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

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We expressed the Arabidopsis thaliana gene for phytochelatin synthase (PCSₐₙ) in Mesorhizobium huakuii subsp. rengei B3, a microsymbiont of Astragalus sinicus, a legume used as manure. The PCSₐₙ gene was expressed under the control of the nifH promoter, which regulates the nodule-specific expression of the nifH gene. The expression of the PCSₐₙ gene was demonstrated in free-living cells under low-oxygen conditions. Phytochelatin synthase (PCS) was expressed and catalyzed the synthesis of phytochelatins [(γ-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 PCSₐₙ 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 PCSₐₙ 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 PCSₐₙ 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 (γ-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; PCSAt) 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 recombiant cells to produce PCs and to accumulate Cd²⁺. 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 DH5α F’ [F Δ(lacZYAarF)-U169 deoR endA1 hsdR17(kaac 80d)] supE44 thi-1 recA1 gyr96 relA1 (29) 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 PCSAt 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 for 5 h, and treated overnight with 70% nitric acid (29). The concentration of Cd²⁺ was measured directly in the soluble fraction by atomic absorption spectrophotometry (model SAS7500A; Seiko, Tokyo, Japan).

Nodule formation and measurement of Cd²⁺ 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 CdCl₂ were harvested, washed, dried, and solubilized with 70% nitric acid (29). Cd²⁺ concentrations were measured directly in the soluble fraction by 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 1 h in the dark.

RESULTS AND DISCUSSION

Expression of PCSAt under control of the nifH promoter. The plasmid with the PCSAt 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 pBBRnHPCS and pMPnHPCS (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 PCSAt 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 PCSAt. The PCSAt-specific product was obtained from B3(pMPnHPCS) cells in the presence of reverse transcriptase. There was no difference in PCSAt-specific product between B3(pMPnHPCS) cells that had been exposed to CdCl₂ and those that had not been exposed. The negative control (without reverse transcriptase) produced no PCSAt-specific product. No PCSAt-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 PCSAt gene. PCs were detected in strain B3 that contained the PCSAt gene and that had been treated with 30 μM CdCl₂ for 40 h (Fig. 2A). However, no PCs were detected in untreated cells (Fig. 2B) or in cells that harbored the pMP220 empty

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[Note: The rest of the text is beyond the scope of this instruction and includes detailed methods and data analysis.]

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The production of PCs was dependent on the presence of a heavy metal, namely Cd\(^{2+}\)/H\(_{11001}\), 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. 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 EcoRI-SalI fragment containing PnifH-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 EcoRI-KpnI fragment containing PnifH-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.

FIG. 2. Accumulation of PCs by M. huakuii subsp. rensei 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.
of CdCl2-treated cells decreased to 0.27 nmol/mg (dry weight) of cells. The GSH content of CdCl2-treated cells was similar to that observed in extracts of seedlings of *A. thaliana* exposed to 100 μM CdCl2 (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 Cd2+ in free-living cells.** Cultures of strain B3 containing pBBRnifHPCS and pMPnifHPCS were supplemented with 30 μM CdCl2 under microaerobic conditions. Strain B3 containing PCs accumulated 9- to 19-fold more Cd2+ than did cells without the PCSα gene (cells transfected with pBBR1MCS-2 or the pMP220 empty vector). The levels of Cd2+ 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 Cd2+/mg (dry weight) of cells, respectively. The accumulation of Cd2+ in strain B3 that expressed PCSα 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 PCSα to accumulate Cd2+ was 12-fold higher than that of strain B3 that expressed *MTL4*, the gene for tetrameric human metallothionein (MTL4) (36). The increased accumulation of Cd2+ by cells that contained PCs might have been due to the fact that the ratio of Cd2+ to sulphydryl groups for PCs is higher than that for vertebrate MIT, namely, 1:2 and 1:3, respectively (11). Moreover, long-

Gly] through PC7 (γ-Glu-Cys)-Gly. Levels of PC4 and PC5 were higher than those of other PCs. We also examined PCs by HPLC for the presence of sulphydryl groups. At 30 μM CdCl2, 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 CdCl2-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 CdCl2, but the level of GSH decreased in cells grown in the presence of CdCl2. Thus, it is likely that GSH is a substrate for the production of PCs and that the expression of the PCSα 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 CdCl2 (7 nmol/mg [dry weight] of cells) (33). This similarity indicates that the level of...
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 tetramer 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.

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