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Article

Inhibitory effect of cadmium on competitive nodulation ability of *Bradyrhizobium japonicum*

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Abstract

Competitive nodulation abilities of *Bradyrhizobium japonicum* strains USDA110ET and A1017ET were significantly depressed by growing the strains in yeast extract-mannitol broth supplemented with 2 μM $CdCl_2$. Soybean seedlings were co-inoculated with each test strain and its competitor strain 138NR, and the bacteria in nodules formed on 21 days old plant roots were identified by the antibiotic resistant markers. Cell surface hydrophobicity of each test strain increased by growing the strains with 2 μM $CdCl_2$, though definite increase in bacterial attachment to plant root surface was hindered by excessive secretion of exopolysaccharides of the strains. Polyacrylamide gel electrophoresis revealed that growing the test strains in the medium with 2 μM $CdCl_2$ induced the production of lipopolysaccharides of small molecular sizes. The results in this study suggest that cadmium of low concentration causes the weakening of competitive nodulation ability of rhizobia through inhibition of the lipopolysaccharide synthesis.

Key Words: cadmium, competitiveness, hydrophobicity, legume, lipopolysaccharide, rhizobium

1. Introduction

Rhizobium is a soil bacterium that infects leguminous plants and produces nodules in the root to fix atmospheric nitrogen symbiotically with their host plant. There is a difference in the root nodule formation ability between strains of rhizobia (Dowling and Broughton 1986). A rhizobium strain should compete with other strains to form a root nodule on their host plant and occupy it.

The competitive nodulation ability (CNA) of a rhizobial strain is genetically determined, but not constant. Environmental factors such as temperature, soil pH, and soil nitrate can affect the CNA of *Rhizobium* and *Bradyrhizobium* (Triplett 1990). We demonstrated that *Bradyrhizobium japonicum* strains changed their CNA if the strains grown in a nutrient broth were introduced into soil environment (Ozawa 1988). We have also reported that fertilization manner affected markedly the CNA of the indigenous *Bradyrhizobium* strains (Ozawa *et al.* 2000). These results suggest that some environmental factors affect the expression of the genes involving the competitive nodulation.

Cadmium (Cd) is one of the heavy metals generally existing as a minor element in soils. Cd is a highly toxic metal that causes deleterious effects at higher concentrations on soil bacteria as well as plants (Sanitá di Toppi and Gabbrielli 1999). Degree of sensitivity of rhizobia to Cd differs from strain to strain. Consequently, contamination with Cd at higher concentrations could change a population structure of rhizobia in the soil (Kinkle *et al.* 1987). Thus, the strain more tolerant to Cd would have more opportunity to form nodules on the host plant. Effect of Cd on the CNA of a rhizobial cell, however, has not been elucidated.

Cell surface hydrophobicity of a rhizobial strain

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is one of the key characteristics that determine the CNA of the strain (Araujo *et al.* 1994). We have demonstrated that *Bradyrhizobium japonicum* strains had a positive correlation between the cell surface hydrophobicity and the CNA (Ozawa *et al.* 1991, Ozawa *et al.* 1992). Cell surface hydrophobicity of Gram-negative bacteria was determined by the amount and nature of exopolysaccharide (EPS) and lipopolysaccharide (LPS) (Nikaido 1976, Nikaido 1994, Triplett 1990). Schue *et al.* (2011) reported that extracellular Cd induces the over-production of EPS. These results suggest that Cd could change the CNA of a rhizobial strain by affecting bacterial metabolic processes of polysaccharides. The objective of this study is to confirm the possible role of extracellular Cd in controlling the cell surface hydrophobicity and the CNA of *Bradyrhizobium japonicum* strains.

2. Materials and Methods

2.1. Bacterial strains

B. japonicum USDA110 and USDA138 were obtained from the U.S. Department of Agriculture, Beltsville, Md. *B. japonicum* A1017 was obtained from Dr. K. Minamisawa, Tohoku University, Japan. Spontaneous mutants of these strains resistant to 100 mg L^{-1} nalidixic acid and 100 mg L^{-1} rifampicin (strain 138NR) or to 100 mg L^{-1} erythromycin and 10 mg L^{-1} tetracycline (strains 110ET and A1017ET) were isolated in our laboratory. The parental strains and the mutants were maintained on yeast extract-mannitol (YEM) agar slants and stored at 4°C (MacGregor and Alexander 1971). Prior to inoculation to soybean, the strains were grown on a shaker (110 rpm) for 5 days at 30°C in 5 mL of YEM broth in a glass tube (18 x 200 mm). One hundred μL of the culture was transferred to another YEM broth (50 mL) in a 200-mL flask, and grown for 7 days under the same conditions as above. In case of examining the effect of Cd, 10 μL of 10 mM $CdCl_2$ was added to the 50 mL of YEM just before the transfer. Concentration of $CdCl_2$ in YEM was 2 μM.

We evaluated here competitive nodulation abilities of the strains USDA110, A1017, 110ET, and A1017ET against the strain 138NR as a competitor. Each culture of the test strains was diluted 10- to 10^3-fold with distilled water, and then mixed with the competitor strain 138NR to prepare five varieties of inoculum. Bacterial cell densities of the strains were examined by the dilution plate count method. Densities of the test strains and the competitor in the inoculum were approximately as follows: 10^6 and 10^8, 10^7 and 10^8, 10^8 and 10^8, 10^8 and 10^7, 10^8 and 10^6 cfu mL^{-1}, respectively.

2.2. Bacterial inoculation to plants

Soybean (*Glycine max* cv. Tamanishiki) seeds surface sterilized with 5% calcium hypochlorite were sown on vermiculite (approximate particle size: 5 mm) with a depth of 10 cm in test tubes (25 x 200 mm), one seed per tube. Each tube was previously supplied with 30 mL of Jensen's mineral solution (Gibson 1980), and sterilized by autoclaving (120°C, 20 min). After 3 days at 30°C in the dark, each seedling was inoculated with 1.0 mL of the mixed cell suspension of a test strain and strain 138NR, which was prepared as mentioned above.

The inoculated plants were grown for 21 days in a growth chamber (23°C; 110 μmol m^{-2} s^{-1}, 16 h light-8 h dark). Forty to 50 nodules (> 1 mmϕ) were detached from 6 plants for each treatment. The nodules were surface sterilized with 5% calcium hypochlorite, and then nodule occupancy was determined by examining the antibiotic resistance of bacteria in each nodule as described elsewhere (Ozawa 1988).

N_{50} value, which is the inoculum ratio of a test strain to the competitor strain when it yields 50% nodule occupancy, was used to express the competitive nodulation ability of each strain. N_{50} was calculated in the same way for estimating ED_{50} (effective dose 50%) by the Probit method (Finney 1952).

2.3. Bacterial adhesion to plant root and vermiculite

Strains 110ET and A1017ET were examined on competitive adhesion to soybean root surface against strain 138NR. Each strain was cultured for 7 days in YEM broth with or without 2 μM $CdCl_2$ at 30°C, and then washed with distilled water as described above. Each of the washed cell suspension of former strains was mixed with the washed 138NR cell suspension. Cell density of each strain in the mixture was adjusted to 1 x 10^8 cfu mL^{-1}. Three days old soybean seed-

lings, which were aseptically germinated on paper in a petri dish at 30°C in the dark, were cut with a razor blade at position about 3 cm above the root tip. Sets of 4 excised root segments were soaked in 50 mL of the mixed cell suspension to remove loosely bound bacteria. After 90 min of incubation at 30°C, the root segments were washed 4 times with distilled water. Bacterial cells attached to plant roots were removed by ultrasonic cleaning with the Bronson Sonifier model 250 (output level 4, 2 min on ice). Bacteria released from the root segments were counted by the dilution plate method with YEM agar containing the antibiotics.

Adhesion ability of strains USDA110, A1017, 110ET, A1017ET, and 138NR to vermiculite used in this study for soybean cultivation was evaluated in the following way using vermiculite columns. Optical density (OD) at 660 nm of cell suspension of each strain obtained as described above was adjusted to 0.2, and then the cell suspension was passed through a column of vermiculite (1.5 x 20 cm) at room temperature. We used in this study the rate of decrease in OD of cell suspension during the filtration as an indication of adhesion ability of the bacterial strain to vermiculite particle.

2.4. Cell surface hydrophobicity

Cell surface hydrophobicity of bacterial cells was measured by the method of Rosenberg *et al.* (1980). This method is based on partitioning behavior of bacterial cells between oil and water. Bacteria with more hydrophobic surfaces tend to be attracted to the oil-water phase boundary (Marshal 1976). The rhizobial cells grown in the YEM broth for 7 days were washed twice with distilled water or 50 mM potassium phosphate (pH 6.5). To 3.0 mL of the washed cell suspension of approximately 10^8 cfu mL^{-1} in a test tube (12 x 100 mm) with a screw cap, 0.3 mL *n*-octane as the water-insoluble phase was added, and then the tube was vertically agitated on a shaker (300 rpm, amplitude of 5 cm) for 2 min. After standing for 2 min, optical density of the lower aqueous phase was determined at 660 nm.

Cell surface hydrophobicity index (CHI) was expressed as the following equation:

$CHI = (OD_b - OD_a)/OD_b$

where OD_a and OD_b are optical density at 660 nm of the lower phase after and before agitation, respectively.

2.5. Determination of EPS

Bacteria grown for 7 days at 30°C in 50 mL of YEM broth were harvested by centrifugation at 14,000 rpm for 3 min, and the precipitate was washed 4 times with 50 mM potassium phosphate (pH 6.5). First two washes were collected, and assayed for hexose by the phenol-H_2SO_4 method (Dubois *et al.* 1956).

2.6. Polyacrylamide gel electrophoresis (PAGE) of LPS

The pelleted cells from 1 mL culture were washed once with 10 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and then suspended in 130 μL of sodium dodecyl sulfate (SDS) sample buffer (12 mM Tris [pH 6.8], 4% SDS, 10% glcerol, 0.2% bromophenol blue, 4% 2-mercaptoethanol). The total SDS extracts of bacterial strains were heated at 100°C for 10 min followed by digestion with proteinase K (150 μg mL^{-1}) at 60°C for 60 min (Cava et al. 1989). LPS in the crude preparations was electrophoresed through 4% stacking and 12.5% separating acrylamide gel with 0.4% SDS. The gels were silver stained by the method of Tsai and Frasch (1982).

3. Results and Discussion

3.1. Cd tolerance

We assessed minimum inhibitory concentration (MIC) of Cd against the *Bradyrhizobium* strains in this study by using YEM broth containing $CdCl_2$. The strains from stock cultures were incubated for 7 days at 30°C in 5 mL of YEM supplemented with 0 − 100 μM $CdCl_2$ on a shaker (110 rpm), then OD at 660 nm of each culture was measured. MIC of Cd was 30 μM against every strains tested. Two μM $CdCl_2$ had no effect on growth rate of the strains used in this study (data not shown).

3.2. Effect of Cd on CNA

A rhizobial strain competes with other strains to infect their host plant and multiply in nodules formed in the host roots. Figure 1 shows nodule-occupancy rates of the test strains USDA110, A1017, 110ET, and A1017ET in competition with strain 138NR after the

co-inoculation to soybean. In case of 110ET and A1017ET, proportion of the nodules containing both the test strain and the reference strain 138NR ranged from 0 to 14%. The double-infected nodules were excluded to calculate nodule occupancy rates in Fig. 1. The rate of nodules occupied by a strain increased with the increase in the ratio of the strain to the competitor strain in an inoculum.

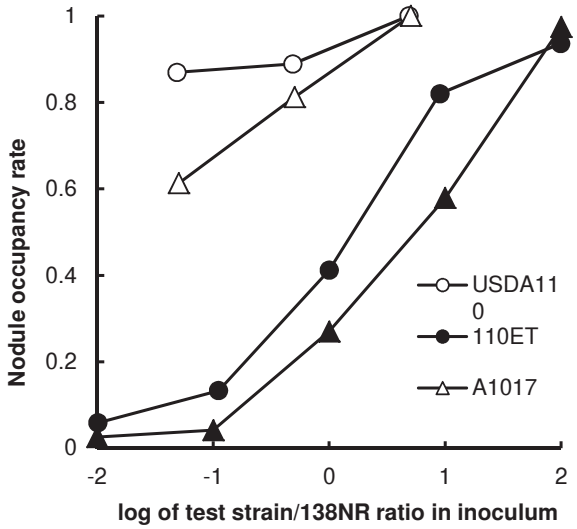

Fig. 1. Nodule occupancy rates of *B. japonicum* strains USDA110, 110ET, A1017, and A1017ET in competition with *B. japonicum* strain 138NR.

From the dose-response curve, we can obtain a N_{50} value, the proportion of a strain to its competing strain in an inoculum when 50% of nodules are formed by the strain (Table 1). A strain showing smaller value of N_{50} is more competitive for nodule formation. Growing in YEM supplemented with 2μM $CdCl_2$ significantly decreased the CNA of strains 110ET and A1017ET. Strains USDA110 and A1017, showing more competitive than the antibiotic resistant mutants, also showed a decreasing tendency in CNA by growing with 2 μM $CdCl_2$, though statistical significance was low.

As both strains USDA110 and A1017 have no antibiotic marker, we were not able to distinguish nodules occupied by single strain from double-infected nodules. We did not use strains USDA110 and A1017 in the following experiments.

Table 1. Effect of cultivation with $CdCl_2$ on the competitive nodulation index (N_{50}) of *B. japonicum* strains. m_U and m_L are upper and lower limit of confidence interval at 95 %, respectively.

Strain	Cd in growth medium	N_{50}	(m_U, m_L)
USDA110	0 μM	7.6×10^{-5}	$(0.20, 2.9 \times 10^{-8})$
	2 μM	1.3	$(560, 3.1 \times 10^{-3})$
A1017	0 μM	0.26	(0.62, 0.11)
	2 μM	0.75	$(18, 3.1 \times 10^{-2})$
110ET	0 μM	1.35	(2.27, 0.80)
	2 μM	7.93	(17.6, 3.58)
A1017ET	0 μM	7.41	(13.3, 4.11)
	2 μM	13.5	(26.8, 8.92)

3.3. Effect of Cd on adhesion of bacterial cells to host roots and vermiculite

As a rhizobial cell density on the host root surface is one of the major factors for competitive nodule formation by the strain (Dowling and Broughton 1986), higher ability to adhere to host roots and lower ability to adhere to vermiculite would both give higher CNA to the rhizobial strain. Cultivation of A1017ET strain in YEM broth with 2 μM $CdCl_2$ caused significantly higher ratio of A1017ET cells to 138NR cells that adhered to soybean root segments, but in case of 110ET there was no effect (Table 2). Adhesion ability of 110ET to vermiculite particles declined by cultivating them in YEM with 2 μM $CdCl_2$ (Table 3).

Table 2. Effect of cultivation with $CdCl_2$ on the adhesion of *B. japonicum* strains to soybean root segments.

Strains	Root-attached cells (cfu per root segment) cultivated with	
	0 μM Cd	2 μM Cd
138NR	$3.6 \times 10^5 \pm 3.0 \times 10^4$	$2.4 \times 10^5 \pm 3.8 \times 10^4$
110ET	$2.4 \times 10^5 \pm 4.5 \times 10^4$	$1.6 \times 10^5 \pm 1.5 \times 10^4$
110ET/138NR ratio	0.68	0.65
138NR	$8.0 \times 10^4 \pm 1.0 \times 10^4$	$4.3 \times 10^4 \pm 2.5 \times 10^3$
A1017ET	$1.0 \times 10^4 \pm 5.0 \times 10^3$	$7.3 \times 10^4 \pm 2.5 \times 10^3$
A1017/138NR ratio	0.13	1.71

Table 3. Effect of cultivation with $CdCl_2$ on the adhesion of *B. japonicum* strains to vermiculite. Values with asterisk are significantly different (n = 3, $p < 0.05$) from the control value.

Strains	Adhesion rate (%) of cells cultivated with	
	0 μM Cd (Control)	2 μM Cd
110ET	25	7 *
A1017ET	28	23
138NR	1	7

Table 4. Effect of cultivation with $CdCl_2$ on the cell surface hydrophobicity of *B. japonicum* strains.
Values (mean ± S.E., $n = 3$) with asterisk are significantly different from the control (0 μM $CdCl_2$) value of each washed cells ($p < 0.05$).

Strain	Wash#	CHI of rhizobium cultivated with	
		0 μM Cd	2 μM Cd
110ET	DW	0.62 ± 0.01	0.49 ± 0.02*
	PB	0.60 ± 0.06	0.90 ± 0.02*
A1017ET	DW	0.56 ± 0.06	0.39 ± 0.04*
	PB	0.69 ± 0.02	0.82 ± 0.03*

DW: distilled water, PB: 50 mM K-phosphate (pH 6.5)

Other strains tested did not show significant change in the adhesion ability after growing in YEM with $CdCl_2$. These results indicate that 2μM $CdCl_2$ in a growth medium does not cause a decrease in rhizobial cell density on the host root surface to bring about the decrease of CNA.

3.4. Effect of Cd on cell surface hydrophobicity

Adhesiveness of bacterial cells to solid surface would be affected by the cell surface hydrophobicity (van Loosdrecht *et al.* 1987). Vesper *et al.* (1987) reported that transposon mutants of a *Bradyrhizobium japonicum* strain reduced both attachment to soybean roots and cell hydrophobicity. Surface hydrophobicity of strains 110 ET and A1017ET in this study that were washed with distilled water significantly decreased by cultivation in YEM broth with 2 μM $CdCl_2$ (Table 4). On the contrary, the Cd-cultured cells of both strains washed with phosphate buffer increased hydrophobicity.

3.5. Effect of Cd on production of EPS and LPS

B. japonicum 110ET and A1017ET produced a large amount of extracellular substances when grown in YEM broth with 2 μM $CdCl_2$ (Fig. 2). When both strains were grown in YEM supplemented with 2 μM $CdCl_2$, significantly larger amount of EPS was detected in the culture than in YEM without $CdCl_2$ (Table 5). As EPS is a hydrophilic molecule, the large amount production of EPS would be responsible for the decrease in cell surface hydrophobicity as shown in Table 4. When the EPSs loosely bound to cell surface were washed out with 50 mM phosphate, the Cd-cultured cells showed larger values of CHI than control cells.

Araujo *et al.* (1994) reported that a Tn-5 mutant of *Rhizobium etli* produced EPS that was indistinguishable from that of its parent. However, the mutant was highly hydrophobic, while greatly reduced in competitive nodulation. Bittinger *et al.* (1997) established that a single gene homologous to a family of transcriptional regulators affected both competitive nodulation and cell surface hydrophobicity of the strain. The results in this study also indicate the correlation between cell surface hydrophobicity and competitive nodulation.

LPS is one of the major components of the outer membrane of Gram-negative bacteria, and is a heterogeneous molecule that varies O-antigen length (Jann *et al.* 1975). LPS that has shorter length of the polysaccharide chain would make more hydrophobic of bacterial cell surface (Nikaido 1976).

Growing in the YEM broth supplemented with 2μM $CdCl_2$ resulted in an alteration in LPS-band patterns of SDS-PAGE (Fig. 3). LPSs of small molecular size appeared in the cells of 110ET and A1017ET when they were grown in YEM broth with 2μM $CdCl_2$. This explains the increase in cell surface hydrophobicity by growing with 2μM $CdCl_2$ as described above.

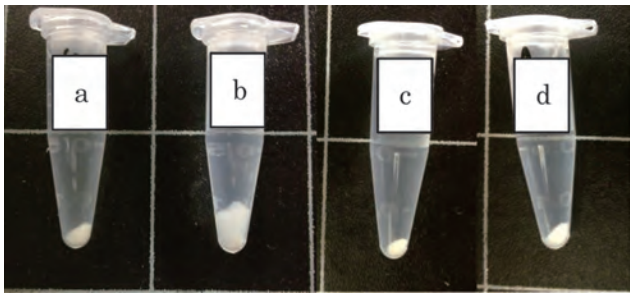

Fig. 2. Production of gel-like substances by *B. japonicum* strains. Strain 110ET (a, b) and A1017ET (c, d) were grown for 7 days in YEM broth supplemented with 0μM (a, c) and 2μM $CdCl_2$ (b, d), then 1.5 mL of the culture was centrifuged (10,000 rpm, 3 min).

Table 5. Effect of cultivation with $CdCl_2$ on the production of EPS by *B. japonicum* strains.
Values are means ± S.E. (n =3). Values with asterisk of each strain are significantly different ($p < 0.05$) from the control values.

Cd in YEM	EPS (μg Glc/mL)	
	110 ET	A1017ET
0μM (Control)	97 ± 10	216 ± 4
2μM	476 ± 56 *	497 ± 57 *

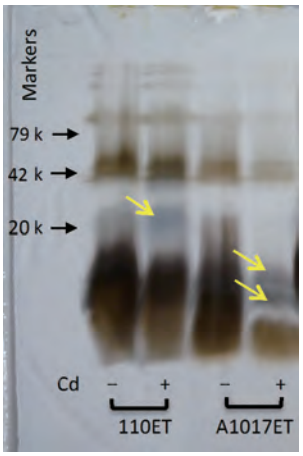

Fig. 3. SDS-PAGE of LPS of *B. japonicum* 110ET and A1017ET. Both strains were grown in the YEM supplemented with 0μM (Cd -) or 2μM (Cd +) $CdCl_2$. The total SDS-extracts from 10 mg (dry weight) of cells of each strain were loaded on each well, and electrophoresed. Slanting arrows indicate characteristically appeared bands in the cells grown with Cd.

LPS molecules have been thought to play various roles in establishing the symbiosis between rhizobia and their host plants (Stacey *et al.* 1982). O-antigen polysaccharide portion of a LPS molecule would be responsible for successful infection of the host plant (Cava *et al.* 1989). Noel *et al.* (1986) reported that reduction or defect of the polysaccharide chains resulted in abortion of infection thread development. The observations in the present study provide a hypothesis that a very small amount (2μM) of Cd causes the decrease in CNA of *B. japonicum* by disturbing the synthesis of polysaccharide chains in LPSs.

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