

Role of the Hrp type III protein secretion system in growth of *Pseudomonas syringae* pv. *syringae* B728a on host plants in the field

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ABSTRACT *hrp* genes are reportedly required for pathogenicity in *Pseudomonas syringae* pv. *syringae* (Pss) and other phytopathogenic bacterial species. A subset of these genes encodes a type III secretion system through which virulence factors are thought to be delivered to plant cells. In this study, we sought to better understand the role that *hrp* genes play in interactions of Pss with its host as they occur naturally under field conditions. Population sizes of *hrp* mutants with defects in genes that encode components of the Hrp secretion system ($\Delta hrcC::nptII$ and $hrpJ::\Omega Spc$) and a protein secreted via the system ($\Delta hrpZ::nptII$) were similar to B728a on germinating seeds. However, phyllosphere (i.e., leaf) population sizes of the *hrcC* and *hrpJ* secretion mutants, but not the *hrpZ* mutant, were significantly reduced relative to B728a. Thus, the Hrp type III secretion system, but not HrpZ, plays an important role in enabling Pss to flourish in the phyllosphere, but not the spermosphere. The *hrcC* and *hrpJ* mutants caused brown spot lesions on primary leaves at a low frequency when they were inoculated onto seeds at the time of planting. Pathogenic reactions also were found when the *hrp* secretion mutants were co-infiltrated into bean leaves with a non-lesion-forming *gacS* mutant of B728a. In both cases, the occurrence of disease was associated with elevated population sizes of the *hrp* secretion mutants. The role of the Hrp type III secretion system in pathogenicity appears to be largely mediated by its requirement for growth of Pss in the phyllosphere. Without growth, disease does not occur.

Pseudomonas syringae pv. *syringae* (Pss) is a common inhabitant of leaves and other aerial parts of a diversity of plant species (1). On snap bean plants (*Phaseolus vulgaris* L.), the bacterium causes bacterial brown spot disease (2). Foliar symptoms are necrotic spots frequently surrounded by a narrow chlorotic margin. Lesions on pods lower the quality of the product and cause economic loss. Although disease causation is of central importance from the perspective of humans, it is only one of several interactions that occur between Pss and its host. In the field, the bacterium is able to multiply and survive for many generations on leaf surfaces of host and nonhost plants, similar to the numerous nonpathogenic bacteria that exist as epiphytes in the phyllosphere (1, 3, 4).

Epiphytic populations of Pss provide inoculum for outbreaks of disease. Two separate studies, using very different approaches, demonstrated that population sizes of Pss on bean leaves are predictive of amounts of subsequent disease (5, 6). Establishment of the large pathogen population sizes that frequently lead to disease is associated with the occurrence of intense rains (7, 8). Rains with sufficient momentum appear to trigger the onset of rapid multiplication of the bacterium (8).

In the absence of intense rains, population sizes of Pss tend to decline, or at least do not increase significantly, and little to no brown spot disease develops.

Substantial progress has been made in the identification of genes in Pss that are reportedly required for pathogenicity (cf. 9–11). Many of the genes participate in one of two major regulons—the *gacS* (global activator sensor kinase; formerly *lemA*) (9) and *hrp* (hypersensitive reaction and pathogenicity) (10, 11) regulons. A phenotype shared in common by mutants bearing defects in *hrp* genes and in *gacS*, *gacA*, and *salA* [key components of the *gacS* regulon (12–15)] is the inability to cause disease in susceptible hosts in laboratory leaf infiltration assays. *hrp*, but not *gacS*, *gacA*, or *salA* mutants, are additionally impaired in their abilities to cause the plant defense-associated hypersensitive response in nonhost plants and to grow when infiltrated into leaves of susceptible plants (9, 12, 16).

hrp genes in Pss and in other major genera of Gram-negative plant pathogenic bacteria are found as clusters of genes spanning ≈ 25 kilobases (kb) (cf. ref. 11). Putative products of *hrp* genes include components of a type III secretion system similar to that found in animal pathogens such as *Yersinia*, *Shigella*, and *Salmonella* spp., proteins that are secreted via the pathway, and regulators that control transcription of *hrp* and *avr* genes (reviewed in ref. 11). In animal systems, virulence effector molecules are exported via the type III secretory pathway and may pass directly into the cytoplasm of the target host cells after pathogen contact (cf. refs. 17 and 18). Similarly, effector molecules required for elicitation of the hypersensitive response and virulence apparently traverse the Hrp type III secretion pathway in the plant systems (19–22).

Most of the research on *hrp* genes has been from the perspective of their function in elicitation of the hypersensitive response, an incompatible reaction that is believed to be an important component of disease resistance. Little is known about the ways in which the Hrp secretion system and its associated effector molecules contribute to pathogenicity. The possibilities that the requirement of the *hrp* cluster for pathogenicity may be attributable to either the inability of *hrp* mutants to grow in host plants or their inability to produce factors necessary for symptom development first were suggested by Lindgren *et al.* in 1986 (16). Subsequent findings confirmed that *hrp* mutants do not grow well in leaves of susceptible hosts (16, 23). However, the possibility that the effect on growth may explain the requirement of *hrp* genes for pathogenicity has been largely overlooked. Additionally, what

Abbreviations: Pss, *Pseudomonas syringae* pv. *syringae*; DAP, days after planting; cfu, colony-forming unit; kb, kilobases; Cm, chloramphenicol; Kan, kanamycin; Spc, spectinomycin.

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is known about the plant-associated phenotypes of *hrp* mutants is based on laboratory assays in which the bacteria are infiltrated into leaves. Such assays circumvent a significant portion of the interactions that occur between bacteria and plants in the field. Thus, the overall objective of this study was to determine the role(s) that *hrp* genes play in interactions of Pss with its host plant in the field. Our data indicate that *hrp* mutants of Pss strain B728a with defects in the Hrp type III secretion system ($\Delta hrcC::nptII$ and $hrpJ::\Omega Spc$) are significantly affected in their ability to grow in association with leaf habitats but, interestingly, not in the spermosphere. Our data also demonstrate that, under certain conditions, *hrp* secretion mutants are able to cause brown spot lesions.

MATERIALS AND METHODS

Bacterial Strains. Pss strain B728a (wild type), isolated from a snap bean leaflet in Wisconsin, is a highly virulent and field-competent strain. B728a carries a spontaneous mutation to resistance to rifampicin. B728aCm is a derivative of the wild-type strain in which an Ω -interposon conferring resistance to chloramphenicol (Cm) (24) was inserted in a null site in the intergenic region between the *gacS* and *cysM* genes (13, 25). Fitness of B728a and B728aCm was indistinguishable in the field (25).

The B728a *hrp* mutants examined included a *hrpZ*, *hrcC*, and *hrpJ* operon mutant. *hrpZ* encodes a protein that is secreted via the Hrp type III secretion system (26). On the basis of sequence similarity to the *yscC* gene in *Yersinia* and biochemical studies, *hrcC* could code for a protein that functions as an outer membrane component of the Hrp secretion system (27, 28). One or more of the genes in the *hrpJ* operon appears to function as inner membrane component(s) of the secretion system (28).

The B728a *hrpZ* mutant ($\Delta hrpZ::nptII$) carries a nonpolar mutation in which the entire *hrpZ* ORF was deleted by oligonucleotide-directed PCR amplification of flanking sequences in a subclone from pHIR11 as described (29). A 1.5-kb *nptII* [neomycin phosphotransferase II conferring resistance to kanamycin (Kan)] cartridge was inserted into the site of deletion such that the downstream genes are driven by the *nptII* promoter. A polar mutation in *hrpJ* ($hrpJ::\Omega Spc$) was constructed by marker exchange of pCPP2319 (28) into the chromosome of B728a. pCPP2319 contains a *BamHI-SacI* 8.6-kb fragment from pHIR11 with an ΩSpc^c interposon [spectinomycin (Spc) resistance] inserted into an *EcoRI* site in *hrpJ*, the first of five genes in the operon. The *hrcC* mutant ($\Delta hrcC::nptII$) carries a nonpolar, ≈ 0.5 -kb deletion mutation in *hrcC*, the third of five genes in the operon. Two subclones from pHIR11 (i.e., a 3.5-kb *KpnI-EcoRI* fragment containing most of the *hrcC* gene and a 1.3-kb *KpnI-Eco0109I* fragment containing the downstream region and 3' portion of *hrcC*) were used to construct the deletion. The *nptII* cartridge was inserted into the deleted site. All mutant constructs were confirmed by Southern blot analysis.

The strains were cultured on King's Medium B (30) supplemented with rifampicin (50 $\mu\text{g/ml}$) (KBR) and the antibiotic specific to the strain. The antibiotics were Kan (30 $\mu\text{g/ml}$) for the *hrcC* and *hrpZ* mutants, Spc (50 $\mu\text{g/ml}$) for the *hrpJ* mutant, Cm (30 $\mu\text{g/ml}$) for B728aCm, and nalidixic acid (Nal, 50 $\mu\text{g/ml}$) for B728aNal (Nal^r spontaneous B728a mutant).

Field Plot Design. Field experiments were conducted in 1995 and 1997 at the University of Wisconsin Arlington Experiment Station using snap bean cultivar Eagle (Asgrow Seed, Kalamazoo, MI). Treatments were arranged in a randomized complete block design with either three (1995) or four (1997) blocks. In 1995, the treatments were single inoculations of B728a, the *hrpZ* mutant, and the *hrpJ* mutant, and co-inoculations of $\approx 1:1$ mixtures of B728aNal with either the *hrpZ* or *hrpJ* mutant. In 1997, the treatments were single inoculations with B728aCm,

the *hrpZ*, *hrpJ*, and *hrcC* mutants. Each plot was 8 m (12 rows) \times 8 m in 1995 and 5.4 m (8 rows) \times 8 m in 1997. Rows were spaced 76 cm apart with ≈ 10 plants/m within a row. The plots were separated from each other by 5 m of bare ground. Seeds were inoculated with the bacterial strains by immersion of the seeds for 1 minute in a bacterial suspension [$\approx 10^7$ colony-forming units (cfu)/ml in water] immediately before planting (31).

Sampling, Enumeration, and Data Analysis of Bacterial Population Sizes. Eight to ten samples were collected at random from each plot at each sampling time, six to eight of which were processed by dilution plating for enumeration of bacterial population sizes. Samples collected before plant emergence were dug from the ground with sterile plastic spoons. The first set of samples after seedling emergence consisted of all above-ground parts (i.e., cotyledons and emerging primary leaves). Subsequent samples were individual primary leaves or leaflets of trifoliolate leaves. Each sample was placed in a test tube containing 9 ml of sterile potassium phosphate buffer (10 mM, pH 7.0) and was stored at -20°C for subsequent processing as described (7).

Population sizes of the bacterial strains were determined by dilution plating of leaflet or seed homogenates as described (7, 31). Each sample and appropriate dilutions were spiral plated (Spiral Biotech, Bethesda, MD) onto four to five different media to enumerate the specific strain (or combination of strains in 1995) inoculated onto the seeds and to detect possible interplot spread of the strains. The samples were additionally plated onto P medium, a semiselective medium for Pss (32), to determine total *P. syringae* population sizes.

Bacterial counts were \log_{10} -transformed and expressed as log cfu per sample before calculation of population statistics (33). Censored observations were assigned the limit of sensitivity of the plating assay (1.95 log cfu/sample for seeds collected immediately after planting; 2.57 log cfu/sample for all other samples). Statistical analyses, including tests for lognormality of bacterial population sizes, analysis of variance, and repeated measures ANOVA (34), were performed with MINITAB (Minitab, State College, PA) and SAS (SAS Institute, Cary, N.C.).

Brown Spot Disease Assessment and Determination of Lesion Occupancy. Rough estimates of disease incidence were made by noting whether brown spot lesions were present on leaves collected for enumeration of bacterial population sizes. In 1997, all plants were additionally scrutinized for the presence of lesions at 14 days after planting (DAP). To determine lesion occupancy, additional leaves were collected, and individual lesions were removed with a 5-mm cork borer. The diseased tissues were individually surface-sterilized with 70% ethanol, were rinsed with sterile water, were macerated in 1 ml of potassium phosphate buffer (10 mM, pH 7.0), and were dilution-plated onto P, KBR, KBRcM, KBRKan, and KBRSpC media. For each sample, two well separated colonies were taken from plates containing KB plus antibiotics. Additionally, mass streakings were made from the P plates seeded with the undiluted homogenate to detect the presence of naturally occurring conspecifics that may have been present at smaller numbers than the mutants. The isolates were tested for pathogenicity by using a leaf infiltration assay.

Growth Chamber Assays. Leaf infiltration pathogenicity and *in planta* growth assays were conducted with cultivar Bush Blue Lake 274 (Rogers Seed, Boise, ID). For most experiments, maximum and minimum temperatures were set at 24°C (14-hr light period) and 20°C (10-hr dark period), respectively. Cells from 2-day-old cultures (grown on KBR plus antibiotic) were suspended in sterile water. For routine pathogenicity assays, suspensions of 10^6 and 10^5 cfu/ml were infiltrated into primary leaves of 10- to 11-day-old plants by using a needleless syringe. The resulting doses were ≈ 2.5 – 3.5×10^3 and 2.5 – 3.5×10^2 cfu per infiltrated site of ≈ 30 – 40 mm². Strains (e.g.,

wild-type B728a) that caused necrosis over the entire infiltrated area in 3–4 days at both doses were considered pathogenic.

For assessing *in planta* growth, bacterial suspensions diluted to 10^6 cfu/ml were infiltrated as described above. A single site was infiltrated on as many leaves as necessary to allow the collection of five samples per sampling time. A cork borer was used to remove a 6-mm (diameter) disk from the infiltrated site. Immediately after inoculation, each disk contained $\approx 3 \times 10^3$ cfu. The leaf disks were macerated and dilution-plated as described above.

RESULTS

Laboratory Characterization of the *hrp* Mutants. The *hrcC* and *hrpJ* mutants did not elicit the hypersensitive response on tobacco nor cause disease on bean when infiltrated into leaves in growth chamber assays, expected phenotypes of *hrp* mutants (data not shown). In contrast, the *hrpZ* mutant was indistinguishable from B728a in these assays. Immunoblot analyses of culture supernatants and cellular fractions using anti-HrpZ antibodies (26, 35) confirmed that the *hrcC* and *hrpJ* mutants produced but did not secrete the HrpZ protein whereas the *hrpZ* mutant did not produce the HrpZ protein (data not shown). The phenotypes of the *hrcC* mutant were restored by pNCHU421, a plasmid that contains the *hrcC* gene from Pss strain 61 (28). The entire *hrpJ* operon carried on cosmid clones was required to restore the phenotypes of the *hrpJ* mutant.

Population Sizes of the Mutants on Preemergent Seedlings. In each of two field experiments, population sizes of the *hrpJ*, *hrcC*, and *hrpZ* mutants on germinating seedlings increased rapidly and were comparable to those of B728a or B728aCm (Fig. 1 *A* and *B*). In the first experiment, the $\approx 1,000$ -fold increases by 3 DAP (Fig. 1*A*) were typical of increases in bacterial numbers measured in previous experiments (31). In the second experiment, plant emergence was delayed by ≈ 2 days, presumably because of below-normal temperatures, and

bacterial population sizes attained on below-ground samples were unusually large (Fig. 1*B*). By 3 DAP, bacterial population sizes had increased nearly five orders of magnitude. At 7 DAP, the seedlings were still below ground, and mean population sizes of the *hrp* mutants and B728aCm were $\approx 10^8$ cfu/sample. There were no significant differences among population sizes of B728a and the mutants on below-ground bean samples collected in 1995 ($P = 0.59$) or in numbers of B728aCm and the mutants in the 1997 experiment ($P = 0.19$). The indistinguishable behavior of the mutants relative to the wild type suggests that the products encoded by *hrpZ*, *hrcC*, and the *hrpJ* operon did not affect growth of Pss B728a on preemergent seedlings.

Population Sizes of the Mutants in Association with Leaves.

Leaf population sizes of the *hrpZ* mutant were not significantly different from B728a ($P = 0.56$, Fig. 1*A*) or B728aCm ($P = 0.13$, Fig. 1*B*) throughout the two experiments. The mutant and wild type both responded to the environment as expected for Pss (7, 8). However, leaf population sizes of the *hrpJ* (Fig. 1*A* and *B*) and *hrcC* (Fig. 1*B*) mutants were significantly lower than those of B728a ($P < 0.0002$). The absence of substantial population increases during rainy periods favorable for growth suggests that *hrcC* and genes in the *hrpJ* operon play critical roles in enabling Pss to grow in the phyllosphere.

Detection of Brown Spot Lesions in Field Plots. As expected, disease was detected in both field experiments in which large numbers of B728a and the *hrpZ* mutant were present. The *hrpZ* mutant was isolated from lesions, as were B728a and B728aCm. In 1997, relatively large population sizes of the *hrcC* and *hrpJ* mutants were detected on individual primary leaves at 12 DAP (Fig. 2). Indeed, some of the leaves harbored population sizes that were sufficiently large (e.g., $>10^6$ cfu/leaf) to be predictive of disease for strains capable of causing brown spot lesions (6). By 14 DAP, $\approx 4\%$ ($n = 64$) of the leaves that were collected from the *hrcC* and *hrpJ* mutant plots for enumeration of bacterial population sizes had brown spot

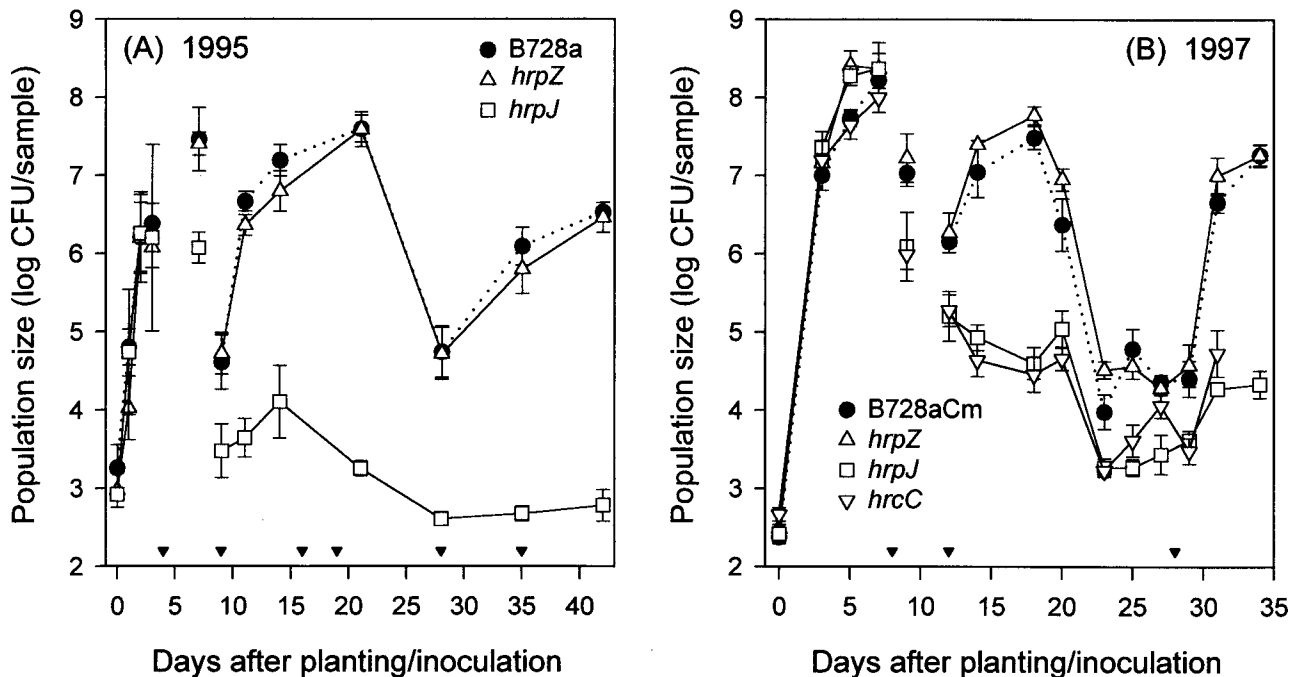


FIG. 1. Population sizes of Pss B728a *hrp* mutants ($\Delta hrpZ::nptII$, *hrpJ*:: ΩSpc^r , $\Delta hrcC::nptII$) in association with field-grown bean plants. The bacterial strains were inoculated onto seeds immediately before planting. Samples were seeds or germinating seedlings collected between 0 and 3 DAP (*A*) and 0 and 7 DAP (*B*); cotyledons and primary leaves were collected at 7 (*A*) and 9 DAP (*B*); primary leaves were collected between 9 and 14 (*A*) and 12 and 20 DAP (*B*); and single leaflet from trifoliolate leaves were collected at all other times. Each datum point represents the mean log cfu/sample and SE based on three (*A*) or four (*B*) replicate plots with six or eight individual samples per plot. Inverted solid triangles indicate rain events with sustained rates >1 mm/min.

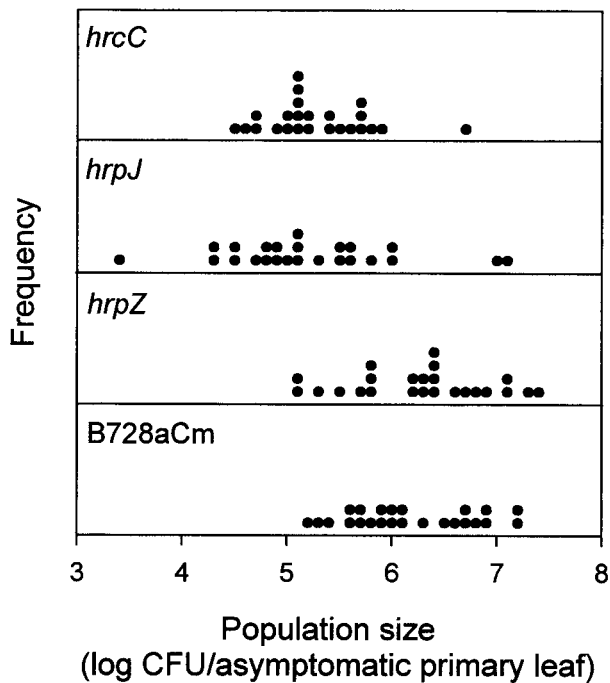


FIG. 2. Distribution of population sizes of B728aCm and the *hrp* mutants in association with individual asymptomatic primary leaves collected at 12 DAP in 1997. Mean bacterial population sizes for the sets of individual leaves are shown in Fig. 1B.

lesions (Fig. 3). At this time, disease incidences were 63 and 75% for B728aCm and the *hrpZ* mutant, respectively.

Additional leaves with brown spot lesions were collected from the *hrcC* and *hrpJ* plots for determination of lesion occupancy. Bacterial population sizes estimated from counts on P medium used to monitor populations of total Pss were similar to those on KBRKan and KBRSpC for lesions collected from the *hrcC* and *hrpJ* inoculated plots, respectively. The results indicated that the mutants were the dominant components within the lesions. None of the bacterial isolates recovered either as single colony isolates from the KBRKan and KBRSpC plates or as mass isolates from the P plates (to detect naturally occurring Pss) caused a pathogenic reaction when subsequently tested in the growth chamber leaf infiltration assay at routine doses of $\approx 3 \times 10^3$ and $\approx 3 \times 10^2$ cfu/infiltrated site. When genomic digests from 10 of these isolates were

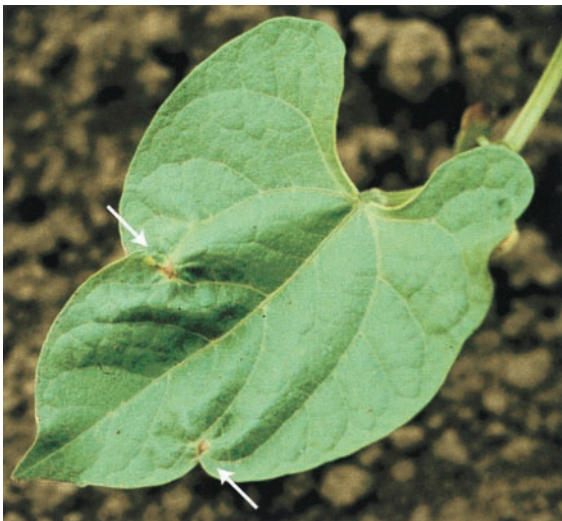


FIG. 3. Brown spot lesions caused by the B728a $\Delta hrcC::nptII$ mutant under field conditions. Lesions were detected 14 DAP in 1997.

probed with either the *nptII* or Ω Spc fragment, the patterns obtained were indistinguishable from those of the *hrcC* or *hrpJ* mutant (data not shown). The results strongly suggest that the lesions detected in the plots inoculated with the *hrcC* and *hrpJ* mutants were caused by these mutants and not by other pathogenic Pss. Further, the mutants had not reverted during passage on plants in the field.

Growth Chamber Assays to Reevaluate the Pathogenic Potential of the *hrp* Secretion Mutants. To determine whether lesion causation by the *hrcC* and *hrpJ* mutants in the field could be reproduced under controlled conditions, the mutants and B728a were inoculated onto bean seeds as was done in the field. Inoculated seeds were germinated under conditions of saturated relative humidity and temperatures mimicking those of the 1997 field experiment (13°C and 22°C during the night and day hours, respectively). Under these conditions, brown spot lesions, similar to those observed in the field, developed on primary leaves. Disease incidences were 2.4% ($n = 163$ leaves) for the *hrpJ* mutant, 4.4% ($n = 159$ leaves) for the *hrcC* mutant, and 30% ($n = 149$ leaves) for B728a. The experiment was repeated with similar results.

Additional experiments were conducted to address the apparent paradox that the mutants were able to cause lesions on primary leaves when inoculated onto seeds but not when infiltrated into leaves. To test the effect of temperature, routine doses of $\approx 3 \times 10^3$ and $\approx 3 \times 10^2$ cfu/infiltrated site were inoculated into plants grown and maintained under the conditions used for the seed-inoculation assay (i.e., lower temperatures than normally used for the leaf infiltration assay). The *hrcC* and *hrpJ* mutants failed to cause a pathogenic reaction under these conditions. To test the effect of dose, the mutants were infiltrated at doses 10- to 100-fold greater (i.e., $\approx 3 \times 10^4$ to $\approx 3 \times 10^5$ cfu/infiltrated site) than routinely used. The *hrcC* and *hrpJ* mutants caused some necrosis when infiltrated with the larger doses (data not shown). The results indicate that the secretion mutants are able to cause a pathogenic reaction in the leaf infiltration assay, albeit reduced relative to wild type, when sufficient doses are used to overcome their inability to grow to numbers necessary to cause disease.

Behavior of the *hrpJ* Mutant in Co-Inoculation Treatments.

When the *hrpJ* mutant was co-inoculated with B728aNal in field experiments and with B728aCm in growth chamber assays, population sizes of the mutant were substantially larger than when inoculated alone (data not shown). Any possible contribution of the *hrp* mutant to disease development could not be ascertained because the B728a derivatives were present in the inoculum and are able to cause disease. We next tested whether growth of the *hrpJ* mutant could be complemented by the non-lesion-forming B728a *gacS* mutant NPS3136 (*gacS1:Tn5*) (12, 13). GacS is similar to the transmembrane histidine kinase protein of a family of bacterial two-component regulatory systems (13). When co-infiltrated with the *gacS* mutant, population sizes of the *hrpJ* mutant were significantly larger than when inoculated alone (Fig. 4). Of interest, a pathogenic reaction developed when the mutants were co-infiltrated into leaves but not when each was inoculated alone (Fig. 5). Co-inoculation of a *gacA* [cognate response regulator of *gacS* (14)] mutant with the *hrpJ* mutant also produced a pathogenic reaction (data not shown). Similar results were obtained when the *hrcC* mutant was co-inoculated with the *gacS* and *gacA* mutants. When the *hrpJ* and *hrcC* mutants were co-inoculated into leaves, growth was comparable to that measured when they were each inoculated alone.

DISCUSSION

Pss may be transmitted from one generation of its host to the next via seeds. Hence, inoculation of bean seeds with relatively small numbers of bacteria at the time of planting was selected

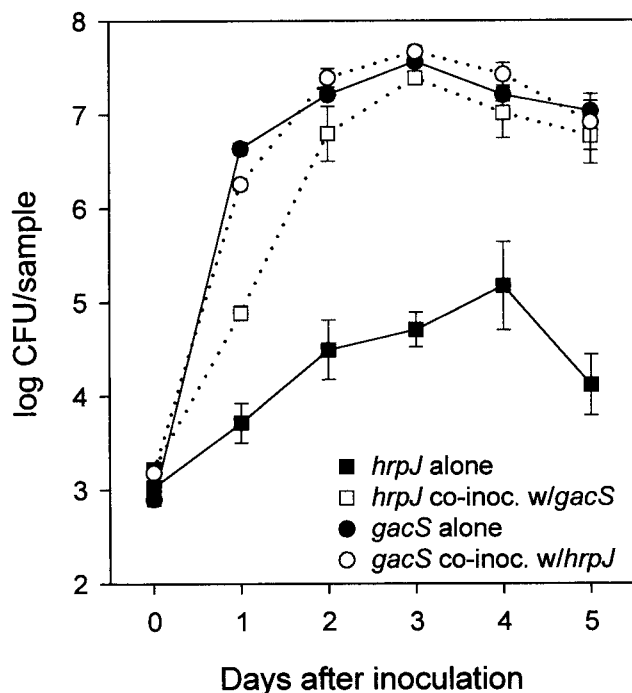


FIG. 4. Rescued growth of the B728a *hrpJ*:: Ω Spc mutant by a non-lesion-forming B728a *gacSI*::Tn5 mutant. The mutants were infiltrated into primary leaves of growth chamber-grown bean plants either alone or in a 1:1 mixture. Approximately 3×10^3 cfu were deposited initially in each of the 6-mm-diameter disks removed for sampling. Samples inoculated with the mixture were dilution-plated onto both KBRSp and KBRKan to enumerate population sizes of the *hrpJ* and *gacS* mutants, respectively. Each datum point represents the mean log cfu/sample and SE based on dilution plating of five individual samples.

as a way to naturally initiate the plant–bacterial interaction in the field. The apparently normal growth of the type III secretion mutants on preemergent bean plants was unexpected given the *in planta* growth defects of *hrp* mutants observed in laboratory experiments (16, 23). After plant emergence, however, leaf population sizes of the *hrcC* and *hrpJ* mutants were

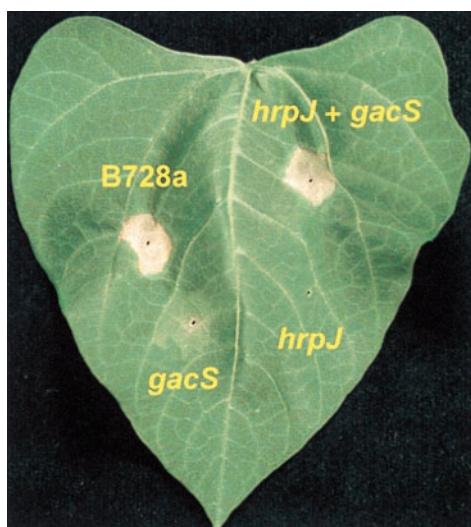


FIG. 5. Leaf infiltration assay for pathogenicity. Necrosis indicative of brown spot disease developed at sites infiltrated with either B728a or a 1:1 mixture of the *hrpJ* and *gacS* mutants. Neither mutant caused the pathogenic reaction when inoculated alone. The dose was $\approx 10^3$ cfu for B728a and the mutants when inoculated alone. In the co-inoculation treatment, the dose for each mutant was $\approx 5 \times 10^2$ cfu.

significantly lower than B728a. Numbers of the mutants tended to remain constant or decline, even under conditions of intense rains, when population sizes of B728a and the *hrpZ* mutant increased significantly. The *hrcC* and *hrpJ* mutants behaved similarly, although the specific genes mutated and the nature of the mutations differed in the two constructs. Thus, mutations in *hrp* genes that affected the Hrp secretion system substantially reduced growth and, possibly, survival of Pss B728a in the phyllosphere. If the behavior of the secretion mutants is attributable primarily to defects in the Hrp type III secretion apparatus, then effector molecules traversing the pathway may be necessary for growth in association with leaves but, interestingly, not with preemergent seedlings.

Our method to remove bacteria from leaves for enumeration precluded a direct assessment of the effect of *hrp* genes on epiphytic versus intercellular growth of Pss. However, several lines of evidence suggest that the diminished population sizes of the *hrcC* and *hrpJ* mutants resulted from their inability to grow on the surfaces as well as intercellular spaces of leaves. Roughly 80–99% of naturally occurring populations of Pss on asymptomatic leaves of field-grown plants are sensitive to a leaf surface sterilization treatment (36), suggesting that a large proportion of the populations is present in unprotected sites on leaves (i.e., epiphytic populations). Hence, if the *hrcC* and *hrpJ* mutants were able to grow epiphytically, we would have expected larger populations than those obtained. Additional evidence for the role of the type III secretion system in epiphytic growth has been obtained by Romantschuk *et al.* (37). After spray inoculation onto tomato plants in the field, epiphytic populations (based on dilution plating of leaf sonicates) of *hrp* mutants of *P. syringae* pv. *tomato* DC3000 were significantly reduced relative to wild type. Models describing the functions of *hrp* genes in interactions of plant pathogenic bacteria with susceptible hosts should be consistent with the scenario that *hrp* genes play a fundamental growth-enabling role that is likely not limited to the intercellular spaces of leaves.

The behaviors of the *hrpZ* mutant and B728a were similar in the spermosphere and the phyllosphere and with regard to lesion formation. Thus, although HrpZ is secreted via the Hrp type III secretory system, and *hrpZ* is located within an operon of the *hrp* cluster, mutation of *hrpZ* had no measurable effect on interactions of B728a with bean plants in the field. The role of HrpZ in interactions of B728a with its susceptible host remains unknown.

In 1997, conditions were highly conducive for growth of the strains before plant emergence. As a result, population sizes of the *hrcC* and *hrpJ* mutants on some newly emerged primary leaves were large enough to be predictive of disease. These relatively large population sizes may have resulted from growth of the mutants while the developing leaves were still enclosed between the cotyledons on the pre- and very early postemergent seedlings. Lesion occupants from the field were recovered and identified as the *hrp* mutants that had been applied to the seed on the basis of their failure to cause a pathogenic reaction in a standard laboratory leaf infiltration assay, their antibiotic resistance marker profiles, and by Southern hybridization analysis. The possibility that naturally occurring pathogenic Pss caused the lesions was eliminated by our inability to isolate bacteria from the lesions that caused a pathogenic reaction when infiltrated into leaves of growth-chamber grown plants. The finding that the *hrcC* and *hrpJ* mutants are capable of causing brown spot lesions on primary leaves after seed inoculation was confirmed in growth chamber experiments.

Necrosis developed when relatively large doses of the *hrcC* and *hrpJ* mutants were infiltrated into leaves of growth chamber-grown plants. Hence, when the requirement for growth was partially alleviated, the secretion mutants were able to produce a pathogenic reaction in the leaf infiltration assay, although reduced relative to B728a. The observation is con-

sistent with the explanation that the apparent requirement for the type III secretion system for pathogenicity in Pss is largely mediated through its role in enabling growth of the pathogen.

Population sizes of the *hrp* secretion mutants were larger when co-inoculated with B728a or the non-lesion-forming *gacS* mutant than when inoculated alone. Because B728a and the *gacS* mutant have functional Hrp systems, it may be that factor(s) secreted by these strains complemented the deficiency in the *hrcC* and *hrpJ* mutants. A similar finding was reported by Kamoun and Kado (38). In their study, growth of a *hrpXc* mutant of the black rot bacterial pathogen, *Xanthomonas campestris* pv. *campestris*, was rescued when the mutant was co-inoculated with its pathogenic parent in cauliflower and radish leaves. An unexpected finding in our experiments was the development of a pathogenic reaction when leaves were co-inoculated with the *gacS* (or *gacA*) and the *hrpJ* or *hrcC* mutants. An explanation for the reaction is that, when co-inoculated, the *gacS* and *hrp* mutants contributed to the pathogenic reaction by providing functions necessary for lesion formation that each alone lacked. Another is that rescued growth of the *hrp* mutant by the *gacS* mutant (either through a direct effect on the mutant or by some plant-mediated mechanism) led to the development of population sizes of the *hrp* mutant that were sufficient to cause disease. Although neither explanation has been ruled out, the second hypothesis is consistent with the findings from field and laboratory experiments that, when growth of the *hrp* mutants in association with plants (preemergent seedlings) leads to establishment of sufficient numbers of the *hrp* secretion mutants in/on primary leaves, brown spot lesions developed.

Although current models for the function of *hrp* genes suggest that pathogenicity or virulence factors may traverse the Hrp type III pathway, none have yet been identified for which effects on disease development have been demonstrated to be distinct from effects on growth in or on leaves. Thus, we cannot rule out the possibility that the role of the type III secretion system in pathogenicity is completely mediated by its requirement for growth of Pss in the phyllosphere. Genes that play a more direct role in symptom development in the Pss-brown spot-snap bean system may reside in the *gacS/gacA* regulon identified by Willis and colleagues (12–15, 39). The role of the Hrp type III secretion system in growth but not symptom development *per se* in Pss is consistent with the recent finding of Shea *et al.* (40) on the role of the type III secretion system within *Salmonella typhimurium* pathogenicity island 2 (SPI2). A mutation in *ssaJ*, a gene encoding a component of the SPI2 type III secretion system, affected growth but not survival of the pathogen in a mouse model system (40).

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