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Ionic Stress and Osmotic Pressure Induce Different Alterations in the Lipopolysaccharide of a *Rhizobium meliloti* Strain

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A halotolerant strain of *Rhizobium meliloti* was isolated from nodules of a *Melilotus* plant growing in a salt marsh in Doñana National Park (southwest Spain). This strain, EFB1, is able to grow at NaCl concentrations of up to 500 mM, and no effect on growth is produced by 300 mM NaCl. EFB1 showed alterations on its lipopolysaccharide (LPS) structure that can be related to salt stress: (i) silver-stained electrophoretic profiles showed a different mobility that was dependent on ionic stress but not on osmotic pressure, and (ii) a monoclonal antibody, JIM 40, recognized changes in LPS that were dependent on osmotic stress. Both modifications on LPS may form part of the adaptive mechanism of this bacterium for saline environments.

It has been estimated that 23% of agricultural soils are affected by problems related to high salinity (1). Most crops are sensitive to relatively low levels of salinity, and in the case of legumes, there is an additional problem because not only the plant but also the symbiotic bacteria (members of the family *Rhizobiaceae*) are sensitive to salinity (13) both at the free-living stage and during the symbiotic process (29).

Among rhizobia, *Rhizobium meliloti* is one of the most halotolerant species, and several strains have been reported to grow at high salt concentrations (7, 10). Studies on salt tolerance in these bacteria relate this ability to metabolic changes that occur mainly in the cytoplasm. In response to salt stress, rhizobia accumulate several compatible solutes to overcome the osmotic stress induced by salt. Examples of these compatible solutes are glutamate, betaines, and the dipeptide NAGGN (8, 15, 22). Although these mechanisms can explain the osmotic tolerance of bacteria by increasing the internal osmotic pressure to avoid cytoplasmic dehydration, the toxic effect of ions must be overcome by other means. It has been shown for *R. meliloti* that there is a toxic effect exerted by ions that is independent from the osmotic stress and that impairment of growth depends upon the specific ion (7).

We are investigating the possible role of cellular envelopes in salt tolerance. Lipopolysaccharides (LPSs) are a major component of the outer membrane of gram-negative bacteria, including *Rhizobium* spp. Rhizobial LPSs are divided into two groups: LPS-1, a complex macromolecule which carries a variable number of repeating oligosaccharides, termed the O antigen; and the simpler LPS-2, which consists of the core oligosaccharide and the lipid A and shows a higher electrophoretic mobility (11, 12). Changes in the structure of LPS have been related to adaptation to different environmental situations such as pH, oxygen concentration, and osmotic pressure (4, 16, 23, 28).

In this paper, we report changes in the LPS structure of a halotolerant strain of *R. meliloti* that are induced by salt. Different techniques have revealed structural changes induced either by ionic stress or by osmotic stress. These changes may represent an adaptive mechanism against the different stresses exerted by saline environments.

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MATERIALS AND METHODS

Isolation of *R. meliloti* EFB1. Nodules from *Melilotus* spp. plants were harvested, surface sterilized, and crushed in TY medium (3). The extracts were spread on TY agar plates and incubated for 48 h at 28°C. Colonies with the typical aspect of rhizobia were tested for nodulation on alfalfa (*Medicago sativa* cv. Moapa). Seeds were surface sterilized with bleach, germinated in the dark, and placed on FP medium (14). Seedlings were inoculated as previously described (6). From one of the effective nodules obtained, the rhizobia were recovered; a bacterial culture was obtained and plated on TY medium supplemented with 200 μg of streptomycin ml^{-1}. A spontaneous resistant mutant was selected and named EFB1.

Bacterial strains and growth conditions. *R. meliloti* EFB1 and *Rhizobium leguminosarum* 3841 (= 300str) were grown either in TY agar or in TY liquid medium vigorously shaken in an orbital shaker, for 48 h at 28°C. When appropriate, media were supplemented with NaCl (25 to 500 mM), 300 mM KCl, 150 mM Na_2SO_4, and 200 mM polyethylene glycol (PEG) 200. No changes of pH were detected after supplementation.

Growth was monitored as optical density at 620 nm with a Hitachi U-2000 spectrophotometer.

Monoclonal antibodies. The monoclonal antibodies were obtained in a fusion experiment (9). Cells of strain EFB1 (grown on TY medium or TY medium plus 300 mM NaCl) were suspended in water, and an aliquot (40 μg of protein) mixed with Freund's incomplete adjuvant was injected subcutaneously into LOU/PAP rats. After 2 weeks, a repeat injection was carried out, and 2 weeks later, 40 μg of bacterial material was injected intravenously. The rats were killed 3 days later, and the spleen cells were fused with the myeloma line IR983F (2). Supernatants were screened by dot immunoassay, and the positive cell lines were cloned.

Nature of epitopes. To test the sensitivity to protease treatment, sonicated bacteria were diluted to a protein concentration of 1 mg · ml^{-1} and treated with proteinase K (protease XI; Sigma) (21). Samples were incubated with proteinase K (50 μg · ml^{-1}) for 90 min at 37°C, incubation was followed by protein denaturation for 10 min at 100°C, and samples were treated with proteinase K to a final concentration of 100 μg · ml^{-1} for 60 min at 100°C. Aliquots (1 μl) of sample with and without proteinase treatment were dotted onto nitrocellulose sheets (Schleicher & Schuell, Dassel, Germany) and immunostained with a goat anti-rat immunoglobulin G antibody conjugated for horseradish peroxidase (25).

In order to confirm that epitopes had carbohydrate components (LPS), the sensitivity to periodate oxidation (26) was tested. One microliter of sonicated cell preparations (1 mg of protein · ml^{-1}) was dotted on nitrocellulose sheets. After drying, they were equilibrated with 50 mM sodium acetate buffer, pH 4.5, for 30 min and treated as previously described (19). Sheets were incubated in the dark for 1 h in the same buffer containing 20 mM sodium metaperiodate and were washed twice with the same buffer for 30 min and once more with TBS (50 mM

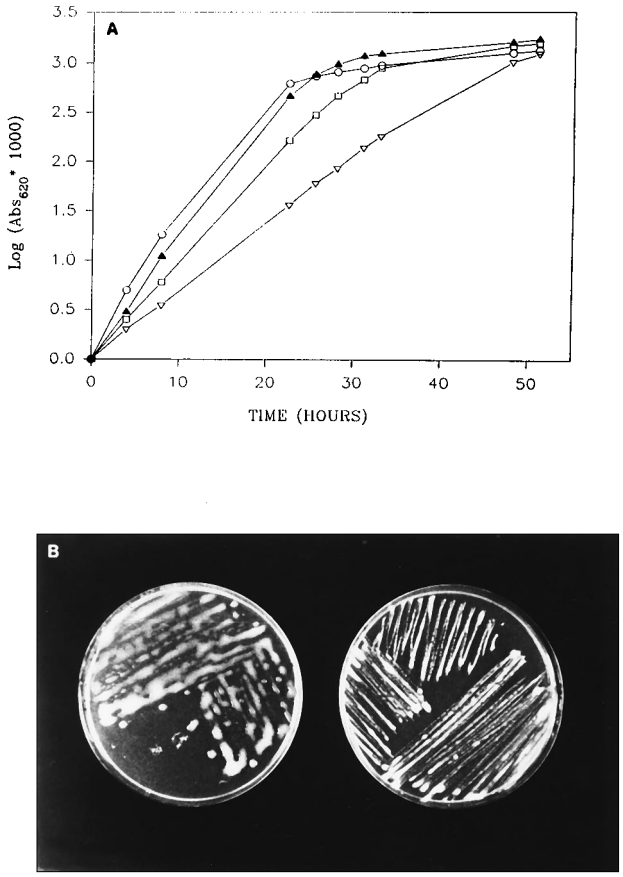

FIG. 1. (A) Growth of *R. meliloti* EFB1 in liquid TY medium with increasing concentrations of NaCl. ○, 0 mM; ▲, 300 mM; □, 400 mM; ▽, 500 mM. (B) TY agar plates of *R. meliloti* EFB1 showing the morphology of the colonies grown without (left) or with (right) a supplement of 300 mM NaCl.

Tris [pH 7.4], 200 mM NaCl). Finally, sheets were incubated for 30 min with 50 mM sodium borohydride in TBS and immunostained as above.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblots. Samples for gel separation were washed three times in TBS and extracted by heating in SDS buffer for 10 min at 100°C. After centrifugation to remove insoluble debris, the extracts (10 μg of protein loaded per lane) were subjected to 15% acrylamide minigels (18). After electrophoresis, gels were stained for carbohydrates (LPS) with periodate oxidation and Bio-Rad silver staining reagents (25). As an alternative to silver staining, gels were transferred electrophoretically to membranes of polyvinylidene difluoride (Immobilon; Millipore, Harrow, United Kingdom) (5). Blots were incubated with 5% bovine serum albumin in TBS containing monoclonal antibody. Immunostaining was visualized with a goat anti-rat immunoglobulin G conjugated to alkaline phosphatase (9).

RESULTS

Isolation and characterization of *R. meliloti* EFB1. *R. meliloti* EFB1 was isolated from pink nodules of *Melilotus* spp. plants growing in a salt marsh in Doñana National Park (southwest Spain). Ex-nodule colonies were tested for nodulation on alfalfa plants which in 2 to 3 weeks developed functional nodules. A spontaneous streptomycin-resistant mutant from one of these nodule-forming colonies was named EFB1.

Although *R. meliloti* strains are often classified as halotolerant in view of their growth at relatively high salt concentrations, in most strains there is an impairment of growth at NaCl concentrations as low as 100 mM (7, 9), with a reduction of 50% growth at about 300 mM NaCl and at lower concentrations for other ions (7, 27). In the case of strain EFB1 (Fig. 1A), concentrations of 300 mM NaCl did not have any effect on growth, and maximum growth was reached, although at a lower growth rate, at 500 mM NaCl, indicating that *R. meliloti* EFB1 is a true salt-tolerant bacterium. Higher concentrations (600 mM) completely inhibited growth.

When the bacterium was grown on solid medium supplemented with NaCl, the morphology of the colonies was different from that of those grown on nonsupplemented medium (Fig. 1B), resembling the semirough colonies produced by LPS mutants. This aspect could indicate that salt induces structural alterations on the LPS. No changes in colony morphology were noticed on cells grown on PEG 200. No decrease in fluorescence on TY-Calcofluor plates was observed after salt addition, indicating that the observed difference in mucoid character was not due to a difference in exopolysaccharide production (data not shown).

***R. meliloti* EFB1 changes LPS structure in the presence of salt.** Cell extracts derived from *R. meliloti* EFB1 growing on TY medium and TY medium plus different NaCl concentrations were subjected to SDS-PAGE and silver stained for LPS detection. As shown in Fig. 2A, salt concentrations that did not affect growth (300 mM NaCl) induced alterations on the LPS electrophoretic profile (lane 3). These alterations consisted of different mobilities of the LPS-1 fraction and in the appearance of new bands on the O antigen. The changes on LPS were evident with NaCl concentrations of 100 mM NaCl (data not shown), much lower than the minimum salt concentration which inhibits growth. These modifications on the LPS of *R. meliloti* EFB1 detected by silver staining were not specific for NaCl. Other salts like KCl and Na_2SO_4 can induce similar effects on the LPS structure (lanes 4 to 6). When a salt-sensitive rhizobial strain, *R. leguminosarum* 3841, was grown on different salt concentrations up to its tolerance limit (75 mM NaCl), no changes on the LPS profile were observed (Fig. 2B), although this strain has been shown to alter its LPS profile in response to several environmental situations (16).

To test whether these alterations were due to the osmotic stress produced by high salt concentrations or to an ionic effect, extracts from *R. meliloti* EFB1 grown in the presence of PEG 200 were analyzed. PEG 200 is a nonionic, nonmetabo-

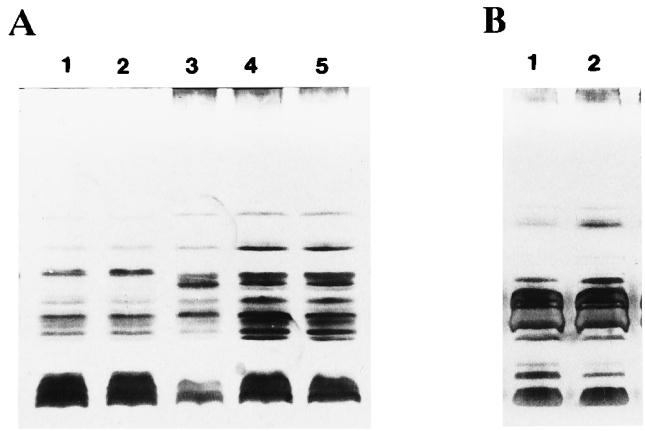

FIG. 2. Electrophoretic analysis of the LPS of *R. meliloti* EFB1 grown in the presence of different salts or a nonionic osmoticum (PEG 200). Cells of strain EFB1 or 3841 were collected and TBS washed after 48 h of growth in TY liquid medium. After centrifugation, SDS extracts were subjected to SDS-PAGE (10 μg of protein loaded per lane) and silver stained following periodate oxidation. (A) *R. meliloti* EFB1. Lane 1, control; lane 2, 200 mM PEG; lane 3, 300 mM NaCl; lane 4, 300 mM KCl; lane 5, 150 mM Na_2SO_4. (B) *R. leguminosarum* 3841. Lane 1, control; lane 2, 75 mM NaCl.

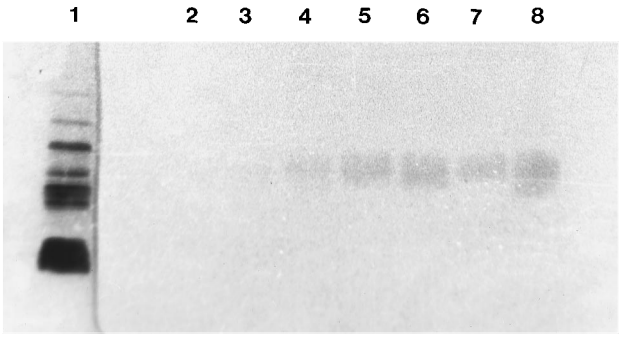

FIG. 3. Western blot (immunoblot) analysis showing osmotically induced alterations of the LPS of *R. meliloti* EFB1. TBS-washed cells of strain EFB1 from TY liquid medium cultures were subjected to SDS-PAGE (10 μg of protein per lane) following solubilization in SDS buffer. Separated material was electroblotted onto polyvinylidene difluoride membranes and probed with monoclonal antibody JIM 40. Lane 1, silver-stained LPS; lane 2, control; lane 3, 50 mM NaCl; lane 4, 100 mM NaCl; lane 5, 300 mM NaCl; lane 6, 300 mM KCl; lane 7, 150 mM Na_2SO_4; lane 8, 200 mM PEG. Antibody binding was detected with anti-rat immunoglobulin G conjugated to alkaline phosphatase.

lizable osmoticum which has been frequently used in osmotic stress experiments. Figure 2A (lane 2) shows that none of the changes in LPS profile that were detectable after growth in high salt could be observed in the presence of PEG 200. These results clearly indicate that these modifications in LPS structure are produced in response to ions and are not related to an increment in osmotic pressure.

A monoclonal antibody recognizes salt-induced changes in the LPS of *R. meliloti* EFB1 which are related to osmotic pressure. A monoclonal antibody, JIM 40, was raised against *R. meliloti* EFB1 cells grown on TY medium and TY medium plus 300 mM NaCl. The affinity of the antibody was checked by dot immunoassay, and no epitope was recognized in *R. meliloti* EFB1 cells grown on TY medium, although a positive reaction was obtained with EFB1 cells grown with 300 mM NaCl (data not shown).

The nature of the epitopes was tested by both protease digestion and periodate oxidation. Epitopes were resistant to the protease treatment but sensitive to periodate oxidation, indicating that carbohydrate components were involved.

Polyacrylamide gels similar to those shown in Fig. 2 were electroblotted on polyvinylidene difluoride membranes and immunostained with JIM 40. As shown in Fig. 3, the antibody recognized epitopes present on more slowly migrating LPS bands, presumably the O antigen of the LPS-1 molecules. No epitope was recognized on the LPS-2 or on control cells grown in the absence of added salt (lane 1). A salt concentration as low as 50 mM NaCl was enough to induce the appearance of the epitope recognized by JIM 40, and the signal was proportional to the amount of NaCl in the growth medium (lanes 3 to 5). The presence of other salts (KCl and Na_2SO_4) in the growth medium also induced the appearance of the epitope (lanes 6 and 7). Conversely in relation to the LPS modifications detected by silver staining, PEG 200 induced the same response (lane 8), indicating that this epitope is induced by osmotic stress. However, this osmotically induced modification of the LPS seems to be too subtle to affect the relative electrophoretic mobility of the LPS as judged from silver staining.

DISCUSSION

In this report, we present evidence showing that high salt concentration induces changes in the structure of the LPS of a highly halotolerant (Fig. 1) strain of *R. meliloti*. Structural changes in the LPS have been reported as adaptations of rhizobia to different environmental and physiological situations, at both the free-living and the symbiotic stages. These changes generally affect the O-antigen portion of the molecule and have been demonstrated either by alterations of electrophoretic mobility after silver staining or by the use of monoclonal antibodies as molecular probes.

Antigenic changes in the LPS of bacteroids of *R. leguminosarum* 3841 (21, 24) have been related to the special environmental conditions found inside nodules. In the same strain, but in free-living bacteria, environmental changes in pH and oxygen partial pressure induced antigenic modifications on the O antigen (16). The LPS antigenic modifications occurring in this strain during nodule development have been described as adaptations of endosymbiotic rhizobia to the surrounding microenvironment (17). Similar results have been reported for another *R. leguminosarum* strain (CNF42), and in this case, the response was also induced by high temperature and low phosphate (23).

Changes in the O antigen of the LPS have been reported in other rhizobial species. In *Rhizobium fredii*, the O antigen is modified in composition when grown in the presence of soybean root extract or apigenin, a flavonoid inducer of *nod* genes (20); these changes were related to an increased mobility of the LPS-1 fraction and a different composition of the O antigen, in which glucose was replaced by xylose and mannose. Similar changes have been reported in *R. leguminosarum* bv. *phaseoli* (4) when this strain was grown on a medium at acid pH (4.8). Silver staining of the LPS revealed a shift in mobility of LPS-1 compared with cells grown at neutral pH. Cells grown at acidic pH did not cross-react with monoclonal antibodies raised against cells grown at neutral pH, and this change in antigenicity was related to the substitution of 2,3,4-tri-*O*-methyl fucose by 2,3-di-*O*-methyl fucose on the O antigen.

We have shown that salt induces two different modifications on the O antigen of *R. meliloti* EFB1. These modifications are similar to other environmentally induced LPS alterations in rhizobia and to our knowledge are the first LPS modifications reported for *R. meliloti*. One of them consisted of an increase of the electrophoretic mobility of certain LPS-1 bands, and it is clearly induced by ionic stress, as a noncharged osmoticum did not induce it (Fig. 2A). The second one is a change in the antigenicity of the O antigen, and it is induced by ionic and nonionic osmotica, indicating that this modification appears in response to high osmotic pressure (Fig. 3). Apparently, this antigenic modification is too subtle to be detected by silver staining. Although salt-induced alterations on the LPS of several salt-tolerant strains of rhizobia have been reported (28), no different effects induced by osmotic or by ionic stress were found. The use of different methodologies allowed us to dissect the two different effects exerted by salt: osmotic and ionic stress. We have found that *R. meliloti* EFB1 responds to both effects, altering the structure of its LPS although in a different way. These alterations, especially the osmotically induced effect, should be complementary to the frequently reported accumulation of compatible solutes. Thus, modification of the bacterial cell wall LPS should also be regarded as an adaptive mechanism of this strain for the special environment from which it was isolated.

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