Acid pH tolerance in strains of *Rhizobium* and *Bradyrhizobium*, and initial studies on the basis for acid tolerance of *Rhizobium tropici* UMR1899[1]

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Received October 12, 1993
Revision received January 5, 1994
Accepted January 6, 1994

GRAHAM, P.H., DRAEGER, K.J., FERREY, M.L., CONROY, M.J., HAMMER, B.E., MARTINEZ, E., AARONS, S.R., and QUINTO, C. 1994. Acid pH tolerance in strains of *Rhizobium* and *Bradyrhizobium*, and initial studies on the basis for acid tolerance of *Rhizobium tropici* UMR1899. Can. J. Microbiol. **40**: 198–207.

Acid pH limits the persistence of *Rhizobium* strains in soil, and the nodulation and nitrogen fixation of legumes. To identify acid-tolerant strains, we tested the ability of 45 *Rhizobium*, *Azorhizobium*, and *Bradyrhizobium* strains to produce isolated colonies on agar medium of pH 4.00 to pH 7.00. Only *Rhizobium tropici* UMR1899 (=CIAT899) grew at pH 4.00 in unbuffered medium, though 6 strains of *R. tropici* and 3 *Bradyrhizobium* strains grew at pH 4.25, and 15 strains grew at pH 4.50. Tolerance to acid pH in *R. tropici* UMR1899 was not an adaptive response, nor was it plasmid mediated, correlated with the production of extracellular polysaccharide, or related to synthesis of polyamines in the cell. When UMR1899 was grown in buffered medium at acid pH, it maintained a ΔpH (measured using ^{31}P-NMR) of up to 1.7 pH units. However, when this strain was subjected to acid shock, it showed only limited ability to regulate cytoplasmic pH in the short term. Cells of UMR1899 accumulated glutamate under pH stress, and were markedly hydrophobic and resistant to the effects of crystal violet, the latter traits raising the possibility that outer membrane composition and structure could also be a factor in pH tolerance.

Key words: *Rhizobium*, pH tolerance, glutamate, hydrophobicity, ^{31}P-NMR.

GRAHAM, P.H., DRAEGER, K.J., FERREY, M.L., CONROY, M.J., HAMMER, B.E., MARTINEZ, E., AARONS, S.R., et QUINTO, C. 1994. Acid pH tolerance in strains of *Rhizobium* and *Bradyrhizobium*, and initial studies on the basis for acid tolerance of *Rhizobium tropici* UMR1899. Can. J. Microbiol. **40** : 198–207.

Un pH acide limite la persistance de souches de *Rhizobium* dans un sol, ainsi que la nodulation des légumineuses et la fixation d'azote. Pour identifier les souches tolérantes aux pH acides, 45 souches de *Rhizobium*, *Azorhizobium* et *Bradyrhizobium* ont été testées pour leur aptitude à produire des colonies isolées sur milieux gélosés de pH 4,00 à 7,00. Le *Rhizobium tropici* UMR1899 (=CIAT899) a été la seule souche capable de croître à pH 4,00 sur un milieu non additionné d'une solution tampon, bien que 6 souches de *R. tropici* et 3 souches de *Bradyrhizobium* ont pu croître à pH 4,25, et 15 autres à pH 4,50. La tolérance aux pH acides de *R. tropici* UMR1899 n'a pas été une réponse d'adaptation, ni a-t-elle été assurée par voie d'un plasmide, corrélée à la production d'un polysaccharide extracellulaire ou reliée à la synthèse de polyamines intracellulaires. Lorsque l'UMR1899 a été cultivée sur un milieu tamponné à pH acide, elle a maintenu un ΔpH (mesuré à l'acide de résonance magnétique nucléaire : NMR-^{31}P) jusqu'à 1,7 unités de pH. Toutefois, lorsque cette souche a été soumise à un choc acide, elle n'a présenté qu'une aptitude limitée à réguler à court terme le pH cytoplasmique. Sous stress de pH, les cellules d'UMR1899 ont accumulé du glutamate et se sont révélées fortement hydrophobes et résistantes aux effets du cristal-violet, soulevant par ces derniers traits la possibilité que la composition et la structure de la membrane externe pourrait aussi être un facteur de tolérance au pH.

Mots clés : *Rhizobium*, tolérance au pH, glutamate, hydrophobicité, NMR-^{31}P.

[Traduit par la Rédaction]

Introduction

Soil acidity constrains symbiotic nitrogen fixation in both tropical and temperate soils (Graham 1981; Munns and Franco 1982; Munns 1986), limiting *Rhizobium* survival and persistence in soils and reducing nodulation (Graham et al. 1982; Hartel and Alexander 1983; Brockwell et al. 1991). Recently, strains of rhizobia with higher tolerance to hydrogen ion concentration have been identified (Date and Halliday 1979; Keyser and Munns 1979*a*, 1979*b*; Cooper 1982; Graham et al. 1982; Lowendorf and Alexander 1983; Howieson et al. 1988; Richardson and Simpson 1989). These strains have usually, but

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not always, performed better under acid-soil conditions in the field. Thus, Keyser et al. (1979) successfully identified about 65% of the cowpea strains that were symbiotically acid sensitive through their inability to grow in culture medium of pH 4.5, and Graham et al. (1982) obtained a strong response to inoculation with an acid-tolerant bean strain under conditions where inoculation with an equally effective acid-sensitive strain gave no response. Host–strain interactions at acid pH have also been reported (Howieson and Ewing 1986; Vargas and Graham 1988).

The basis for differences in pH tolerance among strains of *Rhizobium* and *Bradyrhizobium* is still not clear, though several workers have shown that the cytoplasmic pH of acid-tolerant strains is less affected by external acidity (Gober and Kashket 1984; O'Hara et al. 1989; Goss et al. 1990; Chen et al. 1993*a*, 1993*b*), and Aarons and Graham (1991) reported high cytoplasmic potassium levels in acid-stressed cells. Differences in lipopolysaccharide composition and in proton exclusion and extrusion (Chen et al. 1993*a*, 1993*b*), and the accumulation of cellular polyamines (Fujihara and Yoneyama 1993), have also been associated with the growth of cells at acid pH. Several groups have produced acid-sensitive Tn*5* pH mutants (Goss et al. 1990; Aarons and Graham 1991; Chen et al. 1993*b*).

A range of inoculant cell densities and media differing in composition and buffering capacity were used in these studies, possibly influencing apparent pH tolerance (Date and Halliday 1979; Keyser and Munns 1979*b*). In this study we compare the relative pH tolerance of selected rhizobia and bradyrhizobia, using an agar plate method and low initial cell numbers, then study the characteristics of the most acid-tolerant strain, *Rhizobium tropici* UMR1899 (=CIAT899), under acid stress. Several of the procedures used were modified from studies on acid pH tolerance in *Salmonella typhimurium*. In this organism acid pH tolerance is an adaptive response (Foster and Hall 1990), accompanied by changes in hydrophobicity and tolerance to crystal violet (Leyer and Johnson 1993).

Materials and methods

Rhizobium, Azorhizobium, *and* Bradyrhizobium *strains*

Wild-type *Rhizobium*, *Azorhizobium*, and *Bradyrhizobium* strains used in this study are shown in Table 1. Many of the strains had been used in other studies and previously cited as being acid tolerant. Isolates of *Agrobacterium tumefaciens* GMI9023, harboring plasmids from *Rhizobium tropici* UMR1026, were from the study of Martinez et al. (1987), while the mutants of UMR1899 each cured of specific plasmids and the acid-sensitive Tn*5* mutants of UMR1899 (UMR5005 and UMR5018) were kindly provided by Drs. C. Quinto and S.R. Aarons, respectively. All strains except UMR5005 and UMR5018 were routinely maintained on yeast extract mannitol (YEM) medium (Vincent 1970): these two strains were grown on YEM medium with 50 μg/mL kanamycin.

Evaluation of relative pH tolerance

For the determination of tolerance to acid pH, strains were inoculated into YEM broth and grown for 48 h at 28°C. Cell density was then determined using a Petroff–Hausser bacterial counter (Hausser Scientific, Bluebell, Pa.) and adjusted to only 10^3 cells/mL with sterile water. One drop (25–30 μL) was then added to the surface of plates of modified Keyser–Munns (MKM) medium (Graham et al. 1982), the pH of which had been adjusted after autoclaving, to values from pH 4.00 to 7.00. Five replicate plates were prepared for each pH–strain combination. Plates were incubated at 28°C, and strains were reported as growing at a particular pH on the day when 15–30 individual colonies were discernible on all five plates. When some strains grew at pH levels less than pH 4.5, the experiment was repeated with selected strains, but using MKM medium buffered with either 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), *cis*-5-norbornene-*endo*-2,3-dicarboxylic acid (NEDA, Aldrich Chemical Co., Milwaukee, Wis.; pK_1 4.36; pK_2 7.09), or mellitic acid (Aldrich; pK_4 4.44; pK_5 5.50). The effect of nitrogen source (glutamate, lysine, ammonium nitrate, ornithine, γ-aminobenzoic acid, citrulline, and arginine at equivalent concentration of N) on pH tolerance was also determined.

Adaptive shock response

To determine whether acid pH tolerance in *Rhizobium* strains required a period of adaptation, UMR1899 and UMR1632 were evaluated for adaptation to acid tolerance by means of the procedure of Foster and Hall (1990), with the modification that the survivors of acid treatment were plated on MKM medium.

Determination of hydrophobicity

To determine whether acid-tolerant and acid-sensitive *Rhizobium* cells differ in hydrophobicity, the procedure of Rosenberg et al. (1980) was used. Broth cultures (24 h) of each organism in tryptone yeast broth (TYB) were centrifuged and washed, then resuspended in MKM medium containing 10 mM MES and 10 mM HEPES, at pH values of 4.50, 5.50, 6.50, or 7.50. Although pH 4.5 is beyond the effective buffering range of MES–HEPES, inclusion of these buffers in MKM pH 4.50 medium appeared to reduce the rate of pH change, and meant that the medium composition was the same in all cases. Cells were then grown for 6 h at 24°C, prior to treatment with hexadecane.

Resistance to crystal violet

The procedure used was that of Leyer and Johnson (1993), with crystal violet treated cells plated onto YEM agar and counted after 4 days incubation at 28°C.

Polysaccharide production

Polysaccharide production was determined using the procedure of Cunningham and Munns (1984).

Characterization of cellular lipopolysaccharides

Cells were prepared, subjected to SDS-PAGE, and silver stained as described by Cava et al. (1989).

Glucose and glutamate metabolism

Cells of UMR1899 and UMR1632 were grown overnight in TYB medium (Sambrook et al. 1989), centrifuged, and washed in phosphate-buffered saline, then resuspended in a medium derived from MKM, but with glucose as the energy source in place of glycerol. The medium contained 10 mM MES and 10 mM HEPES, as mentioned above, at pH values of 4.5, 5.5, 6.5, or 7.5. Initial inoculant density was 5×10^8 cells/mL. Cells were incubated at 28°C, with samples taken at 30-min intervals and evaluated for intracellular and extracellular glucose and glutamate concentrations. Glucose concentrations were determined using the glucose oxidase Trinder assay (Sigma Chemical Co., St. Louis, Mo), while glutamate concentrations were determined using a method modified from that of Botsford and Lewis (1990) and employing bovine glutamate dehydrogenase and acetylpyridine dinucleotide.

Polyamine production under acid stress

To determine if polyamine production was a factor in the acid tolerance of UMR1899, cells of UMR1899 and of the acid-sensitive strain UMR1632 were grown overnight in MKM medium, pH 7.0, aseptically centrifuged, and washed, then reinoculated into MKM medium containing 10 mM HEPES and 10 mM MES at pH values of 4.5, 5.0, 5.5, 6.0, or 7.0. Cells were incubated overnight on a rotary shaker at 25°C. Fifteen-millilitre amounts were centrifuged, washed once in phosphate-buffered saline, and extracted overnight in 2 mL 0.4 M perchloric acid (Ponappa et al. 1992), and then centrifuged, and the supernatant was stored at −20°C until assayed. Cell extracts were dansylated using the procedure of T. Ponappa (personal communication). For this procedure, 0.2 mL of the perchloric acid extract was amended with 0.4 mL of a solution containing 5 mg/mL dansyl chloride in acetone and with 0.2 mL saturated sodium carbonate, then briefly vortexed. The mixture was incubated in a shaking water bath for 60 min at 60°C, then 0.1 mL of 100 mg/mL proline was added,

TABLE 1. Days required for visible colony development of selected *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium* strains following growth on agar medium of pH 4.00–7.00

University of Minnesota strain	Synonym	Medium pH							
		4.00	4.25	4.50	4.75	5.00	5.50	6.00	7.00
Rhizobium etli									
1116		—	—	—	10	5	5	4	4
1165		—	—	—	—	4	3	3	3
1173		—	—	—	—	6	4	3	3
1376		—	—	—	9	6	3	3	3
1384		—	—	—	—	6	3	3	3
1632	CIAT632	—	—	—	—	5	5	4	3
Rhizobium tropici									
1026	CNF 299[a]	—	5	4	3	3	3	3	3
1073		—	4	3	3	3	3	3	3
1226[a]		—	4	3	3	3	3	3	3
1291	C05	—	5	5	5	4	3	3	3
1410	CIAT166	—	8	6	5	5	4	3	3
1899	CIAT899[b]	6	4	3	3	3	3	3	2
Rhizobium leguminosarum bv. *trifolii*									
3002	Bald1	—	—	—	7	6	3	3	3
3005	WU 95	—	—	—	8	—	3	3	3
3009	TA1	—	—	—	—	7	3	3	3
3021	HP3[c]	—	—	—	—	10	5	5	4
3022	Bel 1202[c]	—	—	—	—	8	4	4	4
3023	Bel 1192T[c]	—	—	—	—	5	4	4	3
Rhizobium meliloti									
3029	WSM 303	—	—	—	—	—	—	—	4
3031	WSM 419[d]	—	—	—	—	—	—	8	4
3032	WSM 541[d]	—	—	—	—	—	—	10	4
3033		—	—	—	—	—	—	—	6
3039	U45[d]	—	—	—	—	—	—	—	4
3040		—	—	—	—	—	—	10	6
Rhizobium loti									
3018	NZP2014[e]	—	—	8	6	5	5	4	3
3019	NZP2037[e]	—	—	9	6	5	5	5	4
Rhizobium sp. (*Cicer*)									
3006		—	—	13	8	8	6	6	4
Azorhizobium caulinodans									
3015	ORS 51	—	—	—	—	—	6	3	3
3008	ORS 53	—	—	—	—	—	5	3	3
3010	ORS 57	—	—	—	—	—	6	3	3
Bradyrhizobium sp.									
25		—	—	—	—	10	7	7	7
30	USDA 110[f]	—	—	15	8	6	5	5	6
62		—	—	—	—	8	7	7	7
122		—	—	—	11	8	7	7	7
123	USDA 123	—	—	—	—	7	8	8	7
127		—	—	13	8	7	7	7	8
191	INPA37	—	—	13	8	7	6	6	6
213	TAL 174[g]	—	15	9	7	7	6	6	6
217	TAL 1393	—	—	—	—	12	6	5	6
218	TAL 1394	—	—	—	9	7	5	5	5
223	IRC353C[h]	—	15	10	7	7	5	5	5
224	IRC353B1[h]	—	12	9	7	7	5	5	6
3012	CB756	—	—	—	12	8	6	7	5
3020	CC8145	—	—	—	—	6	5	5	5
3024	CB1024	—	—	—	—	12	6	6	6

[a] Previously identified as acid tolerant by Vargas and Graham (1988).
[b] Previously identified as acid tolerant by Graham et al. (1982).
[c] Previously identified as acid tolerant by Dr. J. Cooper (personal communication).
[d] Previously identified as acid tolerant by Howieson et al. (1988).
[e] Previously identified as acid tolerant by Cooper et al. (1985).
[f] Previously identified as acid tolerant by Keyser and Munns (1979*a*).
[g] Previously identified as acid tolerant by Keyser and Munns (1979*b*).
[h] Previously identified as acid tolerant by Drs. A. Ayanaba and D. Munns (personal communication).

and the cells were incubated for a further 30 min at 25°C. Toluene (0.5 mL) was added, the mixture was briefly vortexed, and the dansylated polyamines were removed in the organic phase. The dansylated polyamines were subjected to thin-layer chromatography (TLC) in cyclohexane:ethylacetate (5:4), with production of specific polyamines determined by the exposure of the gels to uv light. Results using TLC were confirmed using high-performance liquid chromatography (HPLC) and the procedure of Slocum et al. (1989).

Determination of cytoplasmic pH

Cytoplasmic pH determinations were made using both ^{31}P nuclear magnetic resonance (NMR) spectroscopy and fluorescent intracellular pH probes.

For ^{31}P-NMR, 100 mL MKM broth culture was centrifuged, washed aseptically in fresh unbuffered MKM medium, and then resuspended in 1 L MKM medium containing 10 mM HEPES, 10 mM MES, and 3 mM dipotassium hydrogen phosphate, at pH values from 4.5 to 7.5. The cells were incubated overnight on an incubator shaker at 25°C, then centrifuged, washed in fresh MKM medium, and resuspended at 10^{11} cells/mL in MKM medium containing 3 mM phosphocreatine. Cells were maintained aerated, but on ice until shortly before use, and in each case the pH of the solutions used to wash and resuspend the cells was the same as that used to culture them. ^{31}P-NMR spectra were determined using a 5-T magnet NMR imaging system, with a 40-cm bore, and a frequency of 87.127 MHz. Repetition rates of 1–4 s were used. For experiments in which the effects of pH change were measured, cells were prepared as above, but were grown and resuspended in unbuffered MKM medium of pH 7.25. Following initial ^{31}P measurements at pH 7.25, the cells were shocked by adding predetermined quantities of 0.1 M HCl, with subsequent cytoplasmic pH measurements made at intervals of as little as 45 s, for periods of up to 1 h thereafter. Cytoplasmic pH values were determined from the chemical shift of the inorganic phosphate peak relative to that of phosphocreatine, and by reference to a calibration curve prepared with uninoculated medium of known pH.

Cytoplasmic pH determinations were also made using 5-carboxyfluorescein diacetate (CFDA) and 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein, pentaacetoxymethyl ester (BCECF-AM) as the pH probes, but modifying the procedure of O'Hara et al. (1989) to limit probe leakage. *Rhizobium* cultures were prepared as for NMR analysis, but resuspended to a concentration of 5×10^8 cells/mL in MKM medium of pH 4.00 to pH 7.50 containing 10 mM MES and 10 mM HEPES, plus CFDA or BCECF-AM to 10^{-5} M. Cells were incubated for 30 min at 28°C, then their fluorescent intensity was determined on a Perkin Elmer LS5B luminescence spectrometer using excitation wavelengths of 450 and 490 nm and an emission wavelength of 525 nm. For cells treated with CFDA or BCECF-AM, cytoplasmic pH was calculated from the ratio of fluorescent intensity at 490:450 nm (Graber et al. 1986), and relative to the absorbance of similarly treated solutions of MKM of known pH.

When cells of UMR1899 were shown to have only a limited ability to regulate cytoplasmic pH in the short term, an additional study to confirm this finding was undertaken. Cell suspensions of UMR1899 were prepared as described above, then suspended in buffered MKM medium, pH 6.0–6.7, and incubated for 120 min at 28°C in the presence of 0–25 mM salicylic acid (Slonczewski et al. 1987). Cytoplasmic and external pH changes were then determined as described above.

A plasmid or chromosomal location for genes associated with pH tolerance

To determine if pH tolerance in *R. tropici* was plasmid encoded, variants of UMR1899 that had been cured of either the symbiotic or second megaplasmid, and isolates of *Agrobacterium tumefaciens* GMI9023 carrying plasmids from a second acid-tolerant bean strain, UMR1026 (Martinez et al. 1987), were evaluated for pH tolerance as detailed above.

This point was further studied using UMR5005 and UMR5018, two Tn*5* pH mutants of UMR1899 from the study of Aarons and Graham (1991). Each shows a reversion frequency to pH tolerance of less than 1 in 10^8, with reversion always associated with loss of kanamycin resistance. Cells of these organisms were grown overnight in TY medium with 50 μg/mL kanamycin, then plasmid DNA was separated using in-well lysis and agarose gel electrophoresis (Eckhardt 1978; Rosenberg et al. 1982). DNA prepared from the plasmid pSUP2021 (Simon et al. 1986) and biotin-labelled *Hin*dIII-digested λ DNA were included as standards on each gel. Gels were blotted onto nylon 66 plus (Southern 1975) and hybridized with the 3.31-kb internal Tn*5* fragment from *Hin*dIII-digested pSUP2021, which had been nick translated in the presence of biotin-labelled dATP (N^6-(*N*-biotinyl-6-aminohexyl)-2′deoxyadenosine 5′-triphosphate). The BRL Blugene nonradioactive nucleic acid detection system (Bio-Rad Laboratories, Gaithersburg, Md.) was used to detect Tn*5* insertions.

Results and discussion

Differences in pH tolerance among strains of *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium* are shown in Table 1. In unbuffered medium only *R. tropici* UMR1899 produced individual colonies in medium of pH 4.00. However, six *R. tropici* strains and 3 *Bradyrhizobium* isolates grew at pH 4.25, and 15 strains including two fast-growing *Lotus* isolates grew at pH 4.50.

Within-species differences in pH tolerance were generally consistent with those reported earlier (Keyser and Munns 1979*a*, 1979*b*; Cooper et al. 1985; Howieson et al. 1988). The exception was that strains of *R. tropici* were at least as tolerant to acid pH as were the *Bradyrhizobium* strains tested. We believe that irrespective of the method of tolerance involved, the ability of an isolated cell to grow and produce an individual colony in medium of a particular pH must be the most rigorous test of acid tolerance possible. It is noteworthy that no strain of *Rhizobium etli*, *Rhizobium leguminosarum*, *Rhizobium meliloti*, or *Azorhizobium* grew at pH 4.5 under the conditions of this test, and that the acid-tolerant *R. meliloti* isolates identified by Howieson et al. (1988) proved to be only slightly more acid tolerant than other members of this species.

Howieson (1985) used MES to buffer media used in the identification of acid-tolerant strains of *R. meliloti*. Unfortunately, MES is only an effective buffer from pH 5.5 to pH 6.7, while several alternative buffers (i.e., citrate or glutamate) may be metabolized by rhizobia. Because our initial studies included pH values outside the effective buffering range for MES, they were undertaken without buffers. Mallette (1967) suggested NEDA and mellitic acid as effective buffers in medium of pH 4.5. In a supplementary study undertaken with a subset of strains, neither substance proved to be an effective buffer at this pH. NEDA had little effect on the minimum pH for growth of *Bradyrhizobium* strains, but markedly inhibited growth of *Rhizobium* strains at low pH, while cells incubated with mellitic acid showed poor growth at pH 5.5 and 6.5, though the growth of acid-tolerant strains at pH 4.5 and 5.0 was similar to that obtained in the absence of buffer (data not included). Similarly, there was no effect of any N source used on growth in medium of pH 4.50, though when ammonium nitrate was used in place of glutamate, the pH of the medium declined even further.

The acid pH tolerance in *R. tropici* did not appear to be an adaptive response, as 58% of UMR1899 cells survived exposure to pH 3.3 without prior adaptation, whereas less than 0.001% of *R. etli* UMR1632 cells survived this treatment even after a period of adaptation at pH 5.8. The finding of superior pH tolerance in a group of organisms that is very poorly represented in most collections of bean rhizobia is of both economic and taxonomic

TABLE 2. Days to visible colony development of selected *Rhizobium* and *Agrobacterium* strains grown on agar medium of pH 4.00–7.00

	Medium pH							
	4.00	4.25	4.50	4.75	5.00	5.50	6.00	7.00
Rhizobium tropici								
UMR 1899	6	4	3	3	3	2	2	2
UMR 5005[a]	—	—	—	—	—	9	3	2
UMR 5018[a]	—	—	—	—	—	8	4	2
UMR 1899(pRlp899a)	8	6	4	3	3	3	3	2
UMR 1899(pRlp899b)	—	5	4	3	3	2	2	2
Agrobacterium tumefaciens								
GMI 9023	—	—	—	6	4	4	3	2
GMI 9023(pRlp1026a)	—	—	—	6	3	3	3	2
GMI 9023(pRlp1026b)	—	—	—	6	3	3	3	2
GMI 9023(pRlp1026c)	—	—	—	10	4	3	2	2

[a]Tn*5* pH mutants derived from UMR1899 (Aarons and Graham 1991).

significance, and attempts to obtain further collections are warranted. The screening of nodule isolates for growth at pH 4.5 might provide a simple test by which putative strains of *R. tropici* could be selected for further study.

Tolerance to acid pH in *R. tropici* does not appear to be plasmid mediated. Loss of individual plasmids from UMR1899 did not significantly change the pH range for growth of this strain, though reduced polysaccharide production did increase the time taken for visible colony detection (Table 2). Similarly, the ability of *Agrobacterium tumefaciens* GMI9023 to grow at pH levels less than 4.75 was not enhanced by the transfer to it of plasmids from the acid-tolerant *R. tropici* UMR1026. Further, when Southern blots of plasmid preparations made from the two Tn*5*-containing pH mutants of UMR1899 were probed with a 3.31-kb *Hin*dIII-digested Tn*5* fragment, there was no hybridization to plasmid DNA. Richardson et al. (1988) reported loss of the clover *Sym* plasmid without change in pH tolerance, while A. Glenn (personal communication, 1993) found pH-tolerance genes in *R. meliloti* to be located on the chromosome. Chen et al. (1991) constructed a more efficient acid-tolerant strain by first curing *R. leguminosarum* bv. *trifolii* of its symbiotic plasmid, then transferring to it the plasmid of a more efficient nitrogen-fixing strain. Loss of a second plasmid in this strain did affect pH tolerance to some degree, perhaps through changes in lipopolysaccharide structure (Chen et al. 1993*b*).

Extracellular polysaccharide (EPS) production has previously been correlated with acid pH tolerance in bean rhizobia (Cunningham and Munns 1984) and *Bradyrhizobium* strains (International Institute of Tropical Agriculture 1980, 1981), but not in *R. leguminosarum* bv. *trifolii* (Chen et al. 1993*b*). In the present study, strain UMR1899, and a variant of this strain lacking the plasmid needed for EPS production showed the same pH range for growth (Table 2). Further, cells of UMR1899 showed decreased ability to produce EPS at acid pH, their production declining from 310 mg dry mass of EPS/L at pH 7.5 to essentially 0 mg dry mass/L at pH 4.5. A light flocculent precipitate was recovered from ethanol-treated cultures of UMR1899 at pH 4.5 but this cross reacted serologically with antiserum to UMR1899 somatic antigen, and in SDS-PAGE gave banding patterns typical of *R. tropici* lipopolysaccharide (data not shown). Kannenberg and Brewin (1989) and Sindhu et al. (1990) each reported surface antigens of *R. leguminosarum* affected by pH. Calcium is needed for cell wall integrity in *Rhizobium* strains (Vincent and Colburn 1961; Vincent and Humphrey 1968) and is involved in the response of *R. meliloti* to pH stress (Howieson et al. 1992; Tiwari et al. 1992). Accumulation of lipopolysaccharide in low pH medium might result from incomplete synthesis of the outer membrane, and studies are underway to further examine this possibility.

We undertook a number of studies to identify traits that could contribute to acid pH tolerance in UMR1899. In the majority of these we also tested the response of the acid-sensitive *R. etli* strain UMR1632. Our justification for using this strain in preference to either of the two pH-sensitive Tn*5* mutants from the study of Aarons and Graham (1991) was twofold: (*i*) we will use UMR1632 in experiments to transfer pH tolerance into other species of *Rhizobium*, and (*ii*) pH-sensitive Tn*5* mutants are likely to differ from the wild type in only a single trait, whereas a number of different traits may contribute to acid pH tolerance, as shown below.

When cells of UMR1899 were grown in buffered MKM medium of pH 4.50–7.00 they demonstrated a capacity for long-term regulation of cytoplasmic pH, measured by both ^{31}P-NMR and fluorescence methods, with a ΔpH of up to 1.70 pH units (Fig. 1). This was in contrast to the response of the acid-sensitive strain UMR1632, in which the cytoplasmic pH declined rapidly as the medium pH was reduced (Fig. 1), and soon passed beyond the portion of the ^{31}P-NMR calibration curve at which chemical shift and cytoplasmic pH showed a linear relationship. For this reason it is likely that the cytoplasmic pH values for cells of UMR1632 grown at pH 6.0 or less significantly overestimate the true cytoplasmic pH. Surprisingly, UMR1899 showed only a limited capacity for the short-term regulation of cytoplasmic pH. Thus, when cells were grown, washed, and resuspended in medium of pH 7.25, but then subjected to acid shock, there was a marked drop in cytoplasmic pH, with little recovery in less than 30 min. Figure 2 shows results with ^{31}P-NMR; results were similar when BCECF-AM was used. Again, the acid-sensitive strain UMR1632 showed little ability to regulate cytoplasmic pH.

The cytoplasmic pH values obtained in this study and the response to pH change are each less than those reported by other workers (Tremblay and Miller 1983; Gober and Kashket 1984, 1985; O'Hara et al. 1989). At least two factors could have contributed to this result. (*i*) In this study an energy source was included in the growth medium, and may have generated additional acid loading for the cells. (*ii*) Buffers were not included in the culture medium of any cell preparation to be used

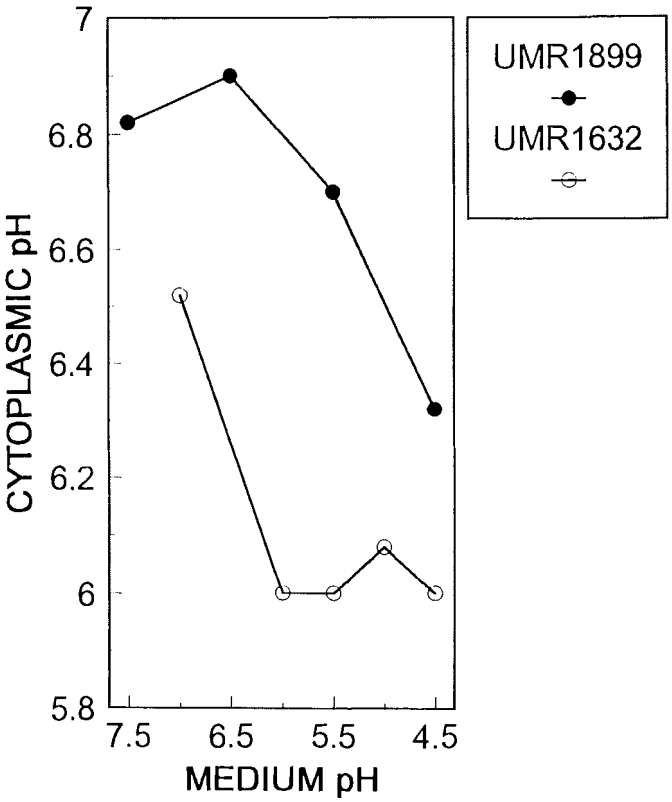

FIG. 1. Cytoplasmic pH values for cells of *R. tropici* UMR1899 and *R. etli* UMR1632 following incubation in buffered culture media differing in pH. The values shown are for ^{31}P-NMR experiments, but similar data were obtained using fluorescent intracellular probes.

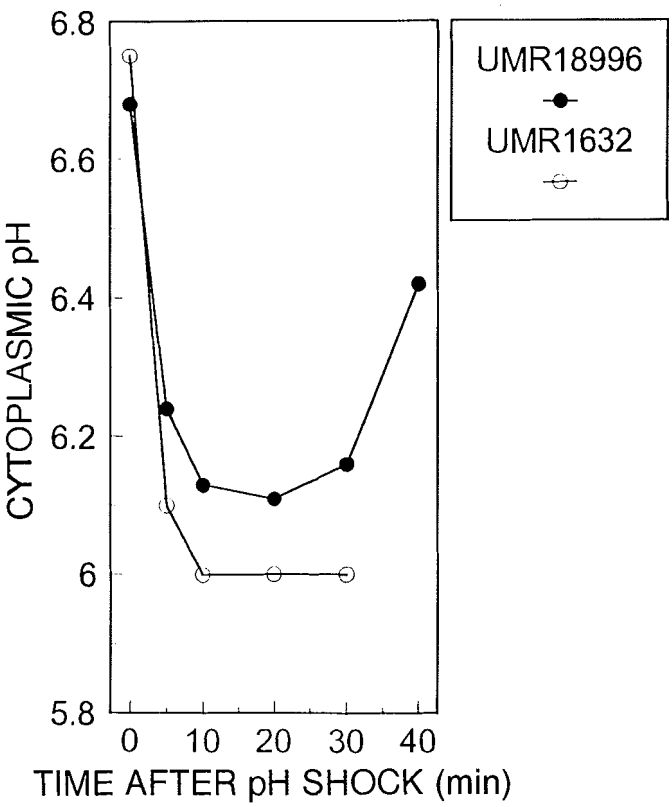

FIG. 2. Influence of acid pH shock on the cytoplasmic pH of *R. tropici* UMR1899 and *R. etli* UMR1632. Cells were grown at pH 7.25, but then were shocked with 0.1 M HCl to a final pH of 4.50. The values shown are for ^{31}P-NMR experiments, but similar data were obtained using fluorescent intracellular probes.

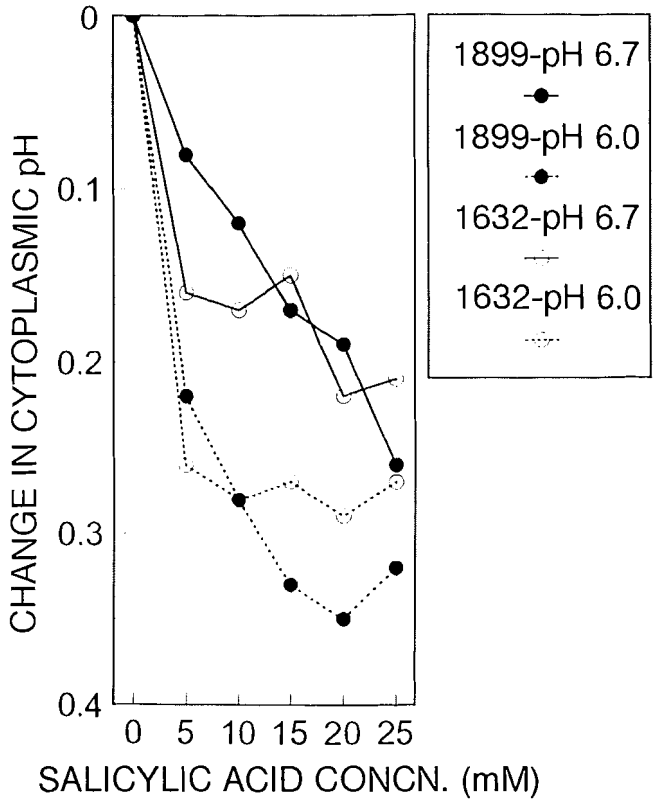

FIG. 3. Influence of salicylic acid addition on the cytoplasmic pH of cells of *R. tropici* UMR1899 (●) and *R. etli* UMR1632 (○) maintained at constant external pH. Cytoplasmic pH values were determined using CFDA as the pH probe. The cells were incubated in media of pH 6.7 (solid lines) and pH 6.0 (broken lines).

for pH shock experiments. Previous studies have assumed that such buffers are not taken up by bacterial cells, but uptake of the Good buffers by procaryotic organisms has been reported (Ferguson et al. 1980), and these buffers may have provided additional buffering capacity within the cell. The limited ability of UMR1899 to regulate cytoplasmic pH in the short term was further evident in the significant pH drop shown with both UMR1899 and UMR1632 following the uptake and intracellular disassociation of salicylic acid (Fig. 3).

Because of the limited ability of UMR1899 to modify cytoplasmic pH in the short term, we sought metabolic changes that could contribute to the ability of UMR1899 to tolerate acid cytoplasmic pH, or permit longer term recovery from pH stress. Aarons and Graham (1991) reported higher potassium levels in cells of UMR1899 following exposure to acid pH. Since glutamate can act as a counter ion for potassium (Booth and Higgins 1990) and has been identified as a compatible solute in osmotically stressed rhizobia (Yelton et al. 1983; Chien et al. 1992), it is not surprising that elevated glutamate levels were found in our studies. Thus, concentrations of cell glutamate in UMR1899 were higher than in UMR1632 at all pH levels, but at pH 4.5 were further increased, reaching levels as much as three times greater than the values obtained with this strain at pH 7.5 (Fig. 4*a*). There was no similar response in UMR1632. Glucose accumulation by cells of UMR1899 was also affected by acidity, and paralleled the data obtained for intracellular glutamate (Fig. 4*b*). Metabolic responses to acid shifts have been reported in other organisms (Goodwin and Zeikus 1987; Marquis et al. 1987; Casiano Colon and Marquis 1988) and are consistent with

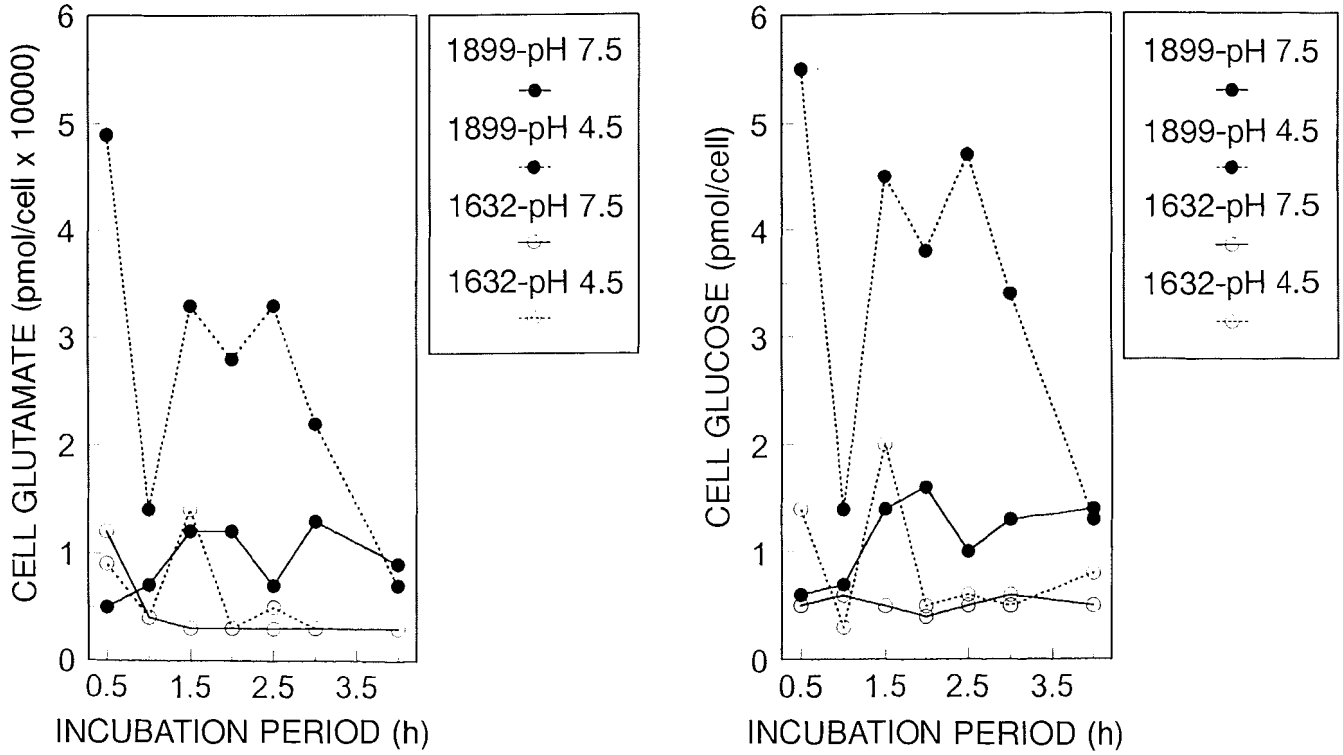

FIG. 4. Influence of medium pH on cell glutamate and cell glucose concentrations for *R. tropici* UMR1899 (●) and *R. etli* UMR1632 (○) incubated at pH 7.5 (solid lines) or pH 4.5 (broken lines).

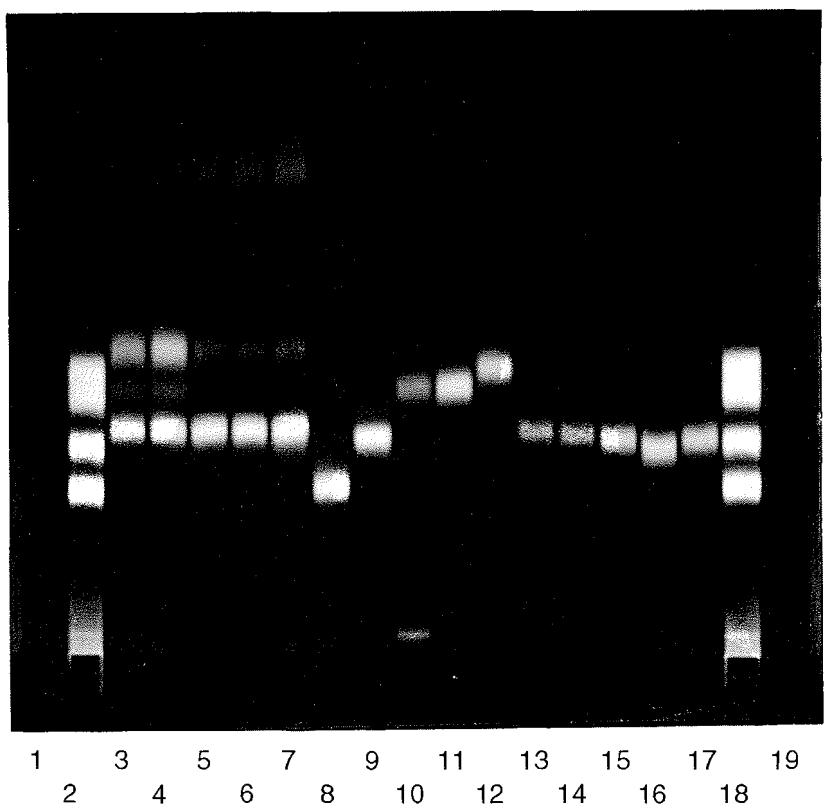

FIG. 5. Influence of medium pH on polyamine production by *R. tropici* UMR1899 and *R. etli* UMR1632. The results are for TLC plates viewed and photographed under uv light. Lane 2 was loaded with a mixture of polyamine standards; lanes 3–7 were loaded with samples from cells of UMR1632 grown at pH 4.5, 5.0, 5.5, 6.0, and 7.0, respectively; lanes 8–12 were loaded with the individual polyamine standards spermine, spermidine, agmatine, cadaverine, and putrescine; lanes 13–17 were loaded with samples from cells of UMR1899 grown at pH 4.5, 5.0, 5.5, 6.0, and 7.0, respectively; and lane 18 was loaded with the polyamine mixture.

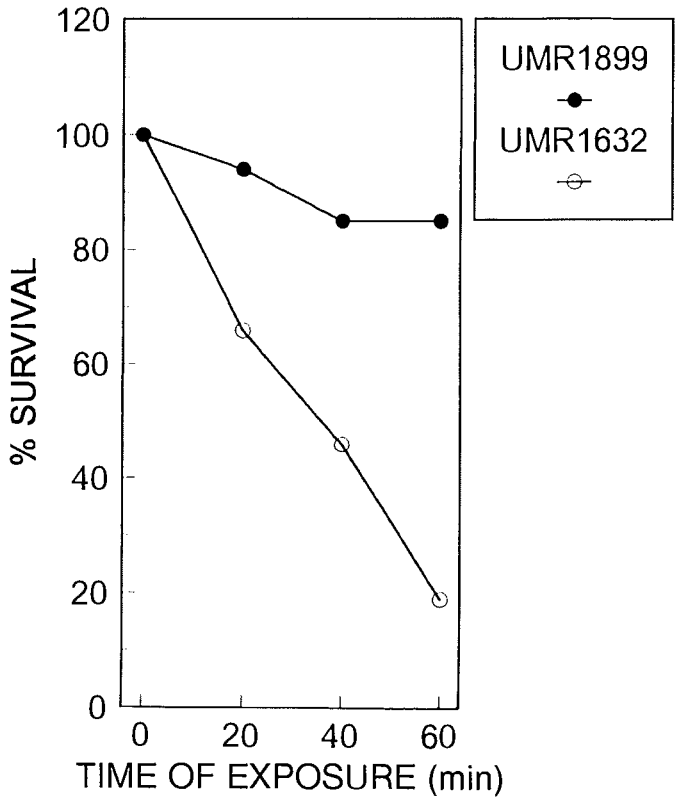

FIG. 6. Percent survival of *R. tropici* UMR1899 and *R. etli* UMR1632 following exposure of cells to crystal violet.

our finding that *R. tropici* UMR1899 has only limited ability to regulate cytoplasmic pH in the short term.

Recent studies in *Escherichia coli* have emphasized the role of lysine decarboxylase in the acidification response (Slonczewski 1992; Watson et al. 1992). Because of these studies and a recent report of enhanced polyamine synthesis by *Rhizobium* cells subjected to acid and osmotic stress (Fujihara and Yoneyama 1993), we undertook studies of polyamine production in UMR1899 and UMR1632 as a function of culture pH. Results are shown in Fig. 5. As reported by Hamana et al. (1990) both UMR1899 and UMR1632 cells contained putrescine, cadaverine, and spermidine or homospermidine, but surprisingly it was the acid-sensitive UMR1632 that showed greater production of polyamines at acid pH. It appears from our results that polyamine production in UMR1632 is a consequence of pH stress, rather than a factor in recovery.

While the deposition of lipopolysaccharide in cultures of UMR1899 that were grown at pH 4.5 and the possibility that lipopolysaccharide incorporation into the cell wall is interrupted at acid pH would argue against the involvement of lipopolysaccaride in the acid pH tolerance of *R. tropici* UMR1899, other outer-membrane components may contribute to the greater pH tolerance of this strain. Thus, when vortexed in hexadecane, UMR1899 cells proved to be highly hydrophobic with 19–25% of cells excluded from the aqueous phase, irrespective of the pH at which the cells were grown. By contrast, less than 1% of UMR1632 cells were not recovered in the water phase. The absence of a pH effect in this study is in contrast with results from *Salmonella typhimurium* (Leyer and Johnson 1993), and again suggests that pH tolerance in UMR1899 is not an adaptive response. Similarly, cells of UMR1899 showed significantly greater tolerance to crystal violet (Fig. 6). The greater hydrophobicity of UMR1899 and its tolerance to crystal violet, a compound whose uptake is affected by the permeability of the outer membrane, indicate that the outer membrane of this organism may play a part in pH tolerance, as reported for acid-adapted cells of *S. typhimurium* by Leyer and Johnson (1993).

This study shows that *R. tropici* strains are a source of pH tolerance in the rhizobia, and provides a precise methodology by which such tolerance can be evaluated. Identification of UMR1899 as a highly acid-tolerant strain opens the way for more detailed genetic and physiological studies of acid pH tolerance in *Rhizobium* strains and for an evaluation of host – *Rhizobium* sp. interactions at acid pH. The identification of glutamate as a compatible solute in acid-stressed cells and the demonstration that cell membrane differences could influence pH tolerance deserve further study. Acid-shock proteins have been identified in UMR1899 (Aarons and Graham 1991), and several clone banks of this organism are available (Vargas et al. 1990; Milner et al. 1992) and could be used for genetic studies on pH tolerance. Rapid advances in our understanding of pH tolerance in the rhizobia are therefore possible, and could lead to significant developments in the resolution of pH stress as a factor limiting nodulation and nitrogen fixation in *Phaseolus vulgaris*.

Acknowledgements

The authors thank J.E. Cooper, R.A. Date, J. Howieson, and H.H. Keyser for *Rhizobium* and *Bradyrhizobium* strains. The senior author is also grateful to F. Sanchez, M. Cevallos, and M. Sadowsky for methodological discussions. This research was funded in part through the U.S. Department of Agriculture – U.S. Agency for International Development (USAID) Limiting Factors program, grant No. 83CRSR-2-2321, and the USAID Title XII Bean–Cowpea Collaborative Research Support program.

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