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Short communication

Electron microscopy analysis of carboxymethylcellulase in rhizobia

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ABSTRACT

Here we analyzed carboxymethylcellulase (CMCase; EC 3, 2, 1, 4), one of the key enzymes in the early symbiotic process, in *Rhizobium*. Specific immunogold labeling of electron microscopy was confirmed in *Sinorhizobium fredii* BCRC15769, ATCC35423, *Sinorhizobium meliloti* ATCC9930, and barely detected in *Bradyrhizobium japonicum* BCRC13528, ATCC10324 and *Rhizobium rhizogenes* ATCC11325. Non-specific labeling was detected in *Rhizobium leguminosarum* bv. *viceae* ATCC10004, *Rhizobium leguminosarum* bv. *trifolii* ATCC10328, and *Mesorhizobium loti* ATCC33669. Treatment of *S. fredii* BCRC15769 in the early log phase with the flavonoid genistein caused relocalization of CMCase. Together our data suggests a role for CMCase in early symbiosis.

The infection of legume roots by rhizobia is a complex process with a high degree of specificity. Carboxymethylcellulase (endo-1,4-β-D-glucanase, or CMCase) is likely involved in the initial infection stage (Bhat and Bhat, 1997), and several hypotheses have been proposed to explain how these events occur. One of the models proposes that wall-degrading enzymes cause localized degradation that completely traverses the root-hair wall, allowing direct penetration by the bacteria (Dazzo and Hubbell, 1982).

During the 1990s, several researchers attempted to develop a new approach for enzyme identification (Mateos et al., 1992; Jimenez-zurdo et al., 1996a,b; Iannetta et al., 1997) using an activity gel overlay detection method. Two CMCase isozymes were determined from a native stain assay, and gel filtration revealed the molecular weights to be approximately 196 and 30 kDa (Hu and Lin, 2003). Michaud et al. (2002) isolated the coding sequence for EndS from *Sinorhizobium meliloti* M5N1CS DNA and compared the deduced amino acid sequence of the mature EndS (337 amino acids, molecular mass 36,418 Da, isoelectric point 4.92) to those of published β-glycanases, and confirmed that EndS belongs to family 5 of the glycoside hydrolases. Furthermore, EndS is similar to the 37-kDa subunit of isozyme 1 in *Sinorhizobium fredii* BCRC15769 (Hu and Lin, 2003). A 1047 bp open reading frame that functions in the hydrolyzation of carboxymethyl cellulose was identified from the *cel*8A gene in *Rhizobium leguminosarum* bv. *trifolii* 1536, and the 3.1-kb genomic DNA fragment from *R. leguminosarum* bv. *trifolii* 1536 was obtained. The *cel*8A gene encodes a glycosyl hydrolase family 8 member of 348 amino acids that exhibits a molecular mass of 35 kDa when induced from *Escherichia coli* DH5α (An et al., 2004).

In our previous study, we used ion-exchange chromatography and electroelution to purify CMCase, and characterized its activity using activity staining (Hu and Lin, 2003). Furthermore, we generated antibodies against CMCase and assessed the specificity of the antiserum by Western analysis, and cell distribution of CMCase was examined using immuno-microscopy. CMCase was demonstrated to be a cellulytic enzyme, consistent with the results of other biochemical studies (Mateos et al., 1992; Jimenez-zurdo et al., 1996b; Hu and Lin, 2003). To extend the findings from immunogold labeling of CMCase distribution, the aim of the research was to treat different species of rhizobia and reveal the different flavonoid compounds present in rhizobia.

The rhizobial strains used in the study were *S. fredii* BCRC15769 (host legume: *Glycine max* L. Merril) isolated from indigenous soil in Taiwan, *S. fredii* ATCC35423, *Bradyrhizobium japonicum* BCRC13528, ATCC10324, *Rhizobium rhizogenes* ATCC11325, *Rhizobium leguminosarum* bv. *viceae* ATCC10004, *Rhizobium leguminosarum* bv. *trifolii*

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ATCC10328, and *Mesorhizobium loti* ATCC33669, *S. meliloti* ATCC9930, which were purchased from Bioresource Collection and Research Center (BCRC), Food Industry Research and Development Institute, Hsinchu City, Taiwan. Bacterial strains were stored in yeast-mannitol agar at 4 °C (Mergaert et al., 1995). Bacteria were maintained on BIII agar (Morales et al., 1984), and cultured aerobically in BIII broth at 28 °C with agitation. Cells were cultivated until late log phase (9×10^8 cells/ml, measured using a Petroff Hausser counting chamber) and 0.5 g (wet weight) was resuspended in 1.0 M potassium phosphate buffer (pH 7.0) and incubated for 30 min at room temperature with continuous stirring. Procedures for pretreatment, immunogold labeling of cells, and examination by transmission electron microscope were followed from Hu et al. (2006). Statistical significance ($p < 0.05$) for the differences between means was determined by one-way ANOVA and Tukey Post Hoc test, and calculations were performed using SPSS 10.07 (SPSS for Windows, Inc., Chicago, IL, USA).

BIII broth was supplemented with the 5 flavonoids (final concentration of 0.2 μM): apigen, chrysin, daidzein, genistein, naringenin, which were purchased from Sigma (St. Louis, MO.). Rhizobial cells were cultivated until late log phase and centrifuged at 8000 × *g* for 30 min at 4 °C. Cultivation and induction methods were followed by Lin et al. (1999). The resting protocols for EM pretreatment and immunolabelling of cells were described by Hu et al. (2006).

Immunogold labeling for electron microscopic observations was performed with ultrathin sections of *S. fredii* BCRC15769 (Fig. 1a). Specific immunogold labeling was barely detected in *B. japonicum*

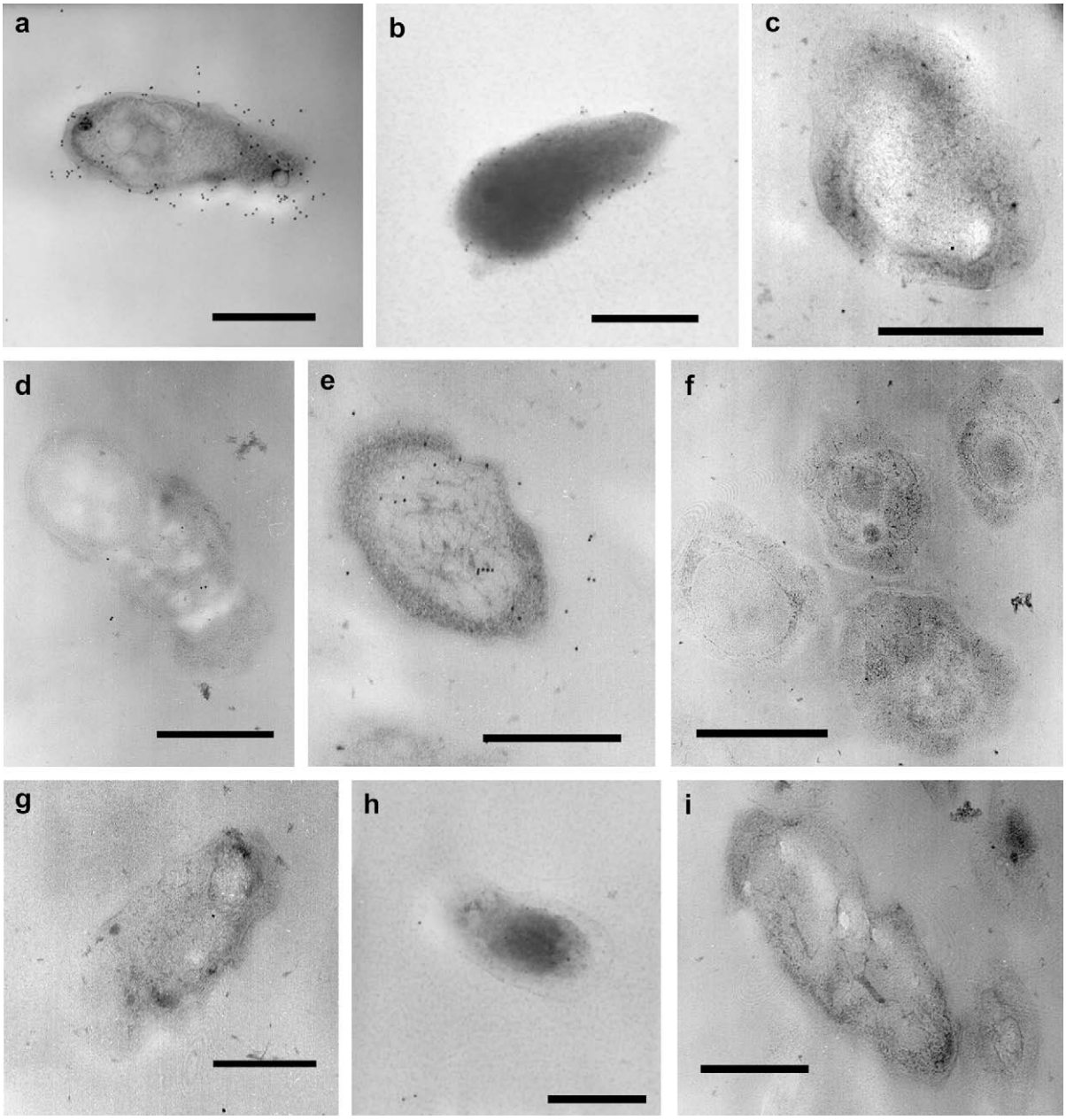

Fig. 1. Transmission electron micrographs of ultrathin sections of different species of rhizobia using immunogold-labeled procedures: (a) *Sinorhizobium fredii* BCRC15769 (RO); (b) *S. fredii* ATCC35423; (c) *Sinorhizobium meliloti* ATCC9930; (d) *Rhizobium leguminosarum* bv. *trifolii* ATCC10328; (e) *Rhizobium leguminosarum* bv. *viceae* ATCC10004; (f) *Mesorhizobium loti* ATCC33669; (g) *Bradyrhizobium japonicum* ATCC10324; (h) *B. japonicum* BCRC13528; and (i) *Rhizobium rhizogenes* ATCC11325 (BCRC13207). Carboxymethylcellulase (CMCase) of the intact gold-labeled (10 nm) bacterial cell. Bar = 500 nm.