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Bacillus subtilis DT134의 카드뮴 저항성

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Characteristics of Cadmium-Resistant *Bacillus subtilis* DT134

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Bacillus subtilis DT134 was resistant to 50-fold higher concentration of cadmium ions (Cd^{2+}) than cadmium-sensitive *B. subtilis* BD224 in Luria Broth (LB) medium. Minimal inhibition concentration tests in LB agar plates also showed similar results. The elevated cadmium resistance of *B. subtilis* DT134 strongly suggested a possible existence of cadmium resistance gene in it. Southern blot with *Staphylococcus aureus cadA* gene fragment (757 bp *Nla*IV-*Xmn*I *cadA* DNA fragment) as probe was carried out to test the existence and similarity of the gene. In high stringency condition, there was no detectable signal, but in low stringency, a strong signal specific to the *cadA* probe could be detected. These results strongly suggested that there was some similarity between total DNA of *B. subtilis* DT134 and *S. aureus* pI258 in terms of cadmium resistance gene and the resistance mechanism might be an efflux mechanism. The subsequent efflux experiment showed that the cadmium resistance mechanism of *B. subtilis* DT134 was also due to the efflux of cadmium.

Key Words : *B. subtilis* DT134, *S. aureus* pI258, *cadA* cadmium resistance gene, efflux mechanism

Introduction

Heavy metal resistances have been studied extensively in a wide range of bacteria (1-3). Many mechanisms are known to help living organisms survive under environmental stresses, including toxic heavy metals. Hg^{2-} is reduced by the enzyme mercuric reductase to Hg^{o}, which volatilizes (2, 4, 5). For Cd^{2+}, Zn^{2-}, Co^{2+}, Cu^{2-}, Ni^{2+}, AsO_4^{2-}, AsO_2^{-}, and CrO_4^{2-} resistances, a wide range of plasmid- or chromosome-governed systems "pump" toxic ions out of the cells (1-3, 6). The cadmium resistance system in the chromosomal determinant of *Staphylococcus aureus* is a cadmium efflux system allowing resistance to lower concentrations of cadmium than the *S. aureus* plasmid pI258 (6). The cadmium resistance system in the plasmid determinant of *S. aureus* pI258 is also a cadmium efflux system (7-10). The *S. aureus* plasmid pI258 has a mercury resistance determinant and operons for cadmium (zinc and bismuth) resistance and for arsenic (and antimony) resistance (11). The efflux (pumping) system of *S. aureus* plasmid pI258 is due to the membrane proteins encoded by genes on bacterial plasmid (12).

The sequence of cadmium resistance determinant of *S. aureus* plasmid pI258 indicated the presence of two open reading frames (12). The product of the longer ORF shows a strong sequence homology with E1-E2 class of ATPases (13, 14), such as the *E. coli* KdpB polypeptide (15). These highly conserved ATPases, which are found in all living cells from bacteria through man (16), have been renamed P-type ATPases (13, 14) because they contain a highly conserved segment with an aspartate residue that is phosphorylated during the process of ATP-driven cation transport. Lebrun et al. (17) showed that there are more than 65% amino acids sequence homology among *S. aureus* CadA, *Bacillus firmus* CadA, and *Listeria monocytogenes* CadA. They also reported very significant amino acid sequence homologies specially among ATPase domains of *S. aureus* CadA. *B. firmus* CadA, *L. monocytogenes* CadA, rabbit Ca^{2+}ATPase, and human Na^{+}/K^{-} ATPase.

In this paper, *B. subtilis* DT134 was shown to be resistant to cadmium in liquid and solid media The degree of resistance was compared among cadmium-resistant *B. subtilis* DT134, cadmium-resistant *S. aureus* pI258, and cadmium-sensitive *B. subtilis* BD224 The sequence similarity of

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cadmium resistance gene between *B. subtilis* DT134 and *S. aureus* plasmid pI258 was examined by Southern blot with *cadA*-specific probe generated from the 757 bp *Nla*IV-*Xmn*I DNA fragment of the *cadA* cadmium resistance determinant of *S. aureus* plasmid pI258. From the hybridization data, a possible role in P-type ATPases was discussed. Efflux experiments were also carried out to understand its resistance mechanism, which further suggested the function of the cadmium resistance gene of *B. subtilis* DT134

Materials and Methods

Bacterial Strains and Plasmids

Table 1 lists the bacterial strains and plasmids used. Cells were grown in LB medium (18) containing ampicillin (100 μg/mL) purchased from Sigma Chemical Co (St. Louis, USA). Procedures for manipulating DNA were as described by Sambrook et al. (18). *B. subtilis* DT134 was generously provided by Dr. S. Silver, University of Illinois, Health Science Center at Chicago.

Materials

$CdCl_2$ and other common chemicals were purchased from Sigma and culture media were purchased from Difco Laboratories (Detroit, USA). Southern-Light™ chemiluminescent detection system (Tropix Inc., Bedford, USA) was used to prepare biotin labeled *cadA*-specific probes $^{109}CdCl_2$ was obtained from New England Nuclear (Boston USA). Restriction nuclease enzymes, calf intestine phosphate, and T4 DNA ligase came from Boehringer Mannheim Biochemicals (Indianapolis, USA)

DNA Fragment Purification

To isolate DNA fragments from agarose gel slices, the Gene Clean kit purchased from BIO101 Co. (La Jolla, USA) was used according to manufacturer's instruction.

Growth Inhibition

The overnight cultures of each microorganism were diluted 100-fold in 5 mL LB medium supplemented with 0, 5, 10, 25 μM $CdCl_2$ (*B. subtilis* BD224); 0, 10, 25, 50, 100, 250, 500, 750 μM $CdCl_2$ (*B. subtilis* DT134); 0, 100, 250, 500, 750, 1000, 2500 μM $CdCl_2$ (*S. aureus* pI258) and shake-cultured at 37℃ for 12 hr. Turbidity (A_{600}) was measured using UV-vis spectrophotometer.

Minimal Inhibitory Concentrations

The overnight cultures were diluted 100-fold in LB media and the diluted cultures were grown for 3 hr, then the cultures were diluted 100-fold again 3 μL of newly diluted cultures was placed on the plates containing $CdCl_2$ (0, 1, 2.5, 5, 10, 50, 100, 250, 500, 1000, 2500 μM). After 20 hr incubation at 37℃, the lowest concentration where cells could not grow at all was determined as a minimal inhibitory concentration.

Construction of Plasmids pKPY26

pKPY21 (Table 1) was cut by *Xmn*I and whole mixture was separated in a 1% agarose gel. A 1.35 kb fragment was purified from the 1% agarose gel with the Gene Clean kit and digested again by *Nla*IV. The digested products were electrophoresed on 1% agarose gel and the purified 757 bp fragment was subcloned into *Sma*I site of pUC19. The resulting construct was named as pKPY26 which has 757 bp fragment of *cadA* (nucleotides 1117-1874 from published sequence, Nucifora et al., 1989).

Preparation of Biotin Labeled Probe DNA

After digestion of pKPY26 with *Eco*RI and *Hin*dIII, the *cadA*-specific 757bp DNA fragments to be labelled were obtained by the Gene Clean kit from a 1% agarose gel. Then the purified fragment was biotinylated with biotin labeling kit (Southern-Light™, Tropix Inc., USA) consisting of dNTP mixture, Biotin-14-dNTP, random octamer primers, and Klenow fragment. The reaction was carried out at 30℃ for 30 min according to the manufacturer's instruction.

Southern Blot Analysis

Total DNAs were isolated by methods described by

Table 1. Strains and plasmids.

Strain or plasmid	Genotype or phenotype	Reference
Strains		
B. subtilis BD224	*cadmium-sensitive trpC2 recE4 thr-5*	8, 12
B. subtilis DT134	cadmium-resistant *B. subtilis*	This study
S. aureus pI258	cadmium-resistant *S. aureus*	1, 8, 9, 12
Plasmids		
pKPY21	The intact 3.0 kb cadmium resistance gene was cloned in *Xba*I site of pKPY20	8, 10
pKPY26	The 757 bp DNA fragment from the 3.0 kb was cloned in *Sma*I site of pUC19	This study

Nucifora et al. (12). 5 μg of isolated total DNAs was partially digested by *Eco*RI (Boehringer Mannheim, Germany), and the digested DNAs were fractionated on a 0.8% agarose gel, and the DNA fragments were transferred to Hybond N^+ (Amersham, USA) according to the procedure of Southern (18). After UV-cross linking (Fluo-link, USA), hybridization was carried out under high or low stringent conditions. In high stringent condition, prehybridization and hybridization were performed at 68℃ for 4 hr and overnight respectively in a standard hybridization buffer (6X SSC, 0.01 M EDTA, 5X Denhardt's solution, 0.5% SDS, 100 μg/mL denatured salmon sperm DNA). For hybridization, the biotin labeled probe (5ng/cm^2) was added to the buffer. The filter was washed twice in 2X SSC, and 0.5% SDS for 15 min at room temperature, and washed twice in 0.1X SSC, and 0.5% SDS for 1 hr at 68℃. In low stringent condition (17), membranes were prehybridized in 4X SET buffer (0.6 M NaCl, 0.12 M Tris hydrochloride [pH 8.0], 4 mM EDTA), containing 10X Denhardt's solution, 0.2% SDS, 100 μg/mL denatured salmon sperm DNA for 5 hr at 30℃. Hybridizations were carried out in fresh prehybridization buffer containing 50% deionized formamide (Sigma Chemical Co. USA) and the biotin labeled probe (5 ng/cm^2) at 30℃ overnight. The membranes were washed with 2X SSC, and 0.5% SDS at room temperature three times. Then, the membrane was treated as described in Southern-Light™ chemiluminescent detection system for detection and exposed to X-ray films (X-Omat AR, Eastman Kodak Co.) overnight at room temperature.

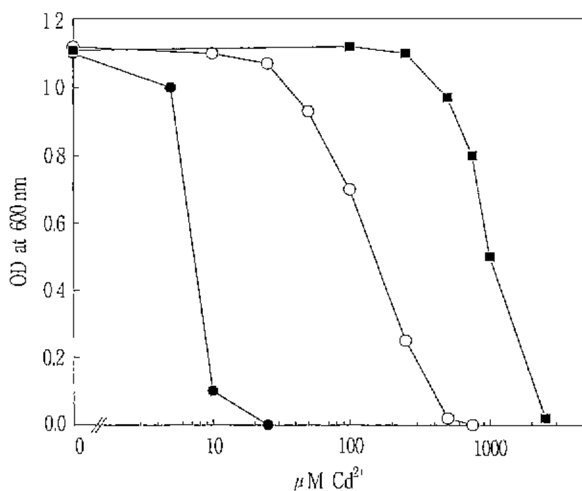

Figure 1. Growth inhibition of *S. aureus* and *B. subtilis* cells harboring different plasmids. Overnight cultures of *S. aureus* and *B. subtilis* were diluted 100-fold into fresh LB broth containing increased amount of $CdCl_2$, and grown for 12 h at 37℃. A_{600} was measured by UV-vis spectrophotometer. Symbols: ●, *B. subtilis* BD224; ○, *B. subtilis* DT134; ■, *S. aureus* pI258.

Efflux Assay

Overnight culture of both *S. aureus* pI258 and *B. subtilis* DT134 were diluted 100-fold in fresh LB medium and the diluted cultures were incubated for 3 hr and induced with 2 μM $CdCl_2$ additional 45 min at 37℃. For uninduced cells all the procedures were the same except the addition of $CdCl_2$ The induced cells (0.3 mL) were incubated with 2 μmol $^{109}CdCl_2$ for at 37℃ for 5 min, and then the cells were kept at 4℃ for 40 min to equilibrate the cells with $^{109}CdCl_2$. Loaded cells were diluted 20-fold in the prewarm LB medium containing 0.15 M sodium acetate (pH 5.5) at 37℃. The first sample was taken after 10 sec and subsequent samples were taken every 5 min. The samples (0.3 mL each) were filtered through filters (0.45 μm, nitrocellulose; Millipore) and rinsed twice with 5 mL of 20 mM $CdCl_2$ in TE buffer (10 mM Tris plus 1 mM EDTA, pH 7.5). Washed filters were counted in a liquid scintillation spectrophotometer.

Results and Discussion

Growth Inhibition

Cadmium resistance can be seen in many different kinds of microorganisms (2, 4, 5). The cadmium resistance system of *S. aureus* pI258 has been the most extensively studied (8-10, 12). Since cadmium resistance mechanism of *B. subtilis* DT134 was not fully understood yet, a series of experiments was carried out to elucidate its resistance mechanism. Growth inhibition experiments were carried out in the presence of increasing amount of $CdCl_2$ to compare the cadmium resistance of *B. subtilis* DT134 with cadmium-resistant *S. aureus* pI258 and cadmium-sensitive *B. subtilis* BD224 (Table 1). Cadmium-sensitive *B. subtilis* BD224 grew well in 5 μM $CdCl_2$ and could grow very poorly in 10 μM $CdCl_2$ But cadmium-resistant *B. subtilis* DT134 grew well in 10 μM $CdCl_2$ and could grow slowly even in 250 μM $CdCl_2$ (Figure 1) In 500 μM $CdCl_2$, the microorganism could not grow. Figure 1 shows that the *B. subtilis* DT134 is indeed about 50-fold more resistant to cadmium than cadmium-sensitive *B. subtilis* BD224. When resistances were compared between *B. subtilis* DT134 and *S. aureus* pI258, *S. aureus* pI258 could grow even in 1 mM $CdCl_2$ as expected (Figure 1). *S. aureus* pI258 is much more resistant than *B. subtilis* DT134 by about 6-fold. *S. aureus* pI258 seemed to have more efficient cadmium-removal system than *B. subtilis* DT134.

Yu (19) reported that cadmium-resistant Yeast (*Hansenula anomala* B-7) could grow in 7 mM $CdCl_2$, and You et al. (20) reported the isolation of *Azomonas agilis* PY101 resistant to 2.7 mM $CdCl_2$. These two isolates have very high cadmium resistance compared to *B. subtilis* DT134. The large difference in the degree of cadmium resistance suggested that the resistance mechanism of *B. subtilis* DT134 might be quite different from that of those microorganisms (*Hansenula*

anomala B-7, *Azomonas agilis* PY101). Yu et al. (21) recently reported that the resistance of *Hansenula anomala* B-7 might be due to the biosorption of heavy metals to the cell wall. Relatively low resistance of *B. subtilis* DT134 strongly suggested that the resistance might be due to other resistance mechanism (e.g., efflux) rather than biosorption.

Minimal Inhibitory Concentration

The lowest $CdCl_2$ concentrations that inhibited cell growth completely were determined on LB agar plates supplemented with increasing amount of $CdCl_2$ as described in Materials and Methods. Colonies of cadmium-sensitive *B. subtilis* BD224 could be seen clearly on agar plate containing 2.5 μM $CdCl_2$, but could not be observed on the agar plates containing 5 μM $CdCl_2$. Colonies of cadmium-resistant *B. subtilis* DT134 could grow well on agar plate containing 100 μM $CdCl_2$, but growth of the microorganism was not detectable on the agar plates containing 250 μM $CdCl_2$. Cadmium-resistant *S. aureus* pI258 could grow well on agar plate even containing 1 mM $CdCl_2$, but could not in 2.5 mM $CdCl_2$ as expected (Table 2). *B. subtilis* DT134 was 50-fold more resistant than *B. subtilis* BD224 and *S. aureus* pI258 was most resistant to cadmium.

These results were in accordance with results of growth inhibition in liquid media. Yoon et al. (8) reported that when *B. subtilis* BD224 was transformed with *cadA* cadmium resistance gene of *S. aureus* pI258, the transformed *B. subtilis* BD224 appeared to have the MIC increased only up to 80 μM $CdCl_2$. *B. subtilis* DT134 showed more resistant (MIC; 250 μM $CdCl_2$) than the transformed *B. subtilis* BD224 (MIC; 80 μM $CdCl_2$). It seemed that *B. subtilis* DT134's cadmium resistance gene functioned more efficiently in its natural host cells than *cadA* cadmium resistance gene of *S. aureus* pI258 in *B. subtilis* BD224 which was not natural host cells. These MIC results also showed the large difference in terms of cadmium resistance between the cadmium-resistant *B. subtilis* DT134 and other cadmium-resistant microorganisms (*Hansenula anomala* B-7 [19], *Azomonas agilis* PY101 [20]). This was another result strongly suggesting the utilization of a different resistance mechanism by cadmium-resistant *B. subtilis* DT134.

Table 2. Minimal inhibitory concentrations of cadmium against *B. subtilis* BD224, *B. subtilis* DT134, and *S. aureus* pI258.

Strains	*B. subtilis* BD224	*B. subtilis* DT134	*S. aureus* pI258
MIC[a] (μM)	5	250	2500

[a] For MIC, exponentially growing cells were diluted 100-fold and 3 μL was placed on the plates containing increasing concentration of $CdCl_2$ as described in materials and methods. Minimal concentration which did not allow cells to grow were selected as MIC.

Construction of Plasmid pKPY26

Since *B. subtilis* DT134 showed a highly elevated cadmium resistance compared with sensitive cells both in liquid media and solid media, it became interesting to compare the DNA sequence similarity of both microorganisms. Since no sequence data of cadmium resistance gene of *B. subtilis* DT134 was available, the sequence similarities of cadmium resistance genes of both microorganisms was examined by DNA-DNA hybridization. To prepare a *cadA*-specific probe for the experiment, the new construction of pKPY26 containing 757 bp *Xmn*I-*Nla*IV fragment of *S. aureus cadA* cadmium resistance gene was carried out as follows (Figure 2). pKPY20 is a derivative of pUC19 which lost *Eco*RI and

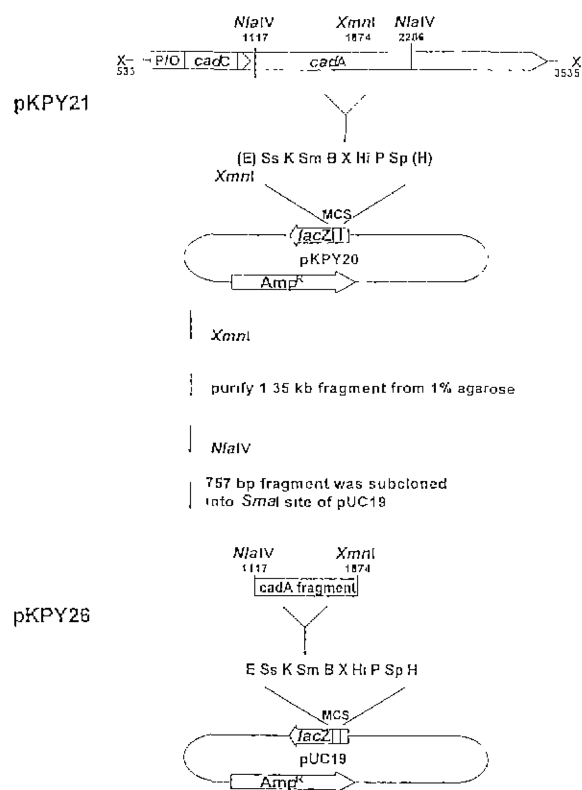

Figure 2. Construction of pKPY26. pKPY21 was cut by *Xmn*I and separated in a 1% agarose gel. A 1.35 kb fragment was purified and digested again by *Nla*IV and electrophoresed on 1% agarose gel. The 757 bp fragment was purified and subcloned into *Sma*I site of pUC19. Operator/Promoter region (P/O), and *cadC* and *cadA* genes are marked by open bars, with arrows inside indicating the direction of transcription. Thin lines flanking *cadA* and *cadC* indicate additionally cloned DNA outside of the genes. Numbering of base pair positions came from the published 3535 bp sequence (12). E, Ss, K, Sm, B, X, Hi, P, Sp, and H represent restriction nuclease sites for *Eco*RI, *Sst*I, *Kpn*I, *Sma*I, *Bam*HI, *Xba*I, *Hinc*II, *Pst*I, *Ssp*I, and *Hind*III respectively within the plasmid multicloning site. See materials and methods for the detailed construction of pKPY26.

*Hin*dIII sites in the multicloning site (Table 1; Figure 2). The *Eco*RI site of pUC19 became a *Xmn*I site after being cut with *Eco*RI, polished with Klenow enzyme, and self-religated with T4 DNA ligase. The *Hin*dIII site was lost in a parallel way (8). pKPY21 was prepared as follows. The 3.0 kb *Xba*I fragment used to make pKPY2 (8) was subcloned into the *Xba*I site of pKPY20 where *cadC* and *cadA* were in orientation opposite of transcription of *lac* promoter of pUC19 to prevent any undesired *cadA* expression in *E. coli* (8). The pKPY21 was cut by *Xmn*I and whole mixture was separated in a 1% agarose gel. A 1.35 kb fragment was purified from the 1% agarose gel and digested again by *Nla*IV. The digested products were electrophoresed on 1% agarose gel and the purified 757 bp fragment was subcloned into *Sma*I site of pUC19. The resulting construct was named as pKPY26 which has 757 bp fragment of *cadA*. (nucleotides 1117-1874 from published sequence, Nucifora et al., 1989).

Southern Blot Hybridization

Hybridization was carried out at high and low stringencies. The subcloned *cadA*-specific gene fragment was used as probe in Southern blot to detect similarities between total DNA of *B. subtilis* DT134 and *cadA* of *S. aureus* pI258. The probe hybridized well with pKPY26 (0.1 μg) restricted with *Eco*RI and *Hin*dIII under the high stringent condition and showed a signal at 757 bp position (Figure 3, lane 1), but the cadmium resistance gene of *B. subtilis* DT134 did not show any hybridization signal under the same condition (Figure 3, lane 2). However, when the stringency was lowered, the *cadA*-specific cadmium resistance gene probe was hybridized to *Eco*RI-digested total DNA of *B. subtilis* DT134 (Figure 3, lane 3). Besides smears generated by non-specific hybridization resulted from the low stringent condition, a signal could be clearly detected. The size of the most prominent signal of hybridization was 2.0kb Since the band did not show up in high stringent condition, the homology between *B. subtilis* DT134 and *S aureus* pI258 seemed to be not quite high enough. Nevertheless there must be some homology between them and strongly suggested that a probable mechanism of cadmium resistance was efflux mechanism.

Tsai et al. (9) demonstrated that cadmium efflux is energized only by ATP by the *cadA* in everted membrane vesicles of *B. subtilis*, proving the model of cadmium resistance mediated by a cadmium-transporting ATPase. Reagents that affect the proton-motive force only partially inhibited transport, whereas the Ca^{2+}/H^{+} antiporter was completely inhibited (9). This was the first report proving that CadA is P-ATPase. The *cadA*-specific probe encompasses the N-terminal half of CadA. This portion was thought to have well conserved metal binding locus and phosphatase domain of ATPase domain among many different organisms such as *L. monocytogenes*, *B. firmus*, *S. aureus*, *E. coli*, rabbit, and human (17). Since the high similarities of cadmium resistance genes present among widely different organisms, the DNA-DNA hybridization results suggested that it was quite feasible for cadmium resistance gene of *B. subtilis* DT134 to share some similarity with *cadA* of *S. aureus* pI258. Lebrun et al. (17) showed the DNA-DNA hybridization between cadmium resistance gene of *L. monocytogenes* and *cadA* of *S. aureus* pI258 and mentioned from the results of sequence analysis that there are 65.8% amino acid sequence similarity between those two investigated genes. They subsequently suggested that *L. monocytogenes* CadA could be considered as P-type ATPase since it shared many basic structural elements and regions found in different organisms, such as CadA *S. aureus*, CadA *B. firmus*, CopA *Enterococcus hirae*, KdpB *E. coli*, and Mcl Human Menkes.

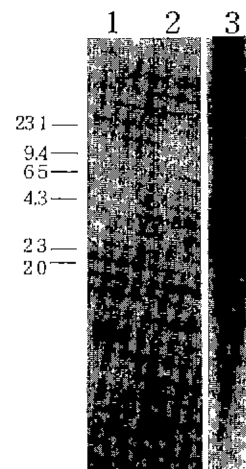

Figure 3. Southern blot analysis for cadmium resistance gene. *B. subtilis* DT134 total DNAs (5 μg/lane) were digested with *Eco*RI restriction enzymes. The fragment were separated on 0.8% agarose gel, transferred to Hybond membrane, and then hybridized with biotin-labeled probe DNA from cadmium resistance *cadA* gene fragment of *S. aureus* pI258 in high stringent condition (lane 2) and low stringent condition (lane 3) Plasmid (pKPY26) digested by *Eco*RI and *Hin*dIII was loaded in lane 1. Sizes of Lambda DNA - *Hin*dIII digest DNA M.W. markers are indicated on the left.

Choi et al. (22) screened 42 *Bacillus thuringiensis* strains by Southern hybridization with *cryI*-specific probe and identified two strains generating weak signals under low stringency hybridization conditions. Lebrun et al. (17) identified restriction fragments of *L. monocytogenes* plasmids hybridizing with an *S. aureus cadAC*-specific probe under low-stringency condition. Cadmium-resistant *B. subtilis* DT134 also showed a hybridizing signal with an *S. aureus cadA*-specific probe under low-stringency condition. Similar strategy would be employed to clone and sequence the cadmium resistance gene

of *B. subtilis* DT134. Exact sequence data of cadmium resistance determinant of *B. subtilis* DT134 would be necessary to assign a cadmium resistance gene of *B. subtilis* DT134 as P-type ATPase.

Efflux Assay

Though the exact mechanism of cadmium resistance was not known at this point, all the results shown above suggested that the resistance mechanism might be efflux mechanism. Lowered cadmium uptake by cells containing *cadA* cadmium resistance determinant had initially been shown to be the basis for resistance (7). But there was other possibility such as the presence of metal-sequestering protein which could bind to Cd^{2+} and sequester it Kim et al. (23) reported recombinant metallothionein protein could remove Cd^{2+} in *Saccharomyces cerevisiae*. Efflux experiments were carried out to further examine resistance mechanism of *B. subtilis* DT134.

To demonstrate the inducible efflux nature of cadmium transport, both *S. aureus* pI258 for a comparison and *B. subtilis* DT134 cells were filtered after 10 sec and every 5 min as described in materials and methods. When the filters were counted in a liquid scintillation spectrophotometer, the radioactivity reflected the remaining $^{109}CdCl_2$ amount still inside cells after efflux during given time. Figure 4 illustrates that when the cells were uninduced, neither *S. aureus* pI258 nor *B. subtilis* DT134 could efflux $^{109}Cd^{2+}$ out of cells even after 15 min There was no significant reduction in amount of $^{109}Cd^{2+}$ inside the cells. But when the cells were induced with 2 μM $CdCl_2$, *B. subtilis* DT134 showed that decreased amount of $^{109}Cd^{2+}$ remained inside cells. After 15 min, *B. subtilis* DT134 could efflux about one-forth of $^{109}Cd^{2+}$ out of cells. *S. aureus* pI258 could remove most $^{109}Cd^{2+}$ out of cells within 10 min. *S. aureus* pI258 showed more efficient efflux activity as expected since *S. aureus* pI258 exhibited much higher resistance to cadmium than *B. subtilis* DT134. Based on these efflux data, the cadmium resistance mechanism of the *B. subtilis* could be explained as the same inducible efflux mechanism as that of *S. aureus* pI258.

Many metals are essential for microbial growth and metabolism at low concentration (e.g., Cu, Fe, Zn, Co, Mn), whereas cadmium is a toxic and biologically inessential heavy metal. For many recent decades, its extensive use in industry such as electroplating, protection against corrosion, plastic stabilization resulted in cadmium contamination of the environment, various kinds of life forms and foods (24). Cadmium ion (Cd^{2+}) exerts its toxicity by binding to thiol groups (-SH) of proteins, which leads to the blockage of several metabolic processes in bacteria, including respiration (7). Despite this, the ability of microorganisms to survive and grow in the presence of heavy metals is a frequent phenomenon (2). Microorganisms can be resistant to heavy

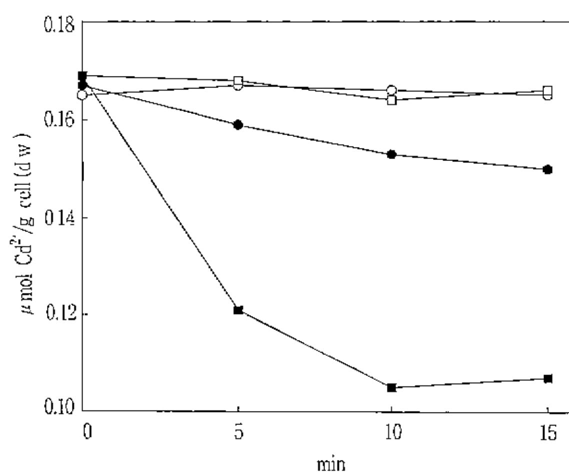

Figure 4. Cadmium resistance is due to inducible cadmium efflux. Induced and uninduced cultures were prepared as described in materials and methods. After induced with 2 μM Cd^{2+} for 45 min for induction. Cells were loaded with $^{109}CdCl_2$ as described in materials and methods. Loaded cells were diluted 20-fold by mixing LB medium and the first 0.3 mL of the sample was taken 10 sec after the dilution of the loaded cells. Subsequent samples (0.3 mL) were taken every 5 min. Washed filters were counted in a liquid scintillation spectrophotometer. Symbols: ○, uninduced *B. subtilis* DT134; ●, induced *B. subtilis* DT134; □, uninduced *S. aureus* pI258; ■, induced *S. aureus* pI258.

metals including cadmium in many ways (2). First, metal-binding protein such as metallothionein isolated from animals, yeast, algae, and fungi (26) was shown to bind and sequester cadmium ions. Second, biosorption by bacterial cell walls can also sequester heavy metal ions, resulting in heavy metal resistance (19, 27). Third, reduction in uptake rate by blocking cellular uptake pathway was demonstrated in *B. subtilis* 168 (28). Fourth, enzymatic reduction by reductase enzyme such as mercuric reductase which reduces Hg^{2+} to volatile Hg^0 was demonstrated (2, 4, 5). Fifth, highly specific efflux systems found in *S. aureus* (9, 12) and *Alcaligenes eutrophus* (3) were shown to pump out rapidly toxic heavy metals. From the efflux result, cadmium-resistant *B. subtilis* DT134 could be explained by the fifth mechanism.

In conclusion, Both Southern blot data and efflux data strongly suggested that cadmium resistance system of *B. subtilis* DT134 might belong to the P-type ATPase as CadA of *S. aureus* pI258 and the resistance was due to the lowered cadmium concentration by inducible efflux mechanism. Cloning of the gene and sequence analysis would be necessary to further elucidate its role in the P-type ATPase.

요 약

카드뮴저항성 *Bacillus subtilis* DT134와 저항성이 없는 *B.*

subtilis BD224를 다양한 농도의 $CdCl_2$를 포함하고 있는 LB 액체배지에 배양하여 성장을 비교하여 본 결과, 카드뮴저항성 *B. subtilis* DT134는 저항성이 없는 *B. subtilis* BD224보다 50배의 더 강한 카드뮴에 대한 저항성을 보였다. Solid agar에서 한 minimal inhibition concentration test에서도 카드뮴저항성 *B. subtilis* DT134는 50배의 더 큰 저항성을 보였다. *B. subtilis* DT134 의 카드뮴 저항성 유전자와 *Staphylococcus aureus* pI258의 카드뮴 저항성 유전자의 유사성을 *S. aureus* pI258의 카드뮴 저항성 *cadA* 유전자 (757 bp *Nla*IV-*Xmn*I *cadA* DNA fragment)를 probe로 사용하여 Southern blot을 한 결과 high stringent condition에선 hybridization signal이 보이지 않았으나 low stringent condition에서는 hybridization signal이 보였다. 이 실험결과로 두개의 유전자가 서로 상당한 유사성이 있다고 사료되었다. Efflux 실험을 한 결과 *B. subtilis* DT134의 카드뮴 저항성 mechanism이 *S. aureus* pI258의 카드뮴 저항성과 같은 efflux mechanism인 것으로 판명되었다.

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