

Identification of gene loci controlling pectate lyase production and soft-rot pathogenicity in *Pseudomonas marginalis*¹

Abstract: *Pseudomonas marginalis* is an important postharvest pathogen capable of causing soft rot in a wide variety of harvested fruits and vegetables. Following transposon mutagenesis, we isolated two groups of *P. marginalis* CY091 mutants deficient in production of pectate lyase (Pel) and soft-rot pathogenicity in plants. The first group, designated Pel⁻, was caused by the insertion of Tn5 into a *pel* structural gene, and the second group, designated LemA⁻, was caused by the insertion of Tn5 into a regulatory locus corresponding to the *lemA* gene previously identified in other Gram-negative bacteria. The LemA⁻ mutants also exhibited alteration in colony morphology and showed deficiency in production of protease (Prt). A cosmid clone pCIC carrying the *P. marginalis lemA* gene was isolated and characterized. pCIC was capable of restoring Pel production and soft-rot pathogenicity in LemA⁻ mutants of *P. marginalis* and *Pseudomonas viridiflava*, indicating that the function of *lemA* gene in these two pseudomonads was similar and interchangeable. Using MudI-mediated mutagenesis, we isolated a third group of *P. marginalis* mutants deficient in production of Pel, Prt, and soft-rot pathogenicity. Mutants in this group (designated GacA⁻¹) contained an insertion of MudI in a locus corresponding to the *gacA* gene of *P. viridiflava*. Like LemA⁻ mutants, GacA⁻¹ mutants also exhibited alteration in colony morphology and showed deficiency in production of Pel and Prt. However, GacA⁻¹ mutants produced much lower levels of levan and fluorescent pyoverdine siderophore than the wild type and LemA⁻ mutants. These results provide the first genetic evidence that *P. marginalis* produces a single alkaline Pel for maceration of plant tissue and demonstrate that production of Pel, Prt, levan, and pyoverdine by this bacterium is mediated by the two-component *lemA/gacA* gene system.

Key words: two-component regulators, pectate lyase, protease, levan, pyoverdine.

Résumé : Le *Pseudomonas marginalis* est un pathogène post-récolte important, qui est capable de causer une pourriture molle chez une grande variété de fruits et de légumes. Par suite d'une mutagenèse par transposon, les auteurs ont isolé deux groupes mutants de *P. marginalis* CY091, qui ne pouvaient produire la pectate lyase (Pel) et la pathogénicité de la pourriture molle chez les plantes. Le premier groupe, désigné Pel⁻, a été causé par l'insertion d'un Tn5 dans un gène structural *pel*; le second, désigné LemA⁻, a été causé par l'insertion d'un Tn5 dans un locus régulateur correspondant au gène *lemA*, antérieurement identifié chez d'autres bactéries gram-négatives. Les mutants LemA⁻ ont aussi présenté des altérations dans la morphologie des colonies et une déficience dans la production de protéase (Prt). Un clone du cosmide pCIC porteur du gène *lemA* de *P. marginalis* a été isolé et caractérisé. Le pCIC s'est révélé capable de restaurer la production de Pel et la pathogénicité de la pourriture molle chez les mutants LemA⁻ de *P. marginalis* et de *Pseudomonas viridiflava*, ce qui fut l'indication que la fonction du gène *lemA* chez ces deux pseudomonades était similaire et interchangeable. Utilisant la mutagenèse via le MudI, les auteurs ont isolé un troisième groupe de *P. marginalis* mutants déficients pour la production de Pel, Prt et de la pathogénicité de la pourriture molle. Les mutants de ce groupe, désignés GacA⁻¹ contenaient une insertion de MudI dans un locus correspondant au gène *gacA* de *P. viridiflava*. Comme les mutants LemA⁻, les mutants GacA⁻¹ ont aussi présenté une altération dans la morphologie de la colonie et une déficience dans la production de Pel et Prt. Toutefois, les mutants GacA⁻¹ ont produit des niveaux bien inférieurs de levanes et du sidérophore fluorescent pyoverdine que les mutants LemA⁻ et le type indigène. Ces résultats sont une première évidence génétique que le *P. marginalis* produit une seule Pel alcaline pour la macération des tissus végétaux et démontrent que la production de Pel, Prt, levanes et pyoverdine par cette bactérie se fait par l'intermédiaire de deux composantes *lemA/gacA* du système génétique.

Mots clés : régulateurs à deux composantes, pectate lyase, protéase, levanes, pyoverdine.
[Traduit par la rédaction]

Introduction

Pseudomonas marginalis is an opportunistic phytopathogen responsible for a large proportion of postharvest rot of fresh fruits and vegetables in cold storage (Lund 1983) and at wholesale and retail markets (Liao and Wells 1987). Apart from its disease-causing ability, *P. marginalis* is physiologically indistinguishable from various biovars of *Pseudomonas fluorescens* (Lelliot et al. 1966). The ability of *P. marginalis* and other soft-rotting bacteria to cause maceration of plant tissue is mainly due to their ability to produce an array of enzymes required for degradation of pectic components in plant cell walls (Collmer and Keen 1986). The pectate lyase (Pel) produced by most if not all strains of *P. marginalis* was generally assumed to be the principal or sole enzyme involved in tissue maceration (see Liao 1989 for review). Previously, we reported detection of two Pel (one alkaline and one neutral) isozymes in culture fluids of *P. marginalis* following isoelectric focusing (IEF) and overlay enzyme-activity staining (Liao 1989). So far, we have been unable to separate the suspected neutral Pel (pI 6.7) from the predominant alkaline Pel (pI 9.7) by biochemical methods and to isolate the gene coding for this neutral Pel from the genomic library of *P. marginalis* CY091 (Liao 1991). The actual number of Pels produced by *P. marginalis* and the role of each Pel in soft-rot pathogenesis are unclear. Several studies have been conducted in the past to investigate the biochemical factors affecting the synthesis of Pel in *P. marginalis* (Zucker and Hankin 1970; Nasuno and Starr 1966; Liao et al. 1993). However, the molecular genetic mechanism by which *P. marginalis* regulates Pel production and soft-rot development in plants has not yet been examined. Molecular genetic techniques such as transposon mutagenesis and gene cloning thus provide a useful tool for identification and characterization of gene loci associated with Pel production and soft-rot pathogenicity in *P. marginalis*. Here, we present the first genetic evidence that *P. marginalis* CY091 produces a single Pel with an alkaline pI for maceration of plant tissue. We also demonstrate that production of Pel and other extracellular compounds by *P. marginalis* is under the control of the two-component *lemA/gacA* gene regulatory system. Furthermore, we show that the *lemA/gacA* gene system in *P. marginalis* and *Pseudomonas viridiflava* is functionally similar and interchangeable.

Materials and methods

Bacteria strains, plasmids, and media

Bacterial strains and plasmids used in the study are listed in Table 1. *Escherichia coli* was grown at 37°C in Luria broth (LB) or Luria agar (LA) (Life Technologies Inc., Gaithersburg, Md.), and *Pseudomonas* sp. was grown at 28°C in LB or *Pseudomonas* agar F (Difco Laboratories, Detroit, Mich.). When required, antibiotics were added at the following concentrations ($\mu\text{g} \cdot \text{mL}^{-1}$): rifampicin (Rif), 100; kanamycin (Km), 50; tetracycline (Tc), 25; ampicillin (Ap), 50; and streptomycin (Sm), 100. For assay of pectolytic and proteolytic activities, the semisolid pectate medium (SSP) and gelatin nutrient agar (GNA) were used as previously described (Liao 1991). For assay of levan and siderophore production, sucrose nutrient agar (SNA) (Difco nutrient agar with 5% w/v sucrose) and blue chrome azurol S (CAS) agar (Schwyn and Neilands 1987) were used, respectively.

Mutagenesis and triparental matings

Transposon (Tn5) mutagenesis was performed by using a suicidal plasmid pSUP1011 (Simon et al. 1983) as previously described (Liao et al. 1988). Rif^r Km^r transconjugants were selected on *Pseudomonas* agar F plates containing rifampicin and kanamycin, and assayed for pectolytic and proteolytic activities on SSP and GNA media, respectively. For generation of transcriptional *lemA/lacZ* fusion with MudI1734, the procedures previously described by Castilho et al. (1984) were followed. MudI1734 was introduced into a cosmid pCIC containing the *P. marginalis lemA* gene by the methods of Castilho et al. (1984), and mutant plasmids unable to complement the LemA⁻ mutants (rep-1 and rep-2) of *P. marginalis* were isolated. Positions of MudI insertions in LemA⁻ mutant plasmids were determined by the standard procedures (Miller 1992). To generate GacA⁻ marker-exchange mutants, plasmid that had MudI1734 inserted in the *gacA* gene of *P. viridiflava* (Liao et al. 1996) was mobilized into *P. marginalis* CY091A by triparental mating (Ruvkun and Ausubel 1981). Km^r Tc^s derivatives deficient in production of Pel and Prt, resulting presumably from the insertion of MudI1734 into the *gacA* locus through homologous recombination, were isolated.

Cloning of *lemA* genes and recombinant DNA techniques

A genomic library of *P. marginalis* CY091A was constructed in the cosmid vector pLAFR3 as previously described (Staskawicz et al. 1987). A cosmid clone carrying the *P. marginalis lemA* gene and capable of restoring enzyme production in LemA⁻ mutants was selected following complementation tests as previously described (Liao et al. 1994). Standard procedures (Sambrook et al. 1989) were used for isolation of chromosome and plasmid DNAs, subcloning, restriction mapping, and preparation of competent cells for transduction and transformation. Southern blot and nonradioactive DNA labeling and detection were conducted as previously described (Liao et al. 1994).

Assays of levan and siderophore production

Bacterial strains were streaked across three culture dishes (100 × 15 mm) containing SNA medium and appropriate antibiotics. The cultures were incubated at 28°C for 3 days and then bacterial cells and exopolysaccharide were harvested by use of a bent glass rod and distilled water. After vigorous stirring, cells were removed by centrifugation (16 300 × *g*, 15 min) and the supernatant fluids were collected. To aid in precipitation, a concentrated solution of KCl was added to the supernatant to give a final concentration of 1% w/v. Levan was then precipitated by addition of two volumes of ice-cold isopropanol. After mixing, the samples were left at 4°C for several hours. Precipitated levan was sedimented by centrifugation as above. The pellet was dissolved in and dialyzed against distilled water, lyophilized, and weighed. The amount of levan present in the samples was estimated by use of a colorimetric assay for ketose sugars (Dische 1962) with D-fructose as the standard. The presence of levan was confirmed by gas-liquid chromatographic analysis. Samples were hydrolyzed with 1 M oxalic acid (2 mg · mL⁻¹) at 70°C for 90 min. The derivatized samples were examined using a Varian model 3400 gas-liquid chromatograph fitted with a Hewlett-Packard HP-5 capillary column and a flame ionization detector with temperature programming from 150 to 250°C at 4° · min⁻¹ (Fett and Dunn 1989). The experiment was repeated twice. For assays of siderophore production, bacterial strains were each grown on the CAS agar medium at 28°C for 2 days (Schwyn and Neilands 1987). Production of siderophores as indicated by the formation of an orange zone surrounding the bacterial growth was determined by measuring the size of the orange zone (diameter in mm). Alternatively, bacteria were grown in the minimal salt medium MY (Liao et al. 1988) supplemented with 0.1% yeast

Table 1. Bacterial strains and plasmids.

Bacteria or plasmids	Relevant properties	Sources
<i>P. marginalis</i>		
CY091A	Spontaneous mutant of CY091, Rif ^r	Liao 1991
rep-1, rep-2	Tn5-induced LemA ⁻ mutants, Km ^r , Pel ⁻ , Prt ⁻	This study
ME #4, ME #18	MudI-induced GacA ⁻ marker-exchange mutants, Km ^r , Pel ⁻ , Prt ⁻	This study
<i>P. viridiflava</i>		
PJ-08-6A	Wild type, Rif ^r	Liao et al. 1994
I-4	Tn5-induced LemA ⁻ mutant, Km ^r , Pel ⁻ , Prt ⁻	Liao et al. 1994
I-10	Tn5-induced GacA ⁻ mutant, Km ^r , Pel ⁻ , Prt ⁻	Liao et al. 1994
<i>E. coli</i>		
HB101	Cloning and subcloning host	BRL
M8820	RecA ⁺ , for Mu-plasmid cointegrate	Castilho et al. 1984
POI1734	Contains <i>Mucts</i> and <i>MudI-lacZ</i>	Castilho et al. 1984
Plasmids		
pLAFR3	Cosmid vector	Staskawicz et al. 1987
pRK415	Cloning vector	Keen et al. 1988
pCIC	pLAFR3 clone containing the <i>P. marginalis lemA</i> gene	This study
pLAI33	pLAFR3 clone containing the <i>P. viridiflava lemA</i> gene	Liao et al. 1994
pLAM2	A pLAFR3 derivative containing the <i>P. marginalis pel</i> gene	This study
pRepX series	Deletion subclones of pCIC	This study
pKPN23A (<i>MudI</i>)	<i>MudI</i> inserted in the <i>gacA</i> region in the 2.3-kb KpnI subfragment of pRepB63	Liao et al. 1996
pLAPH08	0.8-kb <i>PstI-HindIII gacA</i> region of pRepB63 subcloned into pLAFR3	Liao et al. 1996

extract, and the concentration of fluorescent pyoverdinin in the culture supernatant was measured directly at 400 nm and calculated as described previously (Hofte et al. 1993).

Enzymes, protein and tissue-maceration assays

Procedures for determination of Pel (Liao et al. 1988), protease (Prt) (Howe and Iglewski 1984), and β -galactosidase (Miller 1992) activities have been described previously. When needed, protein concentrations were determined using a protein assay kit (Bio-Rad Laboratories, Richmond, Calif.). The ability of bacterial strains to induce soft rot or tissue maceration was assayed on potato tuber slices and detached pepper fruits. Methods for preparation of plant materials and bacterial inocula for testing have been described previously (Liao et al. 1994).

Results

Isolation of Pel⁻ and Prt⁻ mutants

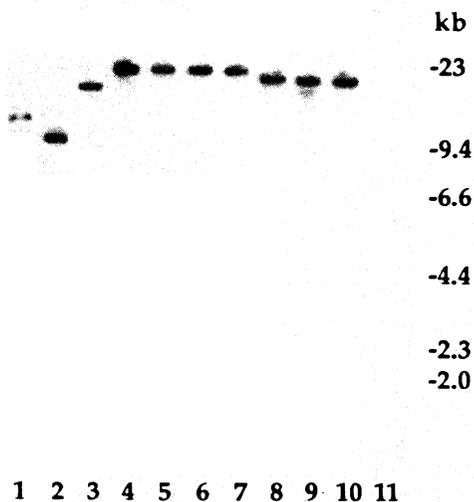
Following Tn5 mutagenesis, approximately 4000 Km^r colonies of *P. marginalis* CY091A were randomly selected and screened for changes in pectolytic and proteolytic activities. Three Pel⁻ isolates showing deficiency in pectolytic activity but exhibiting wild-type levels of proteolytic activity were isolated. When genomic digests of these Pel⁻ mutants were probed with Tn5 and a previously described *pel* gene fragment (0.7 kb) (Liao 1991), Tn5 and *pel* sequences were both detected in the 12.8-kb *EcoRI*-generated genomic fragments of all three mutants (Fig. 1, lanes 8–10). In contrast, the *pel* sequence of the wild-type strain was detected in a 7.1-kb *EcoRI*-generated genomic fragment (data not shown). The insertion of Tn5 (5.7 kb) into the *pel* fragment (7.1 kb) in the

wild type thus led to the formation of a 12.8-kb fragment in the mutant detectable both by Tn5 and *pel* probes. Despite no reduction in protease activity, all three Pel⁻ mutants were unable to cause soft rot in potato tuber slices and bell pepper fruits. A recombinant plasmid pLAM2 containing the *P. marginalis pel* gene restored Pel and soft-rot production of the mutant to wild-type levels. Four Prt⁻ mutants that produced very little or no protease but showed no reduction in production of Pel were also isolated. Southern blot analysis revealed that all four Prt⁻ mutants were possibly caused by the insertion of Tn5 into a 10.2-kb *EcoRI* genomic fragment (Fig. 1, lanes 4–7). Despite the lack of protease activity, all four Prt⁻ mutants induced the same degree of tissue maceration in potato tuber slices and bell pepper fruits as the wild type.

Physical characterization of LemA⁻ mutants

Two mutants of CY091A showing deficiencies in production of Pel and Prt and exhibiting altered colony morphologies were isolated. Because LemA⁻ mutants of *P. viridiflava* are also deficient in Pel and Prt production (Liao et al. 1994, 1996), we determined if Tn5 was in the *lemA* gene of mutants rep-1 and rep-2. Southern blot analysis of these mutants with Tn5 revealed that mutants rep-1 and rep-2 contained Tn5 insertions in 2.7- and 7.5-kb *EcoRI* fragments of genomic DNA, respectively. Previously, we showed that the *lemA* gene of *P. viridiflava* spans the juncture of two *EcoRI* fragments designated *repA1* and *repA2* (Liao et al. 1994). We determined if rep-1 and rep-2 had Tn5 inserted in the genomic fragment of *P. marginalis* corresponding to the

Fig. 1. Southern blot detection of Tn5 sequences in *Eco*RI-generated genomic digests of the Pel⁻, Prt⁻, and LemA⁻ mutants of *P. marginalis* CY091A. pRZ102 containing an intact Tn5 (Simon et al. 1983) or a 2.6-kb *Bgl*III internal fragment of Tn5 was used as a probe. Lane 1, GacA⁻ mutant I-10 of *P. viridiflava* PJ-08-6A (Liao et al. 1994); lanes 2 and 3, *P. marginalis* LemA⁻ mutants rep-1 and rep-2, respectively; lanes 4–7, *P. marginalis* Prt⁻ mutants (isolates 1–4); lanes 8–10, *P. marginalis* Pel⁻ mutants (isolates 1–3); lane 11, the wild-type strain CY091A.



repA1 and *repA2* fragments of *P. viridiflava*. Genomic digests of mutants rep-1 and rep-2 were probed with the *repA1* (1.8-kb *Eco*RI–*Hind*III) and *repA2* (2.8-kb *Eco*RI) fragments of *P. viridiflava* (Liao et al. 1994). Results (not shown) indicated that mutants rep-1 and rep-2 were derived from the insertion of Tn5 into the 2.7- and 7.5-kb genomic fragments of *P. marginalis* CY091A corresponding to the *repA1* and *repA2* counterparts of *P. viridiflava*, respectively.

Cloning of *P. marginalis* *lemA* gene

From a genomic library of *P. marginalis* CY091A constructed in the cosmid vector pLAFR3, only plasmid pCIC restored Pel and Prt production in mutants rep-1 and rep-2. pCIC also restored wild-type level of Pel and Prt production and tissue-macerating ability in LemA⁻ *P. viridiflava* mutant I-4. Southern blot analysis of pCIC with the *repA1* and *repA2* fragments of *P. viridiflava* (Fig. 2) localized the *lemA* locus of *P. marginalis* to a 2.7-kb *Eco*RI fragment and a 2.8-kb *Eco*RI–*Hind*III fragment. pRK415 containing both fragments (pRepX40) restored enzyme production in both mutants, but pRK415 containing either fragment alone did not. Complementation of mutants rep-1 and rep-2 with pRepX40 containing *MudI* insertions further defined the *lemA* gene of *P. marginalis* in the insert. Analysis of β -galactosidase activities in *repA/lacZ* fusion constructs indicated that the *lemA* gene was transcribed from the right (*Hind*III) to the left (*Eco*RI) end of the fragment (Fig. 2).

Isolation and characterization of GacA⁻ mutants

Several studies have suggested that the *lemA* gene may act in concert with the *gacA* gene to mediate the disease-control activity in saprophytic strains of *P. fluorescens* (Corbell and Loper 1995) and to regulate the production of pathogenicity-

related factors in *Pseudomonas syringae* (Rich et al. 1994). Following the identification of *lemA* locus, we suspected that *P. marginalis* might contain a second regulatory component to mediate the production of Pel and other extracellular compounds. To confirm this, a GacA⁻ plasmid (pKPN23A::*MudI*) having *MudI*1734 inserted in the *gacA* (previously designated *repB*) locus of *P. viridiflava* (Liao et al. 1996) was transferred into *P. marginalis* CY091A by triparental matings. All 25 Km^r Tc^s isolates randomly selected were found to be altered in colony morphology (from ripple dry to smooth wet form) and exhibited nonpectolytic and nonproteolytic phenotypes on agar media. When inoculated into potato tuber slices and pepper fruits, all 25 isolates were unable to cause soft rot in both plants. Genomic digests of two representative Km^r Tc^s isolates ME #4 and ME #18 were probed with a specific *gacA* gene fragment (0.8 kb) previously cloned from *P. viridiflava* (Liao et al. 1996). The *gacA* locus of the wild-type strain CY091A was found in an 8.0-kb *Eco*RI fragment (Fig. 3, lane 5), whereas in mutants ME #4 and ME #18 the *gacA* gene was identified in two *Eco*RI fragments (6.3 and 14.3 kb). Since *MudI*1734 (12.6 kb) contains an *Eco*RI site (Miller 1992), integration of *MudI*1734 into the *gacA* region would generate two new *Eco*RI fragments, each containing portions of *gacA* and *MudI*1734 DNA. This was later confirmed by the detection of two *gacA*-containing fragments (6.3- and 14.3-kb fragments) by the *MudI*1734 probe.

Levan and fluorescent siderophore production is affected by the *gacA* gene but not the *lemA* gene

As described above, inactivation of either the *lemA* or *gacA* gene led to the simultaneous loss of pectolytic and proteolytic activities and also to a change in colony morphology from the ripple-dry to smooth-wet form (Fig. 4). However, production of levan and fluorescent siderophores appeared to be affected by the GacA⁻ but not by the LemA⁻ mutation. While the wild type and LemA⁻ mutant (rep-2) produced about the same amount of levan (460–480 mg levan · g dry cells⁻¹), the GacA⁻ mutant (ME #4) produced only 1.9 mg levan · g dry cells⁻¹ (Table 2). Similarly, when bacteria were grown on *Pseudomonas* agar F medium, the wild-type strain and LemA⁻ mutants produced about the same level of fluorescence detectable under ultraviolet light. However, GacA⁻ mutants ME #4 and ME #18 produced very little or no fluorescence (Fig. 5). This result was later confirmed by the conventional chrome azurol S (CAS) assays and measurement of pyoverdine production in culture supernatant by the spectrophotometric method (Table 2). When plasmid pLAPH08 containing the *gacA* gene was mobilized into mutant ME #4, production of wild-type levels of levan, Pel, Prt, pyoverdine, and soft-rot pathogenicity in the mutant was restored.

Discussion

In this study, we employed Tn5- and Mini-Mu-mediated mutagenesis to isolate three classes of *P. marginalis* mutants that were deficient in Pel production and soft-rot pathogenicity in plants. Data presented here show that the alkaline Pel produced by most strains of *P. marginalis* is the principal or sole factor involved in maceration and proteases also produced by all strains do not appear to play an important role in the soft-

Fig. 2. Restriction map of DNA fragments containing the *lemA* gene of *P. marginalis* CY091A. Numbers under the lines represent the sizes of DNA fragments in kilobases (kb). The ability or inability of a plasmid to restore enzyme production in *LemA*⁻ mutants rep-1 and rep-2 is indicated as + or -, respectively. The positions of Mini-Mu-*lacZ* insertions are indicated by triangle flags. Flags pointing to the left indicate that high levels of β -galactosidase activity were detected. Open flags indicate the insertion positions at which the *lemA* gene function was not affected and solid flags indicate positions at which the *lemA* gene function was inactivated. The arrow indicates direction of transcription and approximate location of *lemA* gene.

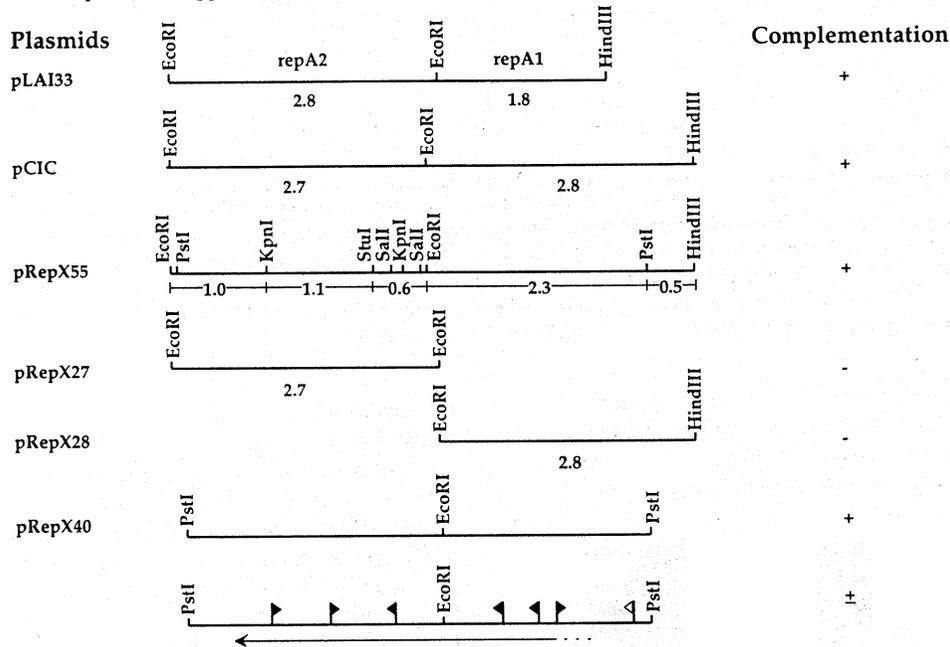


Fig. 3. Detection of homologous *gacA* sequences in marker-exchange *GacA*⁻ mutants of *P. marginalis* CY091A. Two *gacA* fragments previously cloned from *P. viridiflava* (Liao et al. 1996) were used as positive controls. The 1.8-kb *gacA* (*HindIII*-*KpnI*) fragment from pKPN23A and the 1.3-kb *gacA* (*PstI*) fragment from pKPN23B (Liao et al. 1996) are shown in lanes 1 and 2, respectively. In lane 2, the upper band represents the undigested pKPN23A and the bottom band represents the 1.3-kb *PstI*-generated *gacA* fragment. Lane 3, *GacA*⁻ mutant ME #18; lane 4, *GacA*⁻ mutant ME #4; lane 5, the wild type *P. marginalis* CY091A; lane 6, λ -*HindIII* markers. Chromosomal DNAs of mutants ME #4 and ME #18 were digested with *EcoRI* and the blot was probed with the 0.8-kb *gacA* fragment from pLAPH08 (Liao et al. 1996).

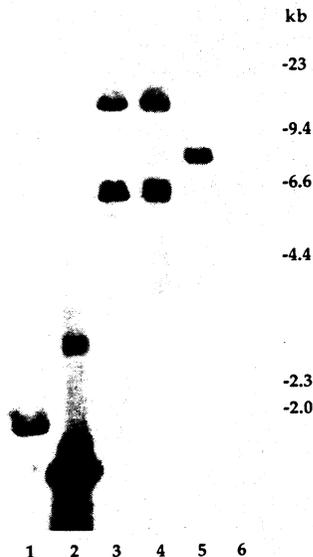
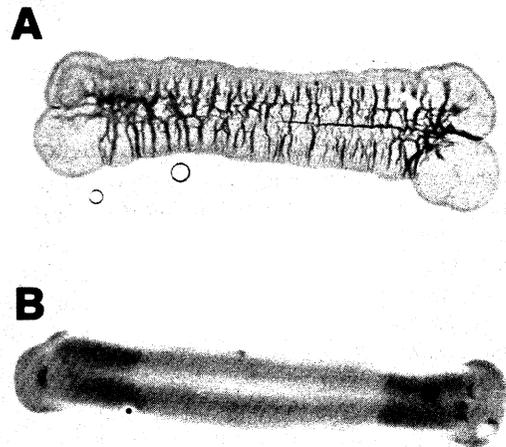


Fig. 4. Effects of *GacA*⁻ mutation on colony morphology of *P. marginalis*. (A) Wild-type strain CY091A; (B) *GacA*⁻ mutant ME #4.



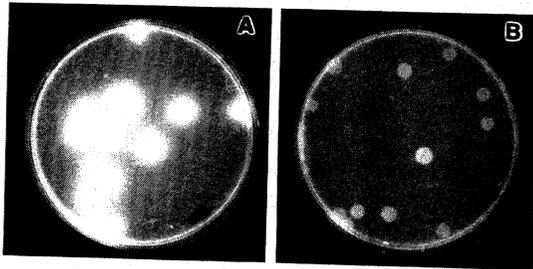
rot pathogenesis. By using the overlay enzyme-activity staining techniques, we previously reported detection of two Pel isozymes in culture fluids of *P. marginalis*. In addition to a predominant alkaline Pel, a minor Pel activity band with a neutral pI was occasionally observed in the overlay enzyme-activity staining gel (Liao 1989). So far, we have been unable to isolate this suspected neutral Pel for further characterization. We have been also unable to identify the gene coding for this enzyme in a genomic library used to isolate the alka-

Table 2. Production of pectate lyase, levan, and fluorescent siderophore by strains of *Pseudomonas marginalis*.

Strain	Diameter of orange halo on CAS medium (mm)	Pel activity ($U \cdot 10^{10} \text{ cells}^{-1}$)	Levan ($\text{mg} \cdot \text{g dry cells}^{-1}$)	Pyoverdin ($\text{mM} \cdot 10^{10} \text{ cells}^{-1}$)
CY091A	6.1	37.5	486.2	16.8
Rep-2	5.6	<0.1	468.3	15.6
Rep-2 (pCIC)	5.7	29.7	471.6	17.3
ME #4	1.9	<0.1	1.9	3.1
ME #4 (pLAPH08)	6.3	32.4	435.4	13.7

Note: The values in the table represent an average of two experiments, two duplicates for each experiment with the exception of levan. General siderophore production was indicated by the formation of an orange halo zone in CAS agar medium (Schwyn and Neilands 1987). Estimations of the fluorescent pyoverdin were determined by the method of Hofte et al. (1993). The molar extinction coefficient of $2 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ and $1.6 \text{ OD}_{600} = 10^9$ cells were used to calculate the pyoverdin concentration. One unit of Pel activity was defined as the amount of the enzyme that caused an increase of 1.0 absorbance unit at 232 nm per minute at 30°C.

Fig. 5. Effects of *GacA*⁻ mutation on fluorescent pigment production by *P. marginalis*. Bacteria were grown in Difco *Pseudomonas* agar F. Photographs were taken under ultraviolet light 2 days after incubation at 28°C. (A) Wild-type strain CY091A; (B) *GacA*⁻ mutant ME #4.



line *pel* gene (Liao 1991) and in a cosmid library used to isolate the *lemA* gene (this study). Successful isolation of *Pel*⁻ mutants by Tn5-mediated mutation, as well as detection of only one *pel* fragment in the genomic digests, indicate that *P. marginalis* CY091 contains a single *pel* gene. Thus, the neutral *Pel* activity band previously observed in the overlay enzyme-activity staining gel likely resulted from sample trailing of the alkaline *Pel* during IEF electrophoresis (Liao 1991). Since all three *Pel*⁻ mutants were unable to cause soft rot in potato tubers and bell pepper fruits, it is unlikely that *P. marginalis* CY091 produces another *Pel* that is inducible only in host plants. Elumalai and Mahadevan (1995) recently reported the cloning of neutral and alkaline *Pels* from an unspecified strain of *P. marginalis*. However, the authors made no attempt to purify the neutral *Pel* from *E. coli* clones carrying this gene and to construct *P. marginalis* marker-exchange mutants deficient in production of one of these two enzymes. It awaits to be further determined if certain strains of *P. marginalis* may contain more than one *pel* gene.

In recent years, an increasing number of reports have shown that expression of virulence factors in plant and animal pathogens is mediated by a large number of regulators in the two-component regulatory protein family (Miller et al. 1989). The *lemA* and *gacA* genes described here not only control the production of pathogenicity factors by pathogenic strains including *P. syringae* (Hrabak and Willis 1992),

P. viridiflava (Liao et al. 1994), and *P. marginalis* (this study) but also regulate the production of factors related to the biocontrol activity by saprophytic strains of *P. fluorescens* (Laville et al. 1992; Gaffney et al. 1994; Corbell and Loper 1995). Presence of conserved domains of sensory and response regulator proteins in the predicted amino acid sequences of LemA and GacA proteins suggests that these two regulatory proteins may act in pairs to mediate the production of various extracellular compounds (Rich et al. 1994). In this report, we provide the first experimental evidence that production of *Pel* and some other extracellular compounds by *P. marginalis* is under the control of the *lemA/gacA* regulatory system. However, we found that two *GacA*⁻ mutants examined in the study produced diminished levels of levan and pyoverdin compared with the wild type and *LemA*⁻ mutants. It is presently unclear if *gacA* is coupled with another non-LemA sensor to mediate production of levan and pyoverdin, as other explanations are possible. For example, mutants *rep-1* and *rep-2* may have resulted from the insertion of Tn5 into two specific sites in the *lemA* region, which led to the formation of a mutant sensor protein affecting the synthesis of *Pel* and *Prt* but not levan and pyoverdin.

In conclusion, by using Tn5- and Mini-Mu-mediated mutagenesis, we have identified three gene loci (*pel*, *lemA*, and *gacA*) in the chromosome of *P. marginalis* CY091 required for production of *Pel* and soft-rot pathogenicity in plants. Unlike the multiple *Pels* produced by soft-rot erwinias, this pseudomonad produces only a single *Pel* with an alkaline pI for maceration of plant tissue. In addition, two of the gene loci (*lemA* and *gacA*) are involved in mediating the production of *Pel*, *Prt*, levan, pyoverdin, and colony morphology in *P. marginalis*.

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