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# Genetic Analysis of *Rhizobium meliloti bacA-phoA* Fusion Results in Identification of *degP*: Two Loci Required for Symbiosis Are Closely Linked to *degP*

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**The function of the *Rhizobium meliloti bacA* gene, which is a homolog of the *Escherichia coli sbmA* gene, is required for an intermediate step in nodule development. A strain carrying the *bacA386*::Tn*phoA* fusion was mutagenized with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, and three mutants that had higher levels of alkaline phosphatase activity were identified. The mutations in these strains were recessive and mapped to the same genetic locus. The gene affected by these mutations was identified and sequenced and was found to be a homolog of the *E. coli degP* gene, which encodes a periplasmic endopeptidase. Although *degP* function is important for the virulence of certain intracellular pathogens of mammals, it is not required for the *R. meliloti*-alfalfa symbiosis. The genetic analyses involving *degP* were complicated by the presence of a locus immediately upstream of *degP* that was lethal when present in multiple copies in a DegP$^-$ background. *R. meliloti* derivatives carrying insertion mutations in this locus displayed an *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine oxidase-negative phenotype, elicited the formation of white cylindrical nodules that did not fix nitrogen, and grew slowly in rich medium, suggesting that the locus was a *cyc* gene encoding a protein involved in the biosynthesis of a component or components of a respiratory chain. The previously identified *fix-382*::Tn*phoA*, which similarly causes the formation of white cylindrical nodules that do not fix nitrogen, was shown to affect a gene that is separate from this *cyc* gene but extremely closely linked to it.**

The gram-negative bacterium *Rhizobium meliloti* enters into a symbiotic association with the legume alfalfa (*Medicago sativa*), in which nodules containing intracellular bacteria that fix nitrogen develop on the plant roots (7, 17). The bacteria induce the formation of these nodules, and then invade them through tubes called infection threads. Upon their release into the plant cells, the bacteria differentiate into the nitrogen-fixing forms called bacteroids. Several classes of *R. meliloti* mutants that affect early or late stages of nodule development have been studied extensively. In particular, *nod* mutants (7, 17) fail to induce nodules; *exo* (16, 23, 24, 31, 32, 34, 35, 42) and *ndv* (8, 13, 19) mutants induce nodules but do not invade them; and *dct* (5, 57), *nif* (26, 27), and *fix* (27) mutants undergo bacteroid differentiation but do not fix nitrogen. In contrast, relatively few *R. meliloti* mutants that are blocked at intermediate stages in nodule development have been isolated (2, 11, 20, 56).

The *R. meliloti bacA* gene is required for an intermediate step in nodule development (20). Like wild-type bacteria, *bacA* mutants invade nodules and are released from infection threads into the host cytoplasm. However, unlike wild-type bacteria, which differentiate into nitrogen-fixing bacteroids, *bacA* bacteria senesce in the plant cell cytoplasm shortly after their release from infection threads (20). The expression of a *bacA*::Tn*phoA* fusion protein in nodules was found to be strongest in the early symbiotic zone, the region where bacteroid differentiation takes place (20). This observation suggested that regulation of *bacA* gene expression according to the developmental state of the bacteria might be important for its function. Interestingly, the *bacA* gene, whose product has been predicted to span the membrane seven times, has been found to be a homolog of the *Escherichia coli sbmA* gene (20). The product of the *sbmA* gene has been implicated in the transport of microcin B17 and bleomycin, both of which contain thiazole rings (4, 39, 58).

In this work, we initially set out to identify genes involved in the regulation of *bacA* by searching for mutants displaying increased levels of alkaline phosphatase in a strain carrying the *bacA386*::Tn*phoA* fusion. Characterization of these mutants unexpectedly led to the identification of the *R. meliloti* homolog of the *E. coli degP* protease gene (20, 36–38, 52, 53). Two genes adjacent to *degP* were found to encode membrane-bound or periplasmic proteins and to be required for effective symbiosis. One of these genes is probably involved in the biogenesis of *c*-type cytochrome(s).

## MATERIALS AND METHODS

**Strains and media.** *R. meliloti* strains used in this study are shown in Table 1. Bacteria were grown in LB medium (43), with 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$ added for *R. meliloti* (LB/MC medium). Antibiotics were used at the following concentrations: neomycin (Nm), 200 μg/ml; gentamicin (Gm), 20 μg/ml; spectinomycin (Sp), 50 μg/ml; streptomycin (Sm), 400 μg/ml; tetracycline (Tc), 10 μg/ml; kanamycin (Km), 25 μg/ml; and chloramphenicol (Cm), 10 μg/ml. 5-Bromo-4-indolyl-β-D-galactopyranoside (X-Gal) and 5-chloro-3-bromo-4-chloro-3-indolyl phosphate (X-P) were used at 40 μg/ml.

**Genetic techniques.** Strain Rm8386 was mutagenized with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) as follows. Cells from 1 ml of a log-phase culture (optical density at 600 nm, 0.2) were washed and resuspended in an equal volume of 0.1 M sodium citrate (pH 5.5). MNNG was added to a final concentration of 200 μg/ml, and the cells were incubated at 30°C for 60 min. They were then washed with 0.1 M potassium phosphate (pH 7.0), resuspended in 5 ml of LB/MC medium, and allowed to grow overnight. These cultures were then diluted and spread on plates containing X-P to screen for mutants. This mutagenesis protocol resulted in 90 to 95% lethality, and 1 to 2% of the survivors were auxotrophs. Generalized transduction was performed as described previously (15). Plasmids were introduced into *R. meliloti* by conjugal transfer in

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TABLE 1. *R. meliloti* strains used in this study

| Strain[a] | Relevant genotype | Source or reference |
|---|---|---|
| Rm8002 | *pho-1* | 41 |
| Rm8386 | *pho-1 bacA386*::Tn*phoA* | 41 |
| Rm8619 | *pho-1 fix-388*::Tn*phoA-233* | 21 |
| Rm8615 | *pho-1 fix-382*::Tn*phoA-233* | 21 |
| Rm8625 | *pho-1 bacA386*::Tn*phoA degP31* (mutagenized background) | This work |
| Rm8626 | *pho-1 bacA386*::Tn*phoA degP32* (mutagenized background) | This work |
| Rm8627 | *pho-1 bacA386*::Tn*phoA degP41* (mutagenized background) | This work |
| Rm8628 | *pho-1 bacA386*::Tn*phoA degP31* 41ΩTn*5-233* | This work |
| Rm8629 | *pho-1 bacA386*::Tn*phoA degP32* 41ΩTn*5-233* | This work |
| Rm8630 | *pho-1 bacA386*::Tn*phoA degP41* 41ΩTn*5-233* | This work |
| Rm8631 | *pho-1 degP31* 41ΩTn*5-233* | This work |
| Rm8632 | *pho-1 degP32* 41ΩTn*5-233* | This work |
| Rm8633 | *pho-1 degP41* 41ΩTn*5-233* | This work |
| Rm8640 | *pho-1 bacA386*::Tn*phoA-233* | This work |
| Rm8647 | *pho-1 bacA386*::Tn*phoA-233 degP31* | This work |
| Rm8642 | *pho-1* Δ*degP65*::Tn*3*HoKm | This work |
| Rm8643 | *pho-1* Δ*degP65*::Tn*3*HoKm *bacA386*::Tn*phoA-233* | This work |
| Rm8648 | *pho-1 degP19*::Tn*5 bacA386*::Tn*phoA-233* | This work |
| Rm8649 | *pho-1 degP23*::Tn*5 bacA386*::Tn*phoA-233* | This work |
| Rm8650 | *pho-1 fix-381*::Tn*phoA-233* | This work |
| Rm8651 | *pho-1 bacA386*::Tn*phoA-233 degP19*::Tn*5* | This work |
| Rm8652 | *pho-1 fix-381*::Tn*phoA-233 degP19*::Tn*5* | This work |
| Rm8653 | *pho-1 fix-388*::Tn*phoA-233 degP19*::Tn*5* | This work |

[a] All *R. meliloti* strains are derivatives of Rm1021 (45).

triparental matings with plasmid pRK600 to provide transfer functions (35). Plasmid pJG90 was mutagenized with Tn*3*HoKm (22) according to the method of Stachel et al. (51). Plasmid pJG93 was mutagenized with Tn*5* by passage through *E. coli* MT614 (41) and with Tn*phoA* by passage through *E. coli* MT621 (*malF*::Tn*phoA*) (44) into *R. meliloti* Rm8002 by triparental mating with selection for $Nm^r$, $Tc^r$, and $Sm^r$ in the presence of X-P to detect alkaline phosphatase-positive insertions. Plasmid-borne insertions were recombined into the genome by homogenotization with plasmid pPH1JI (35), transduced into strain Rm8002 to remove pPH1JI from the strain background, and confirmed by Southern hybridization. The *bacA386*::Tn*phoA-233* and *fix-381*::Tn*phoA-233* alleles were obtained by replacing the $Nm^r$ cassette of Tn*phoA* in insertions *bacA386*::Tn*phoA* and *fix381*::Tn*phoA* with the $Gm^r$ $Sp^r$ cassette of Tn*5-233* by homologous recombination (10).

**Enzyme assays.** For detection of cytochrome *c* oxidase activity, strains were grown on M9 agar (43) containing 15 mM succinate at 30°C for 6 days. Cells were assayed for cytochrome *c* oxidase activity by applying them to filter paper soaked with a 1% solution of *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) as described previously (6). Cells which appeared blue after 5 to 10 s were scored as cytochrome *c* oxidase positive. TMPD was obtained from Sigma (St. Louis, Mo.). Alkaline phosphatase was assayed as described previously (41).

**DNA manipulations.** The pLAFR1 clone bank of Rm1021 DNA (18) was a gift from F. Ausubel. pJG93 was obtained by subcloning the 7.9-kb *Hin*dIII restriction fragment of pJG90 into *Hin*dIII-digested pRK404 (12). Restriction enzymes, Klenow DNA polymerase, and DNA ligase were from New England Biolabs (Beverly, Mass.) and were used according to the instructions of the supplier. Southern hybridizations were performed on Gene Screen Plus membranes (New England Nuclear, Boston, Mass.) with nick-translated probes made with a nick translation kit (Bethesda Research Laboratories, Gaithersburg, Md.). The DNA sequence of the region shown in Fig. 1 was determined by subcloning appropriate restriction fragments from pJG93 into the Bluescript SK+ vector (Stratagene, LaJolla, Calif.) and dideoxy sequencing with a Sequenase kit (U.S. Biochemical Corp., Cleveland, Ohio).

**Alfalfa nodulation assays.** Seeds of *M. sativa* cv. Iroquois were obtained from Agway, Inc. (Plymouth, Ind.). Plants were grown in Jensen's nitrogen-free medium and inoculated with *R. meliloti* as described previously (35). Cylindrical pink nodules on the roots of healthy green plants were judged to be $Fix^+$. White nodules on the roots of blanched and stunted plants were scored as $Fix^-$.

**Nucleotide sequence accession number.** The DNA sequence described in this work was deposited in GenBank under accession number U31512.

## RESULTS

**Isolation and characterization of MNNG-induced mutations that cause elevated levels of alkaline phosphatase activity in a *R. meliloti bacA386*::Tn*phoA* strain.** In the course of our work on the *bacA* locus, we obtained evidence suggesting that the expression of the *bacA* gene in planta is stronger in the region of the nodule where developing bacteroids are found than it is elsewhere in the nodule (20). It therefore appeared possible that there is some regulation of *bacA* expression over the course of nodule development and that this might be important for effective symbiosis. Consequently, we attempted to identify genes that play a role in *bacA* regulation by searching for mutations that cause altered expression of a *bacA*::Tn*phoA*

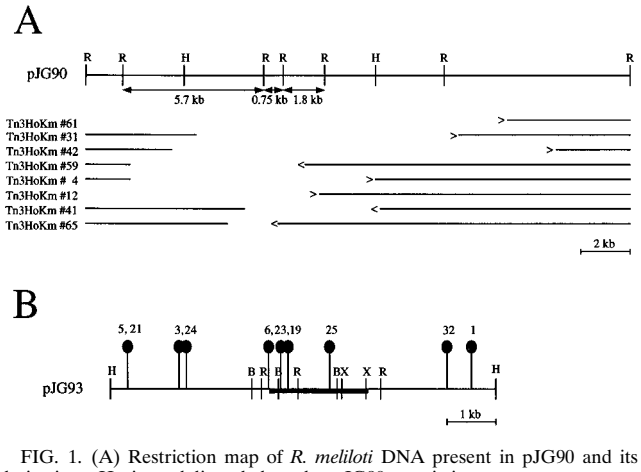


FIG. 1. (A) Restriction map of *R. meliloti* DNA present in pJG90 and its derivatives. Horizontal lines below the pJG90 restriction map represent sequences remaining in Tn*3*HoKm insertion/deletion mutants, and arrowheads indicate the positions of Tn*3*HoKm insertions and the direction of transcription of the *lacZ* gene of Tn*3*HoKm. The 5.7-, 0.75-, and 1.8-kb *Eco*RI fragments discussed in the text are indicated. The positions of *Eco*RI (R), *Bam*HI (B), and *Hin*dIII (H) restriction sites are shown. (B) Restriction map of the 7.9-kb *Hin*dIII fragment that was subcloned from pJG90 to create pJG93. Vertical lines topped by filled circles indicate the positions of Tn*5* insertions in pJG93. The thick horizontal line marks the region that was sequenced. The positions of *Eco*RI, *Bam*HI, *Hin*dIII, and *Xho*I (X) are shown.

fusion. A *bacA386*::Tn*phoA* strain was mutagenized with MNNG, and the mutagenized colonies were screened for those that exhibited a more intense blue color on plates containing the chromogenic alkaline phosphatase substrate X-P. A total of 16,000 colonies from four independent mutagenesis experiments were screened, and three dark-blue colonies were isolated. For reasons discussed below, we will refer to these mutations as *degP31*, *degP32*, and *degP41*. Mutations *degP31* and *degP32* may be identical, because they arose in the same mutagenesis experiment, while mutation *degP41* is probably different, since it arose in an independent experiment.

Several classes of mutations could have caused the increased expression of alkaline phosphatase that we observed in the three mutants we isolated, including (i) *cis*-acting mutations in the *bacA*::Tn*phoA* promoter resulting in increased expression, (ii) mutations that increase the expression of a phosphatase that is not encoded by the *bacA*::Tn*phoA* fusion, and (iii) mutations in genes whose products act in *trans* to increase the level of alkaline phosphatase activity encoded by the *bacA386*::Tn*phoA* fusion. Therefore, we performed a series of genetic experiments to determine the locations and properties of the *degP31*, *degP32*, and *degP41* mutations.

To test for *cis*-acting mutations in the *bacA*::Tn*phoA* promoter, the *bacA386*::Tn*phoA* fusion was transduced from each of the dark blue mutants into the *pho-1* parent strain Rm8002, and the color of the transductants on medium containing X-P was examined. In all cases, the transductants had the same pale blue appearance as colonies of the unmutagenized *bacA386*::Tn*phoA* strain, indicating that mutations *degP31*, *degP32*, and *degP41* were not linked to the *bacA386*::Tn*phoA* fusion and therefore could not be in the *bacA* promoter.

The second possibility, that the phenotype of the mutants resulted from the expression of a phosphatase not encoded by the *bacA*::Tn*phoA* fusion, was eliminated by demonstrating that the dark blue phenotype depended on the presence of the *bacA386*::Tn*phoA* fusion. The *exoH225*::Tn*5-233* mutation is 30% cotransducible with $bacA^+$ (41). As expected, when this insertion was transduced into each of the *degP* mutant strains, 25 to 27% of the transductants were $Nm^s$, indicating that the *bacA386*::Tn*phoA* fusion had been crossed out. All of the $Nm^s$ transductants formed white colonies on plates containing X-P. These observations indicated that the increased levels of alkaline phosphatase observed in the *degP* mutants required the presence of the *bacA*::Tn*phoA* fusion.

Taken together, these experiments strongly suggest that mutations *degP31*, *degP32*, and *degP41* affect one or more *trans*-acting factors that can influence the level of alkaline phosphatase activity in a strain carrying the *bacA386*::Tn*phoA* fusion. In order to facilitate further genetic analyses, we isolated insertions of Tn*5-233* that were cotransducible with the *degP* mutations. This was accomplished by making a transducing lysate from a pool of 5,000 random Tn*5-233* insertion mutants. This collection of Tn*5-233* insertions was then transduced into the *degP* mutants, and the transductants were screened for the pale blue colony phenotype on plates containing X-P. Such a pale blue colony phenotype indicated that the *degP* mutation had been crossed out by the incoming $degP^+$ allele that was cotransduced with a Tn*5-233* insertion. Two such pale blue colonies were identified, which contained insertions referred to as 41ΩTn*5-233* and 31ΩTn*5-233*, respectively, and ϕM12 lysates were prepared from these two derivatives. These lysates were then used to transduce the Tn*5-233* transposon insertions into the *degP* mutants. This resulted in strains containing Tn*5-233* transposon insertions linked to the mutant and wild-type *degP* alleles and allowed us to assess the frequencies at which the transposon insertions and the *degP* alleles were cotransduced. We found that insertions 41ΩTn*5-233* and 31ΩTn*5-233* were 75 and 55% cotransducible with all three *degP* mutations, respectively, demonstrating that the three mutations lie very close to each other and suggesting that they all affect the same genetic locus.

**The MNNG-induced *degP* mutations are recessive.** The frequency with which the *degP* mutations were isolated (1/5,000 at a level of MNNG mutagenesis yielding 1 to 2% auxotrophs) suggested that they are loss-of-function rather than gain-of-function alleles and would thus be recessive to the wild-type $degP^+$ allele. A cosmid clone carrying $degP^+$ was isolated by transferring a genomic library of Rm1021 DNA carried in the broad-host-range cosmid pLAFR1 into a *degP31 bacA386*::Tn*phoA* strain (Rm8628) and screening for pale blue colonies on medium containing X-P. Three different cosmids, pJG80, pJG90, and pJG100, were isolated. The *R. meliloti* DNA contained in pJG90 is shown in Fig. 1. Restriction analysis showed that these cosmids contained overlapping inserts and that two adjacent *Eco*RI fragments (1.8 and 0.75 kb) were common to all three cosmids. Introduction of any of these cosmids into *degP31 bacA386*::Tn*phoA*, *degP32 bacA386*::Tn*phoA*, or *degP41 bacA386*::Tn*phoA* strains restored the pale-blue colony phenotype, suggesting that *degP31*, *degP32*, and *degP41* were all recessive alleles of a gene contained in the 1.8- and 0.75-kb *Eco*RI fragments present in all three cosmids.

***degP* mutants induce effective nodules.** As described above, the chimeric protein encoded by the *bacA386*::Tn*phoA* fusion is expressed in nodules with the highest levels of alkaline phosphatase activity being in the early symbiotic zone, an observation which suggested that *bacA* is subject to regulation (20). Since *degP* mutations altered expression of the *bacA386*::Tn*phoA* fusion, it seemed possible that they would cause abnormal expression of the wild-type *bacA* gene and interfere with nodule development. However, when alfalfa plants were inoculated with $bacA^+$ strains carrying any one of the three *degP* mutations (Rm8631, Rm8632, and Rm8633), $Fix^+$ nodules were induced that were visually indistinguishable from those induced by the $degP^+$ parent strain. This observation indicated that *degP* function is not required for the development of effective nodules.

**A locus adjacent to *degP* is required for nitrogen fixation.** We attempted to obtain an insertion allele of *degP* by mutagenizing pJG90 with the transposon Tn*3*HoKm. This was done by transforming pJG90 into an *E. coli* strain containing the transposon and then transferring pJG90 from 100 independent transformants into a *degP31 bacA386*::Tn*phoA-233* strain (Rm8647), with simultaneous selection for the drug markers on the cosmid and the transposon. The transconjugants were screened for those that were dark blue on X-P medium, indicating that they contained a mutagenized cosmid that failed to complement the *degP31* mutation. Eight such colonies were found. Curiously, all eight of the cosmids isolated from these colonies contained deletions, with insertions of Tn*3*HoKm at one of the deletion endpoints (Fig. 1). All of the deletions removed the *Eco*RI sites between the 1.8- and 5.7-kb *Eco*RI fragments, but they did not have common endpoints. The positions of 16 Tn*3*HoKm insertions in pJG90 that did not abolish *degP* complementation were determined (data not shown). None of these insertions were associated with deletions.

We attempted to introduce the pJG90 insertion/deletion mutations into the *R. meliloti* genome by recombination but were only successful in the case of the smallest insertion/deletion, 65 (Fig. 1). When the 65 mutation was transduced into the *bacA386*::Tn*phoA-233* strain (forming Rm8643), it caused increased expression of the *bacA* fusion (Table 2). Consequently, we named this mutation Δ*degP65*::Tn*3*HoKm. In contrast to

TABLE 2. Alkaline phosphatase activities of *bacA386*::Tn*phoA* strains carrying *degP* mutations

| Strain | Genotype | Alkaline phosphatase activity[a] |
|---|---|---|
| Rm8002 | *pho-1* | 5 |
| Rm8386 | *pho-1 bacA386*::Tn*phoA* | 35 |
| Rm8640 | *pho-1 bacA386*::Tn*phoA-233* | 35 |
| Rm8628 | *pho-1 bacA386*::Tn*phoA degP31* | 103 |
| Rm8629 | *pho-1 bacA386*::Tn*phoA degP32* | 58 |
| Rm8630 | *pho-1 bacA386*::Tn*phoA degP41* | 93 |
| Rm8643 | *pho-1 bacA386*::Tn*phoA-233* Δ*degP65*::Tn*3*HoKm | 94 |

[a] Activity is expressed in arbitrary units defined as 1 U = (optical density at 420 nm/optical density at 600 nm) · $min^{-1}$ · $ml^{-1}$.

the effective nodules induced by the other *degP* mutants, the nodules induced by the Δ*degP65*::Tn*3*HoKm mutant (Rm8642) were cylindrical, white, and did not fix nitrogen. Since none of the other *degP* mutants induced ineffective nodules, the most likely explanation for this result was that the $Fix^-$ phenotype of the Δ*degP65*::Tn*3*HoKm mutant resulted from deletion of sequences encoding a locus other than *degP*.

**Multiple copies of a gene adjacent to *degP* are lethal in a $DegP^-$ background.** We hypothesized that the reason all of the Tn*3*HoKm insertions in pJG90 that interrupted *degP* were associated with deletions of adjacent sequences was that multiple copies of the adjacent gene are lethal in a $DegP^-$ background. To test this idea, we generated Tn*5* insertions in the pJG90 subclone, pJG93 (Fig. 1), in the $degP^+$ strain Rm8002. Tn*5* insertions 6, 23, 19, 25, and 32 (Fig. 1) were recombined into the Rm8002 genome and then transduced into the *bacA386*::Tn*phoA-233* strain. Strains carrying insertion 23 or 19, but not strains carrying insertion 6, 25, or 32, had a dark blue phenotype on X-P plates, indicating that insertions 19 and 23 lay within the *degP* gene. These *degP* alleles were named *degP19*::Tn*5* and *degP23*::Tn*5*, respectively. We then attempted to transfer pJG93 derivatives carrying Tn*5* insertion 6, 23, 19, 25, or 32 into a *degP31* strain (Rm8631) by triparental mating. For pJG93 derivatives carrying insertion 6, 25, or 32, many transconjugants were obtained, but no transconjugants were obtained for pJG93 derivatives carrying *degP19*::Tn*5* or *degP23*::Tn*5*. These results show that sequences on pJG93 are lethal in a $DegP^-$ strain background. The fact that pJG90 containing the Δ*degP65*::Tn*3*HoKm deletion can be introduced into a $DegP^-$ strain background indicates that the sequences that are lethal when present in multiple copies in $DegP^-$ background are removed by the Δ*degP65*::Tn*3*HoKm deletion. The relatively small size of this deletion made it seem likely that the gene responsible for the lethality is the same as the one that is required for effective symbiosis.

**The *R. meliloti* gene that affects the level of alkaline phosphatase activity in *bacA386*::Tn*phoA* strains is a homolog of the *E. coli degP* gene.** To determine the DNA sequence of the *degP* gene, which is defined by the Tn*5* insertions 19 and 23 (Fig. 1), we sequenced the 1,974 nucleotides between the position of Tn*5* insertion 6 in pJG90 and the *Xho*I restriction site indicated in Fig. 1. The GenBank accession number is U31512. This sequence contained an open reading frame of 434 amino acids. The *degP*::Tn*5* insertions 23 and 19 were located at the 3rd and 50th codons of this open reading frame, respectively, so we concluded that it is the *degP* gene. Tn*5* insertion 25, which does not cause a $DegP^-$ phenotype, lay in codon 319, suggesting that the resulting fusion protein contains enough of the DegP protein to confer a $DegP^+$ phenotype.

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RmDegP  1 ..MSTIKAPRPSLKTVLKTTTVAGLAAVPVTTGLPAEVAQSFAEAV.RVQ  47
EcDegP  1 MKKTTLALSRLALSLGLALSPLSATAA.ETSSATTAQQMPSLAPMLEKVM  49

         48 APAVAVSPMWSTPFRRRSSPSACRHVNASATMKATSPSISAAAGSRTCRK  97
         50 PSVVSINVEGSTTVNTPRMPRNFQQFFGDD.......SPFCQEGSPFQSS  92

         98 TIRCGVSSANSLRVKMTVPIVGATAAVRVAKVVSVRGRKAPASSSPKTVT 147
         93 PFCQGGQGGNGGGQQQKFMALGSGVIIDADKGYVVTNNHVVDN....... 135

        148 SSPTTTSSPTARTFTVIMNDGTELDAKLVGKDSRTDLAVLKVDDKRKFTY 197
        136 ..........ATVIKVQLSDGRKFDAKMVGKDPRSDIALIQIQNPKNLTA 175

        198 VSFADDEKVRVGDWVVAVGNPFGLGGTVTAGIISARGR.DIGSGPYDDYL 246
        176 IKMADSDALRVGDYTVGIGNPFGLGETVTSGIVSALGRSGLNAENYENFI 225

        247 QVDAAVNRGNSGGPTFNLSGEVVGINTAIFSPSGGNVGIAFAIPASVAKD 296
        226 QTDAAINRGNSGGALVNLNGELIGINTAILAPDGGNIGIGFAIPSNMVKN 275

        297 VVDSLIKDGTVSRGWLGVQIQPVTKDIAESLGLSEANGALVVEPQAGSPG 346
        276 LTSQMVEYGQVKRGELGIMGTELNSELAKAMKVDAQRGAFVSQVLPNSSA 325

        347 EKAGIKNGDVVTALNGEPVKDPRDLARRVAALRPGSTAEVTLWRSGKSET 396
        326 AKAGIKAGDVITSLNGKPISSFAALRAQVGTMPVGSKLTLGLLRDGKQVN 375

        397 VNLEIGTLPSDAKEPAPATGEAQPDEGQAGEEALADLGLTVTPSEDGKGV 446
        376 VNLELQQSSQNQVDSSSIFNGIEGAE.............MSNKGKDQGV 411

        447 TIASVDPDSDAGDRGLKEGEKIVSVNNQEVNRQTTFSR*........... 485
        412 VVNNVKTGTPAAQIGLKKGDVIIGANQQAVKNIAELRKVLDSKPSVLALN 461
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FIG. 2. Alignment of *R. meliloti* DegP (RmDegP) and *E. coli* DegP (EcDegP). The two proteins are 32% identical and 52% similar. Lines indicate identical residues, double dots indicate conservative substitutions, and single dots indicate less conservative substitutions. The GNSGG motif including the putative active-site serine is underlined.

Comparison of the deduced amino acid sequence of the *R. meliloti* DegP protein to those contained in various databases by the BLAST algorithm (1) revealed that it was closely related to the *E. coli* DegP protease (also called HtrA) (Fig. 2) (36–38). *R. meliloti* DegP was more closely related (52% identical, 67% similar) to a DegP homolog identified in *Brucella abortus* (50) than it was to the *E. coli* protein. This is to be expected, since *R. meliloti* is more closely related to this bacterium than it is to *E. coli* (9). Like all of the deduced DegP sequences reported to date (28, 37, 50), *R. meliloti* DegP includes a GNSGG motif, which is thought to include the active-site serine of this serine endopeptidase (38). For these reasons, we concluded that the gene we had identified is the *R. meliloti degP* gene.

The *E. coli* DegP protease is known to be a periplasmic endopeptidase that degrades certain alkaline phosphatase fusion proteins generated from Tn*phoA* insertions, (52, 53). To determine whether the *R. meliloti degP* mutations stabilized other alkaline phosphatase fusion proteins in addition to that encoded by the *bacA386*::Tn*phoA* fusion, we compared the alkaline phosphatase activities resulting from three different Tn*phoA* fusions in $degP^+$ and *degP19*::Tn*5* strain backgrounds (Table 3). We found that the *degP19*::Tn*5* mutation only caused increased alkaline phosphatase activity in the strain carrying the *bacA386*::Tn*phoA* insertion.

*E. coli degP* mutants do not grow at elevated temperatures (36, 53). We found that the *R. meliloti degP* mutants did grow at the elevated temperature of 38 to 39°C but formed smaller colonies than $degP^+$ bacteria (data not shown).

TABLE 3. Effect of a *degP* mutation on alkaline phosphatase levels in *R. meliloti* strains carrying various Tn*phoA*-generated fusions

| PhoA fusion | Alkaline phosphatase activity[a] | |
|---|---|---|
| | *degP*$^+$ | *degP19*::Tn*5* |
| *bacA386*::Tn*phoA-233* | 26 | 74 |
| *fix-381*::Tn*phoA-233* | 12 | 17 |
| *fix-388*::Tn*phoA-233* | 12 | 12 |

[a] Activity is expressed in arbitrary units defined as 1 U = (optical density at 420 nm/optical density at 600 nm) · $min^{-1}$ · $ml^{-1}$.

**A locus adjacent to *degP* that is required for symbiosis is involved in the biosynthesis of component(s) of a respiratory chain.** Our recognition that we had identified the *R. meliloti* homolog of the *E. coli* DegP periplasmic protease led us to hypothesize that the locus adjacent to the *R. meliloti degP* gene that was lethal when present in multiple copies in a *degP* strain might encode a membrane-bound or periplasmic protein which is degraded by DegP. Furthermore, as suggested above, it seemed likely that this protein might also be required for the formation of nitrogen-fixing nodules on alfalfa. If this hypothesis were true, we felt it might be possible to isolate active Tn*phoA*-generated fusions to the gene encoding this putative membrane-bound or periplasmic protein (44) and that mutations caused by these Tn*phoA* insertions would result in a symbiotic deficiency. Therefore, we mutagenized pJG93 with Tn*phoA*, transferred the mutagenized plasmids into Rm8002, and plated the cells on medium containing X-P. We then successfully identified strains containing plasmids that carry active alkaline phosphatase fusions by screening for blue colonies. The positions of the Tn*phoA* insertions in these plasmids are shown in Fig. 3. These insertions were recombined into the Rm8002 genome. The resulting strains were tested for the ability to induce effective nodules. A cluster of active Tn*phoA* insertions immediately upstream of *degP* caused a Fix$^-$ phenotype (Fig. 3). It should be noted that the chromosomal region defined by these active Tn*phoA* insertions overlaps the sequence that is removed by the Δ*degP65*::Tn*3*HoKm deletion (Fig. 3). Taken together, these observations indicate that the DNA region upstream of the *degP* gene encodes membrane-bound or periplasmic protein(s) essential for effective symbiosis and that this region is lethal when present in multicopies in a DegP$^-$ strain. Like the Δ*degP65*::Tn*3*HoKm mutation, all of the Tn*phoA* insertions in this class caused slow growth on LB/MC medium.

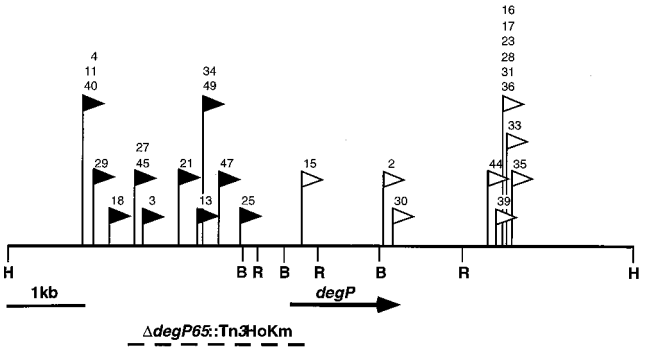


FIG. 3. Positions of Tn*phoA* insertions in the *R. meliloti* sequences contained in pJG93. *Eco*RI (R), *Bam*HI (B), and *Hin*dIII (H) restriction sites are shown, and the position of the *degP* gene is indicated. The approximate range of sequence removed by the Δ*degP65*::Tn*3*HoKm is also shown. Flags point in the direction of transcription of the *phoA* gene. Solid flags indicate insertions that caused a Fix$^-$ and a TMPD-oxidase-negative phenotype. Insertions 15, 2, and 30 were judged to lie within the *degP* gene on the basis of restriction mapping. Furthermore, when they were recombined into the *R. meliloti* genome, they caused slower growth at 38 to 39°C.

In bacteria, most of the protein components of electron transport chains are located in the cytoplasmic membrane or periplasm. Furthermore, some *Bradyrhizobium japonicum* mutants with defects in the electron transport chain form ineffective nodules (40, 46–48, 55). For these reasons, we decided to test the symbiotically defective mutants caused by Tn*phoA* insertions upstream of *degP* for electron transport defects by a cytochrome *c* oxidase test with TMPD as the artificial electron donor. Oxidation of TMPD to a blue compound has been shown to require a membrane-bound, high-potential cytochrome *c* linked to an active cytochrome *c* oxidase (29). Thus, failure to oxidize TMPD indicates the absence of either cytochrome *c*, cytochrome *c* oxidase, or both. All of these Tn*phoA* insertion mutants as well as the Δ*degP65*::Tn*3*HoKm mutant (Rm8642) failed to oxidize TMPD. In contrast, mutants carrying any of the other Tn*phoA* insertions shown in Fig. 3, which did not cause a symbiotic deficiency, oxidized TMPD in the oxidase test. These observations suggest that the Tn*phoA* insertions immediately upstream of *degP*, which result in a symbiotic deficiency and a failure to oxidize TMPD, define a new locus required for the synthesis of *c*-type cytochrome or cytochrome *c* oxidase. For purposes of discussion in this paper, we will refer to this gene as a *cyc* gene. However, we will not assign a letter designation at this point because further work will be required to determine whether this gene corresponds to a particular *cyc* gene that has already been identified in another organism or whether it represents a new class of *cyc* gene.

**pJG90 also complements *fix-382*::Tn*phoA*.** In the course of a chromosomal mapping project (21), we found that the *degP* locus and the *fix-382*::Tn*phoA* mutation described by Long et al. (41) were located very close to each other on the *R. meliloti* chromosome. An *R. meliloti* mutant carrying the *fix-382*::Tn*phoA* allele elicited the formation of white cylindrical nodules similar to those elicited by the *R. meliloti cyc* mutants discussed above. This result led us to wonder whether the *fix-382*::Tn*phoA* mutation was an allele of the *cyc* gene discussed above. To test this idea, genomic DNA from the *fix-382*::Tn*phoA* strain was probed with pJG90 in a Southern hybridization experiment. This showed that the *fix-382*::Tn*phoA* insertion lay in the 5.7-kb *Eco*RI fragment of pJG90 (data not shown). We then tested pJG90 and pJG90 carrying the Δ*degP65*::Tn*3*HoKm deletion for the ability to complement the Fix$^-$ phenotype of the *fix-382*::Tn*phoA* mutant in plant nodulation assays. Both pJG90 and pJG90 carrying the Δ*degP65*::Tn*3*HoKm deletion enabled the *fix-382*::Tn*phoA* mutant to form effective nodules. Furthermore, unlike the mutants deficient in *cyc* function, the *fix-382*::Tn*phoA* mutant was able to oxidize TMPD. These results indicate that the *fix-382*::Tn*phoA* mutation defines a gene different from the *cyc* gene. Thus, in addition to *degP*, the *R. meliloti* DNA carried by pJG90 includes two genes required for nitrogen fixation, the *cyc* gene and the gene defined by the *fix-382* mutation.

## DISCUSSION

All three of the mutations that were identified in a genetic screen for mutations that increased the level of alkaline phosphatase activity in a strain carrying the *bacA386*::Tn*phoA* fusion were found to be alleles of a single gene. On the basis of DNA sequence homology, we concluded that this gene is the *R. meliloti* homolog of the *E. coli degP* gene. *degP* was first identified in *E. coli*, on the basis of the observation that *degP*

mutations stabilized certain alkaline phosphatase fusion proteins synthesized as a consequence of Tn*phoA* insertions (52). It is apparent in retrospect that we inadvertently isolated *R. meliloti degP* mutations in a similar manner. Some of the Tn*phoA* insertions in pJG93 that yielded active alkaline phosphatase were judged to lie in *degP* on the basis of restriction mapping, consistent with the suggestion that the *R. meliloti* DegP protein is located in the periplasm. *E. coli degP*::Tn*phoA* insertions similarly encode active alkaline phosphatase fusion proteins (53). In light of these findings, the most probable explanation for the effect of *degP* mutations in increasing the alkaline phosphatase activity in *bacA386*::Tn*phoA R. meliloti* cells is simply that they reduce proteolytic degradation of the chimeric alkaline phosphatase protein encoded by this fusion. A *degP* mutation had no effect on the levels of alkaline phosphatase activity in strains carrying two other Tn*phoA*-generated fusions. This result is consistent with the results of Lipinska et al. (38), who found that *E. coli* DegP endopeptidase exhibits strong substrate specificity.

In the intracellular pathogens of mammals *Salmonella typhimurium* and *B. abortus*, *degP* mutations cause reduced virulence (3, 14, 28). This may be a consequence of the increased sensitivity to oxidative stress exhibited by these mutants (14, 28). In *R. meliloti*, an intracellular symbiont of plants, *degP* is not required for symbiosis. In *E. coli* and *B. abortus*, *degP* is required for growth at elevated temperatures (14, 36, 53). We found that *R. meliloti degP* mutants could grow at elevated temperatures but formed smaller colonies than the *degP*$^+$ parent.

We found that the *cyc* gene upstream of *degP* was lethal when present on a multicopy plasmid in a DegP$^-$ background. Analysis of the *cyc* locus demonstrated that it encoded a membrane-bound or periplasmic protein. Presumably, overexpression of the *cyc* gene causes accumulation of excessive or poorly folded protein in the cytoplasmic membrane, which requires DegP for its removal. Mutations in the *cyc* gene caused a cytochrome *c* oxidase-negative phenotype, suggesting that the *cyc* mutants we isolated are defective in the biosynthesis of either cytochrome *c* oxidase or *c*-type cytochrome. It is possible that the *cyc* gene we have described encodes one of these components of the respiratory chain. The finding that active Tn*phoA* fusions to the *cyc* gene could be generated is consistent with this possibility. Alternatively, the *cyc* gene may encode a protein required for biogenesis of either of the respiratory chain components.

In addition to the defect in electron transport, mutants defective in the *cyc* function showed a Fix$^-$ phenotype. It is most likely that at least one branch of the electron transport chain blocked by the *cyc* defect is essential for symbiosis, although we cannot exclude the possibility that the *cyc* gene we have identified is also involved in some cellular process other than electron transport and that the cellular process is essential for symbiosis. Electron transport in *Rhizobium* spp. has been studied most intensely with *B. japonicum*. *B. japonicum* has been shown to have a branched respiratory chain terminated by oxidases with different affinities for $O_2$ (25). To determine which branch of the respiratory chain is functional in endosymbiotic bacteroids and is essential for symbiosis, many mutants of *B. japonicum* defective in different branches of the respiratory chain have been isolated and examined for their Fix phenotype. On the basis of the Fix$^-$ phenotype caused by their mutations, several genes are now hypothesized to encode or be involved in the biosynthesis of components of one branch of the respiratory chain that is essential for symbiosis. These genes include *fbcFH* (structural gene for the cytochrome $bc_1$ complex) (55); *cycHJKL*, *cycV*, *cycW*, and *cycX* (genes involved in the biogenesis of *c*-type cytochromes) (47–49, 54); and *fixNOQP* (structural genes for oxidase complex containing a diheme cytochrome) (46). Also, the *tlpA* gene, which encodes a membrane-anchored thioredoxin-like protein involved in the biogenesis of cytochrome $aa_3$, was found to be essential for symbiosis, although the absence of cytochrome $aa_3$ alone cannot account for the Fix$^-$ phenotype of the *tlpA* mutants (40). On the other hand, little is known about the electron transport system of *R. meliloti* that is functional in endosymbiotic bacteroids. So far, the *fixNOQP* operon, which is speculated to encode components of the respiratory chain (30), and the *cycHJKL* gene cluster, which has recently been identified as a gene cluster required for the biogenesis of all cellular *c*-type cytochromes (33), are the only respiration-related genes that have been found to be essential for effective symbiosis. Several lines of evidence suggest that the *cyc* gene we have identified is identical to, or part of, the *cycHJKL* gene cluster. First, the DNA sequence of the region immediately upstream of the *degP* gene that we have determined was found to be almost identical (96% identical along a segment with a length of 164 bp) to the reported sequence of the region downstream of the *cycHJKL* gene cluster found in a different strain of *R. meliloti* (GenBank/EMBL accession number X82560 [33]). Second, like the *cyc* mutant we have described in this paper, mutants carrying a defect in one of the *cycHJKL* genes exhibited a cytochrome *c* oxidase-negative phenotype as well as a Fix$^-$ phenotype. Finally, on the basis of DNA sequences, the *cycHJKL* genes have been predicted to encode membrane-bound proteins, which is consistent with our finding that the active Tn*phoA* fusions to the *cyc* gene we have described could be generated. Although our restriction map of the *cyc-degP* region of *R. meliloti* Rm1021 (Fig. 1) is not identical to that of the *cycHJKL* region of *R. meliloti* 41 (33), this is likely to be a consequence of restriction fragment length polymorphisms that exist in the two different *R. meliloti* strains. The genes in the *cycHJKL* gene cluster have been postulated to encode a multisubunit cytochrome *c* lyase that is essential for the biogenesis of all cellular *c*-type cytochromes (33). At any rate, more work is needed to clarify the function of the *cyc* gene we have described and its role in symbiosis involving *R. meliloti* and alfalfa.

The *fix-382*::Tn*phoA* insertion mutation (41) was also shown to be present in the vicinity of the *cyc* gene and *degP*. This mutation was also shown to affect a gene different from the *cyc* gene on the basis of observations that it does not cause the cytochrome *c* oxidase-negative phenotype and that pJG90 carrying a deletion in the *cyc* gene complements its symbiotic defect. The function in symbiosis of the gene defined by *fix-382* remains to be determined. The proximity of this gene to the *cyc* gene raises the possibility that other genes involved in symbiosis might be located nearby on the *R. meliloti* chromosome. Clearly, this is a region that warrants further study.

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