Morphological Changes of Rhizobia in Peat Cultures

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The inoculation of legume seeds with nitrogen-fixing root-nodulating bacteria (referred to generically as rhizobia) is a well-established technology of major economic and environmental importance. Worldwide, legumes are grown on approximately 250 million ha, and their associated rhizobia fix about 90 million metric tons of N₂ per year (8). A well-nodulated legume crop can fix up to 300 kg of N₂ per ha annually, which is equivalent to 625 kg of urea fertilizer. Successful inoculation of legumes requires the application to seeds of a sizable population of compatible rhizobia and their subsequent survival on seeds and in soil before germination (16). The most widely used method of applying commercially available inocula is from peat cultures, with which seeds are coated prior to being sown. In general, rhizobia survive well in peat, but many species die rapidly after inoculation onto seeds. The reasons for this are not understood but may involve a compromised capacity to resist desiccation stress. Some of the factors that affect survival of rhizobia in peat cultures have been characterized, including properties of the peat (13), storage temperature (14), and moisture potential (5). In an early microscopic study, the cell walls of Rhizobium leguminosarum bv. trifolii TAI were observed to thicken during storage in peat (3). Although the significance of these morphological changes was not established at the time, more recent studies with Acinetobacter and Mycobacterium have indicated that cell wall thickening may be related to enhanced survival of these cells under various types of stress (2, 7). In the present study, morphological changes that take place in peat cultures of several species of rhizobia were examined. These changes seemed to be associated with enhanced survival of cells after inoculation onto plastic beads, which were used as a model for seeds to avoid possible seed coat toxicity affecting the viability of cells (11, 21).

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MATERIALS AND METHODS

Rhizobial strains and culture conditions. Bradyrhizobium lupini WU425 and Rhizobium sp. strain SU343, which have been described (Bio-Care Technology, Somersby, Australia, unpublished results) as good and poor survivors, respectively, after inoculation onto seeds were used for most of the microscopic studies, with R. leguminosarum bv. trifolii TAI, Mesorhizobium ciceri CC1192, and Sino-rhizobium meliloti strain 1080 also examined for comparison. All of the strains were obtained from the culture collection of SUNFix, University of Sydney, Sydney, Australia, except strain 1080, which was provided by Barry G. Rolfe, Genome Interaction Group, Research School of Biological Science, Australian National University. All strains were maintained on yeast-mannitol agar as described by Vincent (22). Sucrose-glucose medium containing 0.75 g of K₂HPO₄, 0.4 g of MgSO₄·7H₂O, 0.4 g of CaCO₃, 2.5 g of sucrose, 2.5 g of glucose, and 3 g of yeast extract per liter was used to grow broth cultures of all strains, with the exception of WU425, which was grown in a glycerol broth containing 0.5 g of K₂HPO₄, 0.3 g of (NH₄)₂HPO₄, 0.8 g of KNO₃, 0.2 g of MgSO₄·7H₂O, 4 g of yeast extract, 0.1 ml of 10% (wt/vol) MnSO₄, 0.1 ml of 10% (wt/vol) FeCl₃, and 10 g of glycerol per liter. Broth cultures were grown at 30°C and aerated by orbital shaking at 200 orbits per min. Cells were harvested from broth cultures by centrifugation at 6,000 × g for 10 min.

Preparation of peat cultures. To prepare peat cultures for use as inoculants, broth cultures in early stationary phase were diluted 1 to 4 with one-fifth strength of the respective fresh medium. Diluted broth (110 ml) was injected aseptically into sealed polyethylene bags containing 150 g (wet weight) of sterilized peat (Bio-Care Technology) and mixed manually. Commercial peat cultures prepared in the same way by Bio-Care Technology were used in some experiments. The moisture content of peat cultures was determined by measuring the weight loss of a sample (5 g) that was heated at 100°C for 16 h. Moisture potential was calculated from moisture content using a calibration curve provided by Bio-Care Technology.

Extraction of cells from peat. Peat culture (10 g) was added aseptically to 90 ml of phosphate-peptone buffer (15) containing glass beads and shaken vigorously in a reciprocal shaker for 15 min. The suspension was centrifuged at 250 × g for 5 min to remove peat particles, and the supernatant was centrifuged at 10,000 × g for 10 min to harvest the cells. The recovery of cells in extracts of peat was estimated by comparing cell counts in a suspension of a peat culture with those in the supernatant after the suspension was centrifuged at 250 × g for 10 min. A comparison of the two counts indicated that the recoveries were 84 and 71% for SU343 and WU425, respectively.

For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (see below), cells extracted from peat were purified by Percoll (Pharmacia, Uppsala, Sweden) density gradient centrifugation as follows. Harvested cells were resuspended in 3 ml of a solution containing 0.4 M sorbitol and 10 mM TES [N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid]-KOH (pH 7.2), loaded onto 30 ml of the same solution containing 70% (vol/vol) Percoll, and centrifuged at 16,000 × g for 15 min. An opaque band containing the cells
formed near the bottom of the tube and was removed using a Pasteur pipette and diluted 1 to 5 with the sorbitol-TES solution. Cells in the diluted suspension were harvested by centrifugation at 10,000 × g for 10 min. Peat culture extracts that were freed of cells were prepared as described above, except that sterile distilled water was used and the suspension was centrifuged at 10,000 × g for 10 min.

Inoculation of beads. The following procedures were performed under aseptic conditions. Peat cultures (2.5 g), cells harvested from 1 ml of broth culture, or cells extracted from 2.5 g of peat cultures were mixed with 10 ml of 1.5% (wt/vol) methycellulose. An aliquot of the mixture (0.2 ml) was inoculated onto 10 g of polypropylene beads (3.5-mm diameter, 2-mm thickness; Bio-Care Technology) and air dried for 30 min at room temperature. To release the cells from the inoculated beads, 99 beads were added to 99 ml of the phosphate-peptide buffer (15) and shaken vigorously in a reciprocal shaker for 10 min. The beads were allowed to settle under gravity, and the supernatant was used for cell counts.

Cell counts. A 10-fold dilution series was made from broth cultures and cell suspensions obtained from peat cultures and inoculated beads. Aliquots (0.1 ml) of each dilution were spread on yeast-mannitol agar plates, and colonies were counted after incubation at 30°C for 3 to 8 days, depending on the strain. The most probable number (MPN) plant infection count was also used for estimating viable cells of *Rhizobium* sp. strain SU343 on inoculated beads according to the method of Somasegaran and Hoben (19), using *Lotus corniculatus* cv. Granger grown as described by Gault et al. (4). The number of nodulated plants was recorded after 6 weeks, and the MPN was calculated using the MPNES computer program (23).

Sample preparation for transmission electron microscopy. Cell pellets and peat cultures were fixed in 2.5% (wt/vol) glutaraldehyde in 0.1 M Pi (pH 7) for 1 to 2 h and washed three times in 0.1 M Pi (pH 7) for 10 min each. The samples were osmicated in 1% (wt/vol) OsO4 in 0.1 M Pi (pH 7) for 1 to 2 h, washed with distilled water for 5 min, and dehydrated by soaking them successively for 10 min each in 30, 50, 70, and 100% (twice) acetone. Dehydrated samples were infiltrated overnight at room temperature with a 1:1 mixture of Spurr’s low-viscosity resin in acetone (20) and then incubated overnight again in undiluted resin. The resin-infiltrated cells were allowed to polymerize at 60°C overnight. The fixed cells were sectioned with a microtome (Reichert-Jung) using a glass knife. Sections were stained with 2% uranyl acetate in water for 15 min, followed by Reynolds’s lead citrate (12) for 10 min. The stained sections were examined with a Philips 400 transmission electron microscope.

**RESULTS AND DISCUSSION**

Survival of rhizobia in peat. After SU343 cells were injected into peat, there was a decrease in the number of viable cells from 4.8 × 10⁶ to 1.3 × 10⁸ g of peat⁻¹ within 1 day, followed by an increase of about sixfold to 7.9 × 10⁸ g⁻¹ between days 2 and 14 (Fig. 1). The number of viable cells decreased subsequently to 4.8 × 10⁵ g⁻¹ after 24 days and then more gradually to 2.8 × 10⁵ g⁻¹ after 85 days (Fig. 1). A similar trend was observed when WU425 cells were injected into peat: the number of viable cells decreased from 7.2 × 10⁸ to 1.4 × 10⁸ g of peat⁻¹ within 1 day, followed by an increase of about twofold to 2.5 × 10⁸ g⁻¹ between days 2 and 14, and then a slow decrease to 1.1 × 10⁸ g⁻¹ after 85 days (Fig. 1). These results are consistent with the cells having entered stationary phase after about 14 days in peat.

To determine whether cells in peat cultures were nutrient limited after this time, a soluble extract from a 14-day-old peat culture was freed of cells and used as a growth medium. When SU343 cells in early stationary phase (1.8 × 10⁶ ± 0.3 × 10⁶ cells ml⁻¹) were added to the autoclaved cell-free peat extract, no further growth of cells was detected. The numbers of viable cells after 3 and 10 days of incubation were 1.8 × 10⁶ ± 0.4 × 10⁸ cells ml⁻¹ and 1.0 × 10⁸ ± 0.2 × 10⁸ cells ml⁻¹, respectively. The failure of cells to proliferate indicated that there was a nutrient limitation in the 14-day-old peat cultures of SU343.

The moisture content of the peat cultures decreased only slightly from an initial value of 52% on a wet basis (moisture potential, −5 × 10⁴ Pa) to 49% (−8 × 10⁴ Pa) after 85 days at 30°C (results not shown). Hence, the rhizobia were unlikely to have been subjected to desiccation stress. The low loss of water from the peat cultures indicated that the polyethylene bags used for packaging have low gas permeability. The O₂ concentration in sealed peat cultures is thought to decrease soon after the introduction of rhizobia, due to a high O₂ demand during the rapid growth phase of cells (Bio-Care Technology, unpublished results). Therefore, it is possible that cells in sealed peat cultures are in an O₂-depleted environment, in which there is a low rate of O₂ diffusion into the polyethylene packets that matches the rate of O₂ uptake for maintenance metabolism of cells in stationary phase.

Transmission electron microscopy examination of rhizobia. Cells were examined microscopically after they were extracted from peat rather than in peat cultures directly. This allowed a much greater number of cells to be observed in each section and avoided difficulties of fixing and sectioning a highly heterogeneous matrix such as peat. The morphology of cells in peat cultures was observed to be the same before and after extraction (results not shown), and in view of the recovery, extracted
FIG. 2. Examination of *Rhizobium* sp. strain SU343 cells by transmission electron microscopy. Cells from broth cultures (A), and cells extracted from peat cultures after 2 (B) and 7 (C) days and 9 months (D) are shown. The arrows indicate representative cells with thickened walls, and PHB granules are marked by asterisks. Bars, 0.5 μm.
FIG. 3. Examination of *B. lupini* WU425 cells by transmission electron microscopy. Cells from broth cultures (A) and cells extracted from peat cultures after 7 (B) and 14 (C) days and 7 months (D) are shown. The arrows indicate representative cells with thickened walls, and PHB granules are marked by asterisks. Bars, 0.5 μm.
cells were considered to be representative of the population of cells in peat cultures.

When cells from stationary-phase broth cultures of SU343 and WU425 were examined microscopically, the cell wall and plasma membrane were seen to be clearly separated and granules of polyhydroxybutyrate (PHB) were abundant in the cells (Fig. 2A and 3A). After 2 days in peat, most of the SU343 cells resembled the broth-cultured cells in appearance, although the PHB granules were less abundant, and in a small number of cells, the periplasmic space appeared to be occluded (Fig. 2B). After 7 days in peat, essentially all of the SU343 cells had lost their PHB and a high proportion of the cells had the periplasmic space occluded (Fig. 2C). Most of the cells observed in multiple electron micrographs of the type shown in Fig. 2C were noted to have undergone cell wall thickening. Based on a random sample of 32 cells in these micrographs, 91% had thickened cell walls. No further morphological changes were evident in cells that were sampled from the peat cultures at different times up to 9 months (Fig. 2D). Similar results were observed in peat-cultured WU425 cells, except that the thickening of the walls was first evident after 7 days in peat and almost all of the cells were altered by 14 days (Fig. 3). The morphologies of TA1, CC1192, and strain 1080 cells in stationary-phase broth cultures were, in general, similar to those observed for SU343 and WU425 cells. Almost all of the TA1, CC1192, and strain 1080 cells from 4- and 5-month- and 11-day-old peat cultures, respectively, were observed to have thickened cell walls (results not shown). Cells from TA1, CC1192, and strain 1080 peat cultures of other ages were not examined.

The cell wall thickening that occurred in peat cultures of SU343 was not observed when stationary-phase cells were maintained in broth cultures at 30°C, with or without shaking, for up to 14 days. PHB granules were lost from the cells that were shaken but not from cells maintained without shaking. Cell wall thickening was not observed in SU343 cells that were examined after 3 and 18 days on sucrose-glucose agar plates at 30°C (results not shown).

In all species examined, no wall thickening occurred in cells cultured in broth, but thickening was evident after the transfer of cells from broth cultures to peat and after the cells entered stationary phase. This suggests that the morphological changes in the cell wall are part of an adaptive response induced by the conditions in peat to increase long-term survival. We suggest that a combination of factors, including nutrient limitation and reduced O₂ tension, are involved in the induction of cell wall changes in peat cultures. These changes were similar to those that were observed in R. leguminosarum bv. trifolii TA1 cells that had been cultured in peat for 1 month (3). Electron-dense material in the periplasmic space was also observed in some soil isolates (1) and several Acinetobacter strains subjected to desiccation stress (7).

**Survival of rhizobial cells on beads.** The survival of SU343 and WU425 cells after inoculation onto plastic beads was estimated by plate counts (Fig. 4). The percentage of viable cells of SU343 and WU425 that survived for 24 h on beads increased significantly when inoculation was from peat cultures that were 14 days old or older compared to younger peat cultures (Fig. 4). Similarly, after inoculation of SU343 onto beads from 21-day-old peat cultures, enhanced survival was observed when viable cells were estimated by MPN analysis (results not shown).

When SU343 cells were extracted from 1- and 14-day-old peat cultures before being inoculated onto beads, the numbers of viable cells recovered after 30 min were estimated to be 4.7 × 10⁵ and 1.1 × 10⁶ bead⁻¹ for SU343 and 1 × 10⁶ and 5 × 10⁶ bead⁻¹ for WU425. The numbers of viable cells after 24 h are expressed as a percentage of viable-cell numbers counted, as described, 30 min after inoculation onto the beads. The survival rate is significantly higher (P < 0.05) for SU343 cells from 14-, 24-, and 51-day-old peat cultures (more than 1%) than for those from 1-, 3-, and 10-day-old peat cultures (less than 0.1%). The survival rate is significantly higher (P < 0.05) for WU425 cells from 14-, 21-, 28-, 56-, and 85-day-old peat cultures (more than 5%) than for those from 1- and 6-day-old peat cultures (less than 0.5%). The data are the means of four samples taken from each of two individual packets of peat cultures. The error bars represent the standard deviation of the mean.

![Fig. 4. Survival on plastic beads of *Rhizobium* sp. strain SU343 and *B. lupini* WU425 cells. The numbers of viable cells that were recovered 30 min after inoculation onto beads from peat cultures were between 1 × 10⁵ and 3 × 10⁵ bead⁻¹ for SU343 and 1 × 10⁶ and 5 × 10⁶ bead⁻¹ for WU425. The numbers of viable cells after 24 h are expressed as a percentage of viable-cell numbers counted, as described, 30 min after inoculation onto the beads. The survival rate is significantly higher (P < 0.05) for SU343 cells from 14-, 24-, and 51-day-old peat cultures (more than 1%) than for those from 1-, 3-, and 10-day-old peat cultures (less than 0.1%). The survival rate is significantly higher (P < 0.05) for WU425 cells from 14-, 21-, 28-, 56-, and 85-day-old peat cultures (more than 5%) than for those from 1- and 6-day-old peat cultures (less than 0.5%). The data are the means of four samples taken from each of two individual packets of peat cultures. The error bars represent the standard deviation of the mean.](http://aem.asm.org/).
Cells in stationary phase are well known to be more resistant to various types of stresses (9, 18). However, simply being in stationary phase did not equip the cells for survival against the stresses of inoculation onto plastic beads. No viable cells were recovered from plastic beads 4 and 24 h after inoculation with SU343 cells from 2- to 3-day-old broth cultures, which contained PHB granules, or from 8- to 10-day-old stationary-phase broth cultures, which had been depleted of their PHB (results not shown). These results also show that the presence of PHB reserves did not improve the survival of rhizobia on plastic beads.

In all of our experiments, increased viable-cell numbers on the beads were noted when cell wall changes had taken place prior to inoculation, suggesting that these changes are related to the enhanced survival. However, fitness for survival is likely to involve additional genetically determined factors, since a much higher number of WU425 than SU343 cells survived on beads, even when both types of cells had thickened walls. Differences in capacity for survival between strains have been attributed to genetic variation (17).

**SDS-PAGE of proteins from broth- and peat-cultured cells.**

Protein extracts of *S. melliloti* strain 1080 were analyzed by SDS-PAGE. This strain was used for SDS-PAGE because it is derived from *S. melliloti* strain 1021, for which the genome sequence is available (http://sequence.toulouse.inra.fr). Differences were observed between the broth- and peat-cultured cells with regard to polypeptides expressed, especially in the 40- to 53-kDa range (Fig. 5). The N-terminal sequence of a 23-kDa polypeptide band that was clearly increased in expression in peat cultures was determined to be AFELPNLFDY, which had 100% homology with an iron-manganese superoxide dismutase (SOD) of *S. melliloti* strain 1021. The increased expression of SOD was noted after 1 to 2 days in peat (data not shown), before the changes in the cell wall morphology occurred, and is unlikely to be related directly to cell wall changes. SOD protects cells against oxidative damage caused by superoxide radicals, which are generated as a byproduct of aerobic metabolism. The increased expression of SOD coincided with the initial rapid growth phase after the cells were transferred into peat, when reactive oxygen species are likely to be formed.

In conclusion, this study has shown that cells from several species of rhizobia undergo changes to their cell wall morphologies when transferred from broth cultures into peat. The cell wall thickening seems to be associated with enhanced survival of rhizobia in peat cultures and may play a role in enhancing survival after inoculation of cells onto surfaces, such as seeds. Nutrient limitation and a lowered O₂ concentration in peat may be factors in the induction of the adaptive morphological changes. Preliminary electrophoretic studies have indicated that a proteomic approach is likely to shed light on the molecular nature of these changes.

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