Synthesis of Exopolysaccharide by *Bradyrhizobium japonicum* during Growth on Hydroaromatic Substrates

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The hydroaromatic acids shikimate and quinate, which may be available as carbon sources in the soil, supported production of low levels of acidic exopolysaccharide by *Bradyrhizobium japonicum*. Exopolysaccharide production (micrograms per 10^9 cells) was 4.9 on quinate and 4.5 on shikimate; in comparison, it was 128 on adipate, 18 on L-arabinose, and 39 on D-glucose.

A number of aromatic compounds are utilized by diverse rhizobia (7, 11, 14, 15). These compounds may also function as chemoattractants (17). It has been proposed (7) that these substances, derived from decomposing plant tissue, may be significant carbon sources in carbon-limited soils. They may also be present in the legume rhizosphere, since Rao (18) found that inoculation of alfalfa and mung bean with rhizobia caused increased root exudation of o-dihydroxyphenols, along with other compounds.

The suggestion that a compound may support the growth of rhizobia raises the question of whether it can also support exopolysaccharide (EPS) synthesis, since EPS may be of importance in infection and nodulation (1, 4, 8), possibly due to its recognition by root hair lectin (19). Rhizobial EPS may also function in competitiveness (12). Previous studies with aromatic compounds have not addressed this aspect of rhizobial physiology.

*Bradyrhizobium japonicum* USDA 110 secretes an acidic EPS in both capsular and soluble forms (13) in quantities that vary with the carbon source, with greater production on pentoses, organic acids, sugar acids, and polyls and lesser production on hexoses and amino acids (21). Additionally, *B. japonicum* secretes a small amount of lower-molecular-weight glucan (9) resolvable by liquid chromatography (22).

In this study, secretion of these EPSs by *B. japonicum* USDA 110 was measured on defined media containing either quinate or shikimate, both hydroaromatic acids. Both compounds are components of the so-called fulvic acid fraction of soil, and both can serve as nontoxic carbon sources for rhizobial growth (15). Quinate, both free and esterified, occurs in the tissues of many plants (10). Shikimate is also present but to a lesser extent (5). For comparison, a pentose (L-arabinose), hexose (D-glucose), and organic acid (adipic acid) were also tested. An attempt was made to test the aromatic acids p-hydroxybenzoate and antranilute, but they were only very poor substrates for growth of *B. japonicum*.

**Cultures.** The defined minimal medium for measuring EPS synthesis comprised 10 mM NH₄Cl, 0.7 mM CaCl₂, 12 mM MOPS (3-(N-morpholino)-2-hydroxypropanesulfonic acid) (brought to pH 6.9 with NaOH), and substrate at a final concentration of 21 mM carbon. The substrates were shikimic acid (3 mM) and quinic acid (3 mM) (each prepared as neutralized, filter-sterilized 0.5 M stock), adipic acid (3.5 mM) (added directly to the medium before autoclaving), and L-arabinose (4.2 mM) and D-glucose (3.5 mM) (prepared as autoclaved concentrates). A 100× concentrate of trace met-als plus phosphate (21) was added after autoclaving, except that citrate was replaced by 5 mM disodium EDTA in the concentrate. Nephelometer flasks containing 200 ml of medium were inoculated with *B. japonicum* 61A89 (=USDA 110) and shaken at 24°C until the stationary phase of culture, when viable counts were taken. Growth was monitored turbidimetrically with a Klett colorimeter equipped with a red filter.

**EPS isolation and chromatography.** Cultures, preserved with 0.01% (wt/vol) thimerosal, were stored at 4°C for 1 week to permit capsular EPS to dissolve without cell lysis (13). After centrifugation at 26,000 × g for 20 min, cells were washed with 25 ml of 12 mM MOPS (pH 6.9) and the supernatants were pooled. The cell pellets were hard and compact, indicating that little residual EPS was left behind. The supernatants were freeze-dried, dissolved in 20 ml of water, brought to pH 8.5 with NH₄OH, and chilled. The resulting precipitate of salts was removed by centrifugation and discarded, the supernatant was freeze-dried and dissolved in water to 3 ml, and EPS was precipitated with 27 ml of absolute ethanol. After chilling, the precipitate was recovered by centrifugation and dissolved in 10 ml of water, and proteins were precipitated with trichloroacetic acid (5% [wt/vol]). After chilling for 1 h, the proteins were pelleted by centrifugation, and the supernatant was freeze-dried. The lyophilized residue was dissolved in water to 3 ml, and EPS was precipitated with 90% ethanol. The EPS was recovered by centrifugation, air dried, and dissolved in 15% (vol/vol) acetic acid to 2.0 to 2.4 ml (final volume). A 1-ml portion was applied to a column (1.5 by 111 cm) of Bio-Gel P-6 (400 mesh; exclusion limit, 6,000 daltons for peptides) and eluted by using 15% (vol/vol) acetic acid as the solvent (22). Fractions were assayed for hexose (20) and uronic acid (3).

Elution profiles of each of the five EPS preparations (Fig. 1) showed a peak of polysaccharide at or near the void volume, in the location where the acidic EPS elutes (22). That this was indeed the acidic EPS was verified by uronic acid assays, which showed molar ratios of hexose to uronate of 4.2 on shikimate, 4.2 on quinate, 4.7 on arabinose, 4.8 on glucose, and 4.7 on adipate. These values are close to the theoretical ratio of 4.0 (13). Although all substrates supported synthesis of the acidic EPS, the quantity varied over a 28-fold range (Fig. 1), with the hydroaromatics being the poorest substrates and adipate the best. The similarity between behavior on shikimate and quinate is not surprising since both compounds are metabolized via the β-ketoadipate pathway through protocatechuate (15, 16).
Production of acidic EPS was not correlated with growth rates. The mid-log-phase doubling times were 7 h on quinate, 13 h on shikimate, 14 h on arabinose, 21 h on adipate, and 39 h on glucose. Thus, the growth rate on quinate was twice that on shikimate or arabinose, whereas EPS production on quinate was the same as on shikimate but only about one-fourth that on arabinose.

Liquid chromatography of EPS from the glucose culture also resolved significant amounts of material of lower molecular weight than the acidic EPS (Fig. 1D). Previous work has shown that this material is glucan (22). Significant glucan levels were observed only in glucose cultures, with little on shikimate, quinate, adipate, or arabinose (Fig. 1).

**Lectin binding.** Although the acidic EPS may exert an effect on host specificity and infection in the soluble, cell-free state (8), some evidence indicates that interaction of root hair lectin with attached capsules may constitute a step in the infection of soybean roots (19); indeed, attachment via a capsule has been observed by electron microscopy (6). The presence of attached capsules was assessed by observing binding of soybean lectin labeled with fluorescein isothiocyanate (FITC-SBL) (2). Cells from cultures at the mid-log phase were treated with FITC-SBL (Sigma Chemical Co.) and observed with a Zeiss Standard 16 microscope equipped with an epifluorescence condenser and mercury lamp, using a 450- to 490-nm exciter filter and a 520- to 560-nm barrier filter. Less than 10% of the cells from the glucose culture, but over 90% of the cells from all other cultures, bound lectin. Galactose inhibited FITC-SBL binding, indicating specificity for galactose residues, as expected (2). Thus, cells on all substrates except glucose produced attached capsules at the mid-log phase of growth.

In summary, the hydroaromatic acids quinate and shikimate served as excellent substrates for the growth of *B. japonicum* but were poor substrates for the production of EPS. Enough capsule was present for the cells to bind lectin. Secretion of large amounts of EPS would occur in the presence of other carbon sources, which would likely be available in most soil environments.

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**LITERATURE CITED**


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**FIG. 1.** Liquid chromatography elution profiles of EPS isolated from culture supernatants of *B. japonicum* USDA 110 grown on shikimate (A), quinate (B), L-arabinose (C), D-glucose (D), and adipate (E). Fractions (2.7 ml) were assayed with anthrone for total hexose. The numbers refer to areas under peaks. *V*<sub>o</sub>, Void volume (determined with dextran 10,000); *V*<sub>r</sub>, included volume (determined with glucose).
Changes with culture age and correlations with binding of soybean seed lectin to the bacteria. Plant Physiol. 66:158–163.


