Molecular Characterization of Two Gene Loci Required for Production of the Key Pathogenicity Factor Pectate Lyase in *Pseudomonas viridiflava*

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Four pleiotropic mutants of *Pseudomonas viridiflava* strain PJ-08-6A that were deficient in production of both pectate lyase (Pel) and protease (Prt) were isolated following transposon mutagenesis. Unlike secretion-defective (Out$^-$) mutants, these four showed no accumulation of enzymes within the cells. Southern hybridization analysis revealed that each mutant had Tn*5* inserted in one of two *Eco*RI genomic fragments. These *Eco*RI fragments (5.2- and 6.3-kb) appeared to contain two distinct gene loci, designated *repA* and *repB*, which were required for production of extracellular enzymes in this bacterium. Cosmid clones carrying the functional *repA* and *repB* DNA fragments were identified in a genomic library of strain PJ-08-6A. After analysis of *repA*$^+$ plasmids by restriction mapping and marker-exchange mutagenesis, the *repA* gene was located in a joint region between the 1.8-kb *Eco*RI-*Hin*dIII and 2.8-kb *Eco*RI fragments cloned. Nucleotide sequence analysis of the *repA* region revealed the presence of an open reading frame consisting of 2,790 bases. The RepA protein predicted from the DNA sequence showed 93% similarity in amino acid sequence to the LemA protein of *P. syringae* pv. *syringae*, which was previously identified as a member of a two-component global regulatory system. A plasmid carrying the *lemA* gene of *P. syringae* pv. *syringae* was capable of complementing the RepA$^-$ mutation in *P. viridiflava*. The functions of the *repA* and *lemA* genes thus appear to be similar and interchangeable. Mutants of *P. viridiflava* strain SF312A deficient in production of Pel, Prt, and the exopolysaccharide alginate also were identified. Cosmid clones carrying the *repA* (but not *repB*) DNA of strain PJ-08-6A were able to restore the enzyme and alginate production in Rep$^-$ mutants of strain SF312A. The *repA* gene is therefore required for production of not only extracellular enzymes but also exopolysaccharides in *P. viridiflava*.

Additional keywords: cloning, extracellular enzymes, gene regulation, soft-rot bacteria.

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Pseudomonas viridiflava (Burkholder) Dowson is an opportunistic phytopathogen that accounts for a large proportion of postharvest losses of fruits and vegetables in storage and during transit (Liao and Wells 1987). Unlike the complex pectic enzyme system demonstrated in *Erwinia* spp. (Collmer and Keen 1986), all *P. viridiflava* strains so far examined produce a single pectic enzyme required for maceration of plant tissue (Liao 1989). This enzyme, identified as pectate lyase (Pel; pI 9.7, 43 kDa), has recently been purified (Liao *et al.* 1988), and the corresponding gene has been cloned and characterized (Liao *et al.* 1992). In addition to Pel, *P. viridiflava* also produces an extracellular protease (Prt) with an approximate pI of 6.0 and of approximately 42 kDa (McCallus and Liao 1992). Although the Prt enzyme does not seem to play a significant role in soft rot pathogenesis (Liao *et al.* 1988), its ecological importance and possible contribution to pathogen fitness have not yet been carefully examined. The bacterium also produces an exopolysaccharide, alginate, contributing to the mucoid colony phenotype in some strains (Fett *et al.* 1989).

During the last few years, global gene regulation of exoprotein production in gram-negative bacteria has been extensively studied. A number of genes that are involved in regulation of exoprotein production, possibly in response to environmental stimuli, have been identified. In *Xanthomonas campestris* pv. *campestris*, seven *rpf* genes that are required for production of Pel, Prt, endoglucanase, amylase, and the exopolysaccharide xanthan gum have been identified (Tang *et al.* 1991). In *P. syringae* pv. *syringae*, a gene (*lemA*) known to mediate disease lesion formation on bean plants and to regulate protease and phytotoxin production in culture media has been isolated and characterized (Hrabak and Willis 1992). In *P. fluorescens*, a gene homologous to the *lemA* has also been cloned and shown to regulate the production of a wide variety of antifungal compounds including chitinase, pyrrolnitrin, and cyanide (Lam *et al.* 1993). So far, there has been no report on how the production of extracellular Pel and Prt in *P. viridiflava* is regulated. In this paper, we describe the isolation of a group of pleiotropic Tn*5* insertion mutants of *P. viridiflava* that are deficient in the synthesis of Pel, Prt, and exopolysaccharide (alginate). We identified and cloned two genes that are involved in the regulation of exoprotein and exopolysaccharide production in this bacterium. We will show that one of these two genes encodes a protein with homology to the LemA protein of *P. syringae* pv. *syringae* (Hrabak and Willis 1992) and to the conserved domains of

both sensor and regulator proteins of other two-component regulatory systems (Ronson *et al.* 1987).

RESULTS

Isolation and characterization of Rep⁻ mutants.

As was reported in our previous study with strain SF312A (Liao *et al.* 1988), mutagenesis of *P. viridiflava* strain PJ-08-6A with a suicide plasmid pSUP1011 (Simon *et al.* 1983) yielded mutants that had Tn*5* randomly inserted in the genome. Approximately 1,500 Rif^r Km^r transconjugants were screened for the loss of pectolytic and proteolytic activities on two diagnostic agar media (SSP and nutrient agar-gelatin) as described in Materials and Methods. (Bacterial strains, plasmids, and the bacteriophage used and constructed in the study are listed in Tables 1 and 2.)

Four pleiotropic mutants (I-2, I-3, I-4, and I-10) deficient in production of both Pel and Prt enzymes were identified. Unlike secretion-deficient (Out⁻) mutants, which usually synthesize and accumulate wild-type levels of enzymes within the cells (Liao *et al.* 1988), mutants I-2, I-3, I-4, and I-10 produced only trace amounts of enzymes that were barely detectable in culture fluids and in cell extracts. This mutant phenotype, tentatively designated Rep⁻, was assumed to be caused by a mutation in gene loci that were involved in

Table 1. Bacterial strains used or constructed in the study

Designation	Description[a]	Reference or source
Pseudomonas viridiflava		
PJ-08-6A	Wild-type, Rif^r, nonmucoid	Liao 1989
SF312A	Wild-type, Rif^r, mucoid	Liao *et al.* 1988
I-2, I-3, I-4	RepA⁻ Tn*5* mutants of PJ-08-6A; Rif^r, Km^r, Pel⁻, Prt⁻	This study
I-10	RepB⁻ Tn*5* mutant of PJ-08-6A; Rif^r, Km^r, Pel⁻, Prt⁻	This study
SF312A1	Spontaneous Rep⁻ mutant of SF312A; non-mucoid, Pel⁻, Prt⁻	This study
MI-13	RepA⁻ Tn*5* mutant of SF312A; Rif^r, Km^r, Pel⁻, Prt⁻, non-mucoid	This study
ME311	Marker-exchanged mutant of PJ-08-6A; *RepA2*::Tn*5*; generated by plasmid pLAI 311; Rif^r, Km^r, Pel⁻, Prt⁻	This study
ME321	Marker-exchanged mutant of PJ-08-6A; *repA1*::Tn*5*; generated by plasmid pRepA321; Rif^r, Km^r, Pel⁻, Prt⁻	This study
ME281	Marker-exchanged mutant of PJ-08-6A; *repA2*::Tn*5*; generated by plasmid pRepA281; Rif^r, Km^r, Pel⁻, Prt⁻	This study
Escherichia coli		
HB101	Cloning/subcloning host	BRL[b]
DH5α	Cloning/subcloning host	BRL
SM10	Contains plasmid pSUP1011; used for Tn*5* mutagenesis; Cm^r, Km^r	Simon *et al.* 1983

[a] Rif^r, Km^r, Cm^r = resistance to rifampicin, kanamycin, and chloramphenicol, respectively. Pel⁻, Prt⁻ = deficient in pectolytic and proteolytic activity, respectively.
[b] BRL = Bethesda Research Laboratory.

Table 2. Plasmids and bacteriophage used or constructed in the study

Designation	Description[a]	Reference or source
Plasmids		
pLAFR3	IncP Tc^r Cos$^+$ rlx$^+$; cloning vector	Staskawicz *et al.* (1987)
pRK2013	IncP Km^r Tra RK2$^+$; helper plasmid used for triparental mating	Ditta *et al.* (1990)
pRK415	Mob$^+$ Tc^r, a derivative of pRK404; used for subcloning	Keen *et al.* (1988)
pRZ102	ColE::Tn*5*; Sm^r; used for preparation of Tn*5* probe	Jorgensen *et al.* (1985)
pEMH97	A derivative of pLAFR3; contains the *lemA* gene of *Pseudomonas syringae* pv. *syringae*	Hrabak and Willis (1992)
pLAI 31, 33, 36, 37	Primary RepA$^+$ clones in pLAFR3; contain *Pseudomonas viridiflava* (PJ-08-6A) *repA1* and *repA2* loci	This study
pLAI 101	Primary RepB$^+$ clone in pLAFR3; contains *Pseudomonas viridiflava* (PJ-08-6A) *repB* locus	This study
pRepA52	*repA1*-containing 5.2-kb *Eco*RI fragment from pLAI 36 cloned in pRK415; Rep⁻, Tc^r	This study
pRepA28	*repA2*-containing 2.8-kb *Eco*RI fragment from pLAI 31 cloned in pRK415; Rep⁻, Tc^r	This study
pRepB2852	A derivative of pRK415; contains the 1.8-kb *repA1* and 2.8-kb *repA2* fragments	This study
pRepB63	*repB*-containing 6.3-kb *Eco*RI fragment from pLAI 101 cloned in pRK415; RepB$^+$, Tc^r	This study
pLAI 311, 312, 313, 314	λ-mediated Tn*5* insertion mutants of pLAI 31; *repA1*::Tn*5* or *repA2*::Tn*5*; Tc^r, Km^r	This study
pRepA321	8.9-kb *Eco*RI fragment from pLAI 312 cloned in pRK415; *repA1*::Tn*5*; Tc^r, Km^r	This study
pRepA281	8.5-kb *Eco*RI fragment from pLAI 311 cloned in pRK415; *repA2*::Tn*5*; Tc^r, Km^r	This study
Bacteriophage		
λ467::Tn*5*	λ6221*rex*::Tn*5* c1857, oam29, pam 80, used for mutagenesis	Ruvkun and Ausubel 1981

[a] Km^r, Tc^r, Sm^r = resistance to kanamycin, tetracycline, and streptomycin, respectively.

regulation of exoprotein production (*rep*) in *P. viridiflava*. To verify that the Rep⁻ phenotype was indeed caused by a Tn*5* insertion, *Eco*RI-generated genomic digests of the wild type and mutants were probed with a specific Tn*5* DNA segment isolated from pRZ102 (Jorgensen *et al.* 1979). A single hybridization band 10.9- or 12.0-kb in size was detected in the genomic digests of Rep⁻ mutants but not in the wild type (Fig. 1A). By substrating 5.7-kb representing the size of Tn*5*, the size of the wild-type *Eco*RI fragment was calculated to be 5.2-kb in mutants I-2, I-3, and I-4, and to be 6.3-kb in mutant I-10. These two *Eco*RI fragments were assumed to contain two distinct gene loci, designated *repA* and *repB*, that were required for production of exoenzymes in this bacterium. Additionally, when the genomic digests of the wild type and Rep⁻ mutants were probed with the *pel* gene fragment from *P. viridiflava* strain SJ074 (Liao *et al.* 1992), similar sizes of the *pel* homologs were detected both in the wild type and mutants (Fig. 1B). This indicated that the Rep⁻ mutation was not caused by the insertion of Tn*5* into structural enzyme genes.

Cloning and characterization of *rep* gene loci.

A genomic library of *P. viridiflava* strain PJ-08-6A was constructed in the broad-host-range cosmid pLAFR3 as described in Materials and Methods. Plasmids from approximately 2,500 tetracycline-resistant (Tc^r) recombinant cells were mobilized en masse into mutants I-3 (RepA⁻) or I-10 (RepB⁻) and examined for restoration of enzyme production in these two mutants. Four recombinant plasmids (pLAI 31, 33, 36, and 37) capable of restoring enzyme production in RepA⁻ mutants (I-2, I-3, and I-4) but not in the RepB⁻ mutant (I-10) were identified. Those four recombinant plasmids designated *repA*$^+$ were found to contain the wild-type allele of *repA* DNA in a 5.2-kb *Eco*RI genomic fragment as predicted from the Southern blot analysis (Fig. 1A). One recombinant plasmid (pLAI 101) capable of restoring enzyme production in RepB⁻ (I-10) but not in RepA⁻ mutants (I-2, I-3, and I-4) was also identified. As described later, pLAI 101 (designated *repB*$^+$) was found to contain the wild-type allele of *repB* DNA in a 6.3-kb *Eco*RI genomic fragment (Fig. 1A). The amounts of Pel and Prt enzymes produced by representative Rep⁻ mutants (I-3 and I-10) carrying pLAFR3, pLAI 31, or pLAI 101 were compared. Results (Table 3) showed that the Rep⁻ mutant carrying pLAI 31 or pLAI 101 produced significantly higher levels of enzymes than its counterpart carrying pLAFR3. However, the amounts of enzymes produced by I-3 (pLAI 31) or I-10 (pLAI 101) were in general lower than those produced by the wild-type strain PJ-08-6A.

All five *rep*$^+$ plasmids were subsequently analyzed with a number of restriction endonucleases. The average size of DNA insert in each plasmid was estimated to be 24 kb. Restriction analysis (Fig. 2) of *repA*$^+$ plasmids with *Eco*RI revealed the presence of three *Eco*RI subfragments in the insert of each plasmid, two of which, 2.8- and 1.1-kb in size, were found in all four *repA*$^+$ plasmids (pLAI 31, 33, 36, and 37). The third *Eco*RI subfragment that varied in size, ranging from 2.2- to 5.2-kb, was located immediately adjacent to the vector pLAFR3 DNA. Further digestion of this third *Eco*RI subfragment with *Hin*dIII revealed the presence of a 1.8-kb region common to all four *repA*$^+$ plasmids. To further locate the DNA region in the insert that was essential for enzyme production, pLAI 31 was mutagenized with λ::Tn*5* as described in Materials and Methods. Four pLAI 31::Tn*5* mutant plasmids (designated pLAI 311, 312, 313, and 314) that lost the ability to restore enzyme production in mutant I-3 were identified. These four Rep⁻ mutant plasmids were digested with four restriction enzymes (*Eco*RI, *Hin*dIII, *Bam*HI, and *Pst*I), and the positions of Tn*5* insertions were mapped both in the 2.8-kb *Eco*RI and in the 1.8-kb *Eco*RI-*Hin*dIII region of pLAI 31 (Fig. 2). The *repA* gene was thus predicted to be located in a joint region between these two fragments, which were designated *repA1* and *repA2*. In addition to *repA*, another gene locus (*repB*) required for enzyme production was identified in pLAI 101. When pLAI 101 was digested with *Eco*RI, three subfragments in the size of 4.2-, 6.3-, and 1.4-kb were generated from the insert (Fig. 2). The 6.3-kb internal fragment was later subcloned into pRK415 to form pRepB63. The pRepB63 in mutant I-10 was able to direct the synthesis of about the same levels of enzyme as pLAI 101 (data not shown), indicating that the *repB* locus is located entirely within the 6.3-kb *Eco*RI fragment.

Southern blot analysis of Rep⁻ mutants with cloned DNAs.

To further confirm that mutants I-2, I-3, and I-4 were caused by the specific insertion of Tn*5* into the *repA* locus, *Eco*RI-generated genomic digests of these mutants were analyzed by Southern blot hybridization with the cloned *repA1* (1.8-kb *Eco*RI-*Hin*dIII) or *repA2* (2.8-kb *Eco*RI) DNA fragments. In the wild type, the *repA1* and *repA2* loci were detected as predicted in the 5.2- and 2.8-kb *Eco*RI fragments, respectively; whereas, in mutants I-2, I-3, and I-4, the *repA1* locus was detected in a 10.9-kb fragment that was also detectable by the Tn*5* probe (Fig. 1A, lanes 4–6). Similarly, in mutant I-10, the *repB* locus was detected in a 12.0-kb frag-

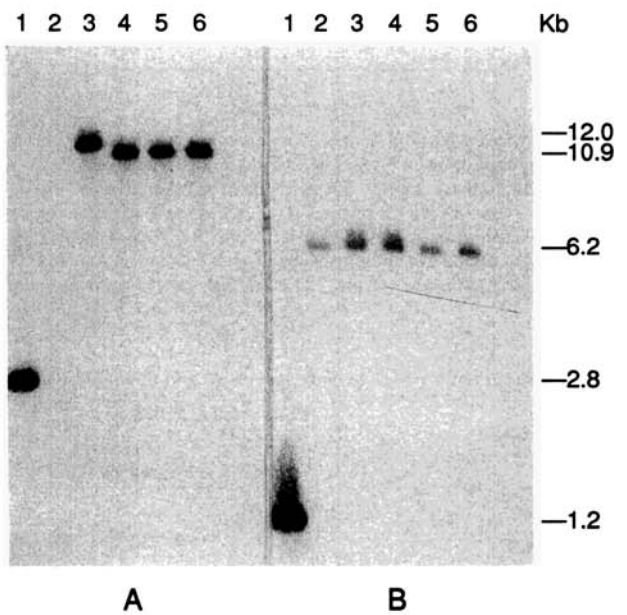

Fig. 1. Southern blot analysis of *Eco*RI-generated genomic DNA digests of the wild-type and Rep⁻ mutants of *Pseudomonas viridiflava* strain PJ-08-6A with **A,** Tn*5* and with **B,** cloned *pel* fragment. Lane 1 (panel A), 2.8-kb internal *Bgl*II fragment of Tn*5* from pRZ102; lane 1 (panel B), 1.2-kb *Pst*I-*Bgl*II *Pel* fragment from strain SJ074; lane 2, wild-type strain PJ-08-6A, lane 3, I-10; lane 4, I-4; lane 5, I-3; and lane 6, I-2 mutant.

ment that was detectable both by the 6.3-kb *repB* gene probe (data not shown) and the Tn*5* probe (Fig. 1A, lane 3).

Characterization of Rep⁻ marker-exchange mutants.

To facilitate the isolation of marker-exchange mutants that had Tn*5* inserted specifically in the *repA1* or *repA2* locus, two specific plasmid vectors designated pRepA 281 and pRepA 321 were constructed. The pRepA 281 was constructed by ligating the 8.5-kb *Eco*RI subfragment (*repA2*::Tn*5*) from pLAI 311 (Fig. 2) into pRK415, and pRepA 321 was constructed by ligating the 8.9-kb *Eco*RI subfragment (*repA1*::Tn*5*) from pLAI 312 (Fig. 2) into pRK415. After that, pRepA 281, pRepA 321, and pLAI 311 were mobilized individually into the wild-type strain PJ-08-6A with the aid of pRK2013. Following a series of subculturings and selections, three marker-exchange mutants (ME311, ME281, and ME321) showing the Rep⁻ phenotype were isolated. When genomic digests of the wild type and marker-exchange mutants ME311, ME281, and ME321 were probed with cloned *repA1* and *repA2* DNAs, the wild-type alleles of *repA1* and *repA2* loci were detected, respectively, in 5.2- and 2.8-kb *Eco*RI fragments as predicted from earlier studies (Fig. 3A, lane 4). In ME311 and ME281 mutants, the *Eco*RI fragments containing the *repA1* locus (5.2-kb) were unaffected (Fig. 3A, lanes 1–2), but the fragments containing the *repA2* locus (8.5-kb) were detectable both by *repA2* and Tn*5* probes (Fig. 3B, lanes 1–2). This result indicates that mutants ME311 and ME281 were likely derived from the insertion of Tn*5* into the *repA2* fragment, and mutant ME321 was derived from the insertion of Tn*5* into the *repA1* fragment (Fig. 3A and B). Complementation studies showed that only *repA*⁺ plasmids (pLAI31, 33, 36, and 37) were able to restore enzyme production in these mutants. Plasmids pRepA28 or pRepA52, carrying the *repA1* or *repA2* locus alone, were insufficient to complement the mutation, but plasmid pRepA2852, containing both *repA1* and *repA2* loci, was capable of restoring enzyme-producing ability in these marker-exchange mutants. These results provide further evidence that the *repA* gene is contained in a joint region between the 1.8- and 2.8-kb fragments.

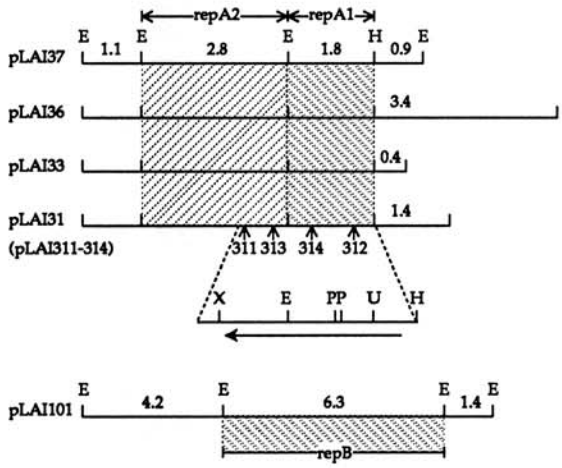

Fig. 2. Restriction map of cloned *Pseudomonas viridiflava* PJ-08-6A genomic regions containing *repA1* and *repB* loci. Numbers above or below the line indicate the size of the fragment in kilobases (kb). Arrows show the positions of Tn*5* insertions that lead to the Rep⁻ phenotype in complementation tests. E = *EcoR*I, H = *Hin*dIII, P = *Pst*I, X = *Xho*I, and U = *Stu*I. The region containing the *repA* open reading frame is shown under the pLAI31. The arrow indicates the possible direction of transcription.

Involvement of *repA2* DNA in alginate production.

Unlike strain PJ-08-6A, *P. viridiflava* strain SF312A was capable of producing in addition to Pel and Prt a large quan-

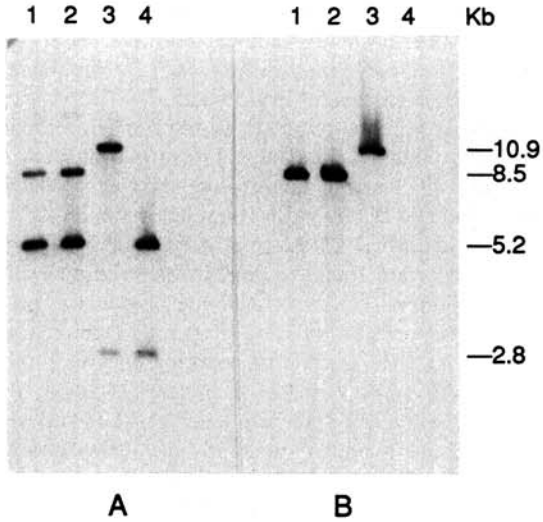

Fig. 3. Southern blot analysis of *EcoR*I-generated genomic digests of the wild-type and Rep⁻ marker-exchanged mutants (ME311, 281, and 321) of *Pseudomonas viridiflava* strain PJ-08-6A with **A,** cloned *repA1* and *repA2* fragments and **B,** 2.8-kb internal *Bgl*II fragment of Tn*5* (panel B). Lane 1, ME311 (*repA2*::Tn*5*); lane 2, ME281 (*repA2*::Tn*5*); lane 3 (*repA1*::Tn*5*); and lane 4, wild type.

Table 3. Production of pectate lyase, protease, and alginate by various strains of *Pseudomonas viridiflava*

Strains	Pectate lyase activity		Protease activity		Alginate	Soft-rotting ability
	Total ($U/10^{10}$ cells)	Percent extracellular	Total ($U/10^{10}$ cells)	Percent extracellular	(mg/g dry wt)	
PJ-08-6A	14.5	93	20.1	95	1.1	+
SF312A	17.3	95	23.2	91	59.3	+
I-3 (pLAFR3)	0.3	87	0.7	89	ND[a]	−
I-3 (pLAI 31)	8.6	91	11.9	93	ND	+
I-10 (pLAFR3)	<0.1	89	0.1	92	ND	−
I-10 (pLAI 101)	9.0	93	9.4	91	ND	+
SF312A1 (pLAFR3)	0.7	95	0.5	87	6.7	−
SF312A1 (pLAI 31)	12.8	89	15.8	85	75.9	+
MI-13 (pLAFR3)	0.5	86	0.4	84	3.4	−
MI-13 (pLAI 31)	10.5	87	11.1	91	42.9	+

[a]Not determined. Other values are the average of three experiments.

tity of alginate in culture. During the course of this study, a spontaneous mutant designated SF312A1 and a Tn*5*-insertion mutant designated MI-13, which were deficient in the synthesis of Pel, Prt, and alginate, were isolated. Since the pleiotropic phenotypic changes as observed with SF312A1 and MI-13 mutants were similar in many aspects to the Rep$^-$ phenotype found in strain PJ-08-6A, studies were then undertaken to determine if the *repA* or *repB* DNA of strain PJ-08-6A was capable of restoring enzyme and alginate production in SF312A1 and MI-13 mutants. Results (Table 3) show that the *repA*$^+$ plasmids (pLAI 31, 33, 36, and 37) restored not only the ability to produce Pel and Prt but also the ability to produce alginate and form mucoid colonies on agar media (Fig. 4).

To analyze the analogous *repA1* and *repA2* loci in strain SF312A further, genomic digests of the wild type and Rep$^-$ mutants were probed with cloned *repA1* and *repA2* DNA from strain PJ-08-6A. The *repA1* and *repA2* loci were identified in the 6.8- and 4.1-kb *Eco*RI genomic fragments, respectively, of strain SF312A instead of the 5.2- and 2.8-kb fragments detected in strain PJ-08-6A. In mutant MI-13, a 9.8-kb *Eco*RI fragment presumably resulting from the insertion of Tn*5* into the *repA2* locus was detected by either the Tn*5* or the *repA2* probe of strain PJ-08-6A (data not shown). However, in mutant SF312A1, no alteration in the size of *repA1*, *repA2*, or *repB* fragment was observed, indicating that this mutant carries a mutation in the locus other than *repA1*, *repA2*, and *repB* described above.

Pathogenicity responses.

Like Pel$^-$ mutants previously demonstrated in strain SF312A (Liao *et al.* 1988), Rep$^-$ mutants of strains SF312A and PJ-08-6A were unable to induce soft rot in potato tuber disks and detached bell pepper fruits. The residual amounts of the Pel enzyme still detectable in culture filtrates of Rep$^-$ mutants (Table 3) were obviously insufficient to initiate disease development. When the plasmid carrying the functional *repA* (or *repB*) gene was mobilized into the RepA$^-$ (or RepB$^-$) mutant, the *rep*$^+$ plasmid was able to restore both the enzyme-producing and disease-causing abilities of the mutant. However, the *rep*$^+$ plasmid in the Rep$^-$ mutant was unable to direct the synthesis of the wild-type level of Pel in culture medium. Despite this, the mutant harboring the *rep*$^+$ plasmid did exhibit the same degree of the tissue-macerating ability as the wild type both in potato tuber disks and in bell pepper fruits. As described below, the *repA* gene of *P. viridiflava* showed a high degree of homology with the *lemA* gene of *P. syringae* pv. *syringae* (Hrabak and Willis 1992). Plasmid pEMH97 containing the *lemA* gene was able to restore the enzyme-producing and disease-causing ability in RepA$^-$ mutant I-2 but not in RepB$^-$ mutant I-10.

DNA sequence of *repA* gene.

The 1.8-kb *Eco*RI-*Hin*dIII and 2.8-kb *Eco*RI fragments containing the essential *repA* region were sequenced on both strands by the dideoxy chain termination method (Sanger *et al.* 1977). An open reading frame consisting of 2,790 nucleotide bases was identified in a region spanning these two fragments. This open reading frame was predicted to encode a protein consisting of 930 amino acids. The guanine plus cytosine (G+C) content of the *repA* gene was estimated to be 64%. A likely Shine-Dalgarno sequence (AAAGG) was identified at nucleotide positions 374 to 378, which was followed by a translational start codon (GTA) at positions 383 to 385. A search of the gene banks revealed that the *repA* gene showed 87% similarity in nucleotide sequence to the *lemA* gene of *P. syringae* pv. *syringae* (Hrabak and Willis 1992) and that the predicted RepA protein showed 93% similarity in amino acid sequence to the predicted LemA protein. Furthermore, similarities in amino acid sequence ranging from 29 to 44% were found between the RepA and several bacterial sensor proteins, including the ArcB proteins of *Escherichia coli* (Inchi *et al.* 1990), PhoR protein of *Bacillus subtilis* (Seki *et al.* 1988), CpxA protein of *E. coli* (Albin *et al.* 1986), NifR2 protein of *Rhodobacter capsulatus* (Jones and Haselkorn 1989), and the DctB protein of *Rhizobium leguminosarum* (Ronson *et al.* 1987). At the carboxyl end of the RepA protein, similarities in amino acid sequence were also found to segments of several response regulator proteins, including the Vir protein of *Bordetella pertussis* (Stibitz *et al.* 1989) and the PhoP proteins of *Salmonella typhimurium* (Miller *et al.* 1989) and *B. subtilis* (Seki *et al.* 1987). When the predicted RepA amino acid sequences were aligned with the amino acid sequences of other regulatory proteins, specific amino acids well conserved in other two-component regulatory proteins were identified in the RepA protein. These include histidine at position 1,233, asparagine at position 1,365, aspartic acid at positions 2,370 and 2,387, and lysine at position 2,667 (Fig. 5). Moreover, analysis of the hydropathicity profile of RepA protein (Kyte and Doolittle 1982) revealed the presence of two hydrophobic transmembrane regions at the amino end of the protein.

DISCUSSION

The data presented here show that at least two gene loci (*repA* and *repB*) are involved in the regulation of the synthesis of the pathogenicity factor, Pel, and other biomolecules in *P. viridiflava*. DNA sequence analysis of the *repA* gene revealed that it has 87% similarity in nucleotide sequence to the *lemA* gene of *P. syringae* pv. *syringae* (Hrabak and Willis 1992). The function of the *repA* and *lemA* genes appears to be

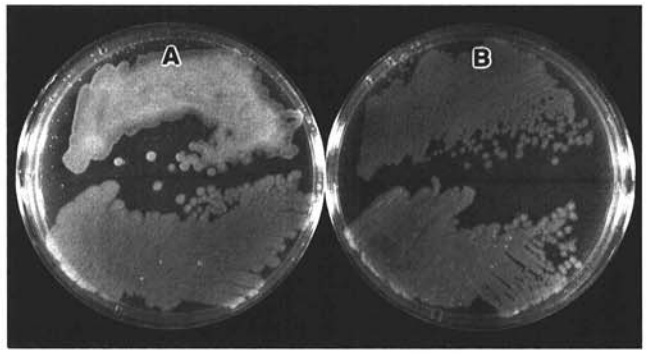

Fig. 4. Restoration of mucoid colony phenotype (or alginate-producing ability) in Rep$^-$ mutant MI-13 of *Pseudomonas viridflava* strain SF312A with plasmid pLAI 31 containing *repA2* DNA of strain PJ-08-6A. Bacteria were grown on *Pseudomonas* agar F supplemented with rifampicin at 100 µg/ml and tetracycline at 25 µg/ml. **A,** Top, MI-13 (pLAI 31); bottom, MI-13 (pLAFR3). **B,** Top, I-3 (pLAI 31); bottom, I-3 (pLAFR3).

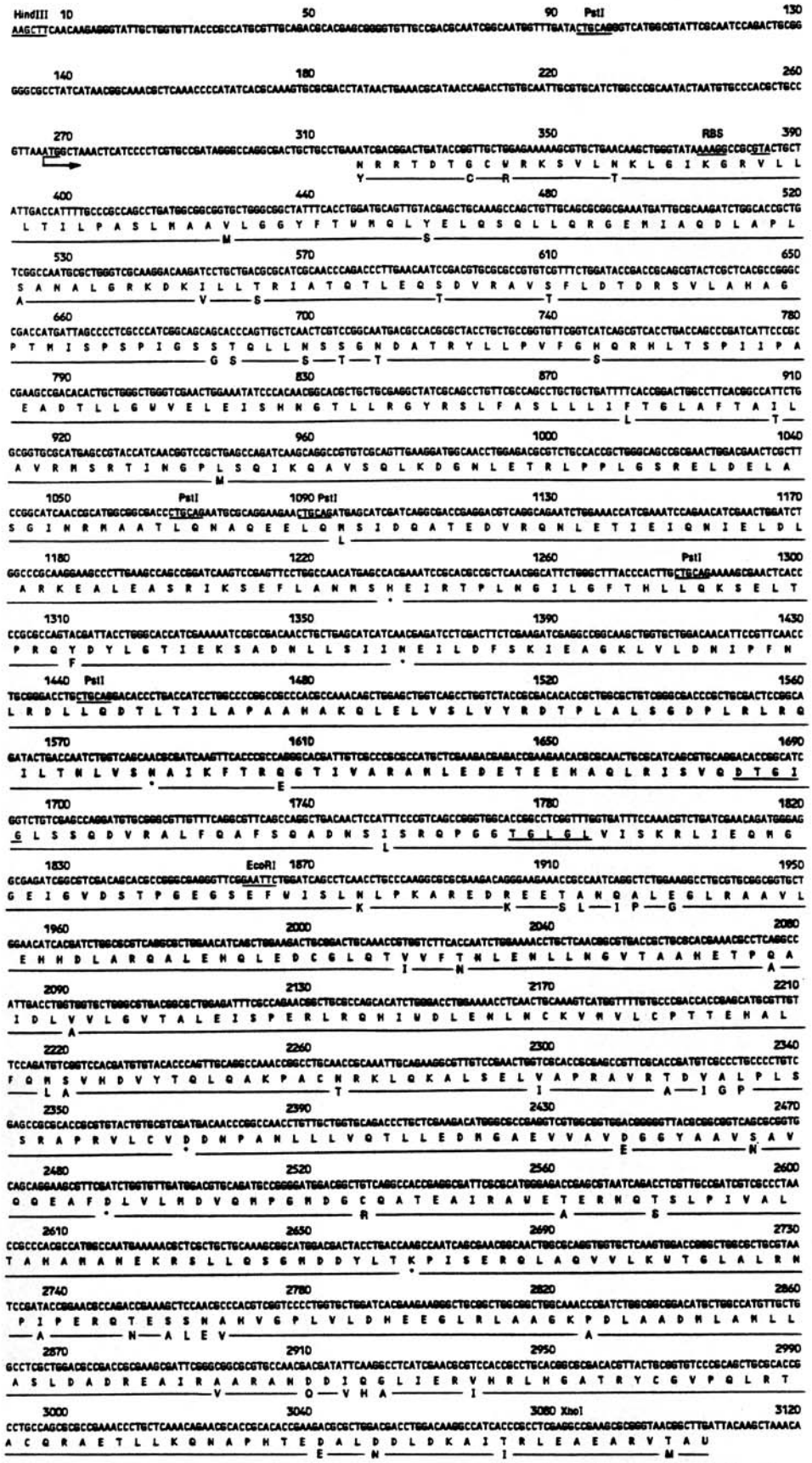

Fig. 5. Nucleotide and predicted amino acid sequences of the *repA* open reading frame. The amino acid sequences of the LemA protein are indicated under the RepA protein. Identical amino acid residues between these two proteins are indicated by a straight line. RBS = ribosome binding site; dot = conserved amino acid residues also found in other sensor or regulator proteins belonging to the two-component regulatory systems; arrow = transcriptional start.

similar and interchangeable. In this study, we determined that the plasmid (pEMH97) carrying the *P. syringae* pv. *syringae lemA* gene is capable of restoring the enzyme-producing and disease-causing ability in RepA$^-$ mutants of *P. viridiflava*. The *lemA/repA* gene family is involved in the regulation of a wide variety of biological or pathological activities in bacteria and appears to be widely distributed in fluorescent pseudomonads. Recently, Rich *et al.* (1992) reported that the *lemA* locus is well conserved within pathovars and strains of *P. syringae* and within *P. aeruginosa*. An independent study conducted at our laboratory also confirmed the presence of *repA/repB* loci in various species and strains of fluorescent pseudomonads including *P. syringae* and *P. putida* (C.-H. Liao, unpublished). In fact, an analogous *repA* gene that mediates production of extracellular enzymes (Pel and Prt) and expression of soft-rot pathogenicity in a phytopathogenic strain (CY091) of *P. fluorescens* has been cloned and partially characterized (McCallus and Liao, unpublished). Lam *et al.* (1993) also reported the identification and cloning of an analogous *lemA* gene from a root-colonizing strain (MOCG134) of *P. fluorescens* that is required for production of various antifungal compounds including chitinase, cyanide, and pyrrolnitrin in this bacterium.

So far, the *lemA/repA* gene has been identified only in fluorescent pseudomonads. By Southern blot analysis, we were unable to detect the *repA/repB* homologs in genomic digests of *Erwinia* and *Xanthomonas* strains (C.-H. Liao, unpublished). It should be noted however that a gene (*aepA*) required for production of various extracellular enzymes (including pectinase, cellulase, and protease) in *E. carotovora* subsp. *carotovora* has been isolated and characterized (Liu *et al.* 1993). The *aepA* gene shows very little or no homology with other prokaryotic regulatory genes and is presumably different from the *repA/lemA* gene described here. In addition, Tang *et al.* (1991) also reported the identification of a regulatory gene (*rpfC*) that is required for production of extracellular enzymes and exopolysaccharides in *X. campestris* pv. *campestris*. The function of the *rpfC* and the *repA* genes appears to be similar. However, we were unable to detect the *repA/repB* homolog in a genomic digest of *X. campestris* pv. *campestris* following Southern blot analysis (C.-H. Liao, unpublished). The *rpfC* gene that regulates enzyme and exopolysaccharide production in *X. campestris* pv. *campestris* is likely distantly related to the *repA/repB* gene of *P. viridiflava*.

In the majority of bacterial two-component regulatory systems, two separate protein components termed sensor and regulator proteins are usually involved. Moreover, the genes encoding both components are often found in close proximity on the bacterial chromosome (Ronson *et al.* 1987). The RepA/LemA protein described here and elsewhere (Hrabak and Willis 1992) is unusual in that it contains conserved domains of both sensor and regulator proteins. So far, a second protein component to the *RepA/LemA* has not been identified. Extensive efforts made by Hrabak and Willis (1992) failed to identify the second component in the DNA region flanking the *lemA* gene. It is possible that the gene coding for the second component may be located far apart from the *repA/lemA* locus on the bacterial chromosome. The *gacA* gene recently identified in *P. fluorescens* strain CHAO (Laville *et al.* 1992) and strain MOCG134 (Lam *et al.* 1993) may represent a possible candidate for the second component to the *repA/lemA* system. The *GacA* protein containing amino acid domains that are well conserved in other response regulator proteins is also required for production of various antimicrobial compounds related to disease-control activities (Laville *et al.* 1992). It is presently unknown if the *repB* gene described in this study shares any homology with *gacA* or if *repB* may act in concert with the *repA* gene to form a global regulatory network in *P. viridiflava*. Recently, we demonstrated that production of both Pel and Prt in *P. fluorescens* is coordinately regulated by divalent cations such as Ca^{2+} and Sr^{2+} (Liao *et al.* 1993). It would be interesting to know if an interaction between the cation in the environment with the *repA/repB* gene is required for activation of the *pel*, *prt*, and *alg* gene expression in *P. fluorescens* and *P. viridiflava*.

Previously, it was reported that certain strains of *P. viridiflava* can rapidly lose pathogenicity in culture (Billing 1970). In our laboratory, we also found that some strains of *P. viridiflava*, such as strain SF312A, spontaneously converted to a nonmucoid phenotype after repeated subculturing on agar media. More importantly, we found that the loss of the mucoid phenotype was always accompanied by loss of the ability to produce Pel and Prt. This earlier observation suggested for the first time that production of extracellular enzymes and exopolysaccharide in this bacterium might be coordinately regulated. In this study, we isolated a Tn*5*-insertion mutant of strain SF312A (designated MI-13) that exhibited pleiotropic phenotype changes resembling those observed with the spontaneous Rep$^-$ mutant SF312A1. Since the *repA* DNA fragments cloned from strain PJ-08-6A are able to restore both enzyme and alginate production in mutants MI-13 and SF312A1, the mechanism regulating exoprotein production in strains SF312A and PJ-08-6A appears similar. Furthermore, when the cloned *repA1* and *repA2* DNA fragments from strain PJ-08-6A were used as probes, we were able to demonstrate analogous *repA1* and *repA2* sequences in strain SF312A in two *Eco*RI fragments (4.1- and 6.8-kb), which were different in size from those detected in strain PJ-8-6A. Although the evidence presented here shows that mutant MI-13 was likely caused by the insertion of Tn*5* into the 4.1-kb *repA2* fragment of strain SF312A, the genetic mechanism that led to the spontaneous mutation in SF312A1 has not yet been determined. The spontaneous phenotypic changes observed with strain SF312A1 are similar to the Phc$^-$ (phenotypic conversion) phenotype of *P. solanacearum* (Brumbley *et al.* 1993). Since the sizes of *repA* and *repB* fragments in strain SF312A1 remain unchanged, this spontaneous mutant may be caused by a mutation in a locus other than *repA/repB*. It would be interesting to know if a gene analogous to *P. solanacearum phcA* is present in *P. viridiflava* and if a mutation in this gene can lead the Rep$^-$ phenotype as observed with the mutant SF312A1.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophage.

Bacterial strains, plasmids, and the bacteriophage used and constructed in the study are listed in Tables 1 and 2.

Media and culture conditions.

Luria broth (LB; BRL/GIBCO, Grand Island, NY) was used for routine cultivation of both *E. coli* and *P. viridiflava*.

When a solid medium was required, Luria agar (LA) or *Pseudomonas* agar F (Difco, Detroit, MI) was used for *E. coli* or *P. viridiflava*, respectively. For detection of pectolytic activity, bacteria were plated on a semisolid pectate (SSP) medium prepared as previously described (Liao 1991). A positive reaction for pectolytic activity was indicated by the formation of a depression surrounding the bacterial growth. For assays of proteolytic activity, bacteria were grown on nutrient agar (Difco Lab.) supplemented with 3% gelatin. A positive reaction in proteolytic activity was indicated by the formation of a clear zone surrounding the bacterial growth in the medium. When antibiotics were required, they were added at the following concentrations (per milliliter): rifampicin (Rif), 100 μg; kanamycin (Km), 50 μg; tetracycline (Tc), 25 μg; chloramphenicol (Cm), 20 μg; and streptomycin (Sm), 100 μg. For assays of enzyme production, bacteria were grown in a minimal salt medium (MYM) containing 0.4% glycerol and 0.1% yeast extract (Liao *et al.* 1988). Unless otherwise indicated, *E. coli* and *P. viridiflava* were incubated at 37° C and 28° C, respectively.

Enzyme assays.

P. viridiflava strains were cultured in MYM medium at 28° C for 60 hr. Cells were harvested by centrifugation and disrupted by ultrasonication. Fractionation of the cells was done by lysozyme/ethylenediamine tetraacetic acid (EDTA) treatment as previously described (Liao 1991). Assays for Pel activity in whole cell extracts or in subcellular fractions were carried out in a 0.5-ml volume containing 100 mM Tris-HCl (pH 8.0), 1 mM $CaCl_2$, 0.2% (w/v) polygalacturonate and enzyme sample (Liao *et al.* 1988). One unit of Pel activity was defined as the amount of enzyme that causes an increase of 1.0 absorbance unit per minute at 232 nm and 20° C. Assays for Prt activity were carried out in 1.5-ml volumes containing 100 mM Tris-HCl (pH 8.0), 1 mM $CaCl_2$, 50 mg of hide powder azure blue (Sigma Chemical Co., St. Louis, MO), and 0.5 ml of enzyme sample (Howe and Iglewski 1984). One unit of Prt activity was defined as the amount of enzyme that causes an increase of 1.0 absorbance unit per hour at 595 nm and 28° C. The specific enzyme activity was expressed in terms of enzyme units per 10^{10} cells. Cell concentrations were estimated from optical density (OD_{600}) readings; an OD_{600} of 1.5 was assumed to contain 10^9 cells per milliliter (Liao *et al.* 1993). When needed, protein concentrations were determined by a protein assay kit (Bio-Rad Laboratories, Richmond, CA).

Assays for alginate production and tissue maceration.

Bacterial strains were grown on *Pseudomonas* agar F containing appropriate antibiotics. After incubation at 28° C for 60–70 hr, bacterial masses were scraped off with a bent glass and water. Cells were removed by centrifugation (16,330 × *g*, 30 min), aqueous KCl was added to the supernatant fluid to give a final concentration of 1%, and three volumes of isopropanol were added. After sitting overnight at 4° C, precipitated material was collected by centrifugation. Pellets were taken up in small volumes of distilled water, dialyzed extensively against distilled water, and then freeze-dried. Uronic acid (alginate) content of the samples was estimated by the method of Blumenkrantz and Asboe-Hansen (1973). The ability of bacterial strains to macerate plant tissue was assayed on potato tuber slices or detached pepper fruits. Methods for preparation of plant materials and inocula for testing have been described previously (Liao and Wells 1987).

Isolation of mutants by transposon Tn*5* mutagenesis.

Transposon Tn*5* mutagenesis was conducted with the suicide plasmid pSUP1011 (Simon *et al.* 1983) in accordance with the method previously described (Liao *et al.* 1988). Following conjugative transfers, Rif^r Km^r transconjugants of *P. viridiflava* strain PJ-08-6A or SF312A were isolated and screened on SSP and nutrient agar-gelatin media for pectolytic and proteolytic activity, respectively. Mutants deficient in pectolytic and proteolytic activities were selected and further tested for their nutritional requirement and chloramphenicol resistance based on the protocol previously described (Liao *et al.* 1988).

Recombinant DNA techniques.

Standard procedures (Sambrook *et al.* 1989) were used for isolation of chromosome and plasmid DNAs, gel electrophoresis, restriction endonuclease digestion, subcloning, and preparation of competent cells for transformation and transduction. Southern hybridization analyses were conducted based on published procedures (Sambrook *et al.* 1989). DNAs were labeled and detected nonradioactively in accordance with the protocol described in the Genius DNA labeling and detection kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). If needed, DNA fragments isolated by electroelution were further purified with mini-Elutip columns from Schleicher & Schuell (Keene, NH).

Cloning of *repA* and *repB* DNA fragments.

The genomic DNA of strain PJ-08-6A was partially digested with the restriction enzyme *Sau*3A and size-fractionated by centrifugation in a 10–40% sucrose gradient. Genomic fragments 15- to 30-kb in size were pooled and ligated to pLAFR3 DNA previously digested with *Bam*HI and dephosphorylated with calf intestine alkaline phosphatase. Recombinant molecules in the ligation sample were packaged *in vitro* using the λ DNA packaging extract obtained from Boehringer Mannheim. The packaged sample was used to transduce *E. coli* HB101, followed by selection on LA-Tc medium. After that, a pool of Tc^r transductants was mobilized into Rep^- mutants (I-3 or I-10) with the aid of pRK2013 (Ditta *et al.* 1980). Transconjugants were selected on nutrient agar-gelatin medium supplemented with rifampicin (Rif) and tetracycline (Tc). Colonies showing restoration of proteolytic activity were isolated and subsequently tested for their pectolytic activity on SSP medium. Recombinant plasmids containing the putative *repA* and *repB* DNA were then isolated from *P. viridiflava* transconjugants and used to transform *E. coli* cells for further analyses.

λ-Mediated Tn*5* mutagenesis and marker exchange.

The λ-mediated Tn*5* mutagenesis was conducted in accordance with the method previously described (Ruvkun and Ausubel 1981). *E. coli* HB101 cells carrying pLAI 31 were infected with λ 467 at a multiplicity of 1.0, followed by selection of Tc^r Km^r transductants on LA-Tc-Km medium. Plasmid DNAs were extracted from a pool of Tc^r Km^r transductants and reintroduced into *E. coli* HB101 to select trans-

formants carrying pLAI 31::Tn*5*. Km^r Tc^r *E. coli* transformants carrying pLAI 31::Tn*5* were mated individually with the *P. viridiflava* I-3 mutant to identify mutant plasmids (pLAI 31::Tn*5*) unable to restore enzyme-producing ability in this mutant. Positions of Tn*5* insertions in Rep$^-$ pLAI 31::Tn*5* mutants were mapped in accordance with standard procedures previously described (Jorgensen *et al.* 1979).

For marker-exchange, three mutant plasmids (pLAI 311, pRepA321, and pRepA281) that had Tn*5* inserted specifically in one of two *Eco*RI fragments were introduced individually into the wild-type strain PJ-08-6A by pRK2013-assisted conjugation (Ditta *et al.* 1980). Rif^r Tc^r transconjugants were isolated by plating on *Pseudomonas* agar F supplemented with rifampicin and tetracycline, followed by repeated subculturing in LB containing Km but lacking Tc. After two to three cycles of subculturing, Rep$^-$ marker-exchange mutants of strain PJ-08-6A were identified by selecting Km^r Tc^s transconjugants on nutrient agar-gelatin medium supplemented with Km or with Km plus Tc (Lindgren *et al.* 1986). Complementation of marker-exchanged mutants with recombinant plasmids carrying the wild-type allele of *repA* DNA fragments was conducted in accordance with the method as described above.

DNA sequencing.

The pNC19 derivatives containing the 1.8-kb *Eco*RI-*Hin*dIII *repA1* fragment or the 2.8-kb *Eco*RI *repA2* fragment were sequenced by the dideoxy chain termination method (Sanger *et al.* 1977). Sequencing primers (18-mer) were synthesized by Labstrand Labs Ltd. (Gaithersburg, MD). Sequenase and 7-deaza nucleotides were obtained from U.S. Biochemical Corp. (Cleveland, OH). DNA sequence analysis and data ase search were conducted by using the micro-genie software from Beckman Instruments (Fullerton, CA).

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