Effects of organic acids and low pH on *Rhizobium meliloti* 104A14

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In the symbiotic relationship between *Rhizobium meliloti* and alfalfa (*Medicago sativa*), the bacteria are enclosed within the plant cell by a membrane that may function like a plant vacuolar membrane and maintain a pH between 5.5 and 6.0. Free-living *Rhizobium meliloti* 104A14 is sensitive to pH in this range and its sensitivity was influenced by the presence of acetate and other monocarboxylic acids. *R. meliloti* can grow at pH 6.0 in 3 mM succinate but does not grow at pH 6.2 if 10 mM acetate is added. The combination of low pH and acetate is bacteriostatic. Measurement of internal pH (pH_i) using 14C-labelled benzoate as a permeant acid showed that growth inhibition occurs when pH_i falls below 7.15.

Keywords: *Rhizobium meliloti*, pH sensitivity, organic acids

INTRODUCTION

Bacterial acid tolerance depends on the cell maintaining an intracellular pH that is satisfactory for cell functions. Symbiotic, dinitrogen-fixing rhizobia are particularly sensitive to low pH and even moderately acid conditions are able to limit bacterial growth and the initiation of nitrogen-fixing nodules on the roots of host plants (Graham & Parker, 1964; O'Hara *et al.*, 1989). Within the plant cell, the symbiotic bacteria or bacteroids may also have to tolerate relatively acid conditions. Bacteroids are enclosed within a plant-derived symbiosomal membrane and similarities between symbiosomes and plant vacuoles have been found (Mellor, 1989). Acidic pH values between 5.5 and 6.0 are characteristic of vacuoles (Roberts *et al.*, 1980). Transport of protons or singly ionized dicarboxylic acids across the symbiosome membrane has been proposed to acidify symbiosomes (Miller *et al.*, 1988; Udvardi & Kahn, 1992). Like vacuoles, symbiosomes contain a number of acid activated hydrolases (Mellor, 1989). *Rhizobium leguminosarum* bacteroids express an antigen that is also detected in free-living cells grown under acidic conditions (Kannenbergh & Brewin, 1989), which also suggests that bacteroid development occurs at acidic pH.

The low oxygen concentration that occurs in root nodules would be expected to alter pathways of bacterial and plant carbon metabolism and under these conditions it is possible that monocarboxylic acids, such as acetic, pyruvic

or β -hydroxybutyric acid would be produced. Although these acids can be used as carbon sources by rhizobia, in other bacteria monocarboxylic acids have been shown to inhibit various functions, including substrate transport (Dijkhuizen *et al.*, 1980), macromolecular synthesis (Cherrington *et al.*, 1990, 1991) and regulation of cytoplasmic pH (Salmond *et al.*, 1984; Baronofsky *et al.*, 1984; Eklund, 1985). Inhibition is strongest at low pH values, suggesting that the unionized form of the acid plays a role in toxicity (Eklund, 1983). The aim of this work was to study the effect of acetic acid and other organic acids that are potential products of plant or bacterial carbon metabolism on the growth of free-living *Rhizobium meliloti* and on its ability to maintain its intracellular pH.

METHODS

Bacterial strains and culture conditions. *Rhizobium meliloti* strains (Table 1) were maintained on a yeast extract mannitol salt medium (Vincent, 1970). Unless stated, rhizobia were grown in MM minimal medium (per litre: sodium succinate, 0.5 g;

Table 1. *Rhizobium meliloti* strains

Strain	Source or reference
104A14	Somerville & Kahn (1983)
1021	Meade <i>et al.</i> (1982)
102F34	Ditta <i>et al.</i> (1980)
102F28	G. Ditta, San Diego
102F50Str	G. Ditta, San Diego

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NH_4Cl , 0.5 g; K_2HPO_4 , 1.0 g; KH_2PO_4 , 1.0 g; Na_2SO_4 , 0.25 g; $\text{FeCl}_2 \cdot 6\text{H}_2\text{O}$, 0.01 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1 g; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.25 g; biotin, 2 mg; thiamin, 2 mg) at 30 °C on an orbital shaker at 200 r.p.m.

Effect of organic acids on growth. Bacteria were grown in MM and various organic acids were added to the cultures at final concentrations of 10 or 25 mM during early exponential phase. Organic acids were dissolved in water, the pH was adjusted with sodium hydroxide and the solutions were filter sterilized immediately prior to addition. A Corning 150 specific ion meter using a Corning combination electrode was used to determine pH values. Growth was measured by optical density using a Klett–Summerson colorimeter equipped with a green filter. The pH of the cultures was measured periodically by withdrawing small samples. All chemicals, acids and buffers were obtained from Sigma.

Internal pH measurement. A modification of the method of Kashket (1981) was used to determine intracellular pH. Bacteria were grown in MM pH 6.7 until late exponential phase (~50 Klett units), collected by centrifugation at 14000 g at 4 °C, washed twice and resuspended at a cell density corresponding to 200 μg protein ml^{-1} (Bio-Rad) in the appropriate buffer containing 3 mM sodium succinate and other organic acids as indicated. Buffers used to establish the external pH at the indicated pH values were: 0.1 M MES (pH 5.50, 5.78 or 6.01), 0.1 M PIPES (pH 6.44 or 6.83) and 0.1 M MOPS (pH 7.33). The suspension was shaken for 30 min at 30 °C. [^{14}C]Benzoic acid (DuPont-NEN) was added at 0.16 $\mu\text{Ci ml}^{-1}$ (5.92 kBq ml^{-1}) and the incubation was continued for 15 min at 30 °C. Samples (200 μl) were taken and the cells separated from the medium by centrifugation at 10000 r.p.m. for 1 min through 100 μl of silicone oil [Fluid 510/Fluid 550 (Dow-Corning); 50:50, w/w] in 400 μl microfuge tubes containing 15 μl 15% (v/v) perchloric acid at the bottom, using a Fisher microfuge equipped with a swing-out rotor. The radioactivity in 100 μl samples of the aqueous upper layer was used to determine the external concentration of [^{14}C]benzoic acid. The microfuge tubes were then frozen in liquid nitrogen and the tips containing the bacterial cells were removed and transferred into liquid scintillation vials. ACS scintillation cocktail (Amersham) (3 ml) was added and the radioactivity was determined in a Packard Tri-Carb 2000CA scintillation counter. To measure radioactivity that was bound non-specifically to the bacteria rather than contained within them, the cell membranes were permeabilized prior to adding radioactive benzoate by incubating the bacteria for 60 min at 30 °C in the corresponding external pH buffer containing 2% toluene.

The intracellular volume was assumed to be $2.4 \pm 0.2 \mu\text{l}$ (mg protein^{-1}) (Tremblay & Miller, 1984) and the internal pH was calculated by substituting the concentration ratio of benzoic acid as the weak acid into the equations of Maloney *et al.* (1975).

RESULTS AND DISCUSSION

The growth of *R. meliloti* 104A14 on succinate as the sole carbon source was similar at initial pH values of 6.0 and 6.7 but was delayed significantly when the initial pH was 5.5 (Fig. 1). Growth observed in the latter culture was dependent on the gradual rise of pH observed as the culture was incubated; growth and the increase in pH were not seen when 10 mM MES buffer was included in the medium. MES buffer did not affect the growth of cultures at pH 6.0 or 6.7 (data not shown). The addition of acetate to similar cultures (Fig. 2) was inhibitory at lower

pH values – no growth was detected during 120 h of incubation at pH values equal to or lower than 6.2. When the pH values at the time acetate was added were 6.8 and 6.5, the pH of the medium increased during the incubation to 8.7 and 8.1, respectively. When acetate was used as the sole carbon source, growth occurred only at pH values greater than 7.3 (data not shown). In all cultures where growth was seen, pH increases were also detected.

Fig. 1. Growth and pH changes observed in cultures of *R. meliloti* 104A14 started at different pH values in MM medium containing 3 mM succinate as carbon source. The initial pH values were pH 6.7 (Δ); pH 6.0 ($\circ$); pH 5.5 ($\diamond$). One culture contained 10 mM MES buffer pH 5.5 ($\square$).

Fig. 2. Growth curves of *R. meliloti* 101A14 in MM medium to which 10 mM sodium acetate was added at pH values of 6.9 ($\bullet$), 6.5 ($\blacktriangle$) and 6.2 ($\blacksquare$) (arrows).

Fig. 3. Growth curves of *R. meliloti* 104A14 in MM medium to which 10 mM (○) or 25 mM (●) sodium acetate was added at pH values of 6.1, 6.5 and 6.8. The time of acetate addition is shown by the arrows.

Table 2. Growth of *R. meliloti* strains in the presence of acetate

Each strain was streaked on a separate plate of solid MM medium (pH 6.8) containing sodium acetate at the indicated concentrations in a way that produced well separated colonies at the end of the streak. —, No growth after 14 d; ±, visible but tiny colonies after 14 d; +, normal sized colonies after 10 d.

Strain	Sodium acetate concn (mM):				
	50	37.5	25	12.5	6.25
104A14	—	—	—	+	+
Rm1021	—	—	+	+	+
102F34	—	—	+	+	+
102F28	—	—	±	+	+
102F50Str	—	—	—	+	+

Growth inhibition also depended on acetate concentration (Fig. 3). At pH 6.1, both 10 mM and 25 mM acetate were inhibitory in media that contained succinate, but at pH 6.5 growth was observed with 10 mM acetate but not with 25 mM. When acetate was added at pH 6.8 the bacterial growth rate between 15 h (time of addition) and 50 h was higher in the presence of 10 mM acetate than in presence of 25 mM acetate. The growth rate in 25 mM acetate increased after 50 h, and this coincided with the time at which the pH rose above 7.4. The increase in the external pH and the simultaneous alleviation of the inhibition suggests that acetate at low pH is bacteriostatic rather than bacteriocidal. The number of viable cells recovered on yeast extract mannitol agar did not decrease during the

Fig. 4. Effect of sodium acetate at 10 mM (○) or 25 mM (●) on the internal pH of *R. meliloti* 104A14 at various external pH values. ▲, Control with 3 mM succinate only. The regression lines have the values (○) $pH_i = 3.40 + 0.60 pH_o$, $R^2 = 0.99$; (●) $pH_i = 3.16 + 0.59 pH_o$, $R^2 = 0.95$; (▲) $pH_i = 5.82 + 0.23 pH_o$, $R^2 = 0.96$.

period when no growth is observed (data not shown). Bacteriostatic inhibition by acetate had been reported for *Clostridium thermoaceticum* (Baronofsky *et al.*, 1984). The additional acetate available for use as a carbon source probably accounts for the higher yield of these cultures (Fig. 3) compared to those grown in MM medium (Fig. 1). The effect of acetate on the growth of *R. meliloti* strains unrelated to strain 104A14 that are used in other laboratories was determined. Like 104A14, these strains are inhibited by acetate (Table 2), indicating that this sensitivity is not limited to a specific strain. All of these strains grow well in similar concentrations of succinate.

Acetate can cross bacterial cell membranes by passive diffusion as the undissociated acid (Visser & Postma, 1973; Salmond *et al.*, 1984; Baronofsky *et al.*, 1984), releasing H^+ and lowering the internal pH by dissociating within the cells. Growth inhibition could therefore be due to decreased intracellular pH. To test this, the internal pH was determined as a function of external pH and acetate concentration (Fig. 4). In the presence of acetate, the internal pH varied more rapidly as a function of external pH than in controls that lacked acetate. This correlated with the stronger inhibitory effect of acetate at both lower external pH values (Fig. 2) and higher concentrations (Fig. 3).

To study the effect of other monocarboxylic acids on *R. meliloti*, growth and internal pH measurements were made at various external pH values in the presence of 10 and 25 mM of the acid (Table 3). Some of these acids were more effective in lowering the internal pH and inhibiting growth than others. In almost all cases, when a combination of pH, acid species and concentration caused the internal pH to drop below 7.15, growth was inhibited. These results are consistent with others in the literature. In their survey of acid tolerant strains of *R. meliloti* and acid sensitive mutants, O'Hara *et al.* (1989) found that growth was inhibited when the internal pH was below

Table 3. Effect of organic acids on the internal pH of *R. meliloti* 104A14 at various external pH values

Numbers are means (and standard deviations) from three determinations. Numbers in **bold type** indicate pH values where no growth was observed.

Acid (mM)	pK	External pH value:					
		5.50	5.78	6.01	6.44	6.83	7.33
Acetic (10)	4.75	6.64 (0.02)	6.86 (0.07)	7.02 (0.03)	ND	7.49 (0.02)	7.74 (0.08)
Acetic (25)		6.32 (0.05)	6.58 (0.08)	6.75 (0.07)	7.14 (0.01)	7.17 (0.13)	7.42 (0.13)
Butyric (10)	4.81	6.69 (0.02)	7.01 (0.02)	7.18 (0.01)	ND	7.66 (0.06)	7.91 (0.03)
Butyric (25)		6.41 (0.01)	6.66 (0.08)	6.92 (0.07)	7.23 (0.07)	7.58 (0.06)	7.89 (0.10)
DL- β -OH Butyric (10)	4.81	6.80 (0.05)	7.03 (0.04)	7.19 (0.06)	7.44 (0.06)	7.60 (0.06)	7.59 (0.10)
DL- β -OH Butyric (25)		6.59 (0.12)	6.73 (0.06)	6.84 (0.07)	7.27 (0.02)	7.64 (0.14)	7.66 (0.21)
Propionic (10)	4.87	6.56 (0.08)	6.84 (0.05)	7.03 (0.02)	7.29 (0.05)	7.51 (0.01)	7.59 (0.11)
Propionic (25)		6.26 (0.06)	6.47 (0.14)	6.78 (0.18)	7.06 (0.05)	7.40 (0.02)	7.51 (0.14)
Formic (10)	3.75	6.67 (0.03)	6.88 (0.03)	7.00 (0.01)	ND	7.43 (0.03)	7.70 (0.08)
Formic (25)		6.41 (0.06)	6.63 (0.09)	6.80 (0.06)	7.09 (0.06)	7.51 (0.17)	7.64 (0.09)
Glycolic (10)	3.83	6.80 (0.09)	7.02 (0.03)	7.09 (0.15)	7.52 (0.17)	7.77 (0.03)	7.85 (0.17)
Glycolic (25)		6.43 (0.03)	6.71 (0.04)	6.90 (0.13)	7.16 (0.02)	7.66 (0.07)	7.62 (0.16)
Acetoacetic (10)	3.58	6.79 (0.02)	7.08 (0.05)	7.27 (0.14)	7.42 (0.12)	7.48 (0.15)	7.67 (0.17)
Acetoacetic (25)		6.28 (0.03)	6.49 (0.17)	7.03 (0.04)	7.31 (0.03)	7.31 (0.16)	7.43 (0.18)
Control*		7.11 (0.04)	7.17 (0.06)	7.20 (0.04)	ND	7.47 (0.09)	7.50 (0.17)

* All incubations were in the presence of 3 mM succinate.

ND, Not determined.

7.0. Our only exception to this rule was 25 mM propionic acid which was inhibitory at an external pH of 6.8 when the internal pH was still 7.4. We suggest that propionate is toxic for some reason other than its ability to lower intracellular pH.

The nitrogenase reaction consumes protons and produces the base, ammonia. It has been suggested that raising the pH of the symbiosome compartment may be an important consequence of rhizobial nitrogen fixation (Udvardi & Kahn, 1992). The results above show that monocarboxylic acids such as acetic, β -hydroxybutyric and acetoacetic acid can act together with mild acidity like that found in symbiosomes to inhibit the growth of *R. meliloti*. Although the concentrations of these monocarboxylic acids in the alfalfa nodule were not reported in the most recent survey of metabolites (Fougère *et al.*, 1991), benzoic acid is present. If pathways of carbon catabolism lead to the production of acids that can move through membranes in their unionized form and if a low symbiosome pH allows a high enough concentration of these forms to be present, then this synergism could significantly affect bacterial growth and may bias bacterial metabolism toward pathways that either produce fewer organic acids or that consume protons.

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