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Hydrolytic Enzyme Production by *Rhizobium*†

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Cellulase and hemicellulase activity was detected in temperate (infective and noninfective) and tropical strains (infective) of *Rhizobium*. Hydrolytic enzymes were initially detected by a cup-plate assay. The presence of cellulase and hemicellulase was confirmed by viscometric assay. Implications of the presence of these enzymes in *Rhizobium* are discussed.

The precise mechanism whereby *Rhizobium* successfully infects (induces infection threads) in temperate legumes is unresolved. The "polygalacturonase" hypothesis of Fahraeus and Ljunggren (10) involves the localized increased activity of plant-synthesized pectolytic enzymes at the site of infection. This increased activity was explained as a result of the inducing activity of the extracellular polysaccharide of the homologous, but not the heterologous, rhizobia. The hypothesis in essence proposes a physical penetration of the root hair cell wall. This hypothesis, however, remains controversial due to negative or variable results obtained in subsequent studies (reviewed by Dart, 7).

Nutman (16) had earlier proposed a hypothesis of infection invoking a process of "invagination" of the root hair cell wall at the site of infection. Supporting evidence for Nutman's hypothesis was provided by Napoli and Hubbell (15). They used electron microscopic examination of serial thin sections of successful infection sites on clover root hairs to reveal the apparent presence of an invagination of the plant cell wall. This report was widely cited as strong support for Nutman's idea that penetration of the plant cell wall did not occur. Subsequently, Callaham (D. A. Callaham, M.S. thesis, University of Massachusetts, Amherst, 1979) detected certain inconsistencies in the interpretation of results by Napoli and Hubbell (15), especially in consideration of the belated report of pectolytic enzymes in *Rhizobium* by Hubbell et al. (12). Callaham (M.S. thesis) repeated the electron microscopic study of infection in greater scope and detail and obtained clear evidence of a zone of cell wall hydrolysis immediately adjacent to adsorbed rhizobia at the infection site. New cell wall material was deposited as a dome over the zone of hydrolysis; the continued growth and extension of this dome formed the infection thread. In serial sections the infection was therefore seen morphologically as an invagination (15). Callaham (M.S. thesis), considering the evidence of hydrolysis and the probable ontogeny of the events, reinterpreted the process as involving localized wall degradation resulting in physical penetration by the bacteria. This supports a basic tenet of the polygalacturonase hypothesis, the main point of divergence being the high probability that the hydrolytic enzymes involved in the process are of microbial rather than plant origin.

The presence of cell wall-hydrolyzing enzymes in plant-infective microorganisms is widespread in nature (3). These enzymes are frequently inducible and occur in the sequence pectinase-hemicellulase-cellulase (6). These generalizations, in addition to the report of the first type of enzyme in the sequence in the *Rhizobium*-legume system (12), prompted a search for the remaining two types of enzymes (hemicellulase and cellulase) that could also reasonably be expected to be present in *Rhizobium* and active in the infection process. We report here the demonstration of low levels of these enzymes in *Rhizobium*.

The strains of rhizobia used in this study were: *R. trifolii* BAL (infective on *Trifolium repens*; from F. B. Dazzo); *R. trifolii* BART-A (noninfective on *T. repens*; from G. Fahraeus); *Rhizobium* sp. (cowpea) CIAT 79 and CIAT 605 (infective on *Stylosanthes*; from P. H. Graham); and CB1650 and CB82 (infective on *Stylosanthes*; from R. A. Date). The cultures were maintained on yeast extract-mannitol (YEM) agar medium (11). Cells to be tested were grown on a basal hemicellulase-cellulase induction medium consisting of (g/liter): KH_2PO_4, 2.3; $MgSO_4$, 0.2; mannitol, 1; and trace elements and vitamins as used by Bergersen (4). Nitrogen sources used were $NaNO_3$, 5 g/liter or sodium glutamate, 1 g/liter. The pH was adjusted to 6.0 by using 10% (wt/vol) Na_2HPO_4. Four carbohy-

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drates were tested for enzyme induction, including (g/liter): cellobiose, 5; carboxymethylcellulose, 1; Walseth cellulose (14), 1; and gum arabic, 1. Flasks of broth were inoculated with a cell suspension from YEM plates and incubated on a shaker at 28°C for 1 week. Cultures were centrifuged at 4°C, suspended in 0.1 M phosphate-citric acid buffer (pH 5.0), and sonicated for 2 min or shaken for 24 h, followed by centrifugation. All three supernatants were assayed for enzyme activity.

Two strains, BAL and CIAT 79, were tested initially for enzyme activity by the cup-plate method (9) using cells induced on cellobiose or Walseth cellulose. Assay plates containing cellobiose or Walseth cellulose (1.0 g/liter) in phosphate-citric acid buffer were solidified with agar (Difco; 1.5%) zones of hydrolysis were developed with 0.1 N iodine in 95% ethanol.

In this initial test, the first supernatant showed no activity unless concentrated (10-fold) by dialysis against polyvinylpyrrolidone. Supernatants from concentrated, shaken, or sonicated pellet suspensions showed definite activity. BAL grown on either substrate produced hydrolysis zones averaging 6 mm in diameter on Walseth cellulose agar. These supernatants from CIAT 79 produced hydrolysis zones of about 9 mm (concentrated) and 18 mm (sonicated), indicating wall-bound nature of the enzyme(s). Control treatments (boiled or pronase-treated) of cellulase (Calbiochem) or sonicated supernatant showed no activity. The positive results of this test could not, however, be attributed to the activity of cellulase enzymes since control plates lacking substrate showed similar zones of hydrolysis. The strains of rhizobia used have enzymes which hydrolyzed some polysaccharide component(s) of agar. The possible similarity of these components to hemicellulose and the unresolved question of cellulase production prompted the more specific viscometric assay for cellulase and hemicellulase.

Cellulase activity was measured by decrease in viscosity of a 0.2% solution of carboxymethylcellulose in phosphate-citric acid buffer at pH 5.0 and 30°C. The solution was prepared by stirring, centrifuged at 5,000 × *g* for 30 min, and allowed to stabilize for at least 48 h at room temperature. Enzyme activity with different concentrations of commercial cellulase (Calbiochem) was determined using both cup-plate and viscometric methods. Hemicellulase activity was assayed by measuring the decrease in viscosity of a solution of 7.5% (wt/vol) gum arabic (Sigma) in phosphate-citric acid buffer at pH 5.0 and 30°C. Sodium azide was added to both carboxymethylcellulose and gum arabic solutions at a concentration of 0.625 g/liter to inhibit microbial growth. By this method (percent reduction in viscosity was calculated according to Fahraeus and Ljunggren [10]), it was shown that the temperate strains had appreciable activity on carboxymethylcellulose when grown with either cellobiose or gum arabic, whereas activity on gum arabic was low for both types of cultures. The tropical strains, grown with either cellobiose or gum arabic, failed to show cellulase activity, but cultures grown with gum arabic did show hemicellulase activity. These results are reported qualitatively due to the limited range of growth and assay procedures employed.

Indications of the involvement of cell wall-degrading enzymes in the *Rhizobium* infection process are found in several electron microscope studies. They have shown cell wall erosion or dissolution surrounding the bacteria (5, 15; Callaham, M.S. thesis). Other steps of the invasion process also suggest hydrolytic enzyme activity, such as the release of bacteria from the infection thread (2, 17).

The differences in enzyme types and activities found among fast-growing and slow-growing strains may reflect the differences in infection pathways (5, 7). Negative results, however, should not be taken as evidence for absence of ability to produce the enzyme(s). Certain strains may require any combination of specific inducer substances, specific substrates, or specific assay conditions to demonstrate presence of the enzymes. The variety of cell wall-degrading enzymes is very large, and some are highly substrate specific (1).

The demonstration of presence of cellulases and hemicellulases in *Rhizobium*, in addition to pectinase (9), strongly supports the view of Callaham (M.S. thesis) that rhizobia infect legumes by hydrolyzing the root cell wall at the highly localized site of infection. Legume infection requires a complex and delicate biochemical interaction between the plant host and its homologous *Rhizobium*. Hydrolytic enzymes have a very wide range of substrate specificity as well as regulatory characteristics (1). Variability in these characteristics may be related to variations in host plant specificity of rhizobia. In essence, *Rhizobium*-legume specificity may be determined at the levels of both cell attachment via specific lectins (8) and penetration via specific hydrolytic enzymes.

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