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The *cadA* Gene in Cadmium-Resistant Bacteria from Cadmium-Polluted Soil in the Zhangshi Area of Northeast China

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Abstract Cadmium-resistant bacteria were isolated from the farmland soil in Zhangshi Irrigation Area in Shenyang of Northeast China, an area has been polluted by heavy metals, especially cadmium, for more than 40 years. The *cadA* gene was detected in 4 *Bacillus* strains and for the first time in one *Flavobacterium* strain. The high sequence identity (93%–99%) of *cadA* gene, shared indels in different bacterial species and genera, and the phylogenetic incongruence between 16S rDNA gene tree and *cadA* gene tree suggested that lateral gene transfer (LGT) occurred among *Bacillus* and *Flavobacterium* spp. The LGT of *cadA* gene might play a vital role in promoting the spread of cadmium-resistant phenotypes throughout soil microbial communities.

Introduction

Soil heavy metals pollution gives a strong stress on soil microbes, resulting in a response of the increase of pre-existed resistant bacteria and the appearance of new resistant microbes via lateral transfer of resistant genes [3, 12]. Cadmium-resistance of soil bacteria is mediated by several genetic systems [9, 13], and the well-studied one is the cadmium efflux system coded by cadmium-resistant gene *cadA* operon [10]. The homologues of *cadA* gene were reported to be widespread in Gram-positive bacteria such as *Staphylococcus aureus*, *Bacillus firmus* [4], *Listeria monocytogenes* [6] and *Lactococcus lactis* [7], and in Gram-negative bacterium *Stenotrophomonas maltophilia* [1].

The farmland soil in Zhangshi Irrigation Area in Shenyang of Northeast China has been polluted by heavy metals, especially by cadmium for more than 40 years [15]. In our previous study, the heavy metals in this soil seemed to have less toxic effects on soil microbial populations, enzymatic activities, and many other bioindicators, suggesting that the microbes in this soil could be already adapted to the severe environmental stress. The objectives of this study were to isolate and identify the cadmium-resistant bacteria in the farmland soil in Zhangshi Irrigation Area, and approach the distribution patterns of *cadA* gene in the isolated bacterial strains.

Materials and Methods

Soil Sampling and Isolation of Cadmium-Resistant Bacteria

Soil samples (Gleyic Cambisols, FAO) of 0–10 cm were collected from 4 sites in the Zhangshi Irrigation Area in Shenyang along a gradient of soil Cd pollution [16]. The soil Cd concentrations in Site 1, Site 2, Site 3, and Site 4 was 2.66 mg kg^{-1}, 2.12 mg kg^{-1}, 2.06 mg kg^{-1}, and 1.85 mg kg^{-1}, respectively. Fresh soil samples were passed

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through a 2-mm sieve, and stored at 4°C for the isolation of cadmium-resistant bacteria. Cadmium-resistant bacteria growing on a meat peptone agar (1% beef extract, 0.5% peptone, 0.5% NaCl and 1.5% agar, pH 7.0–7.2) supplemented with 2.67 mM of Cd^{2+} ($CdCl_2$) were isolated, and morphologically distinct colonies were picked. The isolates were identified genotypically by sequencing ~500 bp of 16S rDNA.

Chromosomal DNA Extraction and PCR Amplification

Bacterial genomic DNA was extracted from whole cell by using a standard method [2], and used for the amplification of 16S rDNA and *cadA* gene. The plasmid pI258 that carries the *Staphylococcus aureus cadA* gene was used as a positive control of the *cadA* gene. The 16S rDNA gene was amplified by using two general bacterial 16S rDNA primers, 341F and 907R [8], and the *cadA* gene was amplified by using primers cad1 (CAAAYTGYGCRGGHAAR TTYGA) and cad2 (AACTAATGCACAAGGACA) [11]. Polymerase chain reaction (PCR) (50 μL) mixture contained 20 pM of each primer, 200 μM dNTP, 2.5 U of Taq DNA polymerase, PCR buffer supplied with the enzyme (Takara), and 1 μL of template DNA. PCR was performed in a PTC-100™ thermal controller (MJ Research Inc., USA). For 16S rDNA gene, PCR was carried out as follows: a single denaturation step (95°C for 5 min) followed by a 35-cycle program (denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min), and a final extension step at 72°C for 10 min. The *cadA* gene was amplified by the program described by Oger [11]. PCR products were examined by electrophoresis on 2% agarose gel.

Cloning and Sequencing of PCR Products

The sequencing of 16S rDNA gene was performed directly with purified PCR products. Primer 341F was used as the sequencing primer. The purified products of *cadA* gene were cloned into a pMD19-T vector, as described by the manufacturer (Takara). DNA sequences of both strands of *cadA* gene were sequenced by using primers RV-M (GAGCGGATAACAATTTCACACAGG) and M13-47 (CGCCAGGGTTTTCCCAGTCACGAC) designed according to the sequence of the vector. DNA sequences were analyzed by using the BlastN programs (http://www.ncbi.nlm.nih.gov/BLAST). The DNA sequences reported in this paper were deposited in the GenBank databases under the following accession numbers: EF590216 ~ EF590252.

Phylogenetic Analysis

The 16S rDNA gene sequences were aligned with published sequences of other closely related microorganisms by using the program ClustalX 1.83 (French Society of Bioinformatics) [14]. A multiple alignment of *cadA* gene sequences was also constructed. The neighbor-joining method was used to construct phylogenetic trees by using Mega 3.1 [5]. Bootstrap confidence values were obtained with 1000 resamplings.

Results

Thirty-two cadmium-resistant bacterial strains were isolated and identified by 16S rDNA sequence. The *cadA* amplicons were found in 5 of 32 strains (data not shown). Four *Bacillus* strains–N7, N9, N10, and N11—out of 5 *Bacillus* isolates, were detected to harbor the *cadA* gene, and for the first time, *cadA* was detected in *Flavobacterium* sp. S1.

Sequence analysis of pMD19-T cloned inserts (accession no. EF590248–EF590252) showed that they were highly identical to each other (93%–99%). According to the BLAST sequence analysis, the *cadA* gene in strains N7, N9, N10, N11, and S1 was 92%–94% identical to that in *Bacillus firmus* (M90750), and 78%–79% identical to that of pI258 of *S. aureus* (J04551). Surprisingly, the *cadA* gene length (1049 bp) between primers cad1 and cad2 in the 5 strains was different from that (1058 bp) in pI258 of *S. aureus*. The insertion and deletions (indels) in *cadA* gene of 5 strains and *B. firmus* explained the differences in gene length. The 9-bp indel existed in all of the 5 strains and *B. firmus*, and consequently, the deletion of 3 amino acids (Lys-92, Ala-93, and His-94), was clearly observed by the alignment of the putative CadA amino acid sequences of 5 strains and *B. firmus* versus that of pI258. The high identity of *cadA* gene sequence and the shared indels in *cadA* genes in different species and genus suggested that there was a possible transfer of *cadA* gene from one source.

A neighbor-joining tree was created for a 1049-bp section of the *cadA* gene (Fig. 1A). It was noticeable that the *cadA* gene of S1 in the tight cluster of *Flavobacterium* spp. (Fig. 1B) (Fig. 2, see supplementary material) (neighbor-joining boot-strap support at the basal node of the clade, 100) was grouped together with the *cadA* gene of *Bacillus* sp. N7. The *cadA* genes of other 4 *Bacillus* strains were also phylogenetically closed to that of *B. firmus*. Therefore, strain S1 might have acquired its *cadA* gene from a *Bacillus* source. The phylogenetic incongruence between the 16S rDNA gene tree and *cadA* gene tree also provided evidence for the lateral gene transfer (LGT) of *cadA* gene in host bacteria of this area.

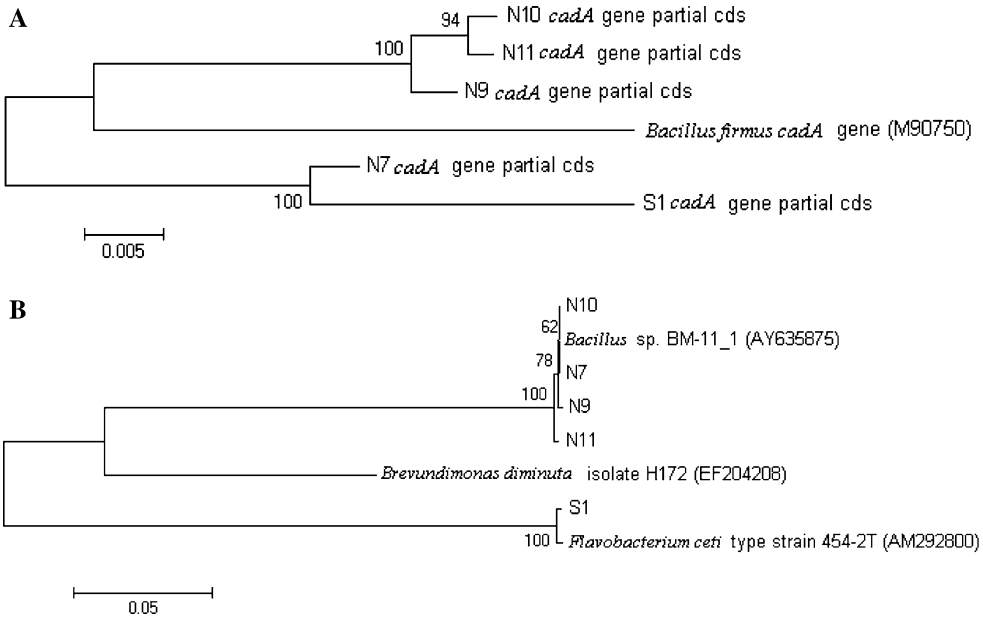

Fig. 1 Phylogenetic tree of 16S rDNA gene **(A)** and *cadA* gene **(B)** of 5 strains and their closest relatives. The numbers at the nodes indicate the percentages of occurrence in 1000 bootstrapped tree. The accession numbers of previously published sequences are given in parentheses

Discussion

The primers cad 1 and cad 2 were designed to amplify gene encoding the Cd^{2+} binding and Cd^{2+} channel domain [11]. The 9-bp indel results in the deletion of 3 amino acids Lys-92, Ala-93, and His-94 in the *cadA* gene of the 5 strains and *B. firmus* (Fig. 3, see supplementary material). This indel is located within a region (residues 86–105 in the *cadA* gene of pI258 *S. aureus*) between the cation binding region and the first transmembrane hairpin segment of Cd^{2+} ATPase [10]. The deletion of 3 amino acids in the region functioned as a hydrophilic stretch, and might not lead to changes of the advanced structure and the function of protein.

The *cadA* gene sequences in the strains isolated from the farmland of Zhangshi Irrigation Area displayed high identity with each other, which might suggest a spread of this gene not only among *Bacillus* species but also toward *Flavobacterium* genus. In our previous study, soil heavy metals showed no toxic effects on many soil microbial indicators such as soil respiration, enzymatic activity, and culturable microbial population [16]. Except for the obscure effects of soil organic matter and nutritive elements, the adaptive ability might also responsible for this result. Here, the genetic factor, lateral transfer of *cadA* gene, could contribute to the diversity of the resistant phenotype, and thus, the LGT of *cadA* gene among different species and genera might play an important role in increasing the genetic diversity of microbes and promoting the spread of cadmium-resistant phenotypes throughout microbial communities.

In conclusion, the *cadA* gene detected in 5 of 32 cadmium-resistant strains isolated from the study area was found to be almost identical, and *Flavobacterium* sp. was first reported to be the *cadA* gene carrier. In addition, the shared indels of the *cadA* gene in different species and genera and the phylogenetic incongruence between a 16S rDNA gene tree and a *cadA* gene tree also provided evidence for the probability of LGT of the *cadA* gene in host bacteria of this area.

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