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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*

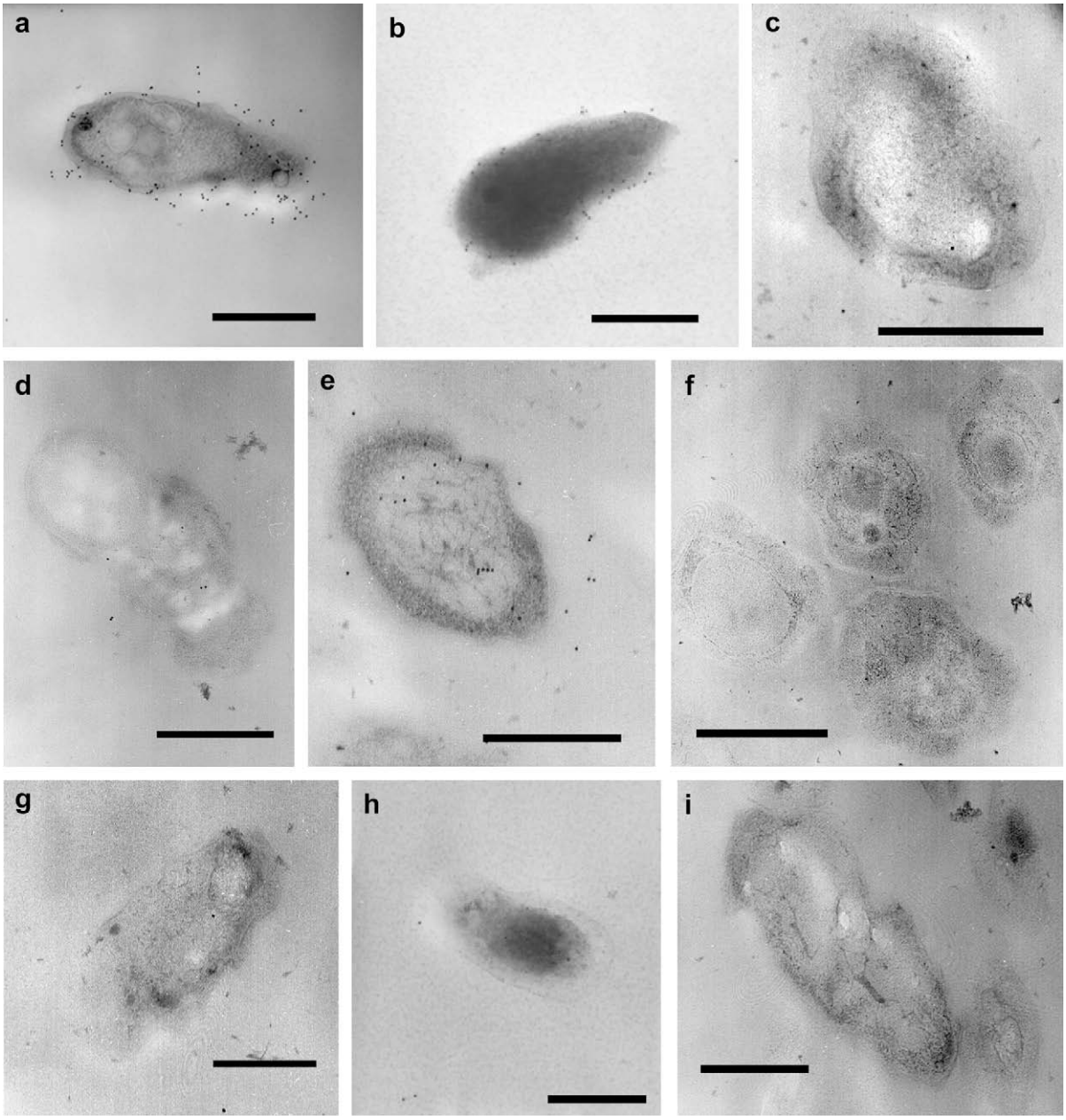

Fig. 1. Transmission electron micrographs of ultrathin sections of different species of rhizobia using immunogold-labeled procedures: (a) *Sinorhizobium fredii* BCRC15769 (RO); (b) *S. fredii* ATCC35423; (c) *Sinorhizobium meliloti* ATCC9930; (d) *Rhizobium leguminosarum* bv. *trifolii* ATCC10328; (e) *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.

Table 1
Results of quantitative evaluations of the distribution of colloidal gold particles as revealed by analysis of ultrathin sections of different species and treatment of rhizobia.

Organism[c]	Pretreatment with 1% OsO_4	Mean (%) of colloidal gold ± SD				Percentage[e] (%)
		Total amount	Membrane associated	Periplasmic space[d]	Cytoplasmic space	
Sinorhizobium fredii BCRC15769	No	97.3 ± 3.1	17.3 ± 1.5[a]	54.0 ± 3.0[a]	26.7 ± 2.1[a]	73.3
	Yes	56.0 ± 1.7	21 ± 1.5[a]	27.7 ± 1.5[a]	7.7 ± 1.5[a]	87
S. fredii ATCC35423	No	51.3 ± 2.1	19.3 ± 1.5[a]	24.7 ± 1.5[a]	6.3 ± 0.6[a]	85.8
Sinorhizobium meliloti ATCC9930	No	4.0 ± 0.0	0.0 ± 0.0[a,b]	2.0 ± 0.0[a]	2.0 ± 0.0[b]	50
Rhizobium leguminosarum bv. *trifolii* ATCC10328	No	5.0 ± 0.0	1.0 ± 0.0[a]	1.0 ± 0.0[b]	3.0 ± 0.0[a,b]	40
Rhizobium leguminosarum bv. *viceae* ATCC10004	No	18.7 ± 1.5	2.0 ± 0.0[a]	5.0 ± 0.0[a]	10.6 ± 1.5[a]	37.4
Mesorhizobium loti ATCC33669	No	12.0 ± 2.0	2.0 ± 0.0[a]	3.0 ± 0.0[a]	6.7 ± 0.6[a]	41.7
Bradyrhizobium japonicum ATCC10324	No	4.0 ± 0.0	0.0 ± 0.0[a]	1.0 ± 0.0[a]	3.0 ± 0.0[a]	25
B. japonicum BCRC13528	No	8.7 ± 0.6	1.0 ± 0.0[a]	0.0 ± 0.0[b]	7.7 ± 1.5[a,b]	11.5
Rhizobium rhizogenes ATCC11325	No	16.3 ± 1.5	1.0 ± 0.0[a]	0.7 ± 0.6[b]	14.7 ± 0.6[a,b]	10.4

[c] For the whole-amount samples tested, each counting was a triplicate evaluation of three experiments. Means in a row of three different locations with the same letter are significantly different, $p < 0.05$.
[d] Periplasmic, associated with cytoplasmic and outer membranes.
[e] Percentage of colloidal gold particles (%): (membrane associated + periplasmic space) × 100/total amount.

ATCC10324 (Fig. 1g), BCRC13528 (Fig. 1h), and *R. rhizogenes* ATCC11325 (Fig. 1i). Non-specific labeling was detected in *R. leguminosarum* bv. *trifolii* ATCC10328 (Fig. 1d), *R. leguminosarum* bv. *viceae* ATCC10004 (Fig. 1e), and *Rhizobium loti* ATCC33669 (Fig. 1f), demonstrating highly contrasting intra- and interspecific CMCase similarities. However, specific labeling was observed in *S. fredii* BCRC15769 (RO) (Fig. 1a), ATCC35423 (Fig. 1b), and *S. meliloti* ATCC9930 (Fig. 1c).

Table 1 presents the results of quantitative evaluations of label distribution. The quantification of colloidal gold in a cell was assessed in three regions: the membrane-associated region, the periplasmic space, and the cytoplasmic space. We determined the percentage of colloidal gold particles using the following calculation:

$$(\text{amount membrane-associated} + \text{amount in the periplasmic space}) \times 100/\text{total amount},$$

Species and treatments could be divided into three groups based on the percentage of particles aggregating in the cell membrane: one group with 100–50% in the membrane, the second from 50% to 30%, and the third group below 30%.

Within the group with the highest percentage of particles in the cell membrane (group 1), the highest percentage of 87.0% was *S. fredii* BCRC15769 with OsO_4 treatment, followed by *S. fredii* ATCC35423 (85.8%), *S. fredii* BCRC15769 without OsO_4 treatment (73.3%), and *S. meliloti* ATCC9930 (50.0%).

The second group included *M. loti* ATCC33669 (41.7%); *R. leguminosarum* bv. *trifolii* ATCC10328 (40.0%), and *R. leguminosarum* bv. *viceae* ATCC10004 (37.4%). Finally, the third group included 2 genera of rhizobia: *Bradyrhizobium* spp. and *R. rhizogenes*, which are slow-growing rhizobia or have different hosts: *B. japonicum* ATCC10324 (25.0%), *B. japonicum* BCRC13528 (11.5%), and *Rhizobium rhizogenes* ATCC11325 (10.4%).

The effects of the flavonoids genistein on CMCase distribution are shown in Fig. 2a,b. Addition of 20 μM genistein in the cultivation stage caused CMCase to aggregate towards two sides of the cell. In contrast, apigenin, chrysin, daidzein, and narigenin had little effect on the distribution of CMCase.

Vessey and Chemining'wa (2006) reported the genetic diversity of *R. leguminosarum* bv. *viciae* in cultivated soils of an eastern Canadian prairie by comparing PCR-RFLP patterns. Aguilar et al. (2006) analyzed symbiotic genes showing 16SrRNA RFLP

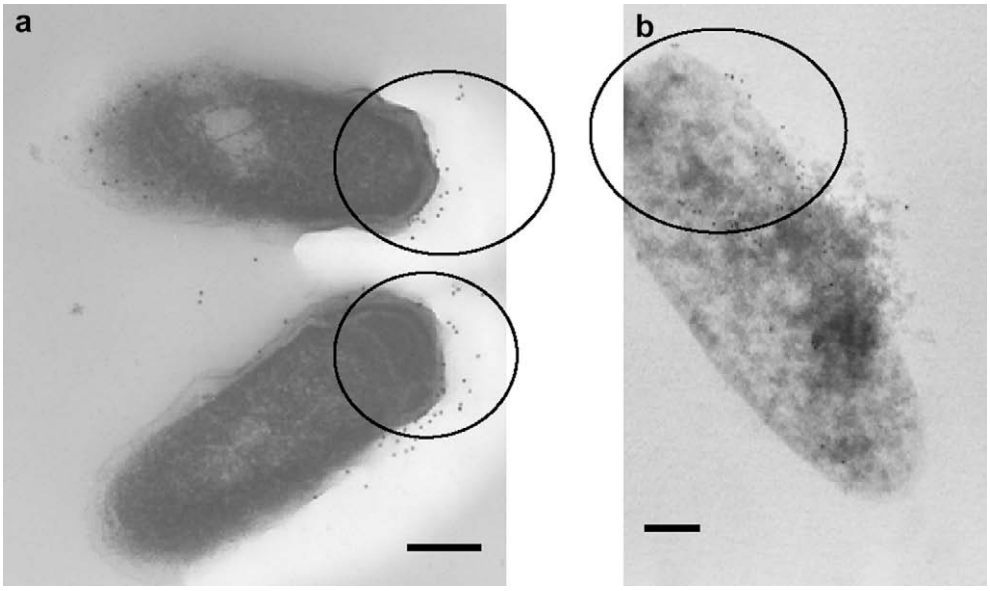

Fig. 2. Transmission electron micrographs of ultrathin sections using immunogold-labeled procedures of flavonoid induction by *Sinorhizobium fredii* BCRC15769. Carboxymethylcellulases (CMCase) of the intact bacterial cell labeled with gold (10 nm), indicated that flavonoid genistein induction of 20 μM in 250 mL of BIII broth at the early log phase of *S. fredii* BCRC15769 caused the aggregation of CMCase towards 2 sites of the cell, as shown in the circles of (a) and (b). Bar = 200 nm.

polymorphisms, and the structure of the nod factor to examine the bean rhizobial community other than the predominant species *Rhizobium etli* present in soils occupied by the host in northwestern Argentina. Our analysis showed a low similarity between the fast-growing and slow-growing rhizobia in terms of CMCase distribution. The fast-growing rhizobia, *S. fredii* BCRC15769 and ATCC35423, had high similarity with *S. meliloti* BCRC9930. The result is consistent with former reports (Chen et al., 1988; Young, 1996). The group with the highest percentages of staining (50–100%; Table 1): *S. fredii* BCRC15769, ATCC35423, and *S. meliloti* ATCC9930, had high similarity in terms of distribution analysis by labeling. Group 2 (30–50%; Table 1): *R. leguminosarum* bv. *viceae* ATCC10004, *R. leguminosarum* bv. *trifolii* 10328, and *M. loti* 33669, was the next group compared with *S. fredii* BCRC15769. Group 3 (10–30%; Table 1): *B. japonicum* ATCC10324, BCRC13528, and *R. rhizogenes* ATCC11325, had low similarity compared with *S. fredii* BCRC15769. Based on our data in Table 1, we found that the trend of percentage of colloidal gold particles around the membrane is specific to the phylogenetic relationships of some rhizobia. According to the conformation (by means of specific binding of antibody) of CMCase distribution in different species, we could preliminarily classify different species of rhizobia, suggesting that the specific labeling of immunogold would be a source to identify the phylogeny of rhizobia.

In a previous study, *S. fredii* USDA 191 was incubated with several flavonoids and its β-galactosidase activity was measured as an indicator of *nod* ABC-lacZ expression. β-galactosidase activity was high in response to addition of genistein, apigenin, chrysin, and naringenin, indicating that these four chemicals can induce effective nodulation (Kosslak et al., 1987). Lin et al. (1999) found that genistein and naringenin also induced 5 extracellular proteins of *S. fredii* USDA257, and they postulated that flavonoids affect some physiological processes. In addition to the function we mentioned above, we demonstrated that the flavonoid extracted from *G. max*, genistein, caused the aggregation of CMCase towards two sides of the cell in *S. fredii* BCRC15769.

In conclusion, our works presented rhizobial diversity in CMCase antibody–antigen interactions, shown in the immunoelectron microscope diagram, and we found that the flavonoid, genistein, caused the aggregation of CMCase in rhizobia, which enhanced the penetration of rhizobia into plant root hairs in the early stage of symbiosis.

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