Water Status of Rhizobia in Relation to their Susceptibility to Desiccation and to their Protection by Montmorillonite

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SUMMARY

The greater susceptibility to desiccation of the *Rhizobium leguminosarum* group of rhizobia compared with the slow-growing rhizobia is related to the different amounts of water retained by these groups of bacteria at any relative vapour pressure, rather than differences in rates of water movement into or out of the bacteria. The higher retention of water by the *R. leguminosarum* group at any relative vapour pressure is related to greater availability of adsorptive surface area, and to higher surface energies resulting in a greater affinity for water. Although the internal osmotic pressure is greater in the slow-growing group than in the *R. leguminosarum* group, it is concluded that differences in internal solute concentrations cannot account for the different adsorption isotherms found for these groups.

Montmorillonitic clay protects the fast-growing *R. leguminosarum* group from the effects of desiccation, but not the slow-growing group. Ca-montmorillonite retains greater quantities of water at any relative vapour pressure than either group of bacteria. Possible mechanisms of protection of the bacteria by the clay are discussed in terms of relative affinities for water under desiccation conditions.

INTRODUCTION

The fast-growing clover, pea and a lotus root-nodule bacteria (referred to subsequently as the *Rhizobium leguminosarum* group) are more susceptible to the effects of desiccation in sandy soil than the slow-growing group of rhizobia. Montmorillonitic clay provides a degree of protection against desiccation damage to the *R. leguminosarum* group but not to the slow-growing group (Marshall, 1964; Bushby & Marshall, 1976). The purpose of the present investigation was to examine these differences between the *R. leguminosarum* and slow-growing groups of rhizobia in terms of the water status of the cells. The two groups of rhizobia have been compared on the basis of the amounts of water retained by the cells over a range of relative vapour pressures. In addition, rates of water loss by rhizobia have been examined and compared with those in other bacteria and a yeast.

METHODS

Organisms and cultural conditions. Cultures used in these experiments are described where appropriate in Results. The rhizobia were grown as described previously (Bushby & Marshall, 1976). *Escherichia coli* M13, *Staphylococcus aureus* M3 and *Bacillus* sp. M70 were grown at 28°C on nutrient broth or agar; *Saccharomyces cerevisiae* M6 was grown at 28°C on malt

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extract broth or agar. *Bacillus* M70 did not form spores after 24 h under the conditions used.

Microbial counts and dimensions. Total counts of micro-organisms were made using a Petroff-Hauser counting chamber and viable counts were made by plating serial decimal dilutions in duplicate or triplicate. The dimensions of individual micro-organisms were measured using a filar micrometer; averages of 20 measurements were used to calculate surface areas and volumes. Rod-shaped bacteria were assumed to be closed cylinders, and the yeast was assumed to be spherical.

Internal osmotic pressures of micro-organisms. The internal osmotic pressures were determined by two methods. (1) Micro-organisms were centrifuged and resuspended in a minimum volume of water. The packed cell volumes were determined by standard haematocrit techniques. Micro-organisms in the suspensions were then disrupted by sonication and/or autoclaving and the cell debris was removed by centrifugation. The osmolalities of the supernatants were determined using a model G62 Fiske osmometer; the results were expressed as milliosmol (mosmol) per unit packed cell volume (PCV). The large amount of extracellular polysaccharide produced by many species of *Rhizobium* gave highly viscous suspensions and resulted in erroneous PCV results.

(2) Micro-organisms were harvested from agar slopes using a minimum volume of distilled water, and the number of viable organisms ml^{-1} was determined. The suspension was frozen at -20°C and then lyophilized. Micro-organisms dried in this way were rehydrated and disrupted in a known volume of the detergent Triton X-100 (BDH), and the cell debris was removed by centrifugation. The osmolalities of the supernatants were measured and expressed as mosmol per unit $\log_{10}$ of the viable count.

Water permeabilities of micro-organisms as determined by nuclear magnetic resonance (n.m.r.) spectroscopy. Water permeabilities of micro-organisms were determined by the method of Conlon & Outhred (1972), using a steady-state, high-resolution JEOL model 4H100 n.m.r. spectrometer operated at 100 MHz. For the reasons outlined by Pople, Schneider & Bernstein (1959), inclusion of particulate matter such as living organisms in the sample leads to errors in the spectra when steady-state n.m.r. is used. Hence the estimates of T_2 (spin-spin relaxation time) in the present investigation are only relative, and not absolute, measurements of the water permeability of cells.

Packed cell volumes were determined for all cultures used and the internal osmotic pressures were measured by method (1) to calculate the concentrations of $\text{MnCl}_2 \cdot 6\text{H}_2\text{O}$ required to yield isotonic solutions. Double-strength stock solutions were used so that the final concentration on mixing with the cell suspensions (50%, v/v) yielded isotonic solutions. Within 2 min of mixing the suspensions with Mn^{2+} , the n.m.r. spectra were recorded using 0.5 ml samples in standard high-resolution n.m.r. tubes.

Water sorption isotherms of rhizobia and montmorillonite. The apparatus used to obtain the sorption isotherms was similar to the silica spring balance described by Bateman *et al.* (1962). The water temperature in the jacket was maintained at 40°C . Bovine serum albumin was used to check that the apparatus was functioning properly and the values obtained were in good agreement with those reported by Bull (1944). A complete adsorption-desorption cycle for *R. japonicum* QA372 showed the hysteresis phenomenon common to such isotherms.

The B.E.T. theory (Brunauer, Emmett & Teller, 1938) has been applied to the data from the water adsorption isotherms for rhizobia and montmorillonite. The B.E.T. equation states that:

$$\frac{P/P_0}{W(1-P/P_0)} = \frac{(C-1)}{W_m C} P/P_0 + \frac{1}{W_m C} \quad (1)$$

in which W is the amount of water vapour sorbed per 100 g dry weight at a pressure P ,

P_0 is the saturation vapour pressure, W_m is the amount of water required to form a monolayer over the adsorbing surface, and C is a constant from which the heat of adsorption for the monolayer can be obtained. Values of W_m and C can be calculated from a plot of $[P/P_0]/W(1 - P/P_0)$ against P/P_0 .

The change in free energy involved when proteins (Bull, 1944) or montmorillonite (Roderick & Demirel, 1966) adsorb water vapour has been determined by application of Bangham's (1937) free-energy equation to the isotherm data. This equation can be expressed as:

$$\Delta F = \frac{-RT}{MA} \int_0^1 \frac{W}{P/P_0} d(P/P_0) \quad (2)$$

in which R is the gas constant, T is the absolute temperature, M is the molecular weight of water, A is the specific surface area of the adsorbing material, W is the weight of water adsorbed per 100 g dry weight at a pressure P , P_0 is the saturation vapour pressure, and ΔF is the change in free energy involved with wetting. The equation states that ΔF is proportional to the area under the curve in a plot of $W/(P/P_0)$ against P/P_0 .

Bull & Breese (1970), Walker, Wolf & Kapsalis (1973) and Steer (1973) noted that the presence of residual salt markedly affected the water sorption isotherms of various proteins. To determine whether this was an important factor in the water sorption characteristics of both groups of rhizobia, the isotherms of salt-free suspensions of the bacteria were obtained. Rhizobia were disrupted using Triton X-100, suspensions were dialysed thoroughly at 2°C and freeze-dried, and the water sorption isotherms were determined.

RESULTS

Osmolality of the internal contents of rhizobia

The internal solute concentrations of various species of *Rhizobium* relative to packed cell volume or viable count measurements are given in Table 1. No correction has been made for the dilution effect of extracellular water, but the results obtained give an indication of the relative differences in internal osmolality between different groups of rhizobia. The osmolality of the internal contents of slow-growing rhizobia is higher than that for either the *R. leguminosarum* group or the fast-growing *R. meliloti* group. The fact that all osmotic activity was removed by dialysis suggests that the osmotically active constituents are mainly ionic and not due to osmotically active glycoproteins (DeVries, Komatsu & Feeney, 1970).

Water permeabilities of various micro-organisms as determined by n.m.r. spectroscopy

The significant features of the steady-state n.m.r. spectra of *Saccharomyces cerevisiae* M6 and *Bacillus* M70 (Fig. 1) are the broad lines, attributable to extracellular water in contact with the paramagnetic ions (Mn^{2+}), and the relatively narrow lines, which are derived from intracellular water not in contact with Mn^{2+} . A comparison of the spectra in Fig. 1 reveals that the line due to intracellular water for *Bacillus* M70 was broader than the corresponding line for *Sacch. cerevisiae* M6. The line widths at half peak height were 7.0 and 2.5 p.p.m. for *Bacillus* M70 and *Sacch. cerevisiae* M6, respectively. Estimates of the relative T_2 values (the water permeability) of these two organisms are presented in Table 2. The ratio of the T_2 values for yeast relative to *Bacillus* M70 was about 3.0, suggesting that the rate of passage of water molecules across the membrane of *Bacillus* M70 was greater than that of *Sacch. cerevisiae* M6.

Unlike the spectra for yeast and *Bacillus* M70, it was not possible to distinguish between intra- and extracellular water for *Escherichia coli* M13, *Staphylococcus aureus* M3 and the

Table 1. *Relative osmolalities of the internal contents of various rhizobia expressed either per unit packed cell volume (PCV) or per unit log₁₀ of the viable count*

Bacterium	Osmolality of internal contents		
	m osmol/PCV*	m osmol/log ₁₀ viable count	
		Before dialysis†	After dialysis
<i>R. leguminosarum</i> group			
<i>R. trifolii</i> SU297/31A	1.00	—	—
<i>R. trifolii</i> SU297/31A	0.67	—	—
<i>R. trifolii</i> SU297/32B	1.56	—	—
<i>R. trifolii</i> SU297/32B	2.33	—	—
<i>R. trifolii</i> SU298/534C	1.69	—	—
<i>R. trifolii</i> TAI	—	2.80	0
<i>R. trifolii</i> TAI	—	2.99	0
<i>R. leguminosarum</i> TAI01	1.50	4.29	0
<i>R. leguminosarum</i> TAI01	2.00	4.85	0
<i>R. leguminosarum</i> TAI01	—	3.51	0
<i>R. leguminosarum</i> TAI01	—	2.71	0
<i>Rhizobium</i> sp. SU343	1.13	2.90	0
Fast-growing <i>R. meliloti</i> group			
<i>R. meliloti</i> SU47A	2.13	—	—
<i>R. meliloti</i> SU47B	2.35	—	—
<i>R. meliloti</i> CCI31	3.29	—	—
<i>R. meliloti</i> CCI31	2.67	—	—
<i>R. meliloti</i> CCI31	2.00	—	—
<i>R. meliloti</i> CCI31	1.73	—	—
Slow-growing group			
<i>R. lupini</i> UT2	—	9.91	0
<i>R. lupini</i> UT2	—	7.30	0
<i>R. lupini</i> UT12	10.00	5.79	0
<i>R. japonicum</i> QA372	18.00	13.16	0
<i>R. japonicum</i> QA372	8.57	11.84	0
<i>R. japonicum</i> QA372	10.00	8.99	0

* Disrupted by autoclaving.

† Disrupted by the detergent Triton X-100.

three species of *Rhizobium* examined. Consequently, relative T_2 values for these bacteria could not be estimated. At a Mn^{2+} concentration of 50 mM, water would have passed across the membranes of these bacteria in about 0.5 to 1.0 ms (R. Outhred, personal communication). It appears that the membrane offers very little resistance to the movement of water molecules between the cell interior and the external environment in rhizobia, *E. coli* and *Staph. aureus*. Comparison of the surface-to-volume ratios of all the bacteria examined (Table 2) suggests that the estimation of water permeabilities of cells by the n.m.r. technique of Conlon & Outhred (1972) is limited to micro-organisms with surface-to-volume ratios approximately equal to or less than that of *Bacillus* M70.

Water adsorption isotherms of rhizobia and Ca-montmorillonite

Adsorption isotherms of representatives of the *R. leguminosarum* and slow-growing groups of rhizobia, as well as the adsorption isotherm of Ca-montmorillonite, are shown in Fig. 2. The results indicate that bacteria of the *R. leguminosarum* group adsorbed more water per gram than those of the slow-growing group at all relative vapour pressures, except 0.90 and above. The results for Ca-montmorillonite indicate that the clay adsorbed more water per gram than both groups of rhizobia at all relative vapour pressures.

Fig. 1. Steady-state n.m.r. spectra of micro-organisms in the presence of paramagnetic (Mn^{2+}) ions. Organisms (with concentrations of $MnCl_2 \cdot 6H_2O$ used in parentheses) were: (a) *Sacch. cerevisiae* M6 (84 mM); (b) *Bacillus* M70 (84 mM); (c) *R. japonicum* QA372 (50 mM).

Table 2. Surface-to-volume ratios and water permeabilities (T_2 values) for various micro-organisms

Organism	Surface-to-volume ratio	Line width at half peak height (p.p.m.)	Relative T_2 values
<i>Saccharomyces cerevisiae</i> M6	1.27	2.5	0.1270
<i>Bacillus</i> sp. M70	2.97	7.0	0.0455
<i>Staphylococcus aureus</i> M3	5.45	*	*
<i>Escherichia coli</i> M13	5.93	*	*
<i>Rhizobium meliloti</i> CC131	5.93	*	*
<i>R. japonicum</i> QA372	6.86	*	*
<i>R. trifolii</i> SU297/32B	9.46	*	*

* Could not be determined.

The amount of water adsorbed by disrupted bacteria was slightly less than that adsorbed by untreated bacteria. Dialysis of the disrupted suspensions caused a change in both the slope and the position of the isotherms, with the dialysed samples adsorbing more water per gram than the disrupted, undialysed samples (Fig. 3). Obviously, the effect of dialysable solutes was to decrease the amount of water adsorbed by macromolecules of these bacteria.

Calculations based on the water adsorption isotherm

Application of equation (1) to the water isotherm data in Fig. 2 gave the constants presented in Table 3. The average W_m value for the *R. leguminosarum* group is approximately twice that for the slow-growing group, whereas the W_m value for Ca-montmorillonite is higher than those of both groups of bacteria. The Ca-montmorillonite result is in close agreement with that reported by Roderick & Demirel (1966).

If it is assumed that the adsorbed water molecules were in the closest packing arrangement, then the average cross-sectional area for a water molecule would be 0.105 nm^2 (Roderick &

Fig. 2. Water adsorption isotherms at 40°C for Ca-montmorillonite and for rhizobia of the *R. leguminosarum* (L) and slow-growing (S) groups. Δ , Ca-montmorillonite; ∇ , *R. trifolii* SU297/32B (L); $\bullet$, *R. leguminosarum* TA101 (L); $\circ$, *R. trifolii* SU297/31A (L); * and $\blacksquare$, *R. lupini* UT12 (S); $\square$, *R. japonicum* QA372 (S); $\blacktriangle$, *R. lupini* UT2 (S).

Table 3. Compilation of the constants derived by applying the B.E.T. equation to water adsorption isotherm data for various rhizobia and Ca-montmorillonite

	W_m [g water sorbed (100 g) $^{-1}$]	C	A (m 2 g $^{-1}$)
<i>R. leguminosarum</i> group			
<i>R. trifolii</i> SU297/32B	4.769	14.563	167.5
<i>R. trifolii</i> SU297/32B	4.710	8.229	165.4
<i>R. leguminosarum</i> TA101	4.564	13.199	160.3
Mean for group	4.681	11.997	164.4
Slow-growing group			
<i>R. lupini</i> UT12	2.707	5.790	95.06
<i>R. lupini</i> UT12	2.313	3.920	81.23
<i>R. lupini</i> UT2	1.974	3.731	69.32
<i>R. japonicum</i> QA372	2.803	1.829	98.43
Mean for group	2.449	3.818	86.01
Ca-montmorillonite	6.916	20.657	242.87

W_m , Quantity of water required to form a monolayer; C , a constant related to the heat of adsorption of the monolayer; A , specific surface area of the adsorbing material.

Demirel, 1966) and, from the W_m values, the adsorptive surface areas (A) can be calculated. There are consistent differences in the sorptive surface areas calculated in this way for the two groups of rhizobia (Table 3). The average surface area available for water adsorption for the slow-growing group is approximately half that available in the *R. leguminosarum* group.

Because of uncertainties about the validity of values of A obtained using the B.E.T.

Fig. 3

Fig. 4

Fig. 3. Water adsorption isotherms at 40°C for Triton X-100 disrupted rhizobia, either dialysed or undialysed. □, *R. leguminosarum* TA101, dialysed; ■, *R. leguminosarum* TA101, undialysed; ○, *R. lupini* UT12, dialysed; ●, *R. lupini* UT12, undialysed.

Fig. 4. Change in surface energy ($A\Delta F$) per g Ca-montmorillonite or g bacterial dry wt with change in relative vapour pressure. ▲, Ca-montmorillonite; ●, *R. leguminosarum* TA101; ▼ and ■, *R. trifolii* SU297/31A; △ and ○, *R. lupini* UT12; □, *R. japonicum* QA372; *, *R. lupini* UT2.

equation for adsorption of polar gases to porous solids, Bull (1944) and Sharma, Hehara & Mann (1969) obtained estimates of the energy involved in wetting various substrates by evaluation of the integral:

$$A\Delta F = \frac{-RT}{M} \int_0^{P/P_0} \frac{W}{P/P_0} d(P/P_0) \quad (3)$$

for porous solids. The values obtained for $A\Delta F$ are the free energy changes per gram of solid or the surface energy associated with the adsorption of water at a particular value of P/P_0 . Values for $A\Delta F$ have been obtained for various P/P_0 levels, and the results show that, at all relative vapour pressures, the surface energies of the *R. leguminosarum* group are greater than those of the slow-growing group (Fig. 4). The surface energy of Ca-montmorillonite is very much greater than those of the rhizobia at all relative vapour pressures.

DISCUSSION

The studies reported here were undertaken to provide some insight into the variation in desiccation sensitivity observed between two groups of root-nodule bacteria, as well as into the protective effects of montmorillonitic clays on desiccated bacteria. With reservation about extrapolating from these results to actual field soil situations, some interesting features on the water status of the rhizobia have emerged.

Differences in susceptibility to desiccation between the two groups of rhizobia cannot be accounted for by rates of water exchange as little resistance to passage of water molecules

was observed in both groups of bacteria. This relatively unrestricted water permeability is probably related to the small size of these bacteria. Dick (1966) has postulated that an inverse relationship exists between cell size and rate of water permeability, and this relationship is evident in the comparison made here between micro-organisms of different size. The order of increasing permeability to water in these micro-organisms is: *Sacch. cerevisiae* M6 < *Bacillus* M70 < rhizobia and other small bacteria.

The data derived from water adsorption isotherms indicate that susceptibility to desiccation in these groups of rhizobia is related to the amounts of water retained by the bacteria during desiccation. Greater amounts of water were retained at lower relative vapour pressures by the desiccation-sensitive *R. leguminosarum* group than by the more resistant slow-growing rhizobia, with calculated surface energies showing that bacteria of the former group exhibit a greater affinity for water than those of the latter group. The results obtained suggest that internal osmotic pressure is not important in determining the characteristic isotherms observed in the two groups of bacteria. Since bacteria of the *R. leguminosarum* group possess a greater adsorptive surface area than those of the slow-growing group, it is probable that the isotherm characteristics are determined by differences in the number of water-accessible polar sites on the internal macromolecules of these bacteria.

A lower internal water content at low relative vapour pressures probably imparts greater survival capacity in bacteria by reducing the activity of those enzymes capable of functioning at relatively low water availabilities. The degree of survival of *R. leguminosarum*-type bacteria might be improved by reducing the internal water content of the bacteria at low relative vapour pressures. Amendment of sandy soils with montmorillonite before desiccation results in improved survival of the *R. leguminosarum* group, but not of the slow-growing group (Marshall, 1964; Bushby & Marshall, 1976).

The suggestion that montmorillonite protects bacteria from the effects of desiccation by altering rates of water loss from the bacteria (Marshall, 1968) is contradicted by the present finding of rapid rates of water permeability that would not be altered significantly by the presence of montmorillonite. Since montmorillonite has a greater affinity for water than the root-nodule bacteria, it is postulated that this clay protects the *R. leguminosarum* bacteria from desiccation by decreasing the internal water content to levels below the equilibrium value attained in the absence of the clay. The equilibrium water content of the slow-growing bacteria may be below some critical value beyond which no further improvement in survival due to reduction of water content can be expected. Obviously, further studies on these bacteria are required before a complete explanation of the observed field response of rhizobia to desiccation and montmorillonite amendments can be satisfactorily explained.

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