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Growth and Nodulation Responses of *Rhizobium meliloti* to Water Stress Induced by Permeating and Nonpermeating Solutes†

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Isolates of *Rhizobium meliloti*, representing antigenically distinct indigenous serogroups 31 and 17, were grown in yeast extract-mannitol broth (YEM) containing NaCl or polyethylene glycol (PEG) to provide external water potentials ranging from −0.15 to −1.5 MPa. Several differences were found between representatives of the two groups in their abilities to adapt to water stress induced by the nonpermeating solute PEG. At potentials below −0.5 MPa, strain 31 had a lower specific growth rate than strain 17 and an irregular cell morphology. In contrast, neither growth nor cell morphology of either strain was affected significantly over the same range of water potentials created by a permeating solute, NaCl. Despite the superior growth of strain 17 at the low water potentials imposed by PEG, upshock of water-stressed cells (−1.0 MPa; PEG) into normal YEM (−0.15 MPa) resulted in a faster recovery of growth by strain 31 than by strain 17. Different responses of the two strains to a water potential increase were also revealed in nodulation studies. Strain 31 required significantly fewer days to nodulate alfalfa than strain 17 did when the strains were transferred from YEM with PEG at −1.0 MPa onto the roots of alfalfa seedlings in plant growth medium (−0.1 MPa). The addition of supplemental calcium (0.1 mM) to growth medium with PEG (−1.0 MPa) reduced the differences between strains in their responses to water stress. The severe growth restriction and morphological abnormalities shown by strain 31 were corrected, and the prolonged recovery time shown by water-stressed cells (−1.0 MPa; PEG) of strain 17 upon transfer to normal YEM was shortened. The latter strain also nodulated earlier and more rapidly after growth in PEG medium at −1.0 MPa in the presence of supplemental calcium ions. These results indicate that the efficacy of osmoregulation can vary among strains of the same species and that the mechanism of osmoregulation may differ depending on the nature of the water stress.

The majority of physiological studies of osmoregulation by heterotrophic bacteria involve the use of permeating solutes to lower the water potential of the suspending growth medium (23, 46). Osmotic adjustment under these conditions depends, in part, on external-solute uptake to regain water potential equilibrium. Under nonsaline soil conditions, water potential is controlled predominantly by matric potential (30). As a result, soil bacteria must osmotically adjust to changes in external water potential without significant assistance from external, permeating solutes (12). Plant and soil scientists have recognized a need to study water relations under conditions in which water stress is induced in a manner similar to that which actually occurs in the soil. Polyethylene glycol (PEG) has been used in plant (9, 18, 47) and bacterial (28) studies because of its ability as nonpermeating solute to lower the external water potential without penetrating the cell wall.

The ability of *Rhizobium* sp. to survive at a low water potential in soil has been established by many studies in which viability was assessed by determining colony-forming ability on agar plates with a high water potential (1, 10, 26, 27, 39). Very few studies have addressed either the efficiency of *Rhizobium* growth at low water potential (35) or growth recovery after a rapid water potential increase brought about by a rewetting process (5, 32). A significant turnover of microbial biomass during rewetting of field soils has been recently established (19). It is unknown what impact a rapid water potential change might have on soil *Rhizobium* populations both under free-living conditions and during the period of establishment of the symbiosis. The objectives of this research were to compare the growth responses of *Rhizobium meliloti* isolates with a decrease in water potential imposed by either PEG or NaCl and to evaluate the influence of a rapid increase in water potential on the growth and nodulating characteristics of the isolates.

MATERIALS AND METHODS

***Rhizobium* strains and growth media.** Representative isolates of *R. meliloti* serogroups 31 and 17 were chosen for study on the basis of their antigenic differences (11) and their competitive (serogroup 31) and noncompetitive (serogroup 17) abilities to nodulate field-grown alfalfa *Medicago sativa* L. (14). The strains were maintained as previously described (15) and were grown routinely in yeast extract-mannitol (YEM) broth to serve as inoculum for the water stress experiments.

Response of *R. meliloti* to a lowering of external water potential. (i) Growth. A range of water potentials was established in YEM broth with the addition of either NaCl or PEG 4000 (lot no. A42733; J. T. Baker, Jackson, Tenn.). The concentrations of potentially toxic elements such as copper, cadmium, and manganese in the PEG were found to be low (<1 mg/kg) after replicate samples were ashed and analyzed by inductively coupled argon plasma spectrometry. The osmolality values of NaCl and PEG solutions were determined with a vapor pressure osmometer (model 5100C; Westcor, Logan, Utah) and converted to water potential values by using the Van't Hoff equation (37). Water potentials were chosen to reflect a range over which alfalfa seeds might germinate and be susceptible to nodulation. Values included were −0.15 (YEM broth minus supplemental NaCl or PEG), −0.25, −0.50, −0.75, −1.0, and −1.5 MPa. All

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PEG solutions were autoclaved separately from YEM. Three replicate growth tubes (20 by 2.5 cm), each containing 35 ml of YEM, were included for each isolate at each water potential, and the experiments were repeated at least twice. Growth was monitored at 30°C in a temperature-controlled water bath, with cultures continuously aerated with a supply of filter-sterilized air. After the cultures were inoculated, the initial cell densities were 10^6 cells per ml, and the turbidity of the cultures was determined at 3-h intervals at 660 nm until late exponential phase. Cultures which failed to show visible growth were monitored for at least 72 h and then plated onto isotonic YEM agar to determine viability of the inocula. Increase in turbidity was due to proliferation of bacteria, since cell densities of $>10^8$/ml were achieved by cultures at late exponential phase regardless of the presence of NaCl or PEG. Cultures which grew at −1.0 MPa (with PEG or NaCl) were back transferred into isotonic YEM, and growth rates were measured to determine whether the growth observed might have resulted from enrichment of spontaneous mutants. The growth responses of isolates to water stress were similar at 25°C and also when KCl was substituted for NaCl.

(ii) Cell morphology. Strains 17 and 31 were observed microscopically to determine whether exposure to low water potentials had resulted in modified cell morphology. Cultures were grown in PEG- or NaCl-supplemented YEM (−1.0 MPa) for 72 h. Portions were diluted with an isotonic NaCl solution to provide 50 to 100 cells per microscopic field when viewed at a magnification of ×1,000. Subsamples (2 ml) were stained for 5 min with 0.05% (wt/vol) acridine orange, filtered onto 0.2-μm-pore-size filters (Nuclepore Corp., Pleasanton, Calif.), and examined by epifluorescence microscopy (6). Cell dimensions were compared with those from cultures grown in YEM without additional solutes.

(iii) Influence of organic and inorganic supplements on growth response under water stress. Cultures growing under PEG-induced water stress failed to achieve the cell densities of control cultures at stationary phase. As a result, supplements were added to YEM broth to determine if inadequate energy or other nutritional factors contributed to the limited growth response under water stress. Substrate supplements compared in separate experiments included mannitol (10, 15, and 20 g/liter), yeast extract (0.4 and 0.8 g/liter), glutamic acid (0.5 and 1.0 g/liter), NH_4Cl (0.5 and 1.0 g/liter), $CaCl_2 \cdot 2H_2O$ (0.025, 0.1, 0.25, and 0.5 mM), $MgSO_4 \cdot 7H_2O$ (0.8, 1.6, and 2.4 mM), and K_2HPO_4 (2.3 and 4.6 mM). The ability of the osmoprotectant glycine betaine to assist in osmoregulation during permeating- and nonpermeating-solute stress was evaluated. Strains 17 and 31 were grown in YEM, with or without glycine betaine (1 mM), at low water potentials created by PEG (−1.0 MPa) or NaCl (−2.0 MPa). It was necessary to reduce the water potential of the NaCl treatment to −2.0 MPa to obtain conditions which sufficiently restricted the growth rate of the isolates. Inoculum density and growth measurements were done as described above.

(iv) Mineral cation content of bacterial cells. The K^+, Ca^{2+}, and Mg^{2+} contents of cells of strains 17 and 31 were measured after growth in PEG- or NaCl-supplemented YEM (−1.0 MPa) or in nonstressed conditions. A minimum of 12 separately grown cultures per treatment were combined to provide sufficient dry weight for elemental analysis by atomic absorption spectrophotometry. Since strain 31 grew poorly at −1.0 MPa (with PEG), 4 liters of culture was grown. All cultures were washed twice in isotonic saline solution, and cell pellets were dried at 55°C for 26 h.

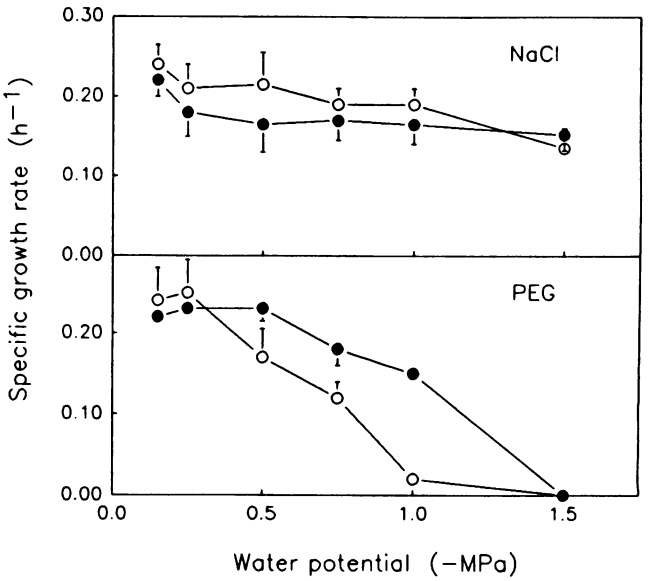

FIG. 1. Growth responses of strains 17 and 31 to water stress imposed by increasing concentrations of either NaCl or PEG. Symbols: ●, strain 17; ○, strain 31.

Replicates of each treatment were retained for dry weight determinations.

***R. meliloti* growth response to an increase in water potential.** The growth responses of strains 17 and 31 were determined after an increase in water potential from −1.0 to −0.15 MPa. Cultures were grown in YEM at −1.0 MPa created by either PEG or NaCl and inoculated into YEM (−0.15 MPa) at final densities of 10^6 cells per ml. Growth was monitored as described above.

Nodulation kinetics of *R. meliloti* following an increase in water potential. Strains 17 and 31 were grown in the following media: (i) YEM, (ii) YEM plus PEG (−1.0 MPa), and (iii) YEM plus PEG (−1.0 MPa) and $CaCl_2 \cdot 2H_2O$ (0.5 mM). Cultures were diluted with sterile, isotonic NaCl solutions and plated on YEM medium to determine cell viability. Portions were then inoculated onto roots of 'Vernal' alfalfa seedlings to provide approximately 10^4 viable cells per seedling. Seven seedlings were included per replication, with three replications of each treatment arranged in a completely randomized experimental design. The seedlings were grown in test tubes (30 by 3.2 cm) containing 25 ml of mineral salts agar (−0.10 MPa) under conditions previously described (4). Nodulation was monitored at daily intervals, and the experiment was repeated twice under similar conditions.

RESULTS

Influence of low water potential on growth kinetics. The specific growth rates of isolates from serogroups 17 and 31 were dependent on the solute used to create the range of water potentials. With the exception of serogroup 31 at −1.5 MPa, lowering the water potential to −1.5 MPa with NaCl did not result in a significant decline in specific growth rate (ca. 0.2/h) of either serogroup (Fig. 1). In contrast, low water potentials created by the nonpermeating solute, PEG, resulted in a more dramatic and differential decrease in the growth rates of both serogroups (Fig. 1). As water potentials were lowered with PEG, serogroup 17 isolates showed consistently higher growth rates than serogroup 31 isolates,

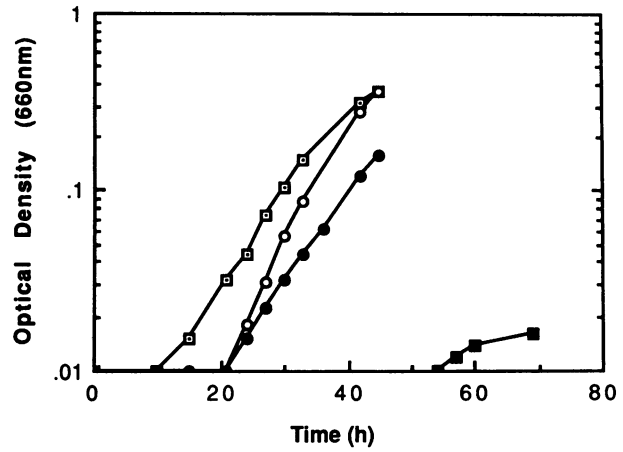

FIG. 2. Influence of calcium (0.1 mM) on the growth responses of strains 17 and 31 in medium with PEG (−1.0 MPa). Symbols: ●, strain 17; ○, strain 17 plus calcium; ■, strain 31; □, strain 31 plus calcium.

TABLE 1. Influence of permeating and nonpermeating solutes on the cation content of *R. meliloti* 17 and 31 grown in YEM

Strain	YEM supplement[a]	Amt of cation (g/kg of dry wt)[b]		
		K^+	Ca^{2+}	Mg^{2+}
17	None	3.53 (0.10)	0.41 (0.01)	1.30 (0.03)
	Calcium	5.08 (0.04)	0.62 (0.02)	1.18 (0.03)
	PEG	1.70 (0.10)	0.49 (0.05)	0.43 (0.02)
	PEG + Ca^{2+}	5.28 (0.45)	1.00 (0.05)	0.60 (0.02)
	NaCl	9.30 (0.10)	0.45 (0.04)	0.83 (0.03)
31	Control	4.80 (0.10)	0.42 (0.04)	1.27 (0.01)
	Calcium	5.15 (0.05)	0.69 (0.02)	1.26 (0.01)
	PEG	5.00 (0.20)	0.38 (0.04)	0.50 (0.02)
	PEG + Ca^{2+}	1.69 (0.10)	1.07 (0.04)	0.66 (0.02)
	NaCl	8.60 (0.10)	0.48 (0.03)	1.00 (0.02)

[a] Calcium (0.1 mM) was added to YEM. PEG and NaCl were added in quantities sufficient to produce water potentials of −1.0 MPa.

[b] Values in parentheses represent the standard errors of the means of composite samples from two separate experiments.

with the differences being most pronounced at −1.0 MPa. Although no isolates from either serogroup were able to grow at −1.50 MPa (with PEG), the initial inocula were known to have retained viability after 72 h of water stress, since the cells were recovered on YEM agar plates containing supplemental NaCl (−1.5 MPa). Back transfer of cultures from −1.0 MPa (with PEG) into the same medium resulted in identical growth kinetics, indicating that growth under water stress was not the result of enrichment of spontaneous mutants during the initial exposure to water stress (data not shown). Identical results were obtained with different isolates representing the two serogroups. In addition, the effects were not influenced by growth, temperature, inoculum size, or different batches of PEG. Similar results were obtained when NaCl was replaced with other permeating solutes.

Influence of low water potential on cell morphology. Microscopic observation of cultures grown at −1.0 MPa (with PEG) indicated that cells of strain 31 had undergone morphological changes. Individual cells were irregular in shape, and their average lengths increased from 1.7 μm for nonstressed cells to 3.9 μm for PEG-stressed cells. In contrast, strain 17 showed a minor change in morphology, increasing from a length of 1.5 μm for nonstressed cells to 2.0 μm for stressed cells. There were negligible changes in cell size resulting from growth of either strain at −1.0 MPa (with NaCl).

A role for supplemental calcium in adaption to water stress. We consistently observed that the optical densities of cultures of PEG-water-stressed cells at stationary phase were substantially less than those of nonstressed cells. We suspected an inadequacy of the YEM medium to meet the growth requirements of the cells under PEG-water-stressed conditions. Supplemental mannitol, yeast extract, glutamate, ammonium, K^+, and trace minerals had no effect on growth (data not shown). The addition of supplemental calcium at concentrations as low as 0.1 mM to YEM at −1.0 MPa (with PEG) dramatically stimulated the specific growth rate of strain 31 during exponential phase to 0.13/h (Fig. 2). The stimulation of growth of strain 17 by calcium was less dramatic; specific growth rates of 0.12 and 0.21/h were measured in the absence and presence of supplemental calcium, respectively. In addition, strain 31 cells, grown at −1.0 MPa (with PEG) with 0.1 mM supplemental Ca^{2+}, were not morphologically altered as previously observed. A general divalent-cation effect was ruled out, since supplemental magnesium ions could not substitute for the Ca^{2+} required by strain 31 (data not shown).

Despite the different growth responses to supplemental calcium, the calcium contents of strains 17 and 31 grown in normal YEM or YEM containing PEG or NaCl (Table 1) were similar. The calcium content was greater in cells of both strains grown at −1.0 MPa with PEG supplemented with 0.1 mM calcium than in cells grown in normal YEM with the same quantity of supplemental calcium. Both strains increased their K^+ concentrations when grown at −1.0 MPa (with NaCl). In contrast, cells grown at −1.0 MPa with PEG did not accumulate K^+ to greater concentrations than control cells did. To further emphasize the differences in osmoregulatory adjustment to permeating and nonpermeating solutes, the presence of glycine betaine allowed the generation times of both strains to be reduced to an average of 34% when they were grown at −2.0 MPa with NaCl, yet it failed to influence their growth rates at −1.0 MPa with PEG (data not shown).

Growth recovery following a water potential increase. The presence of supplemental Ca^{2+} during growth at −1.0 MPa (with PEG) had a small effect on growth recovery by strain 31 following water potential increase but considerably reduced the lag period of strain 17 by approximately 12 h (Fig. 3). No differences between the two strains were found in their growth recovery following an increase in water potential from −1.0 MPa (with NaCl) (data not shown). Although a linear plot of optical density against time clearly reveals the differential influence of upshock on the lag phase of strains 17 and 31, the specific growth rates achieved by both strains during exponential growth were identical at 0.2/h (3.5-h generation time).

Influence of growth at low water potential on nodulation. Only slight differences in the kinetics of nodule formation for the two strains were observed when inocula were grown under nonstressed conditions. A slightly higher percentage of seedlings were nodulated at day 7 by strain 17 than by strain 31. In contrast, cells of strain 31 grown at −1.0 MPa with PEG nodulated the seedlings 48 h earlier, and subsequent nodulation developed more rapidly than when −1.0 MPa-grown cells of strain 17 were used as an inoculum (Fig.

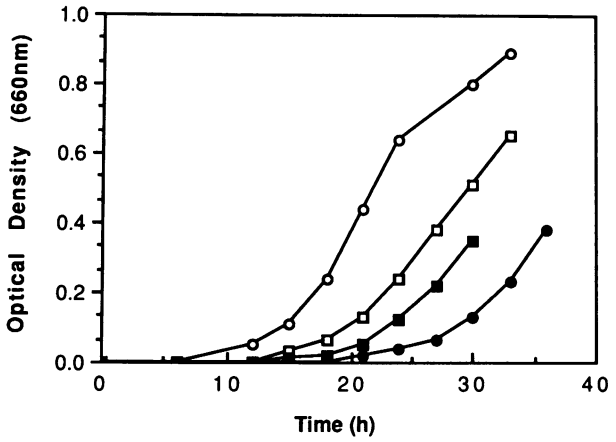

FIG. 3. Growth responses of strains 17 and 31 to a water potential increase from −1.0 to −0.15 MPa. Inocula were grown in YEM with PEG at −1.0 MPa in the absence (closed symbols) or presence (open symbols) of 0.1 mM calcium. Symbols: ● and ○, strain 17; ■ and □, strain 31.

4). The presence of supplemental calcium during growth of strain 17 at −1.0 MPa (with PEG) was sufficient to correct this delay. Cells of strain 31, grown initially at −1.0 MPa with PEG with or without supplemental Ca, were consistently observed to nodulate seedlings approximately 24 h faster than normal YEM-grown cells did.

DISCUSSION

Our findings indicate the importance of soil water potential to the success of microsymbionts both in the free-living state and during the establishment of a symbiosis. Furthermore, these facts have a general applicability to the study of osmoregulation in gram-negative bacteria. Most studies on the molecular mechanisms of osmoregulation by procaryotes evaluate response to water stress induced by solutes that can move freely through the cell wall (23, 46). The ability of isolates of serogroups 17 and 31 to grow at low water potentials induced by NaCl was expected, since *R. meliloti* is credited with growth in medium containing 2% NaCl (16). Additionally, both the increase in intracellular K^+ content (Table 1) and stimulation of growth by glycine betaine are in agreement with other observations of osmoregulatory responses by gram-negative bacteria to NaCl-induced water stress (7, 8, 17, 22, 36, 38). However, our findings that growth, K^+ accumulation, and response to glycine betaine under water stress induced by a nonpermeating solute are different from the responses to a permeating solute suggest that more attention should be focused on water stress situations more typically associated with nonsaline soil environments. Furthermore, the failure of different serogroups within a bacterial species to respond uniformly to nonpermeating-solute-induced water stress suggests that caution is needed in the choice of a bacterial strain when the modeling of osmoregulation is the goal.

A few comments are in order, however, to prevent overzealous extrapolation of these findings to the soil. First, as the matric potential of soil is lowered, restricted diffusion of substrates to bacteria will become a factor of considerable importance in dictating whether a bacterium could take advantage of superior osmoregulatory adjustment (28). Second, although matric potential can be controlled in different soils at constant values, soil texture has been found to play a confounding role in the influence of matric potential on survival and colonization by rhizobia (10, 26). Third, as a soil dries, there is an increase in the contribution of solutes to the overall soil water potential. Viability of rhizobia in soil adjusted to varying water and salt contents did not necessarily correlate with strain tolerance to individual components of soil water potential (35).

The role of calcium in osmoregulation by *R. meliloti* merits comment. Although the calcium requirements for *Rhizobium* growth are low (5 to 25 μM [40]), several reports have shown that calcium requirements for nodulation (24, 25, 29), for growth in the presence of low phosphate (2), and for complete expression of surface antigens (11, 13, 43) can be much higher. Our findings suggest that the requirement of calcium for growth by strain 31 was increased under water stress induced by a nonpermeating solute. Although growth of strain 17 was only slightly affected by supplemental calcium, the tolerance of the strain to a water potential increase was significantly improved after growth with supplemental calcium. Although we did not localize the site of calcium accumulation, Vincent and Humphrey (42) found that calcium was more concentrated in the cell wall of *Rhizobium trifolii* than in the cytoplasm, and calcium-deficient cells of *R. trifolii* have an altered morphology (41). Consistent with these findings, cells of strain 31 stressed with PEG (−1.0 MPa) showed modification in cell wall morphology when grown without supplemental calcium. More recently, a specific role for calcium in stabilizing outer-membrane architecture in gram-negative bacteria has been proposed (3, 44). Taken together, these findings indicate that more attention should be given to the possible relationship of calcium, cell wall architecture, and the osmoregulatory response by *R. meliloti* to nonpermeating solutes.

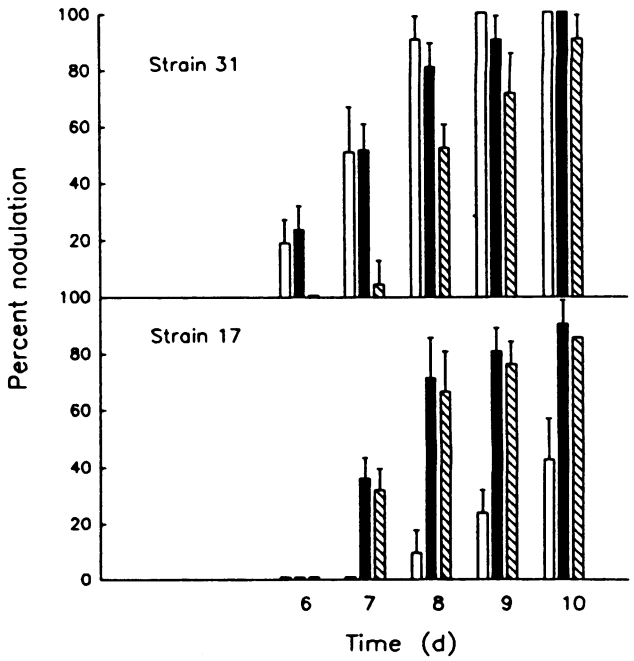

FIG. 4. Nodulation kinetics of strains 17 and 31 following a water potential increase. Inocula were grown in normal YEM (−0.15 MPa) or in YEM with PEG at −1.0 MPa in the presence or absence of 0.1 mM calcium and transferred onto alfalfa seedlings growing on mineral salts agar (−0.1 MPa). Symbols: ▨, YEM; □, YEM plus PEG (−1.0 MPa); ■, YEM plus PEG (−1.0 MPa) and Ca^{2+} ions (0.5 mM).

In conclusion, we would like to discuss our findings in relation to competitive nodulation among *Rhizobium* strains. It is well documented that rhizobia are capable of survival under low water potential (10, 26). However, as Kieft et al. (19) pointed out, upshock events, coincidental with the return of favorable growth conditions for plants and bacteria, are likely to have a more important effect on bacteria which inhabit surface soils. Our data revealed that strain 17 took 48 h longer than strain 31 to nodulate alfalfa seedlings when the strain was exposed to a water potential increase. The magnitude of this time difference is of particular interest since data from split-root experiments have shown that seedlings of soybean (20, 21, 31) and subclover (33), when inoculated with a primary strain 48 h prior to inoculation with a second strain, can suppress nodulation by the latter. Further work in progress will evaluate whether the superior ability of members of serogroup 31 to respond to an increase in soil water potential contributes to their competitive ability to nodulate alfalfa under field conditions (14). On a cautionary note, the impact of water potential on the characteristics of both root hair infections and nodule development of the host plant should not be overlooked (34, 45, 47).

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