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Maintenance of Intracellular pH and Acid Tolerance in *Rhizobium meliloti*

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The development and function of the *Rhizobium meliloti-Medicago* sp. symbiosis are sensitive to soil acidity. Physiological criteria that can be measured in culture which serve to predict acid tolerance in soil would be valuable. The intracellular pH of *R. meliloti* was measured using either radioactively labeled weak acids (5,5-dimethyloxazolidine-2,4-dione and butyric acid) or pH-sensitive fluorescent compounds; both methods gave similar values. Six acid-tolerant strains (WSM419, WSM533, WSM539, WSM540, WSM852, and WSM870) maintained an alkaline intracellular pH when the external pH was between 5.6 and 7.2. In contrast, two Australian commercial inoculant strains (CC169 and U45) and four acid-sensitive strains from alkaline soils in Iraq (WSM244, WSM301, WSM365, and WSM367) maintained an alkaline intracellular pH when the external pH was ≥6.5, but had intracellular pH values of ≤6.8 when the external pH was ≤6.0. Four transposon Tn*5*-induced mutants of acid-tolerant strain WSM419, impaired in their ability to grow at pH 5.6, showed limited control over the intracellular pH. The ability to generate a large pH gradient under acid conditions may be a better indicator of acid tolerance in *R. meliloti* under field conditions than is growth on acidic agar plates.

Soil acidity is a major factor limiting legume growth and nitrogen fixation because of its adverse effects on the growth of the host plant, its root nodule bacteria (*Rhizobium* spp. and *Bradyrhizobium* spp.), and symbiotic development (20). The *Rhizobium meliloti-Medicago* sp. symbiosis is particularly sensitive since acidity, per se, markedly inhibits the growth of *R. meliloti* (6) and the process of nodule initiation (18, 19). The poor survival of many strains of *R. meliloti* in soils with a pH of below 6.0 (24) severely limits *Medicago* pasture establishment on moderately acidic soils (26, 27).

Strains of *R. meliloti* selected for acid tolerance, using laboratory and greenhouse trials, have often not proved to be tolerant in the field (8–10, 16, 17, 22–24). Screening for growth on an acidified medium failed to identify *R. meliloti* strains capable of persisting and nodulating in acid soils (16). Howieson et al. (10) found a poor correlation between growth ratings derived from laboratory screening on acid media and acid tolerance in the field. Acid-tolerant *R. meliloti* strains would be beneficial in improving medic pasture production on acid soils, but selection of these strains remains empirical in the absence of knowledge of the mechanisms involved in acid tolerance.

Bacterial cell function is dependent on the maintenance of an appropriate intracellular pH (pH_i). Although the precise relationship between pH homeostasis and acid tolerance in bacteria is not understood, both genetic and biochemical studies point to a central role for the maintenance of internal pH in acid tolerance. For example, acid-sensitive mutants of *Streptococcus faecalis* deficient in proton extrusion could not generate a pH gradient and maintain near-neutral pH_i (13, 14).

Two acid-tolerant strains of *R. meliloti* isolated from nodules on *Medicago* spp. growing on acid soils in Sardinia (WSM419 and WSM540) have been released commercially in Western Australia (10). Their superiority over the previously used acid-sensitive strains (CC169 and U45) in their ability to colonize, persist, and nodulate *Medicago polymorpha* in these acidic soils has led to the successful establishment of medic pastures on 400,000 ha of acidic soils (10). At present the physiological basis of acid tolerance in these strains is unknown, but a fundamental prerequisite for growth under acid conditions appears to be the regulation of pH_i.

In this study we compared the ability of acid-tolerant and acid-sensitive strains of *R. meliloti* to control pH_i and grow under acid conditions.

MATERIALS AND METHODS

Organisms. The strains and mutants of *R. meliloti* used are listed in Table 1. All nonmutant *R. meliloti* strains were obtained from J. Howieson, Western Australian Department of Agriculture, South Perth, Western Australia 6151, Australia.

Media. *Escherichia coli* was routinely grown at 37°C on 2× YT (16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl per liter). *R. meliloti* was generally grown at 28°C on YM medium (34) or TY medium (1); the latter was also used for mating mixtures of *E. coli* and *R. meliloti* used in transposon mutagenesis. The minimal medium JMM used for the growth of *R. meliloti* WSM419 and its derivatives was adapted from the 6M medium of Howieson (8). The composition of JMM ($liter^{-1}$) was as follows: D-(+)-galactose, 1.8 g; L-(+)-arabinose, 1.5 g; L-glutamate (monosodium salt), 0.507 g; K_2HPO_4, 26.1 mg; KH_2PO_4, 20.4 mg; $FeSO_4 \cdot 7H_2O$, 5.5 mg; $CaCl_2 \cdot 2H_2O$, 147 mg; thiamine hydrochloride, 1.0 mg; pantothenic acid, 1 mg; biotin, 20 μg; $Na_2MoO_4 \cdot 2H_2O$, 0.967 mg; Na_2SO_4, 100 mg; $MgSO_4 \cdot 7H_2O$, 246.4 mg; $MnSO_4 \cdot 4H_2O$, 1.114 mg; $ZnSO_4 \cdot 7H_2O$, 1.08 mg; $CuSO_4 \cdot 5H_2O$, 0.5 mg. It was buffered with either MES (morpholineethanesulfonic acid; pK_a, 6.1; used from pH 5.6 to 6.0) or Bis-Tris (bis[2-hydroxyethyl]iminotris[hydroxymethyl] methane, pKa 6.5; used from pH 6.5 to 7.0) at 20 mM. When appropriate, bacteriological agar was used at 1.5% (wt/vol) and media were supplemented with 50 μg of kanamycin sulfate ml^{-1}.

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TABLE 1. Origin and characteristics of *R. meliloti* strains and mutants

Strain identification	Origin	pH sensitivity profile[a]				Growth on 6M agar plates at pH 5.6
		7.0	6.5	6.0	5.6	
WSM419	Acid-tolerant strain isolated from acid soil in Sardinia	+	+	+	+	+
WSM533	Acid-tolerant strain isolated from Sardinia	+	+	+	+	+
WSM539	Acid-tolerant strain isolated from Sardinia	+	+	+	+	+
WSM540	Acid-tolerant strain isolated from Sardinia	+	+	+	+	+
WSM852	Acid-tolerant strain isolated from Greece	+	+	+	+	+
WSM870	Acid-tolerant strain isolated from Greece	+	+	+	+	+
U45	Commercial inoculum strain from Uruguay	+	+	+	+/−	+
CC169	Commercial inoculum strain from South Australia	+	+	+	+/−	+
WSM244	Acid-sensitive strain isolated from Iraq	+	+	+/−	−	−
WSM301	Acid-sensitive strain isolated from Iraq	+	+	+/−	−	−
WSM365	Acid-sensitive strain isolated from Iraq	+	+	+/−	−	−
WSM367	Acid-sensitive strain isolated from Iraq	+	+	+/−	−	−
TG1-6	Transposon Tn*5*-induced acid-sensitive mutant of WSM419	+	+	−	−	−
TG1-11	Transposon Tn*5*-induced acid-sensitive mutant of WSM419	+	+	+/−	−	−
TG2-6	Transposon Tn*5*-induced acid-sensitive mutant of WSM419	+	+	+/−	+/−	+/−
TG5-46	Transposon Tn*5*-induced acid-sensitive mutant of WSM419	+	+	+	−	−

[a] Shows growth in 6M broth buffered at the given pH: +, growth; +/−, very poor growth; −, no growth.

Batch cultures for physiological studies were grown at 28°C in the 6M minimal salts medium of Howieson (8) with arabinose (2 g liter^{-1}) and galactose (2 g liter^{-1}) as the carbon sources and glutamate (0.4 g liter^{-1}) as the nitrogen source. The medium was buffered at the indicated pH with 30 mM MES. The pH was adjusted approximately before autoclaving and more exactly after the addition of separately sterilized vitamins and phosphorous after autoclaving. Calcium was supplied as $CaCl_2$ at a concentration of 0.1, 1.0, or 2.0 mM.

Tn*5* mutagenesis. Insertion mutagenesis of *R. meliloti* WSM419 was carried out using the "protocol for insertion mutagenesis of *Rhizobium* spp. with pGS9" described by Selvaraj and Iyer (29). The *E. coli* donor harboring suicide plasmid pGS9 was J53 (F$^-$ *proA metF*) (7), which was grown on 2× YT supplemented with kanamycin. The selection of Tn*5* mutants was carried out on JMM (pH 7.0) plates supplemented with kanamycin.

Screen for acid sensitivity. Tn*5* insertion mutants of *R. meliloti* WSM419 were screened for acid sensitivity by using sterile toothpicks to transfer individual colonies onto JMM plates buffered at pH 7.0 and 5.6. Isolates displaying poor growth at an acidic pH, but wild-type growth at a neutral pH (relative to the WSM419 parental strain), were further assessed for acid sensitivity. Mutants and WSM419 were grown at 28°C for 3 days in JMM (pH 7.0) liquid media. The cultures were centrifuged and the pellet was suspended in an equal volume of 0.85% NaCl. After a 10^{-2} dilution in 0.85% NaCl, 1-μl samples (about 10^4 cells) were spotted onto JMM plates adjusted to pHs of 7.0, 6.5, 6.0, 5.8, and 5.6. Plates were incubated at 28°C, and colony growth was compared with that of the WSM419 control.

Assessment of growth and survival in acid media. Starter cultures were grown in 6M medium buffered at pH 6.5 (8), and growth was monitored by measuring the optical density (600 nm). Duplicate flasks containing 6M medium at pH 5.6 were inoculated with a sample of starter culture diluted to give an initial density of 10^3 to 10^4 cells ml^{-1}. Flasks were incubated on a gyratory shaker (200 rpm) at 28°C and periodically sampled for a determination of viable counts by plate count on 6M medium (pH 7).

Determination of pH_i. pH_i in *R. meliloti* was determined by two methods: (i) the distribution of isotopically labeled weak acids (21) and (ii) the pH-sensitive fluorescence of intracellularly produced dyes (30). The former technique can be used with either dilute growing cultures or concentrated cell suspensions, whereas the latter method is only applicable to dense cell suspensions. Data reported for pH_i are means of three replicate determinations.

(ia) Growing cells. pH_i was measured in growing cells of *R. meliloti* WSM419 with the radioactively labeled weak acids [2-^{14}C]5,5-dimethyloxazolidine-2,4-dione (DMO) and [1-^{14}C]butyric acid by following their distribution across the cell membrane (21). Cells were grown for 3 to 5 days in a 2-liter flask containing 200 ml of 6 M medium, with 1 mM calcium contained on a gyratory shaker (200 rpm) at 28°C. A 10-ml volume of a cell suspension (A_{600}, 0.4 to 0.6; 0.1 to 0.2 mg of protein ml^{-1}) was quickly transferred from the growth vessel into a prewarmed (28°C) 100-ml flask containing 0.32 mM DMO (120 μCi mol^{-1}) or 5 μM butyric acid (10 μCi mol^{-1}). The suspension was incubated with continuous shaking at 28°C for 1 min and then filtered through a glass fiber filter (Whatman GFC; diameter, 25 mm). The filters were transferred into toluene-Triton X-100 scintillation liquid and assayed for radioactivity in a Packard Tri-Carb liquid scintillation counter. The radioactivity retained on the filters at pH 7 in the presence of carbonyl cyanide 4-trifluoromethoxyphenyl hydrazone was used as the blank. To calculate the concentration of radioactive probe inside cells, the internal volume of the filtered cells was estimated from the amount of cell protein retained on the filters, using the value of 2.15 ml (g of protein)$^{-1}$ to relate cell water to protein.

(ib) Concentrated cells. Cells were grown as described above and were prepared for pH_i measurements by centrifugation at 10,000 × *g* at 25°C and washing with sterile salts medium of the same pH as the growth medium (8). Washed cells were suspended in 1/10 volume of 6M medium adjusted to the appropriate pH. pH_i was determined by using the weak acid probes [^{14}C]DMO and [^{14}C]butyric acid. Isotopically labeled compounds (10 μl) were dispensed into Eppendorf centrifuge tubes (1.5 ml), to which were added washed cells (500 μl; 2 mg of protein ml^{-1}), and the suspension was incubated at 28°C for 10 min. Samples were centrifuged through silicone oil mixtures (0.5 ml) as described by Kashket (11). The supernatant was carefully removed with a pipette, and the pellet was suspended in 0.2 ml of distilled water. Supernatant and pellet fractions were counted for

TABLE 2. Fluorescent dyes used to determine pH_i in *R. meliloti*

Fluorescent dye	Membrane-permeable derivative	Concn (μM)	λ Excitation (nm)	λ Emission (nm)	Reference
Fluorescein	Fluorescein diacetate	20–100	520	435, 490	31
5-Carboxyfluorescein	5-Carboxyfluorescein diacetate	10	479	580	30
6-Carboxyfluorescein	6-Carboxyfluorescein diacetate	10	479	580	30
2′,7′-*bis*(carboxyethol)-5,6′-carboxyfluorescein	[2′,7′-*bis*-(carboxyethyl)-5,6′-carboxyfluorescein, pentaacetoxymethyl ester]	10	500	530	25
DHPN	1,4-Diacetoxy-2,3-dicyanobenzene	40–80	385	455, 512	15

radioactivity (28). The calculation of pH_i was made by substituting the concentration ratio of the weak acid into the Nernst equation (4). Corrections for extracellular counts of the radioactive probes were made as detailed by Kashket (11). The pK_a used for DMO was 6.32 and that used for butyric acid was 4.81. The intracellular volume was measured by using the combined [^{3}H]water, [^{14}C]sucrose, and [^{14}C]inulin technique of Stock et al. (32).

(ii) Fluorescent probes. Cells were grown and prepared as described above. Washed cells were suspended in fresh sterile salts medium buffered at the appropriate pH with MES (30 mM). The suspension was then incubated for 30 min at 28°C with the membrane-permeable derivative of the appropriate fluorescent dye (Table 2). After incubation the cells were centrifuged (3,500 × *g*) and suspended in 1 ml of fresh salts medium in cuvettes. Fluorescence at the appropriate wavelength was recorded using a Farrand MK-1 spectrofluorimeter. The values for pH_i were determined using calibration curves relating pH to the intensity of dye fluorescence (15, 31). For 3,6-dihydroxyphthalonitrile (DHPN) and fluorescein, pH_i was calculated using the ratio of fluorescence intensity at two wavelengths as a function of pH (Table 2). These measurements are independent of the dye concentration (15, 31). For 5-carboxyfluorescein, 6-carboxyfluorescein, and 2′,7′-*bis*(carboxyethol)-5,6′-carboxyfluorescein, pH_i was calculated using established procedures (25, 30).

Analytical methods. Bacterial protein was measured by the Lowry method with bovine serum albumin as the standard.

Radioisotopes. [^{3}H]inulin (72.5 GBq mmol^{-1}), [^{14}C]inulin (185 MBq mmol^{-1}), [^{14}C]sucrose (17.6 GBq mmol^{-1}), [2-^{14}C]DMO (1.85 MBq mmol^{-1}), and [1-^{14}C]butyric acid (43.5 MBq mmol^{-1}) were obtained from Amersham Corp. ^3H_2O (0.67 MBq nmol^{-1}) was from Dupont, NEN Research Products.

Chemicals. 2′,7′-bis(carboxyethol)-5,6′-carboxyfluorescein, 2′,7′-*bis*(carboxyethyl)-5,6′-carboxyfluorescein, pentaacetoxymethyl ester, 5-carboxyfluorescein, 5-carboxyfluorescein diacetate, 6-carboxyfluorescein, 6-carboxyfluorescein diacetate, DHPN, and 1,4-diacetoxy-2,3-dicyanobenzene were obtained from Calbiochem-Behring (Australia). All other chemicals were of analytical grade and were obtained from Sigma Chemical Co.

RESULTS

Measurement of pH_i. Radioactively labeled DMO and butyric acid were selected as the most suitable weak acid probes for measuring pH_i in *R. meliloti* because the pK_as of these acids allowed for dissociation of the probes in the extracellular and intracellular milieu at the external pHs used in this study. In addition, both acids showed negligible nonspecific binding to *R. meliloti* and neither acid was actively transported by *R. meliloti* (data not shown). In a control experiment *R. meliloti* WSM419, CC169, and U45 did not grow when butyric acid was used as a sole carbon source. The above criteria have been identified as important properties for weak acid probes to measure pH_i (3, 21).

The sensitivity of the weak acid method is reduced when the external pH is less than the pK_a of the acid being used to measure pH_i because of the high proportion of the acid present in the undissociated form (3). In this study we used butyric acid to check the results obtained with DMO when the external pH was 6.5 or less. Both weak acid probes gave similar results. For example, at an external pH of 6.90, we found pH_i values in *R. meliloti* WSM419 of 7.35 with DMO and 7.37 with butyric acid.

Several established techniques for assessing the distribution of weak acids across the membrane were examined in this study: filtration of cells through membrane filters (0.45 μm; Millipore Corp.), separation of cells by centrifugation through oil, and cell filtration through glass fiber filters. The first two methods required concentrated cell suspensions, whereas the last method used either dilute cell suspensions or cells sampled from the growth vessel. Filtration through Millipore filters was an unsatisfactory separation method since the filters became easily blocked by low bacterial mass and the values derived for pH_i by this technique varied widely between replicate samples. The most useful method for monitoring the distribution of weak acids in both growing and concentrated cells was filtration through glass fiber filters. In experiments with WSM419, a pH_i of 7.42 was measured using these filters, compared with a pH_i of 7.45 for the same culture determined by centrifugation through oil.

To confirm the results obtained with the weak acid probes, we also measured pH_i by using pH-sensitive fluorescent dyes. This alternate technique has the advantage of allowing for rapid measurements of pH_i and monitoring changes in pH_i (30). However, one problem encountered with this method is the permeability of the bacterial cytoplasmic membrane to internally generated fluorescent compounds (3). It can be difficult to interpret the results from this system (31) when there is a rapid efflux of the dye from cells. We tested five pH-sensitive fluorescent compounds for their suitability for pH_i measurements. The fluorescein-based compounds [fluorescein, 5-carboxyfluorescein, 6-carboxyfluorescein, and 2′,7′-*bis*(carboxyethol)-5,6′-carboxyfluorescein] were retained within four strains of *R. meliloti* (WSM419, WSM540, CC169, and U45) during a 30-min incubation period, and in these cells the pH_i values measured were similar to those obtained using the weak acid probes. The acid-sensitive strains of *R. meliloti* (WSM244, WSM301, WSM365, and WSM367) and several acid-sensitive mutants of WSM419 (TG1-6, TG1-11, and TG2-6) were

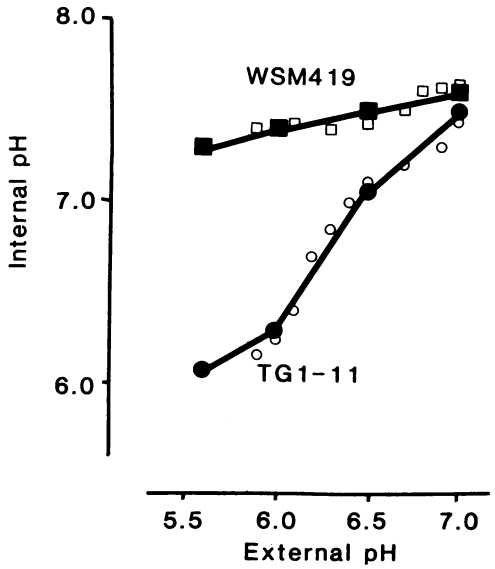

FIG. 1. pH_i in acid-tolerant *R. meliloti* WSM419 and acid-sensitive mutant TG1-11. Cells grown in 6M medium at pH 7.0 were harvested, washed, and suspended in minimal salts medium adjusted to the shown external pH. pH_i was measured using [^{14}C]DMO (■, ●) or the fluorescent dye DHPN (□, ○).

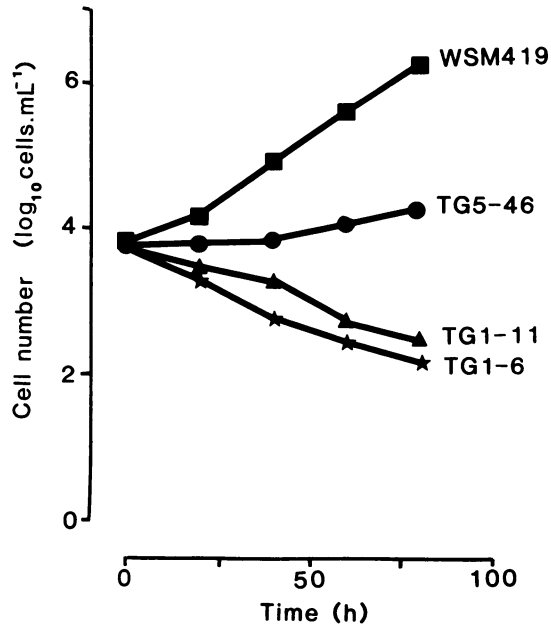

FIG. 2. Growth and survival of *R. meliloti* WSM419 and Tn5-induced mutants (TG5-46, TG1-11, and TG1-6) in solution culture at pH 5.6 as determined by dilution plate counts.

unable to retain these dyes within their cytoplasmic membranes.

The fluorescent dye DHPN was, in contrast, retained inside *R. meliloti* cells with negligible efflux during a 30-min incubation, and this compound was an efficient probe for pH_i in the strains of *R. meliloti* studied here. DHPN was used to validate the results obtained with the weak acid probes, and in all cases similar values for pH_i were recorded by both methods (Fig. 1).

pH_i of acid-tolerant and acid-sensitive strains of *R. meliloti*. We investigated the ability of strains of *R. meliloti* isolated from nodulated *Medicago* spp. growing on acid soils in Sardinia and Greece to control pH_i when exposed to acid conditions (pH 5.6 to 7.0). The four Sardinian strains (WSM419, WSM533, WSM539, and WSM540) and two Greek strains (WSM852 and WSM870) maintained pH_i in the range of 7.20 to 7.64 when the external pH was varied between 5.6 and 7.2 (Table 3).

TABLE 3. Field performance and pH_i of strains of *R. meliloti*

Strain	Field performance[a] in acid soil at pH:		pH_i at external pH of:			
	4.8	5.0	5.6	6.0	6.5	7.0
WSM419	20	36	7.25	7.35	7.43	7.57
WSM533	14	ND	7.21	7.37	7.47	7.51
WSM539	15	ND	7.27	7.31	7.52	7.64
WSM540	40	ND	7.20	7.36	7.41	7.50
WSM852	ND	ND	7.23	7.32	7.38	7.54
WSM870	ND	ND	7.31	7.41	7.48	7.56
U45	ND	16	6.15	6.56	7.21	7.42
CC169	ND	12	6.52	6.65	7.24	7.41
WSM244	ND	9	6.24	6.71	7.18	7.21
WSM301	ND	ND	6.15	6.45	7.25	7.34
WSM365	ND	ND	5.92	6.11	7.20	7.45
WSM367	ND	ND	6.31	6.32	7.12	7.18

[a] Percentage of plants nodulated in the region 11 to 20 cm from the initial point of inoculation 1 year previously (9, 10). Soil pH was measured using a 1:5 (wt/vol) mixture in 0.01 M $CaCl_2$. ND, Not determined.

Two commercial inoculant strains (CC169 and U45) had less control of pH_i under acid conditions than did the acid-tolerant Sardinian and Greek strains. Strain CC169 maintained pH_i between 7.24 and 7.41 when the external pH was in the range of 6.5 to 7.0. However, when the external pH was 5.6 to 6.0, pH_i values in CC169 decreased to 6.52 to 6.65 (Table 3). Strain U45 had similar pH_i levels (7.21 to 7.42) in solutions at pH 6.5 to 7.0, but this strain showed much less control of pH_i in solutions more acidic than pH 6.0 than did strain CC169. Apparently, strains CC169 and U45 are less able to control pH_i under acid conditions than are the acid-tolerant Sardinian and Greek strains.

Many strains of *R. meliloti* are more acid sensitive than is strain U45 (26, 27). We examined the maintenance of pH_i in four of these strains (WSM244, WSM301, WSM365, and WSM367) isolated from alkaline soils in Iraq and which were unable to grow at pH 6.0. Cultures of these four strains were grown in media buffered at pH 7.0, and cells were exposed to moderately acidic buffered solutions. When the external pH was greater than pH 6.5, all four strains showed an alkaline pH_i (7.12 to 7.34) (Table 3). However, when these strains were exposed to media buffered at pH 6.0, their pH_i levels decreased to 6.32 to 6.71 (Table 3). At an external pH of 5.6, the pH_i levels in these four strains were between 5.92 and 6.31. It is evident that these four acid-sensitive strains display only a very limited ability to control pH_i when exposed to acid solutions.

Acid-sensitive mutants of *R. meliloti* WSM419. Four Tn5-induced acid-sensitive mutants of *R. meliloti* WSM419 grew as well as did the parent strain (WSM419) in media buffered at pH 7.0, with mean generation times of 3.4 to 3.7 h. The mutants showed considerable variation in their growth and survival in acid media (Fig. 2). Mutant TG5-46 did not grow in broth culture at pH 5.6, but apparently survived at this pH since the number of viable cells did not decline during a

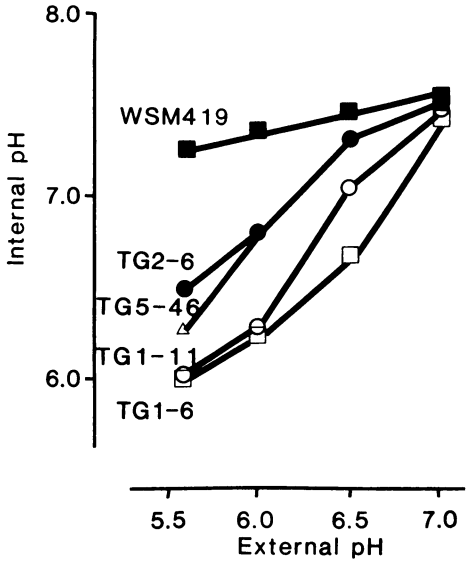

FIG. 3. pH_i in acid-tolerant *R. meliloti* WSM419 and acid-sensitive mutants TG2-6, TG5-46, TG1-11, and TG1-6. Cells grown in 6M medium at pH 7.0 were harvested, washed, and suspended in minimal salts medium adjusted to the shown external pH. pH_i was measured using [^{14}C]DMO and the fluorescent dye DHPN; both probes gave similar values. Points shown are means of three replicate measurements. Standard errors are less than 0.05 pH unit in each case.

4-day incubation (Fig. 2). Two mutants (TG1-11 and TG1-6) were markedly acid sensitive and did not survive at pH 5.6 (Fig. 2).

At pH 7.0 the mutants all had alkaline pH_i levels similar to that of strain WSM419 (Fig. 3). However, all four mutants were impaired in their ability to control pH_i under acid conditions (Fig. 3). None was capable of maintaining a pH_i of 7.0 when the external pH was $\geq$6.0. The two mutants which were unable to survive at pH 5.6 (TG1-11 and TG1-6) had the least control over pH_i, and in these mutants the pH_i levels approached the pH of the external media when the external pH was less than 6.5 (Fig. 3).

Effect of calcium on pH_i in *R. meliloti*. The growth rates of the four acid-tolerant Sardinian strains used in this study were increased by millimolar levels of calcium. Strain WSM419 grown at pH 7.0 with 0.1 mM calcium had a mean generation time of 5.0 h, whereas with 2.0 mM calcium a mean generation time of 2.8 h was observed. The effect of calcium on pH_i control in these strains was investigated; the maintenance of pH_i was not affected by growth in low concentrations of calcium. The growth rates of strains CC169, U45, and WSM244 (3.1, 2.9, and 3.2 h, respectively) are not affected by calcium in the 0.1 to 2.0 mM range (data not shown), and similarly pH_i maintenance was not altered by calcium in this range. In contrast, the acid-sensitive mutants of WSM419 did show an interaction between the calcium concentration and pH_i maintenance. These strains were less able to maintain pH_i when grown with 0.1 mM calcium than with 2.0 mM calcium (Table 4).

TABLE 4. pH_i of *R. meliloti* WSM419 and acid-sensitive mutants grown with different concentrations of calcium

Strain	Calcium concn (mM)	pH_i at external pH of:			
		5.6	6.0	6.5	7.0
WSM419	0.1	7.25	7.45	7.42	7.56
	1.0	7.25	7.35	7.43	7.57
	2.0	7.21	7.42	7.45	7.53
TG1-6	0.1	5.80	6.12	6.15	7.25
	1.0	5.95	6.32	6.51	7.35
	2.0	5.95	6.32	6.51	7.35
TG1-11	0.1	6.01	6.18	6.25	7.41
	1.0	6.07	6.29	7.01	7.45
	2.0	6.05	6.31	7.00	7.40
TG2-6	0.1	6.00	6.51	7.15	7.45
	1.0	6.51	6.82	7.31	7.51
	2.0	6.55	6.85	7.25	7.48
TG5-46	0.1	6.15	6.32	6.95	7.45
	1.0	6.24	6.87	7.27	7.47
	2.0	6.27	6.84	7.20	7.50

DISCUSSION

Acid-tolerant strains of *R. meliloti* of Sardinian and Greek origin generated a pH gradient under acid conditions and always maintained an alkaline interior. In contrast, strains of *R. meliloti* and mutants of WSM419 sensitive to acidity were unable to control pH_i and only maintained a small pH gradient in acid solutions. These results suggest that cellular regulation of cytoplasmic pH is necessary for the growth of *R. meliloti* in acid environments. It appears that the acid sensitivity of many strains of *R. meliloti* may be related to their inability to regulate internal pH.

Seven of the strains used in the present study have been assessed previously for field performance with *Medicago* spp. in acid soils, using the criterion of nodulation on plants sown a year later 11 to 20 cm from inoculated plants (9, 10). A comparison of those results with the work reported here shows that strains of *R. meliloti* incapable of maintaining an alkaline pH_i in acid media also perform poorly with *Medicago* spp. in acid soils. The acid-sensitive Iraqi strain WSM244 does not have an alkaline pH_i when the external pH is less than 6.5, and this strain is a very poor symbiont for medics in acid soils. The two commercial inoculant strains, CC169 and U45, also do not show a tight regulation of cytoplasmic pH, and neither strain performs well in acid soils. In contrast, the four acid-tolerant Sardinian strains (WSM419, WSM533, WSM539, and WSM540), which maintain pH_i at alkaline levels at pH 5.6, have superior field performance with *Medicago* spp. in soils at pH 4.8 (10). The ability of the Sardinian strains to maintain alkaline pH_i may thus be an important characteristic for their survival in acid soils.

Although the Sardinian strains are considerably more acid tolerant in the field than are other acid-sensitive strains, the acid-tolerant strains do show variation in their field performance in acid soils (10): strains WSM419 and WSM540 perform better than do strains WSM533 and WSM539. However, since all four strains maintain similar pH_i levels under acid conditions, other factors must be responsible for the variation in field performance.

Screening for growth on an acidified medium has not identified strains of *R. meliloti* capable of improved symbiotic performance in acid soils (16). The two commercial inoculant strains, CC169 and U45, show growth on agar plates at pH 5.6 (10), but such growth does not indicate useful tolerance of acidity since they grow poorly at pH 5.6 in broth. The growth of these two strains on acidic solid

media may be due to the ability of the growing colony to modify the local environment and protect itself from exposure to the pH at the agar surface. The present results show that CC169 and U45 do not maintain an alkaline pH_i under acid conditions, and the poor control of cytoplasmic pH in these strains may account for their poor growth in broth cultures at pH 5.6 (8). It appears that an understanding of the control of pH_i in *R. meliloti* may provide a better indicator of strain performance in acid soils than does an assessment of colony growth on acidic agar plates.

All strains of *R. meliloti* tested in this study showed alkaline pH_i levels under near-neutral conditions; a similar value (pH_i 7.44) has been reported for an unnamed strain of *R. meliloti* incubated at pH 7.0 (12). These results contrast with the study by Tremblay and Miller (33), who reported a constant pH_i of 6.5 in *R. meliloti* 102 F70 over the range of external pHs of 5.0 to 9.0. The value is lower than those usually reported for gram-negative bacteria (12), and its constancy over such a wide pH range is surprising. In the latter study (33) pH_i was assessed under the ^{31}P nuclear magnetic resonance of intracellular phosphate ions, whereas pH_i was assessed in the work reported here and by Kashket (12) using radioactively labeled weak-acid probes. We also used fluorescent probes as an alternative method and found similar alkaline values for pH_i under neutral conditions. Previous work with *E. coli* has shown good agreement in the values of pH_i assessed by these three methods (21). The finding of a constant pH_i of 6.5 in *R. meliloti* 120 F70 may indicate that this strain has a much tighter control of cytoplasmic pH than do the *R. meliloti* strains studied here and by Kashket (12).

Not all strains of *R. meliloti* examined in the present study maintained a constant pH_i as the external pH varied between 5.6 and 7.2. A similar variation in pH_i with a change in external pH has been shown in *Bradyrhizobium* spp. (3, 5). The pH_i of *Bradyrhizobium* sp. strain 32H1 varied from 7.6 to 8.9 as the external pH increased from 6.1 to 8.45 during batch growth (5). Washed cells of *Bradyrhizobium japonicum* CC705 grown with 5% O_2–95% N_2 and 10 mM KNO_3 had internal pH values of between 7.2 and 8.0 when the external pH ranged between 6.0 and 8.0 (2). Kashket (12) has suggested, on the basis of these results, that *Bradyrhizobium* spp. may have only a limited ability to tightly regulate the internal pH as the external pH varies. Further work is necessary with other *Rhizobium* species to examine their control of cytoplasmic pH before a general assessment can be made about this characteristic in the root nodule bacteria.

The four acid-sensitive mutants of WSM419 studied here were unable to maintain their cytoplasmic pH at alkaline levels under acid conditions. Different systems are thought to be involved in the generation of a pH gradient in neutrophiles under acid or alkaline conditions, and the acid-sensitive mutants appear to be affected in their system that raises pH_i. The mechanisms involved in pH homeostasis are not clearly understood, but studies with a number of organisms have identified potassium uptake as a major means by which a pH gradient, inside alkaline, might be generated (3).

The mechanism by which calcium affects the ability of the acid-sensitive mutants to generate a pH gradient is not known. Calcium is involved in rhizobial cell walls (19), and cytoplasmic calcium may affect pH_i maintenance through effects on ion transport systems (3). In this study the finding in the acid-sensitive mutants of an effect of calcium on pH_i indicates a possible impairment of their calcium transport systems. Further work assessing the effect of pH and calcium on cation transport will be required to determine the role of ion transport systems in pH_i maintenance in *R. meliloti*.

The ability of acid-tolerant strains of *R. meliloti* to regulate their cytoplasmic pH appears to be a necessary requirement for growth under acid conditions. The ability of *R. meliloti* to colonize moderately acid soils is essential for the establishment of medic pastures in these soils. It appears that a vital characteristic for acid tolerance in *R. meliloti* is the ability to control pH_i and maintain an alkaline cytoplasm when the external pH is acidic.

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