APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 2003, p. 1791–1796 Vol. 69, No. 3
0099-2240/03/$08.00+0 DOI: 10.1128/AEM.69.3.1791–1796.2003

Enhanced Accumulation of Cd^{2+} by a *Mesorhizobium* sp. Transformed with a Gene from *Arabidopsis thaliana* Coding for Phytochelatin Synthase

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Received 30 July 2002/Accepted 26 November 2002

We expressed the *Arabidopsis thaliana* gene for phytochelatin synthase (PCS_{At}) in *Mesorhizobium huakuii* subsp. *rengei* B3, a microsymbiont of *Astragalus sinicus*, a legume used as manure. The PCS_{At} gene was expressed under the control of the *nifH* promoter, which regulates the nodule-specific expression of the *nifH* gene. The expression of the PCS_{At} gene was demonstrated in free-living cells under low-oxygen conditions. Phytochelatin synthase (PCS) was expressed and catalyzed the synthesis of phytochelatins [$(\gamma\text{-Glu-Cys})_n$-Gly; PCs] in strain B3. A range of PCs, with values of *n* from 2 to 7, was synthesized by cells that expressed the PCS_{At} gene, whereas no PCs were found in control cells that harbored the empty plasmid. The presence of $CdCl_2$ activated PCS and induced the synthesis of substantial amounts of PCs. Cells that contained PCs accumulated 36 nmol of Cd^{2+}/mg (dry weight) of cells. The expression of the PCS_{At} gene in *M. huakuii* subsp. *rengei* B3 increased the ability of cells to bind Cd^{2+} approximately 9- to 19-fold. The PCS protein was detected by immunostaining bacteroids of mature nodules of *A. sinicus* containing the PCS_{At} gene. When recombinant *M. huakuii* subsp. *rengei* B3 established the symbiotic relationship with *A. sinicus*, the symbionts increased Cd^{2+} accumulation in nodules 1.5-fold.

Widespread pollution by heavy metals that are generated by various industries has serious adverse effects on human health and the environment (24). Decontamination of the soil and water around industrial plants presents major challenges for a long time. Genetic engineering suggests the possible use of specially designed microbial biosorbents with suitable selectivity and affinity for heavy metals. Overexpression of metal-binding peptides, such as metallothioneins (MTs), by bacterial cells results in enhanced accumulation of Cd^{2+} and offers a promising strategy for the development of microbe-based biosorbents for the remediation of metal-contaminated soil (16, 21, 28, 40).

Phytochelatins (PCs), which are naturally occurring metal-binding peptides, are an attractive alternative to MTs since they offer the potential for enhanced affinity and selectivity for heavy metals. PCs are short peptides composed of only three amino acids, namely, Glu, Cys, and Gly, with Glu and Cys residues linked through a γ-carboxylamide bond. The structure of such peptides can be represented by $(\gamma\text{-Glu-Cys})_n$-Gly, where *n* ranges from 2 to 11. PCs have been identified in a wide variety of plant species and in some microorganisms (4, 27, 41). Compared to MTs, PCs offer many advantages that are due to their unique structural characteristics, in particular, the continuously repeating γ-Glu-Cys units. For example, PCs have a higher metal-binding capacity (on a per-cysteine basis) than do MTs (18). In addition, PCs can incorporate high levels of inorganic sulfide, which results in very significant increases in the Cd^{2+}-binding capacity of these peptides (19). Thus, PCs are attractive as metal-binding peptides for the development of microbe-based biosorbents for the remediation of metal-polluted soils.

Rhizobia are gram-negative bacteria that can establish a symbiotic relationship with leguminous plants. They grow slowly for long periods in soil, but if they infect a compatible legume, they can grow rapidly. Successful infection by a single bacterium can lead to the formation, on the root of a legume, of a nitrogen-fixing nodule that contains more than 10^8 bacterial progeny (6). The rhizobium-legume symbiosis is initiated when flavonoids and related plant compounds induce the bacterium to produce molecular signals that stimulate nodule organogenesis (8). Bacteria enter the developing nodule via infection threads (14) and are eventually taken up by the plant host cells by an endocytosis-like process. Each rhizobium undergoes differentiation into its endosymbiotic form, which is known as a bacteroid. Bacteroids can fix atmospheric nitrogen as ammonia, which is exported to and assimilated by the host plant (23).

Mesorhizobium huakuii subsp. *rengei* strain B3 (22, 25) is a bacterium that establishes a symbiotic relationship with *Astragalus sinicus*, a legume that has been used as green manure in rice fields in Japan and southern China, by eliciting the formation of nitrogen-fixing root nodules (3). *A. sinicus* is widely used as a natural fertilizer in rice fields during fallow periods. It would be of considerable interest if we could use this leguminous plant to increase fertilizer nitrogen and to remove heavy metals from soil at the same time.

The presence of 10^6 to 10^8 bacterial progeny of *M. huakuii*

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subsp. *rengei* B3 in each nodule on the roots of *A. sinicus* is advantageous for the expression of foreign genes that help to sequester heavy metals in contaminated soil. Once symbiosis is established, the heavy metals should accumulate in such nodules. In this report, we describe the introduction of the *Arabidopsis thaliana* gene for phytochelatin synthase (PCS; PCS_{At}) into *M. huakuii* subsp. *rengei* strain B3. The gene was expressed under the control of a bacteroid-specific promoter, namely, the promoter of the *nifH* gene (26, 30). The gene for PCS was expressed in free-living cells under microaerobic conditions when the promoter was activated by NifA (the regulatory protein for *nif* and *fix* promoters) (37). We investigated the ability of the recombinant cells to produce PCs and to accumulate Cd^{2+}. Such cells might be useful for the development of a novel plant-bacterium remediation system for the removal of heavy metals from rice fields when genetically engineered *M. huakuii* subsp. *rengei* strain B3 establishes a symbiotic relationship with *A. sinicus*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used were *Escherichia coli* DH5αF′ [F′ Δ(*lacZYA-argF*) U169 *deoR endA1 hsdR17*(r_R^- m_R^+) *supE44 thi-1 recA1 gyr96 relA1* (ϕ80*dlacZ* Δ*M15*)] and *M. huakuii* subsp. *rengei* strain B3, which was isolated from nodules of *A. sinicus* cv. Japan that had been grown in a rice field in Hiroshima, Japan (22, 25). The cloning vector pBluescriptKS was purchased from Stratagene (La Jolla, Calif.). The expression vectors pBBR1MCS-2 (provided by K. M. Peterson, Louisiana State University Medical Center, Shreveport) and pMP220 (35) were used for the expression of the PCS_{At} gene. The *E. coli* cells were grown at 37°C in Luria broth (31) or on agar (1.5%, wt/vol) plates supplemented with appropriate antibiotics. Strain B3 was grown in tryptone-yeast extract (TY) medium (2) and incubated at 30°C with shaking.

Gene constructs. The 1.45-kb PCS_{At} gene (12) was amplified by PCR with primer PCF (5′-ACCATGGCTATGGCGAGTTTATATC-3′) and primer PCT (5′-CGGGATCCCTAATAGGCAGGAGCAGCGAGATCATCC-3′). The template was PCS_{At} in pUC18, and DNA was synthesized by using KOD polymerase (Toyobo, Osaka, Japan) and cloned into pBluescriptKS that had been digested with *Eco*RV, yielding the plasmid pBluescriptKSPCS. The sequence containing the promoter region of *nifH*, including the initiation codon and Shine-Dalgarno ribosome-binding site, was amplified with primers nifH1 (5′-CGGAATTCAGTCGCTATGCC-3′) and nifH2 (5′-ACCTGCCATGGTAGATTTCC-3′). The 240-bp amplified fragment, flanked by *Eco*RI and *Nco*I sites, was digested with *Eco*RI and *Nco*I and ligated into pBluescriptKSPCS that had been digested with *Eco*RI and *Nco*I. The resulting plasmid, pBluescriptKSnifHPCS, contained the PCS_{At} gene fused in frame downstream of the *nifH* promoter. A 1.7-kb *Kpn*I-*Eco*RI fragment of pBluescriptKSPCS was ligated into *Kpn*I- and *Eco*RI-digested pMP220. The resulting plasmid was designated pMPnifHPCS. For introduction of the *nifH-PCS* fusion gene into pBBR1MCS-2, a 1.7-kb *Eco*RI-*Sal*I fragment of pBluescriptKSnifHPCS was ligated into *Eco*RI- and *Sal*I-digested pBBR1MCS-2 to yield plasmid pBBRnifHPCS.

Heterologous expression of His-tagged PCS_{At}. To detect the expression of PCS in bacteroids by immunostaining, the PCS_{At} was fused with a His tag at the C terminus. The resulting plasmid was designated pMPnifHPCSHis.

Introduction by electroporation of pMPnifHPCS and pBBRnifHPCS into strain B3. The PCS_{At} gene in the expression vector pMPnifHPCS or pBBRnifHPCS was introduced into strain B3 by electroporation (13). Transformants were selected on TY medium plates (2) supplemented with tetracycline (20 μg/ml) and kanamycin (100 μg/ml) for pMPnifHPCS and pBBRnifHPCS, respectively. Several clones were isolated, and their sequences were checked by PCR and restriction digestion.

Analysis of gene expression by reverse transcription and PCR. Total RNA was isolated from 3 ml of a microaerobic culture of B3(pMPnifHPCS) (36) with RNAwiz (Ambion, Austin, Tex.) according to the manufacturer's instructions. Then cDNA was synthesized from 0.25 μg of total RNA with antisense primer 5′-CAGGACCTTTGATGCATTTC-3′ and a SuperScriptII RT kit (Invitrogen, Carlsbad, Calif.), according to the instructions from Invitrogen. Independent PCRs with PCS_{At}-specific primers (5′-AGACAGTCTGACTTATGCTG-3′ and 5′-CAGGACCTTTGATGCATTTC-3′) were performed. Each reaction mixture contained 1.5 mM Mg^{2+}, a 0.2 mM concentration of each deoxynucleoside triphosphate (dNTP), a 1 μM concentration of each primer, and 0.25 U of ExTaq polymerase (Takara, Osaka, Japan)

Identification of PCs and intermediates in their biosynthesis. Five hundred milliliters of microaerobically conditioned culture cells in the presence of 30 μM $CdCl_2$ (final concentration) was harvested. The samples were analyzed for reduced glutathione (GSH) and PCs by high-performance liquid chromatography (HPLC) and subsequent reaction with Ellman's reagent in combination with an ion pair method as described elsewhere (7, 11, 15).

Microaerobic culture and quantitation of Cd^{2+}. $CdCl_2$ was added to the microaerobic culture cells to give final concentrations of 1 to 50 μM, and the cells were grown at 30°C with gentle shaking for 40 h. To determine the concentration of Cd^{2+}, bacterial cells were pelleted, washed twice with 0.85% NaCl in 5 mM HEPES (pH 7.1), dried at 65°C for 5 h, and treated overnight with 70% nitric acid (29). The concentration of Cd^{2+} was measured directly in the soluble fraction with an atomic absorption spectrophotometer (model SAS7500A; Seiko, Tokyo, Japan).

Nodule formation and measurement of Cd^{2+} concentration. Nodule formation on *A. sinicus* cv. Japan (Takayama Seed Co., Kyoto, Japan) infected with strain B3 was determined as described previously (22). Nodules from 6-week-old plants cultivated hydroponically in nitrogen-free modified medium (22) supplemented with 50 μM $CdCl_2$ were harvested, washed, dried, and solubilized with 70% nitric acid (29). Cd^{2+}concentrations were measured directly in the soluble fraction by determining atomic absorption with a spectrophotometer (29, 36).

Immunostaining of paraffin sections. The paraffin sections of the nodules were prepared as described previously (36). Deparaffinized and rehydrated sections were incubated with a 1:200 dilution of anti-His antibody (Amersham Pharmacia Biotech, Buckinghamshire, England) in washing buffer (Tris-buffered saline containing 0.1% [wt/vol] bovine serum albumin and 0.1% [vol/vol] Tween 20). An alkaline phosphate-conjugated goat anti-mouse immunoglobulin G was used as a secondary antibody (Promega, Tokyo, Japan). The signal was detected by using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate solution for 30 min to 10 h in the dark.

RESULTS AND DISCUSSION

Expression of PCS_{At} under control of the *nifH* promoter. The plasmid with the PCS_{At} gene fused in frame downstream of the *nifH* promoter was subcloned into the broad-host-range expression vectors pBBR1MCS-2 and pMP220. The resulting plasmids were designated pBBRnifHPCS and pMPnifHPCS (Fig. 1). For the expression of genes under control of the *nifH* promoter in free-living cells, a low level of oxygen is necessary, since the fixation of nitrogen in nodules occurs under anaerobic conditions. A low concentration of oxygen induces expression of the *nifA* gene, and in a cascade, NifA activates the transcription of other *nif* genes (5). Thus, we analyzed the expression of PCS_{At} under the microaerobic conditions by reverse transcription and PCR. A single 120-bp fragment, corresponding to the predicted cDNA product, was observed when we used primers specific for PCS_{At}. The PCS_{At}-specific product was obtained from B3(pMPnifHPCS) cells in the presence of reverse transcriptase. There was no difference in PCS_{At}-specific product between B3(pMPnifHPCS) cells that had been exposed to $CdCl_2$ and those that had not been exposed. The negative control (without reverse transcriptase) produced no PCS_{At}-specific product. No PCS_{At}-specific product was observed in the case of strain B3 that harbored the pMP220 empty plasmid (data not shown).

Identification of PCs and intermediates in their biosynthesis in free-living cells. We performed an analysis by HPLC to examine the synthesis of PCs in strain B3 that harbored the PCS_{At} gene. PCs were detected in strain B3 that contained the PCS_{At} gene and that had been treated with 30 μM $CdCl_2$ for 40 h (Fig. 2A). However, no PCs were detected in untreated cells (Fig. 2B) or in cells that harbored the pMP220 empty

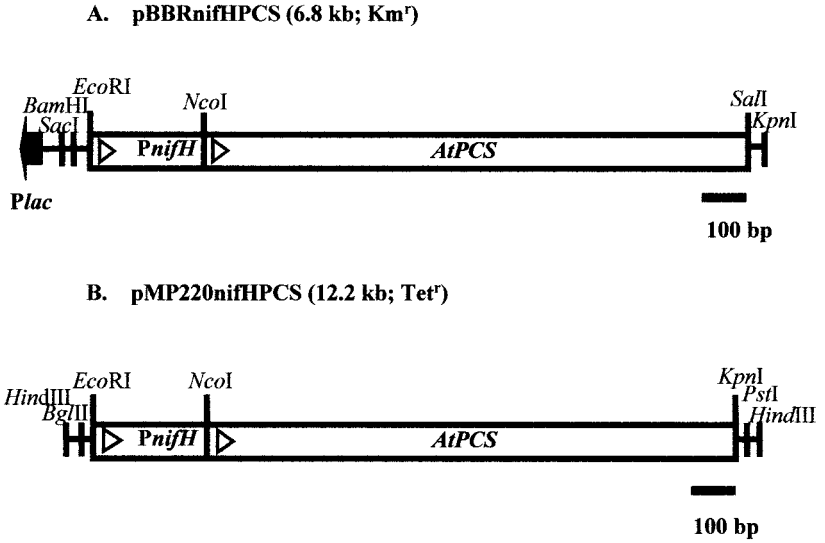

FIG. 1. Construction of the PCS_{At} expression vectors. (A) Structure of the pBBRnifHPCS plasmid that contained the PCS_{At} gene fused, in frame, to the *nifH* promoter. A 1.7-kb *Eco*RI-*Sal*I fragment containing P*nifH-PCS*$_{At}$ was inserted into pBBR1MCS-2. (B) Structure of the pMPnifHPCS plasmid that contained the PCS_{At} gene fused, in frame, to the *nifH* promoter. A 1.7-kb *Eco*RI-*Kpn*I fragment containing P*nifH-PCS*$_{At}$ was inserted into pMP220. The orientations of *nifH* and the PCS_{At} gene are indicated by open triangles, and the orientation of the *lac* promoter (plac) is indicated by an arrow.

plasmid (data not shown). The production of PCs was dependent on the presence of a heavy metal, namely Cd^{2+}, and our results indicated that the PCS_{At} gene was transcribed and translated under the control of the *nifH* promoter. Moreover, the enzymatic activity, as detected by the synthesis of PCs, was enhanced by $CdCl_2$. This observation reflects the production of PCs in *Rauvolfia serpentina*, *Silene cucubalus*, and *A. thaliana*, which contain no or almost no PCs when grown without metal ions but produce PCs upon addition of a variety of metal ions (10, 12, 17).

The profiles of PCs from Cd^{2+}-treated B3(pMPnifHPCS) cells after HPLC revealed the presence of PC_2 [(γ-Glu-Cys)$_2$-

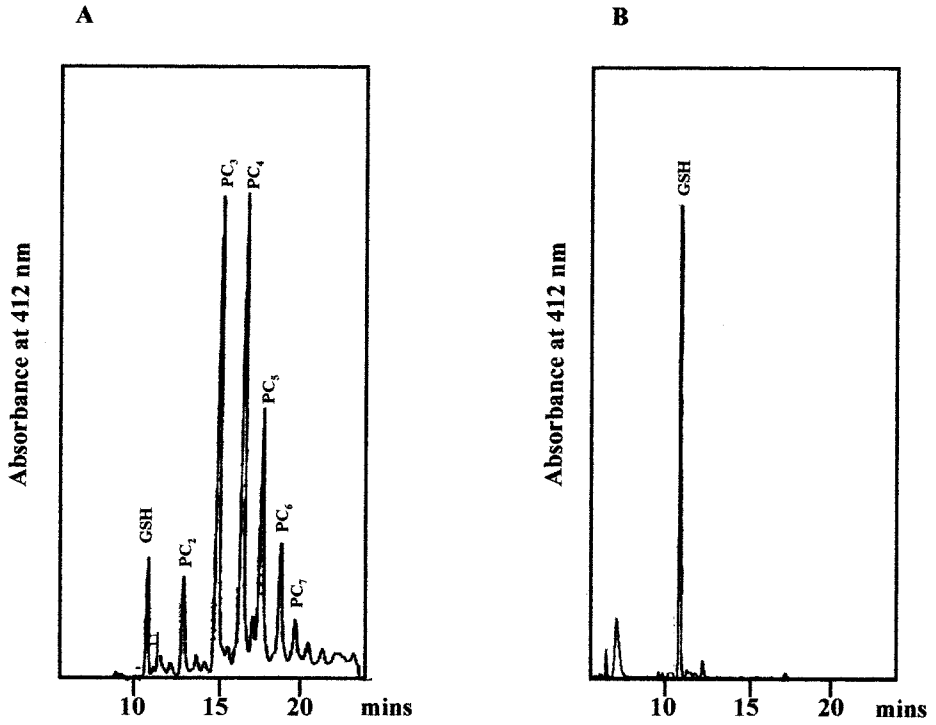

FIG. 2. Accumulation of PCs by *M. huakuii* subsp. *rengei* B3 cells that expressed PCS_{At}. After 40 h of growth under microaerobic conditions in the presence (A) or absence (B) of 30 μM $CdCl_2$, extracts of cells that harbored pMPnifHPCS (A and B) were analyzed by HPLC as described in the text. Peaks that represent GSH, PC_2, PC_3, PC_4, PC_5, PC_6, and PC_7 are identified.

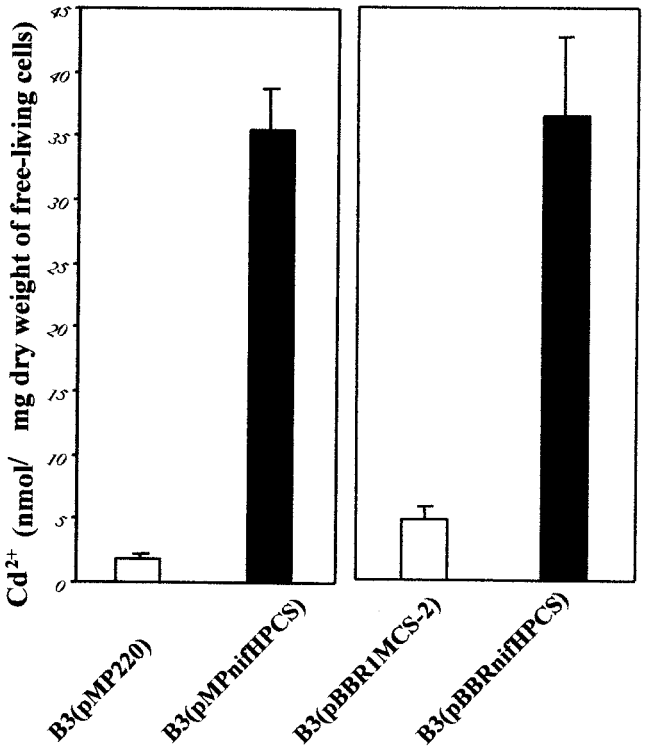

FIG. 3. Accumulation of Cd^{2+} by *M. huakuii* subsp. *rengei* B3 cells that expressed PCS_{At}. The amount of Cd^{2+} that accumulated in cells was determined by atomic absorption spectrometry and is indicated as nanomoles of Cd^{2+} per milligram (dry weight) of cells The values shown are means (± standard deviations) of the results for three independent experiments in each case. (Left) Accumulation of Cd^{2+} by free-living strain B3 that harbored the empty vector, namely, B3 (pMP220) cells, and strain B3 that harbored the PCS_{At} gene, namely, B3(pMPnifHPCS) cells; (right) accumulation of Cd^{2+} by free-living strain B3 that harbored the empty vector pBBR1-MCS2, namely, B3 (pBBR1-MCS2) cells, and strain B3 that harbored the PCS_{At} gene, namely, B3(pBBRnifHPCS) cells. Bacteria were grown under microaerobic conditions in TY medium plus 30 μM $CdCl_2$ for 40 h.

Gly] through PC_7 [$(\gamma$-Glu-Cys$)_7$-Gly]. Levels of PC_3 and PC_4 were higher than those of other PCs. We also examined PCs by HPLC for the presence of sulfhydryl groups. At 30 μM $CdCl_2$, we detected 5.44 ± 2.08 nmol/mg (dry weight) of cells (mean ± standard deviation of results from three independent experiments [$n = 3$]) in terms of SH equivalents. The GSH content of $CdCl_2$-treated cells decreased to 0.27 ± 0.09 nmol/mg (dry weight) of cells ($n = 3$), whereas untreated cells contained a GSH level of 1.0 ± 0.49 nmol/mg (dry weight) of cells ($n = 3$). The GSH content was high in cells of *M. huakuii* subsp. *rengei* B3(pMPnifHPCS) that had been grown in the absence of $CdCl_2$, but the level of GSH decreased in cells grown in the presence of $CdCl_2$. Thus, it is likely that GSH is a substrate for the production of PCs and that the expression of the PCS_{At} gene under control of the *nifH* promoter in bacteroids, which contain large amounts of GSH (20), might allow production of large amounts of PCs. The amount of PCs, in terms of SH equivalence, was similar to that observed in extracts of seedlings of *A. thaliana* exposed to 100 μM $CdCl_2$ (7 nmol/mg [dry weight] of cells) (33). This similarity indicates that the level of expression and the folding or stability of the protein were appropriate in strain B3.

Accumulation of Cd^{2+} in free-living cells. Cultures of strain B3 containing pBBRnifHPCS and pMPnifHPCS were supplemented with 30 μM $CdCl_2$ under microaerobic conditions. Strain B3 containing PCs accumulated 9- to 19-fold more Cd^{2+} than did cells without the PCS_{At} gene (cells transfected with pBBR1MCS-2 or the pMP220 empty vector). The levels of Cd^{2+} were 35.52 ± 3.16 and 36.41 ± 4.03 nmol/mg (dry weight) of cells ($n = 3$) for B3(pMPnifHPCS) and B3(pBBRnifHPCS) cells, respectively (Fig. 3). For cells of strain B3 without PCs, namely, B3(pMP220) and B3(pBBR1MCS-2), we found only 1.84 ± 0.45 and 4.09 ± 1.05 nmol of Cd^{2+}/mg (dry weight) of cells, respectively. The accumulation of Cd^{2+} in strain B3 that expressed PCS_{At} was higher than that in genetically engineered *E. coli* cells that expressed a eukaryotic MT as a fusion protein with a membrane or membrane-associated protein, namely, LamB or peptidoglycan-associated lipoprotein (34, 38, 39). *E. coli* cells that expressed the *PCS* gene from *Arabidopsis* or its analogs had strong PC synthase activity and accumulated heavy metals in cells (1, 12). The ability of *M. huakuii* subsp. *rengei* B3 that expressed PCS_{At} to accumulate Cd^{2+} was 12-fold higher than that of strain B3 that expressed *MTL4*, the gene for tetrameric human metallothionein (MTL4) (36). The increased accumulation of Cd^{2+} by cells that contained PCs might have been due to the fact that the ratio of Cd^{2+} to sulfhydryl groups for PCs is higher than that for vertebrate MT, namely, 1:2 and 1:3, respectively (11). Moreover, long-

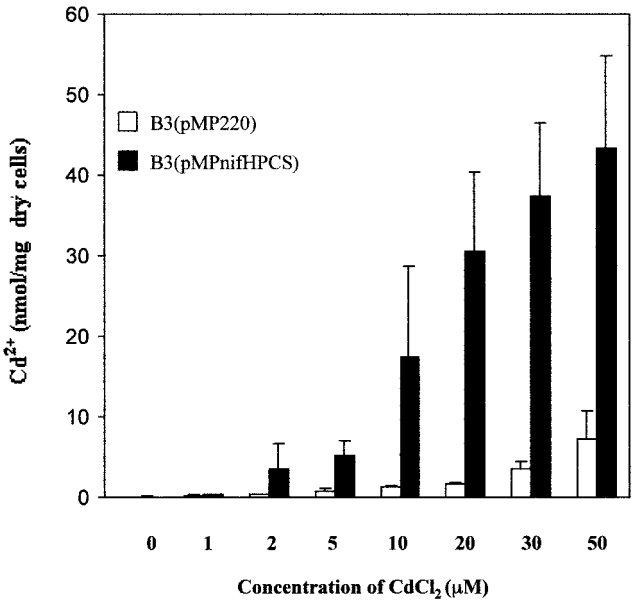

FIG. 4. Effects of $CdCl_2$ on the synthesis of PCs in *M. huakuii* subsp. *rengei* B3. Determinations were made by atomic absorption spectrometry of the amount of Cd^{2+} accumulated in strain B3 that harbored the empty vector, namely, B3(pMP220) cells, and in B3 cells that contained the PCS_{At} gene, namely, B3(pMPnifHPCS) cells. The amount of Cd^{2+} bound to bacterial cells is indicated in nanomoles per milligram (dry weight) of cells. The values shown are means (± standard deviations) of the results for three independent experiments in each case. Bacteria were grown under microaerobic conditions in the presence of various concentrations of $CdCl_2$ (1 to 50 μM) as indicated for 40 h.

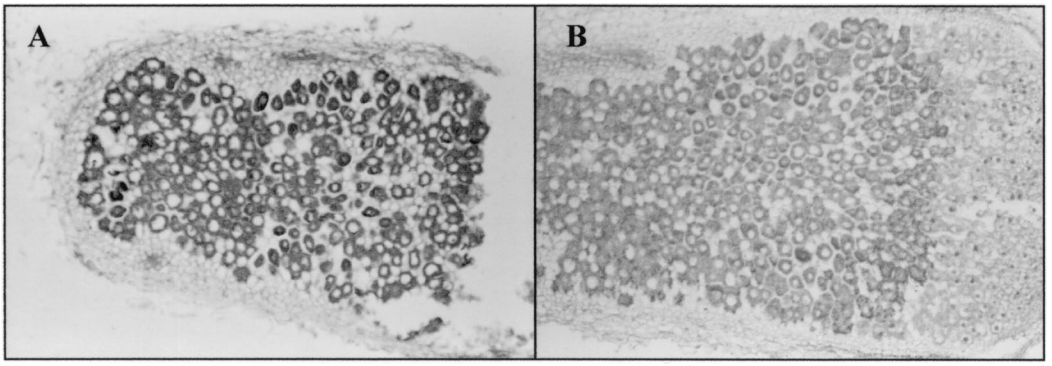

FIG. 5. Immunostaining for detection of PCS protein in the mature nodules of *A. sinicus* containing B3(pMPnifHPCSHis) and B3(pMP220). The accumulation of PCS in bacteroids was detected by using anti-His antibody, followed by alkaline phosphatase-conjugated anti-mouse antibody. (A) The purple color that developed was detected in bacteroids containing PCS_{At}. (B) The purple color was not detected in bacteroids containing pMP220 empty vector.

chain PCs might affect the accumulation of Cd^{2+}. It has been reported that the longer PCs have a higher relative complexing affinity for heavy metals than do shorter ones (17, 32). Therefore, the features of the synthesis of PCs in B3(pMPnifHPCS) cells, which contained longer PCs, such as PC_4 through PC_7, should be advantageous for the removal of Cd^{2+} from soil when B3(pMPnifHPCS) cells establish a symbiotic relationship with *A. sinicus*.

Increased concentrations of $CdCl_2$ increased the accumulation of Cd^{2+} in strain B3 (Fig. 4). In strain B3(pMPnifHPCS), the level was 43.4 ± 11.43 nmol of Cd^{2+}/mg (dry weight) of cells ($n = 3$) after treatment of the cells with 50 μM $CdCl_2$. These observations indicated that higher concentrations of $CdCl_2$ increased the activity of PCS and the synthesis of PCs, with subsequent increased accumulation of Cd^{2+} by the cells. It is possible that Cd^{2+} ions activated PCS and prolonged the synthesis of PCs until all Cd^{2+} ions had been chelated by PCs. Moreover, it has been reported that the presence of metal ions leads to the transpeptidation of the γ-Glu-Cys moieties of shorter PCs to longer PCs (9). Thus, prolonged exposure to $CdCl_2$ results in production of longer PCs, which have a high relative complexing affinity for heavy metals (17, 32).

Expression of PCS_{At} in bacteroids. To assess whether the PCS was expressed in bacteroids within nodules, we performed immunostaining using the anti-His antibody to detect the presence of PCS-His tag in bacteroids. Bacteroids containing pMPnifHPCSHis showed a strong signal of immunostaining (Fig. 5). There was no signal of immunostaining in bacteroids containing pMP220. This result indicated that the PCS_{At} gene was expressed under the control of the *nifH* promoter within the bacteroids.

The accumulation of Cd^{2+} in nodules has been investigated. The content of Cd^{2+} in the nodules containing bacteroids from strain B3(pMPnifHPCS) increased 1.5-fold compared with that of nodules containing bacteroids from strain B3(pMP220). In a previous work (36), the same symbionts expressed tetrameric MT protein to increase Cd^{2+} accumulation in nodules 1.7- to 2.0-fold. The limitation of Cd^{2+} accumulation by bacteroids is currently being investigated. However, a symbiotic relationship between genetically engineered *M. huakuii* subsp. *rengei* B3 and *A. sinicus* might help in the removal of Cd^{2+} from contaminated rice fields.

ACKNOWLEDGMENTS

We thank M. Tansengco and N. Tsuji for valuable discussions.

This work was supported by a grant-in-aid for scientific research from the Ministry of Culture, Education, Science and Technology of Japan (Kaken Houga no. 12875155). R.S. is supported by a scholarship from MonbuKagakusho, Tokyo, Japan.

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