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Location and Survival of Leaf-Associated Bacteria in Relation to Pathogenicity and Potential for Growth within the Leaf

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The growth and survival of pathogenic and nonpathogenic *Pseudomonas syringae* strains and of the nonpathogenic species *Pantoea agglomerans*, *Stenotrophomonas maltophilia*, and *Methylobacterium organophilum* were compared in the phyllosphere of bean. In general, the plant pathogens survived better than the nonpathogens on leaves under environmental stress. The sizes of the total leaf-associated populations of the pathogenic *P. syringae* strains were greater than the sizes of the total leaf-associated populations of the nonpathogens under dry conditions but not under moist conditions. In these studies the surface sterilants hydrogen peroxide and UV irradiation were used to differentiate cells that were fully exposed on the surface from nonexposed cells that were in "protected sites" that were inaccessible to these agents. In general, the population sizes in protected sites increased with time after inoculation of plants. The proportion of bacteria on leaves that were in protected sites was generally greater for pathogens than for nonpathogens and was greater under dry conditions than under moist conditions. When organisms were vacuum infiltrated into leaves, the sizes of the nonexposed "internal" populations were greater for pathogenic *P. syringae* strains than for nonpathogenic *P. syringae* strains. The sizes of the populations of the nonpathogenic species failed to increase or even decreased. The sizes of nonexposed populations following spray inoculation were correlated with the sizes of nonexposed, internal populations which developed after vacuum infiltration and incubation. While the sizes of the populations of the pathogenic *P. syringae* strains increased on leaves under dry conditions, the sizes of the populations of the nonpathogenic strains of *P. syringae*, *P. agglomerans*, and *S. maltophilia* decreased when the organisms were applied to plants. The sizes of the populations on dry leaves were also correlated with the sizes of the nonexposed populations that developed following vacuum infiltration. Although pathogenicity was not required for growth in the phyllosphere under high-relative-humidity conditions, pathogenicity apparently was involved in the ability to access and/or multiply in certain protected sites in the phyllosphere and in growth on dry leaves.

Microorganisms on leaf surfaces are subject to various environmental stresses, including fluctuations in relative humidity (RH), extremes of temperature, and both UV and visible light irradiation. Bacteria that colonize leaf surfaces have apparently evolved diverse adaptations which enhance stress tolerance (3, 23); however, survival in the phyllosphere may also be achieved through stress avoidance. Leaf-associated populations may avoid stress through colonization of sites on leaves that are buffered from the external environment of the leaves; these sites have been referred to as protected sites (21). Phytopathogenic bacteria are known to colonize and invade leaves through natural openings, including stomata, hydathodes, and trichomes, as well as through cuticular wounds (8, 12, 27, 28, 41). While bacterial survival has been reported to occur in the substomatal chamber (36) and in broken trichomes (37), the nature, location, availability, and accessibility of other possible protected sites remain unknown. At least for the phytopathogenic bacteria that have been examined, substantial proportions of the leaf-associated populations appear to reside in protected sites (37, 43).

The study of survival on leaves has been biased by concentration on phytopathogenic species, especially species in the genera *Pseudomonas* (9, 13, 14, 15, 22, 28, 31, 42, 47, 52), *Xanthomonas* (6, 30), and *Erwinia* (5). In particular, the phytopathogenic bacterium *Pseudomonas syringae* has received much attention, both because of the diseases that it causes and because of the role played by ice nucleation-active strains in frost injury (16). Following inoculation of a pathogenic *P. syringae* strain onto bean leaves in the field or in a growth chamber under low-RH conditions, the size of the bacterial population that can be removed by sonication and washing (the so-called epiphytic population [15]) decreases and then increases to a higher, stable level, often termed the carrying capacity of the leaf (1, 46–48). The initial decline in population size may reflect the death of the cells exposed to the harshest physical environment of a leaf (46), while the increase in population size may represent multiplication of the remaining viable cells, presumably in sites more conducive to cell survival.

Phytopathogenic bacteria survive in the phyllospheres of both host and nonhost plants (32); however, the majority of studies that have been conducted have indicated that both colonization (1, 6, 7, 30, 31, 39) and survival (1, 5, 9, 13, 50, 52) in the phyllosphere are greater on compatible host plants than on incompatible or nonhost plants. These studies suggested that pathogenicity (or associated phenotypes) may be involved in colonization of healthy leaves under some environmental conditions (32). In other cases, however, colonization and survival characteristics of phytopathogenic bacteria on compatible and incompatible hosts were similar (11, 28, 35). For example, O'Brien and Lindow (35) observed no difference in the average population sizes of *P. syringae* strains on several compatible and incompatible host plants under controlled environmental

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TABLE 1. Sources of bacterial strains used in this study, pathogenicities on *P. vulgaris*, and isolation media

Strain	Source	Pathogenicity on *P. vulgaris*	Isolation medium[a]
Pseudomonas syringae pv. syringae B728a	Snap bean	Pathogen (brown spot)	KBR
Pseudomonas syringae pv. syringae 9B1	Snap bean	Pathogen (brown spot)	MS
Pseudomonas syringae pv. syringae 5B-530	Snap bean	Pathogen (brown spot)	MS
Pseudomonas syringae pv. syringae 5B-333	Snap bean	Pathogen (brown spot)	MS
Pseudomonas syringae pv. phaseolicola PpSG44	Snap bean	Pathogen (halo blight)	MS
Pseudomonas syringae pv. syringae NPS3136	Snap bean	Non-lesion-forming mutant (*lemA*::Tn*5*)	KBR
Pseudomonas syringae MF714R	Snap bean	Weak pathogen or nonpathogen	KBR
Pseudomonas syringae 5B1143	Snap bean	Nonpathogen	MS
Pseudomonas syringae 1063	Potato	Nonpathogen	MS
Pseudomonas syringae TLP2	Potato	Nonpathogen	KBR
Pseudomonas syringae Cit7	Citrus	Nonpathogen	KBR
Pantoea agglomerans (*Erwinia herbicola*) WHL9	Hawthorn	Nonpathogen	KBN
Stenotrophomonas maltophilia (*Xanthomonas maltophilia*) BP1	Potato	Nonpathogen	KBE
Methylobacterium organophilum SH1PK	Snap bean	Nonpathogen	Minimal + methanol

[a] KBR, KB amended with 100 μg of rifampin per ml; KBN, KB amended with 50 μg of nalidixic acid per ml; KBE, KB amended with 30 μg of erythromycin per ml; MS, selective medium of Mohan and Schaad.

conditions. It has been suggested that the larger populations observed on compatible plant species than on incompatible plant species may have been attributable to the presence of lesions which were not visible and that the bacteria were not entirely superficial and the leaves, therefore, were not entirely asymptomatic (healthy) (35). A recent review of the biology of leaf-associated bacteria identified the locations of cells in or on leaves and the factors that lead to the distribution of the cells as important unanswered questions for this group of organisms (4). It was suggested in this review that pathogens may occupy the interior of a leaf more commonly than nonpathogens occupy the interior. In this study we examined the possible role of pathogenicity in colonization by and survival of leaf-associated bacteria. The goal of this study was to determine whether phytopathogenic bacteria on a compatible host and nonpathogens on the same host are found inside leaves in equal numbers and proportions.

The objectives of this study were (i) to compare the effectiveness of hydrogen peroxide (H_2O_2) and the effectiveness of UV irradiation as sterilants for killing bacteria on leaf surfaces; (ii) to determine whether strains of bacteria that are able to cause disease on bean differ from strains that cannot cause disease with respect to the numbers of cells (i.e., population sizes) or the proportions of cells in exposed sites and nonexposed, protected sites (exposed sites were defined as sites that were accessible to the bactericidal effect of a sterilant, and protected sites were defined as sites that were not accessible to the bactericidal effect of a sterilant); (iii) to compare, under growth chamber conditions, the effect of RH on the number and proportion of cells in protected sites; (iv) to determine whether the number or proportion of cells in protected sites was correlated with the ability of various bacterial strains to grow in leaves following vacuum infiltration; and (v) to determine whether survival under low-RH conditions was correlated with the ability of bacterial strains to grow in leaves following vacuum infiltration.

MATERIALS AND METHODS

Bacterial strains. In this study we used pathogenic strains of *P. syringae* pv. syringae (the causal agent of brown spot of bean) and *P. syringae* pv. phaseolicola (the causal agent of halo blight of bean), nonpathogenic *P. syringae* strains, and the nonpathogenic species *Pantoea agglomerans* (previously *Erwinia herbicola*), *Stenotrophomonas maltophilia* (previously *Xanthomonas maltophilia*), and *Methylobacterium organophilum* (Table 1).

Inoculum preparation and plant inoculation. Most bacterial strains were cultured on King's medium B (KB) (20) for 18 h at 28°C; the only exception was *M. organophilum*, which was grown for 72 h. Bacterial cells were removed from plates and suspended in 0.01 M potassium phosphate buffer (pH 7.0). The concentrations of cells in bacterial suspensions were estimated turbidimetrically, as described previously (35), and were adjusted to the appropriate values by dilution with phosphate buffer. Bean plants (*Phaseolus vulgaris* cv. Bush Blue Lake 274) that were about 2 weeks old and had only primary leaves were spray inoculated to runoff (ca. 1.0 ml/leaf) with suspensions of the bacteria (10^6 CFU/ml for colonization studies and 10^8 CFU/ml for survival studies). Each treatment was replicated with five pots of plants (10 plants per pot). After inoculation, the bean plants were either covered with plastic bags to maintain a high RH or, when appropriate, left uncovered and placed in a growth chamber at a low RH (ca. 45%) and 26°C with constant illumination. To examine bacterial multiplication in the internal spaces of leaves, bacterial suspensions (10^5 CFU/ml) were vacuum infiltrated into leaves as described previously (45). Plants were then incubated on a greenhouse bench at ca. 24°C until samples were collected. Each treatment was replicated with two pots of plants (five plants per pot).

Surface sterilization of leaves. Leaves were surface sterilized by using either H_2O_2 (40) or UV irradiation (19). Surface sterilization with H_2O_2 was accomplished by placing individual leaves (10 leaves per treatment) in a beaker containing 150 ml of a 15% (vol/vol) H_2O_2 solution. The beaker was covered on all sides with aluminum foil to prevent exposure to light. The leaves were treated for 5 min with gentle shaking on a rotary shaker. The leaves were rinsed in sterile distilled water and then dried in a laminar flow hood for 1 h. Surface sterilization with UV radiation was accomplished by exposing both the adaxial and abaxial surfaces of leaves (10 leaves per treatment) to UV (254-nm) irradiation at a flux of 1,000 ergs/m^2/s for 30 s.

Enumeration of bacteria. The sizes of total bacterial populations associated with leaves were determined by homogenizing individual leaves for 15 s in 20 ml of sterile wash buffer (0.1 M potassium phosphate buffer [pH 7.0] amended with 0.1% Bacto-Peptone [Difco]) in a blender. Internal population sizes were determined by homogenizing leaves that had been surface sterilized with H_2O_2. Leaf surface population sizes were determined by placing leaves individually into tubes containing 20 ml of wash buffer, sonicating them for 7 min in an ultrasonic cleaning bath to dislodge the cells, and then vortexing the preparation to suspend the bacterial cells, as described previously (35).

Serial dilutions of leaf homogenates or washes were plated onto selective media. *P. syringae* B728a, MF714R, NPS3136, TLP2, and Cit7 were enumerated on KB amended with 100 μg of rifampin per ml. Other *P. syringae* strains were isolated on the selective medium of Mohan and Schaad (34). *P. agglomerans* WHL9 was enumerated on KB amended with 50 μg of nalidixic acid per ml. *M. organophilum* SH1PK was enumerated on minimal medium containing 0.1% (vol/vol) methanol. *S. maltophilia* BP1 was enumerated on KB amended with 30 μg of erythromycin per ml. All media were also amended with 100 μg of cycloheximide per ml and 50 μg of benomyl per ml to suppress fungal growth.

Bacterial population sizes were log transformed to achieve normality prior to statistical analysis. Mean population sizes were determined by using samples consisting of 10 leaves for each treatment. Mean population sizes of different strains at a given sampling time and the contribution of pathogenicity to the variability in the percentage of cells that survived surface sterilization with H_2O_2 were assessed by performing analysis of variance tests with the PROC GLM procedure in SAS (SAS Institute, Cary, N.C.).

RESULTS

Comparison of methods used to determine cell localization. The numbers and proportions of cells in protected sites on snap bean leaves were determined for two pathogenic *P. syrin-*

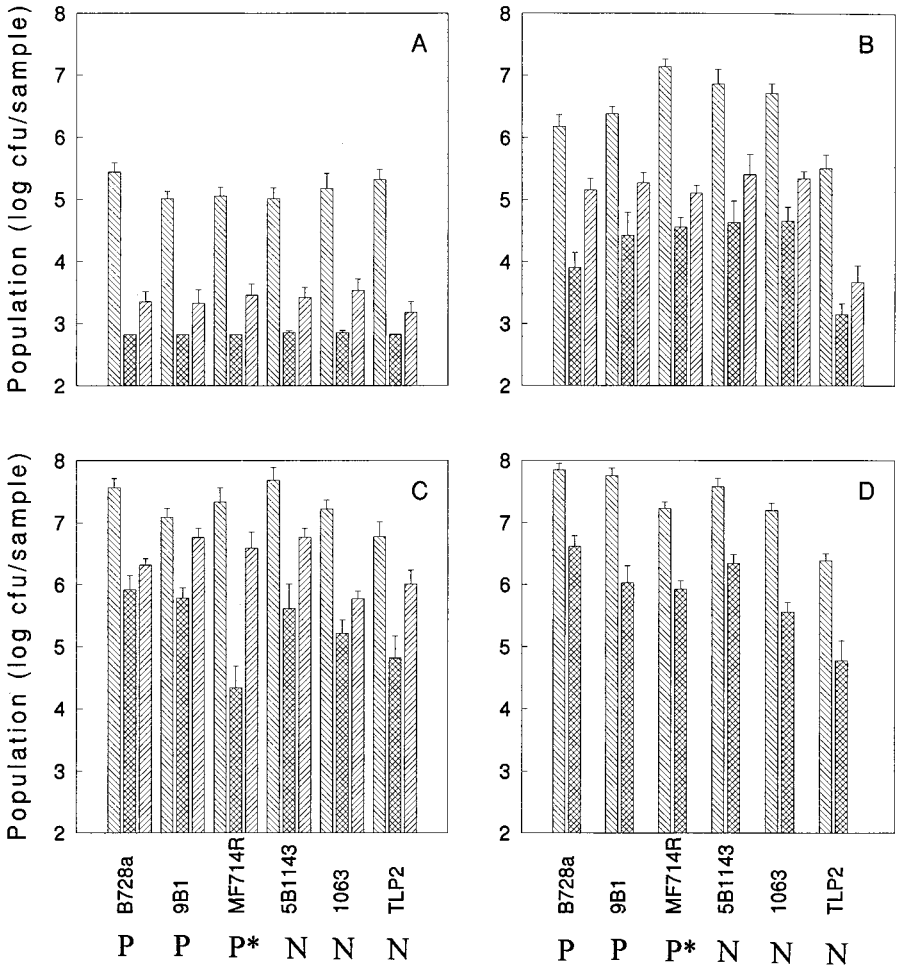

FIG. 1. Total population sizes and population sizes in protected sites of pathogenic and nonpathogenic *P. syringae* strains on bean leaves. Leaves of snap bean were spray inoculated with each *P. syringae* strain (10^6 CFU/ml) and incubated under high-RH conditions. ▧, total population size on bean leaves not treated with sterilants; ▩, population size in protected sites on leaves surface sterilized with H_2O_2; ▨, population size in protected sites on leaves surface sterilized with UV irradiation. (A) Immediately after inoculation. (B) One day after inoculation. (C) Two days after inoculation. (D) Six days after inoculation (no UV irradiation treatment included). P, pathogen; P*, weak pathogen; N, nonpathogen.

gae strains (B728a and 9B1), one weakly pathogenic *P. syringae* strain (MF714R), and three nonpathogenic *P. syringae* strains (5B1143, 1063, and TLP2) by using the H_2O_2 and UV irradiation sterilization techniques (Fig. 1). Treatment with H_2O_2 resulted in the death of a higher proportion of cells than did treatment with UV irradiation for all of the strains (Fig. 1). When assessed 2 days after inoculation, the percentages of total cells on leaves that survived H_2O_2 treatment ranged from 2 to 5% for the strongly pathogenic strains *P. syringae* B728a and 9B1 and from 0.8 to 1.2% for the nonpathogenic strains *P. syringae* 5B1143, 1063, and TLP2. In contrast, the percentages of total cells that survived UV irradiation ranged from 5 to 40% for the strongly pathogenic strains *P. syringae* B728a and 9B1 and from 4 to 16% for the nonpathogenic strains *P. syringae* 5B1143, 1063, and TLP2.

The total population sizes on leaves increased with time and were similar for the pathogenic and nonpathogenic *P. syringae* strains (Fig. 1), although the total population sizes of *P. syringae* TLP2 were consistently lower than those of the other strains. The population sizes in H_2O_2-protected sites and UV irradiation-protected sites also generally increased with time. The mean percentage of *P. syringae* cells that survived surface sterilization of leaves with H_2O_2 increased (for all strains) from 0.5% immediately after inoculation to 0.64, 1.72, and 3.86% 1, 2, and 6 days after inoculation, respectively. Due to great variability in leaves, the mean percentages of cells that survived surface sterilization were not significantly different for the pathogenic and nonpathogenic *P. syringae* strains, except for the samples collected 2 days after inoculation; for the latter samples the percentage of nonpathogenic *P. syringae* cells in protected sites (0.77%) was significantly lower ($P = 0.0353$) than the percentage of pathogenic *P. syringae* cells in protected sites (3.6%).

Effect of RH and pathogenicity on occupancy of protected sites. The numbers and proportions of cells in H_2O_2-protected sites on bean leaves incubated under both high-RH and low-RH conditions were compared further by using four pathogenic strains (*P. syringae* pv. syringae B728a, 5B-530, and 5B-333 and *P. syringae* pv. phaseolicola PpSG44), the Tn*5* non-lesion-forming mutant *P. syringae* pv. syringae NPS3136 (derived from B728a), and the nonpathogenic strains *P. syringae* Cit7, *P. agglomerans* WHL9, *S. maltophilia* BP1, and *M. organophilum* SH1PK (Fig. 2 and 3). The total population sizes increased with time when plants were incubated under

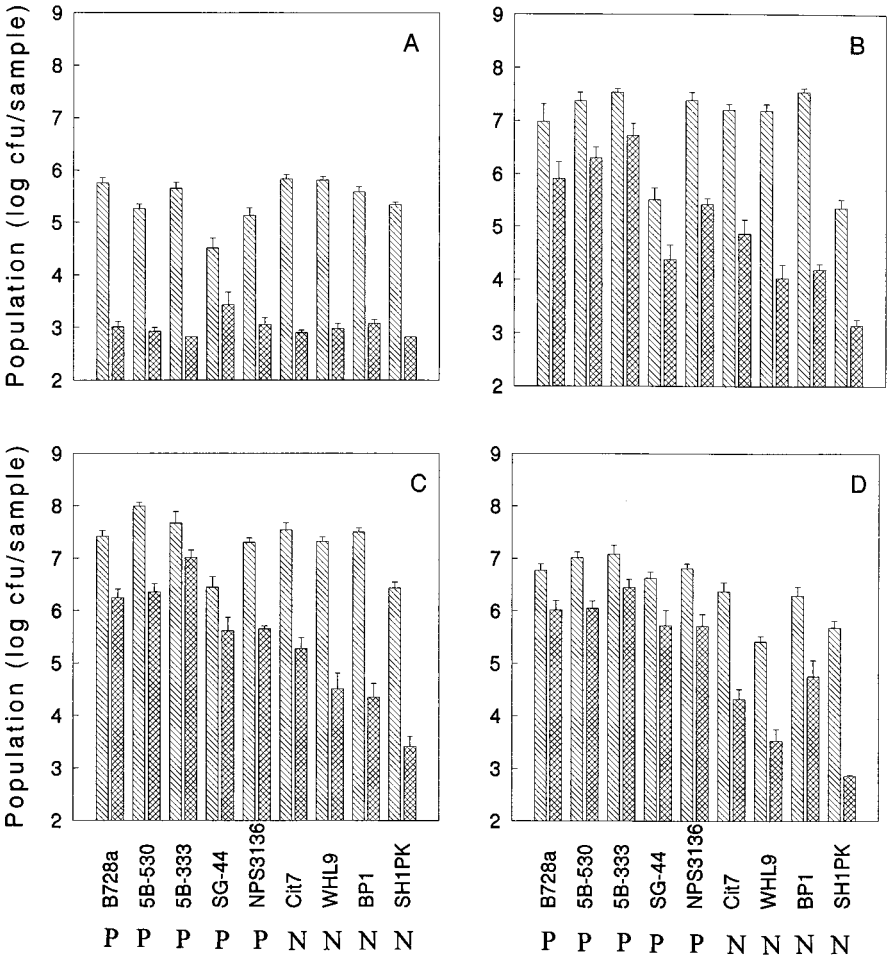

FIG. 2. Total population sizes and population sizes in H_2O_2-protected sites of pathogenic and nonpathogenic *P. syringae* strains and the nonpathogens *P. agglomerans*, *S. maltophilia*, and *M. organophilum*. Leaves of snap bean were spray inoculated with each *P. syringae* strain (10^6 CFU/ml). ▧, total population size on bean leaves not treated with eradicants; ▨, population size in protected sites on leaves surface-sterilized with H_2O_2. (A) Immediately after inoculation. (B) After 2 days of incubation under high-RH conditions. (C) After 3 days of incubation under high-RH conditions. (D) Three days after inoculation (after incubation for 2 days under high-RH conditions and for 1 day under low-RH conditions). P, pathogen; N, nonpathogen.

high-RH conditions (Fig. 2A through C) but decreased when the preparations were exposed to low-RH conditions subsequent to the high-RH conditions (Fig. 2C and D). The population sizes in H_2O_2-protected sites under both high- and low-RH conditions were generally higher for the pathogenic *P. syringae* strains than for the nonpathogens (Fig. 2). The population sizes in H_2O_2-protected sites, however, were similar for the pathogenic *P. syringae* strains and for the non-lesion-forming mutant NPS3136 (Fig. 2).

The percentages of the cells in H_2O_2-protected sites generally increased with time under high-RH conditions (Fig. 3). On average, when all strains were considered, the percentage of all cells that survived surface sterilization increased from 1.16% immediately after inoculation to 4.65 and 5.44% after 2 and 3 days of incubation, respectively, under high-RH conditions (Fig. 3A through C). The percentage of cells in H_2O_2-protected sites under high-RH conditions was significantly greater for the pathogenic *P. syringae* strains than for the nonpathogens (Fig. 3B and C). The average percentage of cells that survived 2 days after inoculation under high-RH conditions was 8.12% for the pathogens, compared to 0.32% for the nonpathogens ($P = 0.0192$), and 3 days after inoculation the average percentage of cells that survived was 9.63% for the pathogens and 0.21% for the nonpathogens ($P = 0.0681$). The mean percentage of cells of all strains in H_2O_2-protected sites was generally lower following incubation under high-RH conditions than following incubation under low-RH conditions (5.44 and 8.57%, respectively) (Fig. 3C and D). The percentage of cells of pathogenic strains that survived surface sterilization under low-RH conditions was also significantly greater ($P = 0.0034$) than the percentage of cells of the nonpathogens that survived surface sterilization under low-RH conditions (14.4 and 1.28%, respectively) (Fig. 3D).

Internal growth of bacteria introduced into leaves. Following vacuum infiltration into bean leaves and incubation in the greenhouse, the pathogenic strains *P. syringae* B728a, 9B1, 5B-333, and PpSG44 exhibited similar initial growth rates inside the leaves (although *P. syringae* pv. phaseolicola PpSG44 seemed to grow a little more slowly than the *P. syringae* pv. syringae strains), and the final sizes of the populations after 3 days of incubation were similar (Fig. 4A). The size of the internal population of the non-lesion-forming mutant NPS3136 was similar to the sizes of the internal populations of the pathogenic *P. syringae* strains when the organisms were vacuum infiltrated (Fig. 4B). The size of the population of the nonpathogenic or weakly pathogenic strain *P. syringae*

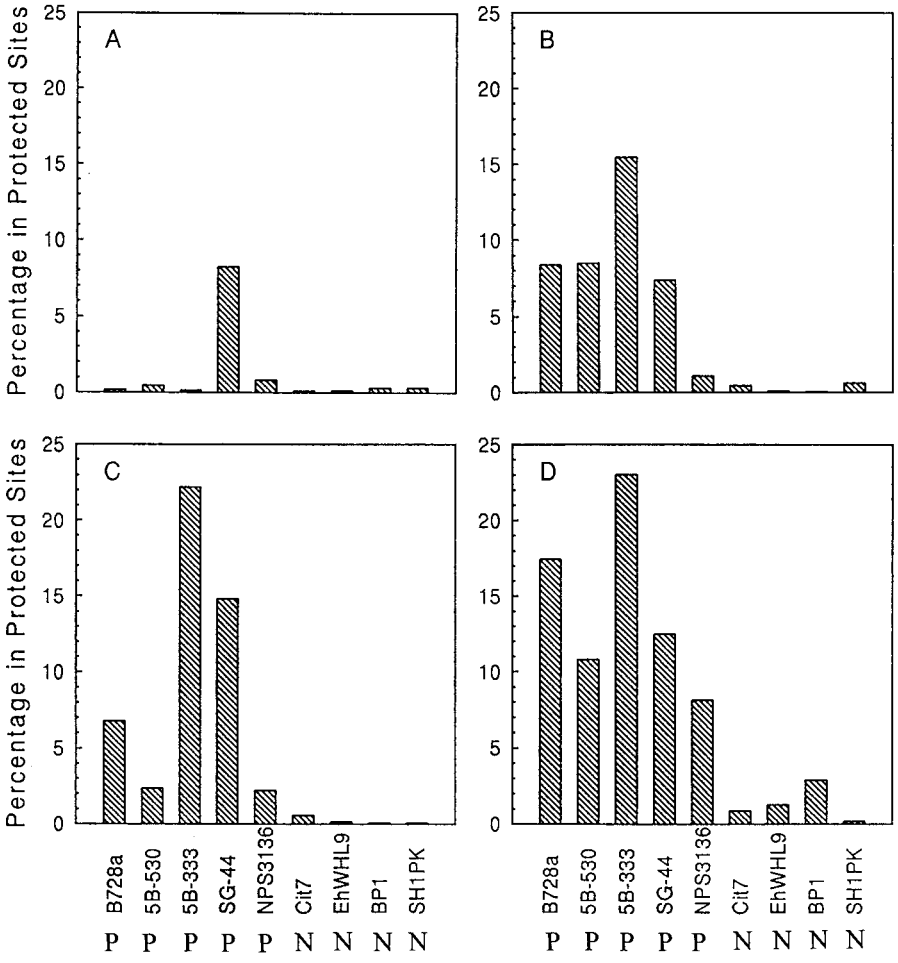

FIG. 3. Percentages of populations of pathogenic and nonpathogenic *P. syringae* strains and the nonpathogens *P. agglomerans*, *S. maltophilia*, and *M. organophilum* in H_2O_2-protected sites on bean leaves. (A) Immediately after inoculation. (B) After 2 days of incubation under high-RH conditions. (C) After 3 days of incubation under high-RH conditions. (D) Three days after inoculation (after incubation for 2 days under high-RH conditions and for 1 day under low-RH conditions). (P, pathogen; N, nonpathogen.

MF714R increased at a similar initial rate, but the final size was significantly smaller than the sizes of the populations of the other pathogenic strains (Fig. 4A). In contrast, the maximum internal population sizes of the nonpathogenic strains *P. syringae* 5B-1143, 1063, and Cit7 were significantly smaller than the maximum internal population sizes of all of the pathogenic strains, and strain TLP2 failed to grow in the leaves (Fig. 4B). The initial population sizes of the other nonpathogenic organisms, *P. agglomerans* WHL9 and *S. maltophilia* BP1, decreased slightly following infiltration, and the initial population size of *M. organophilum* SH1PK decreased to below the detection limit (Fig. 4C).

The numbers of cells in nonexposed sites following spray inoculation were significantly correlated ($R^2 = 0.839$; $P = 0.0005$) with the numbers of cells in nonexposed sites following vacuum infiltration (Fig. 5). However, the population sizes in nonexposed sites did not conform to the expected 1:1 relationship which would have occurred if the nonexposed cells had colonized the same protected sites. The sizes of the populations of the nonpathogens (the organisms with the smallest populations following vacuum infiltration) in nonexposed sites were larger than expected when the organisms were spray inoculated, while the sizes of the populations of the pathogens (the organisms with the largest populations following vacuum infiltration) were smaller than expected when the organisms spray inoculated.

Role of pathogenicity in survival during desiccation stress on leaves. The ability to survive desiccation stress was investigated with the pathogenic strains *P. syringae* B728a and MF714R, the non-lesion-forming mutant NPS3136, and the nonpathogenic strains *P. syringae* TLP2 and Cit7, *P. agglomerans* WHL9, and *S. maltophilia* BP1 (Fig. 6). Bacteria were inoculated onto plants and incubated under low-RH conditions without preincubation under high-RH conditions. The leaf-associated population sizes of the pathogenic *P. syringae* strains (determined following sonication and washing) decreased and then started to increase again within about 6 to 12 h (Fig. 6) in all experiments. The population size of the non-lesion-forming mutant *P. syringae* NPS3136 decreased to the same level as the population sizes of the pathogenic strains but did not subsequently increase to the same extent. The decrease in the number of viable cells of the nonpathogenic *P. syringae* strains with time was similar to the decrease in the number of viable cells of the pathogenic strains, but the population size of the nonpathogenic strains decreased continuously before it reached a lower constant level (Fig. 6). The nonpathogenic strains *P. agglomerans* WHL9 and *S. maltophilia* BP1 exhibited lower apparent death rates than the *P.*

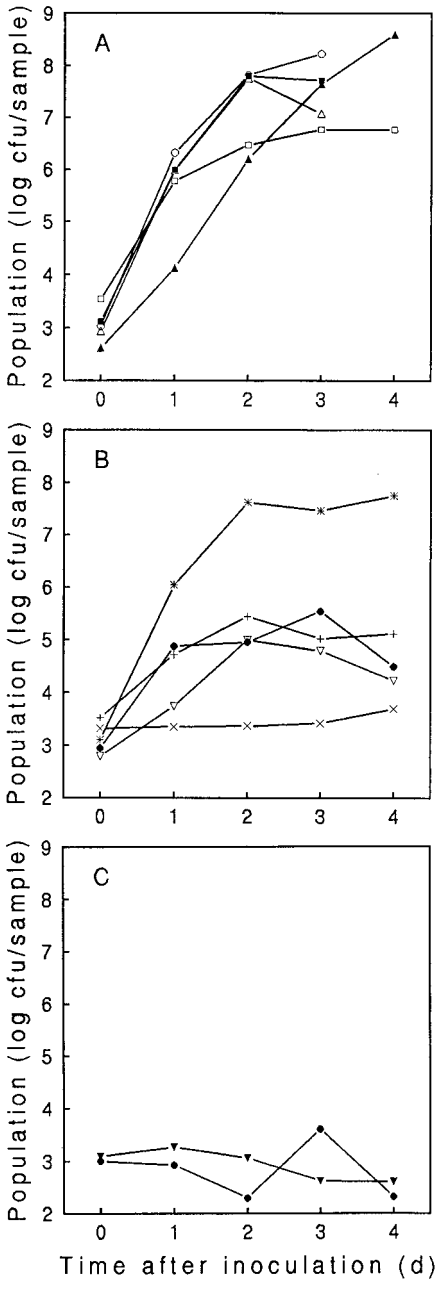

FIG. 4. Internal population sizes of pathogenic and nonpathogenic *P. syringae* strains and nonpathogens following vacuum infiltration into bean leaves and incubation in the greenhouse. (A) Pathogenic strains *P. syringae* B728a (■), 9B1 (○), 5B-333 (△), and PpSG44 (▲) and weakly pathogenic strain MF714R (□). (B) Nonpathogenic strains *P. syringae* 5B1143 (●), 1063 (▽), Cit7 (+), TLP2 (×), and NPS3136 (∗). (C) Nonpathogens *P. agglomerans* WHL9 (●) and *S. maltophilia* BP1 (▼). d, days.

syringae strains exhibited in one experiment (Fig. 6A) but similar death rates in another experiment (Fig. 6B); the population sizes either decreased continuously or reached lower constant levels in different experiments.

Survival on dry leaves was related to the ability of strains to colonize the leaf interior following vacuum infiltration. The number of cells washed from leaves following spray inoculation and 48 h of incubation under dry conditions was correlated ($R^2 = 0.529$; $P = 0.064$) with the population size in nonexposed

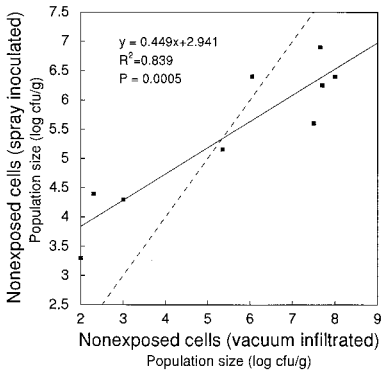

FIG. 5. Correlation between population size of nonexposed, H_2O_2-protected cells in snap bean leaves following vacuum infiltration and incubation in the greenhouse (Fig. 4, day 3) and population size of nonexposed cells following spray inoculation and incubation under high-RH conditions (Fig. 2C, day 3). The dashed line indicates the idealized situation, where population sizes in protected sites in plants following spray inoculation equaled population sizes in plants following vacuum infiltration.

sites following vacuum infiltration and 72 h of incubation in the greenhouse (Fig. 7).

DISCUSSION

Methods for determining cell localization. The use of surface sterilization techniques allowed us to investigate the role of internal populations in survival of and colonization by pathogenic *P. syringae* strains, nonpathogenic *P. syringae* strains, and other nonpathogenic species in the phyllosphere of snap bean leaves. Leaf surface sterilization with H_2O_2 killed a higher proportion of cells than UV irradiation killed, possibly because cells in sites such as trichomes (37) and stomata (36) were shielded from UV irradiation but were exposed to H_2O_2. The reduction in the total leaf-associated bacterial population size (as determined by homogenization) caused by surface sterilization with H_2O_2 was greater than the reduction caused by transferring plants from high-RH conditions to low-RH conditions, suggesting that more cells were located in sites protected from desiccation than in sites protected from H_2O_2 surface sterilization. Alternatively, desiccation stress may be less damaging to cells than exposure to H_2O_2 is, enabling at least some of the exposed cells to survive.

While surface sterilization techniques allowed us to discriminate between exposed and nonexposed populations on leaves, the results gave no indication of the location of surviving cells. Scanning electron microscopy can provide such spatial information (8, 12, 27, 36, 38, 41), but the results may be subject to artifacts resulting from the relocation of cells during preparation procedures. Information on the location of cells protected from surface sterilization could provide insight into the ecology of leaf-associated phytopathogenic bacteria, as well as information on the processes by which these bacteria cause disease (4).

Effect of RH and pathogenicity on occupancy of protected sites and survival in the phyllosphere. Our results suggest that the pathogenic *P. syringae* strains used in this study may have had access to or may have been able to colonize certain parts of the leaves of the compatible host snap bean which were not available to or could not be colonized by the nonpathogenic leaf-associated bacteria. With the bacteria tested, survival and/or multiplication in protected sites in the snap bean phyllosphere was correlated with pathogenicity. Although the total

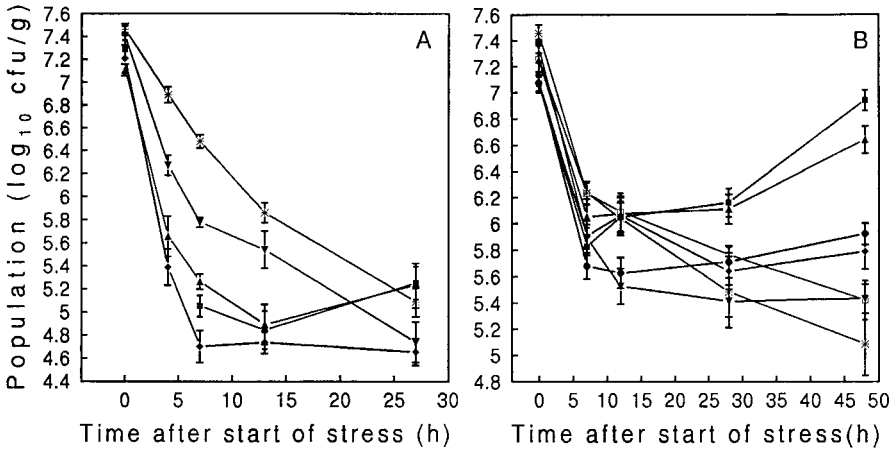

FIG. 6. Survival of pathogenic and nonpathogenic *P. syringae* strains and nonpathogens on bean leaves subjected to desiccation stress in two separate experiments (A and B). Leaves of snap bean were inoculated with a 10^8-CFU/ml suspension of each strain. The plants were then placed in a growth chamber at low (45%) RH. The population sizes of the pathogenic strains *P. syringae* B728a (■) and MF714R (▲), the nonpathogenic strains *P. syringae* NPS3136 (●), TLP2 (◆), and Cit7 (×), and the nonpathogens *P. agglomerans* WHL9 (▼) and *S. maltophilia* BP1 (∗) were determined.

leaf-associated population sizes (as determined by homogenization) were similar for pathogens and nonpathogens under high-RH conditions, under low-RH conditions the total leaf-associated population sizes of the pathogenic strains were consistently greater than the total leaf-associated population sizes of the nonpathogens. Furthermore, only the pathogenic *P. syringae* strains were able to multiply on leaves in the absence of surface moisture (i.e., under continuous low-RH conditions). Much of the multiplication presumably occurred in sites not fully exposed to the surface of the leaf, because the number of cells and the proportion of the total bacterial population in such sites were both greater for the pathogenic *P. syringae* strains than for the nonpathogens. As observed previously (10, 51), the extent of colonization of the internal leaf tissues following vacuum infiltration and incubation was greater for pathogens than for nonpathogens. The size of the nonexposed population following vacuum infiltration, which reflected the internal colonization potential of the strain, was correlated both with the ability to access or multiply in nonexposed sites following spray inoculation (Fig. 5) and with the ability to access and/or multiply on dry leaf surfaces (Fig. 7). Hence, although pathogenicity was not necessary for phyllosphere colonization under high-RH conditions (i.e., in the absence of environmental stress), the ability to multiply and/or survive on leaves under low-RH conditions (i.e., under stressful environmental conditions) was greater for pathogens than for nonpathogens. Differential survival of pathogenic and nonpathogenic *P. syringae* strains on leaves could be one factor accounting for interspecific shifts in communities of *P. syringae* strains on susceptible host plants under fluctuating environmental conditions in the field (16).

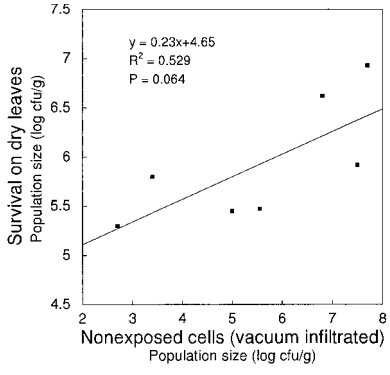

FIG. 7. Correlation between population size of nonexposed, H_2O_2-protected cells in snap bean leaves following vacuum infiltration and incubation in the greenhouse (Fig. 4, day 3) and total population size following spray inoculation and incubation under low-RH conditions (Fig. 6B, 50 h).

While the conclusion that pathogenicity may be correlated with survival in the phyllosphere under stressful environmental conditions conflicts with the results of the previous laboratory studies of O'Brien and Lindow (35), it is supported by the results of several other studies performed with both naturally occurring strains (5, 9, 13, 50, 52) and mutant strains (1, 2, 24, 45, 49). The population sizes of both the compatible pathogen *P. syringae* pv. syringae and the incompatible pathogen *P. syringae* pv. morsprunorum decreased following inoculation onto *Prunus* leaves; however, only the compatible pathogen subsequently multiplied on leaves (52). As in this study, the nonpathogenic epiphytes *Pseudomonas fluorescens* and *Erwinia herbicola* exhibited behavior similar to that of incompatible strains of the pathogen (52). Similarly, a bean isolate of *P. syringae* pv. syringae did not survive as well as a pear isolate of this pathogen after inoculation onto pear leaves under low-RH conditions (50). A comparison of nonpathogenic mutants with nearly isogenic pathogenic parental strains permitted direct assessment of the contribution of pathogenicity to epiphytic fitness. Nonpathogenic mutants of *P. syringae* subsp. savastanoi did not multiply on olive leaves as well as the parental pathogenic strains multiplied (44). Similarly, nonpathogenic mutants of *P. syringae* pv. syringae did not survive as well as the parental strain on pear leaves (49), and mutants of *P. syringae* pv. syringae B728a which were less virulent than the parental strains were less able to multiply on bean leaves both in a growth chamber under low-RH conditions (1, 24) and in the field (2) than the parental strain. Hence, pathogenicity of these phytopathogenic bacteria was apparently related to their ability to multiply and survive on leaves under stressful environmental conditions.

The correlation of pathogenicity with survival in the phyllosphere under stressful environmental conditions suggests that the epiphytic stage (15) of phytopathogenic *Pseudomonas* spp.

on compatible host plants may involve some colonization of the interior of leaves (26, 35, 52); that is, populations of pathogenic strains on compatible hosts apparently include large numbers of cells that are within leaves and are not epiphytic in the sense in which this term is often used, namely, in the sense of colonizing the leaf surface with no invasion of the underlying leaf tissues. Colonization of nonsymptomatic leaves, therefore, may involve at least some internal colonization of plant tissue that does not result in symptoms. In such a scenario, a strain which is able to effectively colonize the interior of leaves but does not necessarily produce lesions might be expected to be found commonly in protected sites during colonization in the phyllosphere. This implies that pathogenicity but not the ability to form lesions is involved in colonizing internal portions of the plant and in survival.

To test this concept, colonization by and survival of the non-lesion-forming *lemA* (*gacS*) mutant *P. syringae* pv. syringae NPS3136 (45) were compared with colonization by and survival of the parental strain B728a. Strain NPS3136 is not able to form lesions on snap bean (45) and does not produce as much syringomycin and protease as the parent strain produces, but it still elicits the hypersensitive response in tobacco (18). While growth in the phyllosphere under high-RH conditions and internal growth of the mutant following vacuum infiltration apparently were not affected (Fig. 2 and 4), the ability of NPS3136 to access and/or multiply in protected sites was reduced compared to the ability of the pathogenic strain B728a (Fig. 2 and 3). While the proportion of the population of NPS3136 in protected sites was less than the proportion of the population of B728a in protected sites, it was greater than the proportions of the populations of the other nonpathogenic *P. syringae* strains in protected sites. The reduced ability of NPS3136 to access and/or multiply in protected sites and to colonize the dry leaf surface compared to the ability of the parental strain may explain the reduced survival of this strain under field conditions (17). However, the multiple phenotypes altered as a result of disruption of the *lemA* (*gacS*) locus in NPS3136 make it difficult to determine which specific phenotypes contribute to acquisition of or multiplication in protected sites on leaves, and further work will be required to address this question.

Implications for biological control of bacterial diseases. To date, relatively few studies have examined the survival of nonpathogenic bacteria on leaves (50, 52). Because of the growing interest in biological control of foliar bacterial pathogens with leaf-associated bacteria, however, such studies will be needed. In this study, the nonpathogenic species *P. agglomerans*, *S. maltophilia*, and *M. organophilum* were able to access and/or multiply in fewer protected sites than the pathogenic *P. syringae* strains were able to access and/or multiply in. Consequently, these nonpathogens were less able to colonize leaves under stressful environmental conditions than the pathogenic strains were (Fig. 6), an observation which has been made previously (50, 52). In this respect the nonpathogens behaved like the nonpathogenic strains *P. syringae* TLP2 and Cit7 in this and other studies (1, 2). The inability of nonpathogenic bacteria to access or multiply in certain protected sites which are accessible to and colonized by pathogenic bacteria could be a serious limitation to the use of these organisms in biological control of bacterial pathogens in the phyllosphere. The high levels of preemptive exclusion of the pathogenic strain *P. syringae* pv. syringae B728a and the subsequent control of brown spot disease by NPS3136 in the greenhouse (25) may have been due to the ability of strain NPS3136 to access some of the same internal sites as the pathogen accessed.

Phyllosphere survival in nonpathogenic species. Although the nonpathogens were not able to access and/or multiply in as many protected sites, these organisms exhibited lower death rates than *P. syringae* under low-RH conditions (Fig. 6). The superior stress tolerance of *P. agglomerans* compared to *P. syringae* has been noted previously (29). It is possible that while pathogenic leaf-associated bacteria survive stress primarily by avoidance in refuges on leaves, nonpathogens have developed superior stress tolerance mechanisms, since they cannot access as many of these sites. While survival has been studied previously in pathogens, the adaptive phenotypes may in fact be better developed in nonpathogenic bacteria and in the future should be studied in these organisms (3, 4, 23).

Terminology and the location of cells. While the widely used functional definition of an epiphyte as any cell washed from the aerial portions of a plant (15) is a convenient way to describe plant-associated bacteria, in some cases a large proportion of the cells may not be on the surface of the plant, as the term often implies when it is used in common parlance. Many of the cells washed from leaf surfaces may be removed from protected sites on or in leaves. The distinction between epiphytic cells and internally located or endophytic cells is unclear in this situation. The habitat of epiphytic bacteria could thus include the substomatal chamber, in which pathogenic bacteria may multiply prior to egress onto the surface (33, 36), and the interiors of broken trichomes, in which cells may reside (37). Such possibilities suggest that the term phyllosphere, which incorporates these locations, is perhaps more appropriate than the term phylloplane, which implies a surface, in discussions of epiphytic bacteria.

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