in length. Every treatment was replicated in each of four blocks. Plants were spray inoculated on the evening of 8 May 1987. At this time, most plants were from 10 to 40 cm in height. Plants were spray inoculated with a suspension (approximately 10^7 cells per ml) of either *P. syringae* TLP2 or Cit7. A higher inoculum concentration was used in the field than in the growth chamber to offset the higher initial mortality anticipated under field conditions. Bacteria were applied to plants to runoff with a CO_2-pressurized hand-held sprayer operated at 40 lb/in^2 as in other studies (2). Plants were subsequently exposed to ambient field conditions. Maximum daily temperatures were between 35 and 40°C for the first 10 days after inoculation, decreased to about 25°C for the next 15 days, and then increased to about 35°C for the remainder of the experiment (Fig. 1). Minimum daily temperatures generally

paralleled the maximum daily temperatures and varied from about 10 to 23°C (Fig. 1). While rain occurred rather frequently prior to inoculation, only 1 and 21 mm of rain fell at 7 and 12 days after inoculation, respectively. At different intervals after inoculation, leaves were sampled randomly from the plants in each replicate block. From 3 to more than 25 leaves were collected from each of the four replicates (mass of each sample was about 25 g) for each plant species. Periodic sampling continued until 16 June 1987. Leaves were sampled into plastic bags that were loosely sealed and transported on ice to the laboratory. Leaves were processed within 24 h of sampling.

A second field study was established at the University of California Intermountain Research and Experiment Station located near Tulelake, Calif., on 10 July 1986. The experimental design of this plot was very similar to that noted above except that only the following plant species were established: 1, 2, 5, 7 to 10, 12, 14, 15, 17, 20, and 22 to 29. Plants in the plot were spray inoculated on 25 August 1986 with either *P. syringae* Cit7 or TLP2 as described above. Bacterial population sizes were estimated on leaves sampled on five subsequent dates, starting on 27 August and ending on 24 September 1986. Leaves were sampled as described above. The maximum daily temperature generally decreased from a high of about 32°C within the first 12 days after inoculation to about 15°C by the end of the experiment (Fig. 1). Daily minimum temperatures generally paralleled the daily maximum temperatures and ranged from about 12 to about 0°C (Fig. 1). Daily rainfall totalling 7, 7, 1, 2, 5, 1, and 1 mm occurred only on days 21, 23, 25, 26, 27, 28, and 29 after inoculation, respectively; little rainfall occurred prior to inoculation.

A third field study in which plant species 1, 4, 7, 17, 24, 26, 28, and 37 were established on 17 September 1987 at the University of California Kearney Agricultural Research and Extension Center was conducted. These plants were spray inoculated on 23 November 1987 as described above and sampled on a single occasion on 30 November 1987. Five individual leaves or leaflets were sampled from each of eight plants of each plant species on this date. The location of each leaf relative to position in the plant canopy was also recorded. The maximum daily temperature during this experiment was about 15°C, while minimum temperatures averaged about 0°C (Fig. 1). Little rainfall occurred prior to inoculation, and no rain fell after inoculation.

Enumeration of bacterium population sizes. Bulk leaf samples used in the first and second field studies were weighed and immersed in 200 ml of washing buffer (0.1 M potassium phosphate buffer, pH 7.0) in a 500-ml Erlenmeyer flask. Individual leaf samples used in the third field study were weighed and immersed in 20 ml of washing buffer in a large test tube. Tubes or flasks were sonicated in an ultrasonic cleaner (Bransonic 52) for 7 min, and appropriate 10-fold serial dilutions were plated onto KB to estimate total bacterium population sizes and onto KBR (KB containing 100 μg of rifampin per ml) to estimate population sizes of *P. syringae* Cit7 or TLP2. Bacterium population sizes for each sample were determined on the basis of bacterial colony counts on plates after 3 days incubation at 20°C.

Statistical methods. All statistical calculations were performed with SAS (version 6.04) (SAS Institute Inc., Cary, N.C.). Analysis of variance, regression analysis, and correlation analysis were done on log-transformed estimates of population size ($\log_{10}$ cells per gram [fresh weight]). The goodness of fit of the normal distribution to the log-transformed and nontransformed estimates of bacterial populations was tested by the Shapiro-Wilk *W* statistic or the Kolomogorov *D* statistic by the Univariate procedure in SAS.

RESULTS

The population sizes of *P. syringae* TLP2 and Cit7 were measured at field locations throughout California on up to 40 different plant species that had been spray inoculated with these strains during the spring and summer months. The experiment was repeated a total of three different times at two locations. Results were consistent among experiments; data presented here are from the largest experiment, which included the full complement of 40 plant species. Population sizes of both total culturable bacteria, hereafter referred to as total bacteria, and introduced strains were quantified on multiple dates starting 3 days after inoculation. In general, population sizes of Cit7 and TLP2 were largest shortly after inoculation and decreased with time. Population sizes of the introduced strains ranged from 10^3 to 10^6 cells per g (fresh weight) on the leaves of all species by 3 days following inoculation. Bacterial populations on many plant species, such as bean, showed modest decreases over time (Fig. 2A and B). On some plant species, such as safflower, population sizes of Cit7 and TLP2 remained constant or increased slowly with time (Fig. 2C and D). However, the population size of the inoculated strains following inoculation decreased more than 100-fold on most plant species, such as corn (Fig. 2E and F), over the 30-day sampling period. Mean population sizes of total bacteria also varied substantially among plant species at a given sample time. Total bacterium population sizes ranged from as high as 10^8 cells per g for some plant species, such as bean and safflower (Fig. 2), to as low as 10^5 cells per g for other species, such as Ponderosa pine and horseradish (data not shown). While total bacterium population sizes on most plant hosts, such as bean and safflower, remained relatively constant over the 39-day experiment (Fig. 2), they decreased significantly on other plant species, such as corn (Fig. 2).

To determine whether the phenological stage or chronological age of the plant species affected the ability of inoculated strains of *P. syringae* to establish populations on leaves, plantings of 40 different plant species, made at two different times, were simultaneously inoculated with strains Cit7 and TLP2. While the total bacterium population sizes on different plant species of the same age were significantly different, the total population sizes on plants of the same species but of different ages were seldom significantly different (Table 1). Since the population sizes of *P. syringae* Cit7 were usually very much smaller than that of the total bacteria on any set of leaves, most of the bacteria recovered from leaves were indigenous strains. For a given plant species, plant age also had no significant influence on population sizes of inoculated *P. syringae* Cit7 (Table 1). However, population sizes of the inoculated strain differed significantly among different plant species of the same age (Table 1). Similar results were observed when plants were inoculated with *P. syringae* TLP2 (data not shown).

Since the population sizes of *P. syringae* Cit7 and TLP2 had been measured on a large collection of plants incubated in microcosms under wet and subsequent dry conditions (31) as well as in the field, it was possible to compare the population sizes maintained after inoculation on the same 40 plant species under controlled environmental conditions and when established in field plots. In the controlled environment, population sizes of a particular bacterial strain on a given plant species incubated under wet and dry conditions were generally significantly positively correlated (Table 2). However, there was seldom a significant relationship among plant species between the mean population size of a given *P. syringae* strain on plants incubated under wet conditions in the mist chamber and the mean population size which could be recovered from the same plant species when inoculated in the field (Table 2). There was also no significant relationship between the rankings of the population size of the inoculated bacteria on plants incubated under wet conditions in the mist chamber and the rankings of mean bacterium population sizes grown on the corresponding plant species in the field (for Cit7, Spearman correlation coefficient = 0.006 and $P = 0.96$; for TLP2, Spearman correlation coefficient = −0.097 and $P = 0.56$). Likewise, there was seldom a significant relationship among plant species between the mean population size of a particular *P. syringae* strain on plants of a given species that had been allowed to dry in the growth chamber for 48 h after an initial 48-h wet incubation period prior to sampling and the population size of that strain on the same plant species grown and inoculated in the field (Table 2). Rankings of the mean population size of the inoculated bacteria attained on each plant species when grown in the growth chamber under dry conditions and in the field were also not significantly correlated (for Cit7, Spearman correlation coefficient = 0.26 and $P = 0.14$; for TLP2, Spearman correlation coefficient = −0.006 and $P = 0.97$).

Since bacterial population sizes were measured at multiple times under field conditions, it was possible to evaluate the relationship among plant species between *P. syringae* popula-

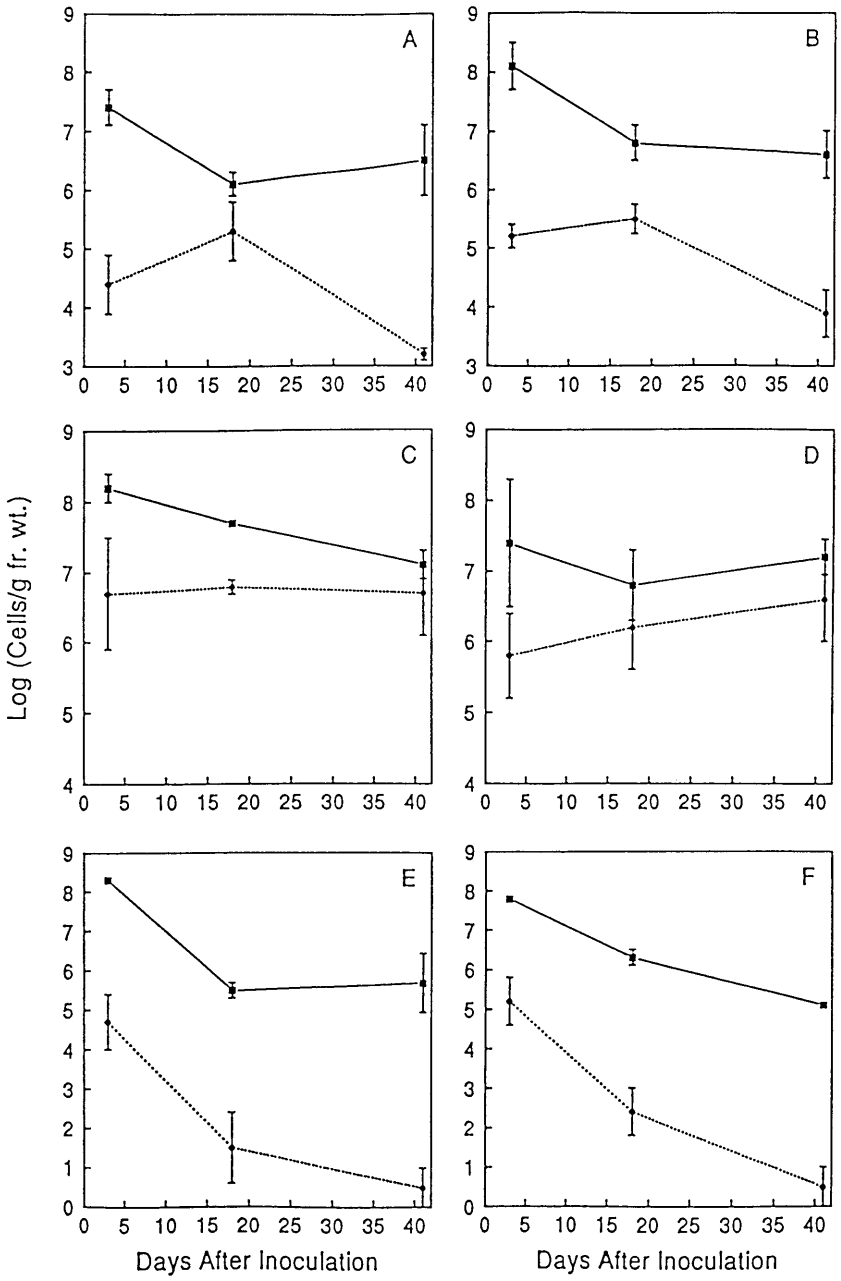

FIG. 2. Population sizes of *P. syringae* Cit7 (A, C, and E) and TLP2 (B, D, and F) (dashed lines) and total bacterial population sizes (solid lines) on leaves of bean (*P. vulgaris* cv. Eagle) (A and B), safflower (C and D), and corn (E and F) at different times after inoculation of plants on 8 May at an experimental site near Fresno. The vertical bars represent the standard error of mean log-transformed population sizes at a given sampling time. fr. wt., fresh weight.

tion sizes in the controlled-environment microcosms and in the field on numerous occasions. For example, neither population sizes nor relative rankings of population sizes of strain Cit7 grown on plants under wet incubation conditions in a mist chamber were ever significantly correlated with field population sizes of the strain on six occasions in the largest field experiment (Table 2; ranked data not shown). Population sizes of strain Cit7 maintained for 48 h under dry conditions were significantly positively correlated with field populations on only one of six occasions in the same experiment (Table 2). Relative rankings of population sizes of strain Cit7 attained in the field were never significantly positively correlated with the rankings observed on the same plant species maintained under dry conditions in the growth chamber (data not shown). When considering all other experiments in which either strain Cit7 or TLP2 was inoculated onto plants in the field, on none of 26 occasions were mean population sizes of either of these strains recovered from greenhouse-grown plants under wet conditions in a mist chamber significantly correlated with the field population sizes of the inoculated strains on the same species. Similarly, on only 1 of 26 occasions was there a significant correlation between population sizes of either of these strains on plant species incubated under dry conditions in a growth chamber and on the same plant species when in the field. Thus,

TABLE 1. Population sizes of total indigenous bacteria and of inoculated *P. syringae* Cit7 on plants of different species of two different ages under field conditions

Plant species	Log bacterial cells recovered/g (fresh wt)[a]			
	Total[b]		*P. syringae* Cit7[c]	
	Old plants	Young plants	Old plants	Young plants
Cucumber	8.83 A a	8.32 A ab	0.00 A e	2.03 A cd
Cheatgrass	8.80 A a	8.12 A abc	4.81 A abcd	5.45 A ab
Petunia	8.54 A ab	4.36 B d	4.65 A abcd	2.76 A bcd
Barley (*H. vulgare* cv. Larker)	8.35 A ab	7.46 A abc	5.05 A abcd	5.26 A abc
Pea	8.30 A ab	8.66 A a	6.08 A ab	6.17 A a
Potato	8.28 A a	7.31 A abc	4.67 A abcd	5.27 A abc
Wild oats	8.26 A ab	8.23 A abc	4.40 A abcd	5.98 A ab
Tomato	8.25 A ab	7.97 A abc	4.43 A abcd	3.87 A abc
Purslane	8.25 A ab	7.40 A abc	6.75 A a	4.58 B abc
Mallow	8.23 A ab	8.85 A a	5.19 A abcd	5.34 A abc
Datura	8.23 A ab	8.21 A abc	6.65 A a	3.32 A abcd
Safflower	8.22 A ab	8.43 A ab	6.63 A a	3.53 A abc
Morning glory	8.15 A abc	8.24 A abc	5.34 A abcd	6.02 A ab
Red clover	8.11 A abc	9.02 A a	2.85 B cde	5.59 A ab
Sudangrass	8.10 A abc	7.99 A abc	4.78 A abcd	5.97 A ab
Quackgrass	8.08 A abc	8.52 A ab	6.32 A ab	6.61 A a
Wheat (*T. aestirum* cv. Modoc)	8.03 A abc	8.62 A a	4.58 A abcd	4.47 A abc
Ponderosa pine	8.03 A abc	7.61 A abc	5.36 A abcd	4.76 A abc
Johnsongrass	8.01 A abc	7.14 A abc	5.92 A abc	5.39 A abc
Sudangrass	8.10 A abc	7.99 A abc	4.78 A abcd	5.97 A ab
Horseradish	7.95 A abc	6.80 A abcd	5.68 A abc	5.07 A abc
Oats	7.89 A abc	7.77 A abc	4.49 A abcd	5.39 A abc
Onion	7.88 A abc	5.58 B cd	3.58 A abcd	4.19 A abc
Atriplex sp.	7.84 A abc	8.01 A abc	6.13 A ab	5.60 A ab
Bean (*P. vulgaris* cv. Red Mexican)	7.80 A abc	7.82 A abc	4.53 A abcd	5.01 A abc
Italian ryegrass	7.76 A abc	8.67 A a	5.90 A abc	6.52 A a
Corn	7.71 A abc	8.46 A ab	5.13 A abcd	5.64 A ab
Barley (*H. vulgare* cv. Steptoe)	7.71 A abc	8.46 A ab	4.81 A abcd	5.50 A ab
Strawberry	7.68 A abc	7.86 A abc	5.39 Aabcd	4.58 A abc
Dandelion	7.44 A abc	8.02 A abc	4.37 A abcd	4.96 A abc
Bean (*P. vulgaris* cv. Eagle)	7.39 A abc	7.05 A abcd	4.39 A abcd	5.21 A abc
Perennial ryegrass	7.32 A abc	6.75 A abcd	4.62 A abcd	5.50 A ab
Alfalfa (*M. sativa* cv. Apollo)	7.31 A abc	8.32 A ab	4.49 A abcd	5.09 A abc
Pepper	7.19 A abc	7.71 A abc	4.44 A abcd	0.00 B d
Orchard grass	7.18 A abc	7.18 A abc	5.88 A abc	5.74 A ab
Alfalfa (*M. sativa* cv. Vernal)	7.11 A abc	6.99 A abcd	4.79 A abcd	5.12 A abc
Beet	6.94 A abc	8.38 A ab	4.47 A abcd	6.45 A a
Zinnia	6.93 A abc	5.85 A bcd	5.68 A abc	3.95 A abc
Pigweed	6.68 A abc	7.92 A abc	4.67 A abcd	6.03 A ab
Nightshade	6.62 A bc	7.87 A abc	5.02 A abcd	4.85 A abc
Soybean	6.04 A c	7.98 A abc	3.46 A bcd	5.06 A abc
Mean	7.76 A	7.74 A	4.90 A	4.98 A

[a] Means in each column followed by the same lowercase letter do not differ significantly ($P = 0.05$) by Fisher's unprotected least significant difference test. Means in each row followed by the same uppercase letter do not differ significantly ($P = 0.05$) by Fisher's unprotected least significant difference test. Old plants were established early in the growing season; young plants were established 30 days after those established early in the growing season.

[b] Population sizes determined from the total number of colonies enumerated on KB.

[c] Population sizes determined from the total number of colonies enumerated on KBR.

bacterial population sizes on plants grown in a controlled environment when incubated under either wet or dry conditions were not predictive of bacterium population sizes on the same plant species in the field.

The population size of *P. syringae* Cit7 recovered from a given plant species inoculated in the field could be either substantially larger or smaller than that recovered from the same plant species when incubated in a controlled environment under wet or dry conditions (Fig. 3). When *P. syringae* Cit7 was recovered 3 days after inoculation onto field-grown plants, its population size on a given species was larger than that recovered from plants incubated in a mist chamber on 20 of 36 plant species and smaller on 15 of 36 plant species (Fig. 3A). In contrast, when the strain was recovered in the field 18 days after inoculation, only 10 of 37 plant species harbored larger population sizes of the inoculated strain than were found under wet conditions in a mist chamber (Fig. 3B). Even when population sizes of strain Cit7 recovered 18 days after inoculation of plant species in the field were compared with those populations on the same species exposed to dry conditions in a growth chamber, population sizes were higher on 20 of 36 species (Fig. 3C).

An index was developed to measure the fidelity with which bacterium population size in a controlled environment predicted the population size of the introduced organisms in the field. This index was calculated as the difference between the

TABLE 2. Correlations between population sizes of *P. syringae* Cit7 on a collection of plant species under greenhouse conditions and population sizes of this strain and of total bacterial population sizes on plants of the same species at different times after inoculation of strain Cit7 onto the plants in the field[a]

Dependent variable	Pearson correlation coefficient (*P*) with independent variable:														
	GW	GD	Ps1-o	Ps1-y	Ps2-o	Ps2-y	Ps3-o	Ps3-y	Tot1-o	Tot1-y	Tot2-o	Tot2-y	Tot3-o	Tot3-y	GW-GD
GD	0.56 (0.0005)														
Ps1-o	−0.03 (0.83)	0.004 (0.97)													
Ps1-y	−0.03 (0.83)	−0.06 (0.72)	0.28 (0.07)												
Ps2-o	−0.02 (0.86)	−0.02 (0.91)	0.11 (0.49)	0.09 (0.57)											
Ps2-y	−0.23 (0.16)	−0.08 (0.63)	−0.02 (0.89)	0.08 (0.60)	0.66 (0.0001)										
Ps3-o	0.08 (0.62)	−0.05 (0.79)	0.007 (0.96)	−0.004 (0.97)	0.78 (0.0001)	0.49 (0.001)									
Ps3-y	−0.09 (0.56)	−0.01 (0.95)	−0.05 (0.75)	0.04 (0.77)	0.62 (0.0001)	0.60 (0.0001)	0.60 (0.0001)								
Tot1-o	−0.09 (0.57)	−0.001 (0.99)	0.003 (0.98)	−0.10 (0.53)	0.12 (0.42)	0.09 (0.58)	0.18 (0.26)	0.22 (0.16)							
Tot1-y	0.10 (0.54)	−0.09 (0.57)	−0.02 (0.90)	0.31 (0.04)	0.24 (0.13)	0.17 (0.29)	0.19 (0.22)	0.16 (0.32)	0.06 (0.71)						
Tot2-o	−0.14 (0.37)	−0.01 (0.93)	0.08 (0.60)	0.29 (0.06)	0.66 (0.0001)	0.62 (0.0001)	0.49 (0.001)	0.64 (0.0001)	0.38 (0.01)	0.15 (0.33)					
Tot2-y	−0.11 (0.48)	−0.11 (0.52)	0.22 (0.16)	0.25 (0.11)	0.22 (0.16)	0.33 (0.04)	0.18 (0.24)	0.08 (0.59)	0.11 (0.48)	0.13 (0.40)	0.30 (0.05)				
Tot3-o	0.07 (0.67)	−0.005 (0.97)	0.03 (0.81)	0.02 (0.88)	0.42 (0.006)	0.27 (0.08)	0.51 (0.0006)	0.58 (0.0001)	0.37 (0.01)	0.05 (0.75)	0.61 (0.0001)	0.08 (0.60)			
Tot3-y	−0.01 (0.96)	0.22 (0.18)	−0.04 (0.78)	−0.08 (0.62)	0.49 (0.001)	0.31 (0.05)	0.36 (0.02)	0.48 (0.002)	0.34 (0.03)	−0.11 (0.49)	0.60 (0.0001)	0.15 (0.34)	0.65 (0.0001)		
GW−GD	0.27 (0.11)	−0.64 (0.0001)	0.005 (0.97)	0.01 (0.94)	0.01 (0.96)	−0.14 (0.42)	0.14 (0.40)	−0.04 (0.83)	−0.08 (0.63)	0.27 (0.11)	−0.14 (0.40)	0.01 (0.96)	0.04 (0.80)	−0.16 (0.36)	
Dif	−0.01 (0.95)	0.002 (0.98)	0.46 (0.003)	0.43 (0.006)	−0.60 (0.0001)	−0.48 (0.002)	−0.77 (0.0001)	−0.74 (0.0001)	−0.22 (0.16)	−0.04 (0.80)	−0.40 (0.01)	0.03 (0.84)	−0.49 (0.001)	−0.43 (0.005)	−0.03 (0.84)

[a] Abbreviations: GW and GD, population size of *P. syringae* Cit7 on greenhouse plants maintained under wet and dry conditions, respectively; Ps1-o and Ps1-y, population size of *P. syringae* Cit7 3 days after inoculation on field plants established on 1 March and 1 April, respectively; Ps2-o and Ps2-y, population size of *P. syringae* Cit7 15 days after inoculation on field plants established on 1 March and 1 April, respectively; Ps3-o and Ps3-y, population size of *P. syringae* Cit7 41 days after inoculation on field plants established on 1 March and 1 April, respectively; Tot1-o and Tot1-j, total bacterial population size 3 days after inoculation of plants established on 1 March and 1 April, respectively; Tot2-o and Tot2-y, total bacterial population size 15 days after inoculation of plants established on 1 March and 1 April, respectively; Tot3-o and Tot3-y, total bacterial population size 41 days after inoculation of plants established on 1 March and 1 April, respectively; GW−GD, difference of logarithm of population sizes of *P. syringae* Cit7 on greenhouse plants incubated under wet conditions and the logarithm of population sizes when incubated under dry conditions; Dif, difference of logarithm of population sizes of *P. syringae* Cit7 on field plants 3 days after inoculation with this strain and the logarithm of population sizes 41 days after inoculation.

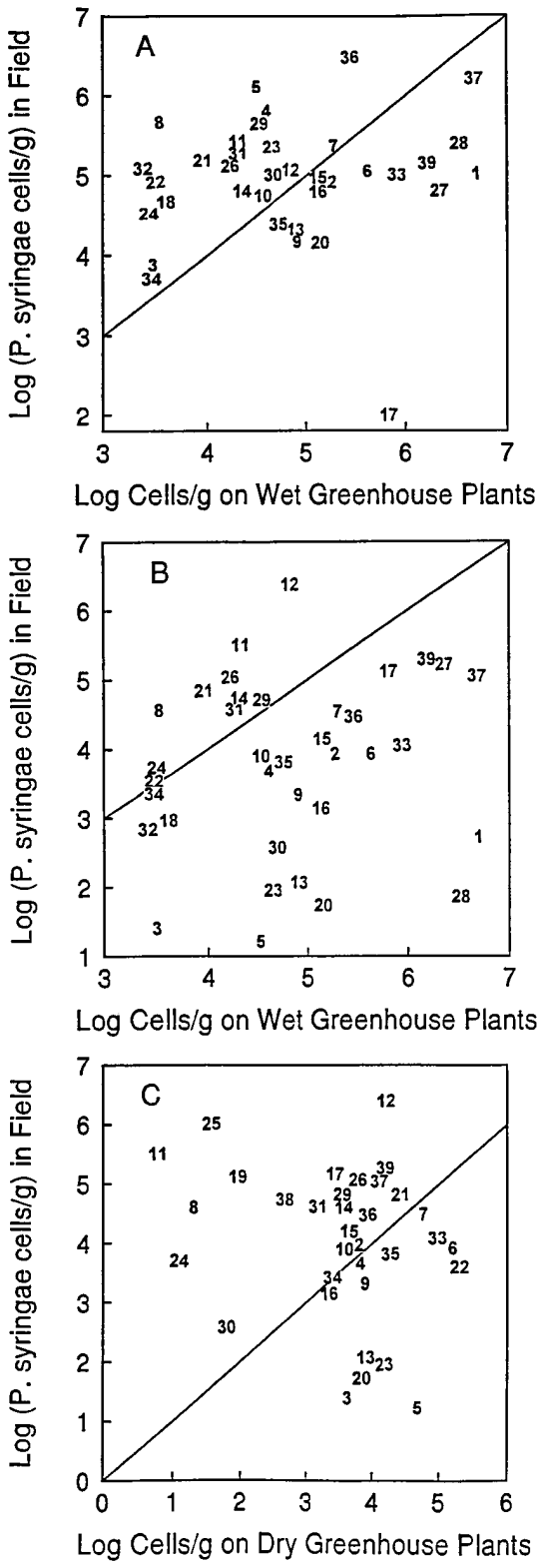

FIG. 3. Relationship between mean population size of inoculated *P. syringae* Cit7 on a given plant species grown in a greenhouse and incubated under wet conditions (A and B) or subsequently under dry conditions (C) (abscissa) and population size of this strain on the same plant species grown in field plots near Fresno and inoculated with this strain on 8 May (ordinate). The relationship with population sizes of strain Cit7 recovered from plants 3 days (A) and 18 days (B and C) after inoculation is shown. Each point represents the mean population size determined from four replicate samples of the same plant species under these two conditions. Plant species are identified by number as described in Materials and Methods. The lines drawn represent the case in which population sizes on leaves in the greenhouse and in the field are equal.

mean log population size measured in the controlled-environment microcosm, under either wet or dry conditions, and that observed in the field for a given plant species. There was a strong positive correlation between the deviation in the predicted population size for a bacterial strain on a specific plant species based on wet incubation conditions in the mist chamber and the field population of the inoculated strain on that species when estimated at different sample times (R ranged from 0.417 to −0.801; $P < 0.009$ in every case). That is, population sizes of the inoculated *P. syringae* strain were consistently higher in the field than predicted by population sizes observed in the mist chamber for some plant species over many samplings. Similarly, population sizes of the inoculated strain in the field on a subset of the plant species were consistently lower than predicted by population size estimates obtained from those plants incubated under wet conditions in the mist chamber (Fig. 3). As was the case for deviations of population sizes in the field from those measured under wet conditions in the mist chamber, the deviation in the sizes of field populations from predictions based on population sizes measured on dry plants in the growth chamber was also consistent among species when considered over numerous sampling times and when considered across many plant species (R ranged from 0.44 to 0.82; $P < 0.01$ in every case). In addition, there were significant positive correlations between the deviations obtained when predicting bacterium population sizes on the basis of wet and dry microcosm conditions among plant species (R ranged from 0.65 to 0.83; $P < 0.0001$ in every case). Thus, those plant species on which *P. syringae* population sizes in the field tended to deviate most from estimates obtained from plants incubated under wet conditions in the mist chamber also had *P. syringae* population sizes that tended to deviate greatly from population estimates from plants incubated in the growth chamber under dry conditions. Finally, not only were deviations consistent among plant species regardless of whether the predicted value was based on wet or dry incubation, but also deviations among predictions for plant species from wet mist chamber incubations were significantly correlated at different field locations (two different Fresno experiments and one Tulelake experiment: $R = 0.494$; $P = 0.0016$). That is to say, those plant species for which inoculated *P. syringae* population sizes were larger at Tulelake than populations observed in the mist chamber following wet incubation also had larger *P. syringae* population sizes in both Fresno experiments than in the greenhouse. Similarly, deviations in population sizes at Tulelake and Fresno from those observed under dry incubation conditions in the growth chamber were also significantly positively correlated ($R = 0.35$; $P = 0.052$).

Population sizes of inoculated *P. syringae* strains were often highly positively correlated with the total bacterium population sizes on the same plant species (Table 2 and Fig. 4). Since *P. syringae* Cit7 was a small fraction of the total bacteria on inoculated plants (Fig. 4), the total bacterial populations comprised nearly exclusively indigenous strains. Plant species which harbored relatively small indigenous bacterium population sizes generally also supported quite small populations of an inoculated *P. syringae* strain (Fig. 4). It is noteworthy, however, that introduced *P. syringae* strains represented a proportionally larger percentage of the total bacteria on plants as the total population size on a given plant species increased (Fig. 4).

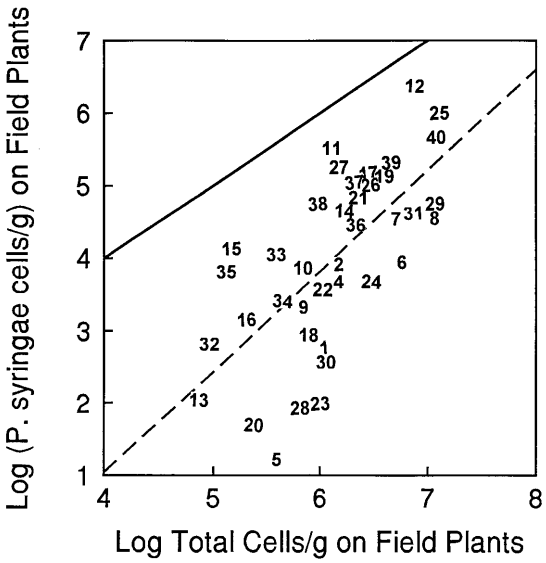

FIG. 4. Relationship between total bacteria recovered 18 days after plants grown in a field plot near Fresno were inoculated with *P. syringae* Cit7 (abscissa) and population sizes of this strain recovered from the same plants at the same time (ordinate). Each point represents the mean population size of indigenous bacteria and of strain Cit7 determined from four replicate samples of the same plant species. Plant species are identified by number as described in Materials and Methods. The solid line represents the condition that the population size of strain Cit7 is equal to the total indigenous bacterial population size. The dashed line represents the best-fit regression line $y = 1.39x - 4.52$ ($R^2 = 0.44$) ($P < 0.0001$) describing the relationship between mean total bacterial population size and population size of strain Cit7 recovered from a given plant species.

That is, on plant species having fewer than 10^6 cells of total bacteria per g, *P. syringae* represented less than 0.01% of the total, while inoculated strains represented as much as 1% of the total on plants supporting communities in excess of $10^{7.5}$ cells per g (Fig. 4).

Population sizes of a particular inoculated strain of *P. syringae* strain recovered at a given time were generally well correlated with population sizes of that same strain at previous and/or subsequent sampling times (Table 2). Additionally, the population sizes of inoculated strains recovered at the same sample date from plants of two different ages were often positively correlated (e.g. Table 2).

An estimate of the ability of a given plant species to maintain epiphytic bacterial populations of *P. syringae* under harsh environmental conditions was obtained by comparing populations recovered from wet-mist-chamber-grown plants with those recovered after allowing plants to dry for 48 h. An analogous survival index was calculated in the field by comparing population sizes determined in the first sampling (shortly after inoculation) with those determined in the second or third sample taken several days later, when most population sizes had declined. There was no correlation between these two survival indices for either *P. syringae* strain in any of the three field experiments. Thus, plant species on which relatively large decreases in *P. syringae* population size occurred upon change of the physical environment from wet to dry conditions in the growth chamber were not necessarily those which exhibited large decreases in population size in sequential samples in the field.

DISCUSSION

A fundamental limitation in assessing the colonization potential of microorganisms, inherent in assessing the potential risks associated with the release of recombinant strains, is the fact that prerelease experimentation must be restricted to contained environments. We lack basic information on the relevance and applicability of data from contained-environment studies to predictions of field behavior. While numerous microcosm studies have been designed to study basic factors, such as survival of genetically modified strains and gene exchange among microbial populations (15, 23), seldom have field studies been conducted to permit validation of microcosm studies (4, 11, 16, 18, 20, 25, 26, 35, 45). In the context of bacteria to be released for the biological control of frost damage or plant diseases, one may wish to predict whether the released strain would successfully establish on the phylloplane of nontarget plants in the vicinity of a test site. There are two general approaches to making such a prediction. In the first approach, the recombinant strains and their isogenic parental strains could be tested on a variety of nontarget plant hosts in a contained environment. This approach was used to predict the relative host preference of recombinant Ice^- deletion mutants of *P. syringae* (31). By this approach, it was predicted that certain plant species, such as quackgrass and dandelion, were relatively good hosts of epiphytic *P. syringae* TLP2 and Cit7 as well as their corresponding Ice^- deletion mutants (31). This method, the use of microcosms or natural-environment simulators, has been used exclusively in prerelease testing of the potential risks associated with the release of genetically engineered microorganisms. An alternative approach would have been to inoculate nontarget plant species with the isogenic parental strains under field conditions. Clearly the latter approach offers no control over the types of environmental conditions under which colonization is assessed. Additionally, one would have to assume that the parental and the recombinant strains would behave similarly in a given environment. This assumption cannot be tested under field conditions but could be tested with microcosms as discussed above. This study was designed to address whether the microcosm-based approach was predictive of the eventual behavior of a set of recombinant strains once released into the field; hence, it represents one of the first rigorous tests of the efficacy of microcosms in predicting the behavior of released recombinant organisms.

Population sizes of bacteria on various hosts in microcosms were not predictive of plant species preferences, or the ability of the bacterium to achieve or maintain a large population size on that species under field conditions, in this study. Although absolute epiphytic population sizes were not correlated on plants inoculated in the field and greenhouse (Table 1), it is not unreasonable to expect that the relative rankings of population sizes on different plant species would be well correlated. In fact, we observed no significant correlation in ranked population sizes in such an analysis. Therefore, one is left with the conclusion that different processes may have been involved in determining the epiphytic population sizes in the greenhouse and field studies conducted here.

It should be noted that microcosm experiments were conducted in a fashion somewhat different from that which was possible in the field. For example, growth chamber-grown plants were inoculated with a relatively low inoculum level of the two *P. syringae* strains (10^5 cells per ml). This inoculum level was selected to ensure that measured *P. syringae* population sizes reflected growth and/or survival on plants. Likewise, the growth chamber plants were exposed to dry conditions (which presumably selected for survival characteristics on a given host) only after large resident populations had been established by growth on the plants. In contrast, field-grown plants were inoculated with a relatively large inoculum dose of these strains (10^7 cells per ml), and ambient field conditions

were not consistently conducive for substantial amounts of net growth of the cells after inoculation and often led to progressive decreases in population size (Fig. 2). Measurements of *P. syringae* population sizes in the field thus may emphasize differences in the ability of host plants to act as refuges for the survival of cells after inoculation. It is likely that epiphytic populations on plants in the mist chamber under wet conditions were determined primarily by resources contributed by the host plant. Because population sizes on field-grown plants were likely primarily a function of microbial survival, features of the plants other than nutritional resources that mediate microbial growth might be proportionally more important in determining field population sizes. However, the lack of significant correlation between bacterial population sizes on plants incubated in the growth chamber under dry conditions and in the field suggests that factors other than the ability of a particular plant species to support microbial survival under adverse conditions differ significantly between the controlled environment and the field.

Perhaps the most substantial difference between microcosm studies and field studies is the presence of large indigenous bacterial populations on the field-grown plants. In fact, these data suggest that a major difference between the field and the controlled-environment-grown plants in terms of their ability to support bacterial populations lies not in the physical environment (wet versus dry) but rather in the potential role of indigenous populations in influencing bacterial abundance. Specifically, on plants maintained in the growth chamber, on which bacterial populations were virtually nonexistent prior to the inoculation of the experimental strains, the ability of a particular plant species to support populations of an inoculated bacterium when incubated under wet conditions was significantly positively correlated with the ability of that plant species to support populations of that bacterium when inoculated under dry conditions. This indicates that while the absolute population sizes attained by bacteria on leaves of a particular plant species differ under wet versus dry conditions in the growth chamber, the inherent ability of a particular plant species to support bacterial populations is relatively predictable across environments. However, the lack of any significant correspondence between the bacterial populations attained on plants incubated in the growth chamber under either wet or dry conditions and the bacterial populations attained in the field suggests that the microcosm data fail to provide adequate information on the role of indigenous populations in influencing the dynamics of the inoculated bacterium on any particular plant species in the field. This is consistent with the findings of researchers working in soil microcosms (21, 27). Thus, though a particular plant species was capable of supporting a relatively large population of a bacterial strain in the absence of any other significant bacterial colonization in controlled-environment trials, that bacterium was not always capable of establishing a similarly substantial population on that plant species in the presence of a large indigenous population. Alternatively, on some plant species, the inoculated bacteria established significantly greater population sizes in the field in the presence of a large indigenous population than on uncolonized plants in the growth chamber.

What is the role of the indigenous populations in influencing bacterial colonization in the field? In fact, the data show that across all plant species the population size of indigenous microorganisms was positively correlated with the ability of inoculated *P. syringae* strains to become established on leaves in the field. The intriguing and unexpected observation that population sizes of introduced *P. syringae* strains were positively correlated with the population size of the indigenous bacterial populations across all plant species, and often positively correlated with the indigenous bacterial populations among samples for individual plant species, is suggestive of two different processes. First, this observation may indicate that the ability of a particular plant species to support large introduced populations of *P. syringae* is strongly correlated with the ability of that species to support large total bacterial populations. In this scenario, a particular plant species might provide large amounts of nutritional resources required for microbial growth and/or survival on the phylloplane; these resources therefore may be shared by the introduced *P. syringae* strains and indigenous bacteria. If this model is correct, it would imply that nutritional resources are not completely exhausted on plants at a given time and thus are available to support at least limited growth of immigrant bacterial strains. This contrasts with what has been shown for beans, potato, and tobacco (51–54). Specifically, previous work has shown that large indigenous bacterial populations can sequester resources required for epiphytic colonization, limiting the growth of subsequent immigrants by the process of preemptive exclusion. Alternatively, consistent with the evidence that resources on the leaf surface can be limiting to microbial population sizes, the data could indicate that on those resource-rich plant species capable of supporting large bacterium population sizes in the field, *P. syringae* tends to be a fairly good competitor. Further data are needed to investigate these possibilities.

A second explanation for the significant positive correlation between indigenous bacterium population sizes and introduced population sizes across all plant species as well as on some individual plant species may be that large indigenous microbial populations are conducive to the growth and/or survival of introduced *P. syringae* strains. Since most inoculated populations either remained stable or declined over time, it seems likely that factors influencing bacterial survival rather than bacterial growth are more critical to determining population size of the introduced organism in the field. It is thus intriguing to consider the possibility that the indigenous microflora mediated the survival of the introduced strains. We have observed that the survival of *P. syringae* strains introduced onto leaves is density dependent (50). It seems possible that only cells impinging on leaves supporting other epiphytic bacteria had a high probability of survival under the stressful conditions that apparently occurred in the field (Fig. 1). This may help to explain why population sizes of bacteria observed were larger in the field than in the greenhouse on some plant species. That is, on those plant species on which introduced population sizes were generally always larger in the field than in the controlled environment, the presence of a large indigenous population, absent in the greenhouse, could have been critical to the survival of the introduced microbes. Such survival may be influenced by the presence of an established biofilm consisting of a microbial assemblage on leaf surfaces which provides a habitat conducive for bacterial survival (17). This assemblage may maintain a hydrated environment in its vicinity by elaborating a polysaccharide matrix. Additionally, several different bacterial phenotypes have recently been shown to be influenced by the exogenous production of autoregulatory compounds such as a family of *N*-acyl-homoserine lactones (HSL) (7, 19, 22, 38). In fact, cell-to-cell interactions among rhizosphere bacteria, apparently involving HSL production, influence the expression of phenazine antibiotics in *Pseudomonas aureofaciens* (54). It is possible that phenotypes expressed by *P. syringae* on leaf surfaces which mediate their survival of environmental stress are exhibited only in a density-dependent fashion, possibly mediated via either HSL, extracellular matrices, or both. The survival of *P. syringae* on plants

already has been shown to be a plant-inducible phenomenon, since cells recovered from the surface of plants exhibit substantially higher survival under desiccation stress when reapplied to leaves than do cells cultured in vitro (49).

Though there was a significant correlation between indigenous and introduced bacterial population sizes when all plant species were considered together, we note that for individual plant species there was no significant correlation in 86 of 141 cases. Thus, the potential role of the indigenous community in enhancing or antagonizing the establishment of an introduced bacterial population is a function of the plant species and the bacterial strain. For instance, on plant species 9 (red clover), 15 (alfalfa [*M. sativa* cv. vernal]), and 33 (petunia), inoculated populations of both Cit7 and TLP2 were smaller in the field than in the growth chamber (wet or dry), and for both bacterial strains there was no significant correlation between indigenous and introduced population sizes in the field. Likewise, though introduced bacterial populations on safflower and barley were greater in the field than in greenhouse trials, there were no significant correlations between indigenous and inoculated population sizes on these plant species. In the first case, the indigenous populations provide no apparent benefit or detriment to the establishment of the inoculated populations. In the second case, however, the indigenous populations implicitly provide some benefit to the survival or growth of the introduced bacteria, since bacterial populations were consistently greater in the presence than in the absence of the indigenous bacterial community, though the benefit to the introduced bacterium is not directly related to the absolute size of the indigenous community. Thus, though the size of the indigenous community may be useful in predicting the survival and/or growth of introduced populations on some plant species, this does not offer a general means for understanding the potential for introduced bacteria to establish leaf surface populations in the field.

While bacterium population sizes measured on a collection of different plant species in a controlled environment were not predictive of either the relative or absolute population sizes measured on these same species in the field, we found that the deviations in population size of an introduced strain on field plants relative to populations on a particular plant species in a growth chamber were consistent among experiments. That is to say, while population sizes of introduced *P. syringae* strains were relatively much larger on some plant species in the field than in the growth chamber, they were always relatively large on that species in the variety of locations and times sampled. This finding suggests that even though the physical environment in these studies varied, the physical conditions were relatively less important in influencing microbial growth or survival than were other species-associated factors in the field. In particular, the unique indigenous microflora supported on a specific plant species at a particular time may be critical to determining the potential for bacteria to successfully establish populations on that plant species. Unfortunately, while the relative departure of population sizes of a given strain in the field from those predicted from controlled-environment studies was relatively constant, the relative population size of a strain among plant species was not consistent among field experiments. These results indicate that pilot studies involving the release of an isogenic parental strain into field sites in advance of the release of a recombinant strain may not accurately predict the relative habitat preference of that strain. Microcosm studies therefore may be most effective at assessing possible differences in behavior of parental and recombinant strains under a given environmental regimen but seem limited in their ability to predict the population dynamics of either under field conditions.

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