

Characteristics of Insertional Mutants of *Pseudomonas syringae* with Reduced Epiphytic Fitness

STEVEN E. LINDOW,* GARY ANDERSEN, AND GWYN A. BEATTIE

Department of Plant Pathology, University of California, Berkeley, California 94720

Received 29 September 1992/Accepted 22 February 1993

Random Tn5 mutagenesis was used to identify genes in *Pseudomonas syringae* which contribute to epiphytic fitness. Mutants were selected on the basis of deficiencies in epiphytic growth or survival on plants rather than deficiencies in predetermined phenotypes exhibited in culture. A sample freezing procedure was used to measure the population sizes of 5,300 mutants of *P. syringae* exposed to alternating wet and dry conditions on bean leaves in growth chambers. Eighty-two mutants exhibited reduced population sizes. Of these mutants, over half exhibited a reduced ability to survive the stresses associated with dry leaves, while others grew more slowly or attained reduced stationary-phase population sizes on leaves. While some epiphytic fitness mutants were altered in phenotypes that could be measured in culture, many mutants were not altered in any in vitro phenotype examined. Only three of the epiphytic fitness mutants were auxotrophs, and none had catabolic deficiencies for any of 31 organic compounds tested. Other mutants that exhibited reductions in one or more of the following were identified: motility, osmotolerance, desiccation tolerance, growth rate in batch culture, and extracellular polysaccharide production. All of the mutants retained the abilities to produce disease symptoms on the compatible host plant, bean, to incite a hypersensitive response on the non-host plant, tobacco, and to produce a fluorescent pyoverdine siderophore.

Bacteria are common inhabitants of leaf surfaces, with individual bacterial species often being present at more than 10^7 cells per g of leaf. These epiphytic bacteria can affect the plants on which they live in many ways, for example, by inciting disease or ice formation and altering the plant's growth. Most phytopathogenic bacteria exist as epiphytes on healthy plants prior to infection, and their epiphytic population sizes can be good predictors of foliar disease (15, 36). Several common epiphytic inhabitants of plants, including *Pseudomonas syringae*, *Pseudomonas viridiflava*, *Pseudomonas fluorescens*, *Erwinia herbicola*, and *Xanthomonas campestris* pv. *translucens*, can catalyze ice formation at temperatures warmer than -5°C (17, 22, 26, 29, 30). Thus, these bacterial species can incite frost injury by limiting the supercooling ability of the water in plant tissues (27). The incidence of frost injury to many plants is related directly to the logarithm of the population size of ice nucleation-active (Ice^+) bacteria on the plant (21, 25). Additionally, many epiphytic bacteria can produce the phytohormone 3-indoleacetic acid, which can alter plant growth (23).

The leaf surface is subject to rapid changes in its physical environment such as changes in water availability, temperature, and intensity of UV light and other ionizing and nonionizing radiation. Epiphytes appear to have an excellent ability to survive harsh environmental conditions on leaf surfaces (33). The adaptations that allow them to survive in this habitat may explain, in part, why epiphytic bacteria are usually distinct from the predominant bacteria in nearby habitats such as soil (35, 40, 41).

There has been considerable speculation on the nature of the adaptive characteristics that allow epiphytic bacteria to exploit leaf surfaces for growth. Unfortunately, only a few of the hypothesized phenotypes have been critically evaluated (23). For example, motility was demonstrated to play a role

in epiphytic fitness by behavioral comparisons of isogenic motile and nonmotile mutants of *P. syringae* (12). In contrast, pigmentation was shown to not play a major role in epiphytic survival since nonpigmented mutants of *E. herbicola* did not differ appreciably in their sensitivity to UV irradiation in culture (9). Such detailed evaluations of bacteria with specific phenotypes that influence epiphytic fitness, which we define as the ability to develop and maintain a population of cells on leaves, have been pursued for only a few traits (23), and these traits have been phenotypes that are observable in culture. It seems likely that bacterial cells that are exposed to heterogeneous environments may exhibit substantial phenotypic plasticity. Epiphytes may have a broad range of phenotypes that they can exhibit, and they may express only a subset of their genes in any given habitat. For example, the genes required for growth and survival on plants may differ from those expressed in culture. Extrapolation of bacterial behavior in laboratory cultures to the behavior in natural habitats therefore is unlikely to address all of the pertinent traits utilized by bacteria in these habitats and may overlook roles for novel or unanticipated phenotypes.

We describe in this report the characteristics of randomly generated insertional mutants of *P. syringae* that exhibited decreased abilities to grow or survive on leaves. A similar random mutagenesis approach was used to identify previously undetected genes required for virulence in phytopathogenic bacteria (20) or for nitrogen fixation (7). Direct screening for altered epiphytic fitness was facilitated by estimating the population sizes of mutants on leaves on the basis of measurements of ice nuclei produced by Ice^+ bacterial strains (24). This powerful screening approach allowed us to identify a large number of individual genes in *P. syringae* that contribute to the epiphytic fitness of this species. We report here the characteristics of these epiphytic fitness mutants.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains and culture media. *P. syringae* B728a has been described previously (28). All bacterial strains were stored in duplicate at -80°C in 15% glycerol and in 7.0% dimethyl sulfoxide and were cultured on King's medium B (KB) (18) containing 100 μg of rifampin per ml (KBR). Unless otherwise specified, bacterial suspensions were prepared by suspending plate-grown cells in 10 mM potassium phosphate buffer (PB; pH 7.0). Bacterial cell concentrations were determined by measuring the optical density at 600 nm of suspensions and relating it to a standard curve of the optical density at 600 nm versus cell concentration.

Evaluation of the randomness of Tn5 insertions. Tn5 mutagenesis was performed as described previously (24). Whole bacterial cells were embedded in a 1% mixture of inCert agarose (FMC Biochemicals) (39) to allow for the purification and subsequent enzymatic manipulation of chromosomal DNA without appreciable shearing. The chromosomal DNA was digested with *EcoRI*, *NotI*, *SfiI*, or *XbaI* in a potassium glutamate buffer amended with 0.5 mM spermidine (Sigma) (13), and the resulting fragments were separated by contour-clamped homogeneous electric field (CHEF) gel electrophoresis for 16 h with a 5-s pulse and 8 h with a 3-s pulse (5) and were transferred to a Zetabind (AMF, Meriden, Conn.) nylon membrane (8). Internal 2.3- and 2.4-kb *XhoI* restriction fragments were gel purified from the transposon Tn5 and were labeled with [^{32}P]dCTP by using a random primer extension kit (Multiprime; Amersham). Southern blotting techniques were performed as described by Sambrook et al. (37).

Quantification of ice nuclei and bacteria on plants. Plant inoculations, tube nucleation assays, and quantification of bacteria on leaves were performed as described previously (24).

Phenotypic characterization of mutants. Motility was determined by touching a sterile toothpick to a bacterial colony and then stabbing the toothpick vertically into semisolid tryptone medium by a procedure similar to that described by Haefele and Lindow (12). Plates were incubated at 28°C for 24 h, and the diameter of the disk of visible cells surrounding the inoculation spot was measured.

The osmotic tolerance of bacterial strains was determined by placing 10- μl drops of suspensions containing about 10^8 cells per ml onto the surface of LB medium (32) containing either 0, 0.2, 0.4, 0.6, 0.8, or 1.0 M NaCl. Three replicate plates containing each strain were incubated at 28°C for 48 h, and the presence of cell growth on each medium was scored by visual examination.

Alterations in cell surface characteristics such as extracellular polysaccharide (EPS) production were determined by inoculating bacterial strains onto different culture media and determining the sizes and appearances of the colonies. The tip of a sterile toothpick was immersed in a bacterial suspension containing about 10^9 cells per ml and was then lightly touched onto the surface of nutrient agar medium containing 2% glucose or 2% sucrose or minimal medium A (32) containing 2% glucose or 2% sucrose to produce inoculated zones of less than about 1 mm in diameter. Plates containing amended nutrient agar media were incubated for 3 days at 28°C , and plates containing amended minimal media were incubated for 7 days at 28°C before colony diameters were measured.

The catabolic characteristics of bacterial strains were determined by spotting 10 μl of a suspension of each bacterial strain onto the surface of minimal medium A

containing 0.2% of one of the following carbon sources: L-alanine, betaine, choline chloride, sodium citrate, L-erythritol, fructose, fumaric acid, D-galacturonic acid, glucose, D-glucuronic acid, L-glutamine, glycerol, *p*-hydroxybenzoic acid, D-malic acid, L-malic acid, malonic acid, mannitol, D-mannose, mesotartaric acid, oleic acid, L-proline, D-saccharic acid, L-serine, sodium acetate, D-sorbitol, L-sorbose, succinic acid, sucrose, *trans*-aconitic acid, lauric acid, maltose, or sodium polypectate. The presence of growth on each medium was determined after 7 days of incubation at 28°C . Prototrophy of bacterial mutants was determined as growth of bacterial suspensions on minimal medium A containing 0.2% glucose as a carbon source.

The growth rates of the bacterial strains in KB broth were determined. Cells of 1-day-old cultures were added to KB broth to a final concentration of about 10^7 cells per ml, and growth was followed by measuring the increase in optical density at 600 nm.

The growth of each bacterial mutant relative to that of the parental strain B728a was determined by coinoculating flasks containing KB broth with approximately equal concentrations (10^7 cells per ml) of each strain. The total bacterial population and the population of the mutant were each enumerated at the time of inoculation and when cells reached the stationary phase of growth by plating appropriate dilutions onto both KBR and KBR containing 30 μg of kanamycin per ml. Population estimates of the mutant were determined directly on KBR containing kanamycin, and total populations were determined from counts on KBR. A change in the mutant-to-total population ratio was considered evidence for a difference in competitive ability in culture.

Desiccation stress tolerance was evaluated by placing 20- μl droplets of a bacterial strain (about 10^7 cells per ml) on six replicate disks of Whatman no. 1 filter paper (about 2 cm^2) and immediately placing the disks in a closed chamber in which the relative humidity was maintained at about 75% over a solution of saturated sodium chloride (45). Disks were incubated for 3 h at 21°C . The numbers of viable bacteria immediately and 3 h after inoculation were determined by suspending the filter paper disks in test tubes containing PB, vortexing them vigorously for 30 s, and plating appropriate 10-fold serial dilutions on KBR. Evidence for altered desiccation tolerance was considered to be a significant change in the fraction of cells which succumbed to desiccation stress during the 3 h of incubation in comparison with the fraction of parental strain cells which succumbed to desiccation stress.

The ability to produce a fluorescent pyoverdine siderophore characteristic of *P. syringae* B728a was assessed by viewing individual bacterial colonies, which were grown for 3 days at 24°C on KB, under incident UV light (366 nm). Greenish blue fluorescence was considered evidence for pyoverdine production in these strains.

The ability of bacterial strains to elicit a hypersensitive response in the non-host plant, tobacco (cultivar Turk), was evaluated by infiltrating suspensions containing about 10^6 cells per ml in PB, using a syringe, into the intercellular spaces of tobacco leaves to produce water-congested areas of at least 2 cm^2 for each bacterial strain. Plants were incubated for 24 h on a greenhouse bench. The presence of discolored and collapsed tissue in the infiltrated area was considered evidence of the hypersensitive reaction, as described by Klement (19).

The nucleation frequency of bacterial strains (the fraction of cells capable of producing an ice nucleus) was determined

by a droplet freezing assay similar to that used by O'Brien and Lindow (34). Cells of each strain were grown on KBR for 2 days at 21°C and were suspended in PB to a concentration of about 10^8 cells per ml. Tenfold serial dilutions of these suspensions were then made in PB and 40 10- μ l droplets of each dilution were placed on the surface of paraffin-coated aluminum sheets which were then floated on the surface of a refrigerated alcohol bath maintained at -3 or -5°C. Drops were allowed to stand on this cooling surface for 2 min to allow temperature equilibration, and the number of droplets from each dilution which had frozen was determined by visual examination. The concentration of ice nuclei was calculated from the fraction of drops which had frozen, using the equation of Vali (43). Ice nucleation frequency was calculated by dividing the number of ice nuclei per milliliter by the number of bacterial cells per milliliter as determined from turbidity measurements. In some experiments, appropriate 10-fold serial dilutions of leaf washings were assayed as described above for their content of bacterial ice nuclei.

Statistical methods. Estimates of bacterial population sizes from individual leaves were log transformed to achieve normality. Analysis of variance was performed by using the General Linear Models procedure of SAS (SAS Institute, Cary, N.C.). Comparison of mean responses from different treatments was performed by Fisher's unprotected least significant difference test. This test controls the comparison-wise error rate. Linear regression analysis was performed by the regression procedure of SAS.

We used the chi-square (χ^2) test to determine whether chance distribution could account for the frequency of Tn5 insertions in each of the *Xba*I restriction fragments. The expected number of Tn5 insertions for each restriction fragment (E) was determined by the formula $(rf/2,309) \cdot 62 = E$, where rf is the length of an individual restriction fragment (in kilobases), 2,309 represents the physical length of the chromosome, and 62 represents the total number of Tn5 insertions. The size of the *P. syringae* B728a genome was estimated by summing the sizes of individual *Xba*I restriction fragments that were separated by pulsed-field gel electrophoresis (Table 1). This value was compared with the observed number (O) of Tn5 insertions for each restriction fragment by the formula $\chi^2 = (O - E)^2/E$. The normality of distributions of sizes of restriction fragments containing Tn5 was determined by calculating the Shapiro-Wilk statistic, W , by using the Univariate Procedure of SAS.

RESULTS

Tn5 transposed into the genome of *P. syringae* B728a in an apparently random manner. Southern blot analysis of genomic DNA from 40 mutants demonstrated that Tn5 occurred in a wide range of *Eco*RI, *Sfi*I, *Xba*I, and *Not*I restriction fragments. The number of *Eco*RI restriction fragments of various sizes that contained Tn5 approximated a normal distribution (Fig. 1). Normal distributions of *Sfi*I and *Not*I restriction fragments were also observed (data not shown). About 1.03% of the 5,300 Tn5-induced mutants screened were auxotrophs, and no one auxotrophic deficiency predominated among these mutants.

Of 5,300 Tn5 mutants of *P. syringae* B728a whose population sizes were estimated on leaves by the leaf freezing assay following sequential exposure to wet and dry conditions, 109 mutants caused a smaller fraction of leaves to freeze compared with the fraction the parental strain caused to freeze. While some mutants exhibited lower ice nucleation activity on leaves after a single test, only these 109

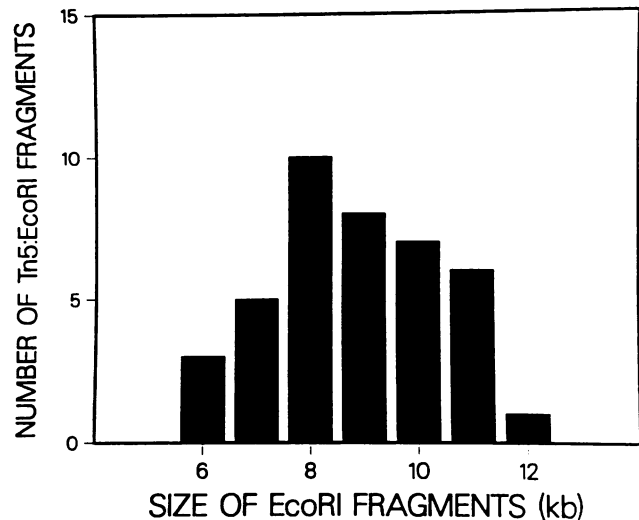


FIG. 1. Distribution of the sizes of *Eco*RI restriction fragments containing Tn5 in genomic DNAs of different mutant derivatives of *P. syringae* B728a. $W = 0.974$; $P < W = 0.57$ (W is the Shapiro-Wilk statistic).

mutants exhibited a lower incidence of nucleation on leaves in at least two of three replicate experiments. These mutants, designated epiphytic fitness mutants, were characterized further, while the former were considered to have false-positive activity because of normal variations in population size.

The pattern of growth and death of parental strain B728a on plants exposed to alternating wet and dry conditions was consistent in different experiments (Fig. 2). When exposed to continuous moisture for 24 h following inoculation, B728a exhibited little if any lag before entering the exponential growth phase on leaves and grew with a generation time of 2 to 4 h. When leaves were allowed to dry, B728a usually exhibited a 10- to 100-fold decrease in viable population size within 8 h (Fig. 2), with the variability occurring even though the plants were exposed to similar ambient air conditions in all experiments. Even though plants remained dry for 24 h, the population sizes of viable cells usually increased during the latter half of this period and then resumed a slightly higher growth rate upon rewetting of the leaves (Fig. 2). The rate of population increase slowed dramatically by 72 h after inoculation, and maximum population sizes of 10^6 to 10^7 cells per g (fresh weight) were attained (Fig. 2).

The 109 epiphytic fitness mutants exhibited a range of differences from the parental strain in their population dynamics on plants exposed to a regimen of alternating wet and dry conditions (Table 1). Nine of the 109 presumptive mutants exhibited stress tolerance on dry leaves similar to that of B728a, but they exhibited a reduced growth rate on leaves in comparison with that of B728a (Fig. 2A). Thirty mutants exhibited a reduction in viable population size during exposure to dry conditions that was two- to eightfold larger than that of the parental strain (Fig. 2C). Of these 30 mutants, 5 also exhibited a reduced growth rate on leaves, while 13 exhibited a slight increase in growth rate on leaves, particularly during moist incubation periods. Fourteen epiphytic fitness mutants exhibited a reduction in viable population size on dry leaves that was much larger (greater than eightfold) than that exhibited by the parental strain. For

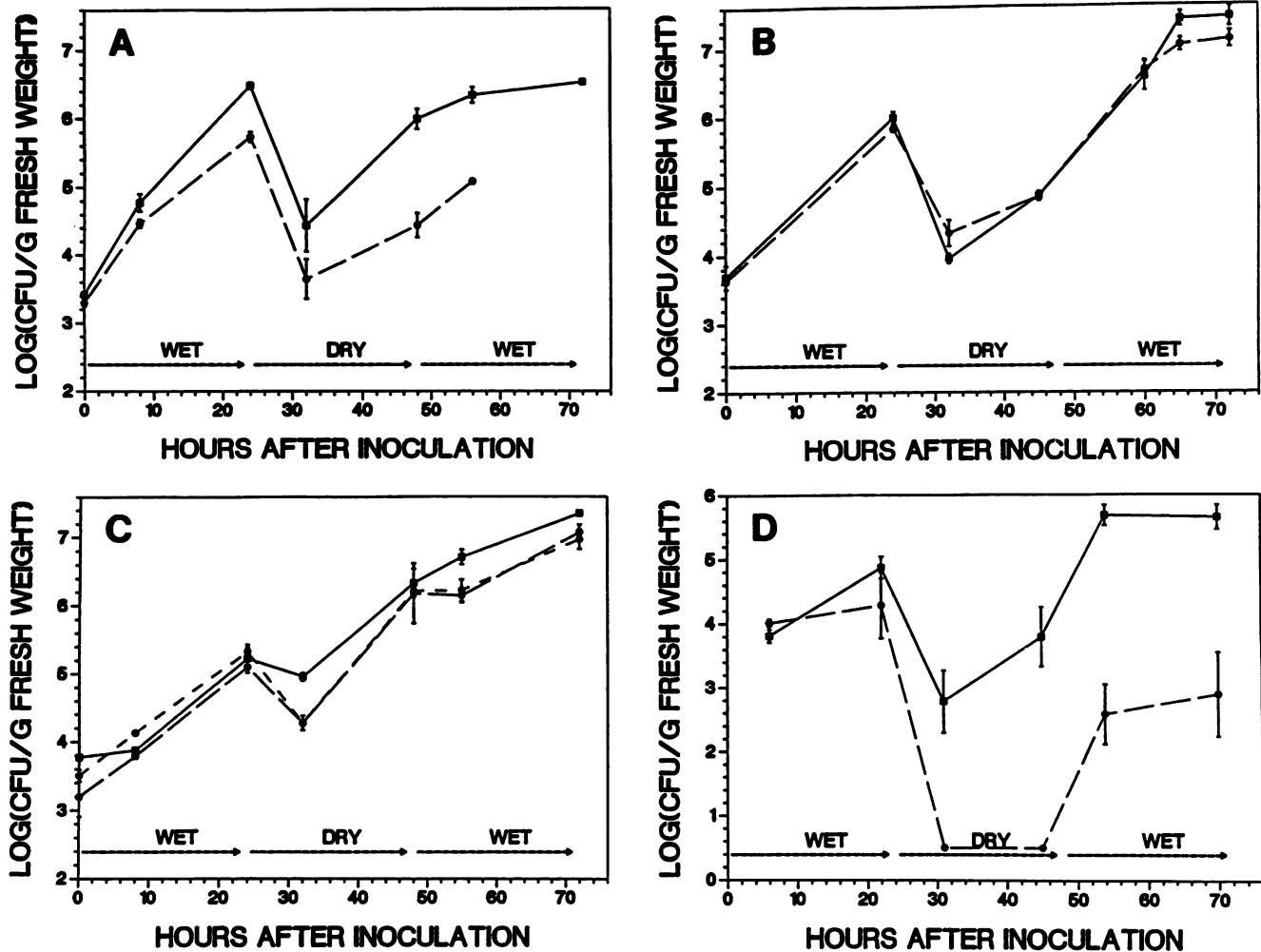


FIG. 2. Population dynamics of *P. syringae* B728a (solid lines) and Tn5 mutant derivatives (dashed lines) 68 (A), 24 (B), 18 (○) and 20 (●) (C), and 42 (D) on bean plants exposed to alternating wet and dry conditions.

example, mutant 42 exhibited up to a 100-fold larger decrease in the number of cells that survived initial dry periods on leaves (Fig. 2D). Of these mutants, six also had a reduced growth rate, while five had an increased growth rate on leaves. In comparison with parental strain B728a, some Tn5 mutants exhibited nearly identical growth rates on leaves during moist incubation periods and similar survival during dry incubation periods but stopped multiplying at a lower population size. For example, mutant 24 achieved a final population size that was sixfold less than that of the parental strain (Fig. 2B).

Although all 109 presumptive epiphytic fitness mutants produced fewer ice nuclei on leaves after exposure to alternating moist and dry conditions, 56 of these mutants did not differ from parental strain B728a in their population dynamics when the populations were enumerated by viable counts at various times during the humidity cycles (Table 1). The possibility that these mutants produced fewer ice nuclei per cell was investigated. While most of the mutants were similar to B728a in the fraction of cells capable of producing an ice nucleus at a given time (usually, about $10^{-4.5}$ ice nuclei were active at -5°C per cell), some strains exhibited ice nucleation activity of as low as about 10^{-6} ice nuclei per

cell (Fig. 3). Twenty-seven mutants had significantly lower ice nucleation activities at an assay temperature of -5°C in vitro. These 27 mutants represented 48% of the 56 presumptive epiphytic fitness mutants that did not exhibit altered population dynamics on plants. Eight mutants that had reduced ice nucleation activity in culture were tested for ice nucleation activity on plants; seven had reduced activity on plants compared with the activity of the parental strain, although the differences were not significant, probably because of the small number of replicate samples tested. Thus, at least half of the presumptive epiphytic fitness mutants that had no detectable growth or survival deficiencies on plants were likely selected because of their reduced ability to express ice nucleation activity, as demonstrated in vitro, and the possibility remains that others may be reduced in their ice nucleation activity in planta but not in vitro.

The population sizes of some of the mutant strains on plants may have been overestimated because of the bulked leaf samples that were used in some studies. Bulk leaf samples were used to minimize the number of samples required. To evaluate the extent to which use of bulked leaf samples allowed for overestimation of population sizes, the population sizes of 18 mutants were estimated by using

TABLE 1. Mutants exhibiting various population dynamics

Parameter	No. of mutants expressing:								Total ^a
	Auxotrophy	Altered motility	Reduced osmotolerance	Reduced desiccation tolerance	Altered EPS production	Altered growth in culture	Reduced competitiveness	No in vitro alterations	
Indistinguishable from B728a	0	2	4	0	6	6	0	43	56
Similar epiphytic stress tolerance to B728a and reduced growth on leaves	0	1	3	2	0	0	0	4	9
Population reduction upon stress moderately greater ^b than that of B728a and:									
Reduced growth rate on leaves	0	0	1	0	0	0	0	4	5
Increased growth rate on leaves	0	2	3	3	1	3	0	5	13
Growth rate similar to that of B728a	0	0	2	4	2	2	1	4	12
Population reduction upon stress much greater ^c than that of B728a and:									
Reduced growth rate on leaves	1	0	1	1	0	1	0	3	6
Increased growth rate on leaves	0	1	0	1	0	1	0	4	5
Growth rate similar to that of B728a	2	0	0	0	0	1	1	1	3

^a The total does not reflect the sum of the entries in each row because some mutants exhibited multiple phenotypic alterations.

^b Moderately greater was two- to eightfold.

^c Much greater was more than eightfold.

individual leaves as well as multiple leaves as samples. The sizes of the populations recovered from dry leaf surfaces were generally smaller when estimated with individual leaves than when estimated with bulked leaf samples; frequently, they were more than 0.5 log units smaller. Only 1 of

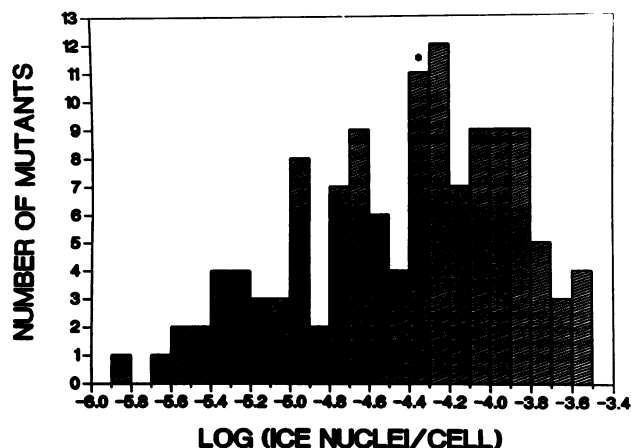


FIG. 3. Distribution of ice nucleation activities of *P. syringae* B728a and 82 Tn5 mutants of this strain that were reduced in epiphytic fitness, as evaluated by their population dynamics on leaves, and 11 Tn5 mutants that showed reduced ice nucleation activity on leaves but whose population dynamics were not measured. All strains were cultured on KB and were assayed at -5°C . The activity of parental strain B728a is denoted with an asterisk.

the 18 mutants evaluated was similar to B728a in its epiphytic growth and survival, and it was deficient in ice nucleation activity in culture. We conclude, therefore, that most, if not all, of the 29 mutants whose population dynamics were indistinguishable from that of B728a when bulked leaf samples were used and which had in vitro ice nucleation activity similar to that of B728a may have differed from B728a in either growth or survival, but these differences were obscured by the bulked leaf samples.

Since 27 of the 109 potential epiphytic fitness mutants had demonstrably reduced ice nucleation activities, only 82 of the mutants were considered to be altered in epiphytic fitness, and these mutants were further characterized. While some epiphytic fitness mutants had alterations in phenotypes that could be measured in culture, many mutants exhibited no alterations in any in vitro phenotype (Table 2). Interestingly, only 3 of the 82 epiphytic fitness mutants were auxotrophs, 1 each for methionine, tryptophan, and both isoleucine and valine. No catabolic deficiency was observed for any of the 31 organic compounds tested, all of which could serve as sole carbon sources for the parental strain. None of the mutants lost the ability to produce disease symptoms on beans, a compatible host plant, nor did any mutant lose the ability to incite a hypersensitive response on the non-host plant, tobacco. All mutant strains were capable of producing a fluorescent pyoverdine siderophore indistinguishable from that produced by the parental strain. While most mutant strains were similarly motile in a semisolid medium, three independent measurements showed that four strains consistently exhibited significantly reduced motility and two strains were completely nonmotile (Fig. 4).

TABLE 2. Characterization of epiphytic fitness mutants

Parameter	No. of mutants expressing:							
	Auxotrophy	Altered motility	Reduced osmotolerance	Reduced desiccation tolerance	Altered EPS production	Altered growth in culture	Reduced competitiveness	No alterations
Auxotrophy								
Altered motility	0							
Reduced osmotolerance	0	0						
Reduced desiccation tolerance	0	2	1					
Altered EPS production	0	0	2	0				
Altered growth in culture	1	2 ^a	2	1	3 ^b			
Reduced competitiveness	1	0	0	0	0	0		
No other alterations	1	2	7	6	4	5	1	41
Total no. of mutants	3	6	14 ^c	11 ^c	9	14	2	41

^a One of these mutants was also reduced in desiccation stress tolerance, and the other had reduced osmotolerance.

^b One of these mutants also had reduced osmotolerance.

^c Total number of mutants includes those described in footnotes *a* and *b*.

Fourteen epiphytic fitness mutants exhibited reduced osmotolerance. While strain B728a could grow in LB containing 1.0 M NaCl, 14 Tn5 mutants grew in LB only when the concentration of NaCl was less than 0.8 M. Although these mutants did not exhibit extreme osmosensitivity, they were consistently less tolerant to high concentrations of salt.

The desiccation tolerance of bacterial mutants was evaluated by measuring the reduction in viable population size as cell suspensions were incubated on dry filter paper disks at 75% relative humidity. In that assay, 11 of the 82 epiphytic fitness mutants exhibited a larger population decrease than B728a did. While the population size of the parental strain decreased an average of 10-fold in replicate experiments, several mutants exhibited a more than 20-fold decrease in viability. In no case, however, did the population size of a mutant fall below the detection limit.

The EPSs produced by the mutants were evaluated semi-quantitatively by measuring colony sizes on media containing one of several carbon sources known to stimulate EPS production in culture. While six mutants produced small colonies with altered colony morphologies indicative of reduced EPS production, three mutants produced larger,

more mucoid colonies than did the parental strain. Small "dry" colony types, characteristic of strains devoid of EPS production, were not observed among the 82 epiphytic fitness mutants evaluated.

The growth rates of several epiphytic fitness mutants differed from that of strain B728a in KB broth. Five mutants grew 10 to 33% faster than B728a; in contrast, two mutants grew 16 to 29% slower than B728a. Also, five mutants entered the stationary phase at lower cell densities than the other mutants or strain B728a did. Of the two mutants that grew more slowly in culture, neither showed a decreased growth rate on leaves. Of the five mutants that grew faster in culture, only one showed an increased growth rate relative to that of the parental strain on leaves.

Since the differences in growth rate between bacterial strains in culture may be sufficiently small that direct comparisons of generation times would not reveal any differences, the relative competitiveness of individual Tn5 mutants with the parental strain in culture was assessed directly. Approximately equal numbers of a given mutant and the parental strain were coinoculated into KB broth at relatively low cell concentrations and were allowed to multiply until the stationary phase. A change in the proportional representation of mutants in culture was considered evidence for a difference in growth rate or relative nutrient acquisition ability. By this procedure, two Tn5 mutants were found to have competitiveness constant values that were larger than the mean by greater than three standard deviations of the mean, thus having reduced competitiveness compared with strain B728a (Fig. 5).

The distribution of Tn5 in the chromosomes of 62 distinct epiphytic fitness mutants was determined by Southern blot analysis of large restriction fragments separated by pulsed-field gel electrophoresis. A single Tn5 insertion was found in each of the 62 mutants evaluated. Tn5 insertions were found in 23 distinct *Xba*I restriction fragments ranging in size from 19 to 270 kb. Tn5 was distributed nonrandomly among these *Xba*I restriction fragments (Table 3); some fragments had substantially more and several had substantially fewer Tn5 insertions than would be expected on the basis of a random spatial occurrence. For example, restriction fragments of 34 and 66 kb had nearly three times as many Tn5 insertions as would be expected, while those of 186 and 270 kb had only half the number expected. Genes determining epiphytic fitness thus appear to be at least partially clustered in the chromosome of *P. syringae* B728a. Mutants with Tn5 inser-

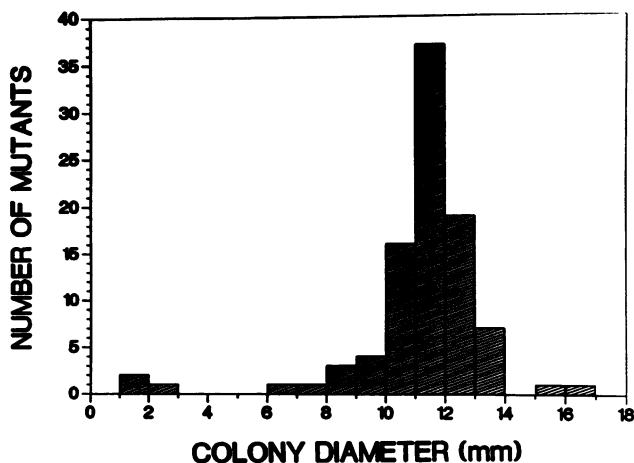


FIG. 4. Motilities expressed by *P. syringae* B728a and the 93 Tn5-induced mutants of this strain described in Fig. 3 when tested in a semisolid agar. The results of one of three replicate experiments are depicted.

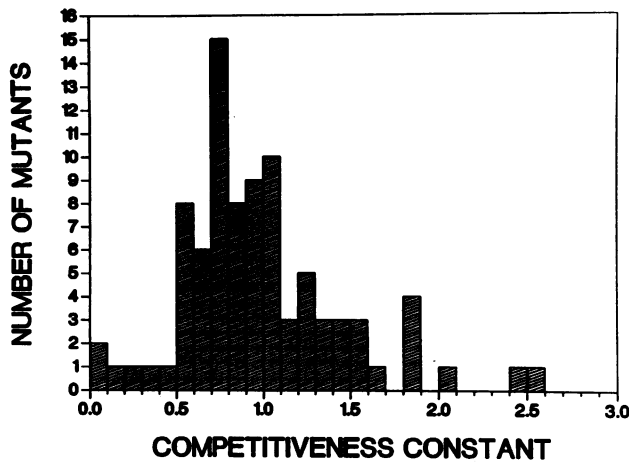


FIG. 5. Distribution of the competitiveness constant of the 93 Tn5-induced mutants described in Fig. 3. Each mutant was coinoculated with the parental strain B728a into KB. The competitiveness constant is the ratio of the proportional representation of the mutant in the mixture at the time of inoculation to its proportional representation after 24 h of growth. Thus, a large value indicates a large reduction in the proportional representation of a mutant.

tions in a given restriction fragment did not all exhibit the same in planta or in vitro phenotypic alterations.

DISCUSSION

Epiphytic bacteria are superior to most other species in their ability to tolerate stresses on leaves (33). This ability to survive physical environmental stresses on leaves may be an adaptive feature common to all epiphytic bacteria. While rhizosphere, aqueous, and enteric bacteria all can multiply on wet leaves, they do not survive as well as *P. syringae*

strains on dry leaf surfaces (33). Since the amount and type of nutrients in the phylloplane may be similar to that in the rhizosphere (42), the catabolic capabilities of epiphytic bacteria might be similar to those of bacteria in the rhizosphere. However, the frequency and extremes of physical conditions that epiphytic bacteria encounter are probably distinct from those experienced by other plant-associated bacteria. For example, leaves may be wetted and then dried several times in a single day.

Epiphytic fitness, the ability of a bacterial strain to develop or maintain a high population size on plants, is dependent on the environmental context. For this reason, genetic lesions causing several distinct growth or survival deficiencies all reduced epiphytic fitness. For example, some insertional mutants were apparently able to multiply normally on wet leaves but were deficient in their ability to survive on dry leaves. In contrast, some mutants were able to survive normally on dry plants but had a reduced ability to multiply on moist leaves. Thus, the former mutants would not be considered epiphytically unfit on leaves that never dried, while the latter might appear as fit as the parental strain under dry conditions. Since leaf surfaces are exposed to fluctuating environmental conditions, both types of mutants would probably be epiphytically unfit in most natural habitats.

About 40% of the epiphytic fitness mutants identified in the present study were intolerant of the stresses associated with dry leaves. The particular stresses that may be sensed by bacteria on dry leaves include, among others, high temperature, high osmolarity, and low matric potential. Since the dry conditions involved elevated temperatures (28°C) that were far lower than those required to kill the bacteria in culture, it seems likely that water stress may have been more detrimental than high temperature to cell survival. Osmotic water stress on drying leaves probably increases because the removal of water causes organic compounds and other small molecules that leak from, and accumulate on, the surfaces of plants (42) to become concentrated and thus increases the osmotic potential of the water that remains. It is unknown, however, whether the osmotic potential of water in bacterial habitats reaches a level such that it has an impact on bacterial survival. The matric stress component of the low water potential on dry leaves may be even more important than osmotic stress (14).

While the distribution of Tn5 insertions into the chromosome of strain B728a was apparently random, as shown by the random distribution of fragment sizes containing Tn5 and by the 1% frequency of auxotrophy among the mutants as in other studies (1, 6, 31, 38), the distribution of Tn5 insertions in the epiphytic fitness mutants was not. Although some chromosomal regions contained several insertions that altered epiphytic fitness, mutations within a particular region usually did not confer the same phenotype. Since Tn5 usually causes polar mutations in genes linked in an operon (2, 3), the absence of clusters of insertional mutations altering identical phenotypes suggests that large operons may not contribute to epiphytic fitness. The low number of insertions, 5,300, relative to the number of genes in this species, approximately 4,200 on the basis of a genome size of 2.8×10^9 Da, may not have been sufficient to reveal such clustering, however. The insertional inactivation of 5,300 randomly located sites in the chromosome yields an approximately 72% chance that any one gene was inactivated by insertion of Tn5 by allowing for the probability of multiple insertions in some genes.

A relatively small part of the genome of *P. syringae*, less

TABLE 3. Sizes of *Xba*I restriction fragments from epiphytic fitness mutants that contain Tn5

Fragment size (kb)	Mutant strains	Expected frequency	Deviation	$(O - E)^2/E^a$
19	51	0.51	0.49	0.47
22	57b	0.59	0.41	0.28
23	6	0.62	0.38	0.23
26	102, 107	0.70	1.30	2.41
34	34, 70, 96, 104	0.91	3.09	10.49
49	28, 49, 89	1.32	1.68	2.13
56	16, 67, 79, 108	1.50	3.50	8.16
62	25, 52	1.66	0.34	0.07
66	17, 18, 72, 83, 97, 100	1.77	4.23	10.11
73	31, 32, 91, 92, 95	1.96	3.04	4.71
76	29, 65, 94, 95a	2.04	1.96	1.88
92	10, 104	2.47	-0.42	0.07
97	20, 68, 105	2.60	0.40	0.06
109	1s, 2	2.93	-0.93	0.29
121	3, 7	3.25	-1.25	0.48
123	23	3.30	-2.30	1.60
130	5, 7, 9, 22, 103	3.49	1.51	0.65
142	43, 57, 101	3.81	-0.81	0.17
155	42, 53	4.16	-2.16	1.12
168	8, 19	4.51	-2.51	1.14
186	13	4.99	-3.99	3.19
210	1, 4, 27, 64, 106	5.64	-0.64	0.07
270	21	7.25	-6.25	5.38

^a $\chi^2 = 45.49$; $P < 0.01$.

than 2%, appears to be required for growth and survival on leaves. If some of the loci inactivated in these epiphytic fitness mutants represent the regulatory genes required for expression of other genes that individually make small contributions to epiphytic fitness, then a substantially higher percentage of the genome may be involved. If transcriptionally active genes were preferred sites for transposition, as has been suggested previously (16), some genes that were not transcriptionally active in culture may have been missed. Also, plant-inducible genes may have been underrepresented if they were transcribed at low levels in culture.

We identified 82 of the 5,300 mutants evaluated as epiphytic fitness mutants. Fifty-three of the mutants exhibited reduced epiphytic fitness in both the original ice nucleation assay and all subsequent assays. While the other 29 mutants exhibited reduced epiphytic populations only in the original leaf freezing assay, we believe that the dilution plating assay was less sensitive than the original assay for detecting small differences in population sizes among bacterial strains. Since we used 20 individual leaves in the original assay, the presence of a large population on 1 or more leaves contributed little to the frequency of ice nucleation events in the collection of leaves. In contrast, since bulked leaf samples were used in the subsequent assay, large variances in population sizes among leaves may have contributed to an overestimation of mean population sizes, even after populations were log transformed. If the variance in population sizes with some mutants was greater than that of parental strain B728a because of the mutants' increased sensitivities to environmental extremes encountered on a subset of leaves, overestimates of the mean population size of the mutants would obscure true population differences. Since all 17 mutants which were compared in studies with single leaf samples had lower population sizes than that of the parental strain, we believe that there were also small differences in population size for those few presumptive epiphytic fitness mutants that did not differ from the population size for strain B728a when bulked leaf samples were used.

An additional 27 mutants appeared to be epiphytic fitness mutants in the original assay, but they were later found to be deficient in ice nucleation activity. These mutants represented those with false-positive activity. Reduced ice nucleation activity in these mutants could result from modifications of the cell membranes, particularly the outer membrane. While the *P. syringae* ice nucleation gene encodes a single protein which is necessary for ice nucleation activity (44), this ice protein apparently must aggregate into large homogeneous structures which catalyze ice formation at temperatures higher than -12°C (4, 10). Lipids and/or a membrane are also required for expression of ice nucleation activity (11). Thus, insertional mutations which alter either lipid metabolism or the protein content of the outer membrane could affect the abundance or the stability of ice protein aggregates. Since aggregates as large as 50 or more proteins are required for ice nucleation activity at -2.5°C (4, 10), which was the assay temperature used in the present study, even subtle changes in membrane structure would greatly reduce the ice nucleation activities of mutants in our assay.

Many of the epiphytic fitness mutants identified in the present study exhibited no obvious alterations in phenotypes that were expressed in culture. Curiously, the osmosensitive mutants identified in the present study were only slightly impaired in this phenotype. If osmotolerance per se were important in epiphytic fitness, we should have selected for highly osmosensitive mutants. The slight osmosensitivity of

these mutants, therefore, may reflect pleiotropic changes resulting from other phenotypic alterations. While some phenotypes such as motility and EPS production were anticipated to contribute to epiphytic fitness (23), mutants altered in these phenotypes were only slightly deficient in growth or survival on leaves. In other studies, nonmotile mutants were slightly reduced in their ability to survive prolonged incubation on dry leaves (12). These nonmotile mutants would have exhibited only a three- to fivefold greater decrease in viable population size than the parental strain under the conditions used in the present study. Two nonmotile mutants were found in the present study, but their tolerance of stresses on dry leaves was much greater than that exhibited by many motile strains. Although the tube nucleation assay discriminated between strains that differed by three- to fivefold or more in population size (24), the contribution to epiphytic fitness of motility, EPS production, or other hypothesized phenotypes expressed in culture may be sufficiently small that they were not easily detected. It is clear, however, that phenotypes that were either not anticipated or which were not easily observable in culture confer large effects on epiphytic growth and survival. The elucidation of these traits will greatly increase our understanding of adaptive characteristics of epiphytic bacteria.

The leaf surface appears to contribute many different nutrients to epiphytic bacteria. Since auxotrophic mutants were not eliminated in the present study, we would have expected that many more strains would have exhibited reduced growth had the leaves not provided the required growth factor. Even the three auxotrophic mutants that were identified did not exhibit a significant reduction in growth rate on leaves. Thus, not only does there appear to be a diversity of amino acids and other growth factors on leaves but their abundance appears to be sufficient to support bacterial growth to a level similar to that of prototrophs. Carbon sources might, therefore, be the most limiting nutrient resource on leaves since dependency on amino acids did not limit growth. This further suggests that any one amino acid may not comprise the major source of nitrogen on leaves.

Since the relative growth of bacterial mutants in culture and on plants was not similar, the resources available for growth on leaves and in KB broth probably differ greatly. The abundance and diversity of nutrients in culture media are probably much greater than those on plants. Blocking of one catabolic step that is important in culture may not reduce the growth rate of the strain on plants if that resource is not available. While an exhaustive study of carbon source utilization by epiphytic fitness mutants was not conducted, we were surprised that no mutants were deficient in their ability to utilize any of 31 common carbon sources that were tested in culture. Acquisition of nitrogen may also be important for growth on leaves. While we evaluated the use of nitrogen-containing compounds as carbon sources, we did not evaluate the use of these compounds as nitrogen sources by these strains in culture. More detailed studies of the nutrient utilization patterns of these mutant strains could provide insight into the chemical resources available, and those which are preferred by bacteria, on leaves.

ACKNOWLEDGMENTS

This work was supported by a grant from the U.S. Department of Energy (DE-FG03-86ER13518).

We thank C. Pierce for technical assistance and B. Rotz for maintaining the plants used in the study. We also thank C. Manceau

for optimizing Tn5 mutagenesis procedures for the bacterial strains used in the study.

REFERENCES

- Anderson, D. M., and D. Mills. 1985. The use of transposon mutagenesis in the isolation of nutritional and virulence mutants in two pathovars of *Pseudomonas syringae*. *Phytopathology* 75:104-108.
- Berg, D. E., and C. M. Berg. 1983. The prokaryotic transposable element Tn5. *Bio/Technology* 1:417-435.
- Berg, D. E., M. A. Schmandt, and J. B. Lowe. 1983. Specificity of transposon Tn5 insertion. *Genetics* 105:813-828.
- Burke, M. J., and S. E. Lindow. 1990. Surface properties and size of the ice nucleation site in ice nucleation active bacteria: theoretical considerations. *Cryobiology* 27:80-84.
- Chu, G., D. Vollrath, and R. W. Davis. 1986. Separation of large DNA molecules by contour-clamped homogeneous electric fields. *Science* 234:1582-1585.
- Cupples, D. A. 1986. Generation and characterization of Tn5 insertion mutants in *Pseudomonas syringae* pv. *tomato*. *Appl. Environ. Microbiol.* 51:323-327.
- Ditta, G. 1986. Tn5 mapping of *Rhizobium* nitrogen fixation genes. *Methods Enzymol.* 118:519-528.
- Ganal, M. W., and S. D. Tanksley. 1989. Analysis of tomato DNA by pulsed field gel electrophoresis. *Plant Mol. Biol. Reporter* 7:17-28.
- Gibbins, L. N., and D. L. Peterson. 1978. Responses of *Erwinia herbicola* Y46 and a non-pigmented mutant to ultraviolet radiation and visible light: survival curves and photoreactivation, p. 443-450. *In* Proceedings of the Fourth International Conference on Plant Pathogenic Bacteria. Institut National de la Recherche Agronomique, Angers, France.
- Govindarajan, A. G., and S. E. Lindow. 1988. Size of bacterial ice nucleation sites measured *in situ* by gamma radiation inactivation analysis. *Proc. Natl. Acad. Sci. USA* 85:1334-1338.
- Govindarajan, A. G., and S. E. Lindow. 1988. Phospholipid requirements for expression of ice nuclei in *Pseudomonas syringae* and *in vitro*. *J. Biol. Chem.* 263:9333-9338.
- Haefele, D. M., and S. E. Lindow. 1987. Flagellar motility confers epiphytic fitness advantages to *Pseudomonas syringae*. *Appl. Environ. Microbiol.* 53:2528-2533.
- Hanish, J., and M. McClelland. 1988. Activity of DNA modification and restriction enzymes in KGB, a potassium glutamate buffer. *Gene Anal. Technol.* 5:105-107.
- Harris, R. F. 1981. Effect of water potential on microbial growth and activity. *In* J. F. Parr, W. R. Gardner, and L. F. Elliot (ed.). *Soils Science Society of America, Madison, Wis.*
- Hirano, S. S., and C. D. Upper. 1983. Ecology and epidemiology of foliar bacterial plant pathogens. *Annu. Rev. Phytopathol.* 21:243-269.
- Hohn, B., Z. Koukolikova, F. Durrenberger, G. Bakkeren, and C. Koncz. 1991. The T-DNA on its way from *Agrobacterium tumefaciens* to the plant, p. 19-27. *In* H. Hennecke and D. P. S. Verma (ed.), *Advances in molecular genetics of plant-microbe interactions*, vol. 1. Kluwer Academic Publishers, Boston.
- Kim, H. K., C. Orser, S. E. Lindow, and D. C. Sands. 1987. *Xanthomonas campestris* pv. *translucens* strains active in ice nucleation. *Plant Dis.* 71:994-997.
- 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.
- Klement, Z. 1963. Rapid detection of the pathogenicity of phytopathogenic pseudomonads. *Nature (London)* 199:299-300.
- Lindgren, P. B., R. C. Peet, and N. J. Panopoulos. 1986. Gene cluster of *Pseudomonas syringae* pv. "phaseolicola" controls pathogenicity on bean plants and hypersensitivity on nonhost plants. *J. Bacteriol.* 168:512-522.
- Lindow, S. E. 1982. Population dynamics of epiphytic ice nucleation active bacteria on frost sensitive plants and frost control by means of antagonistic bacteria, p. 395-416. *In* A. Sakai (ed.), *Plant cold hardiness*. Academic Press, Inc., New York.
- Lindow, S. E. 1983. The role of bacterial ice nucleation in frost injury to plants. *Annu. Rev. Phytopathol.* 21:363-384.
- 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.
- Lindow, S. E. 1993. Novel method for identifying bacterial mutants with reduced epiphytic fitness. *Appl. Environ. Microbiol.* 59:1586-1592.
- Lindow, S. E., D. C. Army, W. R. Barchet, and C. D. Upper. 1978. The role of bacterial ice nuclei in frost injury to sensitive plants, p. 249-263. *In* P. Li (ed.), *Plant cold hardiness and freezing stress*. Academic Press, Inc., New York.
- Lindow, S. E., D. C. Army, and C. D. Upper. 1978. *Erwinia herbicola*: a bacterial ice nucleus active in increasing frost injury to corn. *Phytopathology* 68:523-527.
- Lindow, S. E., D. C. Army, and C. D. Upper. 1982. Bacterial ice nucleation: a factor in frost injury to plants. *Plant Physiol.* 70:1084-1089.
- 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.
- Maki, L. R., E. L. Galyon, M. Chang-Chien, and D. R. Caldwell. 1974. Ice nucleation induced by *Pseudomonas syringae*. *Appl. Environ. Microbiol.* 28:456-460.
- Maki, L. R., and K. J. Willoughby. 1978. Bacteria as biogenic sources of freezing nuclei. *J. Appl. Meteorol.* 17:1049-1053.
- Meade, H. M., S. R. Long, G. B. Ruvkun, S. E. Brown, and F. M. Ausubel. 1982. Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn5 mutagenesis. *J. Bacteriol.* 149:114-122.
- Miller, J. H. 1972. *Experiments in molecular genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- O'Brien, R. D., and S. E. Lindow. 1988. Effect of plant species and environmental conditions on epiphytic population sizes of *Pseudomonas syringae* and other bacteria. *Phytopathology* 79:619-627.
- O'Brien, R. D., and S. E. Lindow. 1988. Effect of plant species and environmental conditions on ice nucleation activity of *Pseudomonas syringae* on leaves. *Appl. Environ. Microbiol.* 54:2281-2286.
- Pieczarka, D. J., and J. W. Lorbeer. 1975. Bacterial populations on basal lettuce leaves and in soil from under lettuce plants. *Phytopathology* 65:509-513.
- 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.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Shaw, K. J., and C. M. Berg. 1979. *Escherichia coli* K12 auxotrophs induced by insertion of the transposable element Tn5. *Genetics* 92:741-747.
- Smith, C. L., P. E. Warburton, A. Gaal, and C. R. Cantor. 1986. *Genetic engineering*, vol. 8, p. 45-71. Plenum Press, Newark, N.J.
- Stout, J. D. 1960. Biological studies of some Tussock-Grassland soils. XV. Bacteria of two cultivated soils. *N.Z. J. Agric. Res.* 3:214-223.
- Stout, J. D. 1960. Bacteria of soil and pasture leaves at Claudlands Showgrounds. *N.Z. J. Agric. Res.* 3:413-430.
- Tukey, H. B., Jr. 1970. The leaching of substances from plants. *Annu. Rev. Plant Physiol.* 21:305-324.
- Vali, G. 1971. Quantitative evaluation of experimental results on the heterogeneous freezing nucleation of supercooled liquids. *J. Atmos. Sci.* 28:402-409.
- Warren, G., and P. Wolber. 1991. Molecular aspects of microbial ice nucleation. *Mol. Microbiol.* 5:239-243.
- Winston, P. W., and D. H. Bates. 1960. Saturated solutions for the control of humidity in biological research. *Ecology* 41:232-237.