Distribution Among Pseudomonads of Sequences Homologous to the Rutin Glycosidase and β-Glucosidase Genes of *Pseudomonas viridiflava*

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Accepted for publication 16 June 1992.

ABSTRACT

Hendson, M., Hildebrand, D. C., and Schroth, M. N. 1992. Distribution among pseudomonads of sequences homologous to the rutin glycosidase and β-glucosidase genes of *Pseudomonas viridiflava*. Phytopathology 82:1230-1233.

The rutin glycosidase and β-glucosidase genes of *Pseudomonas viridiflava* were cloned, and fragments internal to these two genes were used as probes to determine homology with some members of the rRNA homology I group of pseudomonads and other phytopathogenic bacteria. Sequences homologous to these two probes occurred in all fluorescent arginine dihydrolase-negative phytopathogenic pseudomonads tested, regardless of whether the phenotype was expressed. In contrast, the rutin glycosidase probe did not hybridize with any of the rutin glycosidase-negative nonfluorescent or fluorescent arginine dihydrolase-positive pseudomonads, with the exception of *P. marginalis,* which was weakly homologous with the probe. One strain of *P. putida* bv. B that expressed rutin glycosidase activity showed homology with the probe. The nonfluorescent, β-glucosidase-negative strain of *P. alcaligenes* hybridized weakly with the β-glucosidase probe. β-Glucosidase-positive strains belonging to other genera did not share any homology with the β-glucosidase probe. Pathogenicity tests were performed on tomato with a *P. syringae* pv. *tomato* strain and transposon mutants lacking β-glucosidase or rutin glycosidase to determine the possible function of these enzymes. No difference was observed between wild-type and β-glucosidase- and rutin glycosidase-negative strains with respect to numbers and morphology of lesions on tomato leaves.

Two β-glycosidases that hydrolyze naturally occurring glucosides have been described among phytopathogenic fluorescent pseudomonads: one (β-glucosidase) hydrolyzes β-glucosides such as salicin and arbutin (9), which consist of an aglycone attached to the monosaccharide glucose, and the other, rutin glycosidase, hydrolyzes rutin (quercetin-3-*O*-β-D-glucose-α-rhamnose) (8). Plant glycosidases occur at different concentrations in different parts of the plant during growth (6). Salicin, arbutin, and rutin are phenolic glycosides that can occur in high concentrations in plant tissues and are generally compartmentalized in vacuoles (13). All three glycosidases are widely distributed among diverse plant families. For example, salicin has been found in the leaves, flowers, and bark of Salicaceae and the distant family Caprifoliaceae (23), and arbutin occurs in the families Ericaceae, Leguminoseae, Saxifragaceae, Rosaceae, Liliaceae, Pyrolaceae, and Proteaceae (29). Rutin is so widely distributed among plants that its absence is more significant than its presence (6). The buckwheat plant (*Fagopyrum esculentum* Moench) contains 3% rutin when starting to bloom (3).

Rutin glycosidase and β-glucosidase differ in their distribution among phytopathogenic pseudomonads (7–10). Rutin glycosidase activity was exhibited by all phytopathogenic fluorescent pseudomonads that are arginine dihydrolase-negative, with the exception of *Pseudomonas syringae* pv. *glycinea* (8), whereas fewer than 10% of the arginine dihydrolase-positive fluorescent saprophytes were positive. The occurrence of β-glucosidase among fluorescent pseudomonads was more sporadic but still confined mostly to the phytopathogens (7,9–11,14).

The widespread occurrence of these enzymes among phytopathogens in contrast to saprophytes and the broad distribution of their substrates among plants suggest a number of possible roles for these enzymes in pathogenesis. Several cases of plant phenolic compounds as regulators of gene expression in phytopathogens have been documented (5,12,20–22,26). Arbutin and salicin are strong inducers of *syr*B, a gene required for the production of syringomycin in *P. s. syringae* (21). Phenolic compounds stimulate or repress expression of nodulation genes in *Rhizobium* (5) and induce virulence genes of *Agrobacterium tumefaciens* (22,26). A β-glucosidase activity has been associated with virulence of a gymnosperm-specific strain of *A. tumefaciens* (22). The hydrolysis of coniferin (a phenylpropanoid glycoside) to coniferyl alcohol by β-glucosidase affected virulence gene induction.

This study describes the distribution among rRNA group I pseudomonads of DNA sequences homologous with the rutin glycosidase and β-glucosidase genes isolated from *P. viridiflava.* The suggestion that rutin glycosidase and β-glucosidase may be involved in pathogenicity of phytopathogens was tested.

MATERIALS AND METHODS

Bacterial strains. The pseudomonad species and number of strains tested are listed in Table 1. They were obtained from the collection of the Department of Plant Pathology, University of California, Berkeley. Included also were representatives of *Agrobacterium, Erwinia, Escherichia,* and *Xanthomonas.*

Assay for enzyme activity. The hydrolysis of rutin by rutin glycosidase was assayed as described by Hildebrand and Caesar (8). Bacteria were grown on minimal medium (1) containing 0.1% salicin and 0.1% glucose to induce the β-glucosidase enzyme. The method of Hildebrand et al (11) using the chromogenic substrate *p*-nitrophenyl-β-glucoside was used to assay strains for β-glucosidase activity.

Cloning and subcloning rutin glycosidase and β-glucosidase genes. A *P. viridiflava* F62L library, in *E. coli* DH5α (Bethesda Research Laboratories, Gaithersburg, MD) using pLAFR3 (27) as the cosmid vector, was obtained from L. Rahme (Department of Plant Pathology, University of California, Berkeley). Members of the cosmid library were tested for β-glucosidase activity as described above. Partially restricted insert DNA from a β-glucosidase-positive cosmid clone was ligated into pGEM3 (Promega, Madison, WI) and transformed into DH5α. The resulting plasmid, pBG2 (Fig. 1A), which carried gene(s) for β-glucosidase production, contained an 8.6-kb *Eco*RI insert. Since the rutin glycosidase gene(s) was not expressed in *E. coli* DH5α, recombinant clones were tested for rutin glycosidase after mobilizing (4) the entire library to *P. putida* 642rif, a strain that does not produce the enzyme. Plasmid pRut2 (Fig. 1B), which carries the gene(s) for rutin hydrolysis, was constructed by cloning the 6.3-

TABLE 1. Distribution and expression of β-glucosidase and rutin glycosidase genes among pseudomonads of rRNA homology group I and some other bacteria

Species and pathovars	Rutin glycosidase activity	Homology with rutin glycosidase probe	β-Glucosidase activity	Homology with β-glucosidase probe
Pseudomonas syringae pv. *aceris* (1),[a] *antirrhini* (5), *aptata* (11), *coronofaciens* (6), *erobotryae* (3), *helianthi* (2), *lachrymans* (5), *delphinii* (1), *maculicola* (20), *papulans* (1), *passifloriae* (3), *pisi* (8), *syringae* (28), *tabaci* (3), *tomato* (13), *P. cichorii* (6), *P. viridiflava* (3)	+	+	+	+
P. s. cannabina (1), *huszi* (4), *mori* (3), *morsprunorum* (3), *phaseolicola* (6), *pisi* (7), *savastanoi* (3), *sesami* (8)	+	+	−	+
P. putida bv. B (1)	+	+	−	−
P. marginalis (2)	−	−	+	+
P. marginalis (5)	−	W[b]	+	+
P. s. glycinea (3)	−	+	−	+
P. fluorescens (8), *P. aureofaciens* (4), *P. stutzeri* (1), *P. mendocina* (1), *P. pseudoalcaligenes* (1), *P. solanacearum* (5), *P. aeruginosa* (7), *P. putida* bv. A (2), *Escherichia coli* (4)	−	−	−	−
Erwinia amylovora (3), *E. carotovora* (3), *P. cepacia* (8), *Xanthomonas campestris* (23), *Agrobacterium tumefaciens* (8)	−	−	+	−
P. alcaligenes (1)	−	−	−	W

[a] Numbers in parentheses indicate the number of strains tested. All strains of a particular pathovar or species reacted in the same way.
[b] Weakly homologous with the probe.

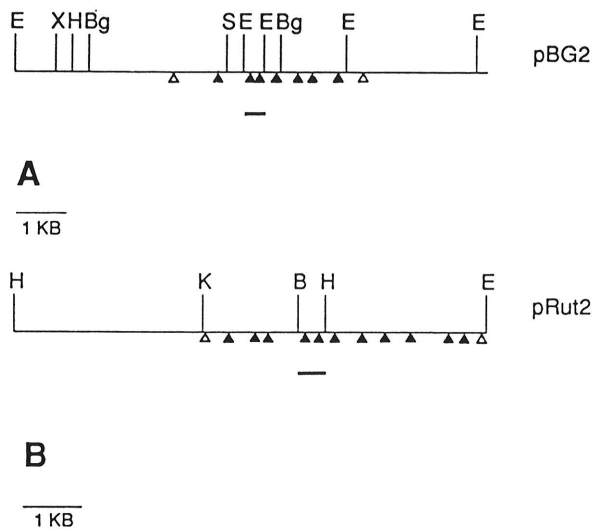

Fig. 1. Location of Tn*3*-spice insertions in **A,** pBG2, and **B,** pRut2. Solid triangles indicate the position of insertion in β-glucosidase and rutin glycosidase mutants. Open triangles indicate the position of insertion that does not affect β-glucosidase and rutin glycosidase activity. The 0.4-kb *Eco*RI fragment of pBG2 and the 0.4-kb *Bam*HI-*Hin*dIII fragment of pRut2 used as probes are indicated by the solid bar beneath the restriction map. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; S, *Sma*I; X, *Xho*I.

kb *Hin*dIII-*Eco*RI fragment of a rutin glycosidase-positive cosmid clone into the broad host range vector pLAFR3.

Tn*3*-spice insertion mutagenesis of pBG2 and pRut2. Tn*3*-spice (18) insertion derivatives of the plasmids pBG2 and pRut2 were constructed by random mutagenesis according to the procedure of Rahme et al (25). Plasmid pBG2:Tn*3*-spice and pRut2:Tn*3*-spice derivatives were tested for β-glucosidase and rutin glycosidase activity, respectively. The positions of insertion of Tn*3*-spice in pBG2 and pRut2 were mapped by single and double restriction enzyme digests of β-glucosidase$^-$ and rutin glycosidase$^-$ mutant plasmids.

Colony hybridization. Fragments within the rutin and β-glycosidase genes were used as probes. The 0.4-kb *Eco*RI fragment of pBG2 (Fig. 1A) and the 0.4-kb *Bam*HI-*Hin*dIII fragment of pRut2 (Fig. 1B) were purified from low-melting-point agarose using Elutip columns (Schleicher and Schuell Inc., Keene, NH) according to the manufacturer's instructions. Fragments were labeled with ^{32}P-dCTP using the Multiprime labeling system (Amersham, Arlington Heights, IL).

Colony blots were prepared using Nytran membranes (Schleicher and Schuell) as recommended by the manufacturer. Hybridizations and washings were performed according to Maniatis et al (19). Filters were incubated for 16–24 h at 65 C with 10^6 cpm of probe DNA in hybridization solution.

Spectrophotometric analysis of the products of rutin hydrolysis. Rutin glycosidase-positive strains and rutin glycosidase-negative strains of *P. s. glycinea, P. fluorescens,* and *P. marginalis* were grown in minimal agar medium (1) containing 0.1% glucose and 0.1% rutin. Plates were incubated at 28 C until the agar medium was dehydrated (approximately 2 mo). The dried medium was homogenized, and ethanol-soluble products in the medium were extracted. The ethanol extract was scanned in a Gilford Response II spectrophotometer (Gilford, Oberlin, OH) at 250–450 nm. Samples were read against a solution of rutin (75 ng/ml in ethanol) as well as against an ethanol extract of a dehydrated rutin plate.

Tn*5* mutagenesis of *P. s. tomato* 3455rif and pathogenicity testing. To determine the effect of a lack of rutin or β-glucosidase on pathogenicity, we selected *P. s. tomato* instead of *P. viridiflava,* because it is easy to quantify lesions in *P. s. tomato* and determine any differences in their morphology. The nature of the disease caused by *P. viridiflava,* a rot, makes it much more difficult to detect small differences in virulence. Tn*5* mutagenesis of *P. s. tomato* strain 3455rif was performed as described by Peet et al (24). The suicide plasmid pUW964 was used to generate Tn*5* mutants (28). Rif^{R}Km^R colonies were screened for rutin and β-glucosidase as described above. Wild-type, β-glucosidase$^-$, and rutin glycosidase$^-$ strains were tested for pathogenicity. Strains were grown on King's B agar (16) with the appropriate antibiotics for 24 h. Cell suspensions of 10^3 cfu/ml in sterile distilled water were infiltrated under vacuum into the intercellular spaces of leaves of 30-day-old tomato plants (*Lycopersicon esculentum* 'Peto 76'). Plants were incubated in a growth chamber at 24 C with 12 h of light. Leaves were analyzed daily for the development of symptoms.

RESULTS

Positions of Tn*3*-spice insertions in β-glucosidase⁻ and rutin glycosidase⁻ mutants of pBG2 and pRut2. The positions of Tn*3*-spice insertions in β-glucosidase⁻ and rutin glycosidase⁻ mutants of pBG2 and pRut2, respectively, are shown in Figure 1. Since Tn*3*-spice insertions were located within and encompassed the 0.4-kb *Eco*RI fragment of pBG2 and the 0.4-kb *Bam*HI-*Hin*dIII fragment of pRut2 in β-glucosidase⁻ and rutin glycosidase⁻ mutants, respectively, these fragments were used to probe all strains for homology.

Homology with the 0.4-kb *Eco*RI fragment of pBG2. All arginine dihydrolase-negative fluorescent pseudomonads had DNA that was homologous with the β-glucosidase probe (Table 1), regardless of whether they exhibited activity in the β-glucosidase assays. In the arginine dihydrolase-positive group, only strains of *P. marginalis* that were positive for β-glucosidase activity hybridized with the probe. Bacteria outside the fluorescent pseudomonad group showed no homology with the β-glucosidase probe even if they exhibited β-glucosidase activity. The only exception was one strain of *P. alcaligenes* that did not exhibit β-glucosidase activity but showed weak homology with the probe.

Homology with the 0.4-kb *Bam*HI-*Hin*dIII fragment of pRut2. All arginine dihydrolase-negative strains of fluorescent pseudomonads had DNA homologous with the rutin glycosidase probe (Table 1). This included all rutin hydrolysis-positive strains of the species *P. viridiflava, P. cichorii,* and *P. syringae* and the rutin hydrolysis-negative *P. s. glycinea.* Among the arginine dihydrolase-positive fluorescent pseudomonads, only the rutin hydrolysis-positive *P. putida* bv. B strain shared homology with the probe; five of seven *P. marginalis* strains were weakly homologous with the probe. None of the nonfluorescent pseudomonads or strains belonging to other genera produced rutin glycosidase or carried sequences homologous to the 0.4-kb *Bam*HI-*Hin*dIII fragment of pRut2.

Spectrophotometric analysis of the products of rutin hydrolysis. Ethanol extracts from cultures of all strains in which rutin glycosidase was expressed gave a peak at the same wavelength as quercetin, the aglycone component of rutin (420 nm), when scanned against the standards. Extracts from plates of *P. s. glycinea, P. fluorescens,* and *P. marginalis* strains where rutin glycosidase was not detected showed a profile similar to that of the ethanol extract of the rutin plate, which served as a control (Fig. 2).

Tn*5* mutagenesis of *P. s. tomato* 3455rif and pathogenicity tests. Two thousand Tn*5* mutants of 3455rif were tested for rutin and β-glucosidase activity. Two rutin glycosidase⁻ and two β-glucosidase⁻ mutants were isolated, and these were tested for pathogenicity. No differences were observed between wild-type and mutant strains in lesion number and morphology.

DISCUSSION

Rutin glycosidase activity and sequences homologous to the rutin glycosidase gene were primarily confined to phytopathogenic fluorescent arginine dihydrolase-negative pseudomonads. In general, sequences homologous to the rutin glycosidase probe occurred only in strains that exhibited rutin glycosidase activity. The exceptions to this were the rutin glycosidase-negative *P. s. glycinea,* which showed strong homology with the rutin glycosidase probe, and five strains of *P. marginalis* that showed weak homology.

The expression of β-glucosidase among phytopathogenic, fluorescent, arginine dihydrolase-negative pseudomonads was not as widespread as rutin glycosidase. However, DNA from all of these phytopathogens hybridized with the β-glucosidase probe. The presence of sequences homologous with a particular gene in strains that do not exhibit activity is not an unusual phenomenon. The pectate lyase gene of *X. campestris* pv. *vesicatoria* hybridized to other xanthomonads whether or not they were able to produce pectate lyase (2). Similarly, sequences homologous with the pectate lyase gene of *P. fluorescens* were

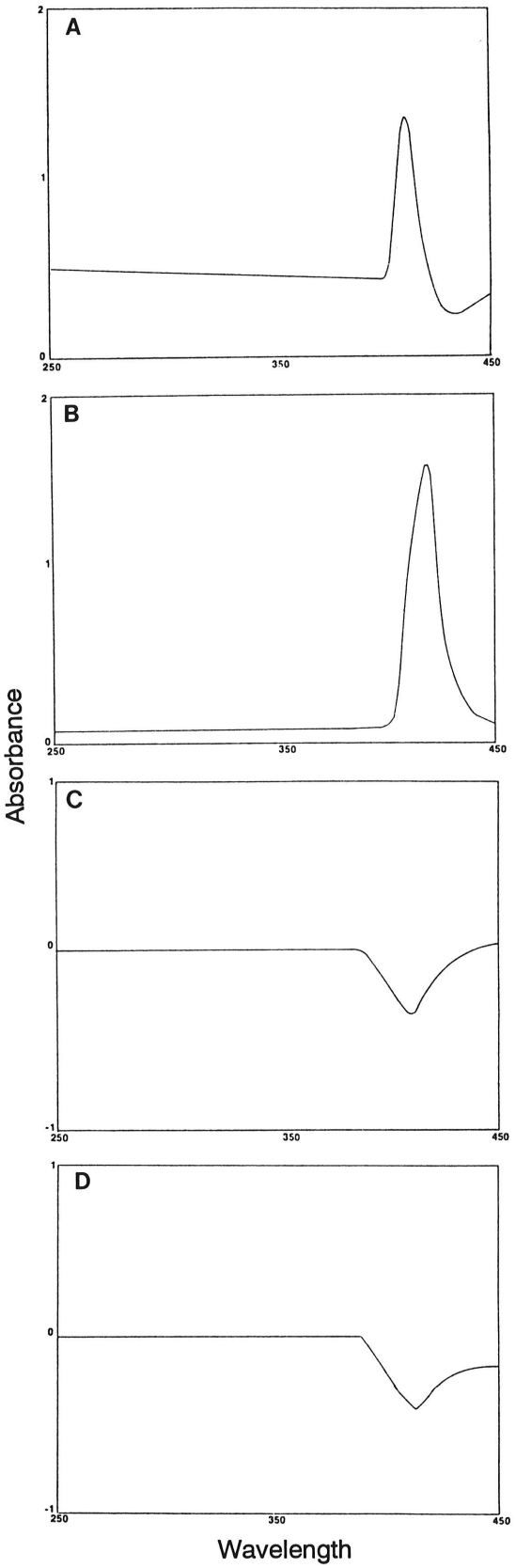

Fig. 2. Spectrophotometric profiles of **A,** an ethanol extract of a quercetin plate; **B,** an ethanol extract of a rutin plate inoculated with *Pseudomonas syringae* pv. *syringae;* **C,** an ethanol extract of an uninoculated rutin plate; **D,** an ethanol extract of a rutin plate inoculated with *P. s. glycinea.* Samples were scanned against a rutin ethanol standard (75 ng/ml).

found in *P. fluorescens* that did not exhibit pectate lyase activity (17). There are several explanations for the presence of sequences homologous to the rutin and β-glucosidase genes in strains that exhibit no activity. A mutation possibly has occurred to render the gene inactive, the sequences that hybridize may encode a glycosidase whose substrate is unknown, the gene may be repressed, or the level of the glycosidase may be too low to be detected. It was not surprising that we detected no homology between our *P. viridiflava* β-glucosidase probes and β-glucosidase-producing species of other genera, since they have widely divergent evolutionary origins.

The association of rutin glycosidase and β-glucosidase enzymes with nearly all phytopathogenic fluorescent pseudomonads, in contrast to their general absence in saprophytes, suggested the involvement of these enzymes in pathogenesis in plants. This, however, was not the case for *P. s. tomato* strain 3455rif. Tn*5* mutants that lacked rutin or β-glucosidase enzymes were not altered in their ability to cause lesions on tomato leaves. β-Glucosidase appears to play varying roles among phytopathogens. In the gymnosperm-specific strain of *A. tumefaciens,* β-glucosidase, which converts coniferin to coniferyl alcohol, plays an essential role in the induction of virulence genes (22). In contrast, the β-glucosidase of *E. amylovora* does not affect pathogenicity (15). It is noteworthy that *P. s. glycinea* exhibits neither β-glucosidase nor rutin glycosidase activity. We are studying other possible functions for these enzymes in pseudomonads such as survival, specificity of β-glucosidases for plant glycosidic substrates, and expression of the genes in host and nonhost plants.

LITERATURE CITED

1. Ayers, S. H., Rupp, R., and Johnson, W. T. 1919. A study of the alkali-forming bacteria in milk. U.S. Dep. Agric. Bull. 782.
2. Bealieu, C., Minsavage, G. V., Canteros, B. I., and Stall, R. E. 1991. Biochemical and genetic analysis of a pectate lyase gene from *Xanthomonas campestris* pv. *vesicatoria.* Mol. Plant Microbe Interact. 4:446-451.
3. Couch, J. F., Naghski, J., and Krewson, C. F. 1946. Buckwheat as a source of rutin. Science 103:197-198.
4. Ditta, D. W., Stanfield, S., Corbin, D., and Helsinki, D. R. 1980. Broad host range cloning system for Gram-negative bacteria: Construction of a gene bank of *Rhizobium meliloti.* Proc. Natl. Acad. Sci. USA 27:7347-7351.
5. Djordjevic, M. A., Redmond, J. W., Batley, M., and Rolfe, B. G. 1987. Clovers secrete specific phenolic compounds which either stimulate or repress *nod* gene expression in *Rhizobium trifolii.* EMBO J. 6:1173-1179.
6. Harborne, J. B., and Williams, C. A. 1975. Flavone and flavonol glycosides. Pages 377-441 in: The Flavonoids. J. B. Harborne, T. J. Mabry, and H. Mabry, eds. Academic Press, New York.
7. Hayward, A. C. 1977. Occurrence of glycoside hydrolases in plant pathogenic and related bacteria. J. Appl. Bacteriol. 43:407-411.
8. Hildebrand, D. C., and Caesar, A. 1989. The widespread occurrence of rutin glycosidase in fluorescent phytopathogenic pseudomonads. Lett. Appl. Microbiol. 8:117-119.
9. Hildebrand, D. C., and Schroth, M. N. 1964. β-Glucosidase activity in phytopathogenic bacteria. Appl. Microbiol. 12:487-491.
10. Hildebrand, D. C., and Schroth, M. N. 1971. Identification of the fluorescent pseudomonads. Pages 281-287 in: Proc. Int. Conf. Plant Pathog. Bact., 3rd. H. P. Maas Geesteranus, ed. Wageningen, Netherlands.
11. Hildebrand, D. C., Schroth, M. N., and Sands, D. C. 1988. *Pseudomonas.* Pages 60-80 in: Laboratory Guide for Identification of Plant Pathogenic Bacteria. 2nd ed. N. W. Schaad, ed. American Phytopathological Society, St. Paul, MN.
12. Horvath, B., Bachem, C. W. B., Schell, J., and Kondorosi, A. 1987. Host specific regulation of nodulation genes in *Rhizobium* is mediated by a plant signal interacting with the *nod*D gene product. EMBO J. 6:841-848.
13. Hrazdine, G., and Wagner, G. J. 1985. Compartmentation of plant phenolic compounds; sites of synthesis and accumulation. Pages 119-133 in: Annual Proceedings of the Phytochemical Society of Europe, vol. 25. C. F. Van Sumere and P. J. Lea, eds. Clarendon Press, Oxford.
14. Joubert, J. J., Hildebrand, D. C., and Schroth, M. N. 1970. Nonutilization of beta-glucosides for growth by fluorescent pseudomonads. Phytopathology 60:502-505.
15. Kerppola, T. K., Serwold-Davis, T., Gross, D., and Kahn, M. C. 1987. Effect of increased β-glucosidase activity on virulence of *Erwinia amylovora.* Applied Environ. Microbiol. 53:677-682.
16. King, E. O., Wood, M. K., and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. 44:301-307.
17. Liao, C.-H. 1991. Cloning of pectate lyase gene *pel* from *Pseudomonas fluorescens* and detection of sequences homologous to *pel* in *Pseudomonas viridiflava* and *Pseudomonas putida.* J. Bacteriol. 173:4386-4393.
18. Lindgren, P. B., Peet, R. C., Govindarajan, A. G., Panopoulos, N. J., Staskawicz, B. J., and Lindow, S. E. 1989. An ice nucleation reporter gene system: Identification of inducible pathogenicity genes in *Pseudomonas syringae* pv. *phaseolicola.* EMBO J. 8:2990-3001.
19. Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
20. Melchers, L. S., Regensburg-Tuink, T. J. G., Schilperoort, R. A., and Hooykaas, P. J. J. 1989. Specificity of signal molecules in the activation of *Agrobacterium* virulence gene expression. Mol. Microbiol. 3:969-977.
21. Mo, Y.-Y., and Gross, D. 1991. Plant signal molecules activate the *syr*B gene, which is required for syringomycin production by *Pseudomonas syringae* pv. *syringae.* J. Bacteriol. 173:5784-5792.
22. Morris, J. W., and Morris, R. O. 1990. Identification of an *Agrobacterium tumefaciens* gene inducer from the pinaceous gymnosperm *Pseudotsuge menziesii.* Proc. Natl. Acad. Sci. USA 87:3614-3618.
23. Paris, R. 1963. The distribution of plant glycosides. Pages 337-357 in: Chemical Plant Taxonomy. T. Swain, ed. Academic Press, New York.
24. Peet, R. C., Lindgren, P. B., Willis, D. K., and Panopoulos, N. J. 1986. Identification and cloning of genes involved in phaseolotoxin production by *Pseudomonas syringae* pv. *phaseolicola.* J. Bacteriol. 166:1096-1105.
25. Rahme, L. G., Mindrinos, M. N., and Panopoulos, N. J. 1991. Genetic and transcriptional organization of the *hrp* cluster of *Pseudomonas syringae* pv. *phaseolicola.* J. Bacteriol. 73:575-586.
26. Stachel, S. E., Messens, E., Van Montagu, M., and Zambryski, P. 1985. Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens.* Nature 318:624-629.
27. Staskawicz, B., Dahlbeck, D., Keen, N., and Napoli, C. 1987. Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea.* J. Bacteriol. 169:5789-5794.
28. Weiss, A. A., Hewlett, E. L., Myers, G. A., and Falkow, S. 1983. Tn*5*-induced mutations affecting virulence factors of *Bordetella pertussis.* Infect. Immun. 42:33-41.
29. Williams, A. H. 1964. Dihydrochalcones: Their occurrence and use as indicators in chemical plant taxonomy. Nature 202:824-825.