Growth-Related Substituent Changes in Exopolysaccharides of Fast-Growing Rhizobia

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Pyrivic acid and O-acetyl groups are the major noncarbohydrate substituents in exopolysaccharides (EPS) produced by fast-growing species of Rhizobium. EPS substituent variations were observed among strains of the same species. The amounts of these substituents also varied with culture age; pyrivic acid increased in the EPS of all four species, whereas O-acetyl increased in Rhizobium trifolii and R. leguminosarum EPS, decreased in R. meliloti EPS, and remained constant in R. phaseoli EPS. The use of glycerol as a substrate for R. meliloti significantly increased EPS yields, whereas mannitol increased those of the other three Rhizobium species.

The notion that rhizobium exopolysaccharides (EPS) participate in the initial stages of recognition leading to host legume nodulation through interaction with lectins in root hairs (3) is currently a subject of active investigation in many laboratories. A corollary of this hypothesis is that the host specificity displayed by a nodulating strain resides in the EPS. However, in a number of studies (16, 19, 20), no significant differences in EPS sugar or substituent composition have been found among Rhizobium leguminosarum, R. phaseoli, and R. trifolii strains. There are two reports that the galactose content in R. trifolii EPS is lower (8, 12). Fast-growing rhizobium (R. trifolii, R. phaseoli, R. leguminosarum, and R. meliloti) EPS contain pyronic acid (PA), O-acetyl groups (Ac), d-glucose, and d-galactose, as determined by composition and structure studies. Polysaccharides from all species except R. meliloti also contain D-glucuronic acid (11, 19).

Studies from this center have shown that the EPS from Xanthomonas campestris NRRL B-1459 (xanthan gum), which also contains PA, is a mixture of low- and high-PA types produced during early and later stages, respectively, of fermentation (4, 17). Other factors, including strain variation and growth medium, affect the levels of PA and Ac in xanthan gum. A recent study by Mort and Bauer (14) has attributed a culture age-related interaction between soybean lectin and EPS from R. japonicum to increasing O-methylation of galactosyl residues. In view of our earlier experience with xanthan gum, it was of interest to determine whether similar substituent changes occur during the production of EPS by fast-growing rhizobia.

The following strains of Rhizobium were obtained from the Agricultural Research Service Culture Collection: R. trifolii NRRL B-327, B-508, and L-158; R. leguminosarum NRRL B-4406 and L-235; R. phaseoli NRRL B-507; and R. meliloti NRRL B-1261 and L-89. Stock agar slant YMS medium (1) contained the following (per liter): 10 g of mannitol, 1.0 g of yeast extract, 20 g of agar, and 200 ml of soil extract (6). The pH was adjusted to 6.7 before sterilization. We prepared fresh cultures at monthly intervals by incubating the inoculated medium for 5 days at 25°C; the cultures were then stored at 4°C.

We prepared culture broths from stock slants by inoculating a loopful of cells into 50 ml of YMS broth in an Erlenmeyer flask (300 ml) and then incubating the flask on a rotary shaker (25°C) until abundant growth was evident (3 to 5 days).

Owen synthetic medium C (15), used as the test medium, contained the following (grams per liter): Casamino Acids (lot B-230; Difco Laboratories), 1.0; mannitol, 10.0; NH4NO3, 0.48; CaCl2, 0.11; MgSO4, 0.25; Tris buffer, 1.21; KH2PO4, 0.04; FeCl3·6H2O, 0.001; MnSO4, 0.0001; and Na2MnO4, 0.0001 (pH 6.8). After sterilization at 121°C (15 min), filter-sterilized thiamine (0.1 mg) and biotin (0.5 µg) were added. This medium was modified by the addition of 3.9 g of KH2PO4.

Polysaccharide-producing cultures were grown in Erlenmeyer flasks (500 ml) that contained 100 ml of synthetic medium. These test flasks (third stage) were inoculated with 5 ml of a 3-day-old inoculum (second stage) which had been started from 5 ml of YMS broth culture.
(first stage). The test flasks were incubated on a reciprocal shaker (100 strokes per min) at 25°C. One flask was prepared for each sample to be taken.

Culture broths were diluted when necessary and centrifuged at 20,000 × g for 30 min to remove cells. After the cell-free broths were concentrated fourfold by lyophilization, KCl was added to give 1% solutions, and the EPS were precipitated by the addition of 3 volumes of 95% ethanol. After two additional precipitations, the EPS solutions were dialyzed against distilled water, adjusted to pH 7.0, and lyophilized. None of the products precipitated with quaternary ammonium halide (18) showed the presence of significant amounts of neutral polysaccharide.

Reducing sugars were determined on EPS acid hydrolysates in an automatic analyzer (Technicon Instruments Corp.) by the potassium ferricyanide method (10); owing to the presence of galactose and glucuronic acid, values for reducing sugars were consistently higher than those for glucose alone. Glucose was analyzed by a glucose-oxidase procedure, uronic acid was measured by the method by Gregory (7), PA was determined by the enzymatic method of Duckworth and Yaphe (5) as described by Jeanes et al. (13), and Ac was measured quantitatively by the procedure of Hestrin (9). Cellulose thin-layer chromatography (2) of acetylhydroxamic acid confirmed the identification of this substituent in all of the EPS. Minor differences in PA and Ac among similar experiments with one strain may have been due to experimental error.

Owen synthetic medium was first used in inoculum and test flasks but was later modified by the addition of 0.39% potassium phosphate. Without the additional buffer, insufficient quantities of EPS (10 mg/100 ml) were produced for analytical purposes, probably owing to the low pH during the fermentation. The yield increase due to buffering was most dramatic for the R. trifolii strains (225 mg/100 ml); culture viscosities became so high that considerable dilution was required before the cells could be removed from the broths. For the other three species of Rhizobium, the effect on yield was less dramatic. Even so, the amount of EPS steadily increased during the course of fermentation (20 to 100 mg/100 ml), and adequate quantities of the product were obtained for analyses.

Samples taken after the first 32 h of incubation showed no further production of EPS; PA and Ac contents of the isolated EPS did not change. Consequently, EPS were recovered from samples taken between 8 and 32 h.

Changes in EPS PA content are illustrated in Fig. 1. PA values are expressed as percentages of total EPS. EPS from R. trifolii L-158 had significantly less PA during the early hours of fermentation, even though the PA level ultimately reached previously reported values (19). The same pyruvlation kinetics were obtained for R. trifolii B-508. The ultimate PA level in the EPS of this strain was about half that in strain L-158 EPS. R. leguminosarum B-4406 and R. phaseoli

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**FIG. 1.** EPS PA content during Rhizobium growth in shaking flasks of synthetic medium at 25°C.

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**FIG. 2.** EPS content during Rhizobium growth in shaking flasks of synthetic medium at 25°C.
produced EPS that exhibited a more gradual increase in PA during the course of fermentation; however, the maximum values differed from each other. Molar ratios of D-glucose to D-glucuronic acid were the same throughout the growth period in the EPS of all of the strains. In the EPS of *R. meliloti* L-89, the PA content diminished during the early hours of fermentation and then increased to a constant level after 32 h.

Changes in EPS Ac content are shown in Fig. 2. Ac levels increased in the EPS of *R. trifolii* L-158 and *R. leguminosarum* B-4406 but remained constant in the EPS of *R. phaseoli*. *R. meliloti* L-89 EPS actually showed decreases in Ac content. These decreases could not be accounted for by alkaline deacetylation because the pHs of the cultures remained below 7 when the cultures were buffered.

In this study, EPS variations were observed among strains of a single species. PA and Ac values for EPS isolated from stationary-phase cultures are given in Table 1. With the exceptions of *R. trifolii* B-508 and B-327, considerable variation in the ratio of PA to Ac can be seen among EPS from strains of a single species.

Changes in EPS composition may not be associated only with culture age and strain variations. In Table 2, glycerol and mannitol are compared as carbon sources. Selected strains of *R. trifolii*, *R. leguminosarum*, and *R. phaseoli* produced more EPS from mannitol than from glycerol. Conversely, the *R. meliloti* strain produced very little polysaccharide from mannitol but satisfactory amounts from glycerol. The EPS PA and Ac values for *R. trifolii*, *R. leguminosarum*, and *R. phaseoli* seem independent of the substrate. PA and Ac were lower in *R. meliloti* EPS isolated from the glycerol medium.

The results show that PA and Ac variation occurs in fast-growing rhizobium EPS. The change in one substituent is not always related to a change in the other. If EPS do indeed function as recognition factors, then such substituent changes could affect the host legume specificity and, consequently, the relative competitiveness of the microsymbiont.

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**LITERATURE CITED**

