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Use of an Intergenic Region in *Pseudomonas syringae* pv. Syringae B728a for Site-Directed Genomic Marking of Bacterial Strains for Field Experiments

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To construct differentially-marked derivatives of our model wild-type strain, *Pseudomonas syringae* pv. syringae B728a (a causal agent of bacterial brown spot disease in snap bean plants), for field experiments, we selected a site in the *gacS-cysM* intergenic region for site-directed insertion of antibiotic resistance marker cassettes. In each of three field experiments, population sizes of the site-directed chromosomally marked B728a derivatives in association with snap bean plants were not significantly different from that of the wild-type strain. Inserts of up to 7 kb of DNA in the intergenic region did not measurably affect fitness of B728a in the field. The site is useful for site-directed genomic insertions of single copies of genes of interest.

Quantitative comparisons of bacterial population sizes in the field are greatly facilitated when each strain carries a unique selectable marker. This is particularly true when dispersal of bacteria between experimental plots in the field is expected or when one strain must be enumerated in the presence of a large excess of the other and measurement by difference or even by distinct colony morphology may give biased results. We are interested in comparisons of phyllosphere population sizes of mutants bearing defects in genes associated with pathogenicity to population sizes of wild-type *Pseudomonas syringae* pv. syringae (causal agent of bacterial brown spot disease) on snap bean (*Phaseolus vulgaris* L.) plants in the field (5, 8). The mutants carry selectable markers present on inserts that disrupt the genes of interest. For example, Tn5 mutants are resistant to kanamycin (Kan^r) due to the presence of the neomycin phosphotransferase gene (*nptII*) on the transposon. The issue that we faced in our studies was how to mark our wild-type strain in a way that would allow facile measurement of the population sizes to unambiguously differentiate them from those of mutant strains in field experiments.

One alternative is to use strains bearing spontaneous mutations that confer antibiotic resistance. These mutants are often easy to isolate, but they are less easy to characterize and may be less fit than the strain from which they were derived. In previous field experiments, we used a spontaneous nalidixic acid-resistant (Nal^r) mutant of *P. syringae* pv. syringae B728a as a surrogate wild-type strain (8). Although Nal^r B728a colonized bean leaves and caused disease in the field (8), we subsequently found that the derivative is less fit than the wild-type B728a. To better mark the wild-type strain, we sought to identify a site in the genome of B728a into which marker cassettes could be inserted in a site-directed manner without affecting fitness of the bacterium in the field. De Leij et al. (2) constructed derivatives of *Pseudomonas fluorescens* SBW25 with site-directed genomic insertions of marker genes (*lacZY, aph-1* conferring Kan^r, and *xylE*) to examine the effects of gene insertions on the fitness of genetically modified bacteria. In their studies, however, bacterial fitness was assessed in greenhouse and growth chamber experiments, not under natural field conditions.

To identify a possible site for insertion of marker cassettes in the genome of B728a, we targeted a site (hereafter referred to as the landing site) located 51 bp upstream of the *gacS* open reading frame and within the roughly 235-bp intergenic region between the well-characterized regulatory gene *gacS* (global activator sensor kinase) (9) and *cysM*, a gene required for cysteine biosynthesis. The genes are transcribed in opposite directions away from the intergenic region (9). In *P. syringae* pv. syringae B728a, *gacS* is required for brown spot lesion formation; production of syringomycin, protease, *N*-acyl-L-homoserine lactone, and alginate; and a swarming behavior on soft agar (9, 10, 12, 13, 17). The landing site in the intergenic region was selected based on the finding that pEMH97 (9.7-kb *Hin*dIII fragment with *gacS* and *cysM* cloned in pLAFR3) containing a Tn*3*Gus insertion in the site retained the ability to restore the phenotype of the *gacS* mutant NPS3136 (*gacS1*::Tn*5*) to wild-type levels, whereas transposon insertions in *gacS* failed to do so (9). The objective of this study was to determine whether antibiotic resistance genes inserted into the landing site in the *gacS-cysM* intergenic region in the genome of B728a affect fitness of the bacterium in its natural habitat, leaves in the field.

Construction of site-directed marked derivatives. To introduce marker cassettes into the genome of B728a, we modified plasmid pEMH97 to contain a unique *Bam*HI restriction sequence at the landing site. The restriction sequence was created by PCR using *Pfu* DNA polymerase (Stratagene, La Jolla, Calif). The modified plasmid pSSH-LS was digested with *Bam*HI, and when necessary, the ends were made blunt with the Klenow fragment prior to ligation with the following marker cassettes: *nptII* (kanamycin resistance) (1), ΩSpc inter-

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poson (spectinomycin resistance) (15), ΩCm interposon (chloramphenicol resistance) (3), and Gm^r (gentamycin resistance) (16). The markers were introduced into the chromosome of B728a by recombinational exchange.

The sequence of the roughly 1-kb region in pSSH-LS that was generated by PCR to introduce the *Bam*HI restriction site differed from the published sequence (9) only by the presence of the six additional bases constituting the newly created *Bam*HI site. The site-directed marked derivatives of B728a were indistinguishable from the wild-type strain in their abilities to produce extracellular protease and syringomycin in plate bioassays (10), to cause brown spot lesions when infiltrated into leaves of growth-chamber-grown bean plants at 10^6 CFU per ml, and to grow on minimal medium in the absence of cysteine with glucose as the carbon source (data not shown). Thus, *gacS* and *cysM* were still functional in the marked derivatives of B728a, and the derivatives were indistinguishable from the wild-type strain in these laboratory assays.

Field experiments. Three field experiments were conducted to determine the relative fitness of the marked derivatives and B728a. The bacterial strains were inoculated onto snap bean seeds (cultivar Eagle; Asgrow Seed Co., Kalamazoo, Mich.) at the time of planting, and bacterial population sizes were monitored on germinating seeds and leaves of emergent plants as described previously (8). The treatments (i.e., bacterial strains) were in a randomized complete block design with three (1996 experiments) or eight (1997 experiment) blocks. Plot sizes were 8 by 8 m and 4 by 6 m in the 1996 and 1997 experiments, respectively. Bacterial population sizes were determined by dilution plating of individual leaf or seed homogenates as described previously (6, 8). The samples were plated on King's Medium B (11) containing rifampin (50 μg/ml) (KBR) supplemented with the appropriate antibiotics (kanamycin, 30 μg/ml; spectinomycin, 50 μg/ml; chloramphenicol, 30 μg/ml; gentamicin, 2 μg/ml; nalidixic acid, 50 μg/ml). Bacterial counts per sample were $\log_{10}$ transformed before calculation of population statistics (7). Samples with no detectable colonies 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). Evaluation of the relative fitness of the bacterial strains was based on the relative changes in population sizes over time. Treatment effects and treatment-by-time interactions were determined over all sampling time points using repeated-measures analysis of variance tests implemented with SAS Procedure Mixed (SAS Institute, Cary, N.C.) (14).

1996 experiments. In 1996, the relative fitnesses of B728a and the marked derivatives were compared in two adjacent experimental plots planted on 25 June. B728aTn*3*Gus, constructed by recombinational exchange of pEMH97::Tn*3*Gus (9) and therefore subjected to fewer molecular manipulations than the site-directed marked derivatives, was included as a control on the construction process. Additionally, the B728aTn*3*Gus construct provided a test of the effect of insert size on fitness. At roughly 7 kb, the Tn*3*Gus insert was over twice the size of any other insert tested.

Population sizes of all strains fluctuated as expected based on previous field experiments with naturally occurring populations of *P. syringae* and other introduced strains derived from B728a (Fig. 1) (4, 5, 8). Comparisons of bacterial population sizes within each of the experiments yielded no significant treatment effects or treatment-by-time interactions (experiment I [Fig. 1A], P [treatment] = 0.947, P [interaction] = 0.805; experiment II [Fig. 1B], P [treatment] = 0.502, P [interaction] = 0.695). Thus, there was no evidence to suggest that insertion of marker cassettes in the *gacS-cysM* intergenic region affected the fitness of B728a in the field.

Although the plots were planted on the same day, bacterial population sizes among replicate plots were more variable in experiment II than in experiment I. This may have been due to the greater spatial heterogeneity noted in the area occupied by experiment II than in that occupied by experiment I.

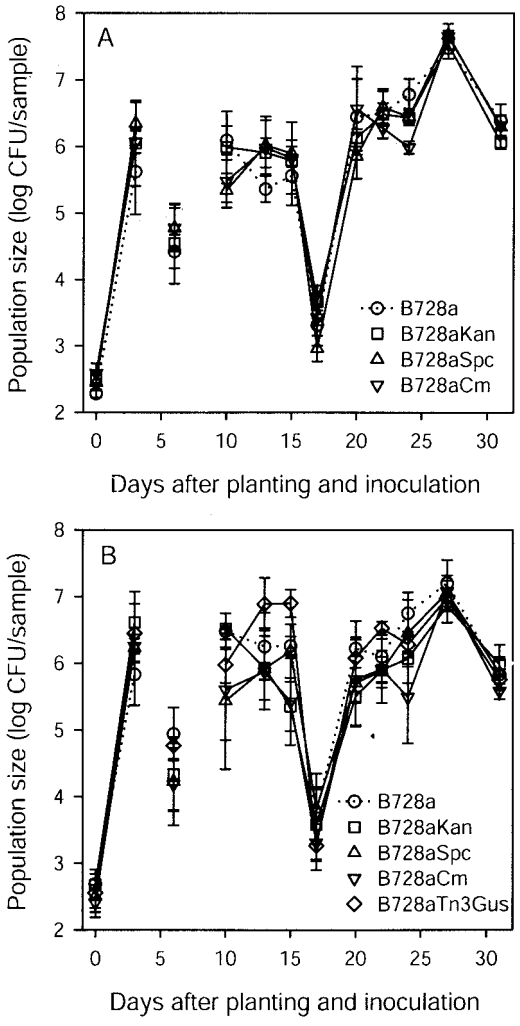

FIG. 1. 1996 field experiments. Mean population sizes of wild-type B728a and site-directed marked derivatives in association with field-grown bean plants are shown. The two experiments (A and B) were initiated on the same day in 1996. The bacterial strains were inoculated onto seeds immediately before planting. The sampling units collected were seeds at 0 days after planting (DAP); germinating seeds at 3 DAP; entire above-ground parts of emergent seedlings at 6 DAP; primary leaves at 10, 13, and 15 DAP; and leaflets from trifoliolate leaves at all other sampling times. Each datum point represents the mean log CFU/sample and standard error based on three replicate plots with six or eight individual samples per plot.

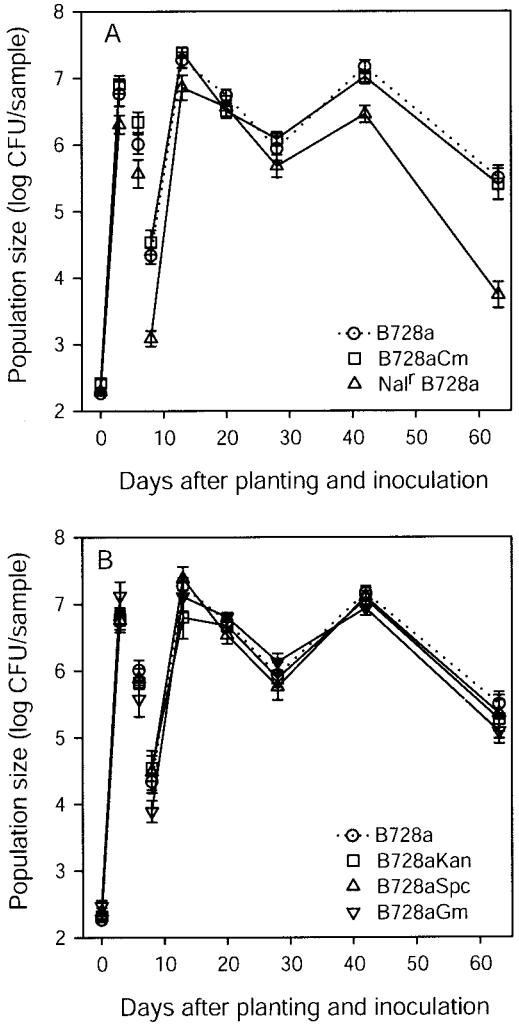

FIG. 2. 1997 field experiment. Mean population sizes of wild-type B728a, Nal^r B728a, and site-directed marked derivatives in association with field-grown bean plants are shown. For clarity, the data are plotted in two groups. (A) B728a, Nal^r B728a, and B728aCm; (B) B728a (same data as in panel A), B728aKan, B728aSpc, and B728aGm. The bacterial strains were inoculated onto seeds immediately before planting. The sampling units collected were seeds at 0 days after planting (DAP), germinating seeds at 3 DAP, entire above-ground parts of emergent seedlings at 6 DAP, primary leaves at 8 and 13 DAP, and leaflets from trifoliolate leaves at all other sampling times. Each datum point represents the mean log CFU/sample and standard error based on eight replicate plots with five individual samples per plot.

1997 experiment. To increase sensitivity in the detection of possible small differences in bacterial population sizes in an inherently variable system, we increased the number of blocks from three to eight in the 1997 experiment and monitored population sizes of the bacterial strains for a longer time (63 versus 31 days). Fewer subsamples were collected from each plot (eight in 1996 and five in 1997), and the plots were sampled less frequently. We included the Nal^r spontaneous mutant of B728a as a control strain with known decreased fitness relative to B728a.

Repeated-measures tests across the nine sampling times and six strains (Fig. 2) yielded a significant treatment effect ($P < 0.0001$). Pairwise comparisons of the strains indicated that, as expected, Nal^r B728a was significantly different from the wild type and each of the site-directed marked derivatives ($P < 0.0001$). None of the site-directed derivatives, however, were significantly different from B728a or each other (P, 0.217 to 0.624). When Nal^r B728a was omitted from the analyses, no significant treatment ($P = 0.218$) or treatment-by-time ($P = 0.145$) effect was found. Thus, we were able to measure a decrease in fitness in a bacterial strain (Nal^r B728a) previously found to be less fit than B728a but found no measurable differences among the site-directed marked derivatives and the wild type.

In three field experiments, population sizes of the site-directed marked derivatives were reproducibly similar to those of B728a. The results strongly suggest that neither the site of insertion (landing site in the *gacS-cysM* intergenic region) nor the inserts themselves affected the fitness of B728a in its natural habitat, bean leaves in the field. We now have a pool of well-defined, differentially marked derivatives of our model strain B728a for field experiments. Additionally, any decreased fitness of a B728a mutant relative to the wild type can be attributed specifically to disruption of the gene of interest and not to the presence of the insert used to mutate the gene, at least for those inserts examined thus far. While insertion of the ΩCm^r interposon in the intergenic region appeared not to affect fitness of B728a, the Cm^r marker was not as satisfactory as the Kan^r, Spc^r, and Gm^r markers due to a reduction in plating efficiency (~20%) of B728aCm on KBR plus chloramphenicol relative to KBR alone.

In laboratory studies, tests of whether a particular phenotype is due to the specific gene mutated are routinely done by complementation in *trans*. Issues of plasmid instability and potential effects of the plasmid itself or of copy number of a plasmid-borne gene render such tests problematic for field experiments that examine field fitness over many hundreds of bacterial generations. We envision that pSSH-LS may be a useful tool to deliver a single copy of a wild-type gene of interest into the genome of the corresponding mutant by marker exchange. Insertion of the wild-type gene into a site such as the landing site in the genome of a mutant background would yield a stable construct for restoration experiments. Because only a single copy of a wild-type gene would be present, potential problems associated with copy number effects encountered with plasmid restoration in *trans* would be circumvented.

While the study described here focused on a specific intergenic region, and well-defined site-directed marked derivatives were constructed for a specific bacterial strain (B728a), there is no reason to suspect that other intergenic regions could not be exploited in B728a and other bacterial strains.

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