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Contribution of the Regulatory Gene *lemA* to Field Fitness of *Pseudomonas syringae* pv. syringae

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In *Pseudomonas syringae* pv. syringae, *lemA* is required for brown spot lesion formation on snap bean and for production of syringomycin and extracellular proteases (E. M. Hrabak and D. K. Willis, J. Bacteriol. 174: 3011–3022, 1992; E. M. Hrabak and D. K. Willis, Mol. Plant-Microbe Interact. 6:368–375, 1993; D. K. Willis, E. M. Hrabak, J. J. Rich, T. M. Barta, S. E. Lindow, and N. J. Panopoulos, Mol. Plant-Microbe Interact. 3:149–156, 1990). The *lemA* mutant NPS3136 (*lemA1*::Tn*5*) was previously found to be indistinguishable from its pathogenic parent B728a in its ability to grow when infiltrated into bean leaves of plants maintained under controlled environmental conditions (Willis et al., Mol. Plant-Microbe Interact. 3:149–156, 1990). We compared population sizes of NPS3136 and B728aN (a Nal^r clone of wild-type B728a) in two field experiments to determine the effect of inactivation of *lemA* on the fitness of *P. syringae* pv. syringae. In one experiment, the bacterial strains were spray inoculated onto the foliage of 25-day-old bean plants. In the other, seeds were inoculated at the time of planting. In both experiments, the strains were inoculated individually and coinoculated in a 1:1 ratio. NPS3136 and B728aN achieved similar large population sizes on germinating seeds. However, in association with leaves, population sizes of NPS3136 were diminished relative to those of B728aN in both experiments. Thus, *lemA* contributed significantly to the fitness of *P. syringae* pv. syringae in association with bean leaves but not on germinating seeds under field conditions. When NPS3136 was coinoculated with B728aN, the mutant behaved as it did when inoculated alone. However, population sizes of B728aN in the coinoculation treatment were much lower than those when it was inoculated alone. Inactivation of the *lemA* gene appeared to have rendered the mutant suppressive to B728aN.

The *lemA* (for lesion manifestation) gene was originally identified by its requirement for formation of bacterial brown spot lesions on snap bean (*Phaseolus vulgaris* L.) by *Pseudomonas syringae* pv. syringae B728a (30). Sequence analysis of *lemA* revealed similarity of the predicted product to the transmembrane histidine kinase portion of a family of bacterial two-component regulatory systems (14). In these systems, the kinase is thought to serve as a sensor of environmental stimuli. The signal is then relayed to a cytoplasmic response regulator which, in turn, mediates changes in transcription upon phosphorylation by the sensor protein. The *gacA* gene has been identified as encoding the cognate response regulator for the *lemA* sensor (28, 31). The *gacA* (for global antibiotic and cyanide control) gene was first described for *Pseudomonas fluorescens* as a regulator of production of a number of secondary metabolites that contribute to its biocontrol activity against a fungal root rot disease of tobacco (18). Homologs of *lemA* and/or *gacA* have since been identified in a number of pseudomonads, including other pathovars of *P. syringae* (1, 27) and other species of *Pseudomonas* (4, 7, 17, 20).

The *lemA* mutant NPS3136 (*lemA1*::Tn*5*) was derived from the wild-type strain B728a by transposon mutagenesis (30). Although NPS3136 is no longer able to cause brown spot disease, the mutant was found to be indistinguishable from B728a in its ability to cause the hypersensitive response in tobacco and to grow when either infiltrated into or mist inoculated onto bean leaves in growth chamber assays (30). Indeed, the retention of unimpaired in planta growth ability was an important criterion in the original search for lesion-minus mutants of *P. syringae* pv. syringae B728a by Willis et al. (30). Thus, unlike mutants with mutations in other genes required for pathogenicity, such as the *hrp* (for hypersensitive reaction and pathogenicity) genes, a *lemA* mutant does not suffer from general defects in its ability to grow in planta. The findings of Willis et al. (30) demonstrated that intercellular growth is not sufficient for disease development. Further, the *lemA* mutant NPS3136 appeared to be as fit as its pathogenic parent on bean plants under those controlled environmental conditions frequently used to assess growth and pathogenicity of this bacterium (30).

As might be expected from the regulatory role of *lemA*, a *lemA* mutant exhibits a pleiotropic phenotype. In addition to being deficient in lesion formation, NPS3136 no longer produces extracellular proteases or the peptide antibiotic syringomycin (15). Syringomycin has been implicated as a virulence factor that is not absolutely required for pathogenicity in disease caused by *P. syringae* pv. syringae on cherry (33). The isolation of other mutants that are similar to B728a in lesion-forming ability but do not produce syringomycin or proteases suggests that these factors are not required by B728a to cause brown spot lesions on bean (15).

Under field conditions, disease causation is but one aspect of the life strategy of *P. syringae* pv. syringae (12). The bacterium is known to grow and survive in association with asymptomatic leaves of snap bean plants (6, 21). As population sizes of the bacterium increase, so does the likelihood of occurrence of bacterial brown spot lesions (11, 21, 22, 29). For example, Lindemann et al. (22) found that the frequency with which population sizes of *P. syringae* pv. syringae exceeded approximately 10^4 CFU per asymptomatic bean leaflet was predictive

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of brown spot disease incidence measured a week after the pathogen population sizes were measured. Fifty percent effective doses of roughly 10^5 CFU per leaflet were estimated for the brown spot disease in field experiments (29). In microclimate modification experiments conducted in the field setting, we found that raindrops with sufficient momentum trigger the onset of rapid growth of the pathogen in association with bean leaves (8). Hence, the development of the relatively large population sizes of *P. syringae* pv. syringae that lead to disease is frequently a response to intense rains.

In general, substantial progress has been made in understanding the molecular and biochemical natures of two-component regulatory systems in many bacterial species (13). However, little is known about the extent to which these genes affect the behavior of bacteria in their natural habitats. Because of the substantial understanding of the interaction of *P. syringae* pv. syringae with bean plants in both laboratory and field environments, this system provides an excellent opportunity to bridge the enormous gap between our understanding of plant-bacterial interactions in the laboratory and that of the autecology of the pathogen and epidemiology of bacterial diseases in the field. Hence, the objective of this study was to determine the contribution of the regulatory gene *lemA* to fitness of *P. syringae* pv. syringae B728a in association with bean plants in the field setting.

MATERIALS AND METHODS

Bacterial strains and culture media. *P. syringae* pv. syringae B728a and *P. syringae* 5B-91 (naturally nonpathogenic on bean) were isolated from snap bean leaflets collected in 1981 and 1985, respectively, from experimental plots at the University of Wisconsin—Madison Arlington Experiment Station. Strain 5B-91 produces syringomycin and protease as assayed according to the methods described by Hrabak and Willis (15) and is able to induce the hypersensitive reaction in tobacco. The nonpathogenic *lemA1*::Tn*5* mutant NPS3136 was derived from wild-type strain B728a by transposon mutagenesis as described previously (30). The Tn*5* insertion appears to affect only the *lemA* coding sequence, since saturation transposon mutagenesis and deletion analyses did not reveal any additional genes in the *lemA* transcriptional unit (14). Strains B728a and 5B-91 bear spontaneous mutations to rifampin (RIF) resistance. NPS3136 is resistant to RIF and kanamycin (KM); the latter resistance is encoded by Tn*5*. The clone of B728a used in the field experiments is a nalidixic acid (NAL)-resistant spontaneous mutant of B728a (referred to as B728aN; provided by S. E. Lindow, University of California, Berkeley). The strains were cultured on King's medium B (KB) (16) or Mohan and Schaad's selective medium for *P. syringae* pv. syringae (P) (25) supplemented with the appropriate antibiotics. Antibiotic concentrations were 50 μg per ml for RIF and NAL and 30 μg per ml for KM. All media were supplemented with cycloheximide (100 μg per ml) to inhibit fungal growth.

Field plot design. The *lemA1*::Tn*5* mutant NPS3136 was field tested in two experiments conducted in 1991 and 1993 at the University of Wisconsin—Madison Arlington Experiment Station, Columbia County. The experiments differed primarily in the mode of bacterial inoculation. In 1991, the test strains were applied to the foliage of 25-day-old plants that were seeded on 13 June. In 1993, the bacteria were applied to the seeds at the time of planting on 24 August. Details of inoculation procedures are given below. Snap bean cultivar Eagle (Asgrow Seed Co., Kalamazoo, Mich.) was used in both experiments. The seeds had had prior commercial treatment with captan. Cultivation of the plots and insect pest management procedures were done as necessary and were similar to those used by commercial snap bean growers in Wisconsin.

The general experimental designs were similar for the two experiments. Treatments were in a randomized complete block design with three blocks. Each plot was 12 rows wide and 7 m in length (1991) or 8 rows wide and 8 m in length (1993). Rows were spaced 76 cm apart with approximately 10 plants per m within a row. The plots were separated from each other by 5 m of bare ground. In 1991, the entire plot area was surrounded on all sides by 8 m of bare ground and 10 rows of bean plants beyond the bare-ground zone. The additional zones were established as a stipulation of the approval from the U.S. Department of Agriculture Animal and Plant Health Inspection Service that some attempt be made to monitor dispersal of the *lemA* mutant from the inoculated plots to "trap" bean plants separated from the plots by a bare-ground zone.

The treatments in common for both experiments consisted of (i) NPS3136 inoculated alone, (ii) B728aN inoculated alone, (iii) NPS3136 and B728aN coinoculated in approximately a 1:1 ratio, and (iv) control plots treated with water. The nonpathogenic *P. syringae* strain 5B-91 was included only in the 1991 experiment. We present data for the treatments listed above (i.e., five for 1991 and four for 1993). However, the field area encompassed by the experiments actually included plots for a total of seven treatments in 1991 and eight in 1993. Data from the additional treatments are not included in this report.

Inoculation of field plots. To prepare inocula for the field experiments, aliquots (0.1 ml) of cell suspensions adjusted turbidimetrically to approximately 10^8 CFU per ml (optical density at 600 nm of ca. 0.1) were spread onto KB plus RIF for all strains included in the 1991 experiment or onto KB plus NAL for B728aN and KB plus KM for NPS3136 in the 1993 experiment. After 2 to 3 days of incubation at 28°C, the lawn of bacterial growth in each petri dish was harvested by adding 5 ml of sterile distilled water and gently scraping the cells from the surface of the medium with an L-shaped glass rod. The suspension was removed from the plate, and a second aliquot of 5 ml of sterile distilled water was added to recover as many of the cells as possible. The suspensions for each strain were combined and dispensed into sterile 16-mm-diameter test tubes at 10 ml per tube with roughly 10^{10} CFU per ml as determined by dilution plating. The suspensions were either stored at −20°C for a day (1991 experiment) or kept on ice for about 3 h (1993 experiment) before being used in the field. At the time of inoculation, the stock suspensions were diluted with water to 10^8 CFU per ml for the foliage treatments (1991 experiment) and to 10^7 CFU per ml for the seed treatments (1993 experiment).

(i) Foliage inoculation. Approximately 7.2 liters of inoculum at 10^8 CFU per ml was applied to the foliage for each treatment (2.4 liters per plot) 25 days after planting. A tractor-mounted research plot sprayer (R & D Sprayers, Inc., Opelousas, La.) equipped with TeeJet no. 8004VS flat spray nozzles was maintained at 30 lb/in^2 with compressed CO_2 during application. Control plots were sprayed with water. To minimize dispersal during application of the bacteria, the inoculation was conducted in the evening (i.e., 2030 on July 8) under calm conditions (i.e., anemometer at stall speed [<0.5 m per s]).

(ii) Seed inoculation. For each treatment, bean seeds were inoculated by immersing 3 kg of seeds in 2 liters of inoculum diluted to 10^7 CFU per ml. After a 1-min treatment time, the inoculum was drained from the seeds through a piece of cheesecloth, and the treated seeds were immediately placed into the hoppers of a plate-type, tractor-mounted planter and planted. The time required to treat and plant a batch of seeds for a treatment with three replicate plots was about 6 to 7 min. All parts of the planter that came in contact with the inoculum were sterilized with 95% ethanol between treatments. Seeds for control plots were treated with water.

Sampling protocol. For both experiments, 10 samples were collected from each plot at each sampling time; 8 of these samples were actually processed by dilution plating for enumeration of bacterial population sizes. For the 1991 foliage inoculation experiment, the samples at all sampling times consisted of individual leaflets taken at random from the top of the canopy. For the 1993 seed inoculation experiment, the samples consisted of individual seeds or germinating seeds for samples collected immediately after planting (<10 min after inoculation) and at 1, 2, and 3 days after planting. At 6 days after planting, entire seedlings were collected. At subsequent times, samples consisted of individual primary leaves or leaflets from trifoliolate leaves taken at random from the top of the canopy.

For both experiments, all leaf samples were placed in no. 5 Kraft paper bags and transported in a cooler to the field laboratory, which was located within 0.8 km of the field plots for immediate processing. Each leaf sample was submerged in 9 ml of sterile potassium phosphate buffer (0.01 M, pH 7.0) in a 16-mm test tube. The entire seedling samples collected at 6 days after planting in 1993 were cut below the cotyledons with a sterile scalpel to yield two sets of samples. The portion of each seedling below the cotyledons was placed in one buffer-containing test tube, while the corresponding cotyledons and emerging primary leaves were placed in a second buffer-containing test tube. In 1993, each seed or germinating seed sample was dug up with a sterile spoon and transferred immediately to a buffer-containing test tube while in the field. All leaf and seed samples were stored at −20°C until they could be processed by dilution plating. Population sizes of *P. syringae* on bean leaves immersed in buffer were found to be stable for at least 3 years when stored at −20°C (9).

Enumeration of bacterial population sizes. Population sizes of the test *P. syringae* strains were determined by dilution plating of leaflet or seed homogenates as described previously (9). Each sample was thawed and transferred to a sterile 50-ml beaker. The test tube was rinsed with 10 ml of sterile potassium phosphate buffer (0.1 M, pH 7.0) supplemented with Bacto Peptone (0.1%, wt/vol). The rinse buffer was added to the beaker for a total volume of 19 ml. The sample was next minced with sterile scissors and then homogenized for 10 s with a Polytron equipped with a model PTA 20 TS probe (Brinkmann Instruments, Westbury, N.Y.). Tenfold serial dilutions were prepared in potassium phosphate buffer (0.01 M, pH 7.0) and plated onto KB with the appropriate antibiotics for the introduced *P. syringae* strains and onto KB without antibiotics to enumerate population sizes of total *P. syringae* (i.e., applied *P. syringae* strains and naturally occurring conspecifics) and total bacteria culturable on KB. For all leaf samples, NPS3136 was monitored on KB plus KM (1991) or KB plus RIF plus KM (1993), B728aN was monitored on KB plus RIF plus NAL (1991 and 1993), and the nonpathogenic 5B-91 was monitored on KB plus RIF (1991). The germinating seed and root samples collected in 1993 were plated onto P medium for total *P. syringae*, P plus KM for NPS3136, and P plus NAL for B728aN. P medium is a semiselective medium for *P. syringae* pv. syringae (25) and was initially used as the basal medium rather than KB to minimize background growth of soil-associated bacteria when below-ground samples had adhering wet soil (i.e., mud

due to rains). We have since found that addition of RIF in combination with either NAL or KM to KB is as effective as P plus NAL or P plus KM in inhibiting background bacterial growth. Plating efficiencies for *P. syringae* pv. syringae on P and KB were comparable.

Measurement of weather parameters. Parameters of the physical environment were sensed and recorded automatically with CR-7 and CR-21X data loggers (Campbell Scientific, Logan, Utah) as described previously (8). In particular, the time of event for accumulation of each millimeter of rainfall was sensed with a Sierra-Misco tipping-bucket rain gauge (Campbell Scientific).

Data analyses. The number of CFU for each sample was $\log_{10}$ transformed and expressed as log CFU per sample prior to calculation of population statistics (10). Censored observations were assigned the limit of sensitivity of the plating assay, which was 2.27 log CFU per sample for samples homogenized in a total volume of 19 ml. Seed samples collected immediately after inoculation and planting in 1993 were homogenized in 9 ml, and samples with no detectable *P. syringae* colonies were assigned a limit of sensitivity of 1.95 log CFU per seed. Statistical analyses, including tests for log normality of bacterial population sizes, analysis of variance, regression, and others, were performed with Minitab (Minitab Inc., State College, Pa.) and SigmaStat (Jandel Scientific, San Rafael, Calif.).

Because fitness is the ability of an individual to transmit its genes to subsequent generations, and relative fitness is the relative amount of transmission of genes to progeny, relative fitnesses of two bacterial strains over a given period of time can be estimated as relative changes in population sizes over that time. Because the bacterial strains were inoculated in approximately equal numbers, differences in population size after some time are a measure of relative fitness during the period between inoculation and measurement.

RESULTS

Behavior of the *lemA* mutant NPS3136 when inoculated individually onto bean foliage and seeds. In the first experiment, conducted in 1991, NPS3136, B728aN, and a naturally nonpathogenic strain of *P. syringae* (5B-91) were applied to the foliage of bean plants at 25 days after planting (Fig. 1A). The general trends in the population size of B728aN were similar to those that have been observed previously for both introduced and naturally occurring populations of *P. syringae* pv. syringae on field-grown bean plants (8, 9). The population sizes of all three strains decreased nearly 100-fold during the initial 12 h following inoculation. Large increases in and persistence of large population sizes of B728aN were associated with occurrence of intense rains; periods during which population sizes of B728aN decreased occurred in the absence of such rains.

The population sizes of NPS3136 declined steadily relative to those of B728aN for more than 2 weeks after inoculation (Fig. 1). At 4 days postinoculation, the difference in the mean population sizes of NPS3136 and B728aN was roughly 7-fold; by 18 days postinoculation, this difference had increased to approximately 1,500-fold (Fig. 1B). When population sizes increased during this period, increases in numbers of B728aN were greater than those of NPS3136. When population sizes decreased, B728aN decreased more slowly than did NPS3136. At 15 days postinoculation, NPS3136 was detected on 50% of the sampled leaflets (n = 24; limit of detection = 2.27 log CFU/leaflet; detectable population sizes ranged from 2.56 to 4.7 log CFU per leaflet). Thereafter, most of the samples collected had no detectable NPS3136. For example, of 24 leaflets sampled, NPS3136 was detected on only 8 samples and 1 sample on days 18 and 24 postinoculation, respectively. During the 2-week period following inoculation, population sizes of NPS3136 were larger than those of the naturally occurring nonpathogen, 5B-91 (Fig. 1A). However, the differences in population sizes of either NPS3136 or 5B-91 relative to those of B728aN plotted against sampling time declined at very nearly the same rate (Fig. 2). The slope of the regression line may be viewed as an estimate of the selection coefficient, s (19). The remarkably similar values for s suggest that the two strains were selected against at similar rates and hence exhibited very similar fitnesses during this period.

In 1993, NPS3136 and B728aN were applied to bean seeds at the time of planting. Dik et al. (5) had previously observed rapid and substantial growth of B728aN on germinating seeds and emerging seedlings when the bacterium was inoculated onto bean seeds at the time of planting. Hence, the design of the second experiment provided the opportunity to determine whether *lemA* contributes to fitness of *P. syringae* pv. syringae during the early stages of development of its host, which occur below ground, as well as in association with leaves. The experiment was initiated on 24 August, relatively late in the growing season, and bacterial population sizes on germinating seeds and in association with leaf habitats were monitored until the plants were killed by an early frost. When NPS3136 and B728aN were inoculated individually onto bean seeds, both strains grew and achieved similar large population sizes in association with below-ground samples (consisting of seeds or entire seedlings before emergence and on the first day after emergence [6 days after planting]) (Fig. 3A). Hence, the mu-

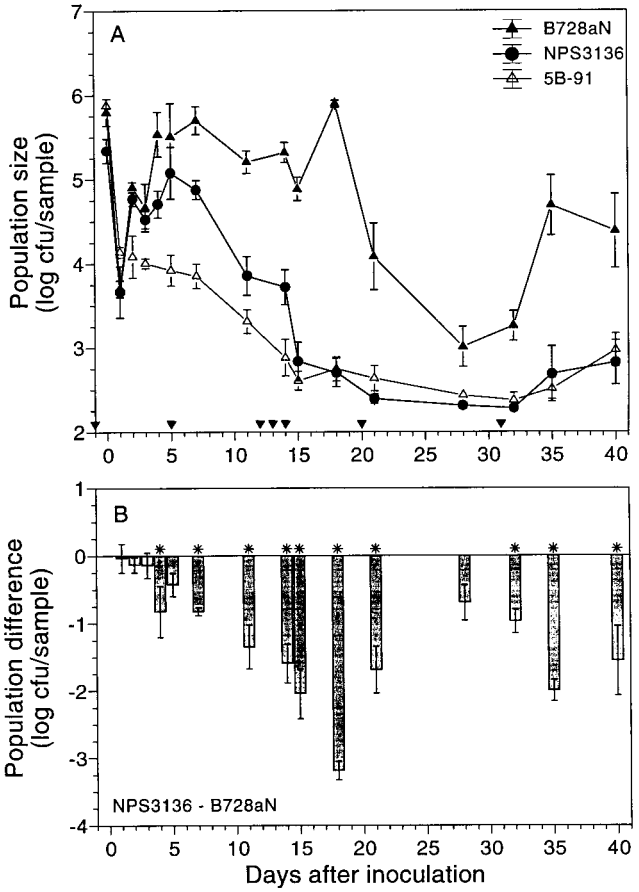

FIG. 1. (A) Mean population sizes of *P. syringae* pv. syringae B728aN, NPS3136 (*lemA1*::Tn*5*), and 5B-91 (naturally nonpathogenic to bean) as a function of days after inoculation. Each bacterial strain was applied alone to the foliage of 25-day-old snap bean plants. Each datum point represents the mean bacterial population size and standard error of the mean based on three replicate plots with eight individual leaf samples per plot. Inverted triangles represent the occurrence of rainfalls with greater than 5 mm of accumulation. Rain amounts were as follows: 29 mm on the day prior to inoculation; 5 mm on day 5 after inoculation (5 DAI); 10, 7, and 11 mm on 12, 13, and 14 DAI, respectively; 12 mm on 20 DAI; and 41 mm on 35 DAI. (B) Differences in mean population sizes of NPS3136 and B728aN as a function of days after foliage inoculation. Times at which population sizes of NPS3136 and B728aN were significantly different are noted with an asterisk (Fisher's protected least-significant-difference test, $P < 0.05$).

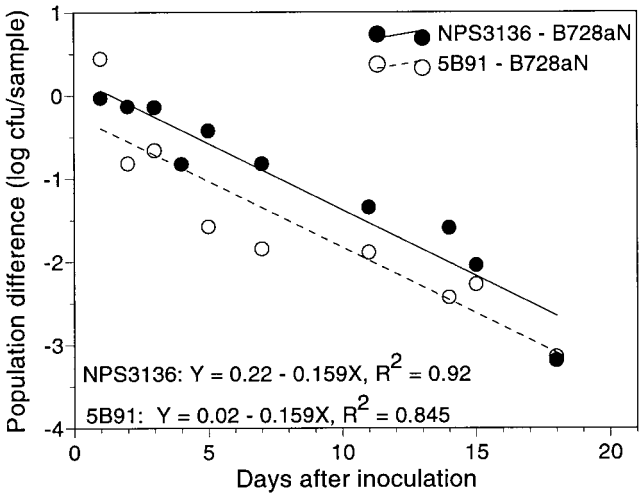

FIG. 2. Fitnesses of NPS3136 and 5B-91 relative to that of B728aN in association with leaf habitats. Each datum point represents the difference in mean $\log_{10}$ population size of either NPS3136 or 5B-91 relative to that of B728aN as a function of days after foliage inoculation.

tant appeared to be as fit as B728aN on germinating bean seeds.

Overall population sizes of B728aN and NPS3136 associated with leaves were much larger in the 1993 than in the 1991 experiment (Fig. 3A and 1A). Despite the much larger overall population sizes in 1993, numbers of NPS3136 declined relative to those of B728aN until about 20 days after planting (Fig. 3B), similar to the results from the 1991 experiment. Indeed, the rate at which differences in population sizes of the strains changed with time was very similar to that observed in the first experiment (Fig. 4), indicating similar relative fitnesses of NPS3136 and B728aN, even at very different absolute population sizes. Although the field plots were not formally evaluated for disease, rough estimates of disease incidence were obtained from the leaflets collected for dilution plating. The samples, which were collected at random regardless of disease status, were scored for the presence of brown spot disease at the time that they were placed in individual test tubes for subsequent processing by dilution plating. As shown in Table 1, disease incidence was much higher in 1993 than in 1991, as expected from the larger pathogen population sizes in 1993.

In both experiments, the steady decline in numbers of NPS3136 relative to B728aN appeared to have stopped after 18 to 20 days after inoculation (1991) or planting (1993) (Fig. 1 and 3). After this time, the relative numbers of the two strains fluctuated substantially. The magnitude of the fluctuation in 1991 may have been due, in part, to the very low numbers of NPS3136, which made precise estimates of numbers of NPS3136 difficult due to censoring. In 1993, the population sizes of both strains were larger, measurements were not confounded by censoring to nearly the same degree, and the amount of fluctuation was much less.

The population sizes of B728aN tended to be larger on diseased than on asymptomatic leaflets (Fig. 5). This was more apparent in 1991, when a smaller proportion of the leaflets was diseased, than in 1993, when nearly all leaves in plots inoculated with B728aN were diseased. Distributions of population sizes of NPS3136 among individual leaflets are included in Fig. 5 for comparison. An exception to the general observation of larger population sizes of B728aN being associated with diseased than with healthy leaves occurred near the very end of

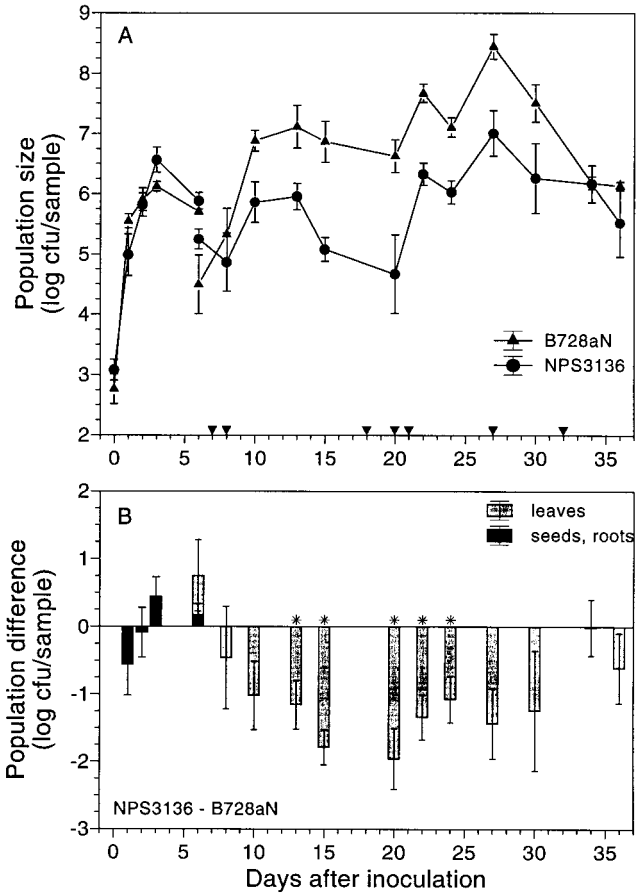

FIG. 3. (A) Mean population sizes of *P. syringae* pv. syringae B728aN and NPS3136 (*lemA1*::Tn*5*) as a function of days after inoculation. Each bacterial strain was applied alone to bean seeds at the time of planting. Each datum point represents the mean bacterial population size and standard error of the mean based on three replicate plots with eight individual samples per plot. Samples collected on days 0, 1, 2, and 3 consisted of seeds or germinating seeds. On day 6, entire seedlings were collected and separated into below-ground (root) and above-ground (cotyledons and emerging primary leaves) samples. Samples collected after day 6 were individual primary leaves or trifoliolate leaflets. Inverted triangles represent the occurrence of rainfalls. Rain amounts were as follows: 4 mm on day 7 after inoculation (7 DAI); 12 mm on 8 DAI; 35 and 44 mm on 20 and 21 DAI, respectively; 7 mm on 27 DAI; and 14 mm on 32 DAI. (B) Differences in mean population sizes of NPS3136 and B728aN as a function of days after seed inoculation. Times at which population sizes of NPS3136 and B728aN were significantly different are noted with an asterisk (Fisher's protected least-significant-difference test, $P < 0.05$).

the second experiment. Population sizes of B728aN peaked at approximately 10^8 CFU per leaflet at 27 days after inoculation (Fig. 3A), following which they declined, while those of NPS3136 tended to stabilize at approximately 10^6 CFU per leaflet. The decrease in population sizes of B728aN during this period coincided with increasing severity of brown spot disease. Many of the leaflets from the B728aN-inoculated plots were almost entirely necrotic; some may have fallen from the plants. Under conditions of severe disease, the pathogen may have destroyed so much of its habitat as to become no more fit than the *lemA* mutant. Similar declines in population sizes of B728a associated with the development of large areas of leaf necrosis have been observed when the pathogen was infiltrated into bean leaves in growth chamber experiments (30).

Behavior of the *lemA* mutant NPS3136 when coinoculated with B728aN onto bean foliage and seeds. The rationale for

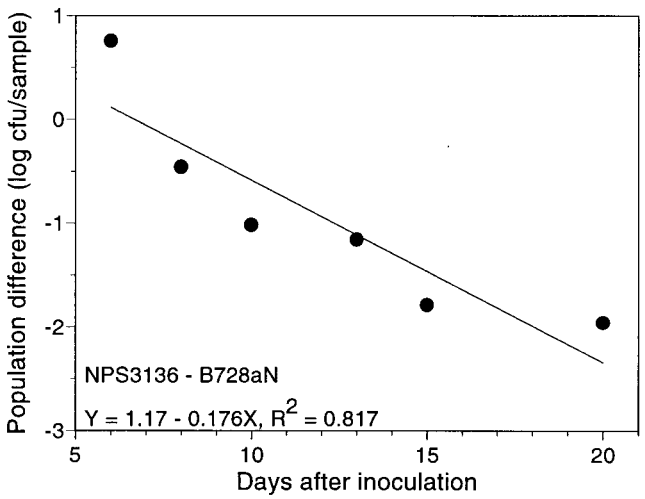

FIG. 4. Fitness of NPS3136 relative to that of B728aN in association with leaf habitats. Each datum point represents the difference in mean $\log_{10}$ population size between NPS3136 and B728aN as a function of days after seed inoculation.

including an NPS3136-B728aN coinoculation treatment was initially based on the assumption that a more precise assessment of the relative levels of fitness of the two strains may be obtained by simultaneous measurements of the strains on the same set of leaflets. Because the strains carried different markers, their population sizes could be monitored directly by dilution plating of each leaflet onto two different media. Unexpectedly, when the strains were coinoculated, the population trends for NPS3136 and B728aN were remarkably similar in both the foliage and seed inoculation experiments (Fig. 6A and B). With the exception of samples collected at 18 days postinoculation in the foliage experiment (Fig. 6A), the population sizes of the *lemA* mutant were not significantly different from those of B728aN when the strains were coinoculated. In the presence of B728aN, the mutant behaved as it did when it was applied alone (Fig. 6C and D). However, population sizes of B728aN were much smaller in the presence of the mutant than when it was applied alone (Fig. 6E and F). Thus, when present on the same leaves, the population trends for NPS3136 and B728aN were remarkably similar in both experiments (Fig. 6A and B). In general, the degree to which population sizes of B728aN were decreased in the presence of NPS3136 was on the order of 10-fold or greater (Fig. 7). Consistent with the lower population sizes of B728aN, brown spot disease incidence tended to be lower in the plots coinoculated with both strains than in plots inoculated with B728aN alone, at least until about 20 days after inoculation in both experiments (Table 1).

Correlation analyses of population sizes of B728aN and NPS3136 on individual leaflets at all sampling times yielded positive relationships for the paired measurements. Hence, on an individual-leaflet basis, smaller population sizes of B728aN were not associated with the presence of larger numbers of NPS3136. Inactivation of *lemA* rendered NPS3136 suppressive to B728aN on bean leaflets. Interestingly, the population sizes of B728aN mimicked those of the *lemA* mutant when both strains were present on the same leaflet.

In both experiments, the population sizes of naturally occurring *P. syringae* were relatively lower than those of the inoculated marked strains. Thus, enumeration of "other" *P. syringae* required determination of small differences between large numbers (that is, between counts on KB and counts on KB supplemented with antibiotics). Such estimates tend to be relatively inaccurate. In addition, at some sampling times during the experiment in which foliar inoculation was used, population sizes of naturally occurring *P. syringae* were near our limit of detection in control (uninoculated) plots. Hence, it was not possible to clearly determine whether NPS3136 suppressed population sizes of naturally occurring *P. syringae.*

DISCUSSION

In each of two field experiments, the fitness of *P. syringae* pv. syringae B728aN in association with bean leaves was clearly diminished by inactivation of *lemA*. The *lemA* mutant NPS3136 decreased in abundance relative to B728aN from the time of inoculation (or from the time of emergence of primary leaves when seed inoculated) to 18 to 20 days after inoculation, at which time it was roughly 100-fold (i.e., in 1993) to 1,000-fold (i.e., in 1991) below the population size of B728aN. Thereafter, differences between NPS3136 and B728aN fluctuated, but they persisted throughout most of the sampling period in the two field experiments. Although population sizes of the *lemA* mutant were somewhat larger than those of the naturally nonpathogenic strain 5B-91 from day 2 through 15 following foliage inoculation, the two strains declined relative to B728aN at similar rates. This is interesting, since it is unlikely that the difference between NPS3136 and 5B-91 is due solely to an inactivated *lemA* gene in the former.

The observation that the behavior of the *lemA* mutant in the field was not predicted from initial growth chamber studies as reported by Willis et al. (30) is noteworthy. If the LemA pro-

TABLE 1. Brown spot disease incidence on leaflets from plots inoculated with B728aN individually or in combination with the *lemA* mutant

Yr	n^a	Days after inoculation	% Diseased leaflets with:	
			B728aN alone	B728aN plus NPS3136
1991	24	1	0	0
		2	0	0
		3	0	0
		4	29.2	0
	30	5	33.3	43.3
		7	23.3	16.7
		11	0	0
		14	20.0	6.7
		15	23.3	3.3
		18	50.0	30.0
		21	16.7	6.7
		28	20.0	23.3
		32	3.3	10
		35	40.0	23.3
		39	60.0	46.7
1993	24	8	4.2	0
		10	62.5	33.3
		13	83.3	58.3
		15	79.2	33.3
		20	75.0	16.7
		24	91.7	100.0
		27	100.0	83.3
		30	100.0	100.0
		34	100.0	100.0
		36	100.0	100.0

[a] n = number of leaflets collected at random per treatment and sampling time. When n = 24, all samples were dilution plated for estimation of bacterial population sizes. When n = 30, a subset of 24 samples (8 samples per plot, three plots) were dilution plated.

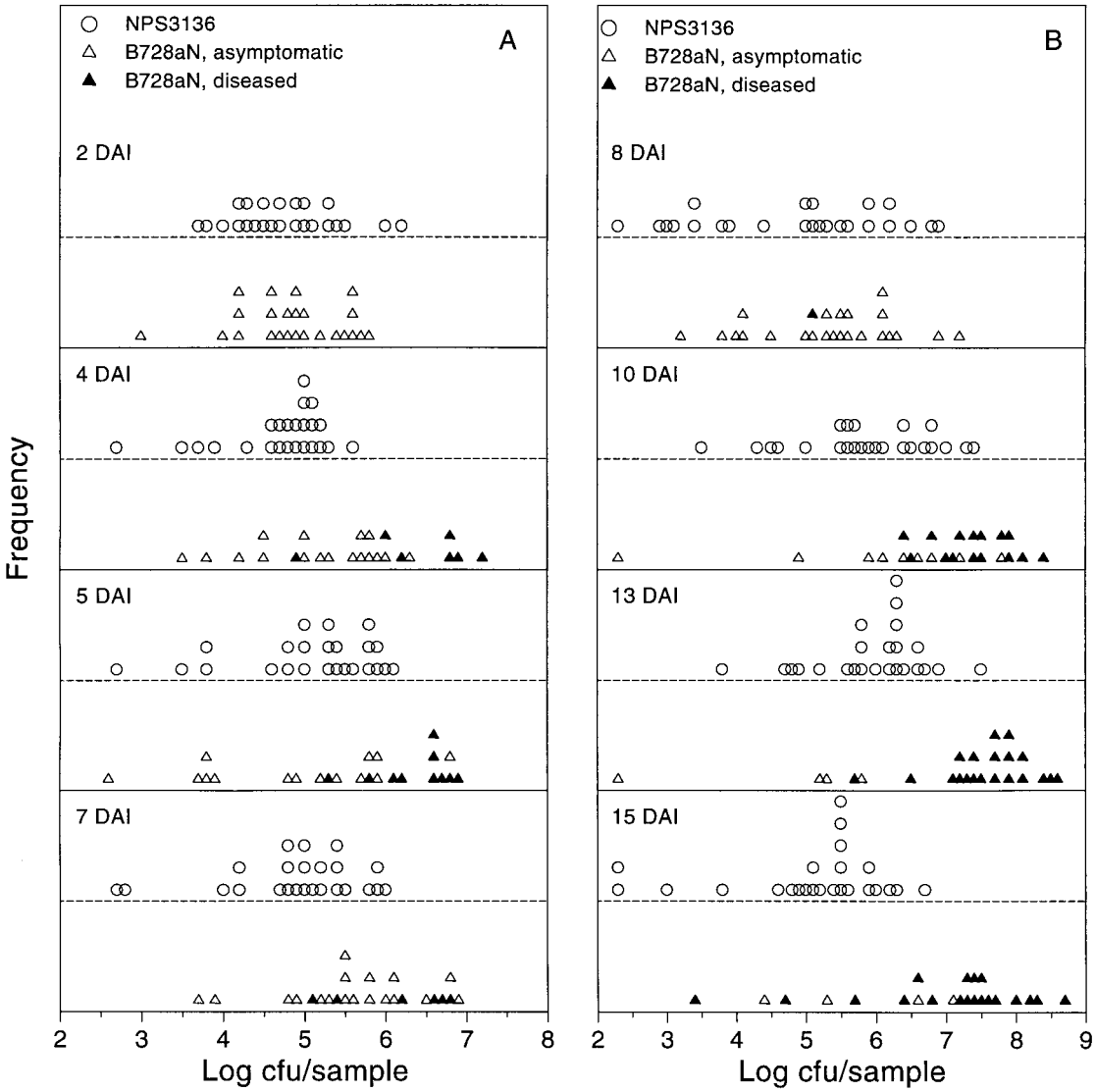

FIG. 5. Distributions of population sizes of B728aN and NPS3136 on individual leaf samples at selected times after foliage (A) and seed (B) inoculations. The 24 individual samples per bacterial strain and sampling day are pooled across the three replicate plots per treatment. Leaf samples from the B728aN-inoculated plots were taken at random from each plot and evaluated for the presence of visible brown spot symptoms at the time that they were placed in test tubes for subsequent processing by dilution plating. DAI, days after inoculation.

tein functions as a sensor in a two-component regulatory system, the signals that it senses in or on bean plants may be different in the field than in the laboratory. Alternatively, requirements for maximum fitness in association with bean plants in the field may differ from those for beans grown in controlled environments. In this sense, *lemA* might be regarded as a field fitness gene. An extensive search by Lindow et al. (2, 3, 23, 24) for genes that affect epiphytic fitness of *P. syringae* has led to the identification of several genes that affect the fitness of *P. syringae* on bean plants in the laboratory. These epiphytic fitness mutants were derived from transposon mutagenesis of wild-type strain B728a, the same parent as for the *lemA* mutant NPS3136. Four of the Tn*5*-induced epiphytic mutants have also been shown to be affected in their abilities to grow and survive on primary leaves of field-grown bean plants (3). However, unlike NPS3136, all four epiphytic fitness mutants were also impaired to different degrees in their abilities to grow when infiltrated into bean leaves in laboratory assays (2). Thus, the epiphytic fitness genes that have been reported to date appear to be necessary for optimum growth and/or survival of *P. syringae* pv. syringae B728a in and/or on leaves. As far as we are aware, *lemA* is the only gene that affects fitness in the field but not growth when infiltrated into leaves in laboratory assays.

The decreased fitness of the *lemA* mutant occurred when weather conditions were highly conducive to growth of *P. syringae* pv. syringae and pathogen population sizes were unusually high (i.e., in 1993), as well as when weather conditions were relatively less conducive to growth of *P. syringae* pv. syringae and population sizes of the pathogen were low to moderate (i.e., in 1991). The amount of bacterial brown spot disease is dependent on the population size of *P. syringae* pv. syringae associated with bean leaves (22, 29). Hence, as expected, disease pressure was much greater during the 1993

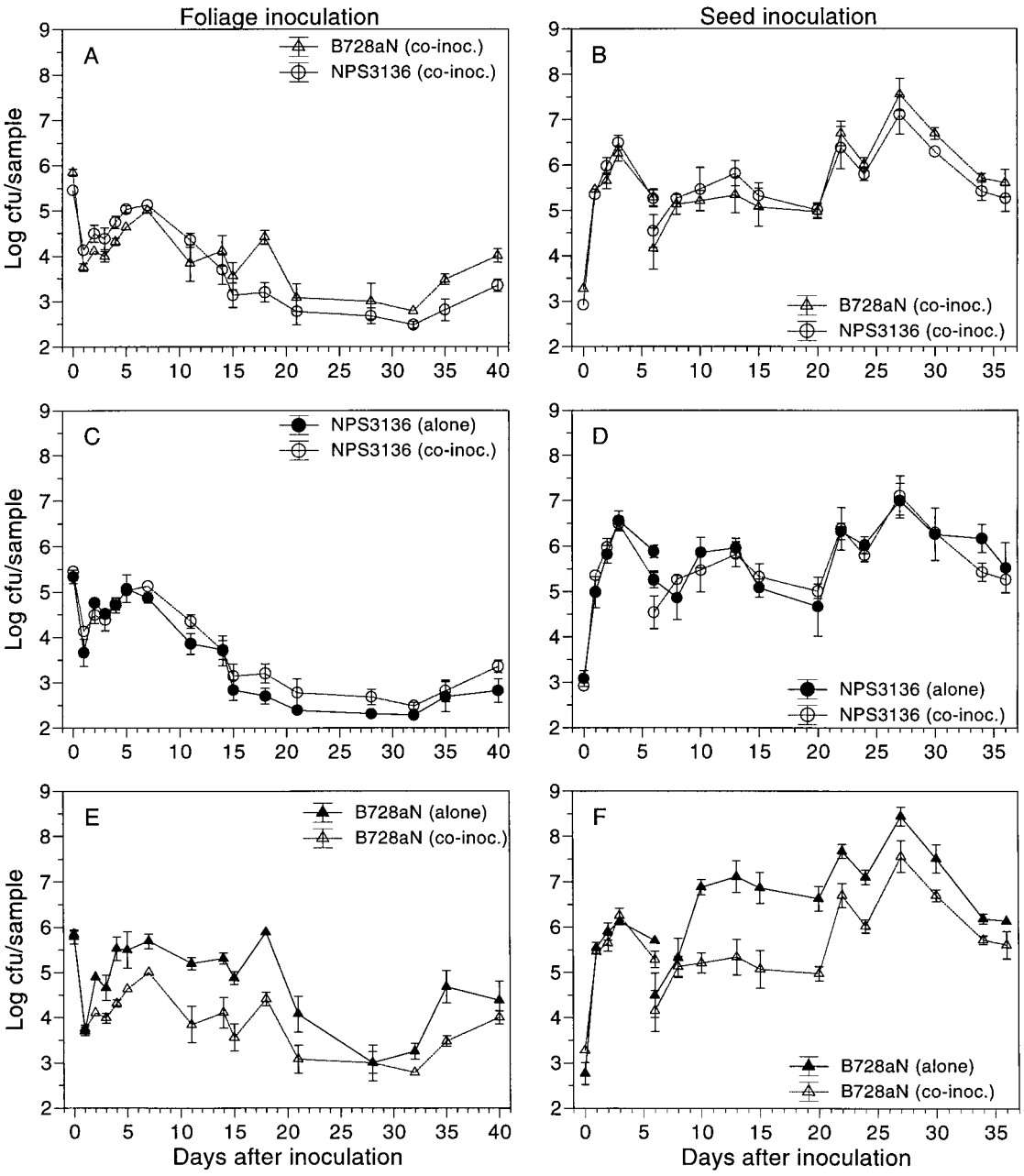

FIG. 6. Comparison of population sizes of NPS3136 and B728aN as a function of days after inoculation either alone or in combination. (A and B) Population sizes of NPS3136 and B728aN when coinoculated (co-inoc.) at a ratio of approximately 1:1. (C and D) Population sizes of NPS3136 when inoculated alone and in combination with B728aN. (E and F) Population sizes of B728aN when inoculated alone and in combination with NPS3136. Each datum point represents the mean bacterial population size and standard error of the mean based on three replicate plots with eight leaf samples per plot.

experiment than during the 1991 experiment (Fig. 5; Table 1). Indeed, the amount of disease was unusually high in 1993. Many of the leaves from plots inoculated with B728a were almost entirely necrotic by the end of the sampling period. In general, despite the large differences in disease incidence and severity in the two experiments, the *lemA* mutant was found to be similarly less fit than B728aN, suggesting that the effect of *lemA* on fitness of *P. syringae* pv. syringae is not strongly influenced by the amount of disease present. On the other hand, when analyzed on an individual-leaflet basis, population sizes of B728aN tended to be much larger on leaflets with one or more brown spot lesions than on asymptomatic samples. The population sizes of B728aN on leaves without lesions were much closer to those of NPS3136 than were the population sizes of B728aN on leaves with lesions. We do not know whether the larger numbers of *P. syringae* pv. syringae organisms associated with leaves bearing lesions arose because of growth of the bacteria in lesions or because of the greater likelihood that a lesion will form when population sizes of the bacterium are very large. Clearly, a role for ability to cause disease in the fitness of *P. syringae* pv. syringae cannot be ruled out on the basis of these data. However, the possibility remains

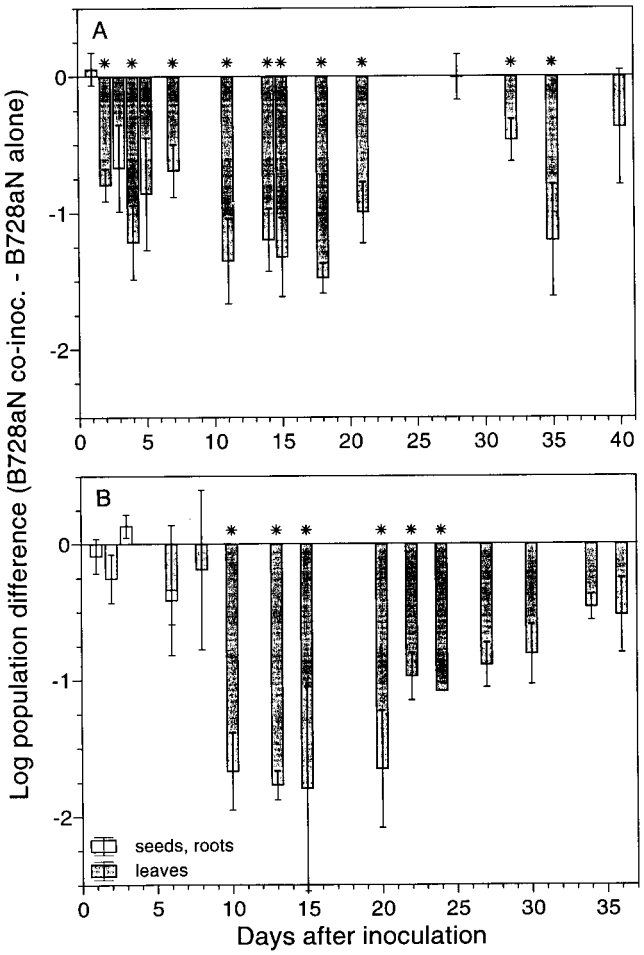

FIG. 7. Magnitudes of the differences in population sizes of B728aN when inoculated alone versus when coinoculated (co-inoc.) with NPS3136 onto bean foliage (A) and seeds (B). Times at which population sizes of B728aN were found to be significantly different (Fisher's protected least-significant difference test, $P < 0.05$) in the single and combination treatments are noted with an asterisk.

that the role of *lemA* in lesion formation may be separate from or coupled to additional roles that affect bacterial population sizes.

The observation that NPS3136 grew at least as well as B728aN on germinating seeds is reminiscent of results of the laboratory experiments, in which growth of the two strains was indistinguishable in or on leaves (30). Quite clearly, the relative levels of fitness of these two strains in association with bean plants are environment dependent. They are equally fit when infiltrated into bean leaves or mist inoculated onto bean plants that are subsequently maintained under conditions of high relative humidity in the growth chamber. They are equally fit in the spermosphere of bean plants. In the seed inoculation experiment, it was only after bean plants emerged and primary leaves began to expand that the population sizes of NPS3136 and B728aN began to diverge, suggesting that the reduced fitness of the *lemA* mutant is unique to the environment associated with leaves of bean plants in the field. Interestingly, Natsch et al. (26) reported that a *gacA* (i.e., the cognate response regulator of *lemA*) mutant of the rhizosphere bacterium *P. fluorescens* CHA0 was indistinguishable from the wild type in its ability to colonize the rhizosphere and roots of wheat and cucumber plants in microcosm assays. The *gacA* mutant, however, was found to be less fit than the wild type when assayed for its abilities to persist in nonsterile soil and to be vertically disseminated in soil columns. Hence, in two very different systems, *lemA* and *gacA* exhibit in common an environment-dependent effect on bacterial fitness.

The surprising result from our field experiments was the effect of NPS3136 on the population size of B728aN when the two were coinoculated in approximately a 1:1 ratio onto bean foliage or seeds. The observation that the mutant, which is itself less fit than B728aN, could render B728aN less fit is both unexpected and somewhat difficult to explain, since we do not know the nature of the possible repertoire of genes regulated by *lemA*. We can, nonetheless, rule out a number of explanations and speculate on others with the information currently available.

We do know that not all mutants derived from B728a by Tn*5* mutagenesis are suppressive to B728a. In the field experiments of Beattie and Lindow (3), the fitnesses of four Tn*5*-induced epiphytic fitness mutants were determined by inoculation of each strain alone and in combination with B728a, similar to the experimental design used in our experiments. None of the mutants affected the behavior of B728a. In fact, one of the mutants, a methionine auxotroph, established larger population sizes when coinoculated with B728a than when inoculated alone. Hence, the suppressive effect of NPS3136 on B728aN is not due to the presence of Tn*5* per se but rather to the specific gene disrupted by insertion of the transposon.

It is unlikely that competition for limiting resources (for example, nutrients [32]) is the mechanism for the suppressive effect of NPS3136 on B728aN. If resource availability were the mechanism, the sum of the NPS3136 and B728aN population sizes in the coinoculation treatment should have approximated the population size for B728aN alone. This was not found to be the case. For example, at 13 days after foliage inoculation, resources were available to support on average 7.2×10^6 B728aN CFU per leaflet when it was singly inoculated. On that sampling day, the sum of NPS3136 and B728aN in the coinoculation treatment was on average 3.26×10^5 CFU per leaflet, over an order of magnitude less than numbers of B728aN alone. Any mechanism in which one strain is displaced by another would seem an unlikely explanation for the phenomenon, because NPS3136 in the single- and co-inoculation treatments was found to be less fit than B728aN inoculated alone.

We have thus far ruled out a number of explanations for the suppressive effect of the *lemA* mutant on B728aN. What are plausible explanations? There are several noteworthy aspects of the phenomenon from which clues may be gleaned. Not only did NPS3136 suppress the numbers of B728aN, but the level of suppression was such that the overall population trends of B728aN in the presence of NPS3136 paralleled very closely those of the *lemA* mutant. In addition, numbers of the mutant and parent were positively correlated on individual leaflets. These observations raise the possibility that one or more genes in the *lemA-gacA* regulon may be up or down regulated by inactivation of *lemA* in some way that results in leaf habitats being less conducive to growth and/or survival or possibly more resistant to *P. syringae* pv. syringae. Another possibility is that *lemA* up or down regulates production and/or secretion of some molecule that affects the bacteria directly and in some way decreases their population sizes. Regardless of the mechanism, it may eventually be possible to mitigate disease hazard by appropriate manipulations of genes such as *lemA* that affect pathogen population sizes. Whether the decreased fitness of NPS3136 in association with bean leaves in the field and its apparent ability to reduce the fitness of a closely related strain when coinoculated are mediated by the same or a different set

of *lemA*-regulated genes remains to be resolved, as does the nature of the genes that are regulated by *lemA*.

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