Cell-Associated Pectinolytic and Cellulolytic Enzymes in
\textit{Rhizobium leguminosarum} Biovar Trifolii

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The involvement of \textit{Rhizobium} enzymes that degrade plant cell wall polymers has long been an unresolved question about the infection process in root nodule symbiosis. Here we report the production of enzymes from \textit{Rhizobium leguminosarum} bv. \textit{trifolii} that degrade carboxymethyl cellulose and pectinolate model substrates with sensitive methods that reliably detect the enzyme activities: a double-layer plate assay, quantitation of reducing sugars with a bichinchoninate reagent, and activity gel electrophoresis-isoelectric focusing. Both enzyme activities were (i) produced commonly by diverse wild-type strains, (ii) cell bound with at least some of the activity associated with the cell envelope, and (iii) not changed appreciably by growth in the presence of the model substrates or a flavone that activates expression of nodulation (nod) genes on the resident symbiotic plasmid (pSym). Equivalent levels of carboxymethyl cellulase activity were found in wild-type strain ANU843 and its pSym-cured derivative, ANU845, consistent with previous results of Morales et al. (V. Morales, E. Martinez-Molina, and D. Hubbell, Plant Soil 80:407-415, 1984). However, polygalacturonase activity was lower in ANU845 and was not restored to wild-type levels in the recombinant derivative of pSym \textsuperscript{Anu} containing the common and host-specific nod genes within a 14-kb HindIII DNA fragment of pSym from ANU843 cloned on plasmid pRt032. Activity gel electrophoresis resolved three carboxymethyl cellulase isozymes of approximately 102, 56, and 33 kDa in cell extracts from ANU843. Isoelectric focusing activity gels revealed one ANU843 polygalacturonase isozyme with a pl of approximately 7.2. These studies show that \textit{R. leguminosarum} bv. \textit{trifolii} produces multiple enzymes that cleave glycosidic bonds in plant cell walls and that are cell bound.

An important event in the infection of white clover roots by \textit{Rhizobium leguminosarum} biovar \textit{trifolii} leading to development of the root nodule symbiosis is the passage of the bacteria across the root hair wall (5, 10, 14, 24, 26, 35). This rigid assemblage of plant polysaccharides and glycoproteins constitutes a barrier to host specificity (1). Several hypotheses have been proposed to explain how this event occurs. Nutman (27) proposed that rhizobia redirect growth of the root hair wall from the tip to the localized site of infection. This would cause invagination rather than penetration of the root hair wall, forming the tubular structure of the infection thread. Ljunggren and Fahraeus (20) proposed that homologous \textit{Rhizobium} strains specifically induce the host plant to produce polygalacturonases, which soften the root hair wall at the site of infection and allow the bacteria to penetrate between microfibrils to the cell membrane and initiate an infection thread. The ability of \textit{Rhizobium melliloti} to induce polygalacturonase production by alfalfa roots is linked to a plasmid carried by the bacterium (28). The third model (5, 10, 16) proposes that wall-degrading enzymes produce a localized degradation that completely traverses the root hair wall, allowing direct penetration by the bacteria.

Electron microscopic studies support the hypothesis that hydrolytic enzymes are involved in various steps in the infection process, including entry of rhizobia into root hairs (5, 14, 26, 31, 35, 40), crossing of root cortical cell walls by rhizobia in the advancing infection thread (3, 6), and release of rhizobia from infection threads into the nodule cell cytoplasm (6, 41). The strongest evidence for involvement of wall hydrolysis in the \textit{R. leguminosarum} bv. \textit{trifolii}-white clover infection process was obtained by Callaham and Torrey (5), who showed a localized degradation of the root hair wall coincident with deposition of a new wall layer, above the site of degradation, which is continuous with the root hair wall. Recently, Baker et al. (2) found that many cells of \textit{R. leguminosarum} bv. \textit{trifolii} attached to the root surface of white clover produce pit erosions in epidermal walls that follow the contour of the bacterium, suggesting that wall-degrading enzymes are associated with the bacterial cell surface itself and/or locally induced in the plant by components of the bacterial surface.

Rhizobial infection of legumes is a delicately balanced process. If wall-degrading enzymes are involved, their production would have to be restricted to account for slow, localized penetration without destruction of the root hair and subsequent abortion of the infection process (10, 16). McCoy (24), in considering the role of hydrolytic enzymes in active penetration of plant cell walls by certain pathogenic microorganisms, was the first to investigate the possible involvement of these enzymes in the infection of legumes by \textit{Rhizobium} species. She found no evidence for these enzymes from rhizobia. However, sensitive procedures to detect minute amounts of cell wall-degrading enzymes were...
not available. More recently, several studies have detected pectinolytic (17, 22, 23, 29, 30, 36), cellulolytic (25, 36), and hemicellulolytic (21, 22) enzyme activities from pure cultures of rhizobia. In general, the activities of these rhizobial enzymes are very low and at the limit of sensitivity of conventional reducing sugar assays; this has hampered research progress in this area. Nucleotide sequences homologous to pectic lyase genes from Erwinia spp. do not hybridize to DNA from R. trifolii (13). A preliminary report of hybridization to DNA from Rhizobium fredii has been made (39). A cloned 2.5-kb fragment of DNA encoding polygalacturonase from Agrobacterium tumefaciens biovar 3 did not hybridize to DNA from R. leguminosarum bv. vicieae or Bradyrhizobium japonicum (33).

In this study, we used assays with improved sensitivity and reliability to verify the production of R. leguminosarum bv. trifolii enzymes that degrade polygalacturonate and carboxymethyl cellulose (CMC) as model substrates of plant cell wall polymers. We also used R. leguminosarum bv. trifolii wild-type strain ANU843 to evaluate (i) the proportion of extracellular activity to cell-associated activity, (ii) methods for releasing these enzymes from cells, and (iii) whether the production of these enzymes is affected by the resident symbiotic plasmid (pSym) (especially its 14-kb nod region), the flavone that enhances nod expression, or the substrates themselves.

(Portions of this work were presented at the 12th North American Symbiotic Nitrogen Fixation Conference [2]).

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains of R. leguminosarum bv. trifolii used in this study were as follows: wild-type ANU843 (34), its pSym-cured derivative, ANU845 (37), a recombinant strain containing the 14-kb HindIII DNA fragment encoding the entire nod region of pSym cloned on plasmid pHr032 and introduced into the pSym-cured derivative (strain ANU845pRhr032A6) with a Tn5 insertion in the vector (38), derivatives of ANU843 containing a Tn5 insert in pSym nodD (ANU851) (11, 37) or nodC (ANU277) (12) (B. Rolfe, Australian National University), and a collection of strains (Fl1 through F9) isolated from nodules of red clover (Trifolium pratense) grown in soils of various pHs near Salamanca, Spain (E. Martinez-Molina, University of Salamanca).

Bacteria were cultured in BHI defined medium (8) with 0.5% (wt/vol) inositol (hereafter called B-INO) instead of mannitol as the carbon source (25). Broth cultures were grown in 75 ml of B-INO medium in 300-ml flasks shaken at 150 rpm at 28°C. Inocula were prepared by suspending cells from 5-day-old plate cultures into sterile B-INO medium, centrifuging aseptically at 4,000 × g for 15 min, and resuspending in B-INO medium to an initial population density of 107 cells per ml. In induction experiments, cells were grown in B-INO broth supplemented with 2 μM 4′,7-dihydroxyflavone (DHF), 0.2% (wt/vol) CMC, or 0.2% sodium polygalacturonate. Plate cultures were grown on B-INO medium containing 4 μM DHF and solidified with 2% purified agar (U.S. Biochemical Corp., Cleveland, Ohio). Kanamycin sulfate (30 μg/ml) was included in culture medium to retain the recombinant plasmid in strain ANU845 pHr032A6. Cultures were routinely checked for purity by streaking on B-INO and CMC agar plates.

Double-layer plate assay. A modification of the Saleh-Rastin et al. (36) plate assay was used. To detect in vivo activity, a bottom layer containing 15 ml of 0.7% agarose in B-INO medium was overlaid with 5 ml of 0.2% (wt/vol) substrate and 0.5% agarose in B-INO medium. To detect in vitro activity, B-INO medium was replaced with water in the bottom layer and 50 mM potassium phosphate–citric acid buffer (PCA; pH 5.2) in the thin overlay; the same concentrations of agarose were maintained. Plates were inoculated by transferring cells from colonies with sterile toothpicks or by adding 10 μl of cell extract containing 15 to 30 μg of protein, and plates were flooded with 0.1% Congo red solution for 4 days, colonies were washed off with water, and discarded. To detect carboxymethyl cellulase (CM-cellulase) activity, plates were flooded with 0.1% Congo red solution for 30 min and then rinsed several times with 1 M NaCl. Areas of degradation of polygalacturonate were detected by adding filtered 0.05% ruthenium red solution to plates for 20 min and then rinsing with water. This procedure revealed clear areas of hydrolysis.

Preparation of culture supernatants and cell extracts. Broth cultures in the early stationary phase (9 × 106 cells per ml) were centrifuged at 3,500 × g for 1 h at 4°C. Ammonium sulfate was added to the culture supernatant to 100% saturation at 4°C with constant stirring. Precipitated proteins in this extracellular fraction were centrifuged at 13,000 × g for 15 min at 4°C, redissolved, and dialyzed for 4 h with three changes of 300 ml of glycine-urea buffer (25) (1% glycine, 1 M urea, 156 mM potassium phosphate, 22 mM citric acid [pH 7.4]) at 4°C. The cell pellet was suspended in 1.5 ml of PCA buffer on ice, sonicated in five cycles of 1-min bursts at 32 W with a microprobe (Fisher model 300 sonic dismembrator), and centrifuged at 11,000 × g for 15 min at 4°C. Alternatively, the cells were washed with 30 mM Tris-HCl (pH 8.1), centrifuged at 11,000 × g for 15 min at 4°C, suspended in 1 ml of 20% sucrose containing 30 mM Tris-HCl (pH 8.1), and incubated in an ice bath for 40 min with 100 μl of 1-mg/ml lysozyme (Sigma Chemical Co., St. Louis, Mo.) in 100 mM EDTA at pH 7.5 to release proteins associated with the cell envelope (18). The lysozyme-EDTA-treated suspension was centrifuged at 11,000 × g for 15 min at 4°C, and the supernatant was dialyzed (Spectrapor membrane tubing; molecular weight cutoff, 12,000 to 14,000) against PCA. The presence of cytoplasmic constituents in these cell fractions was examined by measuring NADP-dependent glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49) (43), a cytoplasmic enzyme marker in R. leguminosarum bv. trifolii (15). All enzyme samples were stored at 4°C.

Quantitative enzyme assays. Substrates for CM-cellulase and polygalacturonase were the sodium salts of CMC (medium viscosity) and polygalacturonic acid (both from Sigma), respectively, at 0.2% (wt/vol) in PCA buffer. Sodium polygalacturonate was dialyzed against 1 liter of PCA buffer with three changes in 3 days. The reaction mixture contained 0.4 ml of substrate, 0.4 ml of enzyme solution, and 0.8 ml of PCA buffer in an Eppendorf tube. After the reagents were mixed quickly very well, an 0.8-ml aliquot (considered t = 0 h) was frozen at −80°C. The rest of the sample was incubated at 30°C for 5 h.

Product formation was measured by using the sensitive 2,2′-bichinonemiate method of Waffenschmidt and Jaenicke (42). This assay measures reducing sugars in the nanomole range without interference from proteins. Samples of 200 μl were mixed with 0.8 ml of water and 1 ml of 2,2′-bichinonemiate reagent (42) in tubes, which were tightly capped and heated at 100°C for 15 min in a heating block. Samples were then cooled to room temperature for 20 min, and their optical density was measured at 540 nm; the blank contained 1 ml of
water and 1 ml of 2,2’-bichinoninate reagent with no sugar. Standard curves for reducing sugar were prepared with glucose and galacturonic acid in the range of 0 to 55 nmol for CM-cellulase and polygalacturonase activities, respectively. One unit of enzyme activity is defined as the amount releasing 1 nmol of reducing sugar equivalent per min at 30°C. The amount of protein in samples was measured by the dye-binding method of Bradford (4) with bovine serum albumin (Sigma) as standard.

Activity stain overlay technique to detect CM-cellulase after PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 0.75-mm gels in a vertical slab unit (Mini-Protein II; Bio-Rad) by a modification of the method of Laemmli (19). The separating gel contained 10% acrylamide, 0.33% Bis, and 20 μg of bovine serum albumin per ml to enhance renaturation of electrophoresed enzymes (32). The stacking gel contained 4% acrylamide/Bis. SDS was excluded from both gels but added at 0.05% to the running buffer. Enzyme samples were mixed with sample buffer (62 mM Tris-HCl [pH 6.8], 10% glycerol, 0.025% bromophenol blue, and 2% SDS) in a ratio of 5:1 (vol/vol) without heating. In native PAGE, the SDS and bovine serum albumin were eliminated and the separating gel contained 7% acrylamide-Bis. Samples were electrophoresed at 200 V until the tracking dye migrated to the bottom of the gel. The proteins in SDS gels were renatured by incubating for 2 h with shaking in three 100-ml changes of 10 mM PCA buffer. CMC-agarose overlays were constructed by using a 0.4-mm layer of 0.2% CMC and 0.5% agarose in PCA buffer on a sheet of GelBond support film as recommended by the manufacturer (FMCE BioProducts, Rockland, Maine). The running gel was placed on the substrate-impregnated overlay and incubated in a moist chamber at 30°C (7 h for native PAGE, 12 h for SDS-PAGE). Then the overlay was immersed in aqueous 0.1% Congo red solution for 30 min and rinsed in 1 M NaCl.

Activity stain for polygalacturonase in isoelectric focusing gels. Agarose gels (10% glycerol, 1% agarose, 5% sorbitol, 2% Bio-Lyte 3/10 or 6/8 ampholytes from Bio-Rad Laboratories) were cast in 0.8-mm trays. Paper electrode wicks were moistened in 100 mM glutamic acid or 500 mM acetic acid (anode) and 500 mM NaOH (cathode). Enzyme samples were prepared by sonication of cells of strain ANU843 as described above but in deionized water; then ampholyte solution was added to a 2% (vol/vol) final concentration. Samples (10 μl) were applied to paper wicks placed near the cathode. A mixture of isoelectric focusing standards (Bio-Rad) with a pH range of 4.6 to 9.6 was applied next to the enzyme sample. Isoelectric focusing was performed at 4°C on a Bio-Phoresis Horizontal Electrophoresis cell (Bio-Rad) at a constant 6 W with a maximum of 1.5 kV until a rate of 1.0 kV·h was reached. After focusing was complete, the lane containing pH standards was excised and stained for protein with Coomassie blue according to the instructions of the manufacturer. The remaining gel was rinsed for 15 min in PCA buffer, placed on the substrate-impregnated overlay (as described above with 0.2% sodium polygalacturonate), and incubated in a moist chamber at 30°C for 3 h. Then the overlay was washed with 0.05% ruthenium red for 20 min, rinsed with water, and photographed.

RESULTS AND DISCUSSION

CM-cellulase and polygalacturonase activities are produced by R. leguminosarum bv. trifolii. CM-cellulase and polygalacturonase activities were detected by the double-layer plate assay in sonicated cell extracts of R. leguminosarum bv. trifolii ANU843 grown on B-INOS plates (Fig. 1A). Both enzyme activities were detected within 4 h of incubation and were destroyed by boiling. No activity was found in this extract when assayed on plates formulated to detect pectate lyase activity (100 mM Tris-HCl [pH 8.5], 1.5 mM CaCl2 [7]) (data not shown). CM-cellulase activity could also be detected directly underneath colonies growing on plates containing B-INOS agar plus 0.2% CM-cellulase. Positive results for CM-cellulase activity from several native strains of R. leguminosarum bv. trifolii isolated from nodules of T. pratense (Fig. 1B). Polygalacturonase activity could not be detected directly by this colony assay on B-INOS-polygalacturonate plates, since the medium beneath the colony stained more intensely with ruthenium red (data not shown). This is probably due to rhizobial acidic exopolysaccharides (9) that have diffused from the colony into the agar medium and are also stained intensely with ruthenium red. Alternatively, polygalacturonase activity was detected in sonicated cell extracts from these same R. leguminosarum bv. trifolii strains spotted onto double-layer plates made with polygalacturonate in PCA buffer (Fig. 1B). These results indicate that the detection method is sensitive enough to detect the low levels of CM-cellulase and polygalacturonase activities produced by a diversity of wild-type R. leguminosarum bv. trifolii strains and should have utility in studies that require sensitive methods to screen for these enzyme activities.

CM-cellulase and polygalacturonase are cell bound in R. leguminosarum bv. trifolii ANU843. Previous studies of R. leguminosarum bv. trifolii 0403 grown in BII medium (15) have shown that cells in the early stationary phase at a population density of ca. 9 × 109/ml have not begun stationary-phase lysis (detected by assaying the cytoplasmic enzyme marker glucose-6-phosphate dehydrogenase in the extracellular fraction). The total and specific activities of polygalacturonase and CM-cellulase were measured by the BCA-reducing sugar method in various fractions of R. leguminosarum bv. trifolii ANU843 grown in B-INOS broth to a density of 9 × 108 cells per ml. Enzyme activities were not found in the extracellular fraction containing the proteins precipitated with ammonium sulfate (Table 1). In contrast, both enzyme activities were detected repeatedly in extracts of pelleted cells produced by sonication or treatment with lysozyme-EDTA (Table 1). Although the specific activity for polygalacturonase was lower in the supernatant of sonicated cells (due to more extraneous protein), total polygalacturonase activity was higher in this fraction. Lysozyme-EDTA treatment released a larger portion of the polygalacturonase activity than the CM-cellulase activity from pelleted cells. As expected, the cytoplasmic enzyme marker NADP-dependent glucose-6-phosphate dehydrogenase was present in the cell sonic extract (0.36 U/mg of protein; 1 U reduces 1 pmol of NADP/min at 30°C and pH 7.8 [43]) but was not detected in the fraction of cell-bound proteins released by lysozyme-EDTA. This indicated that cell lysis did not occur during lysozyme-EDTA treatment and that portions of the polygalacturonase and CM-cellulase activities are associated with the cell envelope of R. leguminosarum bv. trifolii ANU843. These results corroborate an earlier report (25) that the main proportion of the total cellulase activity in broth cultures of R. leguminosarum bv. trifolii remains associated with the cells after centrifugation.

The symbiotic plasmid pSym of R. leguminosarum bv. trifolii ANU843 contributes to its polygalacturonase activity but not to its CM-cellulase activity. Root extracts stimulate
the production of cellulases by *R. leguminosarum* bv. trifolii BAL (25). Expression of *nod* genes on pSym in *R. leguminosarum* bv. trifolii ANU843 is increased significantly by growth in the presence of DHF, a flavone released from white clover roots (11). We therefore examined whether growth with DHF affected the levels of CM-cellulase and polygalacturonase activities of *R. leguminosarum* bv. trifolii ANU843. Growth in the presence of this *nod*-inducing flavone did not significantly alter the levels of polygalacturonase and CM-cellulase activities of *R. leguminosarum* bv. trifolii ANU843, and these enzyme activities remained associated with the cells rather than in the extracellular fraction (Table 1).

Morales et al. (25) showed that curing of pSym did not affect cellulase production in *R. leguminosarum* bv. trifolii 521. We examined the influence of pSym in the CM-cellulase

### TABLE 1. CM-cellulase and polygalacturonase activities in *R. leguminosarum* bv. trifolii wild-type ANU843 and its pSym-cured derivative, ANU845<sup>a</sup>

<table>
<thead>
<tr>
<th>Strain</th>
<th>2 µM DHF in B-1NOS medium</th>
<th>Cell fraction or treatment</th>
<th>Polygalacturonase activity (U)</th>
<th>CM-cellulase activity (U)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total</td>
<td>Specific</td>
</tr>
<tr>
<td>ANU843 (wild type)</td>
<td>-</td>
<td>Extracellular</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>ANU843</td>
<td>-</td>
<td>Sonic extract</td>
<td>5.77 ± 0.35</td>
<td>2.25 ± 0.14</td>
</tr>
<tr>
<td>ANU843</td>
<td>-</td>
<td>Lysozyme-EDTA</td>
<td>2.75 ± 0.03</td>
<td>17.38 ± 0.18</td>
</tr>
<tr>
<td>ANU843</td>
<td>+</td>
<td>Extracellular</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>ANU843</td>
<td>+</td>
<td>Sonic extract</td>
<td>6.17 ± 0.87</td>
<td>2.37 ± 0.33</td>
</tr>
<tr>
<td>ANU843 (pSym&lt;sup&gt;-&lt;/sup&gt;)</td>
<td>+</td>
<td>Lysozyme-EDTA</td>
<td>2.68 ± 0.06</td>
<td>20.40 ± 0.46</td>
</tr>
<tr>
<td>ANU845</td>
<td>+</td>
<td>Extracellular</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>ANU845</td>
<td>+</td>
<td>Sonic extract</td>
<td>1.65 ± 0.20</td>
<td>0.65 ± 0.08</td>
</tr>
</tbody>
</table>

<sup>a</sup> One unit of enzyme activity is the amount releasing 1 nmol of reducing sugar per min at 30°C. The values reported are the means of triplicate samples derived from equivalent numbers of cells ± the standard errors of the means.
and polygalacturonase activities of *R. leguminosarum* bv. trifolii ANU843 by comparison to these enzyme activities in the pSym-cured derivative, strain ANU845. Consistent with the results of Morales et al. (25), CM-cellulase activity associated with cells grown in broth containing DHF was not altered in the pSym-cured strain ANU845 (Table 1). However, the polygalacturonase activity in the sonicated cell extract was ca. 3.5-fold lower in this mutant, indicating a contribution of pSym to the total polygalacturonase activity of wild-type ANU843 (Table 1).

The possible influence of the 14-kb *nod* region of pSym on CM-cellulase and polygalacturonase activities of ANU843 was first examined by using the double-layer plate assay. CM-cellulase activity was detected in sonicated cell extracts of wild-type ANU843, pSym-cured ANU845, recombinant strain ANU845pRt032A6 (containing the pSym *nod* region of ANU843 on plasmid pRt032 in the pSym-cured ANU845 background), and the *nodD::Tn5* (ANU851) and *nodC::Tn5* (ANU277) insertion mutant derivatives of ANU843 (Fig. 2). These results are consistent with those of quantitative assays of CM-cellulase from ANU843 and ANU845 (Table 1). In addition, assay of the same sonicated cell extracts on plates containing sodium polypectate indicated that the polygalacturonase activities of ANU845 and ANU845pRt032A6 were markedly reduced (Fig. 2). These results were confirmed by using the quantitative BCA-reducing sugar assay, which showed a ca. 3.5-fold reduction in polygalacturonase activity in both ANU845 and ANU845pRt032A6 (Table 2).

These data obtained with *R. leguminosarum* bv. trifolii ANU843 grown on B-INOS medium indicate that (i) neither flavone induction of pSym *nod* genes nor the positive regulatory gene, *nodD*, is required for production of the CM-cellulase and polygalacturonase activities detected by these assays; (ii) genes encoding the CM-cellulase(s) are present on either the bacterial chromosome or cryptic plasmids; (iii) pSym does contribute positively to the total polygalacturonase activity; and (iv) the recombinant plasmid pRt032A6 containing the cloned 14-kb pSym *nod* region does not restore the polygalacturonase activity of the pSym-cured strain to that of its ANU843 parent. It is likely that a locus that contributes to wild-type levels of polygalacturonase activity associated with *R. leguminosarum* bv. trifolii ANU843 resides on its pSym outside of the 14-kb *HindIII nod* region.

CM-cellulase and polygalacturonase activities are not affected in *R. leguminosarum* bv. trifolii ANU843 by growth in the presence of substrates. Growth in B-INOS broth supple-

![CM-CELLULOSE Na POLYPECTATE](image)

**FIG. 2.** Influence of pSym and pSym *nod* genes on CM-cellulase and polygalacturonase activities in sonicated cell extracts of *R. leguminosarum* bv. trifolii ANU843 grown on B-INOS plates with 4 μM DHF. Numbered spots correspond to the following extracts: 1, wild-type strain ANU843; 2, pSym-cured derivative ANU845; 3, recombinant strain ANU845pRt032A6; 4, *nodD::Tn5* derivative ANU851; 5, *nodC::Tn5* derivative ANU277.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Polygalacturonase</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANU843 (wild type)</td>
<td>1.90 ± 0.03</td>
</tr>
<tr>
<td>ANU845 (pSym)</td>
<td>0.54 ± 0.10</td>
</tr>
<tr>
<td>ANU845pRt032A6</td>
<td>0.48 ± 0.07</td>
</tr>
<tr>
<td>ANU851 (<em>nodD::Tn5</em>)</td>
<td>2.00 ± 0.20</td>
</tr>
<tr>
<td>ANU277 (<em>nodC::Tn5</em>)</td>
<td>1.80 ± 0.06</td>
</tr>
</tbody>
</table>

* Cells were grown on B-INOS plates containing 4 μM DHF. Specific activity is given as units of enzyme activity per milligram of total protein. Values are means from triplicate experiments ± standard errors of the means.

**TABLE 3.** Polygalacturonase and CM-cellulase activities of sonicated cell extracts of *R. leguminosarum* bv. trifolii ANU843 grown in the presence of polysaccharide substrates

<table>
<thead>
<tr>
<th>Supplement to B-INOS medium</th>
<th>Polyalgalacturonase activity (U)</th>
<th>CM-cellulase activity (U)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Specific</td>
</tr>
<tr>
<td>None</td>
<td>2.24 ± 0.06</td>
<td>2.25 ± 0.06</td>
</tr>
<tr>
<td>0.2% CMC</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>0.2% Polypectate</td>
<td>1.92 ± 0.15</td>
<td>1.93 ± 0.15</td>
</tr>
</tbody>
</table>

* Values reported are the means of triplicate samples derived from equivalent numbers of cells ± standard errors of the means. ND, not determined.
In summary, the improved sensitivity of methods used in this work has permitted reliable detection and quantitation of CM-cellulase and polygalacturonase activities associated with _R. leguminosarum_ bv. _trifolii_, without the handicap of their inherently low activity. These studies are consistent with earlier reports that rhizobia produce enzymes that can break glycosidic bonds present in plant cell walls and provide evidence that these enzyme activities are cell bound. At least a portion of these enzyme activities is released by treatment of cells with lysozyme-EDTA to disrupt their cell envelope without lysis of the protoplast. This is the first report of CM-cellulase and polygalacturonase isozymes in _R. leguminosarum_ bv. _trifolii_ and the contribution of pSym to cell-associated polygalacturonase activity. Further use of these improved methods should make it possible to investigate the role of these polysaccharide-degrading enzymes in the infection process of this nitrogen-fixing _Rhizobium_-legume symbiosis.

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