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Cloning and characterization of a *Rhizobium meliloti* nonspecific acid phosphatase

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Abstract Nodulated legumes require high levels of phosphorus for optimal symbiotic performance. However, the basis for this elevated phosphorus requirement is poorly understood, and very little information regarding bacteroid phosphorus metabolism is available. To develop an understanding of the relative importance of organic and inorganic phosphorus sources for bacteroids, we investigated phosphatase activity in *Rhizobium meliloti*. An *R. meliloti* plasmid library clone that complemented an *Escherichia coli* phosphatase mutant was isolated, and the clone was sequenced. The complementing fragment contained a 337-amino-acid open reading frame that has a potential leader sequence and processing sites characteristic of periplasmic proteins. The phosphatase activity was located in the periplasm of *R. meliloti* and of *E. coli* containing the cloned gene. The subunit molecular mass of the cloned phosphatase was 33 kDa, and gel filtration indicated the active enzyme was a 66-kDa homodimer. Lack of substrate specificity suggests the cloned gene, *napD*, encodes a nonspecific acid phosphatase with a pH optimum of approximately 6.5. An *R. meliloti napD* transposon-insertion mutant was constructed, and its symbiotic phenotype was determined to be Fix^+ regardless of the level of phosphorus provided to the host plant.

Key words Phosphorus · *Rhizobium* · Alfalfa · Phosphatase

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Introduction

In the symbiosis between legumes and rhizobia (used here as a colloquial term to refer to all root-nodule-forming bacteria), the host plant provides the rhizobia with reduced carbon as an energy source. The bacteria use this energy to convert atmospheric N_2 to ammonia, which is used by the plant as a nitrogen source. This agriculturally important plant-microbe interaction has been extensively studied, with much of the work focused on aspects of nodule formation and on carbon and nitrogen metabolism during symbiosis. However, the literature contains many reports showing that nodulated legumes respond dramatically to applied phosphorus fertilizer (e.g., Powell 1977; Cassman et al. 1981; Itoh 1987; Pereira and Bliss 1987). It is well-established that crop plants grow better when the phosphorus supply is adequate, but in legumes the amount of phosphorus required changes with the nitrogen source; plants obtaining nitrogen from symbiosis require higher levels of phosphorus for optimal growth than do plants grown with nitrogen fertilizers (Israel 1987).

Phosphorus often becomes the limiting nutrient when there is adequate nitrogen; therefore, the symbiotic potential of an engineered nitrogen-fixing symbiosis may be limited by phosphorus availability or by the ability of the symbiosis to efficiently utilize available phosphorus. The basis for the higher phosphorus requirement in symbiosis is poorly understood. In particular, relatively little information is available concerning the role of the bacteroid in this phosphorus response. Data concerning phosphorus metabolism in *Rhizobium* and in the symbiosis it forms with legumes is primarily limited to studies conducted by Smart et al. (1984a, b), Sa and Israel (1991), and Al-Niemi et al. (1997a, b), in which responses of legumes and rhizobia to phosphorus stress have been studied.

Our goals are to understand the role of bacteroids in the higher phosphorus requirement of symbiotic nitrogen fixation, and to model phosphorus flow between the symbionts. A *Rhizobium meliloti ndvF* mutant originally reported to have defects in nodule formation and N fixation

(Charles et al. 1991) has recently been discovered to be unable to transport inorganic phosphorus (Bardin et al. 1996). However, it is premature to assume that phosphorus is presented to bacteroids as inorganic phosphorus. Phosphorylated carbon compounds are important carriers of phosphorus in plant cells and might cross the symbiosome membrane and then be hydrolyzed by a phosphatase to release phosphate, which is then transported into the bacteroids.

Several enzymes from different organisms that display phosphatase activity with acidic pH optima have been studied in detail. These include a cyclic diesterase (Anraku 1964a, b), a 5′-nucleotidase (Neu 1967), an inositol monophosphatase (Matsuhisa et al. 1995), an acid glucose-1-phosphatase (Pradel and Boquet 1988), and nonspecific acid phosphatases (Thaller et al. 1994, 1995). Acid phosphatase activity has been reported in periplasmic extracts of soybean bacteroids (Kinnback and Werner 1991), there are acid phosphatases and extremely high (and possibly transient) levels of alkaline phosphatase in *Rhizobium tropici* bean bacteroids (Al-Niemi et al. 1997a), and Lucchini et al. (1990) have reported the presence of one acid phosphatase in free-living cells of *R. meliloti* strain R-41. Based on these reports and indications that the symbiosome may be acidic (Mellor 1989; Brewin et al. 1990), our initial studies with *R. meliloti* have focused on the two acid phosphatases that we have observed in strain 104A14. This report summarizes experiments to characterize one of the acid phosphatases and a mutant lacking this enzyme.

Materials and methods

Bacterial strains and plasmids

The phage, plasmids, and strains of *R. meliloti* and *Escherichia coli* used in this study are shown in Table 1. *R. meliloti* was grown on either minimal mannitol-ammonium chloride ($MMNH_4$) or yeast extract mannitol agar (YMA) (Somerville and Kahn 1983), and *E. coli* was cultured on either LB or M9 mineral salts media (Sambrook et al. 1989). Sucrose (5%, w/v), kanamycin (50 μg/ml), ampicillin (100 μg/ml), or gentamicin (25 μg/ml) were included as required. The M9 medium contained glucose as the primary carbon source and was amended to include per liter: L-leucine (20 mg), L-arginine (20 mg), L-ornithine (10 mg), and thiamine (2 mg).

Cloning of the *R. meliloti* acid phosphatase gene and isolation of the mutant

The *R. meliloti* acid phosphatase gene *napD* was cloned by complementing *E. coli* strain CC118, an alkaline phosphatase deletion mutant. Strain CC118 has normal acid phosphatase activities that could be detected when strain CC118 was grown using LB broth or agar. M9 medium was used in all expression experiments because when strain CC118 was grown on this phosphate-buffered defined medium, all phosphatases were repressed and there was no background activity to interfere with detection of the cloned phosphatase. Strain CC118 was transformed with an *R. meliloti* genomic library constructed in pUC18 as previously described (McDermott and Kahn 1992), and transformants were plated onto M9 agar containing 5-bromo-4-chloro-3-indolyl phosphate (X-P, a chromogenic phosphatase substrate) (40 mg/liter). Periplasmic proteins were extracted from cultures of blue colonies and were assayed for phosphatase activity. Extracts were also fractionated by electrophoresis in native gels and were stained for acid phosphatase activity, using strains CC118 and CC118(pUC19) as controls as described below. Several similar library clones coded for a single acid phosphatase. A representative clone referred to as pAcP3 was chosen for further study.

Plasmid pAcP3, which contained 7.2 kb of *R. meliloti* DNA, was linearized by digestion with *Xba*I and was subcloned into pJQ200KS (Quandt and Hynes 1993) to form pSD1. Plasmid pJQ200KS contains the *sacRB* genes that facilitate positive selection for double recombinants. The *napD* gene was mutated using the methods outlined by De Bruijn and Lupski (1984). Briefly, transposon Tn*5*-B20 (Simon et al. 1989), which contains a promoterless *lacZ*, was transduced from phage λ467 into strain CC118(pSD1) to insertionally inactivate *napD* in pSD1. Plasmid

Table 1 Phage, plasmids, and strains of *Rhizobium meliloti* and *Escherichia coli* used in this study (*Amp*^{r} ampicillin resistant, *Gent*^{r} gentamicin resistant, *Kan*^{r} kanamycin resistant, *Suc*^{s} sucrose sensitive)

Phage, plasmid, or strain	Relevant genotype or characteristics	Relevant phenotype	Reference or source
Phage			
λ467	Tn*5*-B20	Kan^r	Simon et al. (1989)
Plasmids			
pUC18		Amp^r	Yanisch-Peron et al. (1985)
pBluescript KS$^+$	Sequencing vector	Amp^r	Stratagene
pJQ200KS	*gent sacB sacR*	Gent^r Suc^s	Quandt and Hynes ((1993)
pACP3	pUC18 with *R. meliloti* DNA encoding *napD*	Amp^r and *R. meliloti* acid phosphatase	This study
pSD1	pJQ200KS with *R. meliloti napD*	Gent^r Suc^s and *R. meliloti* acid phosphatase	This study
pSD1::Tn*5*-B20	Tn*5*-B20 inserted in *napD*	Gent^r Kan^r Suc^s	This study
R. meliloti			
104A14		Wild-type	Somerville and Kahn (1983)
RmMSU1	*napD*::Tn*5*-B20	Kan^r, acid phosphatase mutant	This study
E. coli			
S17-1		Pro$^-$ Mob$^+$	Simon et al. (1983)
CC118	Δ*phoA*	Alkaline phosphatase mutant	Manoil and Beckwith (1985)

preparations from kanamycin-resistant strain CC118(pSD1) transductants were used to transform strain CC118. Possible *napD* mutants were identified as white colonies on M9 agar containing X-P and kanamycin. Inactivation of the cloned phosphatase was verified using acid phosphatase activity staining in native gels, and the transposon insertion site was mapped using restriction digests and DNA sequencing. The mutated allele (pSD1::Tn*5*-B20) was transferred to *R. meliloti* strain 104A14 via conjugation with *E. coli* strain S17-1(pSD1::Tn*5*-B20), and insertion of the mutated allele into *R. meliloti* by recombination was verified with Southern blot analysis (Sambrook et al. 1989). A random primer labeling kit from Promega and α^{32}P-dCTP(DuPont) were used in probe preparation. DNA isolation and manipulation followed standard methods (Sambrook et al. 1989).

DNA sequencing

Restriction analysis and phosphatase complementation were used to identify a 1.9-kb *Xho*I-*Sac*I fragment of pAcP3 that coded for NapD. This fragment was cloned into pBluescript KS$^+$ (Stratagene), and both strands were sequenced either from nested deletions generated using a commercial kit (Erase-A-Base, Promega) or by using custom synthetic oligonucleotide primers. The dideoxy chain-termination nucleotide sequencing method used a kit purchased from United States Biochemical. The transposon insertion site in pSD1::Tn*5*-B20 was determined by sequencing out from the transposase and *lacZ* ends of the transposon using primers 5′-CCATGTTAGGAGGTCACATGGAAGTCAG-3′ and 5′-AACGACGGGATCCATAAT-3′, respectively. Sequence similarity searches were conducted using the BLAST network service (Altschul et al. 1990), and sequence alignments used the GAP program (Devereux et al. 1984). The *napD* nucleotide sequence can be accessed from GenBank (accession no. U74652).

Enzyme purification and characterization

The periplasmic location of NapD was determined by cell fractionation experiments coupled with the assay of marker enzymes. Periplasmic proteins were extracted by washing and resuspending the cells from a 10-ml culture in 250 µl wash buffer [20 mM MES-NaOH, 20% (w/v) sucrose (pH 6.5; all measurements and adjustments of pH in this and other parts of the study were made at room temperature)]. Lysozyme (0.5 mg/ml) was added, and the cells were incubated for 5 min, then EDTA was added to a concentration of 2 mM, and incubation was continued for 15 min at room temperature. The suspension was centrifuged at 12,000 × *g* for 5 min, and the extracted cell pellet was resuspended in 20 mM MES-NaOH (pH 6.5). Pellets and periplasm extract samples were sonicated three times each at 50% power (1/8-inch tip, 550 Sonic Dismembrator; Fisher Scientific) for 30 s each, and were centrifuged at 12,000 × *g* to pellet unbroken cells and debris. The supernatants were centrifuged again at 100,000 × *g* for 1 h to pellet membranes. Phosphatase enzymes (assays described below), which are periplasmic marker enzymes, and the cytosolic marker enzyme malate dehydrogenase were assayed in all fractions. Malate dehydrogenase was measured using the methods described previously (Al-Niemi et al. 1997a) by monitoring the rate of oxidation of NADH (A_{340}) initiated by addition of oxaloacetate to the assay mix, which contained protein extract, 10 mM oxaloacetate (prior to use, pH adjusted to 7.5 with KOH), 1 mM NADH, and 10 mM K_2HPO_4 (assay pH 7.5).

NapD was partially purified from cells of *E. coli* strain CC118(pAcP3) by a combination of ammonium sulfate precipitation and column chromatography. To the periplasmic extract from 20 l of a late-exponential-phase M9 culture, ammonium sulfate was added to 75% saturation, proteins were allowed to precipitate for 1 h, and then the extract was centrifuged at 15,000 × *g* for 20 min. The supernatant was loaded onto a Phenyl Sepharose (Pharmacia) column (2.5 × 11 cm) pre-equilibrated with 20 mM MES-NaOH (pH 6.5) and 500 mM NaCl. The enzyme was then eluted using a 500–250 mM NaCl gradient, followed by a single wash with buffer containing no NaCl. All active fractions were pooled and loaded onto a DEAE-Sepharose CL-6B (Pharmacia) column (2.5 × 11 cm) equilibrated with 20 mM MES-NaOH (pH 6.5). The enzyme eluted as a single peak in a 0–250 mM NaCl gradient.

To obtain an estimate of the subunit molecular mass, a high-specific-activity DEAE-Sepharose eluate fraction was electrophoresed in a native gel, the activity band was cut out, and the protein was electroeluted and then subjected to SDS-PAGE (Laemmli 1970) and silver staining (Oakley et al. 1980) to visualize the protein band. To electroelute the protein, the gel slice was sealed in a minimal length of dialysis tubing (14,000-Da molecular mass cutoff) containing 0.5 ml 20 mM MES-NaOH (pH 6.5), was submerged in the same buffer in a horizontal electrophoresis cell and electrophoresed for 30 min at 20 mA constant current.

The native molecular mass of NapD was determined using gel filtration through a column of Sephacryl S-200. The 90 × 1-cm column was equilibrated with 20 mM MES-NaOH (pH 6.5) and calibrated using the molecular mass standards blue dextran, albumin, aprotinin, carbonic anhydrase, and cytochrome *c* dissolved in equilibration buffer. Elution of each molecular mass standard protein was determined by protein assays, whereas NapD was detected using acid phosphatase enzyme assays. A linear plot of the elution volumes versus $\log_{10}$ molecular mass of the standards (Reiland 1971) was used to estimate the native molecular mass of NapD.

For routine measurement of acid phosphatase activity, 1.0-ml enzyme assays contained 20 mM MES-NaOH buffer (pH 6.5) and 20 mM *p*-nitrophenylphosphate (Sigma 104). Hydrolysis of *p*-nitrophenylphosphate was determined by recording the rate of change in absorbance at 415 nm. Enzyme specific activity was calculated by converting change in absorbance (A_{415}) per minute in a standard 1.0-ml assay to mol substrate hydrolyzed using the *p*-nitrophenol molar extension coefficient 1.36×10^4 l mol^{-1} (Smart et al. 1984a). Assays for alkaline phosphatase were run similarly in 20 mM Tris (pH 8.5). For substrate-specificity assays, *p*-nitrophenylphosphate and other selected substrates (at concentrations of 100 mM each) were incubated with the enzyme in 20 mM MES-NaOH (pH 6.5) for up to 1 h, and then free phosphate was measured using the methods described by Chen et al. (1956). Protein concentrations were determined using the BioRad protein microassay protocol with bovine serum albumin as the standard.

Activity stains in nondenaturing polyacrylamide gels

The nondenaturing polyacrylamide (native) gels contained 10% acrylamide (9.7% acrylamide, 0.3% bisacrylamide) in the resolving phase, and 5% acrylamide (4.85% acrylamide, 0.15% bisacrylamide) in the stacking phase. Gels were made up in 480 mM glycine and 60 mM Tris-HCl (pH 8.3), and the running buffer was 25 mM Tris-HCl and 192 mM glycine (pH 8.5). Cell extracts were mixed 3:1 (extract volume/sample loading dye volume) with a solution containing 0.04% (w/v) xylene cyanol in 40% (v/v) glycerol, loaded into the well, and electrophoresed at 20 mA constant current until the dye front was approximately 2.5 cm from the bottom of the gel. Gels were equilibrated to pH 6.5 with 20 mM MES-NaOH and then stained for acid phosphatase activity with Fast Blue RR and α-naphthylphosphate monosodium salt (0.5 mg ml^{-1} each) in equilibration buffer.

Plant growth and inoculation

Alfalfa seed surface-sterilization and germination, and plant growth conditions were as previously described (Al-Niemi et al. 1997b). Briefly, contamination-free seedlings were transferred to sterile growth box units [Magenta boxes (Sigma); four seedlings per box] and inoculated immediately with washed cells (to remove medium P) of either the wild-type or the mutant strain. The plants were grown in a mixture of sand and phosphorus-loaded alumina

(Gourley et al. 1993) that served to control phosphorus supply to the alfalfa plants and establish conditions such that the symbiotic phenotype of the NapD⁻ mutant could be tested under conditions of adequate and limiting phosphate concentrations. Plants were cultured in a growth chamber for 5 weeks at 25°C with a photoperiod of 16 h. Plants were then harvested, and acetylene reduction, nodule fresh weight, and plant dry weight were determined.

Nodulation and nitrogen fixation

Nitrogen fixation was estimated by acetylene reduction as outlined by Al-Niemi et al. (1997b). Nodules were then removed, counted, and weighed, and plant shoots were dried at 65 °C and weighed. To determine reporter enzyme levels in bacteroids of the NapD⁻ mutant, triplicate nodule samples were crushed in Z-buffer (Miller 1972) amended with 250 mM mannitol but lacking β-mercaptoethanol. The homogenate was centrifuged at $500 \times g$ for 5 min to pellet large debris, and bacteroids were isolated from the supernatant by centrifugation at $3{,}600 \times g$ for 4 min. The bacteroid pellet was resuspended in Z-buffer and assayed for β-galactosidase activity according to Miller (1972).

Transposon stability during symbiosis was assessed by testing nodule isolates for antibiotic resistance and for the presence of the NapD phosphatase. Fresh nodules were surface-sterilized in 70% (v/v) ethanol for 10 s and in 1.5% (w/v) sodium hypochlorite for 1 min, and were then washed six times with sterile distilled water (2-min soak for each wash). The nodules were crushed in 0.85% (w/v) saline solution, serially diluted, and plated onto $MMNH_4$ agar. After 72-h incubation, isolated colonies were replica-plated onto $MMNH_4$ agar with and without 25 µg kanamycin ml^{-1}. Pure culture isolates obtained from nodule homogenates were grown in $MMNH_4$ broth containing 25 µg kanamycin ml^{-1}. Periplasmic proteins were then extracted, separated by electrophoresis in native gels, and stained for acid phosphatase activity.

Results

Cloning and nucleotide sequence analysis of *napD*

Transformation of *E. coli* strain CC118 with an *R. meliloti* genomic library resulted in several dark-blue colonies on M9 X-P agar containing ampicillin. Analysis of the encoded phosphatase activity in native gels and of restriction digests of the different cloned fragments suggested that a single enzyme had been cloned in these experiments. Strain CC118 bearing plasmid pAcP3 was selected for further work. Additional subcloning experiments determined that the gene coding for this phosphatase was contained on a 1.9-kb *Xho*I-*Sac*I fragment (results not shown). Sequence analysis of the *Xho*I-*Sac*I fragment predicted an 1,014-bp ORF coding for 338 amino acids (see GenBank accession no. U74652). The N-terminal amino acid sequence is consistent with known export signals for protein localization to the periplasm (Oliver 1985), including a positively charged amino terminus, an alanine-rich hydrophobic core, and processing sites that conform to the –3,–1 rule and are located at least six amino acids away from the hydrophobic core. Possible processing sites occur after amino acids 36, 40, 42, and 44. The predicted molecular mass of polypeptides processed after amino acids 36 and 44 are 33.4 and 32.5 kDa, respectively. A putative ribosome-binding site was identified 7 bp upstream of the ATG start codon, and codon usage analysis (Gribskov et al. 1984) was consistent with the gene being highly expressed in *R. meliloti*. Subclones with deletions that remove the first 251 or 521 nucleotides of *napD* did not code for acid phosphatase activity (data not shown).

The predicted amino acid sequence was compared with those for acid phosphatases from *Morganella morganii* (Thaller et al. 1994, 1995), *Providencia stuartii* (EMBL accession no. X64820), *Salmonella typhimurium* (Groisman et al. 1992), and *Zymomonas mobilis* (Pond et al. 1989), as well as with the *E. coli* phosphatases: Agp (Pradel et al. 1990), AppA (Dassa et al. 1990), CpdB (Lui et al. 1986), PhoA (Chang et al. 1986), SuhB (Matsuhisa et al. 1995), and UshA (Burns and Beacham 1986). No significant similarity was found with any of these phosphatases. BLAST searches of major protein and nucleotide databases failed to identify other proteins with sequence similarity.

Characterization of the cloned phosphatase

The EDTA/lysozyme extraction technique used to release phosphatase from the periplasm was effective for both *R. meliloti* strain 104A14 and *E. coli* strain CC118(pAcP3). No phosphatase activity was associated with membranes of either *R. meliloti* strain 104A14 or *E. coli* strain CC118(pAcP3), and malate dehydrogenase activity was found only in sonicated pellets of extracted cells, showing that the EDTA/lysozyme treatment did not result in cell lysis.

In native gels, the activity stain pattern of the cloned phosphatase expressed in *E. coli* strain CC118 depended on the medium in which the host strain was grown (Fig. 1 A). More bands were present when strain CC118(pAcP3) was grown in LB broth as compared to when it was grown in M9 minimal medium, but even with this variability the electrophoretic mobility of the cloned enzyme was distinct from the faintly staining endogenous *E. coli* phosphatases that had much lower electrophoretic mobilities. The cloned phosphatase activity was found only in strain CC118(pAcP3) and not in the parent strain or vector control. The clone-specific phosphatase bands ran at a position that was similar to the faster electrophoresing phosphatase extracted from the *R. meliloti* wild-type strain (Fig. 1 A).

Using *p*-nitrophenylphosphate as substrate in reactions buffered by 20 mM MES-NaOH and ranging in pH from pH 5.1–7.7, the cloned acid phosphatase was found to display a sharp pH optimum at pH 6.5 (results not shown). This was similar to that reported for the *E. coli* 3′-nucleotidase (Anraku 1964a, b), but the cloned enzyme did not hydrolyze 3′-AMP or 3′-UMP. Further, the NapD pH optimum and substrate range were different from those of any *E. coli* phosphatase (Anraku 1964a, b; Neu 1967; Cowman and Beacham 1980; Lui et al. 1986; Pradel and Boquet 1988; Matsuhisa et al. 1995), none of which complicated the purification of NapD, allowing us to study the *R. meliloti* protein as a separate phosphatase enzyme.

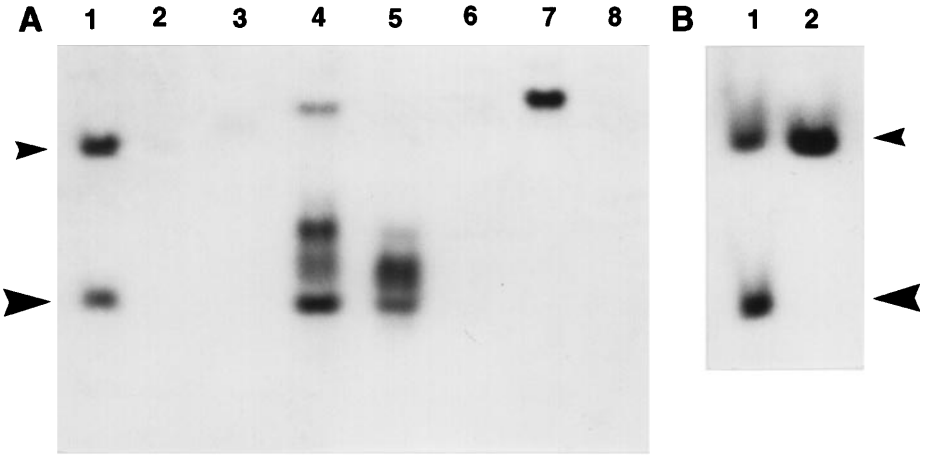

Fig. 1 A, B Acid phosphatase activity in periplasmic extracts from *Escherichia coli* strain CC118 containing different constructs and grown in different media, and from *Rhizobium meliloti* wild-type strain 104A14 and *napD* mutant RmMSU1. Periplasmic proteins were electrophoresed in a native gel and phosphatase activity stained as described in Materials and methods. The polarity of the gel was – at the top and + at the bottom. **A** *Lane 1 R. meliloti* strain 104A14, *2* open, *3 E. coli* strain CC118(pSD1::Tn*5*-B20) grown in M9, *4* strain CC118(pAcP3) grown in LB, *5* strain CC118(pAcP3) grown in M9, *6* strain CC118(pUC19) grown in M9, *7* strain CC118 grown in LB, and *8* strain CC118 grown in M9. Each lane was loaded with 20 μg protein. **B** A separate but identically run gel in which each lane was loaded with 0.04 units of phosphatase activity in periplasmic extract from: *1 R. meliloti* wild-type strain 104A14, and *2* the acid phosphatase mutant RmMSU1. The small arrow indicates the acid phosphatase with the slower electrophoretic mobility and the large arrow indicates NapD, the acid phosphatase with a faster electrophoretic mobility that was cloned in this study

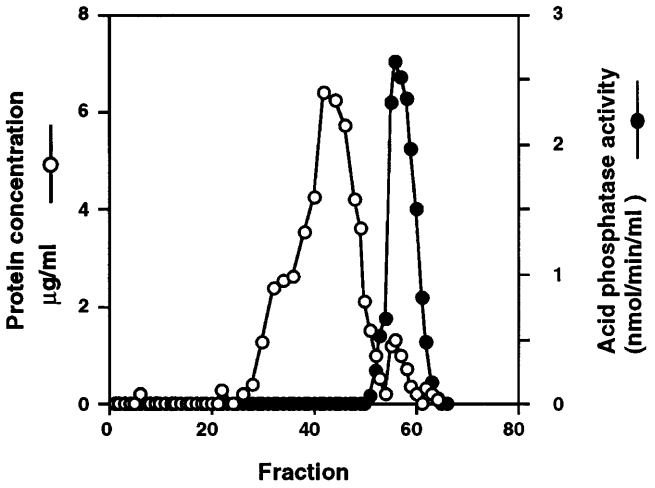

Fig. 2 Elution of NapD from a DEAE-Sepharose CL-6B column. The column was pre-equilibrated with 20 mM MES-NaOH buffer (pH 6.5) and then loaded with fractions from a phenyl Sepharose column that contained acid phosphatase activity. NapD was eluted by a 0–250 mM NaCl gradient. Protein and enzyme activities were measured using the protocols described in the text

NapD eluted as a single peak from a DEAE-Sepharose column (Fig. 2) and was the only phosphatase present in the column eluate as determined by activity stain analysis using native gels. The peak phosphatase specific activity of the DEAE-Sepharose column eluate was 2.01 μmol min^{-1} (mg protein)$^{-1}$, a 34-fold purification from the crude periplasmic extract. NapD eluted from the anion-exchange column was unstable, losing all activity within a few days. Passage through the anion exchange column did not result in complete separation of the different electrophoretic forms of NapD, making N-terminal sequence analysis impossible. To estimate the subunit molecular mass, a sample of the DEAE-Sepharose fraction with the highest activity was fractionated on a native gel and stained for activity. The main band of activity was excised in a thin slice, and the protein in the gel slice was elec-

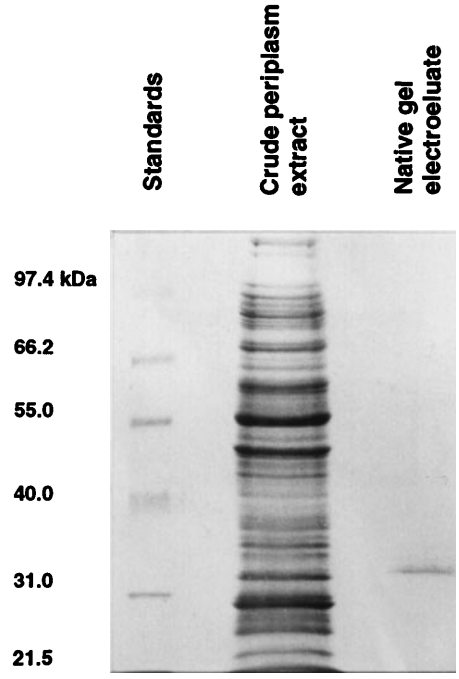

Fig. 3 Molecular mass analysis of NapD. Crude periplasmic extract from *Escherichia coli* strain CC118(pAcP3) and the NapD sample electroeluted from a native gel as described in Materials and methods were separated using SDS-PAGE as described in Materials and methods. Total protein loaded was 1.5 μg in the crude extract lane and approximately 0.8 μg in the NapD lane

Table 2 Specific activity of partially purified NapD with various substrates. Enzyme activity is shown as nmol of PO_4 released per min per mg protein. Results are the mean ± standard error from three independent assays and are corrected for spontaneous acid hydrolysis during the assay used to determine free phosphate (*ND* none detected)

Substrate	Specific activity
p-Nitrophenol-phosphate	479 ± 19
Phenyl-phosphate	312 ± 9
Ribose-5-phosphate	276 ± 17
D, L-Myo-inositol-1-phosphate	250 ± 2[a]
D-Glucose-6-phosphate	192 ± 30
α-Naphthyl-phosphate	118 ± 5
ATP	ND
3′-AMP	ND
5′-AMP	ND
3′-UMP	ND
5′-UMP	ND
Phenolphthalein diphosphate	ND

[a] Results from two assays

troeluted and assayed for acid phosphatase activity to verify the presence of the enzyme. Electroeluate samples had a specific activity of 14.3 μmol min^{-1} (mg protein)$^{-1}$ and showed a single peptide with an estimated molecular mass of 33 kDa (Fig. 3), which is consistent with the predicted molecular mass of the processed polypeptide. When the same DEAE-Sepharose fraction was analyzed by gel filtration, the native molecular mass of NapD was estimated to be 66 kDa (results not shown), implying the enzyme is a homodimer.

Phosphatase activity of NapD eluted from the DEAE-Sepharose column was greatest with *p*-nitrophenylphosphate as substrate, although significant phosphatase activity was also found with several other organic phosphorus compounds (Table 2). No activity was observed with ATP, 3′-AMP, 5′-AMP, 3′-UMP, 5′-UMP, or phenolphthalein diphosphate. The apparent lack of substrate specificity of the phosphatase was the basis for designating the cloned gene as *napD*, for nonspecific acid phosphatase.

Gene mutagenesis and replacement

The cloned *napD* contained in pSD1 was interrupted by Tn*5*-B20, resulting in no NapD activity in enzyme assays or with activity stains in native gels (Fig. 1 A, lane 3). pSD1::Tn*5*-B20 was mobilized into the parent wild-type *R. meliloti* strain, and isolated *R. meliloti* colonies were screened for sucrose sensitivity and kanamycin resistance to identify recombinants that had the *napD*::Tn*5*-B20 allele in the bacterial chromosome. Colony color was also used in the initial screen; the putative mutants were compared to the wild-type strain on media containing X-P or after flooding a replica plate with Fast Blue RR and α-naphthyl phosphate in MES-NaOH buffer (20 mM, pH 6.5). Kanamycin-resistant, gentamicin-sensitive, sucrose-resistant isolates that appeared lighter blue than the wild-type on X-P media and stained light brown (the wild-type stained dark brown) in the presence of Fast Blue RR/α-naphthyl phosphate were identified. The native gel pattern of acid phosphatase activity staining of their periplasmic extracts was determined, and an isolate lacking the faster acid phosphatase band (Fig. 1 B, lane 2) was further characterized by Southern blot analysis. Probing with the 3.7-kb *Xho*I fragment that contains the cloned acid phosphatase gene showed replacement of the wild-type allele with the interrupted gene, resulting in hybridization to 3.2- and 4.6-kb fragments in the mutant strain (results not shown). The isolate that was verified to be a *napD* mutant was named RmMSU1.

The fact that the mutant lacked only the faster-mobility-type acid phosphatase band (Fig. 1 B, lane 2) suggested that there are two acid phosphatase genes. We have now cloned the gene encoding this second acid phosphatase, and preliminary kinetic characterization (K_m and V_{max} determined with *p*-nitrophenylphosphate) of this second acid phosphatase has shown that it differs substantially from the NapD enzyme although its pH optimum is very similar to that of NapD (J. G. Elkins and T. R. McDermott, unpublished work).

Sequence analysis of the Tn*5*-B20 insert site established that the transposon inserted at bp 498/499 in *napD* and was in the correct orientation for reporting *napD* transcription. To test inducibility of the cloned acid phosphatase gene in response to phosphorus stress, strain RmMSU1 was grown in a minimal mannitol medium that was buffered with 5 mM MES-NaOH and 10 mM MOPS (pH 7.0) and amended to 10 mM phosphorus. Exponentially growing cells were washed in the same medium lacking phosphorus and then resuspended in either +P or –P versions of this medium. After 4 h, reporter enzyme levels were 144 ± 10 units (Miller 1972) in the +P cells, and 140 ± 6 units in the –P cells (three replicate samples each), indicating that the gene was not inducible by phosphorus starvation. The *R. meliloti* parent strain 104A14 contained less than 10 units of β-galactosidase activity. No differences were observed when other environmental factors such as carbon and nitrogen source were varied. The unchanged expression of *napD* reporter gene activity in cells grown with the various carbon and nitrogen sources suggests that *napD* is expressed constitutively. To determine if phosphorus levels could influence *napD* expression in nodules, β-galactosidase activity was also measured in NapD$^-$ bacteroids from plants grown under limiting and nonlimiting phophorus conditions. Bacteroids from low-phosphorus-status and high-phosphorus-status plants contained 35 ± 4 and 41 ± 5 units, respectively (mean ± standard error of three replicate samples each).

Symbiotic phenotype of the NapD– strain RmMSU1

Based on plant dry matter production in previous experiments, phosphate-limiting and phosphate-nonlimiting concentrations were 2–5 μM phosphate and ≥ 20 μM

Table 3 Symbiotic phenotype of *Rhizobium meliloti* 104A14 and the NapD$^-$ mutant derivative. The data shown is the overall average from two independent growth chamber experiments. In each experiment, there were five replicates for each treatment. Significant differences between strains and P levels were determined by analysis of variance. Values within the same column followed by the same letter are not significantly different ($LSD_{\alpha = 0.05}$). Low and high phosphorus designations refer to phosphorus concentrations that were limiting and nonlimiting, respectively, for plant growth; low phosphorus starting concentration averaged 2.5 μM, and high phosphorus starting concentration averaged 26 μM

Condition and strain	Plant dry wt. (mg plant^{-1})	Number of nodules per plant	Nodule fresh wt. (mg plant^{-1})	Acetylene reduction activity [μmol h^{-1} (g of nodule)$^{-1}$]
Low phosphorus				
104A14	9.8 a	6.0 a	4.6 a	8.1 a
RmMSU1	11.1 a	5.2 a	4.4 a	8.6 a
Not inoculated	4.9 b			
High phosphorus				
104A14	14.8 c	6.2 a	6.4 b	7.3 a
RmMSU1	15.7 c	9.6 b	8.0 c	6.4 a
Not inoculated	6.2 b			

phosphate (Al-Niemi et al. 1997b), respectively, and were used to determine if phosphorus availability to the host plant would affect the symbiotic performance of strain RmMSU1 as compared to the wild-type strain. Differences in plant dry matter between inoculated plants, and uninoculated and unnodulated controls were significant at both phosphorus levels (Table 3). For plants inoculated with the wild-type strain and strain RmMSU1, dry matter production under phosphorus-limiting conditions was 66 and 71%, respectively, of that of plants grown with nonlimiting phosphorus. Differences in plant dry matter between phosphorus treatments were statistically significant ($LSD_{\alpha = 0.05}$), but there was no inoculant strain influence at either phosphorus level (Table 3). Nodule fresh weight was also significantly affected by phosphorus levels. Interestingly, at the high phosphorus level, strain RmMSU1 consistently formed more nodules – accompanied by significantly greater nodule fresh weight – than did the wild-type strain. Acetylene reduction rates by RmMSU1 were slightly greater than those of the wild-type in the first experiment ($LSD_{\alpha = 0.10}$), but not in the second experiment, and it was concluded that there were no significant effects of the phosphorus treatment on nitrogen fixation activity as measured by acetylene reduction.

Nodule isolates were checked for transposon retention and acid phosphatase profile in native gels. Twenty-five randomly selected isolated colonies from each of the five replicates were all kanamycin-resistant. Of these, 12 randomly selected isolates were checked using acid phosphatase staining in native gels. All the strain RmMSU1 nodule isolates lacked the faster-moving acid phosphatase, indicating that the transposon was stable. No spontaneous resistance mutants of strain 104A14 to the level of kanamycin used in this study were found.

Discussion

Bacteroid phosphate uptake is necessary for development of an effective symbiosis between *R. meliloti* and alfalfa (Bardin et al. 1996), but whether the hydrolysis of organic phosphates might contribute to the imported phosphate pool is unknown. In contrast to a previous study with *R. meliloti* strain R-41 in which only a single acid phosphatase was detected (Lucchini et al. 1990), we have identified NapD and one other phosphatase that give detectable staining activity in low pH buffer using α-naphthyl phosphate or naphthol AS-MX-phosphate as substrates. Based on activity stains in native gels and reporter assays, NapD appears to be expressed in free-living cells grown on various carbon and nitrogen sources. Reporter enzyme was also present in bacteroids, but at levels lower than those observed in free-living cells. *R. meliloti* bacteroids undergo significant morphological differentiation, which results in increased cell volume relative to free-living cells. Therefore, reporter enzyme units based on cell optical density are not directly comparable between free-living cells and bacteroids. However, even though bacteroid Miller units were found to be only approximately one-third that of free-living cells, bacteroid reporter enzyme levels were roughly fivefold that of background β-galactosidase activity that we (data not shown) and others have reported (Uhde et al. 1997) in wild-type *R. meliloti*. This implies that the *napD* gene was expressed in bacteroids in our studies, but expression was not affected by host plant phosphorus nutrition status.

The gene encoding NapD was cloned by its ability to restore phosphatase activity to an *E. coli* alkaline phosphatase mutant under conditions that repressed native acid phosphatases. This cross-species complementation approach has also been used to clone the *phoC* and *napA* acid phosphatase genes from *M. morganii* (Thaller et al. 1994, 1995) and for genes involved with phosphorus metabolism in *Pseudomonas* (Filloux et al. 1988). By manipulating the cloned gene in *R. meliloti* and *E. coli*, we were able to characterize NapD activity and study the role of NapD in free-living and symbiotic bacteria. Yields of NapD cloned on a high-copy-number plasmid in *E. coli* were approximately 50% greater than those from *R. meliloti* and allowed preliminary characterization of the

protein. The data from the purified enzyme and the DNA sequence suggest that NapD is a homodimer of 33-kDa subunits and thus is similar to other bacterial phosphatases that have been characterized [e.g., Pradel and Boquet (1988) and Rothman and Byrne (1963)]. The deduced amino acid sequence of NapD shows no similarity with other sequenced phosphatases, but lack of similarity was also reported for the NapA nonspecific acid phosphatase of *M. morganii* (Thaller et al. 1995).

In *R. meliloti*, the NapD enzyme runs as a single band in native gels, but there are several bands when the same gene is expressed in *E. coli*. Differences in electrophoretic mobility of related proteins in native gels are generally due to different molecular charges of the proteins. A variable activity band pattern has been reported for the *E. coli* alkaline phosphatase and is due to processing of the exported polypeptide at two possible cut sites, coupled with the random formation of homo- or heterodimers (Signer et al. 1961; Piggot et al. 1972; Nakata et al. 1978; Karamyshev et al. 1994). We identified several potential signal peptide processing sites in the *napD* gene sequence. Processing NapD by cutting after amino acid 36 would lead to the most negatively charged monomer species, but cutting after amino acids 40 or 44 would give a protein with one additional positive charge, and cutting after amino acid 42 would give two additional positive charges. Since these three differently charged monomers could give dimers with five different charges, variable processing could account for the pattern of acid phosphatase activity bands observed in periplasmic extracts of strain CC118(pAcP3). Growth conditions affect the number and intensity of alkaline phosphatase bands found in wild-type *E. coli* (Nakata et al. 1978), and we also found that the pattern of NapD bands in strain CC118(pAcP3) changes when the strain was grown in M9 or LB broth (Fig. 3). If this explanation for the heterogeneity of NapD in *E. coli* is correct, the expression of NapD as a single electrophoretic type in *R. meliloti* implies that processing in *R. meliloti* chooses the cleavage site more exactly than in *E. coli* strain CC118.

Strain RmMSU1 formed effective nodules regardless of the phosphorus available to the host plant. Plant dry matter production and nodule fresh weight for mutant and wild-type strains were significantly greater under high-phosphorus conditions. The nodule number for the wild-type strain did not differ between phosphorus treatments, but it was significantly greater at high-phosphorus conditions for RmMSU1. These results imply that with added phosphorus, wild-type nodules increased in mass, while mutant nodules increased in number. Nitrogen fixation efficiency for a given phosphorus treatment was not different between strains as judged by plant dry matter production and acetylene reduction activity.

The Fix^+ symbiotic phenotype of strain RmMSU1 shows that NapD is not required for symbiosis. Indeed, at high phosphorus levels the nodulation characteristics of the mutant were enhanced relative to the wild-type strain, and dry matter production in plants inoculated with strain RmMSU1 was also higher at both phosphorus levels, although the latter differences were not statistically significant. The pH optimum profile of the other *R. meliloti* acid phosphatase overlaps significantly with NapD (data not shown), and it is possible that this second acid phosphatase may at least partially compensate for the loss of NapD. We are currently generating mutants with multiple phosphatase (acid and alkaline) defects in order to completely elucidate the importance of organic phosphorus compounds for alfalfa bacteroid phosphorus acquisition.

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