Journal of Microbiology and Biotechnology
Vol. 1, No. 1, 22-30, 1991

Secretion of *Bacillus subtilis* Cytidine Deaminase by the Aid of Signal Sequences in *Escherichia coli*

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Received 7 January 1991 / Accepted 25 February 1991

In order to secrete the *Bacillus subtilis* cytidine deaminase (CDase, cytidine/2'-deoxycytidine deaminase) encoded by the *B. subtilis cdd* gene in *E. coli* by the aid of signal sequences, the *cdd* gene was fused in-frame to either *amy*E or *pen*P signal sequences and the gene expression and CDase localization were examined. For the *pen*P signal sequence::*cdd* fusion, the *cdd* gene with 9 amino acids truncated from the 5'-terminus was fused in-frame to the signal sequence, then the *cdd*$^+$ colonies were not occurred from the minimal plate by *cdd* complementation. The result suggests that 9 amino acids on the NH_2-terminal of CDase have an essential function in the enzyme activity. The hybrid protein obtained by fused gene *amy*E signal sequence::*cdd* structural gene gave *cdd*$^+$ phenotype and about half of the total CDase activity was found to be secreted in the periplasm of *E. coli* transformant JF611/pSO202. The periplasmic CDase activity of JF611 harboring pSO52 containing the intact *cdd* gene was considerablely lower than that of the cells harboring pSO202 carrying the hybrid *cdd* gene. This suggests that the CDase was secreted to the periplasm through the cytoplasmic membrane by the aid of the *amy*E signal sequence in the *E. coli* transformant.

The cytidine deaminase (cytidine/2'-deoxycytidine aminohydrolase; EC 3.5.4.5, CDase) encoded by the *cdd* gene is a salvage enzyme involved in the deamination of cytidine and 2'-deoxycytidine to uridine and 2'-deoxyuridine, respectively (21). Although the reported data of the nucleotide sequence in *Bacillus subtilis cdd* gene by Song & Neuhard (23) did not recognize the presence of any signal peptide in amino terminal region of CDase, a small amount of CDase was detected in the concentrated culture broth of *B. subtilis*. In order to demonstrate the secretion of CDase by the aid of a signal sequence, the *cdd* gene was fused in-frame to either the *amy*E (25) or *pen*P (9) signal sequences. The 41 amino acids attached to the N-terminal of extracellular α-amylase precursor were known to have a role as the signal sequence (25). The alkalophilic *Bacillus* sp. 170 β-lactamase (*pen*P) also has 30 amino acids of a signal peptide at the N-terminal (9). By using these two signal sequences, the *cdd* gene was fused in-frame downstream to the *amy*E and *pen*P.

The enzyme secretion offers several advantages (20); less degradation from proteolytic attack by intracellular protease, more proper disulfide bond formation, and simplified purification by being released into the medium through osmotic shock or passive leakage processes. For convenient manipulation of the downstream process, many kinds of foreign proteins were secreted through the cytoplasmic membrane by the aid of signal sequences.

In order to accumulate the *B. subtilis* CDase in the periplasm of *E. coli* or to secrete it into the extracellular fluid of *B. subtilis* culture, the *cdd* gene was attached to the *pen*P and *amy*E signal sequence by using restriction and multicloning sites under the condition of no exaggeration of the reading frame, and the hybrid gene expression, the CDase secretion, and localization were examined.

MATERIALS AND METHODS

Strains and Plasmids

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Key words; Protein secretion, cytidine deaminase, *cdd* gene fusion, *amy*E & *pen*P signal sequence, *Bacillus subtilis*.

Bacterial strains and plasmids used are listed in Table 1. *E. coli* JF611, a *cdd*$^-$ *pyr*$^-$ mutant, was used as the host strain. A secretion vector, pBR329ps containing a 0.2 kb fragment for the alkalophilic *Bacillus* sp. 170 β-lactamase (*pen*P) promoter and the signal sequence composed of the coding region for 30 amino acids were used along with another secretion vector, p8A-1, containing the *B. subtilis* α-amylase promoter (*amy*R_2) and the coding region for 41 amino acids as the signal sequece and pro region. The pSO52 containing the *B. subtilis cdd* gene was a subcloned plasmid obtained by minimizing the coding region from our previous study (23).

Media and Growth Conditions

E. coli strains were grown in Luria broth (15) composed of 1% tryptone, 0.5% yeast extract, and 0.5% NaCl. For enzyme assay and transformants isolation, *E. coli* strains were cultured in AB minimal medium consisting of 0.2% $(NH_4)_2SO_4$, 0.75% Na_2HPO_4, 0.3% NaH_2PO_4, 0.3% NaCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 0.003 mM $FeCl_3$, supplemented with appropriate requirements, usually 0.2% vitamin free casamino acid, thiamine (1 μg/ml), antibiotics, glucose (0.2%) or glycerol (0.2%) as a carbon source, and if needed, 1.5% agar was added to the medium. For the selection of *cdd* positive cells, deoxycytidine (40 μg/ml) and ampicillin (50 μg/ml) were added to the minimal medium (23). Most of the reagents were purchased from Takara Shuzo Co., Sigma Co. and Boehringer Mannheim.

Plasmid Isolation and Transformation

For the isolation of the plasmid, a single colony was transferred to 5 ml of L broth supplemented with appropriate antibiotics and cultured overnight at 37℃. Isolation of plasmids from the harvested cells was carried out by a modified alkaline lysis procedure (3).

The *E. coli* transformation was done by the modified method of Maniatis *et al.* (14). An overnight culture of the recipient cells in L broth was inoculated at a 2% concentration into fresh L broth and further cultured with shaking at 37℃ to OD_{550} 1.0. The cultured cells were harvested, washed, suspended with 0.1 M calcium chloride, kept at 0℃ for 20 min, and resuspended in one twentieth volume of 0.1 M calcium chloride. Plasmid DNA was mixed with the competent cells and was incubated for 30 min at 0℃, then heat shocked at 42℃ for 2 min. The cell suspensions were incubated for 1 hour at 37℃ after addition of LB medium, then plated on the selective medium. The transformants were selected by colonization with the complementation of *cdd* mutation in the host cells.

DNA Manipulations

Restriction endonuclease digestion and T4 DNA ligase reactions were performed according to the suppliers' recommendations. Partial digestion with restriction endonuclease was achieved by varying the time of digestion. Klenow filling of 5′ extentions was carried out by using 4dNTP and Klenow fragment at 37℃ for 30 min. DNA elution from the gel was performed by Geneclean kit. DNA was analyzed by agarose gel electrophoresis. The analyses of small DNA fragments were performed by polyacrylamide gel electrophoresis. Gels were stained with 0.5 μg/ml of ethidium bromide (EtBr). DNA was

Table 1. Strains and vectors used

Strains and vectors	Genotypes and properties	Source
E. coli		
JF611	*cdd*1, *pyr*E60, *thi*1, *arg*E3, *his*4, *pro*A2, *thr*1, *leu*6, *mtl*1, *xyl*5, *str*31, *sup*E44, *ara*14, *gal*K 2, *lac*Y1, *rps*L	J. Friesen
MC1061	*hsd*R, *hsd*M$^+$, *hsd*S$^+$, *ara*D139, Δ(ara-leu)7697, Δ(*lac*) λ74, *gal*U, *gal*K, *rps*L(*str*$^+$)	P. Minton
Plasmid		
pBR322	Ap^r, Tet^r	P. Valentin-Hansen
pUC18	Ap^r	Yanisch-Perron
pBR329ps	Ap^r	K. Horikoshi
p8A-1	Ap^r	S.Y. Lee
pSO52	Ap^r	B.H. Song
pSO151	Ap^r	this work
pSO152	Ap^r	〃
pSO201	Ap^r	〃
pSO202	Ap^r	〃
pBR329psMCS	Ap^r	〃
p8A-El	Ap^r	〃
p8A-ElHP	Ap^r	〃

visualized with a red filter on a polaroid camera. Restriction endonuclease, Klenow fragment and T4 DNA ligase etc. were purchased from the KOSCO Co., Takara Shuzo Co. and Sigma Co..

Subcellular Fractionation

Osmotic shock method: Osmotic shock was done as described by Heppel (7). Cells were harvested by centrifugation, washed twice with about 80 parts (w/w) of cold 0.01 M Tris-HCl, pH 8.1. Cells were suspended in 80 parts (w/w) of 20% sucrose-0.03 M Tris-HCl, pH 8, at 24℃. The suspension was treated with 1 mM EDTA-2Na. After incubation for 10 min, the mixture was centrifugated for 10 min at 11,000 rpm. The supernatant fluid was removed, and the well drained pellet was rapidly mixed with a volume of cold water equal to that of the original volume of the suspension. After 15 min, the suspension was centrifugated for 15 min at 11,000 rpm in a cold room, and the supernatant was cautiously removed. The cells were then suspended in 500 μl of 0.2 M Tris-2 mM EDTA, pH 7.5 and an aliquot was sonicated and the spheroplast CDase activity was measured. The periplasmic CDase was found in the supernatant (shock fluid) resulted from the cold water treatment.

Spheroplast formation method: The EDTA-lysozyme spheroplast formation was done according to the procedure by Birdsell and Cota-robles (2). Cells were harvested by centrifugation in a cold room, washed once with an equal volume of 0.01 M Tris-HCl (pH 8.0), and suspended in 80 parts (w/w) of 0.5 M sucrose-0.01 M Tris-Cl (pH 8.0). To prepare the spheroplasts, lysozyme was added to a concentration of 20 μg/ml; the cells were incubated at room temperature for 5 to 10 min, and diluted 1:1 with Tris buffer. For preparation of EDTA-lysozyme spheroplasts, the final EDTA concentration of 10^{-3} M was added to lysozyme spheroplast suspensions. Formation of the spheroplast was completed within 10 to 15 min. After the suspension was centrifugated for 15 min at 11,000 rpm in a cold room, the supernatant was used as source of the periplasmic CDase and the cells sonicated were used as source of the spheroplast CDase.

Cytidine Deaminase Assay

Cytidine deaminase activities were determined by the procedure of Hammer-Jespersen *et al.* (5). 100 μl of appropriately diluted enzyme and 250 μl of Tris-Mg (2.35 ml of 0.1 M Tris-Cl, pH 7.0, and 0.05 ml of 0.2 M $MgCl_2$) were mixed, and then preincubated for 2 min at 37℃. The reaction was started by adding 10 μl of 0.05 M cytidine at 37℃. At 1, 4 and 7 min, 100 μl samples were pipetted into 0.9 ml of 0.5 N perchloric acid, and then the absorbance of the reaction mixture was measured at 290 nm. One unit is defined as the amount of enzyme which will deaminate one nano mole of cytidine per min at 37℃. Protein determination was performed by the method of Lowry *et al.* (12) using bovine serum albumin as a standard.

RESULTS

β-Lactamase Signal Sequence:*cdd* Gene Fusion

Constructions of the secretion vector are shown in Fig. 1 and 3. The pSO52 carrying *B. subtilis* cdd gene was constructed originally by Song & Neuhard (23). One *Eco*RI and three *Hin*dIII sites were located in the *cdd* gene and its vicinity. The *Hin*dIII sites are located just before the stop codon, 28 nucleotides downstream from the start codon, and far upstream from the *cdd* promoter region. The pBR329ps carries the β-lactamase promoter and signal sequence. This plasmid contains one *Hin*dIII site downstream to the signal sequence. The 380 bp *Hin*dIII fragment from pSO52 containing most of the open reading frame of the *cdd* gene was inserted into the same site of the pBR329ps and designated as pSO 151 (Fig. 1a). The pSO151 carries a hybrid gene which consists of the β-lactamase promoter+signal sequence and the *cdd* gene with 9 amino acid residues deleted in the amino terminal and the stop codon.

The 690 bp *Eco*RI/*Hin*dIII fragment obtained from pSO52 by digestion with *Eco*RI prior to partial digestion with *Hin*dIII was substituted with the *Eco*RI/*Hin*dIII fragment in pBR329ps. The resulting plasmid is denoted as pSO152 (Fig. 1b) and is identical to the pSO151 except that it contains the *cdd* gene's own stop codon.

The pBR329psMCS was constructed by substitution of the *Eco*RI/*Hin*dIII fragment of pBR329ps with the multicloning sites of pUC18. The pSO52 has a unique *Kpn*I site at 29bp upstream from the *cdd* translational initiation site. Therefore, the *Eco*RI/*Kpn*I fragment of pSO52 was inserted into the same sites of pBR329ps-MCS (pSO201, Fig. 1c) by tagging the *cdd* structural gene to the β-lactamase promoter and signal sequence through the multicloning sites fitting the reading frame. However, two stop codons were generated by the multicloning sites in a linker DNA region as shown in Fig. 2.

α-Amylase Signal Sequence:*cdd* Gene Fusion

The *Eco*RI site of the p8A-1 carrying the *amy*E promoter and signal sequence was blocked by cutting, filling, and recircularizing it with Klenow fragment and T4 DNA ligase. After subsequent substitution of the *Hin*dIII/*Pst*I fragment in p8A-E1 with the same restriction fragment of pBR322, the resultant plasmid p8A-E1HP was introduced to the *Rsa*I/*Eco*RI fragments of pSO52 for inserting the intact *cdd* structural gene into the *Eco*RI/*Hin*cII sites of the plasmid and the pSO202 was constructed (Fig. 3).

To identify the correct insertion of the intact *cdd* frag-

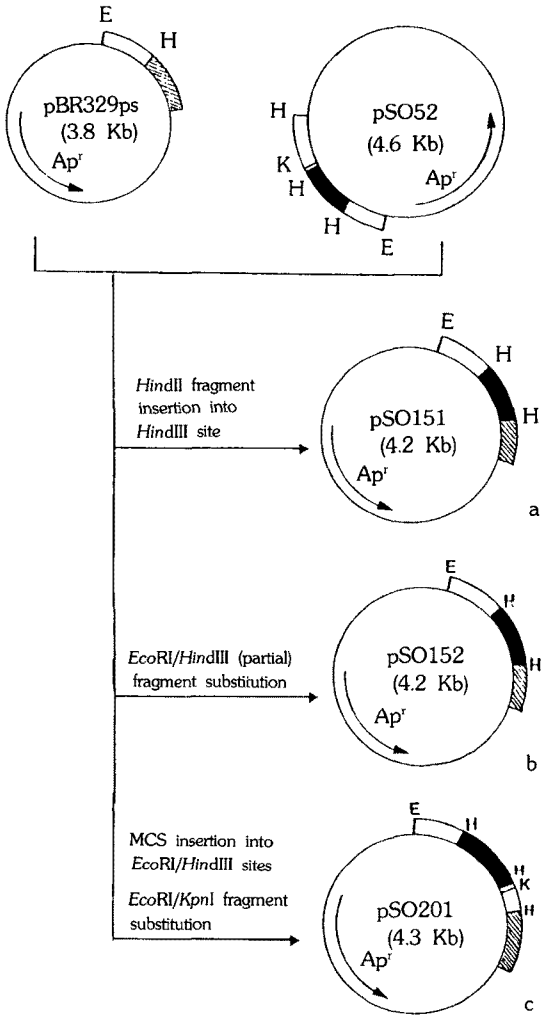

Fig. 1. Construction of hybrid plasmids of β-lactamase promoter and signal sequence::*cdd* gene.
The pSO201 carries the full sequence of *B. subtilis cdd* gene, pSO151 carries a deficient *cdd* gene with 5′ terminal 25 nucleotides and stop codon deleted, and the pSO152 carries the *cdd* gene with only the 5′ terminal 25 nucleotides deleted. The hatched bars represent *pen*P promoter and signal sequence and the filled bars represent the *cdd* gene. Restriction endonucleases are represent as follows: E; *Eco*RI, H; *Hin*dIII, and K; *Kpn*I.

ment in the resulting plasmid pSO202, as shown in Fig. 4, the inserted *Eco*RI/*Rsa*I fragment was estimated after complete double digestion with the *Eco*RI and *Rsa*I. Between the −4 or −29 position of *Rsa*I sites from the translational initiation site, if the −29 *Rsa*I site was ligated to the *Hin*cII site of p8A-E1HP, the 116 bp *Rsa*I fragment should have been generated from the gel as shown in Fig. 5. The absence of the 116 bp fragment indicated that the −4 *Rsa*I site must have been ligated to the *Hin*cII blunt end. Consequently, the α-amylase signal sequence was attached to the intact *cdd* gene in pSO202 as shown in Fig. 4. The reading frame was connected *via* 5 amino acids from the α-amylase signal sequence tail to the start of the *cdd* gene.

Expression of the *cdd* Gene with the Fused Signal Sequence

Expression of the *cdd* gene in the hybrid plasmid was determined in the transformed *E. coli* JF611. The hybrid gene expression from the resulting plasmid after signal sequence fusion was compared to the original *cdd* gene expression carrying its own promoter in pSO52. The transformed JF611 cells carrying pSO151 and pSO152 were not grown on the selective plate for *cdd*$^+$ cells. This result may indicate that the 9 amino acids at the amino terminal of the CDase are essential for the enzyme activity instead of having a function of signal peptide. However, with the pSO201 in which the *Kpn*I/*Eco*RI fragment had a full sequence of the gene attached to the *pen*P signal sequence, the CDase activity was found to be 7572 units, almost the same level as that of the pSO52 (8315 units). With the *amy*E signal sequence vector, the activity in cells carrying pSO202 was 5364 units, a little bit lower than that of the cell extract carrying pSO502 (Table 2).

Localization of *B. subtilis* CDase in *E. coli* Transformant Harboring the Hybrid Genes

The localization of the CDase in *E. coli* JF611 harboring pSO202 and pSO52 was determined. The CDase activity of *amy*R$_2$::*cdd* fusion protein in spheroplast and periplasmic fractions was 2745 (51%) and 2544 units (47%), respectively, and the total activity of whole cell extract was 5364 units as determined by osmotic shock method. This activities were not seem to vary much by the extraction methods, since the activities were almost the same level at 2674 units (50%) in the shperoplast and 2424 units (45%) in the periplasm as determined

Table 2. Expression of *B. subtilis cdd* gene in *E. coli*

Strains/Plasmids[a]	Relevant genotypes	Specific activity[b]
E. coli		
JF611/pSO52	cdd/pcdd$^+_{Bsb}$[c]	8315
JF611/pSO151	cdd/pcdd[d]	0
JF611/pSO152	cdd/pcdd	0
JF611/pSO201	cdd/pcdd$^+_{Bsb}$	7572
JF611/pSO202	cdd/pcdd$^+_{Bsb}$	5364

[a] Strains were grown in minimal medium containing 0.2% glucose, 0.2% casamino acids and 40 μg/ml deoxycytidine
[b] nmole/min/mg protein
[c] Cdd$^+_{Bsb}$ indicates the wild type *B. subtilis cdd* gene
[d] pcdd indicates plasmid containing the *cdd* gene deleted 5′ terminal 25 nucleotides

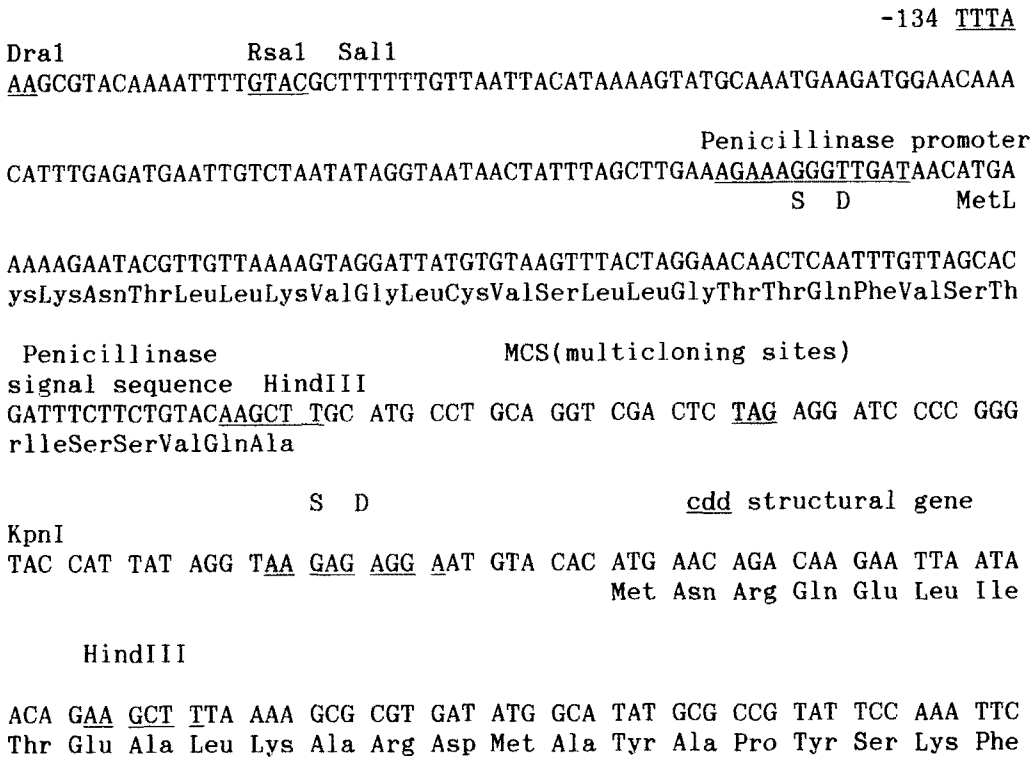

Fig. 2. Nucleotide sequence of the hybrid plasmid of β-lactamase promoter and signal sequence::*cdd* structural gene.

The 30 amino acid residues (indicated from +1 to +30) are the *penP* signal peptide. The *cdd* structural gene was tagged to the signal sequence *via* multicloning sites by fitting the reading frame, but two stop codons were generated from the linker DNA.

by spheroplast formation method (Table 3). This data may indicate that about 50% of the CDase synthesized by the *E. coli* JF611 harboring pSO202 was secreted into the periplasm by the aid of the *amy*E signal sequence, while the CDase activity in the cells harboring pSO 52 was 6619 units (80%) in the spheroplast and 1696 units (20%) in the periplasm out of the total activity of 8322 units as determined by the osmotic shock method. The activities determined by the spheroplast formation method were 4509 units (54%) and 2904 units (35%), respectively. The reason why the activity in the spheroplast fraction was reduced so much by this method is not yet known.

Localization of the CDase During the Time Course of Culture

The CDase activity in transformant JF611 harboring the pSO52 and pSO202 was measured in the spheroplast and periplasmic fractions during the time course of culture. In the early phase of the culture, the CDase activity in the periplasmic fraction was much higher that of the spheroplast one in JF611/pSO202 (Fig. 6). During 7 hrs of culture, the CDase activity was decreased gradually in the periplasm, while that in the spheroplast was increased. In addition, the time course of the activity in the periplasm of JF611/pSO52 was remained the same as that in JF611/pSO202 in the early phase of growth, whereas the patterns in the spheroplast were different from the other.

Consequently, the periplasmic CDase activity in JF611/pSO52 was almost the same level as that in JF611/

Table 3. Localization of CDase in *E. coli* JF611 haboring plasmid containing *B. subtilis cdd* gene

Strain/ Plasmids	Total activity nm/min/mg(%[a])	Spheroplast		Periplasmic	
		O.S[b]	S.F[c]	O.S	S.F
E. coli					
JF611/ pSO52	8322 (100)	6619 (80)	4509 (54)	1696 (20)	2904 (35)
JF611/ pSO202	5364 (100)	2745 (51)	2674 (50)	2544 (47)	2424 (45)

[a]relative activity

[b]The harvest cells were fractioned by the osmotic shock method

[c]The harvest cells were fractioned by the spheroplast formation method

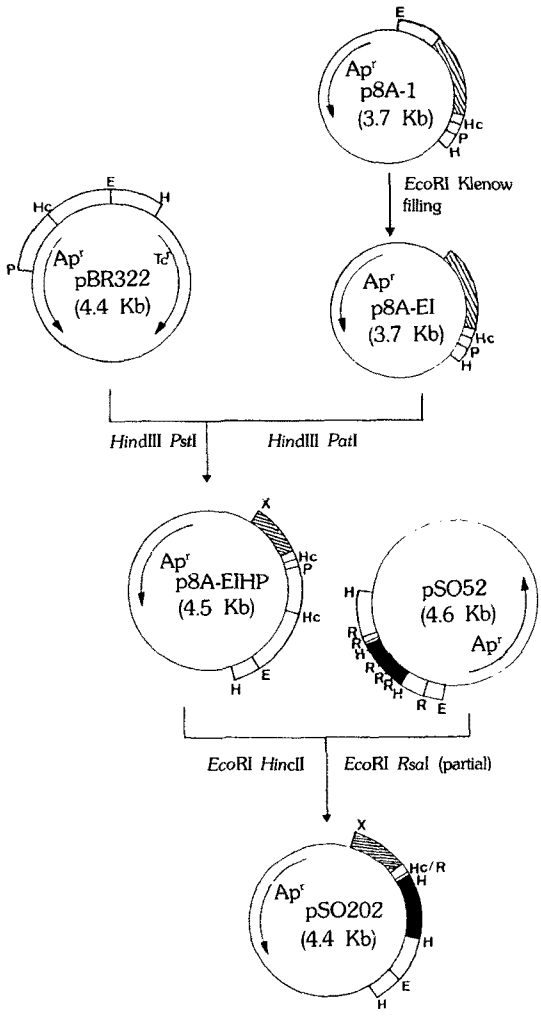

Fig. 3. Construction of the hybrid plasmids of *B. subtilis amyE* promoter and signal sequence::*cdd* structural gene.

The hatched bars represent promoter and signal sequence of the *amyE* gene and the filled bars represent *B. subtilis cdd* structural gene.

Restriction sites are as follows: E; *Eco*RI, H; *Hind*III, Hc; *Hinc*II, P; *Pst*I, R; *Rsa*I, and X; *Xba*I.

pSO202, while the spheroplast activities were far different from the each other in the early phase of growth.

DISCUSSION

For the *B. subtilis cdd* gene expression and for the secretion of *B. subtilis* CDase in *E. coli* JF611, *cdd*$^-$, *pyr* double mutant, the construction of chimeric genes by fusing the *cdd* gene in-frame to either *amyE* or *penP* signal sequences was attempted. When *E. coli* JF611 was transformed with the hybrid plasmid pSO151 and pSO152 which fused the *cdd* gene to the β-lactamase signal sequences with deletion of 9 N-terminal amino acids in both plasmids, prior to deletion of the *cdd* stop codon attached to the C-terminal or not, the *cdd*$^+$ cells were not colonized from the *cdd* complementation in the minimal plate supplemented with the deoxycytidine and ampicillin. These results suggested that the deleted fragment encoding 9 amino acids at the N-terminal might be essential for enzyme activity. This needs further study for determining whether the hybrid protein resulting from a fusion of the β-lactamase signal sequence with the N-terminal truncated CDase was secreted or not, because the CDase activity in the fusion disappeared. In our previous study, the CDase itself was secreted to the extracellular fluid at the late logarithmic phase in *B. subtilis* cells. Our prior theory was that a secretion of the CDase itself occurred with the aid of CDase's own signal sequence which attached to the amino terminal of the polypeptide. However, from these results, it could be concluded that the CDase polypeptide does not contain the signal peptide because the enzyme activity disappeared by truncating 9 N-terminal amino acids with *Hind*III site fusion. There are several ways of secretion by N-terminal signal peptide's independent mechanism, such as C-terminal signal directing export of *E. coli* hemolysin (11), processing with C-terminal cleavage export in penicillin binding protein 3 of *E. coli* (6, 17), and secretion by an unknown mechanism in the alkalophilic *Bacillus* xylanase A in *E. coli* (4, 8), etc. The CDase secretion process independent of the N-terminal signal peptide is largely empirical and not predictable with current knowledge.

In contrast, the *cdd* genes in *E. coli* JF611 harboring pSO201 and pSO202, in which the full *cdd* structural gene was fused to the signal sequences of extracellular β-lactamase and α-amylase, were expressed and revealed to have almost the same level of CDase activity as the cells harboring pSO52 carrying the intact *cdd* gene only. Although the copy number per cells carrying pSO202 originated from the pUC series was 20 folds higher than that carrying pSO201 which originated from the pBR series, the CDase activity in pSO202 was not amplified so much. The reason is that the hybrid polypeptides do not react as enzymes so much with appropriate tertiary structures like the original CDase in the folding process and another reason is that the polypeptide from pSO201 generates the intermediate two stop codons that were produced by pairing triplates from multicloning sites deduced from the sequence in fusion process, and the normal translation as a hybrid protein might be disturbed. The localization of the *amyE::cdd* hybrid protein from the transformant JF611/pSO202 was determined by osmotic shock and spheroplast formation methods. About half of the total CDase activity was recovered

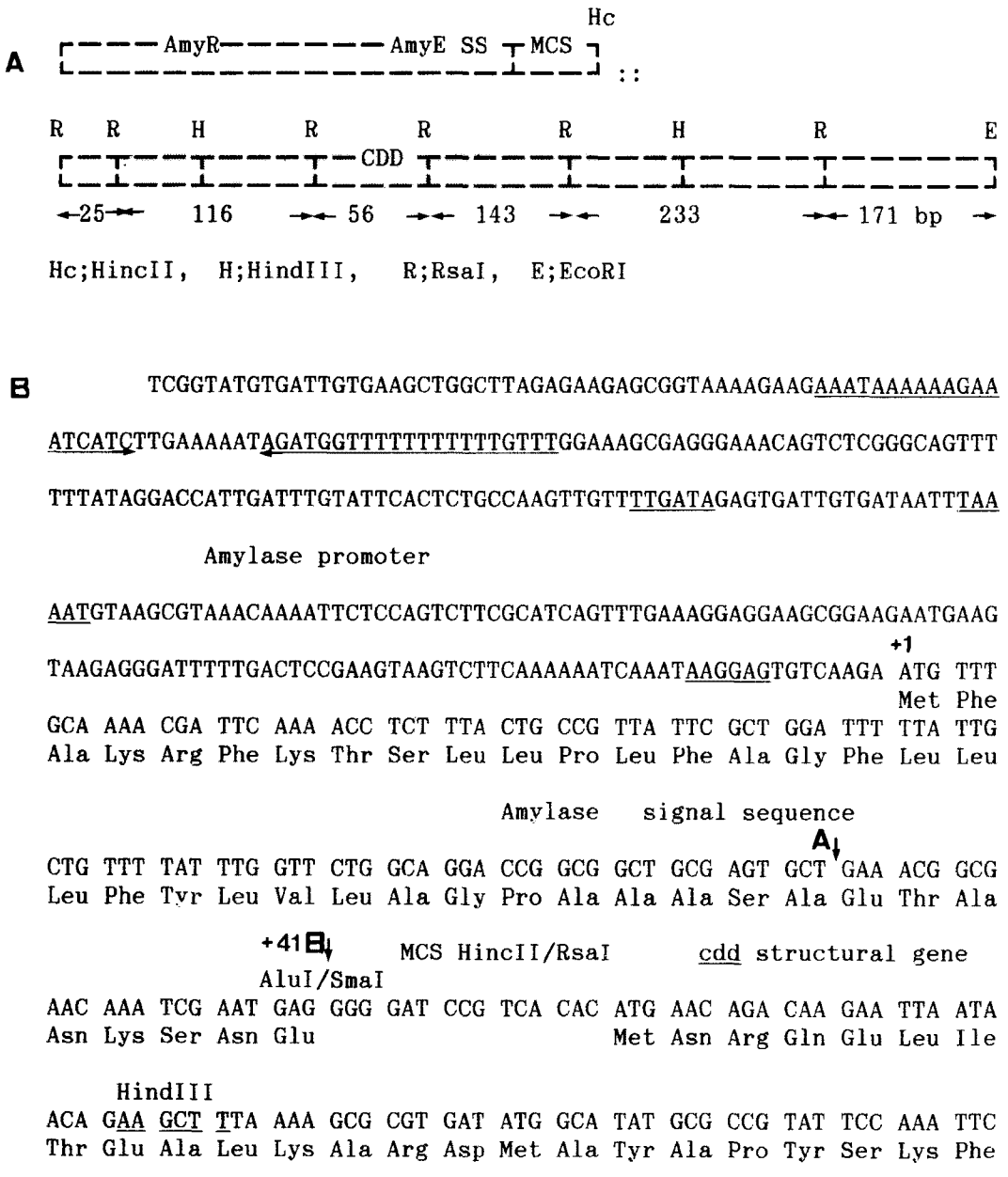

Fig. 4. Structure of the fused *B. subtilis amy*E promoter and signal sequence::*cdd* structural gene.
A: Multiple restriction endonuclease *Rsa*I sites are located in the *cdd* gene and the vicinity. The 2nd *Rsa*I site was fused in frame *via Sma*I/*Hin*cII fragment of multicloning sites to the *Alu*I site of *amy*E signal sequence and the *Rsa*I site of the upstream of the *cdd* open reading frame. B: Numbering of the nucleotides and amino acids begins at the initiator codon ATG. The *amy*E signal sequence is composed of 123 nucleotides (residues +1 to +41). The putative −35 region (TTGATA), Pribnow box (TAAAAT), and a Shine-Dalgarno sequence (AAGGAG) are indicated by solid lines. A sequence containing an inverted repeat structure upstream to *amy*E is designated by →←. The cleavage site between the signal sequence and the extracellular mature protein is indicated by the arrow labeled B, and a possible first cleavage site (25) is indicated by the arrow labeled A.

from the periplasmic fraction and the remaining part was detected from the spheroplast fraction. Nakamura *et al.* (18) reported that the *B. subtilis* α-amylase signal peptide was functional in *E. coli* cells and the hybrid proteins with *amy*E signal sequence were translocated through cytoplasmic membrane.

The CDase activity of JF611/pSO202 carrying *amy*E:*cdd* fusion fragment is at a higher level in the periplasmic fraction than in the spheroplast fraction in early growth phase, but by continuing the culture, the CDase activity

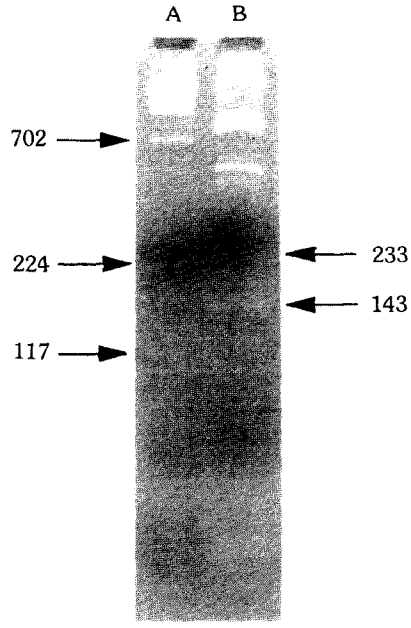

Fig. 5. Identification of the *Rsa*I fusion site in pSO202.
The pSO202 was digested completely by restriction endonulease *Rsa*I, and the fragments were identified in polyacrylamide gel electrophoresis. Lane A: λ-*Bst*EII as a marker, and B: pSO202 cut with *Rsa*I.

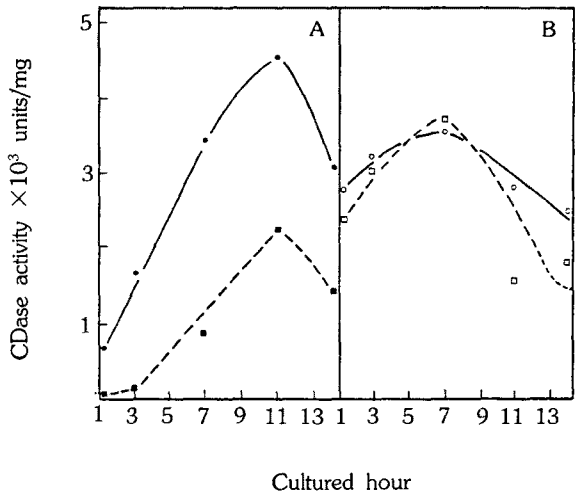

Fig. 6. Secretion of *B. subtilis* CDase in *E. coli* JF611 harboring pSO52 and pSO202.
Strains were cultured in minimal medium containing 0.2% glucose, 0.2% casamino acids and 40 μg/ml of deoxycytidine and harvested. The activity in the periplasm (B ○—○: pSO52; □—□: pSO202) and the spheroplast (A ●—●: pSO52; ■—■: pSO202) were assayed after fractionation by osmotic shock method.

in the periplasmic fraction was reduced, while that in the spheroplast fraction was increased gradually (Fig. 6). This hybrid protein which was detected in the spheroplast fraction in *E. coli* cells could be ascribed to the following reasons. Wong *et al.* (23) reported that the processing by signal peptidase and the secretion into the growth medium depend on the host strain used. The host strain JF611 used in this work is the *mtl* mutant, the product is an integral membrane protein which completely spans the membrane, participating in the PEP-dependent sugar transport and catalyzing exchange group phosphorylation (22). This may be disturb the secretion process through the *B. subtilis* membrane. And, the other reason could be the assumption that overproduction of the hybrid protein in cells carrying the pSO202 leads to the saturation of export sites resulting in a slight accumulation of the β-galactosidase precursor in the spheroplast (15).

Periplasmic proteins were released by several methods such as spheroplast formation (2, 13), osmotic shock (7, 10, 19) and chloroform treatment (1). According to the extraction method, the amount of secretory proteins was varied as if some kinds of proteins were secreted easily into the periplasmic space, while in the other extraction methods they used, the same kinds of proteins were not secreted any more. Thus, the two methods of osmotic shock and spheroplast formation were employed in this work. As shown in Table 3, the CDase activity in JF611/pSO52 was revealed to be significantly higher in the spheroplast fraction compared to that in the periplasmic space from the extract by both methods of osmotic shock or spheroplast formation. However, the activity in the strain JF611/pSO202 was less than that and was revealed to be almost the same level in both methods of extraction. In the time course of the CDase production, the spheroplast CDase activity was found to a peak after 11 hrs of culturing, while the periplasmic CDase activity was found to a peak after 7 hrs. The periplasmic activity in the strain harboring pSO202 was revealed to have a remarkably higher level than the strain harboring pSO52, while the spheroplast activity was revealed to be almost same level in both strains. This means that the CDase was secreted exclusively through the cytoplasmic membrane in strain JF611/pSO 202 by the aid of the α-amylase signal sequence which fused to the *cdd* gene in pSO202.

Acknowledgement

We thank Dr. Toshiaki Kudo and Prof. Koki Horikoshi, Institute of Physical and Chemical Research, Wako, Saitama, Japan, for providing plasmid 329ps. This work was supported by grant from the Korea Science and Technology Foundation and partly from the Ministry of Education in Korea.

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