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The *repB* gene required for production of extracellular enzymes and fluorescent siderophores in *Pseudomonas viridiflava* is an analog of the *gacA* gene of *Pseudomonas syringae*¹

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Abstract: Two genes, designated *repA* and *repB*, are involved in the regulation of the synthesis of extracellular pectate lyase, protease, and alginate in *Pseudomonas viridiflava*. The *repA* gene has been shown to encode a protein highly homologous to several bacterial sensors in the two-component regulator family including the LemA of *Pseudomonas syringae*. In this study, the *repB* locus, initially identified in a 6.3-kb *EcoRI* genomic fragment of *P. viridiflava*, was further characterized. Results obtained from restriction mapping, deletion subclonings, and mini-Mu-*LacZ* fusions indicated that the *repB* gene was contained within a 0.8-kb *HindIII*–*PstI* region. Sequence analysis of this *repB* region revealed the presence of an open reading frame, which was predicted to encode a protein similar or identical to the *gacA* response regulator found in *P. syringae* and *Pseudomonas fluorescens*. The *repB* gene of *P. viridiflava* also regulated the production of fluorescent siderophores, in addition to the aforementioned extracellular enzymes and alginate. The *repB* or *gacA* homologs were detected in the genomes of nine other strains of *P. viridiflava*, *P. fluorescens*, and *P. syringae* included in the study. The data presented here and earlier indicate that the *repA/repB* gene regulatory system of *P. viridiflava* is analogous to the *lemA/gacA* system of *P. syringae* and *P. fluorescens*.

Key words: response regulator, signal transduction, soft-rot bacteria, enzyme production.

Résumé : Les gènes *repA* et *repB* sont impliqués dans la régulation de la synthèse de la lyase pectate extracellulaire, la protéase et l'alginate chez *Pseudomonas viridiflava*. Il a été démontré que le gène *repA* code pour une protéine fortement homologue avec divers détecteurs bactériens faisant partie de la famille de régulation à deux composantes qui inclut le gène *LemA* de *Pseudomonas syringae*. Dans cette étude, le locus *repB*, qui a été initialement identifié dans un fragment génomique *EcoRI* de 6.3 kb chez *P. viridiflava*, a été caractérisé. Les résultats obtenus à l'aide de la cartographie avec des enzymes de restriction, du sous-clonage de délétion et des fusions mini-Mu-*LacZ* ont indiqué que le gène *repB* était présent dans la région *HindIII*–*PstI* de 0,8 kb. L'analyse de séquence de la région *repB* a révélé la présence d'un cadre de lecture ouvert qui pourrait coder pour une protéine similaire ou identique au gène régulateur de la réponse *gacA* qui est détecté chez *P. syringae* et *Pseudomonas fluorescens*. Le gène *repB* de *P. viridiflava* pouvait aussi réguler la production de sidérophores fluorescents en plus de l'alginate et des enzymes extracellulaires mentionnées ci-dessus. Les homologues de *repA* ou *gacA* ont été détectés dans le génome de neuf autres souches de *P. viridiflava*, *P. fluorescens* et *P. syringae* qui ont été analysées dans cette étude. Ces observations et des résultats antérieurs suggèrent que le système *repA/repB* de régulation génique de *P. viridiflava* est similaire au système *lemA/gacA* de *P. syringae* et *P. fluorescens*.

Mots clés : régulateur de la réponse, transduction de signaux, bactérie «soft-rot», production enzymatique.

[Traduit par la rédaction]

Fig. 2. Nucleotide sequence of the *repB* gene and deduced amino acid sequence of the RepB protein. Restriction sites corresponding to those shown in Fig. 1 are underlined. A putative translational start codon (TTG, bases 111–113), a translational stop codon (TGA, bases 754–756), and a putative ribosome binding site (RBS, bases 100–103) are observed as indicated.

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10      20      30      40      50      60      70      80      90      100
AAGCTTCCTGCGGGCGGTTTCGAGCGAAAACGGCAACATCCCTAGGTCGCTCGCCGTTTTTGTATGGTGAATAGCATTGTTATGTCCTGCGCG
HindIII

RBS 110      120      130      140      150      160      170      180      190      200
AGTGTCTGCTTGATTAGGGTGTCTAGTTGTCTGATGACCATGATCTTGTTCGGACAGGCATCACACGGATGCTCGCCGATATAGATGGCTTGCAAGTGGTA
M I R V L V L V D D H D L V R T G I T R M L A D I D G L Q V V

210      220      230      240      250      260      270      280      290      300
GGTCAGGCCGATTCCGGTGAGGAGTCTTGAAGAAGGCGCGGAACTCAAACCCGACGTCGTGCTGATGGACGCTCAAGATGCCGGGCATCGGCCGCTTG
G Q A D S G E E S L K K A R E L K P D V V L M D V K M P G I G G L

310      320      330      340      350      360      370      380      390      400
AAGCCACCCGCAAGCTTTGCGCAGCCATCCGGATATCAAGGTCGTGGCCGTGACGGTCTGCGAAGAAGATCCATTTCCGACCGCGCTGCTTCAGGCCGG
E A T R K L L R S H P D I K V V A V T V C E E D P F P T R L L Q A G
EcoRV

410      420      430      440      450      460      470      480      490      500
AGCGGCCGGTTACATGACCAAGGGCGCAAGCCTTGGCGAAATGGTTCAGGCGATCCGCTGGTCTTTGCGGTCAGCGCTACATCAGCCCGCAGATTGCC
A A G Y M T K G A G L A E H V Q A I R L V F A G Q R Y I S P Q I A
SstI

510      520      530      540      550      560      570      580      590      600
CAACAGCTGGCGCTAAAGTCATTTGAGCCGAGGTCAACAATTCACCGTTTGACCTGTGTCGAGCGCAAAATCCAGATTGCATTGATGATGTCGGTT
Q Q L A L K S F Q P Q V N N S P F D L L S E R E I Q I A L M I V G

610      620      630      640      650      660      670      680      690      700
GCCAGAAGTCCAGACCATCTGGCAAGCTGTGCTGCGCGAAAACCGTGAATACTTACCGCTACCGAATCTTTGAAAAGCTCCTCAGCAGTGA
C Q K V Q T I S D K L C L S P K T V N T Y R Y R I F E K L S I S S D

710      720      730      740      750      760      770      780      790      800
TGTGCAATTGGCGTGTGCGCAGTACGTCACGGCATGGTGGACGCCAGCGCCTGAACATGACCCAGACCTTTGATCCAAGTGCCTTTCTGCGCAGCTGCA
V E L A L L A V R H G M V D A S A U → uvrC PstI

810      820      830      840      850      860      870      880      890      900
GTGGTCGTCGCCGGCGTCTATCGCATGTTGATGCGAAGCCAGGCTTCTGTATGTGGGCAAAGCCAAGAATCTGAAGAAGCGCCTCGCCAGTATTTCG

910      920      930      940      950      960      970      980      990      1000
CAAGACCGGGCACGCGCCAAAGACTGGCGCTCTGGTGGCGCATCGCGCAGATTGAAACCACCATCACCGGTAACGAAAACGAAAGCGCTGTTGCTCGAA

1010     1020     1030     1040     1050     1060     1070     1080     1090     1100
CAGACGCTGATCAAGGAATGGCGACCTCCTTACAACATTCTGCTGCGCGACGATAAGTCTTATCCGTATGTGTTCTGTGCGACGGCAAAGTTTCGCGCC

1110     1120     1130     1140     1150     1160     1170     1180     1190     1200
TGAGCATCCATCGTGGCGCAAAGAAAGCAAAAGCCGGTATTGTCCTTATCCGAGCGGGTGCATCAGGGAAAGTCTTAGTCTGCTGCAAAAAGAC

1210     1220     1230     1240     1250     1260     1270     1280     1290     1300
CTTCTGGTGGCTCAGTGCGAAGACAGTTACTTCAAGAACCCTAACCACCCCTGTCTGACAGTACCAGATCAAGCGCTGCAAAAGGGCGTGGCTGATCTG

1310     1320     1330     1340     1350     1360     1370     1380     1390     1400
GTCGAGCCTGAGGTGATCGCAGAAGATGTGCGTCACTCGGTGATGTTCTGGAAGGGCGCAGTAATGCCCTTGAGCGACGAGTTGAACGCTGCAATGGAAA

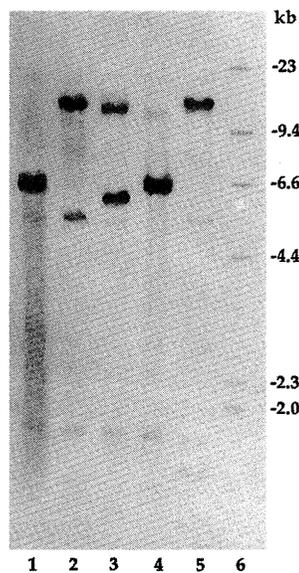
1410     1420     1430     1440     1450     1460     1470     1480     1490     1500
AGGCGCCATGGCCCTGGATTTGAGCGCGCGGCGAACTGCGCATCAGGTCGCTTGTGCGCGTGTGACAGGATCAGCAGAGCATGGAAGCGGCAC

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(Km^r) pKPN23A::MudI1734 derivatives were examined for their abilities to restore the enzyme production in RepB⁻ mutant I-10. Seven pKPN23A::MudI1734 mutants failing to restore the enzyme production in mutant I-10 were digested with four specific restriction enzymes (*EcoRI*, *HindIII*, *SstI*, and *PstI*) to map the positions of MudI1734 insertions in this 2.3-kb *repB*⁺ fragment (Fig. 1). The insertions that led to the

inactivation of *repB* function were all contained within the 0.8-kb *PstI*–*HindIII* region. To further confirm that this 0.8-kb fragment indeed contained the intact *repB* gene, this fragment was cloned into pLAFR3 (Staskawicz et al. 1987) to form pLAPH08. When mobilized into mutant I-10 by triparental matings (Ruvkun and Ausubel 1981), pLAPH08 was capable of restoring the production of wild-type levels of Pel and Prt in

Fig. 3. Localization of the *repB*-containing DNA fragments in *EcoRI*-generated genomic digests of the wild type and RepB⁻ mutants of *P. viridiflava* PJ-08-6A. Lane 1, wild type; lanes 2 and 3, marker exchange mutants ME #7 and ME #2 generated by pKPN23A (*repB*::*MudI1734*) mutagenesis; lane 4, Tn5-induced RepA⁻ mutant I-4, lane 5, Tn5-induced RepB⁻ mutant I-10; lane 6, digoxigenin-labeled λ *HindIII* DNA size markers.



this mutant. Later, the β-galactosidase activities of *E. coli* cells carrying the RepB⁻ or RepB⁺ pKPN23A::*MudI1734* derivatives were analyzed by the standard procedures (Miller 1992) to determine the direction of *repB* transcription. Results as depicted in Fig. 1 indicate that the direction of *repB* transcription is initiated from the *HindIII* to the *PstI* end of the 0.8-kb fragment.

For DNA sequencing of the *repB* gene, a 1.8-kb *repB HindIII* fragment was isolated from pKPN23A and cloned into pUC19 to form pRepBH2. The complete nucleotide sequence of this 1.8-kb insert in pRepBH2 was determined by the dideoxy chain termination method and a portion of the result (first 1500 bp from the *HindIII* end) is shown in Fig. 2. Analysis of this sequence by the PC/GENE programs (Intelligenetics Inc., Mountain View, Calif.) revealed the presence of two open reading frames (ORFs). The ORF1 (bases 111–755) was located within the 0.8-kb *HindIII–PstI* region as predicted earlier by physical and genetic analysis. The overall G+C content of this 1.8-kb fragment is 57.4%. A potential ribosome-binding site (bases 100–103) and putative translational start (bases 111–113) and stop (bases 753–755) codons were identified. The ORF1 representing the putative *repB* gene was predicted to encode a protein consisting of 210 amino acids (a.a.) and with a molecular mass of 24 kDa. The ORF2 immediately following the *repB* gene was predicted to encode a protein homologous to the UvrC protein required for DNA repair function in *E. coli* (Moolenaar et al. 1987). The RepB protein was scanned for similarities in a.a. sequence to proteins deposited in the SWISS-PRO data base (PC/GENE release 6.8). Similarities in a.a. sequence ranging from 51 to 93% were

found between the RepB and several RR proteins in the two-component regulator family (Stock et al. 1990). The RepB protein exhibited 93 and 100% identity in a.a. sequence to the GacA proteins of *P. fluorescens* (Laville et al. 1992) and *P. syringae* (Rich et al. 1994), respectively. Several reports (Lam et al. 1993; Willis et al. 1993; Gaffney et al. 1994; Rich et al. 1994) have suggested that the *gacA* and *lemA* genes constitute a HK–RR pair to mediate the production of pathogenicity factors and other extracellular compounds in *P. syringae* pv. *syringae*. We (Liao et al. 1994) reported that the RepA protein of *P. viridiflava* shared 93% similarity in a.a. sequence to the LemA protein of *P. syringae* (Hrabak and Willis 1992). Together, these results suggest that the *repA/repB* and *lemA/gacA* gene regulatory systems demonstrated in *P. viridiflava*, *P. syringae*, and *P. fluorescens* are analogous. However, the targeted genes under the control of these two systems are somewhat different. The LemA/GacA system has been shown to control the production of disease lesion, protease, and phyto-toxin in *P. syringae* (Hrabak and Willis 1992, 1993) and to control the production of antibiotics, protease, and phospholipase C in two biocontrol strains of *P. fluorescens* (Laville et al. 1992; Sacherer et al. 1994; Gaffney et al. 1994). In contrast, the RepA/RepB system of *P. viridiflava* described here and earlier (Liao et al. 1994) regulates the production of pectate lyase, alginate, and fluorescent siderophores (to be discussed below).

During the course of the study of Rep⁻ pleiotropic mutations, mutant I-10 was the only RepB⁻ isolate obtained after a series of transposon mutagenesis experiments. To further confirm the presence of *repB* locus in the chromosome of *P. viridiflava*, several marker-exchange mutants having *MudI1734* inserted specifically in the *repB* locus were constructed. This was done by mobilizing a RepB⁻ pKPN23A::*MudI1734* derivative into the wild-type strain PJ-08-6A by triparental matings (Ruvkun and Aulsebrook 1981). Transconjugants resistant to both kanamycin and tetracycline (Km^r Tc^r) were randomly selected and subcultured in the medium containing kanamycin but lacking tetracycline for four passages. After that, 25 kanamycin-resistant but tetracycline-susceptible (Km^r Tc^s) derivatives were randomly isolated and tested for their ability to produce Pel and Prt. Like mutant I-10, all 25 Km^r Tc^s derivatives were deficient in production of Pel and Prt. To confirm that the observed phenotypic change in Km^r Tc^s derivatives was caused by the insertion of *MudI1734* into the *repB* locus, the 0.8-kb *HindIII–PstI repB* fragment described above was used to probe the *EcoRI*-generated genomic digests of two representative isolates (ME #2 and ME #7). Results (Fig. 3) show that the *repB* is located in a single 6.3-kb genomic fragment of the wild type as expected. However, in the genomic digests of mutants ME #2 and ME #7, the *repB* hybridization bands were detected either by the *repB* or *MudI1734* gene probe in two separate *EcoRI* fragments. Since the *MudI1734* had been shown to contain an *EcoRI* site approximately 4.5 kb apart from the 5' end of *LacZ* gene (Miller 1992), distribution of *repB* homologous sequence in two *EcoRI* fragments in mutants ME #2 and ME #7 resulted likely from the insertion of *MudI1734* into the wild-type 6.3-kb *EcoRI repB* fragment.

Like Tn5-induced RepB⁻ mutant I-10, all 25 Km^r Tc^s marker-exchange mutants obtained as described above were defective in production of Pel and Prt and unable to cause soft-rot in potato tuber slices and green pepper fruits as determined by the methods previously described (Liao and Wells 1987).

Table 1. Effect of RepB⁻ mutation on fluorescent siderophore production by *Pseudomonas viridiflava*.

Strains ^a	Orange halo on CAS medium, mm diameter ^b	Pel activity, U/10 ¹⁰ cells ^c	Soft-rotting ability
PJ-08-6A (pLAFR3)	6.8	12.2	+
PJ-08-6A (pLAPH08)	7.1	13.3	+
I-10 (pLAFR3)	2.3	<0.1	-
I-10 (pLAPH08)	6.8	8.6	+
ME #2 (pLAFR3)	2.1	<0.1	-
ME #2 (pLAPH08)	6.4	11.1	+
I-4 (pLAFR3)	6.7	<0.1	-
I-4 (pLAI33)	7.0	10.8	+

^aStrain PJ-08-6A, wild type; I-10, RepB⁻ Tn5 mutant; I-4, RepA⁻ Tn5 mutant; and ME#2, RepB⁻ marker-exchange mutant. pAFR3, cloning vector; pLAI33 and pLAPH08, pLAFR3 derivatives carrying the *repA* and *repB* gene, respectively.

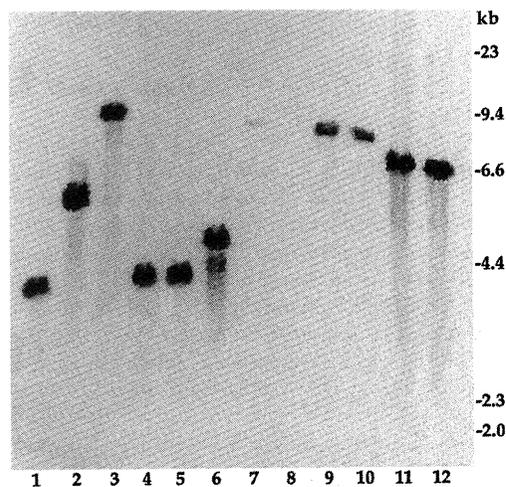
^bThe value represents an average of two experiments; there were two samples taken for each experiment. Soft-rotting ability was assayed on potato tuber slices. Siderophore production was indicated by the formation of orange halo zone on chrome azurol S (CAS) agar medium (Schwyn and Neilands 1987). Orange halo zones were measured from the edge of bacterial growth.

^cOne unit of activity is defined as the amount of enzyme that cause an increase of 1.0 absorbance unit at 232 nm at 30°C per minute.

Furthermore, all of these mutants failed to produce fluorescent pigments in the *Pseudomonas* agar F (equivalent to King's medium B, Difco Lab., Detroit, Mich.). To compare the effect of RepB⁻ and RepA⁻ mutations on fluorescent pigment and extracellular enzyme production in *P. viridiflava*, the following experiments were conducted. Four bacterial strains including the wild type, RepA⁻ Tn5 mutant I-4 (Liao et al. 1994), RepB⁻ Tn5 mutant I-10, and RepB⁻ marker-exchange mutant ME #2 were used. The bacterial strain carrying the vector plasmid pLAFR3 (Staskawicz et al. 1987), the repB⁺ pLAPH08 (described above), or the repA⁺ pLAI33 (Liao et al. 1994) was grown on chrome azurol S (CAS) agar plate medium as previously described (Schwyn and Neilands 1987). Production of both fluorescent and nonfluorescent siderophores, as indicated by the formation of an orange halo surrounding the bacterial growth, was quantitated by measuring the diameter of the orange halo zone on CAS medium. Production of Pel was determined by the methods previously described (Liao et al. 1994). Results as summarized in Table 1 show that production of siderophores is markedly reduced in RepB⁻ mutants I-10 and ME #2, but not in RepA⁻ mutant I-4. The repB⁺ plasmid pLAPH08 was able to restore the synthesis of the wild-type level of siderophores in RepB⁻ mutant I-10 or ME #2. This result provided an unequivocal evidence that reduction in fluorescent pigment and Pel production in RepB⁻ mutants was caused by the insertion of MudI1734 into the *repB* gene. It is presently unclear, however, why production of siderophores was affected by the RepB⁻ but not by the RepA⁻ mutation. We speculate that the RepB RR may act in pairs with another non-RepA HK sensor to mediate the production of fluorescent siderophores in *P. viridiflava*.

Willis and associates (Willis et al. 1994; Rich et al. 1992) previously reported that the sensory *lemA* gene was widely distributed among pathovars of *P. syringae*. Since the *lemA* component was proposed or demonstrated to act in pairs with *gacA* until very recently (Lam et al. 1993; Willis et al. 1993; Gaffney et al. 1993; Rich et al. 1994), it was not known if the *gacA* homologs were present concurrently with the *lemA* in

Fig. 4. Detection of *repB* homologs in the genomic digests of various species or strains of phytopathogenic bacteria. Lanes 1–6, *Pseudomonas syringae* pv. *glycinea*, pv. *lachrymans*, pv. *tomato*, pv. *tabaci*, pv. *phaseolicola*, and pv. *syringae*, respectively. Lane 7, *Erwinia carotovora* subsp. *carotovora*; lane 8, *Xanthomonas campestris* pv. *campestris*; lanes 9 and 10, *Pseudomonas fluorescens* strains PJ-08-30 and CY091; lanes 11 and 12, *Pseudomonas viridiflava* strains PJ-08-9 and PJ-08-6A.



other groups of fluorescent pseudomonads. In this study, the 0.8-kb *repB* HindIII–PstI fragment contained within pLAPH08 was isolated, labeled, and used to detect the presence of *repB* homologs in 12 different strains of phytopathogenic bacteria by Southern hybridization procedures (Sambrook et al. 1989). The *repB* or *gacA* homologs were detected in the genomic digests of all 10 strains of fluorescent pseudomonads included in the study, including two strains each of *P. viridiflava* and *P. fluorescens* and five pathovars of *P. syringae* (Fig. 4). A very weak hybridization band of about 9.4 kb in size was

detected in the genomic digest of *Erwinia carotovora* subsp. *carotovora* (Fig. 4, lane 7). No *repB* homolog was detected in the genomic digest of *Xanthomonas campestris* pv. *campestris* (Fig. 4, lane 8). However, it should be noted that the *lemA* homolog has been detected in other strains of *X. campestris* pv. *campestris* (Willis et al. 1994). The *gacA* gene homologs have been previously found in other groups of bacteria including *E. coli* (Gaffney et al. 1993) and *P. aeruginosa* (Laville et al. 1992). The results presented here further confirmed the wide distribution of this gene in various groups of soft-rotting bacteria. The *repB/repA* or *gacA/lemA* thus represents a common gene regulatory system and appears to be very well conserved in pathogenic and nonpathogenic fluorescent pseudomonads.

The *repB* gene sequence reported here has been deposited in the GenBank (March 22, 1994) where the accession number is L30102.

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