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Symbiotic Loci of *Rhizobium meliloti* Identified by Random Tn*phoA* Mutagenesis

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We have developed a system for using Tn*phoA* (Tn*phoA* is Tn*5* IS*50*$_L$::*phoA*), which generates fusions to alkaline phosphatase (C. Manoil and J. Beckwith, Proc. Natl. Acad. Sci. USA 82:8129–8133, 1985), in *Rhizobium meliloti*. Active fusions expressing alkaline phosphatase can arise only when this transposon inserts in genes encoding secreted or membrane-spanning proteins. By confining our screening to 1,250 Tn*phoA*-generated mutants of *R. meliloti* that expressed alkaline phosphatase, we efficiently identified 25 symbiotically defective mutants, all of which formed ineffective (Fix$^-$) nodules on alfalfa. Thirteen of the mutants were unable to synthesize an acidic exopolysaccharide (*exo*::Tn*phoA*) that is required for nodule invasion. Twelve of the mutations created blocked at later stages of nodule development (*fix*::Tn*phoA*) and were assigned to nine symbiotic loci. One of these appeared to be a previously undescribed locus located on the pRmeSU47a megaplasmid and to encode a membrane protein. Two others were located on the pRmeSU47b megaplasmid: one was a new locus which was induced by luteolin and encoded a membrane protein, and the other was *dctA*, the structural gene for dicarboxylic acid transport. The remaining six loci were located on the *R. meliloti* chromosome. One of these was inducible by luteolin and encoded a membrane protein which determined lipopolysaccharide structure. Three additional chromosomal loci also appeared to encode membrane proteins necessary for symbiosis. The remaining two chromosomal loci encoded periplasmic proteins required for symbiosis.

Nodulation of leguminous plants by rhizobia requires a complex series of cooperative interactions between the rhizobia and the plant. Both organisms induce specific genes in response to signals from the other and undergo ordered developmental changes during establishment of the symbiotic relationship. The development of sophisticated genetic tools for certain rhizobia, such as *Rhizobium meliloti*, which nodulates alfalfa, has made possible the identification of certain bacterial genes required for symbiosis (9, 20, 30, 40).

Two major types of approaches have been used for the primary identification of *R. meliloti* mutants that have defects in their ability to form effective (nitrogen-fixing) nodules. The first has been to randomly mutagenize the rhizobia and then to carry out a brute-force screen in which cultures of individual members of the mutagenized population are used to inoculate alfalfa seedlings before their symbiotic phenotype is determined (3, 18, 36). Although general and therefore powerful, this approach has the disadvantage of being tedious and inefficient. For example, in a screen of a population of *R. meliloti* derivatives containing random Tn*5* insertions, Meade et al. (35) found that only 1 of 300 strains had a defective symbiotic phenotype. In the second approach, some relatively convenient phenotype is used for primary identification of mutants with symbiotic defects. For example, we used the fact that *R. meliloti* synthesizes a Calcofluor-binding exopolysaccharide to identify symbiosis-defective mutants that either fail to synthesize the exopolysaccharide (29) or synthesize an altered exopolysaccharide (28). Although more facile and convenient, this second approach is restricted to certain classes of symbiotic mutants.

Both because of the labor involved in primary identification of mutants with symbiotic deficiencies and because of a propensity for certain genes with related function to cluster in the bacterial genome, many studies have focused on fine-structure mapping of regions of DNA containing genes known to be required for symbiosis. Usually this fine-structure mapping involves the isolation of cosmid clones that complement the mutation of interest, saturation mutagenesis of the flanking DNA with transposons, and subsequent homogenotization of these transposon insertions into the bacterial genome. By this means, clusters of genes affecting nodulation and host range (*nod*) (21, 25), nitrogen fixation (*nif*) (44), nodule development (*fix*) (5, 11, 39), and exopolysaccharide synthesis (*exo*) (31a) have been identified. Although important, this type of approach does not lead to the discovery of new unlinked genes required for nodulation.

In order to better understand the molecular events underlying nodulation, we were interested in identifying additional genes required for symbiosis. We therefore decided to use the transposon Tn*5* derivative Tn*phoA* (Tn*5* IS*50*$_L$::*phoA*) (32), which can be used to create fusions between target genes and *phoA*, the gene for *Escherichia coli* alkaline phosphatase. Such in-frame gene fusions encode hybrid proteins composed of an amino-terminal portion of a target gene product fused to a carboxyl-terminal portion of alkaline phosphatase. Since the alkaline phosphatase encoded by the *phoA* gene of Tn*phoA* lacks the amino-terminal sequences necessary for its own secretion and since the cytoplasmic precursor of alkaline phosphatase has no enzyme activity, active fusions arise only when the target gene codes for a secreted or membrane-spanning protein. It seems likely that the components of the surface of rhizobia are important for the recognition of their hosts and the subsequent development of nitrogen-fixing nodules. By confining our search for symbiotic deficiencies to *R. meliloti* Tn*phoA* insertion mutants expressing alkaline phosphatase, we hoped to identify new bacterial genes required for the critical bacteria-plant interactions necessary for symbiosis.

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
E. coli		
MM294A	*pro-82 thi-1 endA1 hsdR17 supE44*	G. Walker
SM529	MC1000 *phoR* F⁻ *araD* Δ*ara leu* Δ*lac galE galK phoR* Sm^r	J. Beckwith
R. meliloti		
Su47	Wild type	48
Rm5000	Rif^r derivative of Su47	T. Finan
Rm1021	Sm^r derivative of Su47	F. Ausubel
Rm8002	*pho* derivative of Rm1021	This work
Rm6879	Rm5000 *recA*::Tn*5*-Tp	S. Klein
Rm5321	Rm1021 pRmeSu47a::Tn*5-11*	15
Rm5300	Rm1021 pRmeSu47b *thi-502*::Tn*5-11*	15
Rm7225	Rm1021 *exoH*::Tn*5-233*	29
Rm5810	Rm1021 *ndvB*::Tn*5-233*	M. Williams
Plasmids		
pRK600	Cm^r pRK2013 Nm::Tn*9*	15
pRK609	pRK600 ΩTn*phoA*	This work
pT8	Tc^r pLAFR1 cosmid containing *thi* and *dct* loci of Rm1021	15
pPN101	Tc^r pLAFR1 cosmid containing *dct* loci of *R. leguminosarum*	41
pPN108	Tc^r pLAFR1 derivative of pPN101	41
pPN103	Tc^r pSUP104 derivative of pPN101	41
pPN104	Tc^r pSUP104 derivative of pPN101	41
pRMSL26	*nodC-lacZ*	37

MATERIALS AND METHODS

Bacteria, phage, and plasmids. Bacterial strains and plasmids are listed in Table 1. λTn*phoA*-1 was obtained from C. Manoil. φM12 is a *Rhizobium* transducing phage. φM-1, -5, -6, -7, -9, -10, -11, -12, and -14 were from E. Signer.

Media, growth conditions, and enzyme assays. LB and M9 media have been described (29). The carbon sources were added to M9 at a concentration of 0.2% (wt/vol). Unless otherwise indicated, antibiotics were added at the following concentrations: neomycin, 200 μg/ml; gentamicin, 25 μg/ml; rifampin, 75 μg/ml; spectinomycin, 50 μg/ml; streptomycin, 500 μg/ml; kanamycin, 30 μg/ml; chloramphenicol, 25 μg/ml; tetracycline, 10 μg/ml; and trimethoprim, 1,000 μg/ml. Calcofluor White M2R (Cellufluor; Polyscience, Warrington, Pa.) (29) and 5-bromo-4-chloro-3-indolyl phosphate (XP) (Sigma) were added to media at concentrations of 200 and 40 μg/ml, respectively. The induction of *lacZ* and *phoA* fusions by luteolin (K + K Laboratories, Plainview, N.Y.) was performed under the conditions described by Mulligan and Long (37). Alkaline phosphatase assays were performed as described by Brickman and Beckwith (4) with *p*-nitrophenyl phosphate (Sigma) except that assay volumes were scaled down by one-half and after the assays were terminated, cells were pelleted in a microcentrifuge and the OD_{420} was measured in cell-free supernatants, where the OD_{550} was assumed to be zero. Assays were done on cultures grown in LB medium except in the induction experiments with luteolin, when M9 succinate was used. β-Galactosidase assays were performed as described before (37).

Chemical mutagenesis. To obtain a *pho* mutant of *R. meliloti* Rm1021, exponentially growing cells were exposed to 2.5% (vol/vol) ethyl methanesulfonate in M9 medium at 30°C for 1 to 2 h. The mutagenized cells were then washed, resuspended in fresh medium, and allowed to grow for 2 h. Alkaline phosphatase mutants were identified by diluting and plating the mutagenized cells on LB agar containing XP.

Genetic manipulations. To select for the transposition of Tn*phoA* onto pRK600, λTn*phoA*-1 was used to infect *Escherichia coli* MM294A carrying the plasmid. The culture was plated onto LB agar containing chloramphenicol and kanamycin (300 μg/ml). The high concentration of kanamycin favored cells carrying insertions of Tn*phoA* on the multicopy plasmid. Plasmid DNA was prepared from pools of colonies which had been grown in this medium and was used to transform *E. coli* MM294A recipient cells. Transformants were selected on medium containing chloramphenicol and kanamycin (30 μg/ml). Plasmids were analyzed by restriction digests and tested for conjugation and mutagenesis in Rm8002. Mutagenesis with Tn*phoA* with one such plasmid (pRK609) was achieved by conjugal transfer of the transposon-containing plasmid from *E. coli* into Rm8002. Neomycin-resistant transconjugants were selected on LB plates containing XP. One hundred independent conjugations were performed to minimize the isolation of siblings. To identify Tn*phoA* insertions in the megaplasmids, the origin of transfer (*oriT*) of RK2 inserted in pRmeSu47a (Rm5321) or pRmeSu47b (Rm5300) was transduced into each Tn*phoA*-generated mutant strain. The megaplasmids were then conjugally transferred from the mutants into Rm6879 in triparental matings with pRK600, which provides IncP *tra* functions for mobilization of the megaplasmid. Transconjugants (Rif^r Gm^r Sp^r) from these matings were screened for Nm^R and alkaline phosphatase activity to test for the coinheritance of Tn*phoA* with the mobilized megaplasmid. Transduction of genetic markers with φM12 was performed as described previously (29).

Plant nodulation tests. Seedlings of alfalfa were nodulated in tubes on slants of Jensen agar (48). Plants were inoculated with approximately 10^7 bacteria added to each tube in 0.5 ml of water. Nitrogen fixation was determined by plant growth, and acetylene reduction was determined with whole plants at 4 and 5 weeks after inoculation. To determine bacterial content, nodules were sterilized for 2 min in 20% (vol/vol) Chlorox, rinsed well in water and LB medium, and crushed in LB medium containing 0.3 M glucose. Diluted samples were then plated onto LB medium. Nodules crushed in the same way were viewed under the light microscope to determine the presence of bacteroids. Nodules were examined after 2 and 4 weeks.

Phage sensitivity tests. Phage resistance patterns were determined in spot tests (23). Samples (5 μl) of phage lysates were spotted onto lawns of bacteria in soft agar. The absence of a spot of lysis indicated resistance and corresponded to a plating efficiency of $<10^5$. Phage absorption profiles were determined by absorbing phage and cells at 30°C. Samples were removed at 0, 20, 40, and 60 min, and titers of unabsorbed phage were determined on a lawn of wild-type cells.

Southern blotting and DNA hybridization. Chromosomal DNA was isolated from *R. meliloti* by the method of Marmur (34). Restriction enzyme digestions were performed according to the specifications of the supplier (New England Biolabs, Beverly, Mass.). The DNA probe was labeled with ^{32}P by using a nick translation kit (Bethesda Research Laboratories, Gaithersburg, Md.). Southern blotting and hybridization to GeneScreen Plus (New England Nuclear, Boston, Mass.) were performed according to the manufacturer's instructions.

SDS-polyacrylamide gel electrophoresis and immunoblotting. Wild-type and mutant strains were grown in LB broth to an OD_{600} of 1. Cells were collected by centrifugation and lysed in sample buffer, and proteins were separated by

sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis as described before (27). Proteins were blotted onto Immobilon PVDF transfer membranes as described by the suppliers (Millipore Corporation, Bedford, Mass.). The primary antibody was directed against *E. coli* alkaline phosphatase protein. Immunostaining of alkaline phosphatase protein was performed with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) as the detection antibody (46).

Cell fractionation. The methods of de Maagd and Lugtenberg (7) were used for the separation of membrane, periplasmic, and cytoplasmic fractions. The procedure was adapted so that in Rm1021 greater than 80% of the endogenous alkaline phosphatase activity was recovered in the periplasmic fraction and 90% of the β-galactosidase activity was found in the cytoplasmic fraction. Cells were grown in LB broth (35 ml) to an OD_{600} of 0.2 to 0.3 and harvested by centrifugation. The pellet was resuspended in 5 ml of 50 mM Tris (pH 8.0)–20% (wt/vol) sucrose–2 mM EDTA–200 μg of lysozyme and incubated at room temperature for 45 min to release periplasmic proteins. To isolate the cytoplasmic proteins, the remaining cells were resuspended in 50 mM Tris (pH 8.0) and sonicated briefly. Unbroken cells were pelleted, and the supernatants containing cytoplasmic proteins and cell envelopes were diluted and KCl (0.2 M) was added. Cell membranes were separated from the cytoplasmic fraction by ultracentrifugation at 145,000 × *g* for 1 h.

RESULTS

Development of a system for the isolation of Tn*phoA* fusions in *R. meliloti*. Although the alkaline phosphatase activity in the parental strain Rm1021 (Su47) is induced by phosphate starvation, Rm1021 cells express alkaline phosphatase constitutively and colonies on LB agar appear blue in the presence of the chromogenic phosphatase substrate XP. In order to obtain a mutant with reduced levels of endogenous alkaline phosphatase activity so that we could use Tn*phoA* fusions in *R. meliloti*, we mutagenized Rm1021 cells with ethyl methanesulfonate and obtained several mutants that formed white colonies on XP plates. One such mutant, Rm8002, showed a sevenfold reduction in alkaline phosphatase activity (Tables 1 and 2). This level of activity made Rm8002 easily distinguishable from the wild-type strain on XP plates and was a suitable background for the isolation of Tn*phoA* fusions. In LB medium the Pho^- strain grew as well as its wild-type parent, Rm1021. In addition, Rm8002 was able to effectively nodulate alfalfa plants.

To introduce Tn*phoA* into *R. meliloti*, we constructed a derivative (pRK609) of plasmid pRK600, which carried an insertion of Tn*phoA* (Materials and Methods). pRK600 is a hybrid plasmid with the ColE1 replicon, which allows it to replicate in *E. coli* but not in *R. meliloti*. Furthermore, it can be conjugally transferred from *E. coli* to *R. meliloti* because it contains broad-host-range (IncP) *tra* functions. Although the plasmid does not replicate in *R. meliloti*, it persists for a sufficient period of time to allow Tn*phoA* transposition (8). Biparental matings between Rm8002 and the *E. coli* strain MM294A(pRK609) yielded neomycin-resistant (Nm^r) rhizobia at a frequency of 2×10^{-4} to 4×10^{-4} per recipient, which is comparable to the frequency obtained when pRK600 was used for the delivery of Tn*5* (8). Approximately 1% of the Tn*phoA* insertion mutants formed blue colonies on LB medium containing XP. This frequency was similar to that observed when Tn*phoA* inserts into the *E. coli* genome and suggested that, as in *E. coli*, the only mutants that expressed alkaline phosphatase activity were those which generated in-frame fusions between *phoA* and genes encoding secreted or membrane-spanning proteins.

TABLE 2. Determination of alkaline phosphatase activity in Tn*phoA*-generated fusion strains

Strain	Genotype	Alkaline phosphatase activity (U)
Rm1021	Wild type	14.5
Rm8002	Rm1021 *pho*	2.1
Rm8378	Rm8002 *fix-378*::Tn*phoA*	8.4
Rm8379	Rm8002 *fix-379*::Tn*phoA*	93.5
RM8380	Rm8002 *fix-380*::Tn*phoA*	87.1
RM8381	Rm8002 *fix-381*::Tn*phoA*	12.0
Rm8382	Rm8002 *fix-382*::Tn*phoA*	16.5
Rm8383	Rm8002 *fix-383*::Tn*phoA*	4.0
Rm8384	Rm8002 *fix-384*::Tn*phoA*	28.9
Rm8385	Rm8002 *fix-385*::Tn*phoA*	8.1
Rm8386	Rm8002 *fix-386*::Tn*phoA*	27.3
Rm8387	Rm8002 *fix-387*::Tn*phoA*	26.9
Rm8388	Rm8002 *fix-388*::Tn*phoA*	12.2
Rm8389	Rm8002 *fix-389*::Tn*phoA*	4.2
Rm8365	Rm8002 *exoP365*::Tn*phoA*	11.8
Rm8366	Rm8002 *exoP366*::Tn*phoA*	12.1
Rm8367	Rm8002 *exoF367*::Tn*phoA*	21.9
Rm8368	Rm8002 *exoF368*::Tn*phoA*	21.1
Rm8369	Rm8002 *exoF369*::Tn*phoA*	22.1
Rm8370	Rm8002 *exoF370*::Tn*phoA*	24.2
Rm8371	Rm8002 *exoF371*::Tn*phoA*	22.1
Rm8372	Rm8002 *exoF372*::Tn*phoA*	28.6
Rm8373	Rm8002 *exoP373*::Tn*phoA*	12.1
Rm8374	Rm8002 *exoP374*::Tn*phoA*	12.2
Rm8375	Rm8002 *exoF375*::Tn*phoA*	21.1
Rm8376	Rm8002 *exoF376*::Tn*phoA*	24.2
Rm8377	Rm8002 *exoF371*::Tn*phoA*	24.6

Screening Tn*phoA*-generated mutants that express alkaline phosphatase for symbiotic deficiencies. Since it is likely that certain *Rhizobium* genes that encode proteins associated with the membrane and periplasm are involved in symbiotic nitrogen fixation, Tn*phoA* provided a useful method for obtaining symbiotic mutants. Our strategy for identifying *R. meliloti* mutants with symbiotic deficiencies was to mutagenize the Pho^- strain Rm8002 with Tn*phoA*, identify mutants that expressed alkaline phosphatase activity, and then screen for mutants with symbiotic deficiencies. We therefore carried out 100 independent matings to introduce Tn*phoA* into Rm8002 and collected a set of 1,250 Tn*phoA*-generated mutants that expressed alkaline phosphatase activity on LB plates containing XP. To examine the symbiotic phenotypes of the Tn*phoA* fusion strains, we inoculated these in duplicate onto alfalfa seedlings. The appearance of the mutant-infected plants was monitored for 4 to 5 weeks and compared with that of wild-type-infected and uninfected control plants. In this first screen, 38 of the 1,250 strains tested formed ineffective (Fix^-) nodules, as judged by the stunted and yellow appearance of the plants; the remaining, 1,212 strains formed effective (Fix^+) nodules. To establish whether the symbiotic deficiencies of these 38 mutants were due to Tn*phoA* insertions, we first transduced the Tn*phoA* mutations present in these strains into Rm8002. The transductants were then inoculated onto at least 10 alfalfa seedlings each, and their symbiotic phenotypes were examined more stringently. For 25 of the 38 mutants, we found that the symbiotic defect was due to the Tn*phoA* insertion. These mutant strains expressed levels of alkaline phosphatase activity in

TABLE 3. Symbiotic phenotypes of Rm8002 and Tn*phoA*-generated mutants

Strain or allele	Nodule color and shape	Nitrogen fixation (% of wild-type level)	Bacteroid recovery (% of wild-type no.)
Rm8002	Pink, cylindrical	100	100
fix-378::Tn*phoA*	White, cylindrical	<1	19
fix-379::Tn*phoA*	White, cylindrical	<1	16
fix-380::Tn*phoA*	White, cylindrical	<1	20
fix-381::Tn*phoA*	White, cylindrical	10	10
fix-382::Tn*phoA*	White, cylindrical	<1	20
fix-383::Tn*phoA*	White, cylindrical	<1	12
fix-384::Tn*phoA*	White, cylindrical	<1	20
fix-385::Tn*phoA*	White, cylindrical	<1	14
fix-386::Tn*phoA*	White, cylindrical	<1	10
fix-387::Tn*phoA*	White, cylindrical	<1	8
fix-388::Tn*phoA*	White, cylindrical	1	63
fix-389::Tn*phoA*	White, cylindrical	35	37
exoP365::Tn*phoA*	White, round	<1	<1
exoF369::Tn*phoA*	White, round	2	<1

TABLE 4. Localization of Tn*phoA* insertions to megaplasmids by mobilization

Allele	No. of transconjugants			
	pRmeSu47a		pRmeSu47b	
	Gm^r Sp^r	Nm^{ra}	Gm^r Sp^r	Nm^{ra}
fix-378::Tn*phoA*	22	22	57	0
fix-379::Tn*phoA*	5	0	38	0
fix-380::Tn*phoA*	3	0	12	0
fix-381::Tn*phoA*	12	0	21	0
fix-382::Tn*phoA*	31	0	19	0
fix-383::Tn*phoA*	57	0	17	0
fix-384::Tn*phoA*	64	0	25	25
fix-385::Tn*phoA*	80	0	20	0
fix-386::Tn*phoA*	10	0	90	90
fix-387::Tn*phoA*	60	0	75	75
fix-388::Tn*phoA*	48	0	24	0
fix-389::Tn*phoA*	72	0	85	0
exoP365::Tn*phoA*	12	0	18	18
exoP369::Tn*phoA*	23	0	30	30

[a] Transconjugants (Gm^r Sp^r) were tested for Nm^r from Tn*phoA*.

LB medium which were 2- to 45-fold above the background level in Rm8002 (Table 2).

Symbiotic phenotypes of Tn*phoA* strains. The parent strain, Rm8002, formed effective nitrogen-fixing nodules on alfalfa. The nodules were pink and cylindrical, as seen with other wild-type *R. meliloti* strains. In contrast, the nodules induced by the 25 Tn*phoA* fusion strains were white and severely deficient in nitrogen fixation as (i) they had little or no ability to reduce acetylene (Table 3) and (ii) the plants were yellow and stunted. Two nodule shapes were observed. Nodules formed by 12 of the 25 mutants (group I) were initially small and round but eventually became more elongated and after 4 weeks were similar in shape to nodules elicited by wild-type *R. meliloti*. However, the nodules induced by the group I mutants were generally smaller than those induced by the wild type and were often found in clusters. The nodules formed by the remaining 13 mutants (group II) were small and had an abnormal round shape.

Bacteria were isolated from surface-sterilized nodules and tested for genetic markers and alkaline phosphatase activity. We found that for each mutant, the bacteria recovered from crushed nodules were Nm^r and showed alkaline phosphatase activity characteristic of the inoculated strain. We also found that all the mutants were able to grow on minimal glucose medium, demonstrating that the symbiotic defect was not due to an auxotrophic lesion. We recovered up to 3×10^4 bacteria from single nodules induced by the parental strain Rm8002. In general we found that the number of bacteria recovered from nodules induced by group I mutants was 10 to 63% of the number recovered from nodules induced by Rm8002. Thus, it would appear that the group I mutants are not severely affected in the early steps of nodule invasion. However, relative to the effective nodules formed by Rm8002, the ineffective nodules formed by group I mutants contained fewer bacteroids and many more starch granules, as determined by light microscopy, suggesting that the mutants are deficient in later stages of nodule development. In contrast, the number of bacteria recovered from nodules elicited by the group II mutants was less than 1% of the number recovered from wild-type nodules. Furthermore, light-microscope analysis showed that these nodules also appeared to completely lack differentiated bacteroids. This symbiotic phenotype was the same as that of *exo* mutants of *R. meliloti* (29), and all the group II mutants were subsequently shown to fail to synthesize the Calcofluor-binding exopolysaccharide. They all contained insertions of Tn*phoA* in the cluster of *exo* genes located on the second megaplasmid (pRmeSU47b) of *R. meliloti* (31a) and will be described in detail elsewhere (S. Long, T. L. Reuber, and G. C. Walker, manuscript in preparation). In this paper only the representative group II mutants Rm8365 (*exoP365*::Tn*phoA*) and Rm8369 (*exoF369*::Tn*phoA*) were included in the further characterization of the symbiosis-deficient Tn*phoA* strains.

Mapping of Fix^- Tn*phoA* insertions by mobilization of pRmeSu47a and pRmeSu47b. Rm1021 has two endogenous megaplasmids, both of which have genes coding for symbiotic functions. Nodulation (*nod*) and nitrogen fixation (*nif*) genes have been localized to pRmeSu47a (25, 31), while loci involved in exopolysaccharide synthesis (*exo*) are present on pRmeSu47b (15). In order to determine whether any of the group I Tn*phoA* insertions mapped to either megaplasmid, we constructed derivatives of each Tn*phoA* mutant that carried the origin of transfer (*oriT*) of the broad-host-range plasmid RK2 inserted in pRmeSu47a or pRmeSu47b (15). When we provided mobilizing functions by introducing plasmid pRK600, either megaplasmid could be selectively transferred to a *recA* derivative of strain Rm5000. Transconjugants were screened for the acquisition of alkaline phosphatase activity and the Nm^r marker of Tn*phoA* (Table 4). Using such mobilization studies, we mapped the group I mutation *fix-378*::Tn*phoA* to pRmeSu47a and the group I mutations *fix-384*::Tn*phoA*, *fix-386*::Tn*phoA*, and *fix-387*::Tn*phoA* to pRmeSu47b. As mentioned above, the *exoP365*::Tn*phoA* and *exoF369*::Tn*phoA* mutations also mapped to pRmeSu47b. Preliminary mapping studies indicated that *fix-378*::Tn*phoA* was not transductionally linked (0 of 300 transductants) to the *nifH* locus on pRmeSu47a and therefore appears to be different from the *fix* loci clustered around *nifHDK* (11). Another cluster of *fix* genes located on pRmeSu47a approximately 100 kilobases (kb) distant from *nifHDK* has been described (5, 39). This region contains only one locus which, like *fix-378*::Tn*phoA*, is expressed in free-living bacteria. However, in *R. meliloti* 2011 (which is related to Rm1021), the sequences containing this locus are reiterated in the bacterial genome (5); a single Tn*phoA* insertion in this locus therefore might not be expected to result in a symbiotic deficiency. Further detailed mapping will be required to establish whether *fix-378*::Tn*phoA* defines

a new symbiotic locus on pRmeSu47a. We found *fix-386*::TnphoA and *fix-387*::TnphoA to be approximately 30% linked (26 of 100) to *exoH* (28) on pRmeSu47b. Both mutations were isolated from the same mutagenesis and could be siblings of a single insertion. This hypothesis is supported by the identical behavior of the two mutants in the cell fractionation and Western blotting experiments described below. Since no Fix$^-$ loci were previously found with this linkage, these insertions appear to define a new symbiotic locus on pRmeSu47b. The *fix-384*::TnphoA mutation was 80% linked (17 of 21) to *thi-503*::Tn*5-11*, one of two thiamine-biosynthetic loci on pRmeSu47b (15); experiments presented below indicated that this insertion was in the *dctA* gene.

Our results suggest that the remaining eight TnphoA insertions which are linked to neither pRmeSu47a nor pRmeSu47b are chromosomally borne. Southern blot analysis of these mutants with a TnphoA probe showed that the eight chromosomal insertions represented mutations in *Hin*dIII fragments of six different sizes: 1.6 kb (*fix-381*::TnphoA, *fix-385*::TnphoA), 3.5 kb (*fix-379*::TnphoA, *fix-380*::TnphoA), 7.2 kb (*fix-383*::TnphoA), 7.7 kb (*fix-389*::TnphoA), 10.0 kb (*fix-388*::TnphoA), and 11.4 kb (*fix-382*::TnphoA). Two of the chromosomal insertions (*fix-379*::TnphoA and *fix-380*::TnphoA) which showed identical *Hin*dIII digestion patterns were isolated from the same mutagenesis with TnphoA and probably represent siblings. Mutants containing these insertions also showed similar levels of alkaline phosphatase activity (Table 2). The *fix-381*::TnphoA and *fix-385*::TnphoA insertions also appeared to be in the same size *Hin*dIII fragment. In addition, these two insertions mapped to the same size *Eco*RV fragment (7.4 kb). Mutants containing these insertions were isolated from two different mutagenesis experiments. They showed similar levels of alkaline phosphatase activity (Table 2), and as both mutants appeared to contain fusions to periplasmic proteins, it is possible that *fix-381*::TnphoA and *fix-385*::TnphoA are different insertions in the same gene. Thus, it is possible that the eight chromosomal TnphoA insertions we characterized define as many as six different loci. None of the chromosomal insertions were linked by transduction to *ndvB* (0 of 50), a symbiotic locus which affects nodule development (10, 19), nor do they appear to be in the *exoC* or *exoD* genes (29; J. W. Reed and G. C. Walker, unpublished results), since the TnphoA-containing strains exhibited a normal phenotype on plates containing Calcofluor. We do not know whether any of these insertions correspond to any of the *fix* alleles identified on chromosomal regions in a different *R. meliloti* strain, Rm41 (18).

Bacteriophage resistance patterns of TnphoA fusion strains. Some other previously identified symbiotic mutants have been found to have altered spectra of bacteriophage sensitivity. These mutants either lack the wild-type extracellular polysaccharide (*exoB*) (14, 29, 31a) or show changes in a galactose-containing antigen which is probably the phage receptor (47). Phage resistance patterns for the 12 group I *fix*::TnphoA mutants and the two group II *exo*::TnphoA mutants were tested in spot tests (23) with a collection of nine *R. meliloti* bacteriophages (φM1, -1, -5, -6, -7, -9, -10, -11, -12, and -14). Most of the *fix*::TnphoA mutants were sensitive to all of the phages. The *exoP*::TnphoA and *exoF*::TnphoA mutants were also sensitive to all nine phages. However, we observed an altered sensitivity pattern for two of the *fix*::TnphoA mutants. The *fix-378*::TnphoA mutant showed a 10-fold-lower plating efficiency and a reduced rate of phage absorption with φM6. The *fix-389*::TnphoA mutant was resistant to all but two of the phages (φM11 and φM12).

TABLE 5. Cellular location of TnphoA fusion proteins

Strain or allele	Alkaline phosphatase activity[a] (U)	Activity distribution (%)		
		Periplasm	Membrane	Cytoplasm
Rm1021 (wild type)	215	81	6	13
Rm8002	29	70	9	21
fix-378::TnphoA	143	72	21	7
fix-379::TnphoA	1,697	6	89	5
fix-380::TnphoA	1,371	9	90	1
fix-381::TnphoA	114	68	5	27
fix-382::TnphoA	239	20	79	1
fix-383::TnphoA	39	77	3	20
fix-384::TnphoA	1,157	12	78	10
fix-385::TnphoA	82	90	2	8
fix-386::TnphoA	321	20	79	1
fix-387::TnphoA	389	8	90	2
fix-388::TnphoA	111	50	27	23
fix-389::TnphoA	29	28	28	44
exoP365::TnphoA	286	53	44	3
exoF369::TnphoA	739	56	28	16

[a] Values were corrected for endogenous alkaline phosphatase activity present in the Rm8002 background.

Interestingly, the resistance pattern of the latter strain matched the pattern established for a group of symbiotic mutants of Rm1021 which make an altered lipopolysaccharide (LPS) and are also resistant to monoclonal antibodies to the *R. meliloti* surface (24). A complementing clone, pIA, which restores wild-type phage resistance phenotypes to the LPS mutants also complemented the phage resistance pattern of the *fix-389*::TnphoA mutant (24).

Localization of the TnphoA-generated fusion proteins. The location of the different hybrid alkaline phosphatase proteins was examined by cell fractionation (Table 5). As expected, Rm1021 and Rm8002 showed the majority of the endogenous alkaline phosphatase activity in the periplasmic fraction. For three of the *fix*::TnphoA mutants (*fix-381*, *fix-383*, and *fix-385*), the majority of the alkaline phosphatase activity appeared in the periplasmic fraction, with <6% in the membrane fraction (Table 5). These mutants would appear to contain fusions of alkaline phosphatase to periplasmic proteins. For another six *fix*::TnphoA mutants (*fix-379*, *fix-380*, *fix-382*, *fix-384*, *fix-386*, and *fix-387*), the majority of the alkaline phosphatase activity appeared in the membrane fraction, with <20% in the periplasmic fraction. These mutants would appear to contain fusions to proteins located in either the inner or outer membrane. In the case of the remaining *fix*::TnphoA mutants (*fix-378*, *fix-388*, and *fix-389*) and the two *exo* mutants (*exoP365*::TnphoA and *exoF369*::TnphoA), the alkaline phosphatase activity was distributed between the periplasmic and membrane fractions.

Manoil and Beckwith (33) have reported that, in *E. coli*, fusions to integral membrane proteins that have their alkaline phosphatase domains in the periplasm are often degraded to release intact alkaline phosphatase. We separated total cell proteins by SDS-polyacrylamide gel electrophoresis and used a Western blotting procedure to look for proteins that would cross-react with antibodies raised against *E. coli* alkaline phosphatase (Fig. 1). For all of the *R. meliloti* mutants that contained TnphoA-generated fusions, we observed two cross-reacting protein bands: a fragment approximately the size of *E. coli* alkaline phosphatase (47 kilodaltons [kDa]) and a hybrid protein larger than alkaline phosphatase. The ratios of the two bands varied among strains. The appearance of the higher-molecular-weight cross-reacting protein confirmed that hybrid alkaline phos-

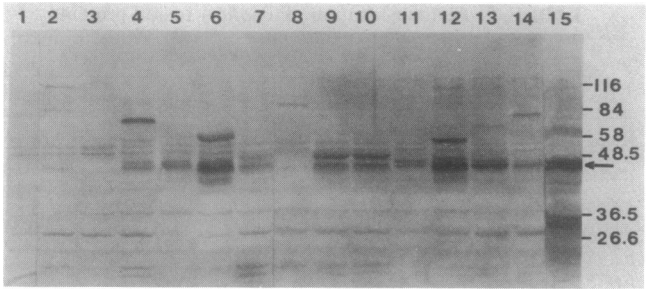

FIG. 1. Western blot of Tn*phoA* fusion proteins with antibodies against *E. coli* alkaline phosphatase as described in Materials and Methods. The approximate sizes of the fusion proteins are shown in parentheses after the allele number for each mutant. Lane 1, Rm8002; lane 2, *fix-388*::Tn*phoA* (109 kDa); lane 3, *fix-383*::Tn*phoA* (90 kDa?); lane 4, *fix-382*::Tn*phoA* (71 kDa); lane 5, *fix-381*::Tn*phoA* (48 kDa); lane 6, *fix-379*::Tn*phoA* (56 kDa); lane 7, *fix-378*::Tn*phoA* (49 kDa); lane 8, *fix-389*::Tn*phoA* (84 kDa); lane 9, *fix-386*::Tn*phoA* (49 kDa); lane 10, *fix-387*::Tn*phoA* (49 kDa); lane 11, *fix-385*::Tn*phoA* (48 kDa); lane 12, *fix-384*::Tn*phoA* (56 kDa); lane 13, *exoF369*::Tn*phoA* (48 kDa); lane 14, *exoP365*::Tn*phoA* (76 kDa); lane 15, *E. coli* SM529. SM529 contains a *phoR* mutation and expresses alkaline phosphatase (arrow) constitutively. Detection of the hybrid alkaline phosphatase produced by the *fix-383*::Tn*phoA* strain was difficult, but on some gels a faint 90-kDa band was visible. Molecular size standards (in kilodaltons) are indicated.

phatase proteins were indeed being produced by these *R. meliloti* strains. However, the appearance of substantial fractions of a ca. 47-kDa cross-reacting band in some of these strains suggests that certain of these hybrid proteins were very susceptible to proteolytic cleavage at or near the junction of the hybrid protein. This phenomenon complicates the interpretation of the cell fractionation experiments described above. However, we tentatively suggest that in the *fix*::Tn*phoA* and *exo*::Tn*phoA* mutants in which alkaline phosphatase activity was distributed across both membrane and periplasmic fractions, the fusion was to a membrane protein but that cleavage of the hybrid protein at or near its junction released alkaline phosphatase to the periplasm.

Cytoplasmic alkaline phosphatase is generally quite unstable (32). Therefore, we were surprised to find a relatively high level of alkaline phosphatase activity in the cytoplasmic fraction of one *fix*::Tn*phoA* mutant (*fix-389*). It is possible that in this case the fusion protein is weakly associated with the membrane and consequently released into the cytoplasmic fraction during sonication, as in the case of a *phoA* fusion to *E. coli* hemolysin (12).

Response of Tn*phoA*-generated fusions to luteolin. Since substituted flavone or flavonone molecules have been identified in root exudates as biologically active components which induce *nod* gene expression (22, 38),.we examined the expression of alkaline phosphatase activity in cultures of group I and group II mutants containing luteolin, the plant-specific inducer of *nodABC* expression in *R. meliloti*. As a control, we used a plasmid-borne *nodC-lacZ* fusion (37) which displayed a 20-fold increase in the level of β-galactosidase activity in the presence of luteolin (Table 6). The majority of the Tn*phoA* fusion strains (for example, *fix-379*, *fix-382*, and *exo-365*) showed no significant induction of alkaline phosphatase activity by luteolin (Table 6). Three fusion strains, *fix-389*, *fix-386*, and its sibling *fix-387*, routinely exhibited a very modest induction of alkaline phosphatase in the presence of luteolin (2.0- and 1.3-fold, respectively). Interestingly, the level of induction of a chromosome-borne *nodC-lacZ* fusion was two- to threefold (37).

TABLE 6. Fusion activity expressed by *nod*::*lacZ* and *fix*::Tn*phoA* strains in response to luteolin

Strain or allele	Mean enzyme activity (U) ± SD	
	Without luteolin	With luteolin
Rm1021(pRm57)	13.8 ± 0.9	273 ± 16.3
Rm8002	1.3 ± 0.2	1.2 ± 0.1
fix-379::Tn*phoA*	70.0 ± 3.8	70.1 ± 1.6
fix-382::Tn*phoA*	6.4 ± 1.2	6.6 ± 0.8
fix-386::Tn*phoA*	13.8 ± 1.2	18.0 ± 0.2
fix-389::Tn*phoA*	2.2 ± 0.6	4.6 ± 1.0
exoP365::Tn*phoA*	13.3 ± 0.5	13.1 ± 0.9

Carbon transport by the Tn*phoA*-generated mutants. We were able to assign one of the group I Tn*phoA*-generated mutations located on pRmeSU47b to a known gene. A number of studies have suggested that the plant-derived carbon compounds which are used by rhizobia for bacteroid development and nitrogen fixation are C_4-dicarboxylic acids (1, 16, 17, 42). Mutants defective in dicarboxylate transport (Dct^-) form ineffective (Fix^-) but structurally normal nodules containing differentiated bacteroids. Our group I Tn*phoA*-generated mutants produced a Fix^- phenotype similar to that of the reported *dct* mutants. In addition, transport mutants with altered membrane or periplasmic components might be expected to be found as active fusions from random Tn*phoA* mutagenesis. We therefore tested the 12 group I mutants for their ability to utilize a number of carbon sources, including the dicarboxylic acids, succinate, and fumarate. We found that all 12 strains were able to utilize glucose, sucrose, maltose, and lactose. Mutants defective in the utilization of these sugars have generally been found to be effective in symbiosis (45). One particular Tn*phoA* mutant, Rm8384 (*fix-384*::Tn*phoA*), failed to grow on minimal medium containing either C_4-dicarboxylic acid as the sole carbon source and therefore was a *dct* mutant.

We were able to complement the *dct* mutation in Rm8384 by using a cosmid, pT8, which contains *R. meliloti dct* genes (15). Furthermore, we were able to characterize the *dct* mutation by using three *Rhizobium leguminosarum dct* plasmids (41). A pLAFR1 derivative (pPN108) which encoded *dct* structural and regulatory functions of *R. leguminosarum* enabled the *R. meliloti* mutant Rm8384 to utilize succinate. We obtained a similar growth phenotype on succinate medium when Rm8384 contained the plasmid pPN104, a pSUP104 derivative carrying only the *R. leguminosarum* structural gene for dicarboxylic acid transport (*dctA*). The *dct* regulatory region of *R. leguminosarum* on the pSUP104-derived plasmid pPN103 did not complement the deficiency of Rm8384 in dicarboxylic acid transport. It therefore seems likely that Rm8384 contains a Tn*phoA* insertion in the structural gene for dicarboxylic acid transport, *dctA*. Together with our mobilization studies (Table 4), these results further demonstrate that the *R. meliloti dctA* gene is located on pRmeSu47b, approximately 80% linked to *thi-503*::Tn*5-11* (15). A 4-kb region of pRmeSu47b containing genes required for dicarboxylic acid transport has been reported (49).

DISCUSSION

In this paper we have described the establishment of a system for the isolation of Tn*phoA* mutations in *R. meliloti*. We used this transposon to isolate a number of different mutants which were affected in symbiosis. Our results

demonstrate that by using Tn*phoA* to identify mutations in genes encoding membrane and periplasmic proteins only, it is possible to enrich for certain mutations which interfere with critical plant-bacterium interactions necessary for symbiosis. By limiting our mutant screen only to those strains which showed alkaline phosphatase activity, we obtained Fix$^-$ mutants at frequency of 2%. This was about fivefold higher than the frequency obtained when *R. meliloti* derivatives containing random Tn*5* insertions were screened for symbiotic deficiencies (18, 35, 36). This Tn*phoA*-based strategy for screening for symbiotically defective mutants combines certain of the elements of convenience associated with approaches based on a second phenotype with certain of the elements of generality associated with brute-force screens and enabled us to identify new classes of symbiotic genes. We therefore feel that this Tn*phoA*-based approach represents a useful alternative to the screening of random mutations.

Of the 25 Tn*phoA*-generated mutations causing symbiotic defects that we obtained, 17 were located on the megaplasmids of *R. meliloti* Rm1021. The *fix-386*::Tn*phoA* and *fix-387*::Tn*phoA* insertions defined a new symbiotic locus on pRmeSU47b, the megaplasmid encoding a cluster of *exo* genes. Expression of this locus was modestly induced (ca. 1.3-fold) by luteolin, the inducer of the *nod* genes, and the product of the locus appeared to be located in the membrane. This is the first locus on pRmeSU47b reported to be inducible by luteolin. The *fix-384*::Tn*phoA* insertion was also located on pRmeSU47b and was in *dctA*, the structural gene for dicarboxylic acid transport. This assignment is consistent with our observation that the hybrid alkaline phosphatase created by the *fix-384*::Tn*phoA* mutant was a membrane protein. In addition, 13 of the 25 symbiotically defective Tn*phoA* insertions were in *exo* genes located on pRme SU47b, and our analyses of the fusions created by the *exo P365*::Tn*phoA* and *exoF369*::Tn*phoA* mutations suggest that ExoP and ExoF are membrane proteins. As discussed above, the *fix-378*::Tn*phoA* mutation may define a new locus on the other megaplasmid, pRmeSU47a, which encodes the *nod* and *nif* genes. The product of this locus seems to be a membrane protein, and its absence interferes with bacteriophage ϕM6 absorption to *R. meliloti*.

The remaining eight Tn*phoA* insertions were located on the chromosome in at least six different *Hin*dIII fragments. One of these (*fix-389*::Tn*phoA*) made the cell resistant to a number of bacteriophage and appeared to be in a locus which determines LPS structure (24). Our analysis of the *fix-389*::Tn*phoA* fusion indicated that expression of the locus was induced by luteolin and that its produce may be a membrane protein. Three other chromosomal loci encoding membrane proteins required for symbiosis were defined by the *fix-379*::Tn*phoA* (and *fix-380*::Tn*phoA*), *fix-382*::Tn*phoA*, and *fix-388*::Tn*phoA* mutations. In addition, three insertions (*fix-381*::Tn*phoA*, *fix-383*::Tn*phoA*, and *fix-385*::Tn*phoA*), defining two chromosomal loci, appeared to encode periplasmic proteins required for symbiosis. These six loci did not correspond to the chromosomally encoded symbiotic loci *ndvB*, *exoC*, or *exoD*, nor did they cause any additional growth requirements in the free-living state. Thus, they may represent five previously unidentified symbiotic loci on the chromosome of *R. meliloti* Rm1021.

We obtained no Nod$^-$ mutants by Tn*phoA* mutagenesis. However, this finding may not be too surprising, since *R. meliloti nod* genes are induced by luteolin (38) and the level of expression of fusions to these genes may have been too low for us to detect in the absence of inducer. Three of the *fix*::Tn*phoA* fusions we did isolate were expressed at higher levels in the presence of luteolin and may be regulated similarly to the *nodABC* genes (38). It would be interesting to determine whether additional classes of symbiotic mutants can be obtained by including luteolin or root exudate in the medium during a primary screen for mutants that express alkaline phosphatase. In addition, the fusions we isolated will prove useful in a variety of other physiological and genetic studies of the possible regulation of these various *fix* loci.

All the symbiotic mutants we obtained were Fix$^-$. The group II mutants (which were all *exo* mutants) induced nodules lacking differentiated bacteria and had deficiencies in the early stages of the nodulation process, as already reported (14, 29). The exact stages of nodule invasion and nodule development at which the various group I mutants are blocked have not yet been determined. These mutants appear to penetrate plant cells and must be at least partially proficient in the early stages of nodulation. The facts that the nodules are white, contain limited numbers of bacteroids, and fail to fix nitrogen indicate that the mutants have defects in later stages of nodulation. In our discussion we have been assuming that it is the loss of function of symbiotically important genes that is responsible for the Fix$^-$ phenotype. However, we have not been able to rule out the formal possibility that it is the presence of abnormal proteins in the membranes or periplasm that blocks symbiotic development, but we consider this the less likely explanation.

The alkaline phosphatase enzyme activity associated with the Tn*phoA* hybrid proteins may provide a useful tool for localizing the fusion proteins in cell fractionation studies. The usefulness of this approach is presently complicated, however, by the fact that certain of the fusion proteins undergo substantial proteolytic cleavage at or near their fusion junction. It might be possible to circumvent this limitation by isolating a mutant that does not carry out this cleavage.

Tn*5* mutagenesis has been used in a considerable number of different gram-negative microorganisms (2, 6, 26), all of which are therefore also potentially amenable to genetic manipulation with Tn*phoA*. Because of its ability to facilitate the identification of mutation in secreted and membrane-spanning proteins, Tn*phoA* should prove generally useful in studying a variety of other bacterium-host interactions.

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