October, 1994

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APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1994, p. 3790–3798
0099-2240/94/$04.00+0

Vol. 60, No. 10

Survival, Growth, and Localization of Epiphytic Fitness Mutants of *Pseudomonas syringae* on Leaves

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Received 18 January 1994/Accepted 17 June 1994

Among 82 epiphytic fitness mutants of a *Pseudomonas syringae* pv. syringae strain that were characterized in a previous study, 4 mutants were particularly intolerant of the stresses associated with dry leaf surfaces. These four mutants each exhibited distinctive behaviors when inoculated onto and into plant leaves. For example, while none showed measurable growth on dry potato leaf surfaces, they grew to different population sizes in the intercellular spaces of bean leaves and on dry bean leaf surfaces, and one mutant appeared incapable of growth in both environments although it grew well on moist bean leaves. The presence of the parental strain did not influence the survival of the mutants immediately following exposure of leaves to dry, high-light incubation conditions, suggesting that the reduced survival of the mutants did not result from an inability to produce extracellular factors in planta. On moist bean leaves that were colonized by either a mutant or the wild type, the proportion of the total epiphytic population that was located in sites protected from a surface sterilant was smaller for the mutants than for the wild type, indicating that the mutants were reduced in their ability to locate, multiply in, and/or survive in such protected sites. This reduced ability was only one of possibly several factors contributing to the reduced epiphytic fitness of each mutant. Their reduced fitness was not specific to the host plant bean, since they also exhibited reduced fitness on the nonhost plant potato; the functions altered in these strains are thus of interest for their contribution to the general fitness of bacterial epiphytes.

Large populations of bacteria are commonly found on aerial leaf surfaces. Many of these epiphytic bacteria can affect plant health under suitable conditions, such as by inciting disease or ice formation or by producing plant hormones that affect plant growth. However, many epiphytes have no known influence under any conditions. It has long been recognized that large epiphytic populations of phytopathogenic bacteria can develop in the absence of disease. These populations can contribute to an increased probability of disease incidence as well as frost injury (15, 30, 41). Understanding the factors that contribute to the establishment and maintenance of large epiphytic populations may be critical to strategies for disease and frost injury control as well as to a basic understanding of the ecology of epiphytes.

The bacteria that are found on leaf surfaces probably have particular adaptations that allow them to exploit epiphytic environments. They are distinct from bacteria found in other habitats, such as the nearby soil (19, 44, 45), and are superior to those from other habitats at tolerating harsh environmental conditions on leaf surfaces (37, 38). Interestingly, such adaptive characteristics may have developed at the expense of those promoting survival in other habitats, since several epiphytes survive poorly in soil (25, 31). We are just beginning to understand the factors that contribute to epiphytic fitness. For example, by comparing a nonmotile mutant with its motile parent, Haefele and Lindow (12) demonstrated that motility can contribute to the fitness of *Pseudomonas syringae* on leaves. In similar mutational analyses, siderophore production (32), antibiotic production (26), and ice nucleation activity (23) appeared not to contribute to epiphytic fitness under the conditions tested.

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While the approach of constructing mutants altered in individual traits is powerful for evaluating the role of those traits in epiphytic fitness, it is restricted to traits that are hypothesized to be involved in fitness and mainly to traits that are identifiable in culture. In a previous study, we screened randomly generated *P. syringae* mutants directly for alterations in epiphytic fitness and identified 82 that exhibited reduced abilities to grow or survive on leaves (29). While some of these mutants were altered in phenotypes that could be measured in culture, many were not altered in any of the 40 in vitro phenotypes examined, suggesting that traits not easily observable in culture may contribute to epiphytic growth or survival. Only a small proportion of these mutants were altered in phenotypes that were hypothesized to condition epiphytic fitness, such as osmotolerance, motility, and the production of extracellular polysaccharides, and those that were altered often exhibited only small changes in their epiphytic behavior. Thus, unanticipated traits not only are likely to contribute to epiphytic fitness but also may be among the largest contributors.

In this report, we investigate several aspects of epiphytic fitness by examining the plant-associated behavior of four epiphytic fitness mutants. First, a behavioral comparison is used to evaluate the possibility that a single, specific stress response mediates tolerance to a range of environmental stresses in bacterial epiphytes. Second, the role of pathogenicity in epiphytic fitness is explored. Although numerous studies have shown that pathogens grow to larger population sizes on susceptible than on resistant varieties of the host plant species (see, e.g., references 9, 33, 34, and 43), the role of pathogenicity in the successful establishment and maintenance of those populations is not clear. Third, we evaluate the extent to which bacteria survive on leaf surfaces by residing in sites that offer protection from exposure to harsh physical conditions, such as in the crevices where bacteria are commonly observed by scanning electron microscopy (6, 10, 40). Fourth, we examine

the potential contribution to epiphytic fitness of extracellular factors produced by the bacteria in planta.

MATERIALS AND METHODS

Bacterial strains and culture media. *P. syringae* pv. syringae B728a was isolated from an asymptomatic bean leaf (32). Strains 14, 22, 42, and 94 were constructed by random insertion of the transposon Tn*5* into B728a, as described previously (29). Strain MX7 was a marker exchange mutant of strain B728a, which was constructed by introducing the Tn*5*-containing region from strain 42 into strain B728a by marker exchange mutagenesis (2). MX7 was identical to strain 42 in every phenotype examined. All strains were cultured on King's medium B (KB) (22) containing 100 μg of rifampin per ml (KBR). Bacterial suspensions were prepared by suspending cells grown on KBR agar in 10 mM potassium phosphate buffer (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.

Characterization of Tn*5* insertions. Genomic DNA of each strain was digested with *Eco*RI, subjected to electrophoresis, and transferred to a nylon membrane. A plasmid containing Tn*5*, pUW964 (48), was labeled with digoxigenin by using a DNA-labeling and detection kit (Genius Kit; Boehringer Mannheim) and hybridized to the genomic DNA under low-stringency conditions, as specified by the manufacturer.

External inoculation of plants. Plant inoculations were performed as described previously (28). Bacterial suspensions of a given strain were applied to 5 to 10 pots of bean plants (*Phaseolus vulgaris* cv. Bush Blue Lake 274), with each pot containing about 10 plants, or to 20 pots of potato plants (*Solanum tuberosum* cv. Russett Burbank), with each pot containing about 4 plants. For experiments involving only single-strain inocula, bean plants were immersed for about 3 s in a suspension of 10^3 cells of a given strain per ml while potato plants were immersed for about 3 s in a suspension of 10^4 cells of a given strain per ml. For experiments involving dual-strain inocula, bacterial suspensions of 10^5 cells of each strain per ml were mixed immediately before plant inoculation. A plastic bag was placed over the plants in each pot and was sealed loosely at the base of the pot to produce a tent.

For evaluation of epiphytic growth, the plants were incubated at 21°C for 4 days with a 12-h photoperiod (light = 1,000 ft-c [10,760 lx]). The leaves remained moist throughout this incubation period. For evaluation of epiphytic stress tolerance, the plants were incubated at 21°C for 24 h, during which they were provided with 12 h of light (1,000 ft-c). The plastic bags were then removed, and the plants were allowed to dry at 21°C and ambient humidity (about 60% relative humidity) for 30 min. All pots were placed in a growth chamber maintained at 28°C and 45% relative humidity for 24 h, during which they were provided 12 h of light (10,000 ft-c [107,600 lx]). The plants were removed from the growth chamber, rewetted by spraying distilled water onto the leaves (without causing runoff), and again enclosed in a plastic bag and incubated for 24 h at 21°C.

For each incubation regime, one primary bean leaf or one potato leaf was sampled from each pot at each sampling time. The bacteria were quantified on each individual leaf by viable counts on KBR containing 100 μg of cycloheximide per ml and 50 μg of Benlate per ml (KBRC) to inhibit fungi, as described previously (28). The bacteria on leaves inoculated with two strains were quantified on both KBRC and KBRC containing 30 μg of kanamycin per ml.

Internal inoculation of plants. Bacterial suspensions (10^4 cells per ml) were vacuum infiltrated into the intercellular spaces of 12-day-old susceptible bean plants (cv. Bush Blue Lake 274) (52). For coinoculation experiments, cell suspensions (10^4 cells per ml) of each strain were mixed immediately before infiltration. After infiltration, the plants were allowed to dry and then were transferred to a growth chamber maintained at 24°C and 80% relative humidity with a 12-h photoperiod. At each sampling time, two 6.5-mm leaf disks were removed from each pot, which contained about four plants. The two leaf disks were combined and homogenized, and the bacteria were quantified in the mixture by plating appropriate 10-fold serial dilutions onto KBRC. After 5 days, lesions were counted.

The ability of the strains to induce a hypersensitive response in tobacco was tested by the method of Rahme et al. (39). Qualitative evaluation of the presence or absence of a hypersensitive response was made after 24 h.

Survival in protected sites. Bacterial suspensions (7×10^4 cells per ml) of a given strain were applied to 10 pots of bean plants, each containing about 10 plants, as described above. The plants were incubated for 43 h in moist conditions and then subjected to low-moisture, high-light conditions, as described above. At each sampling time, two primary leaves were removed from each pot. The total bacterial population was determined on one leaf by homogenizing the leaf in a blender for 20 s and then plating the appropriate 10-fold serial dilutions on KBRC. The other leaf was immersed in 15% hydrogen peroxide for 5 min, dried in a laminar-flow hood with each side facing upward for 30 min, rinsed twice with double-distilled water, and then homogenized and plated in a similar fashion to the first leaf. Tolerance to hydrogen peroxide in vitro was evaluated by treating cell suspensions with various concentrations of hydrogen peroxide, from 0.005 to 0.5%, for 10 min and enumerating the surviving cells on KB medium.

Statistical methods. Estimates of bacterial populations were log-transformed to achieve normality. On the basis of the lognormal distribution of the epiphytic populations (14), a maximum-likelihood procedure (13, 41) was used to obtain estimated means for data sets that included data from leaves harboring population sizes below the limit of detection (approximately 600 CFU/g [fresh weight]). The mean population sizes among the treatments were compared by using Fisher's least-significant-difference test. Doubling times were calculated from regressions of log(CFU per gram [fresh weight]) on time.

RESULTS

Characterization of mutants. The presence of a single Tn*5* insertion in each mutant was confirmed by probing *Eco*RI-digested genomic DNA from each strain with a labeled plasmid containing a Tn*5* element (data not shown). The Tn*5*-containing fragment was a different size for each mutant, indicating that Tn*5* was inserted at a different site in each mutant. While mutants 14 and 22 were prototrophic, mutants 42 and 94 were auxotrophic for methionine and tryptophan, respectively.

Epiphytic stress tolerance. In a previous study, we identified 82 Tn*5* mutants of *P. syringae* pv. syringae B728a that had reduced epiphytic fitness on beans (29). After bean plants were transferred from conditions of high moisture to low moisture and high light, 44 mutants showed a larger population decrease than did B728a. Mutants 14, 22, 42, and 94 were among those showing the greatest population decreases (Fig. 1). In three separate experiments, when bean plants were subjected to stressful conditions the epiphytic population size of B728a decreased 50- to 350-fold but remained detectable on 70 to

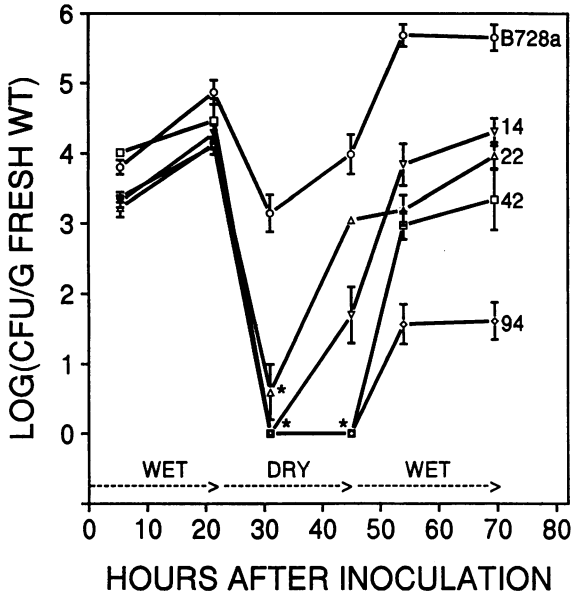

FIG. 1. Population dynamics of *P. syringae* pv. syringae B728a (○) and Tn*5* mutant derivatives 14 (▽), 22 (△), 42 (□), and 94 (◇) on bean plants exposed to alternating wet and dry conditions. Each point represents the mean ± standard error of the mean (SE) of 10 leaf samples. Because means could not be estimated for data sets with eight 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.

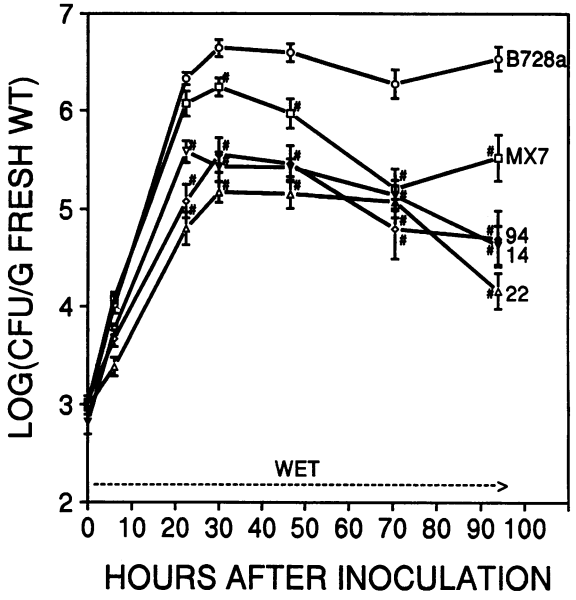

FIG. 2. Population dynamics of *P. syringae* pv. syringae strain B728a (○) and Tn*5* mutant derivatives 14 (▽), 22 (△), MX7 (□), and 94 (◇) on bean plants incubated under constantly moist conditions. Each point represents the mean ± SE of 10 leaf samples. For each sampling time, mean populations (indicated by #) were significantly smaller than the mean population of B728a ($P < 0.05$) when compared by Fischer's least-significant-difference test on the log-transformed data.

100% of the leaves and the population size increased between 8 and 24 h after the plants were transferred to the dry, high-light conditions. In contrast, the epiphytic population sizes of strains 14, 22, 42, and 94 typically decreased to undetectable levels on 90 to 100% of the leaves. While mutants 14 and 22 remained undetectable on 60 to 90% of the leaves until after the leaves were rewetted, mutants 42 and 94 remained undetectable on 100% of the leaves. Furthermore, when relatively high inoculum concentrations were used, the population size of B728a again decreased 50-fold within 9 h after exposure to the stress conditions, while the population sizes of mutants 14, 22, and MX7, which is a marker exchange derivative of strain 42, decreased 132-, 151-, and 204-fold, respectively, and the population size of mutant 94 again decreased below the detection limit (see Fig. 5). Thus, all of the mutants were greatly reduced in their ability to survive and/or grow on leaf surfaces under dry, high-light conditions.

Epiphytic growth of mutants. All four mutants were able to establish large populations on wet bean leaves (Fig. 2). In two separate experiments, MX7 appeared to grow at a wild-type rate in the first 24 h; however, after 24 h the population size of MX7 became increasingly smaller than that of B728a. The population sizes of strains 14, 22, and 94 were significantly smaller than that of B728a as early as 6 h after inoculation and remained at levels typically 15- to 30-fold lower than those of B728a.

Internal growth of mutants. The four mutants were all reduced, but to different degrees, in their ability to multiply in the intercellular spaces of leaves after vacuum infiltration. The results of one representative experiment are shown in Fig. 3. Under these conditions B728a grew with a doubling time of 2.6 to 3.2 h, compared with 1.4 h in a rich medium in culture (data not shown). Strain MX7 grew more slowly than B728a, with doubling times of 3.2 to 5.4 h, and attained population sizes that were 10- to 20-fold smaller than those of B728a. The population sizes of strains MX7 and 14 increased at similar rates until 70 h, resembling the population dynamics of B728a; however, after 70 h the population size of strain 14 decreased dramatically, as did that of MX7 in a separate experiment (see Fig. 6). Although strain 22 grew at a similar rate to B728a in a rich medium (data not shown), it grew much more slowly in leaves, with a doubling time of 6.7 to 9.1 h, and achieved a population size that was 35-fold smaller than that of B728a at 94 h. Mutant 94 was severely reduced in its ability to multiply in the intercellular spaces of leaves (Fig. 3).

The mutants retained the ability to form lesions after vacuum infiltration into bean leaves; however, they formed fewer lesions than did B728a (Fig. 3). This was probably a result of their reduced population sizes. In three separate experiments, a strong correlation was found between the logarithm of the bacterial population size per square centimeter and the logarithm of the lesion number per square centimeter (Pearson's correlation coefficients were 0.897, 0.952, and 0.996). Although their reduced internal growth was similar to the reported behavior of several *hrp* mutants (3, 5, 17, 20, 24, 39), mutants 14, 22, MX7, and 94 each induced a hypersensitive reaction in tobacco identical to that induced by B728a.

Survival in protected sites. It has been speculated that epiphytic bacteria reside in microsites that are protected from exposure to harsh physical conditions. The number of epiphytic bacteria that survived topical application of hydrogen peroxide to leaves was assumed to reflect the number of cells in such microsites, since B728a and the mutants exhibited a similar tolerance to hydrogen peroxide in vitro (data not shown). After 43 h of incubation on wet leaves, only a small proportion (about 0.5%) of the population of B728a survived

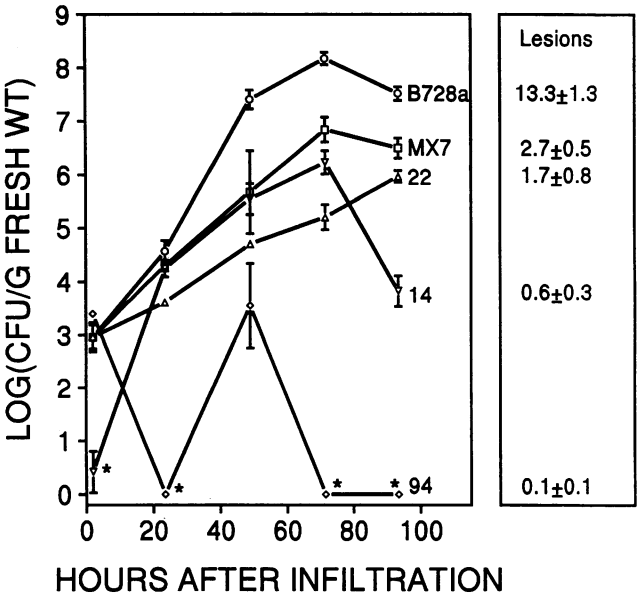

FIG. 3. Population dynamics of *P. syringae* pv. syringae B728a (○) and Tn*5* mutant derivatives 14 (▽), 22 (△), MX7 (□), and 94 (◇) after vacuum infiltration into the intercellular spaces of leaves. Each point represents the mean ± SE of eight samples each composed of two 6.5-mm leaf disks. Because means could not be estimated for data sets with six 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. The values indicating the number of lesions induced by each strain are the mean ± SE of seven plants, with the lesion number per plant represented by the mean number of lesions enumerated in three randomly chosen 1.2-cm^2 leaf regions.

TABLE 1. Survival of B728a and mutant strains in protected sites

Strain	Time p.i. (h)[a]	Population: Total[b]	In protected sites[c]	In exposed sites[d]	% in protected sites[e]
B728a	43	5.12×10^6	2.44×10^4	5.10×10^6	0.48
	51	1.37×10^6	5.06×10^4	1.32×10^6	3.71
	68	3.02×10^6	6.75×10^5	2.35×10^6	22.34
14	43	1.96×10^6	1.36×10^3	1.96×10^6	0.07
	51	1.52×10^5	9.82×10^2	1.51×10^5	0.65
	68	3.02×10^6	2.45×10^4	3.00×10^6	1.66
22	43	2.70×10^6	1.92×10^3	2.70×10^6	0.07
	51	9.79×10^4	2.42×10^3	9.55×10^4	2.47
	68	1.63×10^6	9.33×10^3	1.62×10^6	5.71
MX7	43	1.71×10^6	9.10×10^2	1.71×10^6	0.05
	51	3.51×10^4	2.40×10^2	3.49×10^4	0.69
	68	2.36×10^4	8.32×10^2	2.28×10^4	3.52
94	43	2.95×10^5	3.66×10^2	2.95×10^5	0.12
	51	7.19×10^3	2.33×10^0	7.19×10^3	0.03
	68	8.69×10^3	3.85×10^0	8.69×10^3	0.04

[a] p.i., postinoculation. After application of the bacteria, the plants were incubated under moist conditions for 43 h and then transferred to conditions of low moisture and high light for the remainder of the experiment.

[b] Values represent the CFU per gram (fresh weight). These values were derived from the mean of the log-transformed population estimates from 10 leaves. Leaf populations were estimated in homogenized leaf tissue.

[c] Same as for footnote *b*, except that the leaves were treated with a topical application of 15% hydrogen peroxide before leaf homogenization.

[d] Values are derived by subtracting the average population size in protected sites from the average total population size.

[e] Values are the (average population size in protected sites/average total population size) × 100.

surface sterilization (Table 1), suggesting that most (99.5%) of the epiphytic cells were in exposed sites on a wet leaf surface. An even smaller proportion of each mutant population (generally less than 0.1%) survived surface sterilization, suggesting that the mutants were poorer at reaching, multiplying in, and/or surviving in protected sites. For all strains except strain 94, the percentage of cells that survived peroxide treatment increased with extended incubation under low-moisture, high-light conditions, indicating that exposure of leaf surfaces to these conditions favored multiplication or survival in protected sites. The number of cells of mutant 94 that were in protected sites actually decreased with time, indicating that mutant 94 was unable to multiply and probably unable to survive in these sites.

For B728a, the population decrease upon exposure to low-moisture, high-light conditions was composed almost solely of cells in exposed sites, since the magnitude of the decrease in the total population (3.75×10^6 CFU/g [fresh weight]) was far greater than the number of cells in protected sites before exposure (2.44×10^4 CFU/g [fresh weight]) and the absolute number of cells in protected sites did not decrease. However, a large number of cells in exposed sites survived (1.32×10^6 CFU/g [fresh weight]). For every mutant the total number of cells surviving on dry leaves was far greater than the number of cells in protected sites before stress (at least 20-fold), indicating that the reduced ability to reach, multiply in, and/or survive in protected sites was not sufficient to explain their reduced survival on dry leaf surfaces.

Survival and growth on potato. The influence of plant species on the epiphytic behavior of the mutants was evaluated by comparing the ability of the mutants to grow and survive on bean plants (described above) with their behavior on the nonhost plant potato. When plants were transferred to the low-moisture, high-light conditions, the population size of B728a decreased 611-fold on potato leaves (Fig. 4A), which was 2- to 12-fold more than it did on bean leaves (Fig. 1). Similar to the results on bean plants, immediately following exposure of potato plants to the stressful conditions, B728a was detected on 70% of the leaves while the mutants were detected on less than 10% of the leaves. During further incubation under these conditions, the population size of B728a measurably increased while the population sizes of the mutants remained below the limit of detection. Thus, the mutants exhibited reduced epiphytic fitness on the nonhost plant potato as well as the host plant bean.

Under moist conditions, the relative behavior of the individual mutants on potato leaves was different from that on bean leaves. Mutant 14 exhibited the most striking differences. Immediately after inoculation, the population size of strain 14 declined to undetectable levels and remained relatively small (Fig. 4B), suggesting that it was unable to grow or survive on moist potato leaves; however, its recovery when the leaves were rewetted after dry incubation (Fig. 4A) demonstrated that it was capable of growth. The conditions that favor or disfavor epiphytic growth of strain 14 on potato leaves have not been examined further. Whereas mutants 22 and MX7 grew to levels that significantly differed both from each other and from B728a on moist bean leaves, they were not significantly reduced in growth on moist potato leaves. Both mutants experi-

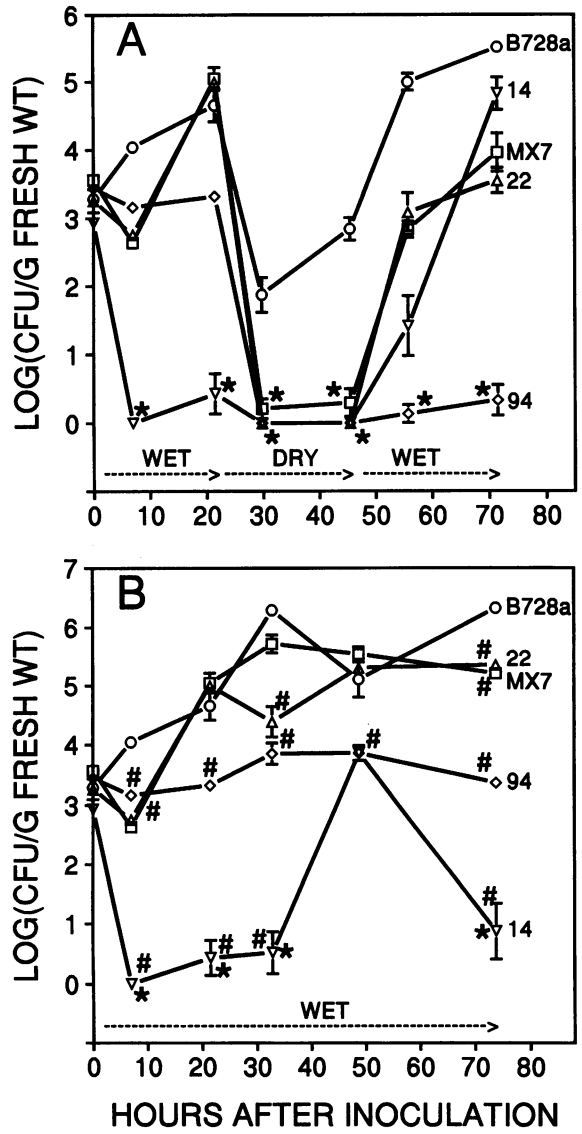

FIG. 4. Population dynamics of *P. syringae* pv. syringae B728a (○) and Tn*5* mutant derivatives 14 (▽), 22 (△), MX7 (□), and 94 (◇) on potato plants incubated under alternating wet and dry conditions (A) and constantly moist conditions (B). Each point represents the mean ± SE of 20 leaf samples. Sample means at times 0, 7, and 22 h are identical in panels A and B. Because means could not be estimated for data sets with 17 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. For each sampling time, mean populations (indicated by #) were significantly smaller than the mean population of B728a ($P < 0.05$) when compared by Fischer's least-significant-difference test on the log-transformed data.

enced an initial decline in population size but then grew to sizes similar to that of B728a on potato leaves (Fig. 4B). In contrast to a 100-fold increase on moist bean leaves, the population size of mutant 94 remained fairly constant for over 70 h on moist potato leaves (Fig. 4B), suggesting that mutant 94 was unable to grow on moist potato leaves.

Coinoculation studies. To determine if the presence of the parental strain B728a influenced the epiphytic survival of the mutants, each mutant was applied to bean plants in a mixture with B728a and the population sizes of the strains were measured during a wet-dry-wet incubation regimen. We inoculated a relatively high concentration of cells to improve the probability that any given microsite that was occupied contained cells of both the mutant and the wild type. Upon exposure of the plants to low-moisture, high-light conditions, the proportional decrease in the mutant population was similar in the presence and the absence of B728a for all of the mutants (Fig. 5), with a few exceptions. In one replicate experiment (results not shown), mutant 14 survived significantly better in the presence of B728a than in its absence; however, this result was not reproducible (Fig. 5). Similarly, the population sizes of MX7 and 94 declined more in the absence of B728a than in its presence in one experiment (Fig. 5) but not in a replicate experiment (results not shown). Thus, the presence of the parental strain B728a did not consistently improve the survival of any of the mutants.

Interestingly, the presence of mutants MX7 and 94 appeared to improve the survival of the parental strain B728a. Upon exposure of the plants to low-moisture, high-light conditions, the population decrease of B728a was consistently at least 10-fold smaller in the presence of mutant MX7 or 94 than in the absence of either of these mutants. Such a large or repeatable effect was not found with mutant 14 or 22, suggesting that this ability to improve the stress survival of B728a may be specific to mutants MX7 and 94. Hirano et al. (16) also observed a nonreciprocated influence of a mutant derivative of B728a on B728a behavior; whereas their mutant attained similar population sizes in the presence and absence of B728a under field conditions, B728a grew to much smaller population sizes in the presence of their mutant than in its absence.

To determine if the presence of B728a influenced the growth of the mutants in the intercellular spaces of leaves, population sizes were measured after each mutant was mixed with B728a and vacuum infiltrated into leaves. For all of the mutants, the growth rate and the size of the populations in the first 70 h after inoculation were similar in the presence and absence of B728a (Fig. 6).

DISCUSSION

Survival on dry leaf surfaces may be critical to the unique success of epiphytes (38). Of the 82 *P. syringae* pv. syringae epiphytic fitness mutants that we previously identified (29), about 40% were reduced in their ability to tolerate the stresses associated with dry leaf surfaces; mutants 14, 22, 42, and 94 were among those showing the least tolerance. The reduced fitness of these mutants was not specific to the host plant bean, since the mutants also exhibited reduced fitness on the nonhost plant potato. The functions altered in these mutants are thus of interest for their contribution to the general fitness of bacterial epiphytes. Furthermore, since they exhibited attenuated pathogenicity on beans, their altered functions are of interest for their contribution to virulence. Their reduced virulence was probably a result of their reduced population sizes in planta, as suggested by previous studies (5, 17, 18, 20, 24, 39, 42). In this study, we found that these four mutants, two of which were auxotrophs, each exhibited a distinctive range of plant-associated behaviors. These differences indicate that there are multiple traits that influence survival on dry leaf surfaces. If these traits are integrated into a coordinated response to the stresses encountered on dry leaf surfaces, the response must involve at least two distinct loci, specifically those inactivated in the prototrophs 14 and 22.

The localization of epiphytes in microsites that are protected from exposure to harsh physical conditions may contribute to

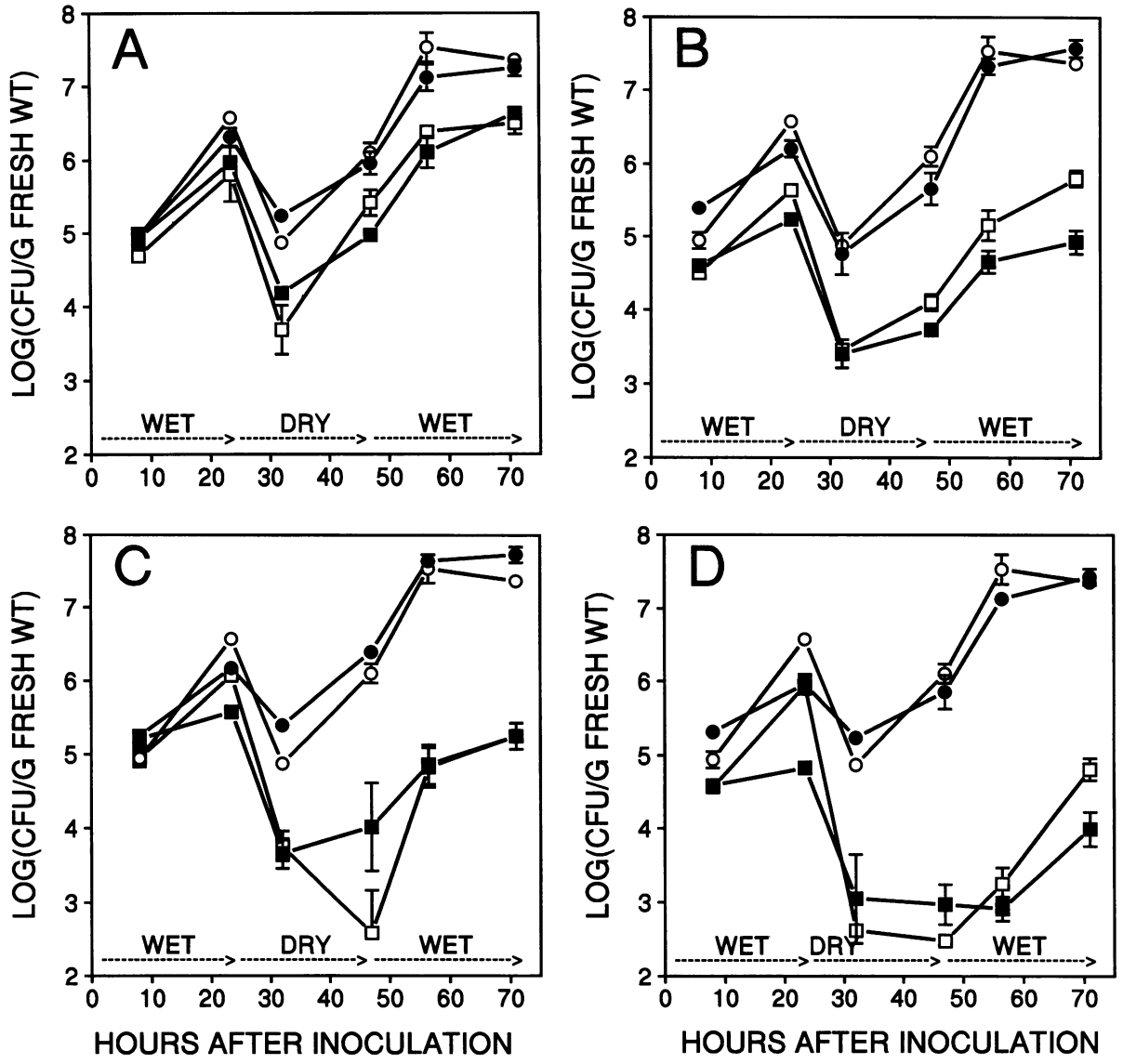

FIG. 5. Population dynamics of *P. syringae* pv. syringae B728a (○, ●) and Tn*5* mutant derivatives (□, ■) 14 (A), 22 (B), MX7 (C), and 94 (D) on bean plants exposed to alternating wet and dry conditions. Population sizes were estimated after each strain was applied alone (open symbols) or in a mixture of the parental strain B728a and a mutant strain (solid symbols). Each point represents the mean ± SE of five leaf samples.

epiphytic fitness. Supporting this hypothesis, one study found a larger proportion of an epiphytic population in internal sites on dry leaves than on wet leaves (38), while others found that on leaves exposed to UV or low relative humidity, surface populations decreased while populations in internal sites remained constant or increased (46, 47). Also, motile cells survived better than nonmotile cells on leaf surfaces exposed to UV radiation (12, 21), probably because motility allowed for active acquisition of protected microsites. Such microsites may be internal spaces, such as intercellular spaces or substomatal cavities, or surface sites, such as crevices. In this study enumeration of the bacteria on bean leaves with and without surface sterilization with peroxide showed that all four of the mutants were poorer at reaching, multiplying in, and/or surviving in sites that were protected from the surface sterilant. This reduced ability is therefore likely to be one factor contributing to their reduced epiphytic fitness. The extent of this contribution depends on the extent of overlap between the sites that were protected from hydrogen peroxide and the sites that were protected from exposure to the dry incubation conditions. The increase in the percentage of cells recovered after peroxide treatment with extended incubation under dry conditions indicates that the sites protected from peroxide were at least a subset of the total protected sites. If the sites protected from peroxide reflected the total number of protected sites, the differences in the sizes of the mutant and the wild-type populations in protected sites before exposure to the dry incubation conditions (approximately 10^4 CFU/g [fresh weight]) were not sufficient to explain the differences in the sizes of the mutant and the wild-type populations after exposure (approximately 10^6 CFU/g [fresh weight]). If, however, the sites protected from peroxide made up only a subset of the total number of protected sites, the size of the populations in protected sites may have been underestimated and the reduced

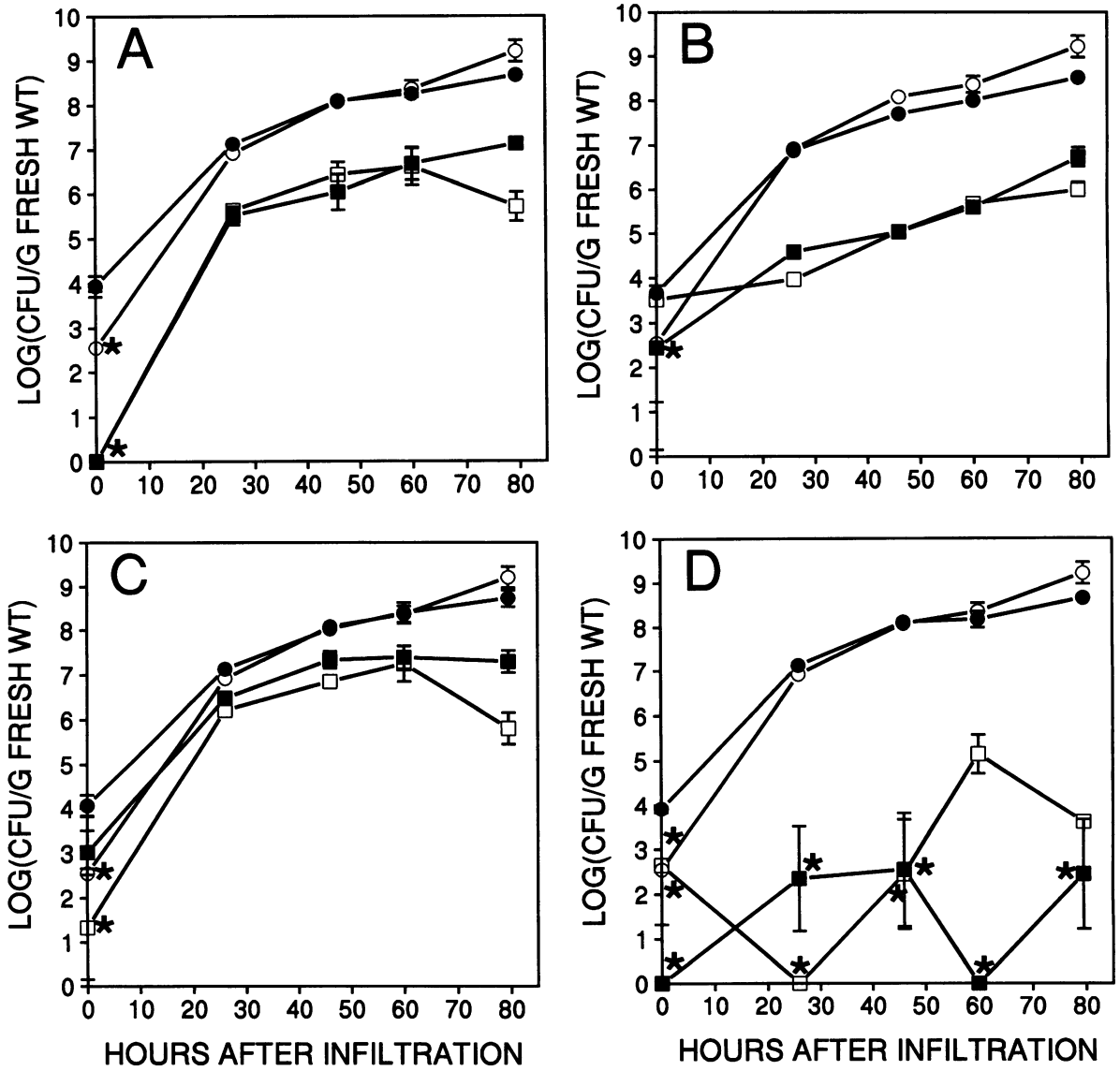

FIG. 6. Population dynamics of *P. syringae* pv. syringae B728a (○, ●) and Tn*5* mutant derivatives (□, ■) 14 (A), 22 (B), MX7 (C), and 94 (D) after vacuum infiltration into the intercellular spaces of leaves. Population sizes were estimated after each strain was applied alone (open symbols) or in a mixture of the parental strain B728a and a mutant strain (solid symbols). Each point represents the mean ± SE of three samples each composed of two 6.5-mm leaf disks. Means indicated by an asterisk were calculated by using log(CFU per gram [fresh weight]) values of zero for leaves with no detectable population.

abilities of the mutants to locate, multiply in, and/or survive in protected sites may, in fact, play a large role in their reduced fitness.

The reduced population sizes of the mutants on moist leaves and in the intercellular spaces could have resulted from a nutritional limitation due to an inability to obtain or synthesize a required nutrient. While mutants 14 and 22 do not have any known anabolic or catabolic deficiencies, mutants 42, MX7, and 94 are amino acid auxotrophs. A methionine limitation could have been responsible for the reduced population sizes of the methionine auxotrophs 42 and MX7. Similarly, a tryptophan limitation was probably responsible for the reduced population sizes of the tryptophan auxotroph 94, since Morgan and Tukey (35) found that tryptophan was among the least abundant amino acids in leaf exudates from many plant species. The substantial growth of mutant 94 on moist bean leaves compared with its poor growth in the intercellular spaces of bean leaves and on moist or dry potato leaves may reflect differences in the available tryptophan levels in those environments. In the 8 h following exposure of the leaves to dry, high-light conditions, all of the strains exhibited net decreases in epiphytic population size; these decreases may have been tempered by the simultaneous growth of subpopulations. It is possible that the decrease in the population size of B728a was smaller than those of mutants MX7, 42, and 94 because B728a grew more than did the mutants during this period and because the growth of the mutants was restricted by an amino acid limitation. However, exogenous application of methionine did not affect the survival of MX7 (2), as would be expected if a methionine limitation restricted growth. A second

possibility is that epiphytic cells of B728a respond to exposure of the leaves to dry, high-light conditions by initiating high levels of protein synthesis that mediate survival; small intracellular pool sizes of methionine and tryptophan in MX7 and 94, respectively, could restrict their ability to synthesize proteins either fast enough or in large enough quantities to mediate survival. These pool sizes may not be affected by exogenous methionine applied at the time of exposure of the plants to the dry, high-light conditions.

Of the many bacterial traits that have been hypothesized to influence epiphytic survival, a large number, including pigments, siderophores, phytotoxins, and exopolysaccharides, are extracellular factors (27). To evaluate whether the mutants were deficient in the in planta production of any extracellular factor that contributes to the epiphytic fitness of strain B728a, we evaluated the fitness of each mutant after inoculation onto leaves in a 1:1 mixture with B728a. Previous studies have demonstrated successful extracellular complementation in planta of a growth-deficient *Xanthomonas campestris* pv. campestris mutant (20). Although there may be barriers that prevented such complementation, the fact that the presence of B728a did not improve the epiphytic fitness of any of the mutants suggests that their reduced fitness was not caused by a deficiency in the production of an extracellular factor.

Pathogenicity may play a role in epiphytic fitness by influencing both bacterial multiplication and survival. Pathogens tend to grow to larger population sizes on host plants than on nonhost plants (1, 11, 37). Supporting these findings, we found that B728a grew to larger epiphytic population sizes on bean leaves than on potato leaves. We cannot, however, distinguish between the possibilities that expression of pathogenicity, or traits correlated with pathogenicity, was causal to abundant epiphytic growth and that the host provided an environment more conducive for bacterial growth than the nonhost did. Similar to previous studies that found that pathogens grew or persisted better on dry leaf surfaces of a host than a nonhost plant (7, 11, 38), we found that B728a survived drying of the leaf surface better on bean leaves than on potato leaves. Thus, the existence of a potential pathogenic relationship was correlated not only with abundant epiphytic growth but also with survival on dry leaf surfaces. In support of this finding, two previous studies showed that pathogenicity-deficient *P. syringae* pv. syringae mutants grew to population sizes similar to those of their parental strains on wet leaves but to reduced population sizes on dry leaves (16, 51).

The behavior of mutants 14, 22, 42, and 94 was strikingly similar to the behavior of several weakly pathogenic and nonpathogenic epiphytes found by Wilson et al. (49, 50). In comparisons among pathogenic *P. syringae* strains and several weakly pathogenic or nonpathogenic strains of *P. syringae*, *Erwinia herbicola*, *Xanthomonas maltophilia*, and *Methylobacterium organophilum*, the weakly pathogenic or nonpathogenic strains were poorer than the pathogens at surviving on dry leaf surfaces, at growing in the intercellular spaces of leaves, and at localizing in sites protected from surface sterilization with peroxide. Young (52) also found that nonpathogenic *Pseudomonas putida* and *Pseudomonas fluorescens* strains grew poorly in the leaf intercellular spaces. The strong similarity between these naturally occurring strains and our mutants suggests that the traits that were inactivated in our mutants may be similar to traits that were absent or poorly expressed in naturally occurring weakly pathogenic or nonpathogenic epiphytes. Such traits could include a low tolerance to plant-produced inhibitory compounds (8, 36), a poor ability to modify the intercellular environment (3), or a poor ability to reach sites favorable for growth or survival.

Among the 82 epiphytic fitness mutants originally identified (29), many exhibited only slight reductions in fitness compared with the large reductions exhibited by mutants 14, 22, 42, and 94. The altered traits in these four mutants thus may be particularly important to epiphytic fitness in the laboratory, as well as under natural conditions, as described in the accompanying report (4). The present study demonstrated the lack of host specificity in the reduced fitness of these mutants, as well as a likely role for an ability to localize in protected sites in the fitness of the parental strain. This study further suggested that extracellular factors do not play a role in the reduced fitness of the mutants. Further studies, currently in progress, will focus on the molecular bases underlying the altered fitness of these mutants.

ACKNOWLEDGMENTS

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

We thank B. Rotz for maintaining the plants used in this study. We also thank M. Wilson for his critical review of the manuscript.

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