Kor. J. Appl. Microbiol. Bioeng.
Vol. 17, No. 4, 334-342 (1989)

Molecular Cloning of *Bacillus stearothermophilus cdd* Gene Encoding Thermostable Cytidine/Deoxycytidine Deaminase

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Bacillus stearothermophilus 의 내열성 시티딘/디옥시시티딘 디아미나제를 코드하는 *cdd* 유전자의 클로닝

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The *Bacillus stearothermophilus cdd* gene encoding cytidine deaminase (cytidine/2′-deoxycytidine aminohydrolase; EC 3.5.4.5) was isolated through shot gun cloning by complementation of an *E. coli cdd* mutation. Primarily 3.0 kbp of the exogenote was cloned into the *Pst*I site of pBR322 (pJSC101). By subsequent deletion and subcloning from the insert of pJSC101 with *cdd*$^+$ and tetracycline resistancy, about 1.35 kbp of the *Eco*RI$_1$/*Pst*I$_2$ fragment containing the *cdd* gene was isolated as pJSC201. The minicell experiment revealed a molecular mass of 33,000 dalton for polypeptide from the cloned DNA fragment complementing the *cdd* gene. From the *lacZ* fusion of 550 bp fragment of the *Eco*RI$_1$/*Ava*I as a putative promoter region, the transcription direction of the *cdd* gene on pJSC201 is from *Eco*RI towards the *Pst*I sites, When the *cdd* gene was expressed in *B. subtilis* ED40 (*cdd*$^-$, *pyr*$^-$) by transformation with the *E. coli-B. subtilis* shuttle vector, the gene expression occured more efficiently than in *E. coli* and the gene appears to be stably maintained in *B. subtilis* as well as in *E. coli.*

The cytidine/deoxycytidine deaminase (cytidine/2'-deoxycytidine aminohydrolase; EC 3.5.4.5) in the pyrimidine salvage pathways in *Escherichia coli* and *Salmonella typhimurium* has been established through studies of mutant strains with single of multiple blocks in the pathways (1).

The enzyme encoded by the *cdd* gene converts cytidine and deoxycytidine to uridine and deoxyuridine, respectively. These pathways are the predominant route for cytidine utilization compared to the pathway in which cytidine is converted to cytidine monophophate directly by uridien kinase (2, 3). Pyrimidine auxotrophic *cdd* mutants of *Bacillus subtilis* are unable to grow normally on cytidine/deoxycytidine as the sole pyrimidine source; they require a uracil compound (4). The synthesis of cytidine deaminase is coregulated by unlinked *cyt*R coded repressor (5), and is highly inducible in *E. coli* (6). The molecular mass has been determined from homogenous preparation of the enzyme (7) and the promoter region was sequenced by inserting it into the *lac*Z fusion vector (8).

The *cdd* gene in *Bacillus subtilis* was studied extensively by cloning and sequencing the whole open reading frame and the promoter region (9, 10). It exists as a tetrameric enzyme and the molecular mass of the monomer was 14,837 dalton by deduction from the coding region of the *cdd* gene. The expres-

Key words: Cloning, *cdd* gene, thermostable cytidine deaminase, *Bacillus stearothemophilus,* pyrimidine salvage pathway.
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Table 1. Bacterial strains and plasmid vectors.

Strains	Genotypes	Sources
E. coli		
JF 611	*pyr*E60, *cdd*, *thi*-1, *arg*E3, *his*-4 *pyr*A2, *thr*-1, *leu*-6, *mtl*-1, *xyl*-5 *ara*-14, *gal*-K2, *lac*Y1, *str*31, λ -	J. Friesen
BD 1854	*min*A, *min*B, *thi*, *rps*L, *his*, *lac* *ton*A, *mtl*, *man*, *mal*, *xyl*	B. Diderichsen
JM 109	*rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17 *sup*E44, *rel*A1, (*lac-pro*AB)/F [*tra*D36, *pro*A$^+$, *pro*B$^+$, *lac*I^q, *lac*Z Δ M15]	
MC 1061	*hsd*R, *hsd*M$^+$, *hsd*S$^+$, *ara*D139 *rps*L (*str*^{r}), Δ(*ara-leu*) 7697 *lac*X74, *gal*U, *gal*K	
B. stearothermophilus IFO 12550	wild type	K F C C
B. subtilis ED 40	*pyr*-2, *cdd*-1, *lys*	I. Takahashi
Plasmid vectors pBR 322	Ap^r, Tet^r	
pGB215-110ΔB	Ap^r, Km^r *B. subtilis-E. coli* shuttle vector	P.M. Andreoli
pRAK 80	Ap^r promoter probe vector	J. Neuhard

sion of *cdd* is not induced by cytidine and it is coregulated by double RNA polymerase σ43 and σ29 holoenzymes (10, 11).

To compare the sequence homology with *Bacillus subtilis* especially in the promoter region, we cloned the *cdd* gene of *Bacillus stearothermophilus* encoding thermophilic cytidine deaminase, and expressed it in *E. coli* and in *B. subtilis cdd*$^-$, *pyr*E$^-$ double mutant through the insertion of the *cdd* gene to shuttle vector, pGB215-110ΔB. The molecular mass and the restriction map of the vicinity area of the open reading frame in *Bacillus stearothermophilus* may be far different from that in *B. subtilis*.

Materials and Methods

Strains

A *Bacillus stearothermophilus* was used as a donor strain of the *cdd* gene. *Escherichia coli* JF611 as a strain of *cdd*$^-$, *pyr*$^-$ was used as a cloning host. *B. subtilis* ED40 was used as a recipient host of the *Escherichia-Bacillus* shuttle vector for *cdd* expression. *E. coli* K12 derivatives with genotypes as well as plasmid vectors used are listed in Table 1.

Media and reagents

Usually Luria broth (12) was used for bacterial growth. For enzyme assay, *E. coli* was cultured in AB medium (13) and *B. subtilis* in MG1 and MG2 (14) supplemented with 5 μM $MnSO_4$, 0.2% L-glutamate and 0.2% glycerol was employed. Most of the minimal medium were supplemented with appropriate requirements,antibiotics and glucose (0.2%) or glycerol (0.2%) as a carbon source. For the selection of *cdd* positive cells, cytidine and deoxycytidine (40 μg/ml) and antibiotics (ampicillin 50 μg/ml, tetracycline 15 μg/ml, and kanamycin 10 μg/ml) were added to the minimal medium. When required, 0.2% vitamin free casamino acid was added to the minimal medium. Most of the reagents were purchased from Takara Shuzo Co., Sigma Co., and Boehringer Mannheim.

DNA techniques

All of restriction endonuclease digestions and T4 DNA ligase reactions were performed according to the manufactures specifications. For the isolations of the plasmid in μg quantities from *E. coli* transformants, single colonies were transferred to 5 ml of L-

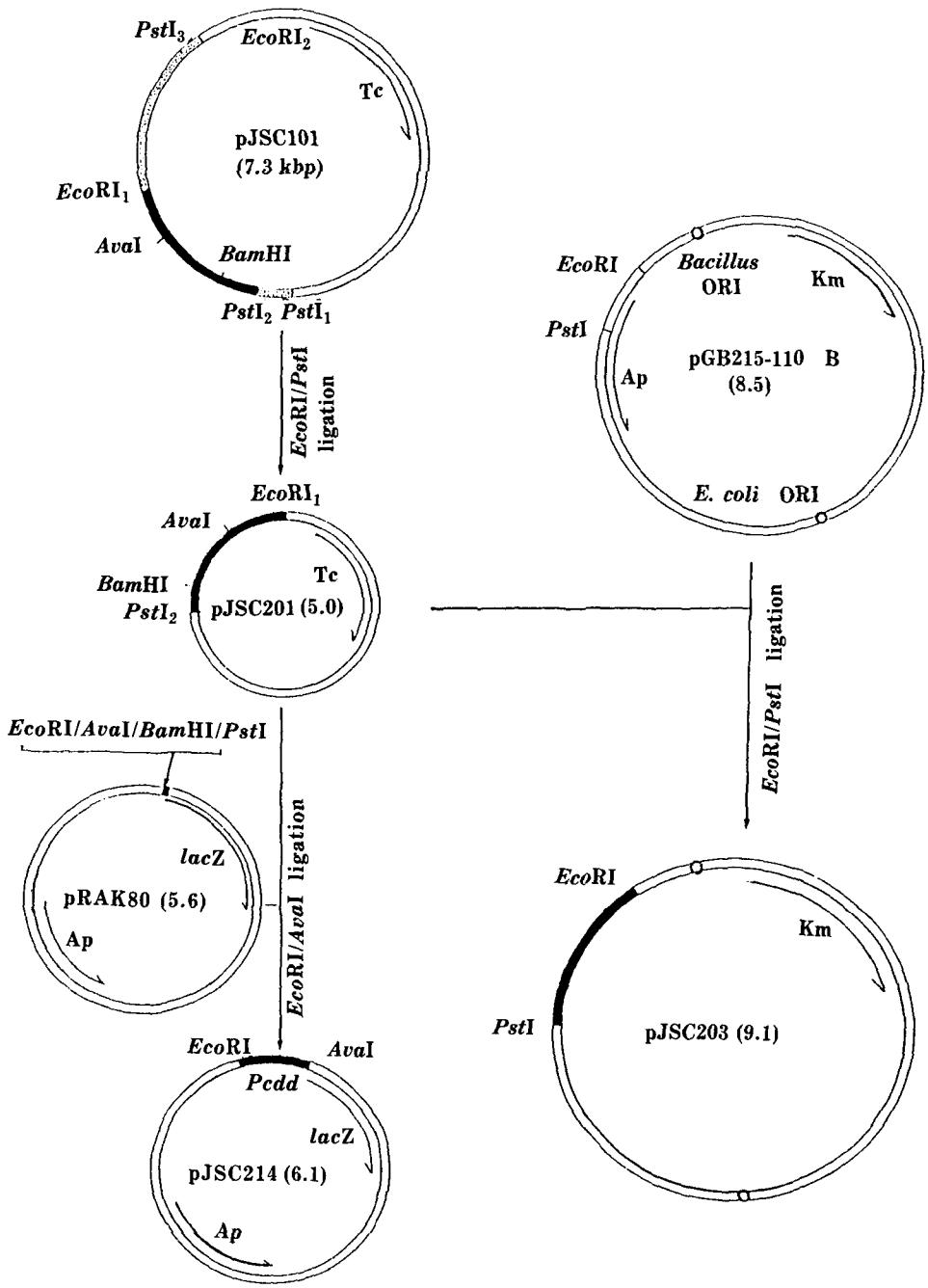

Fig. 1. Schematic diagram showing the construction of plasmids.

Open segments represent pBR322, shuttle vector pGB215-110ΔB and pRAK80 DNA. Filled bars represent the inserted exogenote containing *B. stearothermophilus cdd* gene. Dotted bars represent the inserted DNA fragment as a vicinity area of the exogenote. Arrows indicate the reading direction of ampicillin (Ap), tetracycline (Tc), kanamycin (Km) resistance gene and *lac*Z. The pJSC201 was derived from pJSC101 by *Pst*I$_2$/ *Eco*RI$_1$ insertion to the pBR322 after deletion of *Pst*I$_2$/*Pst*I$_1$ and *Eco*RI$_1$/*Pst*I$_3$ fragments by selecting *cdd*$^+$ and tetracycline resistance. The pJSC203 was constructed by *Pst*I$_1$/*Eco*RI$_2$ insertion to the shuttle vector and pJSC214 by *Ava*I/*Eco*RI$_1$ insertion into the polylinker cloning sites of pRAK80.

broth supplemented with antibiotics and cultured overnight at 37°C. Isolation of plasmids from the harvested cells was followed by a modified alkaline lysis procedure (15). The 3-10 kbp of *Pst*I fragments of *B. stearothermophilus* chromosomal DNA were isolated by the electroelusion with DEAE ion exchanger paper (Bio-Rad Co.) from 0.8% agarose gel. The isolated fragments were supplied to the shotgun cloning with pBR322. When the fragment containing *cdd* gene was transferred to *B. subtilis,* shuttle vector pGB215-110ΔB was used (16). The isolated fragment containing the *cdd* gene from pJSC201 by double digestion with *Eco*RI and *Pst*I was inserted into the corresponding sites of the shuttle vector (Fig.1), and transferred into the *cdd*⁻ mutant of *B. subtilis* ED40. The transformants were isolated by colonization on the AB minimal plate supplemented with cytidine as a sole pyrimidine source and vitamin free casamine acids. *E. coli* was made competent for transformation as described by Maniatis (17).

Restriction endonuclease, RNase, alkaline phosphatase, proteinase K, and T4 DNA ligase *etc.* were purchased from the KOSCO Co., Takara shuzo Co., and Sigma Co. Radioisotopes were obtained from Amersham Co.

Plasmid coded protein detection

For the determination of the molecular weight of the *cdd* gene product, the plasmid carrying *cdd* gene was transformed into the minicell strain, *E. coli* BD1854 which is defective in cell division, but at some frequency cell division occurs asymmetrically which results in some not carrying chromosome but being plasmids only containing the *cdd* sturctural gene. Thus only the plasmid encoded proteins will be synthesized. The minicell containing the *cdd* gene was labeled with (^{35}S)-methionine for 1 hr after preincubation for 1.5 hr, and then the synthesized *cdd* gene product was extracted by heating at 90°C for 10 min in buffer containing SDS and 2-mercaptoethanol. The cytidine deaminase polypeptide was detected by autoradiography after 12.5% SDS-polyacrylamide gel electrophoresis (18).

Enzyme activity assay

Crude cell extracts prepared from sonic disruptions were used as an enzyme source. Cytidine deaminase activities were determined by the procedure of Hammer-Jespersen *et al* (19). One unit is defined as the amount of enzyme which will deaminate 1 μmole of cytidine/min at 60°C. Protein determination was performed by the method of Lowry *et al* (20) using bovine serum albumin as a standard.

Results

Cloning of the *cdd* gene into pBR322

3-10 kbp *Pst*I fragments of *Bacillus stearothermophilus* chromosomal DNA were cloned into the plasmid vector pBR322, and *cdd*⁺ transformants were selected by shotgun complementation of *cdd* mutation of *E. coli* JF611. Three *cdd*⁺ transformants were selected by tetracycline resistance and *cdd*⁺ in the minimal medium supplemented with tetracycline and cytidine as a sole pyrimidine source.

From one of the three transformants, named *E. coli* KM601, the pJSC101 was extracted as a hybrid plasmid. The pJSC101 contains a 3.0 kb of passenger DNA fragment containing the *cdd* gene in the pBR322. After restriction mapping of the inserted fragment as shown in Fig. 1 & 3, subsequent deletion and religation were continued for reducing the size of hybrid plasmid by searching for the location of the *cdd* gene in the insert.

Subcloning of *cdd* gene

To localize *cdd* gene, deletion and complemenation were continued. The plasmid pJSC201, derived from pJSC101 as shown in Fig. 2, coded active cytidine deaminase. However, the plasmid pJSC200 containing *Eco*RI$_1$/*Pst*I$_3$ fragment of the pJSC101 did not express the *cdd* activity. As shown in Fig. 2, another plasmid pJSC208, composed of pUC18 vector and the *Bam*HI/*Eco*RI fragment did not express the *cdd* gene. And, the pJSC214 formed by inserting 550 bps fragments of *Ava*I/*Eco*RI$_1$ into the polylinker cloning sites of pRAK80 was not colonized on the AB minimal plate supplemented with cytidine. These results suggest that the *cdd* gene expression requires the complete fragment of the *Pst*I$_2$/*Eco*RI$_1$ sites in pJSC201 as shown in Fig. 2. By subsequent deletion of *Pst*I$_2$/*Bam*HI from pJSC201, the enzyme activity disappeared. This suggests that at least the *Pst*I$_2$/*Bam*HI fragment of pJSC201 is needed for the expression of *cdd* essentially, and also that it requires 550 bps of the *Ava*I/*Eco*RI$_1$ fragment in the pJSC201 because it has a promoter activity in the

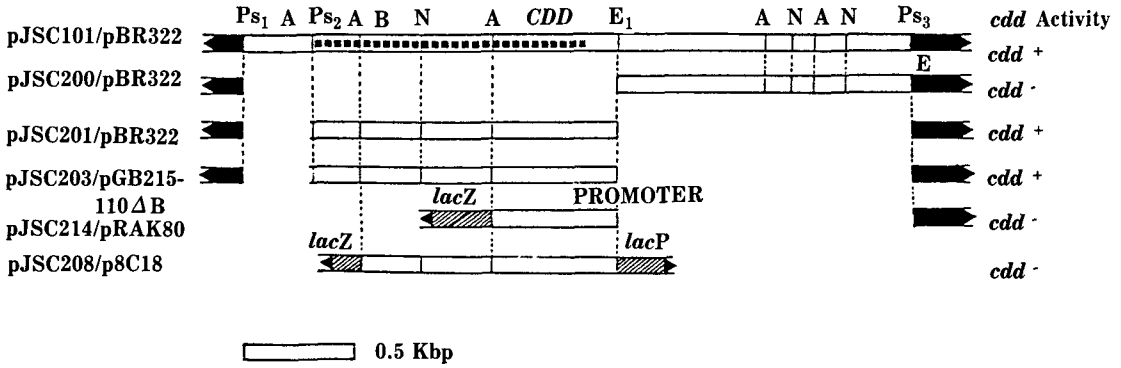

Fig. 2. Identification of *B. stearothermophilus cdd* gene location by subcloning.
The hatched bars represent pRAK80 and pUC18 DNA. Filled bars represent the pBR322 and pGB215-110ΔB DNA and open bars represent the inserts. The arrows indicate the direction of transcription; *lac*P is the *lac* promoter on pUC18. Each plasmid was tested for complementation of an *Escherichia coli cdd* mutation and the results are indicated on the right. a) indicates the designated plasmids/cloning host plasmids. Abbreviations; Ps, *Pst*I; A, *Ava*I; B. *Bam*HI; N, *Nru*I; E, *Eco*RI.

Table 2. Expression of *B. stearothermophilus cdd* gene in *E. coli* and *B. subtilis*[a].

Strains/Plasmids		Relevant Genotypes[b]	Specific Activity (unit) nm/min/mg protein
E. coli	MC 1061	*cdd*$^+$ (wild type)	46
	JF 611/pJSC101	*cdd*/*pcdd*$^+_{Bst}$	1188
	JF 611/pJSC201	*cdd*/*pcdd*$^+_{Bst}$	1212
	JF 611/pJSC203	*cdd*/*pcdd*$^+_{Bst}$	926
B. subtilis ED 40/pJSC203		*cdd*/*pcdd*$^+_{Bst}$	1424
B. stearothermophilus	IFO 12550	*cdd*$^+$ (wild type)	56

[a] Strains were grown in minimal medium containing 0.2% glycerol, 0.2% casamino acids and 40 μg/ml cytidine
[b] *cdd*$^+_{Bst}$ indicates the wild type *B. stearothermophilus cdd* gene

direction from *Eco*RI$_1$ to *Ava*I sites in the pRAK80.

Subcloning of the *cdd* gene into pUC plasmids

The *Pst*I$_2$/*Eco*RI$_1$ fragment of pJSC201 was transferred into the corresponding restriction sites of the pUC18 and pUC19 which have polylinker cloning sites in between the promoter and structural gene of *lacZ*. From the hybrid plasmids pJSC204 and pJSC205 carrying the *Eco*RI$_1$*Pst*I$_2$ fragment in opposite orientation, the cytidine deaminase activity in the transformed *cdd*$^+$ cells was compared. The pJSC204 carrying the *Eco*RI$_1$*Pst*I$_2$ direction had much higher levels of the enzyme activity compared to the reverse direction (Data not shown). To confirm the direction of the reading frame, the putative promoter region of the *Eco*RI$_1$*Ava*I fragment was also cloned into same site of the pRAK80 polylinker sites, the *lacZ* vector deleted its own promoter. Then, the colonies carrying the hybrid vector containing the *cdd* promoter with right orientation and correct reading frame with the *lacZ* sturctural gene appeared as blue colonies. Many blue colonies appeared in the LB plate supplemented with X-gel and ampicillin, and the hybrid vector containing the fragment with *Eco*RI$_1$*Ava*I direction was isolated from the blue colonies. These results suggest that the *cdd* promoter may be contained in the 550 bp fragment of *Eco*RI$_1$/*Ava*I and the reading orentation of it may occur from *Eco*RI$_1$ towards *Ava*I.

Expression of the cloned *cdd* gene in *E. coli* and *B. subtilis*

The specific activity of the cytidine deaminase was determined from the crude extracts of strain carry-

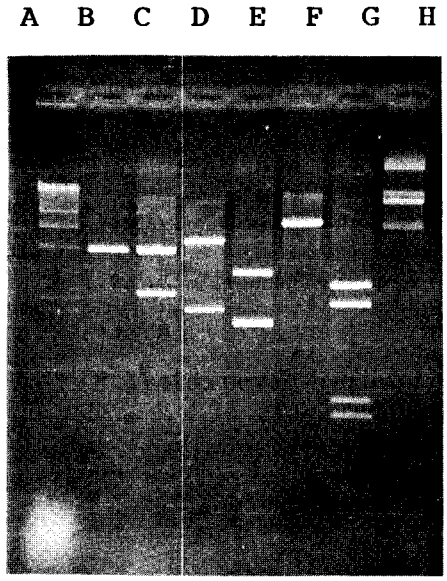

Fig. 3. Restriction patterns of pJSC101.

A; λ -*Hin*dIII
B; pBR322, *Pst*I
C; pJSC101, *Pst*I
D; pJSC101, *Eco*RI
E; pJSC101, *Nru*I
F; pJSC101, *Hin*dIII
G; pJSC101, *Ava*I
H; pJSC101, *Pvu*I

ing *B. stearothermophilus cdd* gene either in a single copy on the chromosomal DNA or in multicopy on various plasmids. As a reference the level of cytidine deaminase in *E. coli* MC1061 and native *B. stearothermophilus* IFO12550 are given as a single copy on the chromosomal DNA.

From the results of Table 2, it can be seen that a single copy of the *B. stearothermophilus cdd* gene is expressed at the same level as the wild type of *E. coli* MC1061. The enzyme acitivity was revealed to be about 20 times more levels in the cell extracts carrying the *cdd* gene in multicopy plasmids compared to the single copy in wild type chromosome. The *B. stearothermophilus cdd* gene carried in the *B. subtilis-E. coli* shuttle vector was expressed at a somewhat higher level in *B. subtilis* cells than in *E. coli*.

Optimal temperature and the thermostability of the cytidine deaminase

To confirm the gene originated from *B. stearothermophilus,* the optimal temperature and thermal stability of the cytidine deaminase were de-

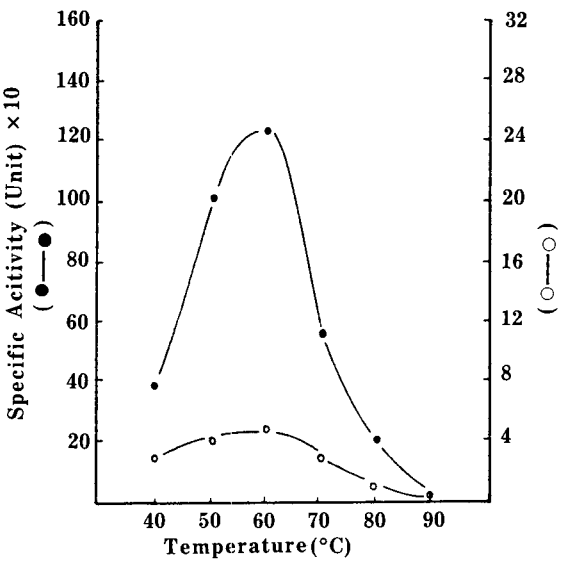

Fig. 4. Optimal temperature for *B. stearothermophilus* cytidine deaminase.

Cytidine deaminase from the sonic extract of *B. stearothermophilus* wild type (○----○) and *B. subtilis* ED40 (●----●) carrying the plasmid pJSC203 were assayed. Cells were recovered at OD.1.0. after culturing in Spizizen minimal medium supplemented with cytidine and kanamycin at 37°C and then the cytidine deaminase acitvity was assayed.

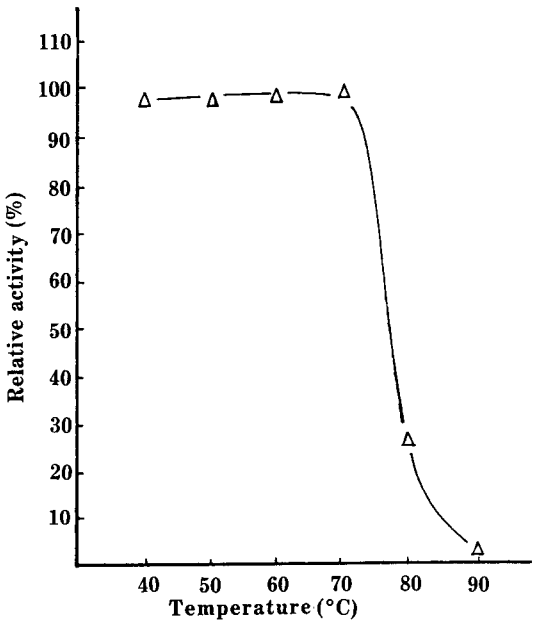

Fig. 5. Thermostability of *B. Stearothermophilus* cytidine deaminase.

The sonic extract of *B. subtilis* ED40/pJSC203 was treated at various temperatures for 20 min after suspending the cells in 0.1 M Tris-Cl (pH 7.5) containing 2 mM EDTA, the remaining cytidine deaminase activity was assayed at 60°C.

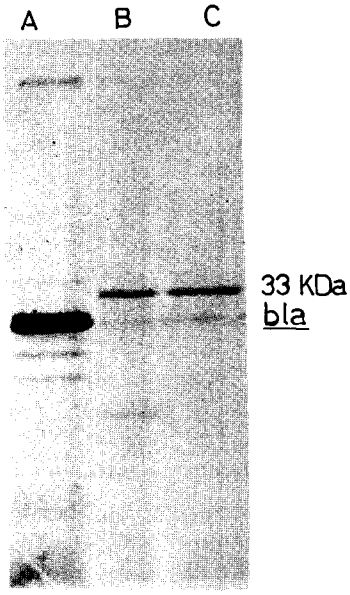

Fig. 6. Identification of the polypeptide encoded by the *cdd* gene.

^{35}S-Methionine labelled polypeptides from the extracts of minicells harboring the plasmid pJSC101 and pJSC201 were analysed in 12.5% SDS-polyacrylamide gel electrophoresis and then autoradiographed. *bla* indicates the plasmid encoded β-lactamase. Lane A, pBR322, B, pJSC101, C, pJSC201.

terminded. Fig. 4 shows that the optimal temperature was revealed to be 60°C. The cytidine deaminase acitvity was decreased about 20% and 55% by carrying out the reaction 10 degrees below and above the optimal temperature, respectively.

Sonic extracts of *B. subtilis* ED40/pJSC203 cells were treated for 20 min at various temperature for determining the thermal stability after suspending the cells in 0.1 M Tris-Cl (pH 7.5) containing 2 mM EDTA, and then the remaining enzyme activity was determined at 60°C. Fig. 5 shows that the enzyme was reasonably stable at high temperatures, because there was no loss of the activity observed by heating for 20 min below 70°C. However, the activity was reduced markedly below the original activity by treatment above 70°C. Combined data with the southern hybrization (unpublished data), the result means that the cloned *cdd* gene may originate from the *B. stearothermophilus* chromosome.

Molecular mass determination from the minicell experiment

In order to determine the molecular mass of the *cdd* gene product, plasmids pJSC101, 201, and pBR322 as a control, were transformed into the minicell of *E. coli* BD1854, and ^{35}S-methionine labelled proteins synthesized by the minicells were analyzed by autoradiography after SDS-polyacrylamide gel electrophoresis. As shown in Fig. 6, one polypeptide with a molecular mass of about 33,000 Da appeared specifically in cells harboring the *cdd* complementing plasmid pJSC101, and 201. This polypeptide is assumed to be the cytidine deaminase subunit encoded by *cdd*. One more extraband of 22,000 Da in molecular mass appeared in pJSC101, but it didn't exist in pJSC201. This means that the band may be another polypeptide coded by the gene located in the *Eco*RI$_1$/*Pst*I$_3$ fragment of pJSC101. The molecular weight of the native form in gel filteration has not been determined yet. Therefore, it has not been clarified yet if the cytidine deaminase was composed of multimer or not.

Discussion

A *cdd* gene of *B. stearothermophilus* encoding thermostable cytidine deaminase was cloned into the high copy number plasmid pBR322. About 3 kbp *Pst*I fragment of *B. stearothermophilus* chromosomal DNA was cloned into the pBR322 by shot gun selection of *cdd*$^+$ colonies. After clarifying the restriction sites in 3.0 kbp of the original insert combination with pBR322, subsequent deletion and subcloning from the pJSC101 were continued.

The pJSC201 was derived from the pJSC101 by *Pst*I$_2$/*Eco*RI$_1$ sites ligation to the pBR322 as a *cdd*$^+$ cells selection and tetracycline resistance. Further deletion was tried, but in any further deletion from this pJSC201 the *cdd* gene expression was blocked.

Considering the results of the minicell experiment, the coding region of *cdd* may be composed of 900 bps minimally. From these two experiments, it is suggested that the *B. stearothermophilus cdd* gene containing its promoter and structural gene region may be located from the *Eco*RI$_1$ to the *Pst*I$_2$ sites.

By mean of directional switch of the *Eco*RI$_1$/*Pst*I$_2$ orientation in pUC18, the *cdd* activity was increased several times above levels of that reverse one in pUC19. The reason why the activity in the reverse direction was reduced is that in the right

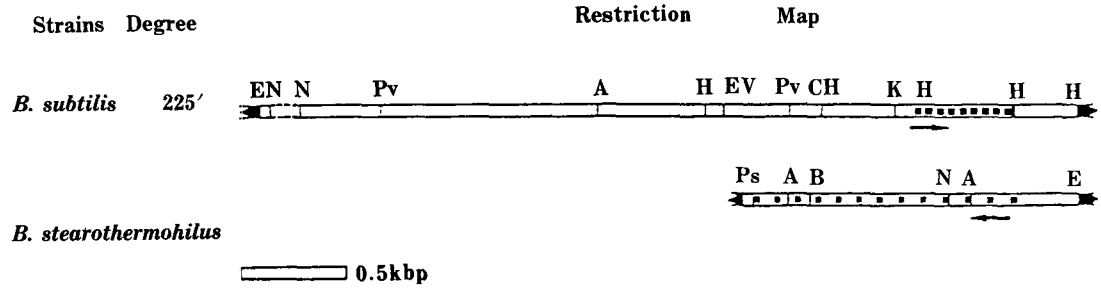

Fig. 7. Comparisons of *B. Stearothermophilus* and *B. subtilis* in restriction map and vicinity area of *cdd* gene. Abbreviations; E, *Eco*RI; A, *Ava*I; H, *Hin*dIII; C, *Cla*I, K, *Kpn*I; P, *Pst*I.

orientation in *Eco*RI$_1$/*Pst*I$_2$ direction, the *cdd* gene expression may occur by the aid of the *lacZ* promoter.

In the minicell experiment, the cytidine deaminase expressed a molecular mass of 33,000 Da. The native molecular mass from gel filteration or sucrose density gradient centrifugation has not been determined yet, however, the polypeptide of 33,000 Da in molecular mass may act as the cytidine deaminase itself or act as a subunit of the enzyme in *B. stearothermophilus*. The molecular mass is very similar to the 33,000 Da or 35,000 Da calculated from gel electrophoresis of the homogeneous preparation of the subunit from cytidine deaminase of *E. coli*. However, it is far different from that in *B. subtilis* which revealed a tetrameric enzyme composed of a 14,837 Da monomer (9, 10).

In the comparison of restriction sites in the vicinity area of the *cdd* gene (Fig. 7) and of the molecular mass in minicell experiment (10), the *B. stearothermophilus cdd* gene was revealed to be a different pattern from the *B. subtilis* one.

Acknowledgment

We would like to express our gratitude to Prof. Jan Neuhard, Institute of Biological Chemistry B, Copenhagen University, Denmark, for his kind gifts of mutant strains and vectors. This work was supported by grants from the Korea Science and Engineering foundation and by a scholor stipendium to Chang, Jong-Soo from the Foundation.

요 약

*Bacillus stearothermophilus*의 cytidine deaminase (cytidine/2′-deoxycytidine aminohydrolase ; EC 3.5.4.5)를 코딩하는 *cdd* 유전자를 *E. coli cdd*$^-$ 결손변이주를 cloning host로 하여 3-10 Kbp의 *B. stearothermophilus* DNA 단편으로부터 shot gun 법으로 클로닝하였다. 고 복제수 플라스미드 pBR 322의 *Pst*I 부위에 3.0 Kb의 *B. stearothermophilus* DNA 단편을 함유한 pJSC 101이 *cdd*$^+$와 tetracycline 내성으로서 cloning되었으며, 이어서, 결실 및 subcloning을 연속 수행한 결과 약 1.35 Kbp의 *Eco* RI$_1$/*Pst* I$_2$ 단편이 동일 부위의 pBR 322에 삽입된 *cdd* 양성의 pJSC 201을 얻었다. Mini 세포 실험결과, 이 단편에서 합성되는 polypeptide는 약 33 KDa이었기에 이 polypeptide가 cytidine deaminase로 추정되었다. 또한 이 단편에 함유한 550 bp의 *Eco* RI/*Ava* I 부분을 *lacZ* 프로모터 영역에 삽입한 경우 프로모터 활성을 나타내었기에 이 단편의 *Eco* RI 부위에서 *Pst* I 부위로 *cdd* 유전자가 전사됨을 알 수 있었다.

*B. subilis*와 *E. coli*에서 발현이 가능한 shuttle vector에 *cdd*가 함유된 단편을 삽입한 후 이를 양세포에서 동시 발현시켰을 때 *B. subtilis*에서 발현시킨 경우가 *E. coli*에서 보다 높은 cytidine deaminase 활성을 나타내었으며 이 유전자는 *B. subtilis*에서도 *E.coli*에서와 같이 안정하게 유지됨을 알 수 있었다.

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(Received June 8, 1989)