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Studies of the Physiological and Genetic Basis of Acid Tolerance in *Rhizobium leguminosarum* biovar trifolii

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Acid-tolerant *Rhizobium leguminosarum* biovar trifolii ANU1173 was able to grow on laboratory media at a pH as low as 4.5. Transposon Tn*5* mutagenesis was used to isolate mutants of strain ANU1173, which were unable to grow on media at a pH of less than 4.8. The acid-tolerant strain ANU1173 maintained a near-neutral intracellular pH when the external pH was as low as 4.5. In contrast, the acid-sensitive mutants AS25 and AS28 derived from ANU1173 had an acidic intracellular pH when the external pH was less than 5.5. The acid-sensitive *R. leguminosarum* biovar trifolii ANU794, which was comparatively more sensitive to low pH than mutants AS25 and AS28, showed a more acidic internal pH than the two mutants when the three strains were exposed to medium buffered at a pH of less than 5.5. The two acid-sensitive mutants had an increased membrane permeability to protons but did not change their proton extrusion activities. However, the acid-sensitive strain ANU794 exhibited both a higher membrane permeability to protons and a lower proton extrusion activity compared with the acid-tolerant strain ANU1173. DNA hybridization analysis showed that mutants AS25 and AS28 carried a single copy of Tn*5* located in 13.7-kb (AS25) and 10.0-kb (AS28) *Eco*RI DNA fragments. The wild-type DNA sequences spanning the mutation sites of mutants AS25 and AS28 were cloned from genomic DNA of strain ANU1173. Transfer of these wild-type DNA sequences into corresponding Tn*5*-induced acid-sensitive mutants, respectively, restored the mutants to their acid tolerance phenotypes. Mapping studies showed that the AS25 locus was mapped to a 5.6-kb *Eco*RI-*Bam*HI megaplasmid DNA fragment, whilst the AS28 locus was located in an 8.7-kb *Bgl*II chromosomal DNA fragment.

Soil acidity can adversely affect the formation of symbiotic associations between *Rhizobium* species and their host legume plants (22). Both the growth of *Rhizobium leguminosarum* biovar trifolii and the process of nodulation of *Trifolium* spp. have been shown to be sensitive to nutritional factors that are commonly associated with acid soils (27, 28, 37–39). The development of inoculant strains of *R. leguminosarum* biovar trifolii with enhanced capacity for growth at low pH may provide a means by which nodulation and growth of *Trifolium* spp. in acid soils can be improved. For instance, acid-tolerant strains of *Rhizobium meliloti* have recently been used to successfully establish *Medicago polymorpha*-based pastures on more than 350,000 ha of acidic soils in Western Australia, soils which were previously considered too acidic to support the growth of this legume (11). Inoculant strains of *R. leguminosarum* biovar trifolii presently used in Australia (e.g., strains WU95 and TA1) have been shown to be sensitive to low pH, as reflected by their poor growth in acidified laboratory media (28). However, strains of *R. leguminosarum* biovar trifolii with increased acid tolerance have been isolated (28, 37, 40, 41), but such strains commonly exhibit poor levels of symbiotic effectiveness in association with *Trifolium subterraneum* (28). Transfer of acid tolerance from these strains to inoculant strains of *R. leguminosarum* biovar trifolii may be desirable for the development of superior inoculant strains of *R. leguminosarum* biovar trifolii. It is apparent that an understanding of the mechanisms involved in acid tolerance in rhizobia is a prerequisite for the construction of such acid-tolerant inoculant strains.

Little is known about the physiological and genetic bases of acid tolerance in rhizobia. It has been shown that acid-tolerant strains of *R. meliloti* can more readily generate a pH gradient when grown in acid conditions and can subsequently maintain a more constant internal pH (24). In contrast, acid-sensitive strains of *R. meliloti* failed to maintain a pH gradient in acid conditions and were, thus, unable to control intracellular pH (24). Repeated subculturing of *R. leguminosarum* biovar trifolii and *Bradyrhizobium* strains on stress media or progressive subculturing on media of decreasing pH failed to increase levels of tolerance to low pH and did not give rise to spontaneous variants with increased levels of acid tolerance (5, 23).

In this study, we have used the transposon Tn*5* in an attempt to identify genes that are involved in the tolerance for growth of *R. leguminosarum* biovar trifolii ANU1173 on defined laboratory medium at a low pH. We show that at least two loci, of either plasmid or chromosomal location, are necessary for the growth of this strain on media at a pH of less than 4.8. We also show that the acid-sensitive mutants have restricted abilities to control cytoplasmic pH, which may be related to their plasma membranes being "leaky" to protons.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. Bacterial strains and plasmids used in this study are listed in Table 1. The compositions of growth media used, TY (1), LB (21), and the acid stress medium (ASM) used to examine the growth of *R. leguminosarum* biovar trifolii strains at low pH (7), have been described elsewhere.

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TABLE 1. Strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
R. leguminosarum biovar trifolii		
ANU1173	Sm^r derivative of the wild-type strain NA3001, Nod^+ Fix^+, acid tolerant	29
AS25	Tn*5*-induced acid-sensitive mutant of strain ANU1173	This work
AS28	Tn*5*-induced acid-sensitive mutant of strain ANU1173	This work
P22	ANU1173 derivative cured of both pSym and the second-largest megaplasmid	6
ANU794	Sm^r derivative of the wild-type strain TA1, Nod^+ Fix^+, acid sensitive	9
E. coli		
HB101	*leu proA2 rpsL20* (Sm^r) *hsdS20*	2
NM522	Δ(*lac-pro*) F′ *lacZ*M15 lac^q *hsd-5*	10
SM10	Chromosomally integrated RP4-2::Mu(Tc^s) derivative, Km^r	33
VCS257	Lambda host strain	ST[a]
Plasmids		
pSUP1011	pSUP101 (pACYC184-Mob) carrying Tn*5*, Km^r Cm^r	33
pKan2	pBR322 containing 3.5-kb inner fragment of Tn*5*	32
pRK2013	Helper plasmid *tra oriT* ColE1	8
pLAFR3	Cosmid cloning vector, Tc^r	36
pMP220	Broad-host-range cloning vector, Tc^r	35
Bluescribe	Multiple cloning vector, Amp^r	VCS[b]
pAT25	13.7-kb *Eco*RI Tn*5*-containing fragment cloned from mutant AS25 into Bluescribe	This work
pAT28	10.0-kb *Eco*RI Tn*5*-containing fragment cloned from mutant AS28 into Bluescribe	This work
pAT26	8.0-kb *Eco*RI wild-type fragment spanning the Tn*5* insertion site in mutant AS25 cloned from strain ANU1173 into Bluescribe	This work
pAT6	Cosmid able to complement mutant AS28	This work

[a] ST, Stratagene, La Jolla, Calif.
[b] VCS, Vector Cloning Systems, San Diego, Calif.

Transposon mutagenesis. The transposon Tn*5* was introduced into *R. leguminosarum* biovar trifolii ANU1173 by conjugal transfer from *Escherichia coli* SM10 containing pSUP1011 (33). Donor and recipient strains were freshly grown on LB and TY media, respectively, and were mated overnight on nitrocellulose filters at 28°C (31). Transconjugants were selected on ASM medium (pH 6.5) containing 200 μg of kanamycin ml^{-1}. The purified kanamycin-resistant (Km^r) isolates were patch plated (30 isolates $plate^{-1}$) on the same selective medium. Acid tolerance of putative Tn*5*-containing mutants was then determined after a 3-day growth by replicate plating on (i) ASM medium at pH 4.6, (ii) ASM medium at pH 6.5 containing 120 μg of chloramphenicol ml^{-1}, and (iii) ASM at pH 6.5 containing 200 μg of kanamycin ml^{-1}. Selected isolates, which exhibited either no growth or very poor growth on ASM medium at pH 4.6, had normal growth on ASM medium at pH 6.5 containing kanamycin, but did not grow on ASM medium at pH 6.5 containing chloramphenicol, were further compared in growth ability with the acid-tolerant parent strain ANU1173 and the acid-sensitive *R. leguminosarum* biovar trifolii ANU794 on ASM solid medium at a range of pH between 4.5 and 6.5. Bacterial cells, freshly grown on solid ASM medium at pH 6.5, were suspended at $\sim 1 \times 10^8$ cells ml^{-1} in sterile water and were diluted to $\sim 5 \times 10^2$ cells ml^{-1}. For each *Rhizobium* strain examined, 0.1 ml of dilution was spread in triplicate on 9-cm petri dishes containing solid ASM medium at a specified pH, and growth was scored by measuring colony diameter 7 to 12 days after incubating plates at 28°C. Colony diameter was measured under a microscope with a 10× calibrated eyepiece. The minimum pH value that permitted growth of the bacteria from a small inoculum ($<10^3$ cells ml^{-1}) was termed the critical pH.

Plasmid visualization. A modified Eckhardt gel method (26) was used to separate and visualize plasmids on agarose gels by electrophoresis.

Recombinant DNA techniques. Total DNA and plasmid DNA were isolated by methods previously outlined (30, 31). Other DNA manipulations were done by the method of Maniatis et al. (20). Selected fragments of digested DNA were isolated from agarose gels (0.8 to 1.0%) with a DEAE-cellulose membrane (Schleicher & Schuell, Inc., Keene, N.H.) according to the manufacturer's recommendations. Recombinant plasmids were transformed into *E. coli* NM522. Transformed colonies and DNA in agarose gels were transferred to nylon membranes (Hybond nylon, Amersham) according to the manufacturer's specifications and were hybridized overnight at 65°C with ^{32}P-labelled DNA probes.

Cloning of acid-tolerant loci of strain ANU1173. DNA fragments containing Tn*5* were cloned from the Tn*5*-induced acid-sensitive mutants (mutants AS25 and AS28 [Table 1]) into *Eco*RI-cleaved Bluescribe vector DNA by selection of transconjugants on LB medium containing 75 μg of kanamycin ml^{-1}. Cloned regions were verified to contain Tn*5* by Southern blot hybridization of digested DNA with a ^{32}P-labelled 3.5-kb *Hin*dIII inner DNA fragment of Tn*5*. These Tn*5*-containing clones (flanked for *Rhizobium* DNA) were subsequently used as hybridization probes to obtain the respective wild-type sequences from total DNA isolated from the parent strain ANU1173. The wild-type acid tolerance loci (AS25 and AS28) were isolated from restriction

TABLE 2. Growth of acid-tolerant and acid-sensitive strains of *R. leguminosarum* biovar trifolii on ASM solid medium at a range of pHs

Strain	Mean colony diam (mm) ± 1 SD[a] after 10-day growth on solid ASM medium at following pH:							
	6.5	5.0	4.9	4.8	4.7	4.6	4.5	4.4
ANU1173	5.4 ± 0.3	4.0 ± 0.3	3.7 ± 0.4	3.5 ± 0.4	2.8 ± 0.5	2.0 ± 0.3	0.9 ± 0.4	NG
AS25	5.0 ± 0.5	3.9 ± 0.4	3.0 ± 0.3	1.3 ± 0.2	NG	NG	NG	NG
AS25(pAT1)	5.2 ± 0.6	3.8 ± 0.4	3.7 ± 0.5	3.3 ± 0.5	2.8 ± 0.4	1.9 ± 0.4	1.0 ± 0.3	NG
AS28	5.2 ± 0.5	3.8 ± 0.5	2.8 ± 0.4	1.0 ± 0.3	NG	NG	NG	NG
AS28(pAT6)	5.5 ± 0.4	4.0 ± 0.5	3.8 ± 0.4	3.2 ± 0.5	2.6 ± 0.2	2.0 ± 0.4	1.0 ± 0.3	NG
ANU794	4.8 ± 0.3	2.0 ± 0.3	0.7 ± 0.2	NG	NG	NG	NG	NG

[a] Mean colony diameter of 12 single, but isolated, colonies. NG, no bacterial growth on solid medium.

fragment lengths of between 7.0 and 9.0 kb of *Eco*RI-digested genomic DNA cloned into Bluescribe (AS25) and from a cosmid library constructed in the broad-host-range cosmid vector pLAFR3 (AS28). The cosmid library of strain ANU1173 was constructed by partially cleaving total genomic DNA with *Mbo*I and isolating fragments of between 20 and 30 kb by sucrose gradient centrifugation. These fragments were cloned into the *Bam*HI site of pLAFR3. The recombinant cosmids were subsequently encapsulated in vitro with a lambda DNA packaging system (Promega) according to the manufacturer's recommendations. The phage particles packaged in vitro were transduced into *E. coli* VCS257. Transconjugants were selected on LB medium containing 15 μg of tetracycline ml^{-1}. Selected DNA fragments were subsequently subcloned into the broad-host-range vector pMP220.

Bacterial matings. Recombinant plasmids were transferred to the acid-sensitive mutants by means of the helper-mobilizing plasmid pRK2013 by a triparental patch-mating technique (8). Transconjugants were selected by replica plating on ASM medium containing 20 μg of tetracycline ml^{-1}. Acid tolerance of transconjugants was determined by examining growth of strains on ASM solid medium at a pH of between 4.5 and 6.5.

Determination of internal pH. Internal pH of *R. leguminosarum* biovar trifolii was determined by the fluorescent probe method (34). Cells grown in TY liquid medium at 28°C for 3 to 4 days were harvested, washed twice with the salt medium of ASM, and resuspended in the same salt medium buffered at the appropriate pH with either 30 mM MES (morpholineethanesulfonic acid) for the pHs between 5.5 and 6.5 or 30 mM malic acid for the pHs between 4.0 and 5.4. The suspension was then incubated for 30 min at 28°C with 20 to 100 μM fluorescein diacetate (Sigma). Cells were then thoroughly washed and resuspended in the original volume of buffer. Samples of 1 ml were placed in the cuvette of a fluorescence spectrophotometer (model F-3000; Hitachi), and the fluorescence intensity was recorded at 520 nm after excitation at 490 and 435 nm. The values of internal pH were read from a calibration curve prepared as described by Slavik (34).

Measurement of proton influx and efflux. Cells grown in TY liquid medium for 3 to 4 days were collected by centrifugation at 3,000 × *g* for 5 min, washed with 2 mM $MgSO_4$, and suspended in a 10 mM KCl–2 mM $MgSO_4$ solution (ca. 2 mg of protein ml^{-1}). Proton influx of bacterial cells was measured after a proton pulse at 28°C in a 15-ml temperature-controlled reaction chamber with an O ring combination pH electrode connected to an O ring pH meter and recorded with a linear recorder. The pH of the reaction mixture, which consisted of 9.8 ml of 10 mM KCl–2 mM $MgSO_4$ and a 0.2-ml suspension of cells, was adjusted to 6.9 with HCl or NaOH, and change in the cell suspension pH was then recorded after the addition of 40 to 50 μl of HCl (0.05 N) to the cell suspension. The alkalization rate of the medium reflected the rate of the net proton influx.

For measurement of H^+ efflux, the pH of the reaction mixture, which consisted of 9.0 ml of solution containing 10 mM KCl, 10 mM $CaCl_2$, 2 mM $MgSO_4$, and 0.5 mM PIPES (piperazine-*N*,*N*′-bis-2-ethanesulfonic acid) and a 1-ml suspension of cells, was adjusted to 7.5 with NaOH. The pH-adjusted reaction mixture was transferred to a 13-ml Falcon tube and incubated at 28°C for 60 to 90 min after addition of glucose (20 mM). Determination of the rate of pH change of the cell suspension through back-titration with alkali allowed calculation of the net rate of H^+ efflux as nanomoles of H^+ milligram of $protein^{-1}$ $minute^{-1}$ (4). The Bio-Rad protein assay kit was used for the measurement of protein content of the intact bacteria according to the manufacturer's instructions.

RESULTS

***R. leguminosarum* acid-sensitive mutants.** Following Tn*5* mutagenesis of acid-tolerant *R. leguminosarum* biovar trifolii ANU1173, kanamycin-resistant colonies arose at a frequency of about 10^{-4} per recipient cell. Under the same growth conditions, spontaneous Km^r mutants of ANU1173 were found to occur only at a low frequency ($\sim 5 \times 10^{-9}$). Two acid-sensitive mutants (AS25 and AS28) were isolated after the examination of 1,000 Km^r colonies of ANU1173 for their acid sensitivity. Strain ANU1173 was able to grow on solid medium at pH 4.5 (Table 2). In contrast, mutants AS25 and AS28 were unable to grow on solid medium at a pH of less than 4.8 (Table 2). However, the growth of mutants AS25 and AS28 was comparable to that of the parent strain ANU1173 at a pH of greater than 5.0. Whilst the mutants AS25 and AS28 were more sensitive to low pH than the parent strain ANU1173, they were comparatively more acid tolerant than strain ANU794, a streptomycin-resistant derivative of the commercial inoculant strain of *R. leguminosarum* biovar trifolii TA1 (Table 2). The growth of strains ANU1173, AS25, AS28, and ANU794 was completely inhibited on solid medium at pH values of 0.1 pH unit below their critical pHs, the minimum pH values that permitted the growth of the bacteria (Table 2).

Internal pH of acid-tolerant and acid-sensitive strains of *R. leguminosarum*. The acid-tolerant parent strain ANU1173 was able to maintain a near-neutral internal pH (6.8 to 7.2) when the external pH was varied between 4.5 and 6.5 (Fig. 1). In contrast, the mutants AS25 and AS28 were able to maintain a near-neutral pH (6.8 to 7.1) only when exposed to

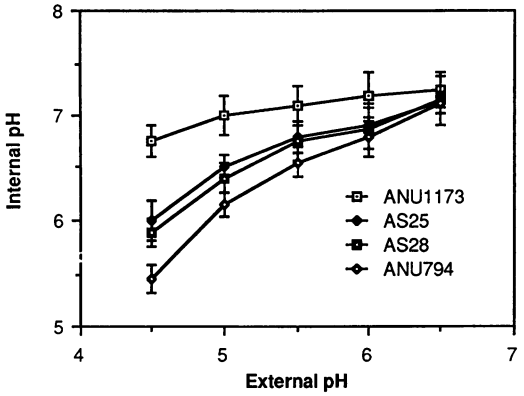

FIG. 1. Internal pH of *R. leguminosarum* biovar trifolii strains ANU1173, AS25, AS28, and ANU794 when exposed to the medium buffered at pH between 4.5 and 6.5. All values and points plotted represent the means ± 1 standard deviation of four observations.

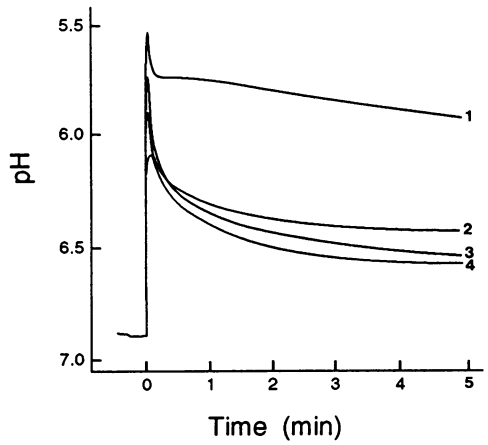

FIG. 2. Proton influx after a proton pulse. A tracing of observed changes in the cell suspension pH after a proton pulse is shown. The cell suspension pH was adjusted to 6.9, and change in the cell suspension pH was recorded after addition of 50 μl of 0.05 N HCl to the cell suspension at time zero. Line 1, acid-tolerant strain ANU1173 (0.2 mg of protein ml^{-1}); line 2, acid-sensitive mutant AS25 (0.21 mg of protein ml^{-1}); line 3, acid-sensitive mutant AS28 (0.19 mg of protein ml^{-1}); line 4, acid-sensitive strain ANU794 (0.18 mg of protein ml^{-1}).

the media buffered at a pH of greater than 5.5. At an external pH of 5.0, mutants AS25 and AS28 had an acidic intracellular pH of 6.5 and 6.4, respectively, which fell to 6.0 and 5.9 when the external medium pH was 4.5 (Fig. 1). The internal pH level of the acid-sensitive strain ANU794 was shown to be similar to that of the acid-sensitive mutants when the external pH was between 6.0 and 6.5. However, the internal pH level of strain ANU794 was about 0.3 to 0.5 pH units below that of mutants AS25 and AS28 when the external pH was less than 5.5 (Fig. 1).

Rates of proton influx and efflux of acid-tolerant and acid-sensitive *R. leguminosarum*. The restricted ability of mutants AS25 and AS28 in regulation of cytoplasmic pH could be due to either low efficiency in proton extrusion or their plasma membranes being more permeable to protons. Thus, the proton permeability of the acid-tolerant and acid-sensitive strains was measured by monitoring the change of the medium pH after a proton pulse. Figure 2 shows a representative trace of observed changes in the medium pH after a proton pulse in a number of experiments. In all cases, an influx of protons was observed for 5 min or more after a proton pulse (Fig. 2). The acid-sensitive mutants AS25 and AS28 and the acid-sensitive strain ANU794 had similar proton influx rates which were greater than that of the acid-tolerant strain ANU1173. Although the mutants AS25 and AS28 altered their membrane permeabilities to protons, their proton extrusion activities were similar to that of the parent strain ANU1173 (Table 3). However, the rate of proton extrusion of the acid-sensitive strain ANU794 was about 37% of the level of the acid-tolerant strain ANU1173 (Table 3).

Location of transposon Tn*5*. Hybridization analysis of the Tn*5*-specific DNA probe to *Eco*RI-digested total DNA of the mutants AS25 and AS28 indicated that Tn*5* was present as a single copy and had inserted into 13.7-kb (AS25) and 10.0-kb (AS28) *Eco*RI DNA fragments (Fig. 3A). The parent strain ANU1173 had four megaplasmids (Fig. 4, panel 1) with molecular masses (in megadaltons) of about 450 (p1173-1), 300 (p1173-2), 240 (p1173-3), and 160 (pSym1173). Further hybridization analysis to native plasmid DNA showed that Tn*5* hybridized to the chromosomal DNA material in the mutant AS28 and to a megaplasmid in the mutant AS25 (Fig. 4, panel 2). The megaplasmid in mutant AS25 which was positively hybridized with the Tn*5* probe appeared to be p1173-3, according to plasmid migration rate in the gel and location of the hybridization band in the X-ray film exposed to the plasmid gel blot.

The 13.7- and 10.0-kb *Eco*RI fragments of DNA carrying Tn*5* were cloned from the mutants AS25 and AS28, respectively, into the plasmid Bluescribe. These cloned fragments were shown to specifically hybridize to an 8.0- and a 4.4-kb wild-type *Eco*RI fragment in strain ANU1173, as well as to a 13.7- and a 10.0-kb *Eco*RI fragment from the two corresponding mutant strains AS25 and AS28 (Fig. 3B and C), respectively. These differences in fragment sizes between wild-type and mutant strains are consistent with the known size of Tn*5*, 5.7 kb (12). Southern blot hybridizations of the 13.7-kb *Eco*RI fragment (containing the Tn*5* of mutant AS25) indicated that this fragment specifically hybridized to an 8.0-kb *Eco*RI DNA fragment in strains ANU1184 (ANU1173 derivative cured of pSym1173) and P22 (ANU1173 derivative cured of both pSym1173 and p1173-2) (Fig. 3C). These results indicate that the plasmid-located acid tolerance locus in the mutant AS25 that was identified by Tn*5* mutagenesis was located on the third-largest megaplasmid (p1173-3) of strain ANU1173.

Cloning of the wild-type DNA sequence complemented to mutant AS25. Clones containing an 8.0-kb *Eco*RI fragment of the wild-type strain ANU1173, homologous to the radioac-

TABLE 3. Proton extrusion in acid-tolerant and acid-sensitive *R. leguminosarum* biovar trifolii strains

Strain	Proton extrusion[a] (nmol min^{-1} mg of $protein^{-1}$)
ANU1173	49.3 ± 8.2
AS25	46.7 ± 6.8
AS28	51.2 ± 9.7
ANU794	18.1 ± 7.5

[a] Mean values of three independent experiment data ± 1 standard deviation.

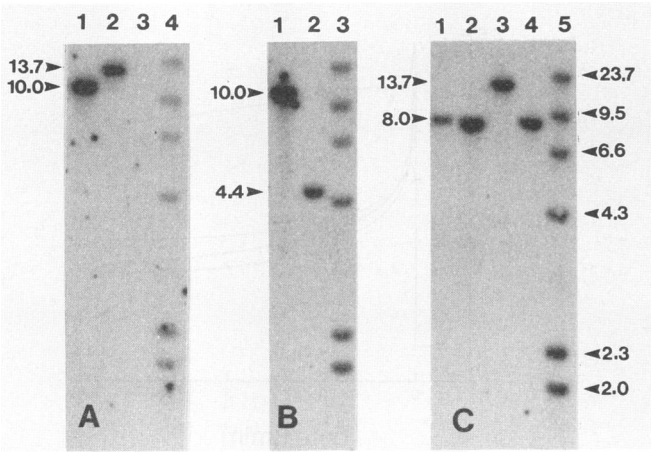

FIG. 3. Southern blot analysis of DNA isolated from wild-type and mutant *R. leguminosarum* biovar trifolii strains. (A) Hybridization of *Eco*RI-digested total genomic DNA of Tn*5*-induced acid-sensitive mutants AS28 (lane 1) and AS25 (lane 2) and the wild-type strain ANU1173 (lane 3) with the ^{32}P-labelled 3.5-kb *Hin*dIII inner DNA fragment of Tn*5*. (B) Hybridization of *Eco*RI-digested total genomic DNA of mutant AS28 (lane 1) and strain ANU1173 (lane 2) with the ^{32}P-labelled 10.0-kb *Eco*RI Tn*5*-containing fragment cloned from mutant AS28. (C) Hybridization of *Eco*RI-digested DNA of strain ANU1184 (ANU1173 derivative cured of pSym1173) (lane 1), strain P22 (ANU1173 derivative cured of both pSym1173 and p1173-2) (lane 2), mutant AS25 (lane 3), and strain ANU1173 (lane 4) with the ^{32}P-labelled 13.7-kb *Eco*RI Tn*5*-containing fragment cloned from mutant AS25. Size standards (panel A, lane 4; panel B, lane 3; and panel C, lane 5) shown are λ*c*I857 DNA digested with *Hin*dIII.

tively labelled 13.7-kb *Eco*RI Tn*5*-containing fragment, were isolated from a partial Bluescribe DNA library containing inserts of ~7.0 to 9.0 kb. The 8.0-kb *Eco*RI fragment was cloned into the broad-host-range plasmid pMP220, resulting in plasmid pAT1. The introduction of this plasmid into mutant AS25 restored to the mutant an acid tolerance

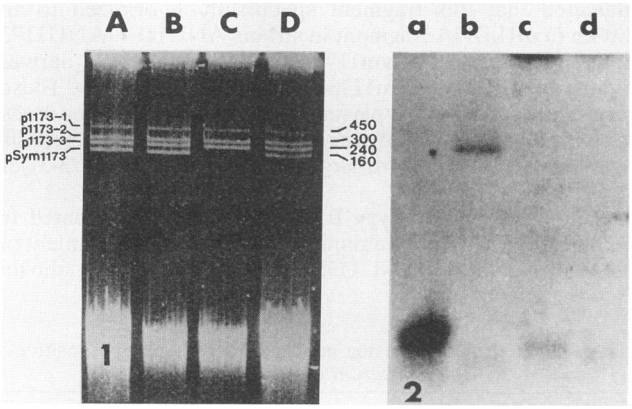

FIG. 4. Localization of Tn*5* in mutants AS25 and AS28. Shown is a plasmid gel analysis of *R. leguminosarum* biovar trifolii DNA (panel 1) and Southern blot hybridization with the radioactively labelled 3.5-kb *Hin*dIII inner DNA fragment of transposon Tn*5* (panel 2). Lanes A and lane B, Tn*5*-induced acid-sensitive mutants AS28 and AS25; lane C, strain ANU1184; lane D, parent strain ANU1173. The Tn*5* probe hybridized to chromosomal material of mutant AS28 (lane a) and a megaplasmid material of mutant AS25 (lane b). Plasmid sizes are in megadaltons.

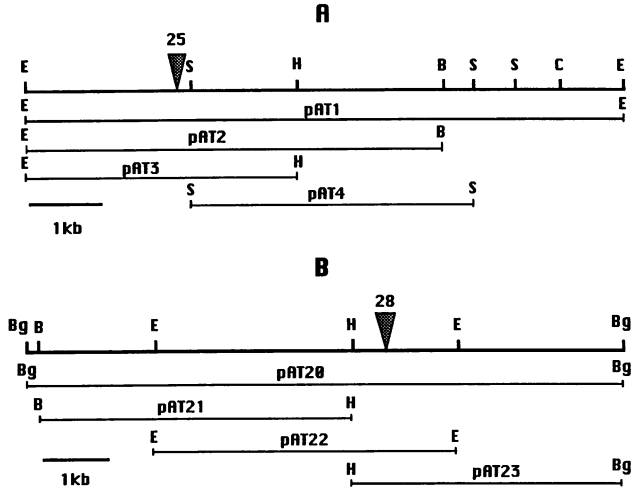

FIG. 5. Restriction maps and subclones of the 8.0-kb *Eco*RI (A) and the 8.7-kb *Bgl*II (B) fragments containing loci involved in the acid tolerance phenotype of strain ANU1173. Plasmids pAT1 (8.0-kb *Eco*RI) and pAT2 (5.6-kb *Eco*RI-*Bam*HI) complemented the Tn*5*-induced acid-sensitive mutant AS25, whereas plasmids pAT3 (3.7-kb *Eco*RI-*Hin*dIII) and pAT4 (3.6-kb *Sph*I) resulted in no complementation when these plasmids were introduced into the mutant. Plasmid pAT20 (8.0-kb *Bgl*II) was able to restore an acid tolerance phenotype to the Tn*5*-induced acid-sensitive mutant AS28, whereas plasmids pAT21 (4.6-kb *Bam*HI-*Hin*dIII), pAT22 (4.4-kb *Eco*RI), and pAT23 (4.0-kb *Bgl*II-*Hin*dIII) failed to do so when these plasmids were transferred into the mutant. Plasmid pMP220 was used as the cloning vector. The shaded triangles show the location of Tn*5* in the cloned DNA fragments as determined by endonuclease restriction analysis of the 13.7- and 10.0-kb *Eco*RI Tn*5*-containing DNA fragments cloned from mutants AS25 and AS28 with *Hpa*I. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; S, *Sph*I.

phenotype similar to that observed for the parent strain ANU1173 (Table 2; Fig. 5). Three subfragments (3.7-kb *Eco*RI-*Hin*dIII, 5.6-kb *Eco*RI-*Bam*HI, and 3.6-kb *Sph*I [Fig. 5]) of the 8.0-kb region were also subcloned into plasmid pMP220, resulting in pAT3, pAT2, and pAT4, respectively (Fig. 5). Mutant AS25 was complemented by plasmid pAT2 but not plasmids pAT3 and pAT4 (Fig. 5).

Cloning of the wild-type DNA sequence complemented to mutant AS28. The 10.0-kb *Eco*RI DNA fragment containing the Tn*5* of mutant AS28 was used as a hybridization probe to identified one cosmid (pAT6). Mutant AS28 containing the cosmid pAT6 restored its acid tolerance phenotype (Table 2). Subcloning and complementation analysis showed that the functional acid tolerance locus corresponding to mutant AS28 was located in an 8.7-kb *Bgl*II wild-type DNA fragment subcloned from the cosmid pAT6 (plasmid pAT20 [Fig. 5B]). However, cloned subfragments of the 8.7-kb *Bgl*II region (plasmids pAT21, pAT22, and pAT23) failed to complement mutant AS28 (Fig. 5B).

DISCUSSION

Transposon mutagenesis was used to identify and to isolate two loci (mutants AS28 and AS25) which were involved in the acid tolerance phenotype of *R. leguminosarum* biovar trifolii ANU1173. These two loci were mapped to an 8.7-kb *Bgl*II chromosomal fragment (AS28) and a 5.6-kb *Eco*RI-*Bam*HI megaplasmid fragment (AS25) in the parent

acid-tolerant strain ANU1173. Strain ANU1173 contained four indigenous megaplasmids (Fig. 4), the smallest of which (pSym1173) is the symbiotic plasmid (29). Hybridization analysis indicated that the wild-type acid tolerance locus (homologous to the Tn*5*-containing region cloned from mutant AS25) was located on the third-largest megaplasmid (p1173-3) of strain ANU1173 (Fig. 3 and 4). The second-largest megaplasmid (p1173-2) of strain ANU1173 has already been shown to carry genes involved in acid tolerance (6). These findings indicate that at least two of the megaplasmids present in strain ANU1173 contain genes required for growth of the bacterium in laboratory media at low pH and there are, at least, three separated loci involved in the complex acid tolerance phenotype of strain ANU1173.

Mutants AS25 and AS28 were changed in their abilities to grow under lower pH conditions and became acid sensitive. The two mutants had an increased critical pH which was 0.3 pH units higher than the acid-tolerant parent strain ANU1173. However, the growth of the acid-tolerant and the acid-sensitive *R. leguminosarum* biovar trifolii strains was completely inhibited on solid medium at pH values 0.1 pH unit below their critical pHs. It has been reported that growth of *R. meliloti* and *R. leguminosarum* with different critical pH values can be totally inhibited in laboratory media at pH values 0.1 to 0.2 pH unit below their individual critical pHs (18, 19, 28, 29). These results indicate that the range of the critical pHs for different *Rhizobium* strains is similarly narrow and is independent of their individual critical pHs. Thus, small changes in medium pH below or above the critical pH of a given *Rhizobium* strain could have a dramatic effect on the bacterial growth.

Bacteria that grow at extreme pH values encounter a variety of biological and, specifically, bioenergetic challenges that are derived from a central problem of pH homeostasis (16). Most major groups of microorganisms have at least some representatives that grow at extremely low pH values (17). It has been reported in a number of acidophiles and neutrophiles that the maintenance of an internal pH near neutrality appears to be necessary for the bacteria to grow at a low pH (3, 17). This implies that regulation of cytoplasmic pH plays a crucial role in acid tolerance in bacteria. The acid-tolerant *R. leguminosarum* biovar trifolii ANU1173 was able to maintain a near-neutral internal pH when exposed to the medium buffered at a pH as low as 4.5, whereas its acid-sensitive mutants AS25 and AS28 had an acidic internal pH when three of the strains were exposed to the medium buffered at a pH of less than 5.5. In addition, strain ANU794, which was comparatively more sensitive to low pH than mutants AS25 and AS28, exhibited a more acidic internal pH compared with the two acid-sensitive mutants under the same low-pH conditions (Fig. 1). Similar results have been obtained by O'Hara et al. (24), who showed that acid-tolerant strains of *R. meliloti* could more readily generate a higher pH gradient when grown in acid conditions and can subsequently maintain a relatively constant alkaline internal pH. In contrast, the acid-sensitive mutants of *R. meliloti* or native acid-sensitive isolates of *R. meliloti* generated a much lower pH gradient in acid conditions than that of the wild-type acid-tolerant strain and thus were unable to control intracellular pH (24). Furthermore, *R. meliloti* mutants, which were more sensitive to low pH, also had more acidic internal pH values under low-pH conditions. These results indicate that the ability of *Rhizobium* bacteria to tolerate acidity is correlated with their ability to control cytoplasmic pH.

In essence, regulation of cytoplasmic pH implies control over the movement of protons crossing the cytoplasmic membrane of the cell. Acidophilic bacteria grow optimally at pH 3.0 or less and maintain an internal cellular pH far less acidic than the external cellular pH (3, 25). The bacteria achieve this in two ways: first, by effective pumping of protons outward, and second, by possessing a cytoplasmic membrane impermeable to protons (3, 25). It has been shown for *Streptococcus faecalis* that the H^+ ATPase is a major factor in the control of the cytoplasmic pH (13–15). It was proposed that a K^+/H^+ antiporter has played an important role in the regulation of internal pH in *E. coli* grown in the acid medium (25). The acid-sensitive mutants AS25 and AS28 exhibited a higher proton permeability of their cell membranes but did not alter their proton extrusion activities compared with that of the acid-tolerant parent strain ANU1173. We suggest that the restricted abilities of mutants AS25 and AS28 to regulate the cytoplasmic pH result from their increased membrane permeability to protons.

However, the acid-sensitive strain ANU794 showed not only a higher proton permeability of the cell membrane but also a lower proton extrusion activity compared with that of the acid-tolerant strain ANU1173. Furthermore, it has been shown that the megaplasmid-deleted, acid-sensitive derivative of strain ANU1173, strain P22, which is more sensitive to low pH and has a more restricted ability to regulate cytoplasmic pH than mutants AS25 and AS28, has an increased membrane permeability to protons and a decreased proton extrusion activity (6). Thus, the more restricted abilities of the acid-sensitive strains ANU794 and P22 to control their cytoplasmic pH are related to both their leaky membrane to protons and less efficient proton pumps. The leaky membrane of the acid-sensitive strains of *R. leguminosarum* biovar trifolii to protons may result from a relatively loose control of the activity of proton transport systems (located in the membrane) which facilitate proton entry. These results have indicated that the membrane permeability and proton pumping activity are involved in regulation of cytoplasmic pH of *R. leguminosarum* biovar trifolii grown at low pH. The acid-tolerant *R. leguminosarum* biovar trifolii, such as strain ANU1173, has a cytoplasmic membrane less permeable to protons and has an efficient proton pump, such as the H^+ ATPase, that allow the bacterium to be able to maintain a near-neutral internal pH and therefore survive under low-pH conditions.

The extent of increased sensitivity to low pH for individual mutations, whether in the chromosome (mutant AS28) or on a megaplasmid (mutant AS25) of *R. leguminosarum* biovar trifolii, was not greater than 0.3 pH units. Similarly, the increased sensitivity to low pH for Tn*5*-induced acid-sensitive mutants of *R. meliloti* is not greater than 0.4 pH units (24). Thus, it will be important to create mutants with double and triple mutations between strains AS25, AS28, and P22 to see if a compounding of their acid sensitivities can occur. Future analysis should more clearly define the various components affecting proton permeability and proton extrusion mechanisms and thus the acid tolerance phenotype of *Rhizobium* bacteria. Such information could be used to construct strains with an enhanced ability in acid tolerance.

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