APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 2001, p. 1400–1403 Vol. 67, No. 3
0099-2240/01/$04.00+0 DOI: 10.1128/AEM.67.3.1400–1403.2001

Genetic Evidence that Loss of Virulence Associated with *gacS* or *gacA* Mutations in *Pseudomonas syringae* B728a Does Not Result from Effects on Alginate Production

DAVID K. WILLIS,[1]* JEREMY J. HOLMSTADT,[2] AND THOMAS G. KINSCHERF[2]

Plant Disease Resistance Research Unit, U.S. Department of Agriculture—Agricultural Research Service,[1] and Department of Plant Pathology, University of Wisconsin—Madison,[2] Madison, Wisconsin 53706

Received 30 August 2000/Accepted 12 December 2000

Mutations in the global regulatory genes *gacS* and *gacA* render *Pseudomonas syringae* pv. syringae strain B728a completely nonpathogenic in foliar infiltration assays on bean plants. It had been previously demonstrated that *gac* genes regulate alginate production in *Pseudomonas* species, while other published work indicated that alginate is involved in the pathogenic interaction of *P. syringae* on bean plants. Together, these results suggested that the effects of *gacS* and *gacA* mutations on virulence in B728a might stem directly from a role in regulating alginate. In this report, we confirm a role for *gac* genes in both *algD* expression and alginate production in B728a. However, B728a mutants completely devoid of detectable alginate were as virulent as the wild-type strain in our assay. Thus, factors other than, or in addition to, a deficiency of alginate must be involved in the lack of pathogenicity observed with *gacS* and *gacA* mutants.

Alginate production has long been studied in the genus *Pseudomonas* due to the link between cystic fibrosis symptoms in humans and the isolation of mucoid *Pseudomonas aeruginosa* from patient lungs (2). Since the production of extracellular polysaccharide had also been implicated in the virulence of several phytopathogenic bacteria such as *Erwinia stewartii* (3) and *Ralstonia solanacearum* (10, 22), the strong foundation of the molecular genetics of the alginate biosynthetic pathway in *P. aeruginosa* (23) made a natural starting point for studies in closely related plant pathogens such as *P. syringae* that were also known to produce alginate (6). The alginate biosynthetic genes in *P. syringae* are highly conserved compared to the *P. aeruginosa* biosynthetic cluster (5, 7, 17). A role for alginate production in pathogenic interactions of *P. syringae* pathovars on their plant hosts had been suggested (4, 21, 27). Mutant screens in another plant pathogen, *Pseudomonas viridiflava*, had demonstrated a link between the *gacS-gacA* regulon in that organism and the production of alginate and pathogenicity (15). These results were reinforced by the finding that *algD*, a biosynthetic gene central to alginate production, requires *gacS* for its efficient expression in *Azotobacter vinelandii* (1). The *gacS-gacA* two-component system is widely distributed in gram-negative bacteria and regulates diverse gene systems involved with moderating the bacterial interaction with the extracellular milieu. The two members of this gene pair, either singly or together, have been implicated in the expression of a wide variety of phenotypes in a number of bacterial genera. Of particular significance have been results that showed that *gacS* and *gacA* play a role in regulating virulence factors in animal pathogens such as *Salmonella* spp. (9) and *Vibrio cholerae* (26). In *P. syringae* pv. syringae B728a, a causal agent of bacterial brown spot of the snap bean, *gacS* and *gacA* have been shown to regulate bacterial swarming, the production of syringomycin, protease, and *N*-acyl-L-homoserine lactone, in addition to the pathogenicity defects originally described (12; see also reference 13). However, none of these individual phenotypes have been directly related to the effects of *gac* mutations on pathogenicity. The requirement for *gacS* and *gacA* in alginate production (1, 14) and the reported involvement of alginate in pathogenic interactions on bean plants (27) made it seem possible that alginate was a significant contributing factor to the lesion-minus phenotype exhibited by *gacS* and *gacA* mutants of B728a. Here we report that this is probably not the case.

An ongoing project in our laboratory uses the reporter transposon Tn*lacZ* to directly search for genes that are affected for expression in a B728a *gacA* mutant background (15; T. G. Kinscherf, J. J. Holmstadt, E. M. Ostertag, A. K. Savage, C. A. Hinkley, T. Kitten, J. L. McEvoy, and D. K. Willis, unpublished results). During the course of this work, two transposon insertions were isolated, cloned, and characterized as being in the *algD* gene. This locus encodes GDP-mannose dehydrogenase (2) and is the first gene in the alginate biosynthetic operon of *P. syringae* pv. syringae (17). Partial sequence analysis indicated appropriately high similarities to *Pseudomonas algD* genes already in the GenBank database. A cosmid containing the Tn*lacZ* insertion in *algD* was mated into strains B728a (wild type), BGACΩ1 (*gacA*), and BSAL1 (*salA*). Potential kanamycin-resistant, tetracycline-sensitive chromosomal exchanges were isolated in all three backgrounds and were checked by Southern blotting for the recombinational inheritance of the Tn*lacZ* mutation (data not shown).

B728a was tested over a range of sorbitol and NaCl concentrations on mannitol-glutamic acid-yeast extract (MGY) plates at 28°C, and maximal alginate production (as evidenced by visual mucoidy) occurred in the presence of 0.6 M sorbitol (data not shown), in agreement with the previously published data (17). In contrast, the *algD* exchange mutant BALG1 was

* Corresponding author. Mailing address: Plant Disease Resistance Research Unit, U.S. Department of Agriculture—Agricultural Research Service, University of Wisconsin, 1630 Linden Dr., Madison, WI 53706. Phone: (608) 262-5063. Fax: (608) 262-1541. E-mail: dkwillis@facstaff.wisc.edu.

TABLE 1. Alginate production by *P. syringae* pv. syringae B728a and various mutant strains

Strain	Description	Alginate production[a] (μg/mg of total protein)[b]
B728a	Wild type	+ (495)
NPS3136	*gacS1*::Tn*5* mutant of B728a (25)	− (<20)
BGACΩ1	*gacA2*::Ω mutant of B728a; the Ω cassette (18) was cloned into the *gacA* gene and exchanged into the chromosome of B728a (Kitten et al.)[c]	− (<20)
BALG1	*algD1*::Tn*lacZ* insertion exchanged into B728a	− (<20)
BALG1(pSK2)	Alginate biosynthetic region (17) mated into BALG1	+ (442)
BHSL	*ahlI*::*lacZ* mutant of B728a (12)	+ (372)
BSAL1	*salA1*::Ω mutant of B728a (13)	+ (588)
BGAC1.313	*algD1*::Tn*lacZ* reporter exchange into BGACΩ1	− (ND)
BGAC2.313	Independent *algD*::Tn*lacZ* reporter exchange into BGACΩ1	− (ND)
BSAL.313	*algD*::Tn*lacZ* reporter exchange into BSAL1	− (ND)
B728a(pLAFR3)	B728a containing plasmid vehicle pLAFR3 (24); used as a wild-type plasmid-containing control	+ (ND)
BALG1(pLAFR3)	BALG1 containing plasmid vehicle pLAFR3 (24); used as a plasmid-containing control	− (ND)
BGAC1.313(pLAFR3)	BGAC1.313 containing plasmid vehicle pLAFR3 (24); used as a plasmid-containing control	− (ND)
BGAC1.313(pSyrgac23)	BGAC1.313 containing a wild-type *gacA* gene in pLAFR3 to yield plasmid pSyrgac23 (20)	− (ND)

[a] The plus or minus sign indicates whether mucoidy was produced on MGY (17) plus 0.6 M sorbitol plates.
[b] Alginate was quantitated using a modified version of a previously described assay (16); the standard errors were ±120, ±78, ±66, and ±70 for B728a, BALG1(pSK2), BHSL, and BSAL1, respectively. Other strains tested too low for meaningful standard error or were not done (ND).
[c] T. Kitten, E. M. Ostertag, T. G. Kinscherf, and D. K. Willis, unpublished data.

nonmucoid and did not produce detectable alginate under these same conditions (Table 1). Mucoidy was restored to BALG1 by the introduction of plasmid pSK2 containing the alginate biosynthetic cluster from the *P. syringae* pv. syringae strain FF5 (17), indicating that the insertion in *algD* was causal to the nonmucoid phenotype. Maximal expression of the chromosomal Tn*lacZ* reporter in strain BALG1 also occurred at 0.6 M sorbitol in MGY liquid medium (data not shown). Figure 1A shows the expression of the chromosomal *algD1*::Tn*lacZ* reporter in various genetic backgrounds (wild type, *gacA2*::Ω, and *salA1*::Ω) in the presence or absence of 0.6 M sorbitol. The addition of 0.6 M sorbitol to the medium resulted in an approximately twofold induction of *algD* expression from all strains tested regardless of the mutational background. This twofold effect of sorbitol was independent of the presence or absence of the *gacA* gene (Fig. 1A), although the lack of an intact *gacA* gene by itself caused a dramatic reduction of expression (12.7- to 16.7-fold) within both of the *gacA* mutant reporter strains tested. Expression of the *algD* reporter was restored completely by introduction of the *gacA* gene on a plasmid (Fig. 1B). Table 1 shows that neither the *gacS* mutant NPS3136 nor the *gacA* mutant BGACΩ1 produces alginate, confirming the regulation of the alginate pathway by this two-component regulator. The *salA* gene is a regulator of antibiotic production and virulence in B728a that is dependent upon *gacS* and *gacA* for its expression (13). The *salA* mutant BSAL1 produced normal levels of alginate (Table 1), and a mutation in *salA* did not significantly affect either the basal expression of the *algD* reporter or the twofold sorbitol induction of expression (Fig. 1). In addition, the alginate production of the acyl-homoserine lactone-deficient mutant BHSL (12) was not affected (Table 1). This indicates that alginate production lies in a separate branch of the *gac* regulon from *salA*. We also tested a collection of field strains of *P. syringae* pv. syringae and their respective *gacS* mutants (19) and, in all cases, alginate production (as judged by mucoidy on MGY medium containing sorbitol) was found to be *gacS* dependent (data not shown).

The effect of Tn*lacZ* insertions in *algD* on the virulence of B728a on the snap bean (*Phaseolus vulgaris*) was tested in the same bean leaf infiltration assay we used to define the nonpathogenic phenotype of *gacS* and *gacA* mutants (Fig. 2). Primary bean leaves were inoculated with our standard assay range of cell concentrations, i.e., 10^7, 10^6, and 10^5 CFU/ml. No difference in the virulence level was observed between B728a and BALG1 in three experiments, with lesion manifestation occurring with both strains at all of the tested inoculum levels. When the *gacA* mutant BGACΩ1 was inoculated along with the other strains in the same experiment, no disease symptoms were observed for that strain, as always (Fig. 2). Bacterial growth following infiltration of primary bean leaves was assayed using B728a(pLAFR3), BALG1(pLAFR3), and the alginate-restored mutant BALG1(pSK2) in a leaf infiltration growth assay (25). The in planta population growth of the three strains was essentially identical over a period of 3 days (data not shown), at which time the level of necrosis made further collection of leaf disks problematic with all three strains.

The data presented here clearly support the inference from earlier work (1, 14) that *gacS* and *gacA* regulation of alginate production is general among pseudomonads and probably among other gram-negative bacteria. The profound effects of *gacA* mutations on the expression of *algD* would appear to be sufficient to explain the requirement for functional *gacS* and *gacA* genes in alginate production. The *algD* gene is the first gene in the *P. syringae* biosynthetic cluster and encodes GDP-mannose dehydrogenase, an essential synthetic enzyme in the alginate pathway (17). However, it is important not to oversimplify the situation, since it is clear that multiple levels of regulation are involved in this system. While sorbitol had only

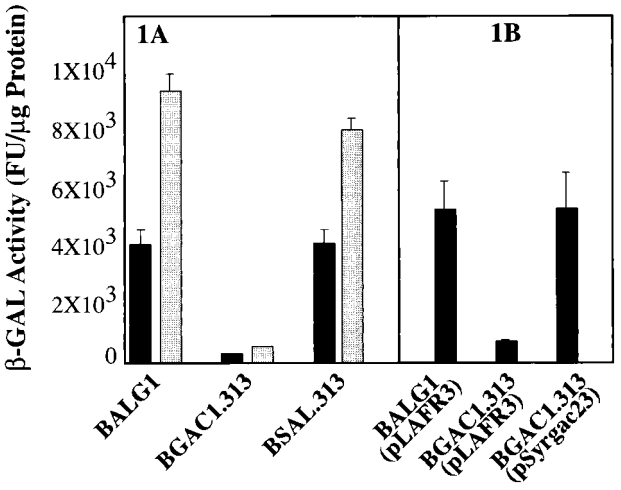

FIG. 1. *algD* Tn*lacZ* reporter activity in B728a mutants. Six individual β-galactosidase assays were performed (FluorReporter *lacZ*/Galactosidase Quantitation Kit F-2905; Molecular Probes) with each culture of strains grown in MGY (17) for 16 h at ambient temperature with aeration. Shown is the mean β-galactosidase activity as fluorescence units (FU)/microgram of total cellular protein for the mean of two repetitions of the experiment. The error bars represent the standard errors of the means. (A) Expression of the reporter in *algD* in various genetic backgrounds without (black column) or with (gray column) 0.6 M sorbitol added to the MGY medium. The β-galactosidase activity from BGAC1.313 was 322 (±21) FU without or 566 (±23) FU with sorbitol in the medium. The independent exchange mutant BGAC2.313 gave 310 (±27) FU and 563 (±23) FU, respectively (data not shown). (B) Restoration of *algD* expression by wild-type *gacA* on a plasmid (pSyrgac23). Strains were grown in MGY containing 0.6 M sorbitol and 10 μg of tetracycline per ml. The β-galactosidase activity from BGAC1.313(pLAFR3) was 677 (±99) FU. The cosmid pLAFR3 was used as the vector control for pSyrgac23.

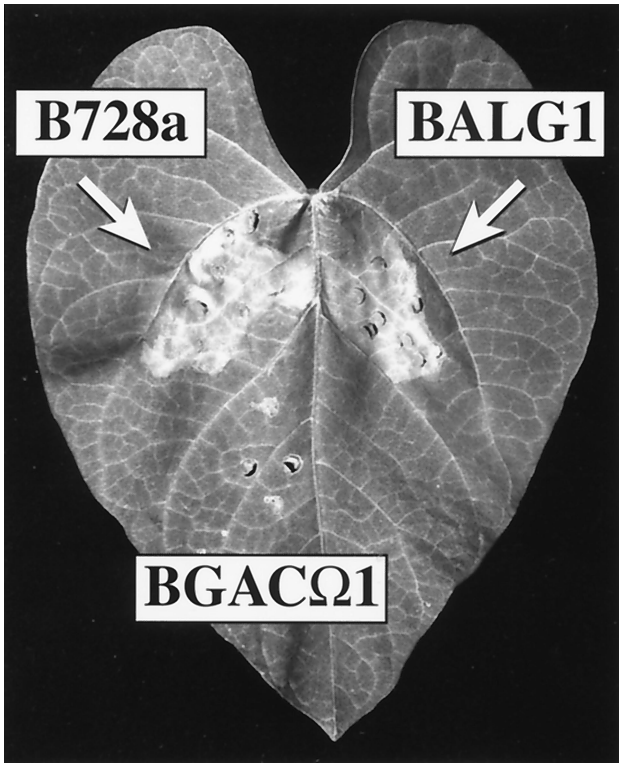

FIG. 2. Lesion formation by B728a and mutant derivatives on bean leaves. Bacterial suspensions were made from King's medium B plates grown for 2 days at 28°C. Bacteria were suspended in water and diluted to 10^6 CFU per ml in 10 mM phosphate buffer (pH 7.2). Bacteria were infiltrated locally into the leaves of 14-day-old bean plants. The photograph was taken 3 days postinoculation using a Kodak DCS420 digital camera. The arrows indicate the pathogenic reaction caused by infiltration of B728a or BALG1. The small circular wounds within the area infiltrated by BGACΩ1 were caused by the infiltration process.

relatively small effects on *algD* expression, it was still absolutely required for the manifestation of mucoidy on plates. This suggests that osmolarity might be affecting the expression of other genes involved in alginate production. The *algT* gene has been shown to be osmoregulated and encodes a sigma factor (σ^{22}) that is required for alginate production (11). The conditional expression of this sigma factor would have the capacity to affect multiple loci within the biosynthetic cluster, and it may be the cumulative effects of this regulation that produces the requirement for osmotic stress that we observed. Previous studies measuring expression of an *algD* reporter on a plasmid reported significantly greater effects for sorbitol induction, possibly reflecting differences in plasmid copy number (17). We found that a chromosomal location for the reporter was critical for the accurate determination of *algD* expression. Our preliminary tests using the *algD* reporter on a plasmid in *P. syringae* resulted in only a threefold *gac* effect on expression and no significant induction by sorbitol (data not shown). This effectively masked the separation of the *gac* and sorbitol effects that our chromosomal reporter demonstrated, with the twofold sorbitol induction occurring independently of the presence or absence of an intact *gacA* gene. The environmental signal(s) to which the *gacS-gacA* system responds remains unknown, and our results indicate that it is neither sorbitol nor the osmotic effects of sorbitol that provide this signal. This finding was reinforced by other experiments that showed that the expression of a *salA* reporter fusion, previously demonstrated to be strongly regulated by *gacA* (13), was also unaffected by 0.6 M sorbitol (data not shown).

The lack of effect by a *salA* mutation on either *algD* expression or alginate production demonstrates that alginate production lies in the *salA*-independent branch of the *gacS-gacA* regulon. The *salA* gene was originally isolated as a copy number suppressor of *gacS* phenotypes in B728a and was subsequently found to encode a regulator that was dependent on the presence of intact *gacS* and *gacA* genes for its expression (13). Phenotypes associated with *salA* mutations in B728a are defects in antibiotic production and severe attenuation of pathogenicity in laboratory assays. These represent a subset of known *gacS* and *gacA* phenotypes in B728a, and thus, *salA* effectively defines the branch point in the *gac* regulon leading to the manifestation of plant disease. From this perspective it is not too surprising that alginate production, being in the *salA*-independent part of the regulon, does not appear to play a major role in virulence. Repeated infiltration experiments failed to demonstrate deficiencies in lesion forming ability by the *algD* mutant relative to the wild type (Fig. 2). Previous work reported by other investigators described a small decrease in lesion number in growth chamber experiments with a

P. syringae mutant affected in the *algL* gene (27), as well as the absence of satellite lesions in infiltrated leaves. We have never observed satellite lesions around sites infiltrated with B728a (19), nor have others that regularly perform bean infiltration assays with *P. syringae* pv. syringae (S. Hirano, personal communication). The leaf infiltration assay that is used in our laboratory is very reproducible and has shown a high degree of correlation with results from large-scale field studies (8; S. S. Hirano and C. D. Upper, unpublished data). This is the method that we have routinely used to define the pathogenicity phenotype in *gacS* and *gacA* mutants of B728a (13, 20, 25). Thus, the virulence exhibited by *algD* mutants in this assay would appear to make it unlikely that the *gacS-gacA*-mediated loss of alginate production is responsible for the complete lack of pathogenicity exhibited by *gacS* and *gacA* mutants (Fig. 2). It remains to be determined if defects in alginate production contribute to a loss of fitness under field conditions such as has been demonstrated with a *gacS* mutant (8). This possibility is currently under investigation.

We thank Susan Hirano and Chris Upper for helpful suggestions on the manuscript.

This work was briefly supported by NSF grant MCB-9419023 to D. K. Willis, and J. J. Holmstadt was supported by the NSF/DOE/USDA *Arabidopsis* Training Grant at UW—Madison.

REFERENCES

1. **Castaneda, M., J. Guzman, S. Moreno, and G. Espin.** 2000. The GacS sensor kinase regulates alginate and poly-beta-hydroxybutyrate production in *Azotobacter vinelandii*. J. Bacteriol. **182:**2624–2628.
2. **Deretic, V., J. F. Gill, and A. M. Chakrabarty.** 1987. *Pseudomonas aeruginosa* infection in cystic fibrosis: nucleotide sequence and transcriptional regulation of the *algD* gene. Nucleic Acids Res. **15:**4567–4581.
3. **Dolph, P. J., D. R. Majerczak, and D. L. Coplin.** 1988. Characterization of a gene cluster for exopolysaccharide biosynthesis and virulence in *Erwinia stewartii*. J. Bacteriol. **170:**865–871.
4. **Fett, W. F., and M. F. Dunn.** 1989. Exopolysaccharides produced by phytopathogenic *Pseudomonas syringae* pathovars in infected leaves of susceptible hosts. Plant Physiol. **89:**5–9.
5. **Fett, W. F., C. Wijey, and E. R. Lifson.** 1992. Occurrence of alginate gene sequences among members of the pseudomonad rRNA homology groups I–IV. FEMS Microbiol Lett. **78:**151–157.
6. **Fett, W. M., S. F. Osman, M. L. Fishman, and T. S. Siebles III.** 1986. Alginate production by plant-pathogenic pseudomonads. Appl. Environ. Microbiol. **52:**466–473.
7. **Fialho, A. M., N. A. Zielinski, W. F. Fett, A. M. Chakrabarty, and A. Berry.** 1990. Distribution of alginate gene sequences in the *Pseudomonas* rRNA homology group I-*Azomonas-Azotobacter* lineage of superfamily B procaryotes. Appl. Environ. Microbiol. **56:**436–443.
8. **Hirano, S. S., E. M. Ostertag, S. A. Savage, L. S. Baker, D. K. Willis, and C. D. Upper.** 1997. Contribution of the regulatory gene *lemA* to field fitness of *Pseudomonas syringae* pv. syringae. Appl. Environ. Microbiol. **63:**4304–4312.
9. **Johnston, C., D. A. Pegues, C. J. Hueck, A. Lee, and S. I. Miller.** 1996. Transcriptional activation of *Salmonella typhimurium* invasion genes by a member of the phosphorylated response-regulator superfamily. Mol. Microbiol. **22:**715–727.
10. **Kao, C. C., E. Barlow, and L. Sequeira.** 1992. Extracellular polysaccharide is required for wild-type virulence of *Pseudomonas solanacearum*. J. Bacteriol. **174:**1068–1071.
11. **Keith, L. M., and C. L. Bender.** 1999. AlgT (σ^{22}) controls alginate production and tolerance to environmental stress in *Pseudomonas syringae*. J. Bacteriol. **181:**7176–7184.
12. **Kinscherf, T. G., and D. K. Willis.** 1999. Swarming by *Pseudomonas syringae* B728a requires *gacS* (*lemA*) and *gacA* but not the acyl-homoserine lactone gene *ahlI*. J. Bacteriol. **181:**4133–4136.
13. **Kitten, T., T. G. Kinscherf, J. L. McEvoy, and D. K. Willis.** 1998. A newly-identified regulator is required for virulence and toxin production in *Pseudomonas syringae*. Mol. Microbiol. **28:**917–930.
14. **Liao, C.-H., D. McCallus, and W. Fett.** 1994. Molecular characterization of two gene loci required for production of the key pathogenicity factor pectate lyase in *Pseudomonas viridiflava*. Mol. Plant-Microbe Interact. **7:**391–400.
15. **Manoil, C.** 1990. Analysis of protein localization by use of gene fusions with complementary properties. J. Bacteriol. **172:**1035–1042.
16. **May, T. B., and A. M. Chakrabarty.** 1994. Isolation and assay of *Pseudomonas aeruginosa* alginate. Methods Enzymol. **235:**295–304.
17. **Peñaloza-Vázquez, A., S. P. Kidambi, A. M. Chakrabarty, and C. L. Bender.** 1997. Characterization of the alginate biosynthetic gene cluster in *Pseudomonas syringae* pv. syringae. J. Bacteriol. **179:**4464–4472.
18. **Prentki, P., and H. M. Krisch.** 1984. In vitro insertional mutagenesis with a selectable DNA fragment. Gene **29:**303–313.
19. **Rich, J. J., S. S. Hirano, and D. K. Willis.** 1992. Pathovar-specific requirement for the *Pseudomonas syringae lemA* gene in disease lesion formation. Appl. Environ. Microbiol. **58:**1440–1446.
20. **Rich, J. J., T. G. Kinscherf, T. Kitten, and D. K. Willis.** 1994. Genetic evidence that the *gacA* gene encodes the cognate response regulator for the *lemA* sensor in *Pseudomonas syringae*. J. Bacteriol. **176:**7468–7475.
21. **Rudolph, K. W. E., M. Gross, F. Ebrahim-Nesbat, M. Nollenburg, A. Zomorodian, K. Wydra, et al.** 1994. The role of extracellular polysaccharide as virulence factors for phytopathogenic pseudomonads and xanthomonads, p. 357–378. *In* C. I. Kado and J. H. Crosa (ed.), Molecular mechanisms of bacterial virulence. Kluwer Academic Publishers, Dordrecht, The Netherlands.
22. **Saile, E., J. A. McGarvey, M. A. Schell, and T. P. Denny.** 1997. Role of extracellular polysaccharide and endoglucanase in root invasion and colonization of tomato plants by *Ralstonia solanacearum*. Phytopathology **87:**1264–1271.
23. **Shankar, S., R. W. Ye, D. Schlictman, and A. M. Chakrabarty.** 1995. Exopolysaccharide alginate synthesis in *Pseudomonas aeruginosa*: enzymology and regulation of gene expression. Adv. Enzymol. Relat. Areas Mol. Biol. **70:**221–255.
24. **Staskawicz, B. J., D. Dahlbeck, N. Keen, and C. Napoli.** 1987. Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea*. J. Bacteriol. **169:**5789–5794.
25. **Willis, D. K., E. M. Hrabak, J. J. Rich, T. M. Barta, S. E. Lindow, and N. J. Panopoulos.** 1990. Isolation and characterization of a *Pseudomonas syringae* pv. *syringae* mutant deficient in lesion formation on bean. Mol. Plant-Microbe Interact. **3:**149–156.
26. **Wong, S. M., P. A. Carroll, L. G. Rahme, F. M. Ausubel, and S. B. Calderwood.** 1998. Modulation of expression of the ToxR regulon in *Vibrio cholerae* by a member of the two-component family of response regulators. Infect. Immun. **66:**5854–5861.
27. **Yu, J., A. Peñaloza-Vázquez, A. M. Chakrabarty, and C. L. Bender.** 1999. Involvement of the exopolysaccharide alginate in the virulence and epiphytic fitness of *Pseudomonas syringae* pv. syringae. Mol. Microbiol. **33:**712–720.