Comparison of the Behavior of Epiphytic Fitness Mutants of *Pseudomonas syringae* under Controlled and Field Conditions

GWYN A. BEATTIE* AND STEVEN E. LINDOW

*Department of Environmental Science, Policy and Management,
University of California, Berkeley, California 94720*

The epiphytic fitness of four Tn*5* mutants of *Pseudomonas syringae* that exhibited reduced epiphytic fitness in the laboratory was evaluated under field conditions. The mutants differed more from the parental strain under field conditions than under laboratory conditions in their survival immediately following inoculation onto bean leaves and in the size of the epiphytic populations that they established, demonstrating that their fitness was reduced more under field conditions than in the laboratory. Under both conditions, the four mutants exhibited distinctive behaviors. One mutant exhibited particularly large population decreases and short half-lives following inoculation but grew epiphytically at near-wild-type rates, while the others exhibited reduced survival only in the warmest, driest conditions tested and grew epiphytically at reduced rates or, in the case of one mutant, not at all. The presence of the parental strain, B728a, did not influence the survival or growth of three of the mutants under field conditions; however, one mutant, an auxotroph, established larger populations in the presence of B728a than in its absence, possibly because of cross-feeding by B728a in planta. Experiments with B728a demonstrated that established epiphytic populations survived exposure of leaves to dry conditions better than newly inoculated cells did and that epiphytic survival was not dependent on the cell density in the inoculum. Three of the mutants behaved similarly to two nonpathogenic strains of *P. syringae*, suggesting that the mutants may be altered in traits that are missing or poorly expressed in naturally occurring nonpathogenic epiphytes.

Bacteria are natural residents on leaf surfaces of most, if not all, plant species. The bacteria that are commonly found on leaves probably have adaptations that allow them to both tolerate and exploit epiphytic environments. Epiphytic fitness, which is inferred by the ability to establish and maintain epiphytic populations, is probably conditioned by these adaptive traits. Wilson and Lindow (54) demonstrated that epiphytic fitness traits are induced in planta, since bacterial cells harvested from plants survived better than cells cultured in vitro after inoculation onto plants under field conditions. Thus, plant factors that are not present in laboratory media appear to be required for maximal expression of epiphytic fitness traits. On the basis of these results, there should be large differences in the epiphytic survival of established populations of bacterial cells and newly inoculated cells that have been grown in laboratory media. Plant signals have been found to induce genes involved in other plant-associated phenotypes, including pathogenicity and induction of a hypersensitive reaction (2, 9, 22, 23, 30, 44), virulence (24, 47), phytotoxin production (39), and nodulation of leguminous plants (13). Interestingly, plant factors have also been found to induce many genes whose functions are not known (10, 42); possibly some of these are involved in epiphytic fitness.

Recently, Wilson and Lindow (55) found that epiphytic survival of a *Pseudomonas syringae* strain increased with increasing cell density in the inocula, suggesting that epiphytic survival can be influenced by cooperative effects of extracellular factors produced on laboratory media. Several extracellular

factors have been hypothesized to be involved in epiphytic fitness, including pigments, siderophores, phytotoxins, and extracellular polysaccharides (7, 33). Recently, the involvement of extracellular signals, specifically autoinducers, in cell density-dependent control of gene expression has been demonstrated to occur in a variety of microorganisms (see, e.g., references 5, 14, 21, 40, 43, and 51). Such regulation may explain the density dependence of epiphytic survival (55) and bacterial growth in leaves (12, 16, 27, 50, 58).

It is often assumed that bacterial behavior in a laboratory or greenhouse is a good predictor of its behavior in the field environment (3, 15); however, this assumption is rarely tested. In fact, of at least 150 genes that have been found to influence the behavior of phytopathogenic bacteria in the laboratory, including *hrp*, *vir*, *avr*, *pel*, and *out* genes (11, 26, 45, 52), to date only two types, *ice* genes (28, 29, 32, 36) and *lemA* (20), have been directly examined for their role in bacterial behavior in a field environment. The fact that greenhouse-grown plants typically support larger epiphytic populations of an inoculated strain than do field-grown plants (54) indicates that controlled and field conditions differ in their influence on the phyllosphere as a habitat for bacteria. Such differences could be critical to the expression of epiphytic fitness determinants.

In a previous study we identified and characterized several Tn*5* mutants of a *P. syringae* strain that showed a reduced tolerance to drying of the leaf surface under controlled conditions in the laboratory (8, 35). In that study, epiphytic fitness was examined only after the strains had been allowed to adapt to the epiphytic environment. The role of this adaptation period in their fitness is addressed in this report, as is the role of extracellular factors. In previous studies we found no evidence that these mutants had lost the ability to produce in planta extracellular factors that were critical to epiphytic

* Corresponding author. Mailing address: Department of Environmental Science, Policy and Management, University of California, 108 Hilgard Hall, Berkeley, CA 94720-3110. Phone: (510) 643-7750. Fax: (510) 642-3845. Electronic mail address: gbeattie@violet.berkeley.edu.

fitness under laboratory conditions; this report extends this finding to include field conditions as well. Most importantly, we present evidence that the epiphytic fitness of these mutants was reduced under field conditions, suggesting that an analysis of their behavior in the laboratory is relevant to their behavior in at least some natural environments.

MATERIALS AND METHODS

Bacterial strains and culture media. The source and characteristics of *P. syringae* pv. syringae B728a and its Tn5 derivatives 14, 22, MX7, and 94 were described previously (8, 37). The source and characteristics of the nonpathogenic epiphytic *P. syringae* Cit7 and TLP2 were also described previously (31, 36). All strains were cultured on King's medium B (25) containing 100 μg of cycloheximide per ml and 50 μg of Benlate per ml (KB) or containing 100 μg of rifampin per ml, 100 μg of cycloheximide per ml, and 50 μg of Benlate per ml (KBR). Bacterial suspensions were prepared by suspending cells grown on KB agar in 10 mM potassium phosphate buffer (PB [pH 7.0]). Bacterial cell concentrations in the suspensions were determined by measuring the optical density at 600 nm and relating it to a standard curve of the optical density at 600 nm versus cell concentration.

Plant inoculations in the laboratory. Plant inoculations were performed as described previously (34). Bacterial suspensions of various concentrations from 10^6 to 10^9 cells of a given strain per ml were applied to five pots of bean plants (*Phaseolus vulgaris* cv. Bush Blue Lake 274), with each pot containing about 10 plants. The bacteria were applied by immersing plants in the bacterial suspension for about 3 s. The pots were immediately placed in a growth chamber maintained at 18°C and 45% relative humidity with 12 h of light (10,000 ft-c [107,600 lx]). One primary leaf was sampled from each pot at various times during the subsequent 4 days. The bacteria were quantified on each individual leaf by viable counts on KBR, as described previously (35).

Field plot design. The field experiments were conducted at the University of California Russell Reservation near Lafayette, Calif. A permit for field testing the recombinant strains was obtained from the U.S. Department of Agriculture Animal and Plant Health Inspection Service. Plots were arranged in a randomized complete block design with nine treatments and four replications. Each plot consisted of approximately 100 bean plants occupying a region 2 m long and 0.25 m wide. The plots were separated by a 1-m unplanted zone to minimize interplot contamination, and the entire experimental area was surrounded by a 2-m unplanted zone. The plants were watered by a drip system to minimize moisture on the leaves and thus to minimize the size of the indigenous epiphytic populations.

Two field experiments were conducted, one from 1 to 8 June 1993 and the other from 2 to 9 August 1993. In the June experiment, the nine treatments consisted of five single-strain inocula (B728a, 14, 22, MX7, and 94) and four dual-strain inocula (14 and B728a, 22 and B728a, MX7 and B728a, and 94 and B728a). In the August experiment, the nine treatments consisted of a PB control, seven single-strain inocula (B728a, 14, 22, MX7, 94, Cit7, and TLP2), and one dual-strain inoculum (MX7 and B728a).

Plant inoculations in the field. The plants were inoculated when the majority had fully expanded primary leaves and newly emerging first trifoliate leaves. The inocula contained 10^8 cells per ml in the June experiment and 10^9 cells per ml in the August experiment. For the coinoculation experiments, bacterial suspensions of each strain, containing 10^8 and 10^9 cells per ml in June and August, respectively, were mixed immediately before plant inoculation. Bacterial suspensions, as well as the PB control, were sprayed onto plants from hand-held spray bottles. To limit aerial dispersal, the plants were inoculated under calm weather conditions at sunrise and the spraying was performed within a moveable box placed over the plants.

Leaves were collected at various times after spraying, with the first leaves taken while they were still visibly wet after spraying. Twelve leaves, three from each replicate plot, were collected for each treatment at each sampling time. Sampling times included morning, midday, and evening on day 1; morning and evening on days 2, 3, and 4; and morning on days 5 and 8. Bacteria on individual leaves were enumerated by plating on the appropriate media, as described previously (35). The background epiphytic populations were determined by enumerating on KB the bacteria in washings from leaves sampled immediately before inoculation. Bacteria on leaves in the PB control treatment were quantified on both KB and KBR. Bacteria in the single-strain treatments were quantified on KBR. Bacteria in the dual-strain treatments were quantified on both KBR and KBR containing 30 μg of kanamycin per ml.

Evaluation of interplot contamination. The absence of interplot contamination was verified by the absence of rifampin-resistant bacteria on the PB-treated leaves, as well as by the appropriate antibiotic resistance markers in selected isolates from washings of the single-strain-inoculated leaves. The presence of the expected mutant in each plot at 3 days after inoculation was verified by extracting chromosomal DNA from selected isolates, digesting it with *Eco*RI, probing with a labeled plasmid containing Tn5 as described previously (8), and comparing the sizes of the hybridizing fragments with the size of the fragment that hybridized in each mutant.

Statistical methods. Estimates of bacterial populations were log-transformed to achieve normality. On the basis of the lognormal distribution of the epiphytic populations (18), a maximum-likelihood procedure (17, 46) was used to obtain estimated means for data sets that included leaves harboring population sizes below the limit of detection. The limit of detection was approximately 365 CFU/g (fresh weight) in the laboratory experiment and the June field experiment and, depending on the sampling time, 90 or 365 CFU/g (fresh weight) in the August field experiment.

The mean population sizes among the treatments at each sampling time were compared by using Fisher's unprotected least-significant-difference test. Changes in the population size of the strains over time were compared by using contrasts based on the pooled standard error derived from a repeated measures analysis.

RESULTS

Survival immediately after inoculation in the laboratory. In previous experiments, four Tn5 mutants of *P. syringae* pv. syringae B728a that were reduced in their epiphytic fitness were identified (8). In those experiments, the ability of the strains to survive on leaves exposed to dry, high-light conditions was examined only after the strains had first colonized the leaves under moist conditions. To evaluate their behavior under conditions more closely resembling those encountered in a field release experiment, survival experiments were performed in the absence of prior leaf colonization (Fig. 1). Several inoculum concentrations were used, since Wilson and Lindow (55) found that inoculum density influenced the survival of a *P. syringae* strain following exposure of plants to stressful conditions. The behavior of the strains immediately
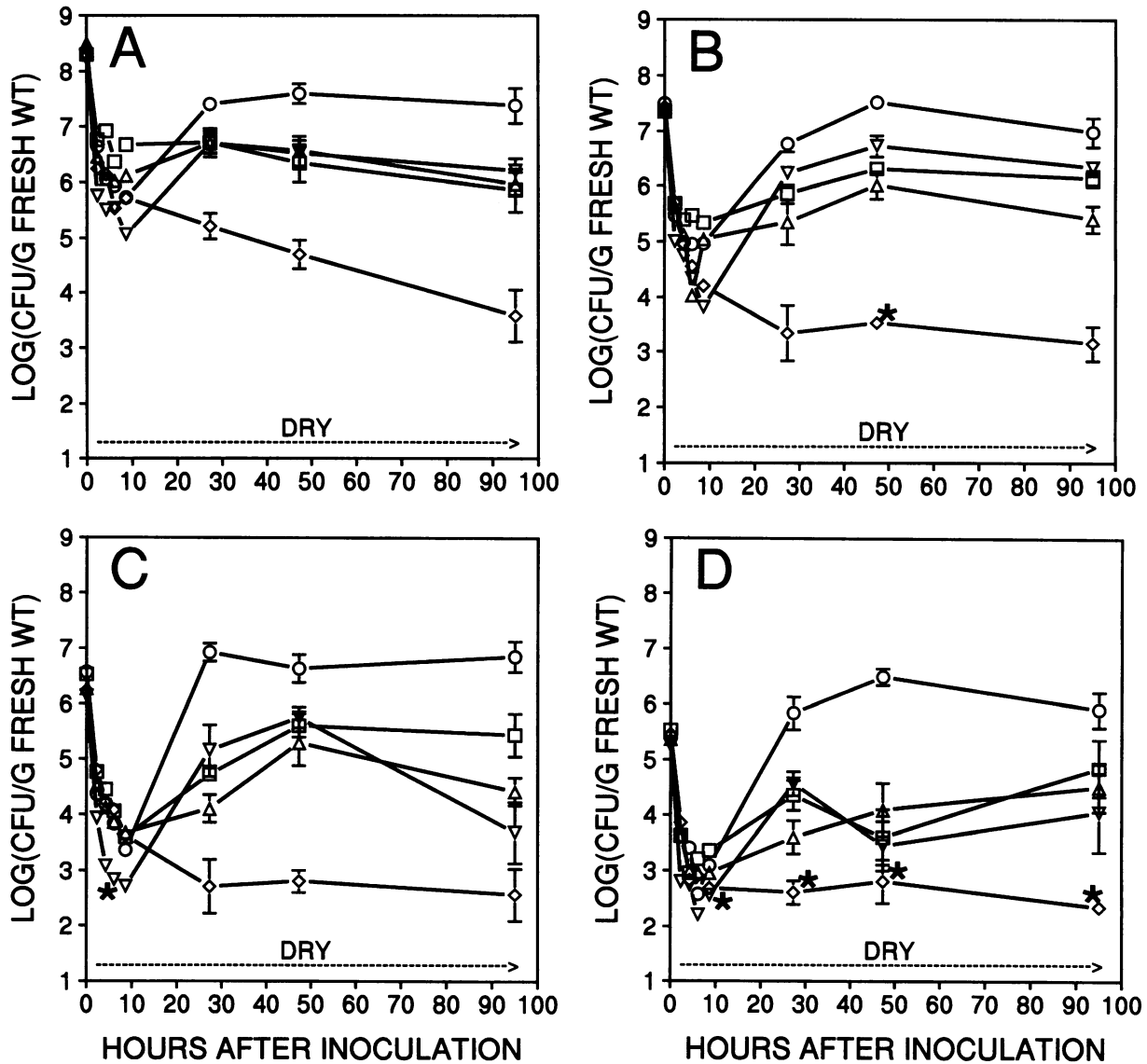
FIG. 1. Population dynamics of *P. syringae* pv. syringae B728a (○) and Tn5 mutant derivatives 14 (▽), 22 (△), MX7 (□), and 94 (◇) on bean plants incubated under conditions of low moisture (45% RH) and high light (10,000 ft-c) after inoculation with cell suspensions containing 10^9 cells per ml (A), 10^8 cells per ml (B), 10^7 cells per ml (C), and 10^6 cells per ml (D). Each point represents the mean ± standard error of the mean (SE) of five leaf samples. Error bars were omitted for clarity for means measured before 10 h. Because means could not be estimated for data sets with 10 or more leaves harboring undetectable populations, means for these data sets (∗) were calculated by using log(CFU per gram [fresh weight]) values of the limit of detection (225 CFU/g [fresh weight]).

following inoculation will be discussed separately from their behavior during an extended incubation on dry leaves.

In the 4 h following plant inoculation in two independent experiments, the half-life of the wild-type strain B728a was 30 to 33 min (Table 1). During this time, the population size of B728a typically decreased about 200- to 400-fold. This decrease was much larger than the 4- to 50-fold decrease in population size that was observed when leaves containing established populations of B728a were exposed to the dry, high-light conditions (8). On the basis of the results of a study by Wilson and Lindow (53) these decreases in the measured population size reflected decreases in the number of viable cells rather than decreases in the culturability of those cells. There was no evidence that the inoculum concentration influenced the survival of B728a (Fig. 1; Table 1).

Under these conditions, only mutant 14 consistently experienced both a shorter half-life and a larger proportional population decrease than did B728a at all inoculum concentrations (Fig. 1; Table 1). As early as 2 h after inoculation, the population size of mutant 14 was significantly smaller than that of B728a ($P < 0.05$), and it generally remained significantly smaller for 4 to 6 h. The half-lives and population decreases of mutants 22, 94, and MX7 were generally similar to those of B728a, although the variability among experiments and among the various inoculum densities were high.

Epiphytic growth during extended incubation on dry leaves in the laboratory. Mutants 14, 22, and MX7 were able to grow on dry leaves but to different extents (Fig. 1). Estimated doubling times for the strains are shown in Table 2; these times were based on the few samplings taken during the primary

TABLE 1. Half-lives and proportional population decreases of strain B728a and several Tn5 derivatives after application at various inoculum concentrations onto plants incubated under low-humidity, high-light conditions

Expt[a]	Inoculum concn (cells/ml)	Half-life (0–4 h) of[b]:					Population decrease (0–8 h) of[c]:				
		B728a	14	22	94	MX7	B728a	14	22	94	MX7
1	10^9	32	31	43	ND[d]	34	195	646	93	ND	316
2	10^9	33	27	30	34	53	427	1,514	245	380	44
	10^8	30	29	34	30	38	347	3,162	204	1,738	105
	10^7	31	25	35	37	36	1,585	2,692	407	417	851
	10^6	33	30	32	29	29	204	1,097	257	589	371

[a] Experiments were performed independently.

[b] Half-life (minutes) = $-\log(2)/m$ where m is the slope of the equation from the regression of the log(CFU per gram [fresh weight]) on time, expressed in minutes.

[c] Proportional population decrease = (CFU per gram [fresh weight] at 0 h)/(CFU per gram [fresh weight] at 8 h).

[d] NT, not tested.

period of growth. Mutant 14 grew with doubling times comparable to those of B728a at all inoculum levels, while mutants 22 and MX7 grew much more slowly. The exceptionally slow growth of MX7 after inoculation with 10^9 cells per ml may have been due to its population being at the carrying capacity of the leaf for that strain at 8 h. In contrast to the other mutants, strain 94 did not show evidence of growth after its initial population decline.

As in previous experiments, B728a established and maintained large populations, approximately 10^7 CFU/g (fresh weight), on dry leaves (Fig. 1; Table 2). With all of the strains, lower inoculum concentrations generally resulted in smaller equilibrium populations after the initial population decline (Table 2). Mutants 14, 22, and MX7 maintained populations on dry leaves that were typically 5- to 30-fold smaller than those of B728a at inoculum densities of 10^9 and 10^8 cells per ml but were as much as 1,400-fold lower at densities of 10^7 or 10^6 cells per ml (Fig. 1). Although apparently incapable of epiphytic growth, mutant 94 maintained a relatively small population size through the course of the experiment.

Survival immediately after inoculation under field conditions. The background epiphytic populations, measured immediately preceding inoculation, were larger in June than in August, perhaps because of the cooler and moister weather conditions (Table 3). The log(CFU per gram [fresh weight]) for uninoculated leaves was 5.67 ± 0.11 (mean ± standard error of the mean [SE]) in June and 3.48 ± 0.11 in August.

Very little interplot contamination of inoculated strains was detected in the field experiments in either June or August.

After spray application of strains B728a, 14, 22, MX7, and 94 onto bean leaves in June, the population size of all of the strains immediately decreased from approximately 10^6 cells per g (fresh weight) to less than 400 cells per g (fresh weight) by 11 h (Fig. 2). The ambient air reached a maximum temperature of 20°C and a minimum relative humidity (RH) of 48% during this time (Table 3). Although these temperature and RH extremes were similar to the constant temperature and RH level in the laboratory experiment (18°C and 45% RH), B728a experienced a 55-fold-larger population decrease in the field than in the laboratory after starting at similar population sizes. Of the mutants, only strain 14 was significantly reduced in its ability to survive in the 24 h following inoculation ($P<0.001$). Whereas mutant 14 experienced a 9-fold-larger population decrease than did B728a in the laboratory, it experienced a 45-fold-larger population decrease under the field conditions in June, indicating that its fitness was reduced more in the field than in the laboratory.

The weather conditions on the day of inoculation in August were even less favorable for bacterial survival than were those in June; the ambient air reached a maximum temperature of 36°C with less than 10% RH (Table 3). In the 7 h following inoculation, the population size of B728a decreased at least ninefold more in August than it did in June. Furthermore, the rate of the population decrease was much higher (Fig. 3); the half-life of B728a was 25 min in June and 18 min in August (between 0 and 4 or 5 h). The size of the epiphytic populations on the PB-treated leaves also decreased on this day (Fig. 3B). Immediately following inoculation in August, the size of the population decrease was significantly larger for mutants 14 and 22 ($P < 0.001$) and MX7 ($P < 0.05$) than for B728a (Fig. 3A). The population sizes of strains 14, 22, MX7, and 94 decreased 449-, 416-, 11-, and 7-fold more, respectively, than did that of B728a. The population sizes of two nonpathogenic epiphytes, Cit7 and TLP2, decreased 10- and 14-fold more than did that of B728a, respectively (Fig. 3B). The half-lives, based on the population sizes at 0 and 5 h, were about 12 min for strains 14 and 22; 15 min for strains MX7, 94, Cit7, and TLP2; and about 18 min for B728a. Thus, in August the mutants and two nonpathogenic epiphytes all exhibited a reduced ability to survive immediately following spray inoculation compared with strain B728a. Furthermore, similar to the June experiment, mutant 14 experienced a much larger population decrease than B728a in the field in August (400-fold) than it did in the laboratory (4-fold) with a comparable inoculum concentration (Table 1).

TABLE 2. Doubling times and final population sizes of strain B728a and several Tn5 derivatives after application at various inoculum concentrations onto plants incubated under low-humidity, high-light conditions

Expt[a]	Inoculum concn (cells/ml)	Estimated doubling time of[b]:				Final population size of[c]:				
		B728a	14	22	MX7	B728a	14	22	MX7	94
1	10^9	4.3	5.8	57.6	92.1	3×10^7	5×10^6	8×10^5	3×10^6	NT[d]
2	10^9	3.4	3.4	9.7	140.7	2×10^7a	2×10^6a	9×10^5a	7×10^5a	4×10^3a
	10^8	3.1	2.3	12.0^e	11.0	1×10^7a	2×10^6a	3×10^5a	1×10^6a	1×10^3a
	10^7	1.6	2.3	7.2^e	5.0	7×10^6a	5×10^3b	3×10^4b	3×10^5a	4×10^2a
	10^6	2.0	2.8	8.8	5.7	8×10^5b	1×10^4b	3×10^4b	7×10^4a	2×10^2a

[a] Experiments were performed independently.

[b] Doubling times (hours) were calculated from growth between 8 and 23 h after inoculation for experiment 1 and between 8 and 27 h for experiment 2, except as indicated.

[c] Population size (CFU per gram [fresh weight]) at 46 h in experiment 1 and 95 h in experiment 2. In each column, values followed by the same letter do not differ significantly at $P = 0.05$ when compared by Fischer's least-significant-difference test on the log(CFU per gram [fresh weight]) values.

[d] NT, not tested.

[e] Doubling times were calculated from the growth between 8 and 47 h after inoculation.

TABLE 3. Weather data during the June and August field experiments

Expt	Conditions during daytime					Conditions during nighttime				
	Time (h) p.i.[a]	Max temp (°C)	Min RH (%)	Avg wind speed (km/h)	Rain (CM)	Time (h) p.i.[a]	Min temp (°C)	Max RH (%)	Avg wind speed (km/h)	Rain (CM)
June	0–12	20	48	ND[b]	0	12–24	10	94	ND	0
	24–36	20	60	ND	0	36–48	11	89	ND	0
	48–60	19	46	5.4	0.13	60–72	12	90	1.9	0
	72–84	16	78	4.8	0.58	84–96	9	94	3.5	0
	96–108	16	62	8.9	0	108–120	11	95	3.1	0
	120–132	16	76	11.7	0	132–144	11	95	5.4	0
	144–156	21	55	3.7	0	156–168	12	94	1.7	0
	168–180	24	48	2.4	0					
Aug	1–11	36	0	0.2	0	11–25	13	40	0.0	0
	25–37	31	1	1.5	0	37–49	14	45	3.3	0
	49–61	22	18	6.1	0	61–73	15	46	1.7	0
	73–85	25	17	2.0	0	85–97	12	56	1.9	0
	97–109	25	21	2.2	0	109–121	13	72	4.4	0
	121–133	22	35	5.0	0	133–145	12	62	3.3	0
	145–157	24	20	2.2	0	157–169	12	58	1.3	0

[a] p.i., postinoculation.
[b] ND, not determined.

Epiphytic growth under field conditions. B728a was able to establish and maintain large epiphytic populations on bean leaves in both the June and August field experiments. In June, the population size of B728a decreased for about 11 h and then increased slightly between 12 and 24 h (Fig. 2), possibly because of the moist conditions that occurred at night (Table 3). On day 2, its population size decreased during the morning, i.e., 25 to 30 h after inoculation, and then rapidly increased,

FIG. 2. Population dynamics of *P. syringae* pv. syringae B728a (O) and Tn5 mutant derivatives 14 (▽), 22 (△), MX7 (□), and 94 (◇) on bean plants under field conditions in June. Each point represents the mean + SE of 12 leaf samples. Periods of daylight are indicated by dashed lines. Because means could not be estimated for data sets with 10 or more leaves harboring undetectable populations, means for these data sets (*) were calculated by using log(CFU per gram [fresh weight]) values of zero for leaves with no detectable population.

resulting in a net increase for the day. The collection of only morning and evening samples on days 3, 4, and 8 may have precluded detection of similar population dynamics on those days. The exceptionally large population increase on day 4 (72 to 84 h after inoculation) may have been triggered by rain (19), which occurred four times during the experiment, at approximately 59, 76, 80, and 85 h after inoculation (Table 3). B728a established and maintained a population size of about 5×10^5 cells per g (fresh weight), which was 20-fold smaller than that established in the laboratory with a comparable inoculum concentration (Table 2). The population dynamics of B728a during the August experiment followed the same general pattern (Fig. 3). In both experiments, B728a grew rapidly, with doubling times of 3.8 h in June (between 30 and 59 h) and 3.3 h in August (between 24 and 35 h).

Although reduced in its initial survival, mutant 14 was capable of establishing and maintaining large epiphytic populations. It grew on leaves with doubling times comparable to those of B728a in both field experiments and established the largest population sizes among the mutants. In both June and August, the population size of mutant 14 was significantly smaller (about 100-fold) than that of B728a 8 days after inoculation ($P < 0.05$), compared with only 5- to 15-fold in the laboratory at comparable inoculum concentrations (Table 2). Interestingly, whereas B728a exhibited fairly consistent growth after 24 h, strain 14 exhibited large fluctuations in its population size on days 3 and 4 in both experiments (Fig. 2 and 3). These changes did not correlate well with known environmental parameters, namely rain, temperature, humidity, or solar radiation (Table 3). Mutants 22 and MX7 established populations significantly smaller than those of B728a ($P < 0.05$), and their ability to maintain those populations was variable. In June, their population sizes decreased rapidly on days 4 and 5, possibly because of strong winds (5 to 20 km/h) during the periods 80 to 86 h and 97 to 110 h after inoculation, but in August they remained fairly constant from days 2 to 8. While their epiphytic population sizes were only 8- to 40-fold smaller than those of B728a in the laboratory after inoculation with 10^8 or 10^9 cells per ml (Table 2), they were 40- to 30,000-fold
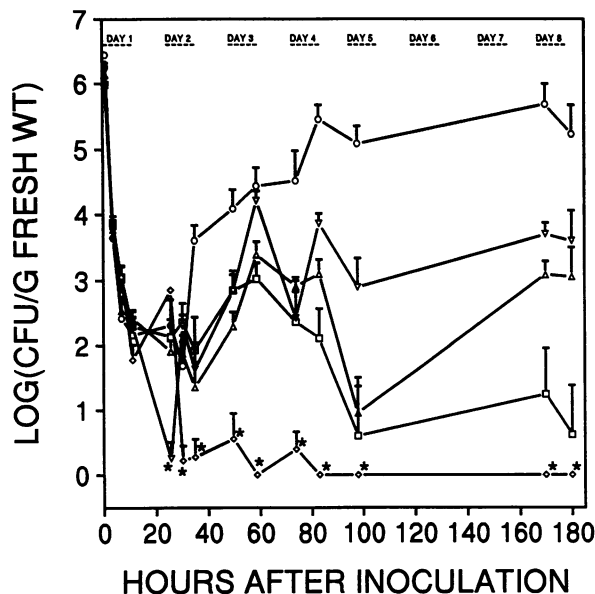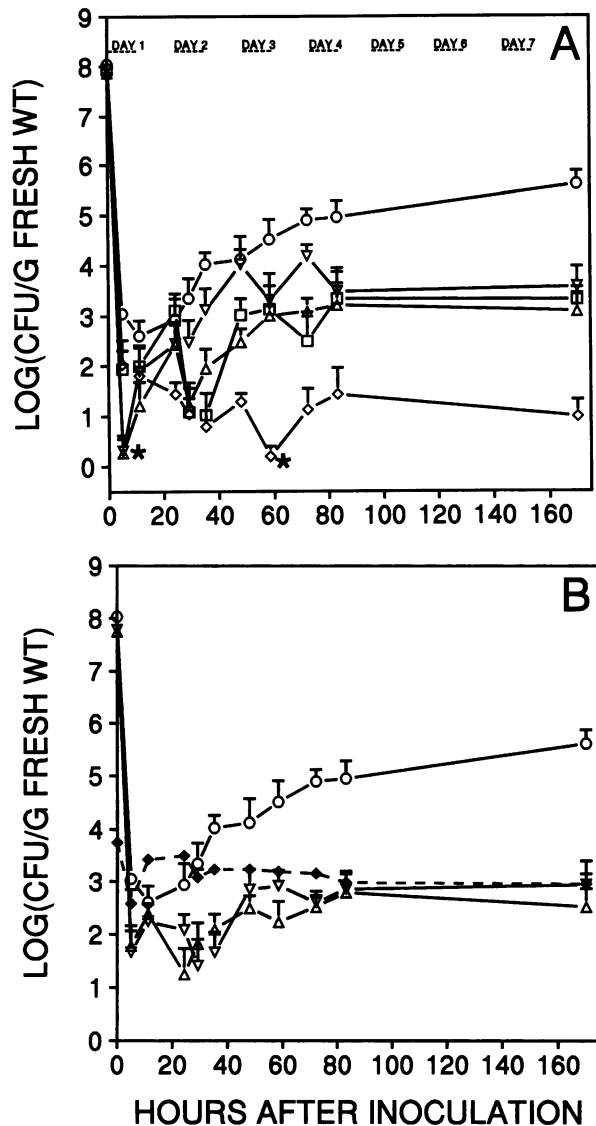
FIG. 3. Population dynamics of *P. syringae* pv. syringae B728a (○) and either Tn5 mutant derivatives 14 (▽), 22 (△), MX7 (□), and 94 (◇) (A) or strains Cit7 (△), TLP2 (▽), and total bacterial population (◆) (B) on bean plants under field conditions in August. Other symbols are the same as those described in the legend to Fig. 2.

smaller in the field studies. Mutant 94 grew poorly, if at all, in both June and August (Fig. 2 and 3).

Effect of coinoculation on survival and growth under field conditions. The behavior of the mutants after they were inoculated in mixtures with the parental strain B728a onto bean plants was strikingly similar to their behavior after they were inoculated alone (Fig. 4). After inoculation in approximately a 1:1 ratio with B728a, each mutant made up far less than 50% of the recoverable population after only 5 h, confirming that each mutant was reduced in epiphytic fitness under these conditions. Mutant 14 consistently made up only 1 to 10% of the total population. Mutants 22 and MX7 made up about 10% of the total population at 30 h after inoculation, 0.1 to 1% at 80 h, and less than 0.01% by 180 h. Mutant 94 made up less than 0.0001% of the total population by 180 h.

The presence of B728a did not appear to influence the

fitness of mutants 14, 22, and 94 (Fig. 4). Although at two or three sampling times the population sizes of mutants 14, 22, and 94 in the mixtures differed significantly from their populations on single-strain-inoculated leaves, no consistent trends were observed. In contrast, in both June and August, the population sizes of MX7 on dual-strain-inoculated leaves were consistently larger than those on single-strain-inoculated leaves after 60 h (Fig. 4 and 5); these differences were significant at 83 and 98 h after inoculation in June and at 29, 35, and 72 h in August ($P < 0.05$).

DISCUSSION

Epiphytic microbial populations must have adaptations that allow them to withstand rapid changes in the leaf surface environment, such as changes in temperature, water availability, and intensity of UV radiation. To begin to understand these adaptations, we identified four mutants of a *P. syringae* pv. syringae strain that were strongly reduced in their epiphytic fitness under laboratory conditions (8). In the present study, we found that the mutants were reduced in their fitness under field conditions as well. Furthermore, their fitness was reduced even more in the field than in the laboratory, as indicated by the larger differences in epiphytic survival and population size between the mutants and the parental strain under field conditions compared with controlled conditions. This difference suggests that the bacteria were exposed to harsher environmental stresses in the field. The evaluation of the behavior of the mutants under field conditions distinguishes this study from most studies of bacterial mutants, which do not examine the behavior of mutants under natural conditions, and assures us that the mutations are relevant to the behavior of the strains.

Although the mutants were all reduced in fitness, they exhibited distinctive behaviors. Mutant 14 appeared to be extremely sensitive to the stresses associated with dry leaf surfaces, since it consistently exhibited larger population decreases and shorter half-lives than did B728a immediately following inoculation. Its strong fluctuations in population size in the field studies may have been a result of this extreme sensitivity to changes in its environment. Mutants 22, MX7, and 94 were only slightly more sensitive than B728a to the stresses on dry leaves, since they showed reduced survival following inoculation under particularly warm conditions, i.e., 28°C in the laboratory (8) and 36°C in the August field experiment, but not under the cooler conditions of 18 to 20°C in the laboratory and the June field experiment. The four mutants also differed in their epiphytic growth; most notably, mutant 14 grew epiphytically at wild-type rates whereas mutants 22 and MX7 generally grew at reduced rates and mutant 94 did not grow at all. Differences among the mutants in their plant-associated behavior under controlled conditions are also described in the accompanying report (8). The existence of differences among the mutants provides evidence that multiple bacterial traits are involved in epiphytic fitness.

The presence of B728a did not influence the survival or growth of mutants 14, 22, or 94 under field conditions, indicating that their reduced fitness was not caused by a deficiency in the production of an extracellular factor in planta. These results are in complete agreement with those in the laboratory (8). However, in both the June and August field experiments, MX7 established larger populations in the presence of B728a than in its absence. The simplest explanation for this population increase is that MX7, a methionine auxotroph (1), was limited for methionine on leaves under field conditions and B728a alleviated this limitation by supplying methi-
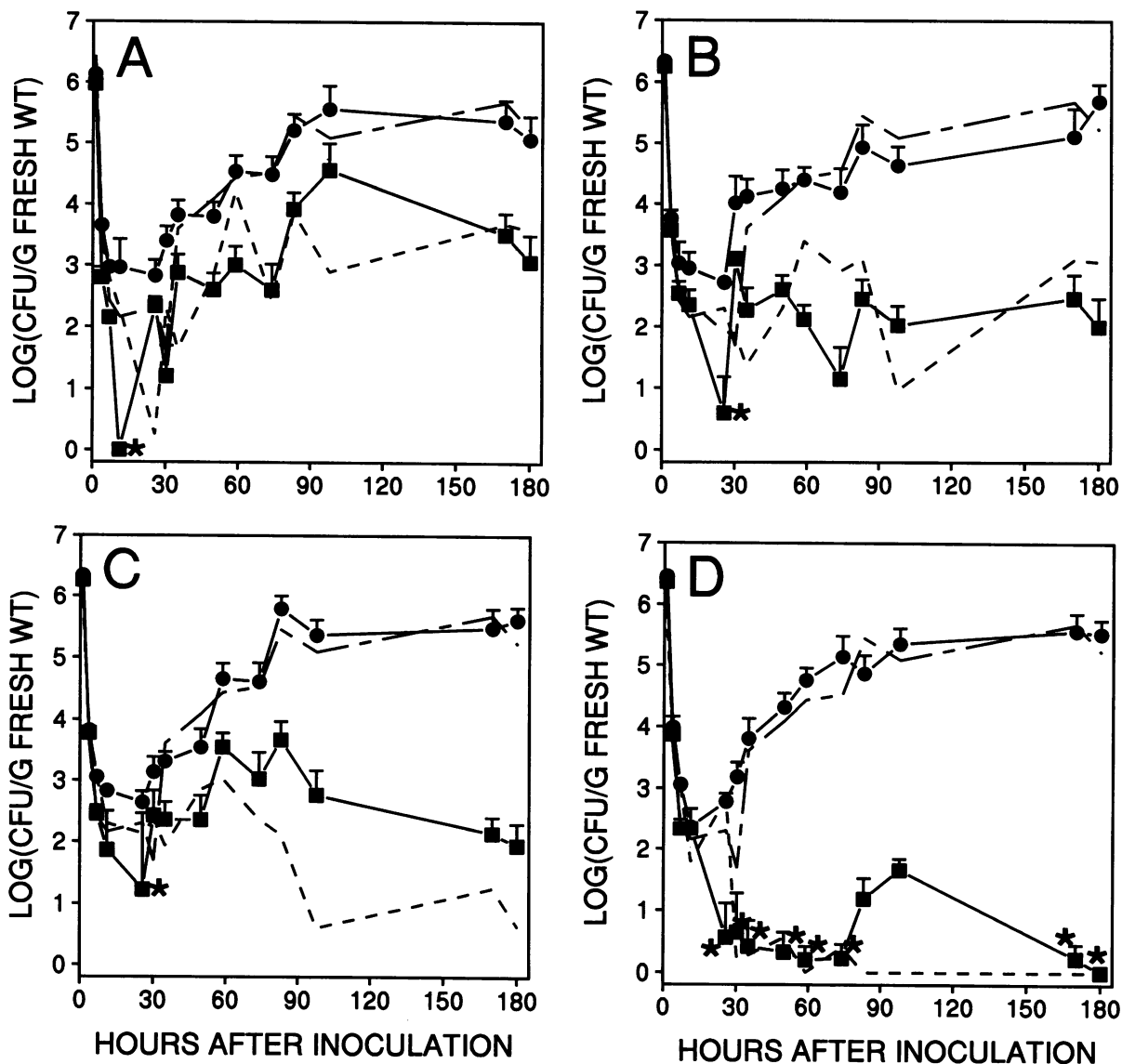
FIG. 4. Population dynamics of *P. syringae* pv. syringae B728a (●) and Tn*5* mutant derivatives (■) 14 (A), 22 (B), MX7 (C), 94 (D) on bean plants under field conditions in June. Population sizes were estimated after B728a was applied alone (- — -), after each mutant strain was applied alone (– – –), or after B728a and a mutant strain were applied in a mixture (————). Each point represents the mean + SE of 12 leaf samples. Other symbols are the same as those described in the legend to Fig. 2.

onine. Such cross-feeding among bacteria in planta has been observed with a *P. syringae* pv. phaseolicola tryptophan auxotroph and a tryptophan-overproducing mutant in bean leaves (38). Under laboratory conditions, application of exogenous methionine caused slight increases in the population sizes on dry leaf surfaces and in the leaf intercellular spaces (1), suggesting that methionine may be limited in and on leaves under these conditions.

The population sizes established on dry leaves under laboratory conditions after inoculation with 10^8 or 10^9 cells per ml were consistently larger than those established from lower-density inocula. This relationship has been observed previously on dry leaves (55) and in the intercellular spaces of leaves (12, 16, 27, 50, 58). It is unlikely that the final population sizes were limited by bacterial growth cessation resulting from a plant response, since the time of growth cessation varied with the

inoculum concentration rather than occurring simultaneously, as with a plant response to the presence of the bacteria. It is more likely that bacterial growth cessation resulted from nutrient exhaustion, and the dependence of the final population size on inoculum density reflected differences in nutrient availability, such as would result if nutrients were localized in discrete compartments. Interestingly, inoculum concentrations of B728a larger than about 10^7 cells per ml did not result in significantly larger final population sizes, whereas about 10^8 cells of mutants 14 and 22 per ml were required to achieve maximum final population sizes. These mutants were therefore either distributed less uniformly across the leaf surface, thus having access to fewer "nutrient compartments," or required more cells to colonize a given microsite. A nonuniform distribution could result from flocculation in culture; however, no evidence for flocculation of either of the strains was found. The
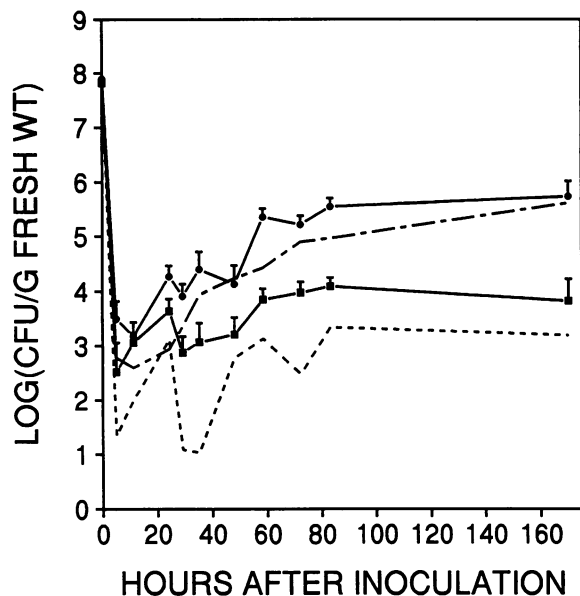
FIG. 5. Population dynamics of *P. syringae* pv. syringae B728a (●) and Tn*5* mutant derivative MX7 (■) on bean plants under field conditions in August. Other symbols are the same as those described in the legend to Fig. 4.

need for a minimum number of cells to colonize a microsite implies the involvement of cooperatively acting extracellular factors, such as for modifying the environment; however, as described below, these strains do not appear to be deficient in the production of such factors. Further studies are thus required to understand the basis for this observation.

Whereas the survival of *P. syringae* MF714R on leaves following exposure to dry, high-light conditions depended on the cell density in the inoculum (55), the survival of B728a did not; thus, density-dependent epiphytic survival is strain specific. This density dependence probably results from the accumulation in the inoculum of extracellular factors, such as extracellular polysaccharides (33, 55) or autoinducing compounds (51), that can act in a cooperative manner to increase epiphytic survival under dry conditions. Our results indicate that B728a does not produce such extracellular factors in a laboratory medium, but the possibility remains that it produces them in planta.

Established epiphytic populations of B728a survived exposure of leaves to stressful conditions better than did newly applied inoculum. Timmer et al. (49) made a similar observation with *Xanthomonas campestris* pv. vesicatoria on tomato leaves. There are several possible explanations for the superior survival of established epiphytic populations. First, during epiphytic colonization bacteria may adapt to the leaf surface, as demonstrated by Wilson and Lindow (54), by inducing traits in response to specific signals in the leaf surface environment. Second, bacteria may modify the leaf surface environment to make it more conducive for survival, such as by altering the pH or the concentration of available nutrients (4). If environmental modification is involved in the fitness of B728a, a deficiency in this modification ability is probably not responsible for the reduced fitness of mutants 14, 22, and 94, since the presence of B728a during epiphytic colonization did not influence the behavior of these mutants. Finally, during epiphytic colonization, bacteria may localize in microsites that are protected from exposure to harsh environmental conditions. Several

studies suggest that epiphytes survive in protected sites during leaf exposure to UV radiation or low moisture (6, 41, 48, 49); furthermore, a reduced ability to establish or maintain populations in such sites appears to be one factor contributing to the reduced survival of mutants 14, 22, MX7, and 94 under laboratory conditions (8).

The mutants retained pathogenicity functions, since they induced lesions after infiltration into bean leaves and induced lesions in the field experiment (data not shown); however, their virulence was severely attenuated (8). Their behavior under laboratory conditions (8) was quite similar to the reported behavior of several weakly pathogenic and nonpathogenic epiphytes. All were poor at surviving on dry leaf surfaces, at growing in the intercellular spaces of leaves, and at localizing in protected microsites (56, 57). In this study, we found that in a direct comparison, the behavior of two nonpathogenic epiphytic *P. syringae* strains, Cit7 and TLP2, was very similar to that of the mutants under field conditions. Cit7 and TLP2 both exhibited half-lives and population decreases that were almost identical to those of MX7 and 94 and established populations that were similar in size to those of mutants 14, 22, and MX7. The strong similarities in behavior between mutants 14, 22, and MX7 and these naturally occurring strains support the hypothesis that the traits that were inactivated in our mutants may be similar to traits that are absent or poorly expressed in naturally occurring weakly pathogenic or nonpathogenic epiphytes (8).

Understanding the genetic and physiological characteristics that influence the ecology of microorganisms requires studies performed under natural conditions; however, studies involving bacterial mutants are performed almost exclusively in the laboratory. This work demonstrates that four *P. syringae* mutants identified as epiphytic fitness mutants in the laboratory were reduced in their epiphytic fitness in a "real-world" field environment as well. These results are critical to interpreting the results of laboratory studies with these mutants.

REFERENCES

1. **Andersen, G. L.** 1993. Molecular characterization of an epiphytic fitness locus in *Pseudomonas syringae* pv. *syringae*. Ph.D. thesis. University of California, Berkeley.
2. **Arlat, M., P. Barberis, A. Trigalet, and C. Boucher.** 1990. Organization and expression of *hrp* genes in *Pseudomonas solanacearum*, p. 419–424. *In* Z. Klement (ed.), Plant pathogenic bacteria. Akadémiai Kiadó, Budapest.
3. **Armstrong, J. L., G. R. Knudsen, and R. J. Seidler.** 1987. Microcosm methods to assess survival of recombinant bacteria associated with plants and herbivorous insects. Curr. Microbiol. **15:**229–232.
4. **Atkinson, M. M., and C. J. Baker.** 1987. Association of host plasma membrane K^+/H^+ exchange with multiplication of *Pseudomonas syringae* pv. *syringae* in *Phaseolus vulgaris*. Phytopathology **77:**1273–1279.
5. **Bainton, N. J., B. W. Bycroft, S. R. Chhabra, P. Stead, L. Gledhill, P. J. Hill, C. E. D. Rees, M. K. Winson, G. P. C. Salmond, G. S. A. B. Stewart, and P. Williams.** 1992. A general role for the *lux* autoinducer in bacterial cell signalling: control of antibiotic biosynthesis in *Erwinia*. Gene **116:**87–91.
6. **Barnes, E. H.** 1965. Bacteria on leaf surfaces and in intercellular

leaf spaces. Science **147**:1151–1152.

7. **Beattie, G. A., and S. E. Lindow.** Epiphytic fitness of phytopathogenic bacteria: physiological adaptations for growth and survival. *In* J. L. Dangl (ed.), Molecular and cellular mechanisms in bacterial pathogenesis of plants and animals, in press. Springer-Verlag, New York.

8. **Beattie, G. A., and S. E. Lindow.** 1994. Survival, growth, and localization of epiphytic fitness mutants of *Pseudomonas syringae* on leaves. Appl. Environ. Microbiol. **60**:3790–3798.

9. **Beaulieu, C., and F. van Gijsegem.** 1990. Identification of plant-inducible genes in *Erwinia chrysanthemi* 3937. J. Bacteriol. **172**: 1569–1575.

10. **Cirvilleri, G., and S. E. Lindow.** 1994. Differential expression of genes of *Pseudomonas syringae* on leaves and in culture evaluated with random genomic *lux* fusions. Mol. Ecol. **3**:249–257.

11. **Clarke, H. R., J. A. Leigh, and C. J. Douglas.** 1992. Molecular signals in the interactions between plants and microbes. Cell **71**:191–199.

12. **Daub, M. E., and D. J. Hagedorn.** 1980. Growth kinetics and interactions of *Pseudomonas syringae* with susceptible and resistant bean tissues. Phytopathology **70**:429–436.

13. **Denarie, J., and J. Cullimore.** 1993. Lipo-oligosaccharide nodulation factors: a new class of signaling molecules mediating recognition and morphogenesis. Cell **74**:951–954.

14. **Eberhard, A., A. L. Burlingame, C. Eberhard, G. L. Kenyon, K. H. Nealson, and N. J. Oppenheimer.** 1981. Structural identification of autoinducer of *Photobacterium fischeri* luciferase. J. Biochem. **20**:2444–2449.

15. **Elliot, E. T., H. W. Hunt, D. E. Walter, and J. C. Moore.** 1986. Microcosms, mesocosms and ecosystems: linking the laboratory to the field, p. 472–480. *In* F. Megusar and M. Gantar (ed.), Perspectives in microbial ecology. Slovene Society for Microbiology, Ljubljana, Slovenia.

16. **Ercolani, G. L., and J. E. Crosse.** 1966. The growth of *Pseudomonas phaseolicola* and related plant pathogens *in vivo*. J. Gen. Microbiol. **45**:429–439.

17. **Harter, H. L., and A. H. Moore.** 1966. Iterative maximum-likelihood estimation of the parameters of normal populations from singly and doubly censored samples. Biometrika **53**:205–213.

18. **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.

19. **Hirano, S. S., C. B. Tanner, and C. D. Upper.** 1987. Rain-triggered multiplication of *Pseudomonas syringae* on snap bean leaflets. Phytopathology **77**:1694.

20. **Hirano, S. S., D. K. Willis, and C. D. Upper.** 1992. Population dynamics of a Tn5-induced non-lesion forming mutant of *Pseudomonas syringae* on bean plants in the field. Phytopathology **82**: 1067.

21. **Horinouchi, S., and T. Beppu.** 1990. Autoregulatory factors of secondary metabolism and morphogenesis in actinomycetes. Crit. Rev. Biotechnol. **10**:191–204.

22. **Huang, Y., P. Xu, and L. Sequeira.** 1990. A second cluster of genes that specify pathogenicity and host response in *Pseudomonas solanacearum*. Mol. Plant-Microbe Interact. **3**:48–53.

23. **Kamoun, S., and C. I. Kado.** 1990. A plant-inducible gene of *Xanthomonas campestris* pv. *campestris* encodes an exocellular component required for growth in the host and hypersensitivity on nonhosts. J. Bacteriol. **172**:5169–5172.

24. **Kanemoto, R. H., A. T. Powell, D. E. Akiyoshi, D. A. Regier, R. A. Kerstetter, E. W. Nester, M. C. Hawes, and M. P. Gordon.** 1989. Nucleotide sequence and analysis of the plant-inducible locus *pinF* from *Agrobacterium tumefaciens*. J. Bacteriol. **171**:2506–2512.

25. **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.

26. **Kotoujansky, A.** 1987. Molecular genetics of pathogenesis by soft-rot *Erwinias*. Annu. Rev. Phytopathol. **25**:405–430.

27. **Leben, C., G. C. Daft, and A. F. Schmitthenner.** 1968. Bacterial blight of soybeans: population levels of *Pseudomonas glycinea* in relation to symptom development. Phytopathology **58**:1143–1146.

28. **Lindemann, J., and T. V. Suslow.** 1987. Characteristics relevant to the question of environmental fate of genetically engineered

INA-deletion mutant strains of *Pseudomonas*. Curr. Plant Sci. Biotechnol. Agric. **4**:1005–1012.

29. **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.

30. **Lindgren, P. B., R. Fredrick, A. G. Govindarajan, N. J. Panopoulos, B. J. Staskawicz, and S. E. Lindow.** 1989. An ice nucleation reporter gene system: identification of inducible pathogenicity genes in *Pseudomonas syringae* pv. *phaseolicola*. EMBO J. **8**:1291–1301.

31. **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. 23–35. *In* H. O. Halvorson, D. Pramer, and M. Rogul (ed.), Engineered organisms in the environment. American Society for Microbiology, Washington, D.C.

32. **Lindow, S. E.** 1990. Design and results of field tests of recombinant ice *Pseudomonas syringae* strains, p. 61–69. *In* J. J. Marois and G. Bruening (ed.), Risk assessment in agricultural biotechnology. Division of Agriculture and Natural Resources, University of California, Oakland.

33. **Lindow, S. E.** 1991. Determinants of epiphytic fitness in bacteria, p. 295–314. *In* J. H. Andrews and S. S. Hirano (ed.), Microbial ecology of leaves. Springer-Verlag, New York.

34. **Lindow, S. E.** 1993. Novel method for identifying bacterial mutants with reduced epiphytic fitness. Appl. Environ. Microbiol. **59**:1586–1592.

35. **Lindow, S. E., G. Andersen, and G. A. Beattie.** 1993. Characteristics of insertional mutants of *Pseudomonas syringae* with reduced epiphytic fitness. Appl. Environ. Microbiol. **59**:1593–1601.

36. **Lindow, S. E., and N. J. Panopoulos.** 1988. Field tests of recombinant Ice⁻ *Pseudomonas syringae* for biological frost control in potato, p. 121–138. *In* M. Sussman, C. H. Collins, and F. A. Skinner (ed.), The release of genetically engineered microorganisms. Academic Press, Inc., New York.

37. **Loper, J. E., and S. E. Lindow.** 1987. Lack of evidence for *in situ* fluorescent pigment production by *Pseudomonas syringae* pv. *syringae* on bean leaf surfaces. Phytopathology **77**:1449–1454.

38. **Miller, W., and N. J. Panopoulos.** Personal communication.

39. **Mo, Y. Y., and D. C. Gross.** 1991. Expression *in vitro* and during plant pathogenesis of the *syrB* gene required for syringomycin production by *Pseudomonas syringae* pathovar *syringae*. Mol. Plant-Microbe Interact. **4**:28–36.

40. **Nealson, K. H., T. Platt, and J. W. Hastings.** 1970. Cellular control of the synthesis and activity of the bacterial luminescent system. J. Bacteriol. **104**:313–322.

41. **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.

42. **Osbourn, A. E., C. E. Barber, and M. J. Daniels.** 1987. Identification of plant-induced genes of the bacterial pathogen *Xanthomonas campestris* pathovar *campestris* using a promoter-probe plasmid. EMBO J. **6**:23–28.

43. **Piper, K. R., S. Beck von Bodman, and S. K. Farrand.** 1993. Conjugation factor of *Agrobacterium tumefaciens* regulates Ti plasmid transfer by autoinduction. Nature (London) **362**:448–450.

44. **Rahme, L. G., M. N. Mindrinos, and N. J. Panopoulos.** 1991. Genetic and transcriptional organization of the *hrp* cluster of *Pseudomonas syringae* pv. *phaseolicola*. J. Bacteriol. **173**:575–586.

45. **Reeves, P. J., D. Whitcombe, S. Wharam, M. Gibson, G. Allison, N. Bunce, R. Barallon, P. Douglas, V. Mulholland, S. Stevens, D. Walker, and G. P. C. Salmond.** 1993. Molecular cloning and characterization of 13 *out* genes from *Erwinia carotovora* subspecies *carotovora*: genes encoding members of a general secretion pathway (GSP) widespread in Gram-negative bacteria. Mol. Microbiol. **8**:443–456.

46. **Rouse, D. I., E. V. Nordheim, S. S. Hirano, and C. D. Upper.** 1985. A model relating the probability of foliar disease incidence to the population frequencies of bacterial plant pathogens. Phytopathology **75**:505–509.

47. **Stachel, S. E., E. W. Nester, and P. C. Zambryski.** 1986. A plant cell factor induces *Agrobacterium tumefaciens vir* gene expression.

Proc. Natl. Acad. Sci. USA **83:**379–383.

48. **Sztejnberg, A., and J. P. Blakeman.** 1973. Ultraviolet-induced changes in populations of epiphytic bacteria on beetroot leaves and their effect on germination of *Botrytis cinerea* spores. Physiol. Plant Pathol. **3:**443–451.

49. **Timmer, L. W., J. J. Marois, and D. Achor.** 1987. Growth and survival of Xanthomonads under conditions nonconducive to disease development. Phytopathology **77:**1341–1345.

50. **Turner, J. G., and R. R. Taha.** 1984. Contribution of tabtoxin to the pathogenicity of *Pseudomonas syringae* pv. *tabaci*. Physiol. Plant Pathol. **25:**55–69.

51. **Williams, P., N. J. Bainton, S. Swift, S. R. Chhabra, M. K. Winson, G. S. A. B. Stewart, G. P. C. Salmond, and B. W. Bycroft.** 1992. Small molecule-mediated density-dependent control of gene expression in prokaryotes: bioluminescence and the biosynthesis of carbapenem antibiotics. FEMS Microbiol. Lett. **100:**161–168.

52. **Willis, D. K., J. J. Rich, and E. M. Hrabak.** 1991. *hrp* genes of phytopathogenic bacteria. Mol. Plant-Microbe Interact. **4:**132–138.

53. **Wilson, M., and S. E. Lindow.** 1992. Relationship of total viable and culturable cells in epiphytic populations of *Pseudomonas syringae*. Appl. Environ. Microbiol. **58:**3908–3913.

54. **Wilson, M., and S. E. Lindow.** 1993. Effect of phenotypic plasticity on epiphytic survival and colonization by *Pseudomonas syringae*. Appl. Environ. Microbiol. **59:**410–416.

55. **Wilson, M., and S. E. Lindow.** 1994. Inoculum density-dependent mortality and colonization of the phyllosphere by *Pseudomonas syringae*. Appl. Environ. Microbiol. Submitted for publication.

56. **Wilson, M., S. E. Lindow, and S. S. Hirano.** 1991. The proportion of different phyllosphere bacteria in sites on or within bean leaves protected from surface sterilization. Phytopathology **81:**1222.

57. **Wilson, M., S. E. Lindow, and S. S. Hirano.** Unpublished data.

58. **Young, J. M.** 1974. Development of bacterial populations *in vivo* in relation to plant pathogenicity. N. Z. J. Agric. Res. **17:**105–113.