Kor. J. Appl. Microbiol. Biotech.
Vol. 18, No. 6, 640-646 (1990)

Escherichia coli 의 시티딘/디옥시시티딘 디아미나제를 코드하는 cdd 유전자의 클로닝

권택규 · 김태호 · 황선갑 · 김종국 · 송방호[1] · 홍순덕*

경북대학교 자연과학대학 미생물학과, [1]사범대학 생물교육과

Molecular Cloning of *Escherichia coli cdd* Gene Encoding Cytidine/Deoxycytidine Deaminase

Kwon, Taeg-Kyu, Tae-Ho Kim, Seon-Kap Hwang, Jong-Guk Kim, Bang-Ho Song[1] and Soon-Duck Hong*

Department of Microbiology, College of Natural Sciences,
[1]Department of Biology, Teachers College, Kyung-Pook University, Taegu 702-701, Korea

We have cloned the *cdd* gene from *E. coli* C600 using (*cdd*⁻) as a host. From the sequenced promoter region of *E. coli cdd* gene which has been determined by Valentin-Hansen P. (1985), we synthesized the 23 mer oligonucleotides corresponding to the transcription initiation region and used as a probe for cloning of the *cdd* gene by Southern blotting. The isolated fragments in the blotting were introduced to the colony hybridization after transforming it into the *E. coli* JF611 (*cdd*⁻, *pyr*⁻ double mutant), and we identified the hybridized band at 27 kb long. From the original insert of 27 kb fragment in the *Bam*HI site of pBR322, the 5.3 kb fragment containing the *cdd* gene was isolated by subsequent deletion and subcloning. From the derived plasmid pTK509, further deletion and subcloning were performed and clarified that the *cdd* gene was located in the 2.1 kb of *SalI*/*DraI* segment in the insert of pTK605. The polypeptide encoded by the cloned DNA was appeared to be a molecular mass of 33,000.

Cytidine deaminase (cytidine/2'-deoxycytidine aminohydrolase EC. 3.5.4.5) catalyzes the deamination of cytidine and 2'-deoxycytidine to uridine and 2'-deoxyuridine, respectively (1). The enzyme is widely distributed in microorganisms with the exception of *Pseudomonase acidovorans* and *Neisseria meningitidis* (2). This deamination pathway is the predominant route compared to the converting route of cytidine directly to cytidine monophosphate by uridine kinase (3). Therefore, a mutant defective in *pyr*G coding CTP synthetase can not grow on cytidine unless it contains a *cdd* mutation. Pyrimidine auxotrophic *cdd* mutants are able to grow on cytidine as a sole pyrimidine source. This enables *Escherichia coli* to grow rapidly with cytidine, although the growth rate is somewhat reduced. This residual growth is abolished by mutations inactivating either cytosine deaminase (*cod*A) or cytidine kinase (*udk*). However, *E. coli* and *Salmonella typhimurium* are devoid of deoxycytidine kinase activity, and they are unable to convert deoxycytidine to cytosine. Therefore, deoxycytidine instead of cytidine was used as a sole pyrimidine source for the selection of *cdd* complementing colonies of *E. coli*.

As one of the most extensively studied *cdd* genes, *Bacillus subtilis cdd* gene was cloned and characterized by Song and Neuhard (4), and its enzymatic properties were also studied (5). The *cdd* gene of *Bacillus stearothermophilus* was cloned in our previous studies (6).

The cytidine deaminase of *E. coli* was purified homogeneously and enzymatic properties were charac-

Key words: *cdd* gene, cytidine/deoxycytidine deaminase.
*Corresponding author

terized extensively (7). And the *cdd* gene of *E. coli* was also cloned, and sequenced in the promoter region only by Valentin-Hansen (8). The gene was mapped at 45 min in the *E. coli* chromosomal map (9). The expression of the *cdd* gene encoding cytidine deaminase is controlled by two regulatory proteins. One is a repressor coded by the *cyt*R gene, and the other is the cyclic AMP-binding protein. In the presense of cAMP the latter acts as a positive controlling element (10). The synthesis of the enzyme in *E. coli* is inducible, whereas that in *B. subtilis* is not.

For elucidating the structural gene, regulating this gene's expression, and producing a high level of the enzyme, the *E. coli cdd* gene was cloned and the gene product was characterized in *E. coli* mini cells.

Materials and Methods

Strains

A *E. coli* C600 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. *E. coli* K-12 derivatives with genotypes as well as plasmid vectors used are listed in Table 1.

Media and reagents

Usually Luria broth was used for bacterial growth.

Table 1. Bacterial strains and plasmid vectors.

Strains	Genotypes or Phenotypes	Source
E. coli C600	*thi*1, *thr*1, *leuB*6, *lacY*, *ton*21, λ-*supE*44	
JF611	*cdd*1, *pryE*60, *thi*1, *argE*3, *his*3, *proA*2, *thr*1, *leu*6, *mdl*1, *xyl*5, *ara*14, *galK*2, *lacY*, *str*31, λ-*supE*44	J. Friesen
JM109	*recA*1, *supE*44, *endA*1, *hsdR*17, *gyrA*96, *relA*1, *thi* Δ *(lac-proAB)*	
HB101	*supE*44, *hsdS*20,(rB-mB-), *recA*13, *ara*14, *proA*2, *lacY*1, *galK*2, *rpsL*20, *xyl*5, *mtl*-1	
Plasmid vectors		
pBR322	Ap^r, Tet^r	
pUC18,19	Ap^r *ori*, *lacPOZ*	

ALB(amplicillin (50μg/m*l*), Luria broth) medium was used for the plasmid isolation. For enzyme assay, *E. coli* was cultured in AB medium (4). Most of the minimal media were supplemented with appropriate requirements, antibiotics and glucose or glycerol (0.2%) as carbon sources. For the selection of *cdd* positive cells, cytidine/deoxycytidine (40μg/m*l*) and antibiotics (ampicilin 50μg/m*l*, tetracycline 15μg/m*l*) 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. (Kyoto, Japan), Sigma Co. (St. Louis, USA), and Boehringer Mannheim GmbH (W-Germany). Restriction endonucleases, RNase, alkaline phosphatase, proteinase K and T_4 DNA ligase were purchased from the KOSCO Co. (Seoul, Korea), Takara shuzo Co. and Sigma Co., Radioisotopes were obtained from Amersham Co. (Buckinghamshire, England).

Preparation of DNA and gel electrophoresis

E. coli chromosomal DNA was isolated from exponentially growing cells according to the preparative method described by Rodriguez and Tait (11). For rapid isolation of plasmids from the bacteria, the alkaline lysis method described by Birnbom and Doly (12) was employed. Chromosomal DNA, plasmid DNA and their restriction digests were analyzed on vertical or horizontal 0.7 to 1.2% agarose gels.

DNA techniques

Probe DNA synthesis: Transcription-initiation region of *E. coli cdd* gene was reported by Valentin-Hansen *et al.* (8). A 23 mer of synthetic oligonucleotide synthesized by the Genetic Engineering Center, KAIST, Korea, was used as a probe. The probing sequence was derived from the transcriptional initiating region of the Valentin-Hansen's promoter sequence (8) (Fig. 1.)

Colony hydribization and southern hybridization: Transformation of *E. coli* JF611 was carried out using the $CaCl_2$ method (11). Transformed cells were colonized on ampicillin plates, the colonies were toothpicked onto two LB plates containing 50μg/m*l* ampicillin. The replica plates were incubated for 10-12 hours at 37°C and the nitrocellulose filter papers were overlaid on one of the plates and stored at 4°C (in an inverted position until the results of the hybridization reaction were available). This probe was end-labeled

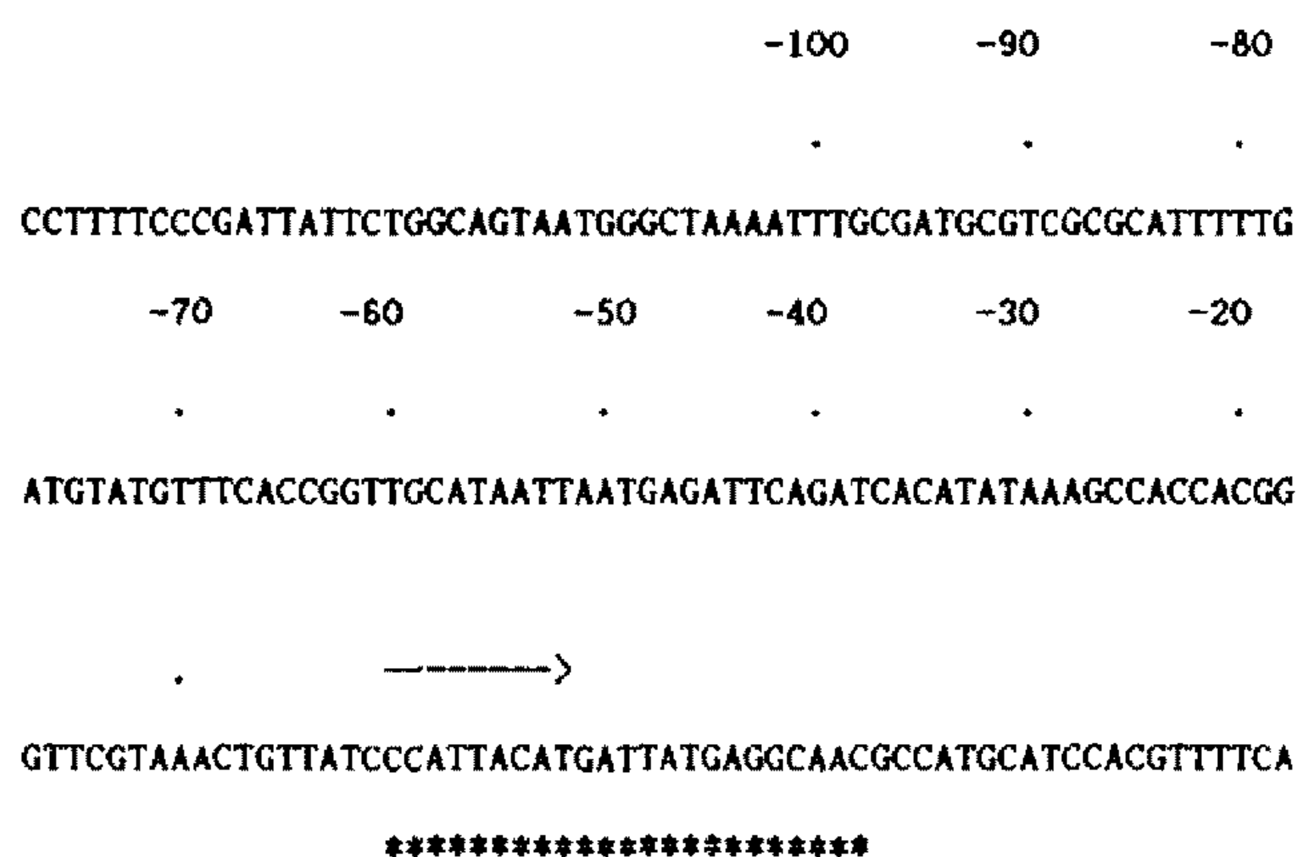


Fig. 1. The nucleotide sequence of the *E. coli cdd* promoter region.
The start point of transcription (+1) is indicated by an arrow (--->), and the sequence for a probe (TK-1) is indicated by asterisks (*). The sequence shown in Fig. 1, was determined by Valentine-Hansen (8), but the complete sequence covering the *cdd* gene was not determined yet.

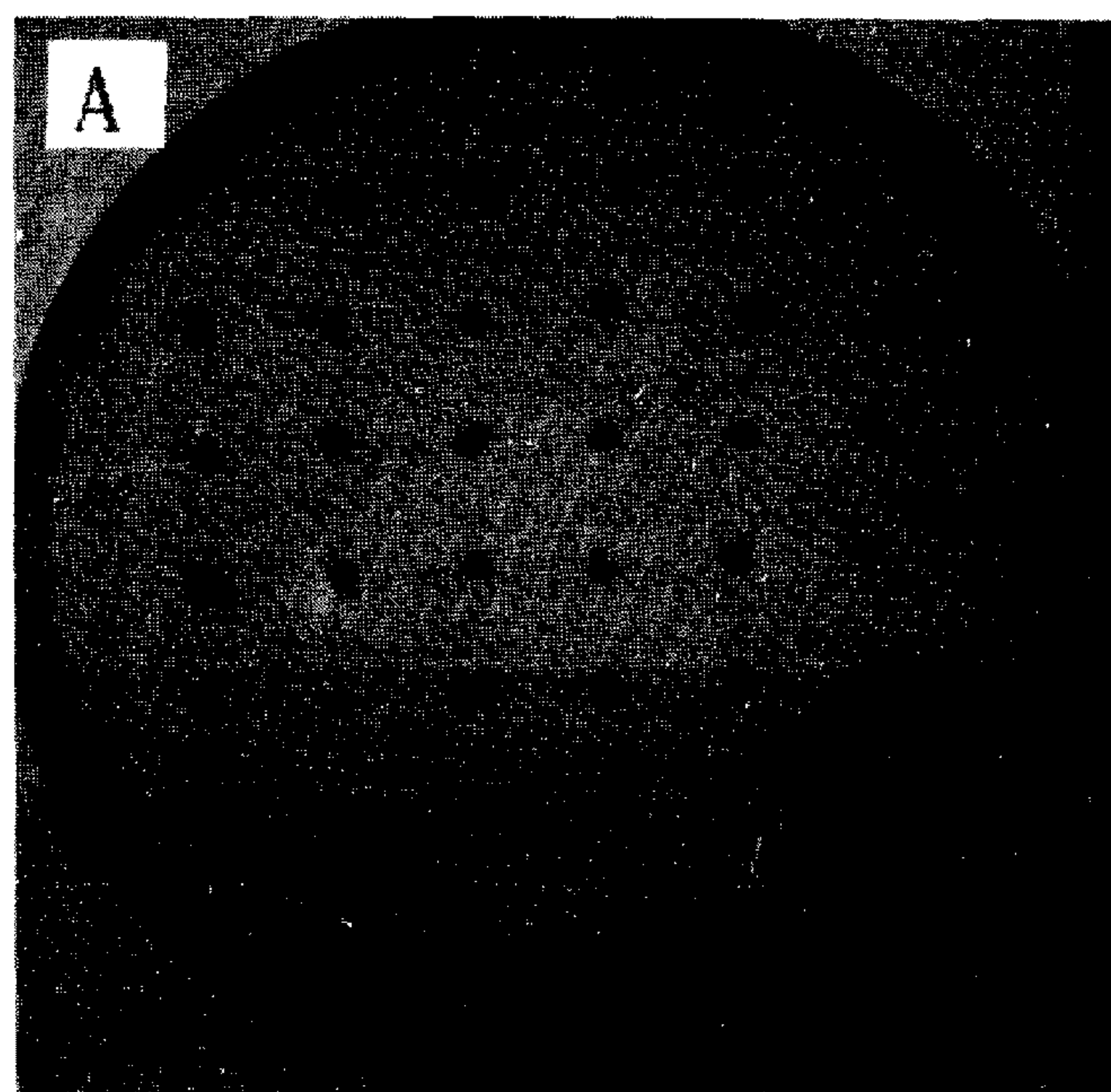
with [γ^{32}-P] dATP by incubation with polynuclotide kinase for 1 hour at 37°C. Processed filters were hybridized with end-labeled probe in 20 m*l* of hybridization buffer overnight at 68°C with gentle shaking (13).

Detection of plasmid-encoded protein

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* structural gene. Thus, only the plsmid which encoded proteins will be synthesized (14). The minicells containing the *cdd* genes were labeled with ^{35}S-methionine for 1 hour after preincubation for 1.5 hours, 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 (15).

Enzyme activity assay

Crude cell extracts prepared from sonic disruptions were used as the enzyme source. Cytidine deaminase activities were determined by the procedure of Hammer-Jesperson *et al.* (16). One unit is defined as the amount of enzyme which will deaminate 1 μmole of cytidine/min at 37°C Protein determination was preformed by the method of Lowry *et al.* (17) using bovine serum albumin as a standard.



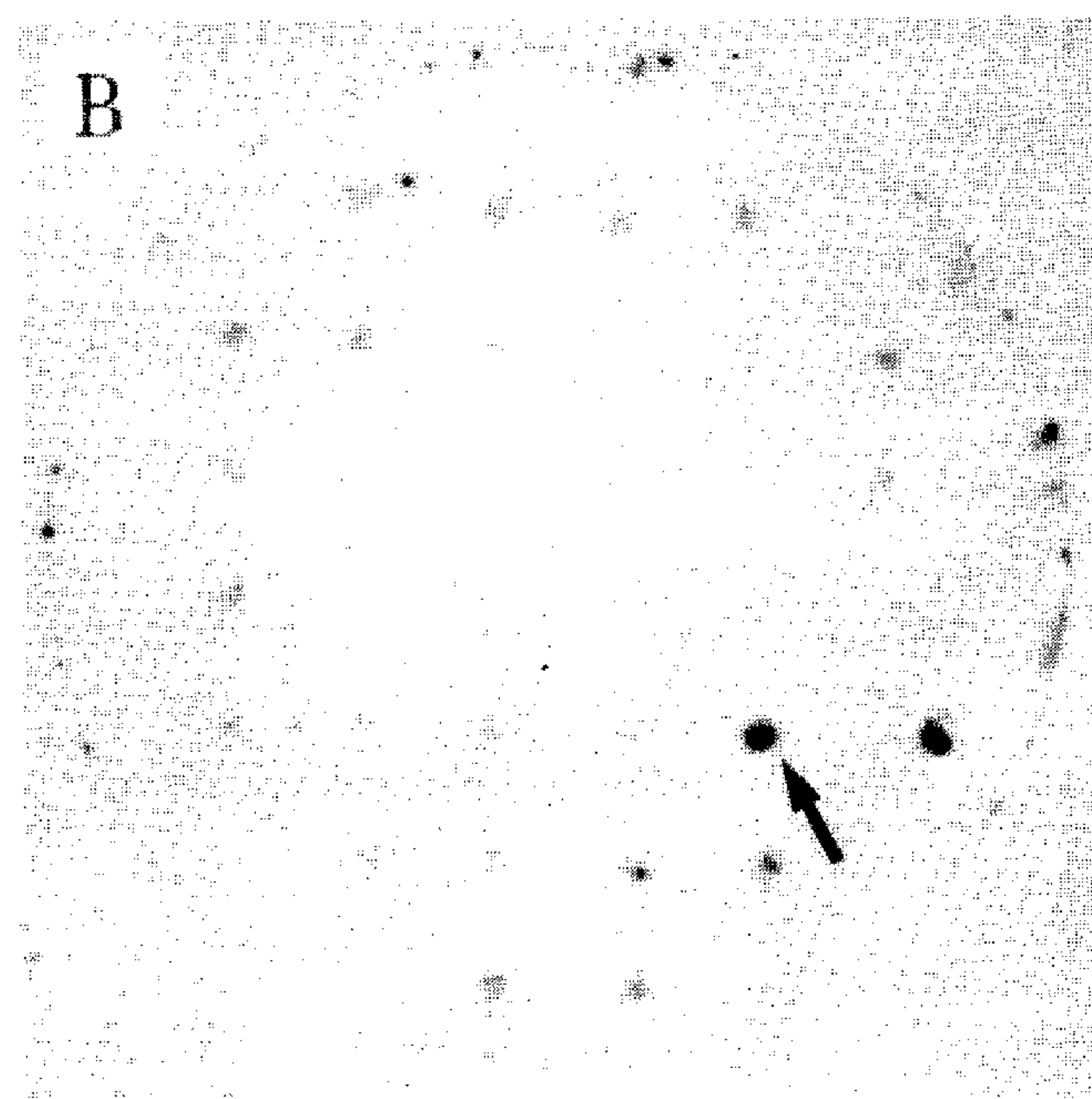


Fig. 2. Selection of transformed colonies with the hybrid pBR322 containing the fragment detected by the probe TK-1.
Colonies grown in A plate containing ampicillin and chloramphenicol were transferred to nitrocellulose filter (B), lysised by 0.5 N sodium hydroxide, and hybridized with the probe TK-1. Arrow indicates the signal hybridized by TK-1, and the plasmid contained in hybridized colony was named pTK100

Results and Discussion

Cloning of the *E. coli cdd* gene into *E. coli* JF611

E. coli chromosomal DNA was partially digested

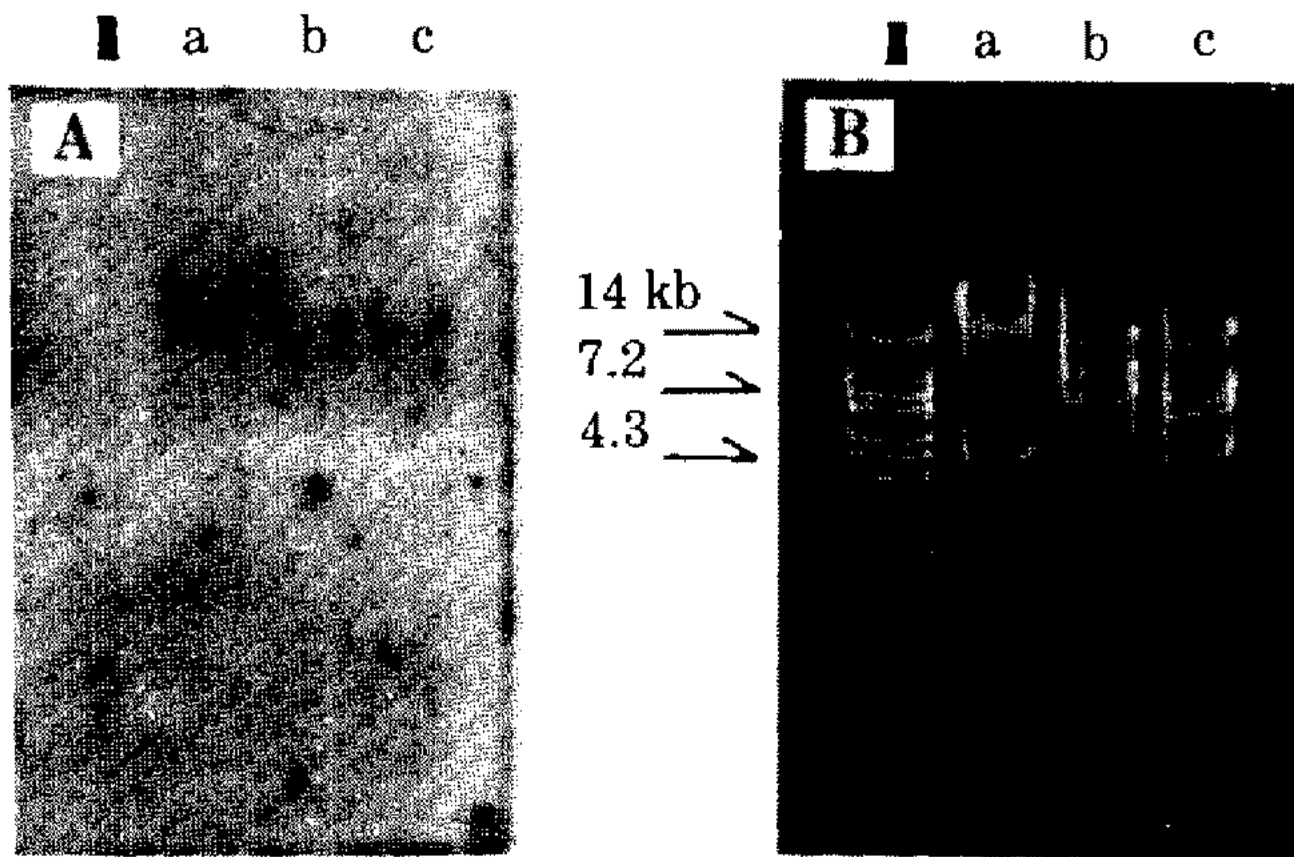


Fig. 3. Southern hybridization of the plasmid pTK100.
The pTK100 selected by colony hybridization was digested by several restriction endonucleases in agarose gel (Fig. 3.B), transferred to nitrocellulose filter, and southern hybridization was carried out with the probe TK-1
A: Hybridization patterns B: Restriction patterns, lane; m: marker (λ-*Bst*EII), a: pTK-100, *Bam*HI b: pTK-100, *Eco*RI c: pTK-100, *Hind*III

with *Bam*HI, *Eco*RI and *Pst*I, and hybridized with the end labeled synthetic oligonuclotide probe. The hybridized signal was shown to be more than 24 kb (data not shown).

More than 20 kb *Bam*HI fragments of *E. coli* chromosomal DNA were ligated with the plasmid vector pBR322. The ligation mixture was used to transform competent *E. coli*JF611 and the ampicillin resistant colonies were introduced for screening the cdd^+ cells by the hybridization with the end labeled synthetic oligonucleotide probe. Out of 2,000 colonies screened, only one colony was hybridized to the probe. The autoradiogram of this positive clone is shown in Fig. 2.

This positive clone was cultured in 100 m*l* ALB for the purification of plasmid. The isolated plasmid from the recombinant was tentatively designated as pTK100. Restriction analysis with *Bam*HI, *Hind*III, and *Eco*RI to the pTK100 showed that the size of inserted DNA

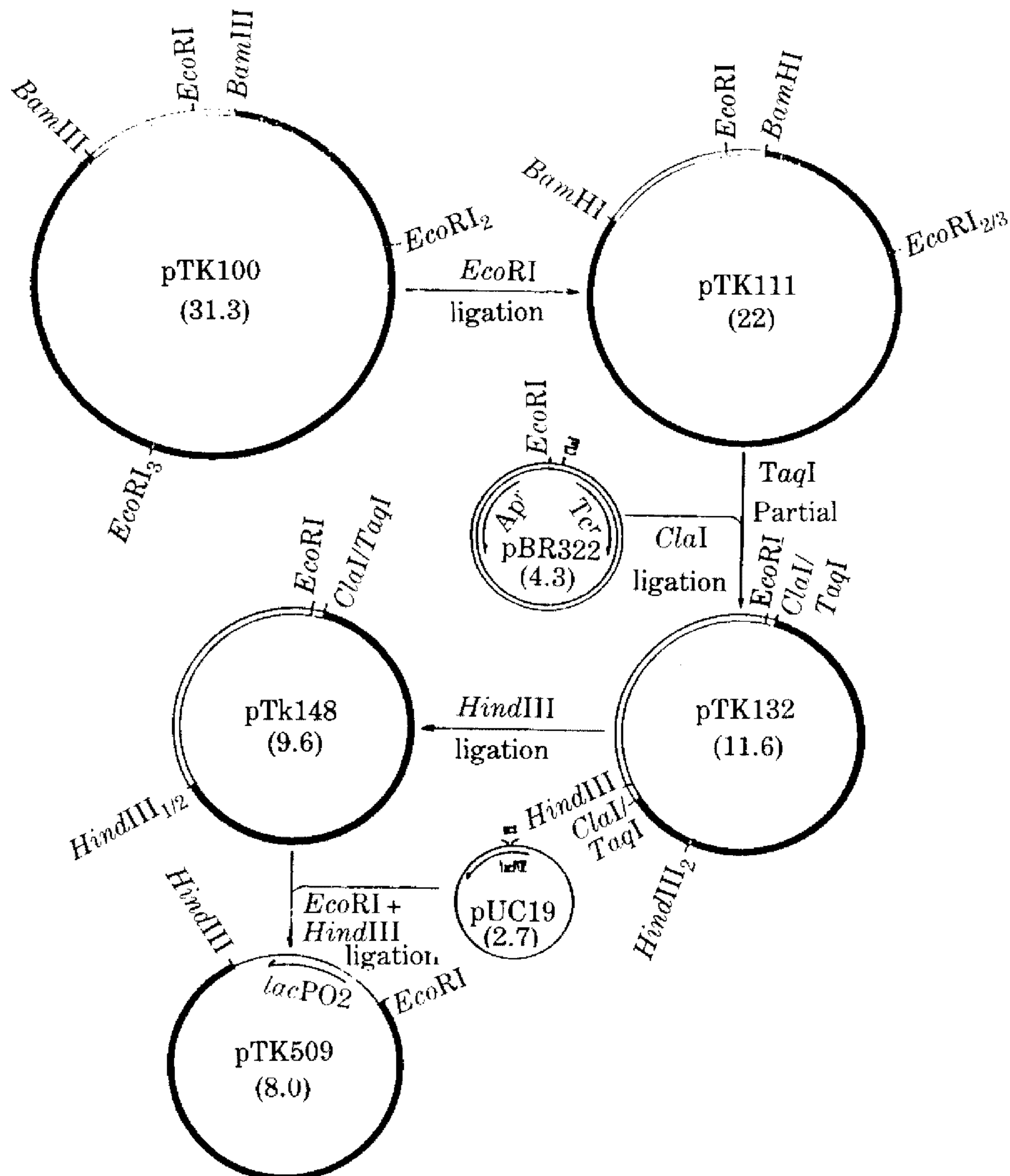


Fig. 4. Schematic diagram showing the construction of pasmids.
Open segments and line represent pBR322 and pUC19, respctively. Filled bars represent the inserted exogenote containing *E.coli cdd* gene. Arrows indicate the reading direction of ampicillin (Ap) and tetracycline (Tc) resistance genes.

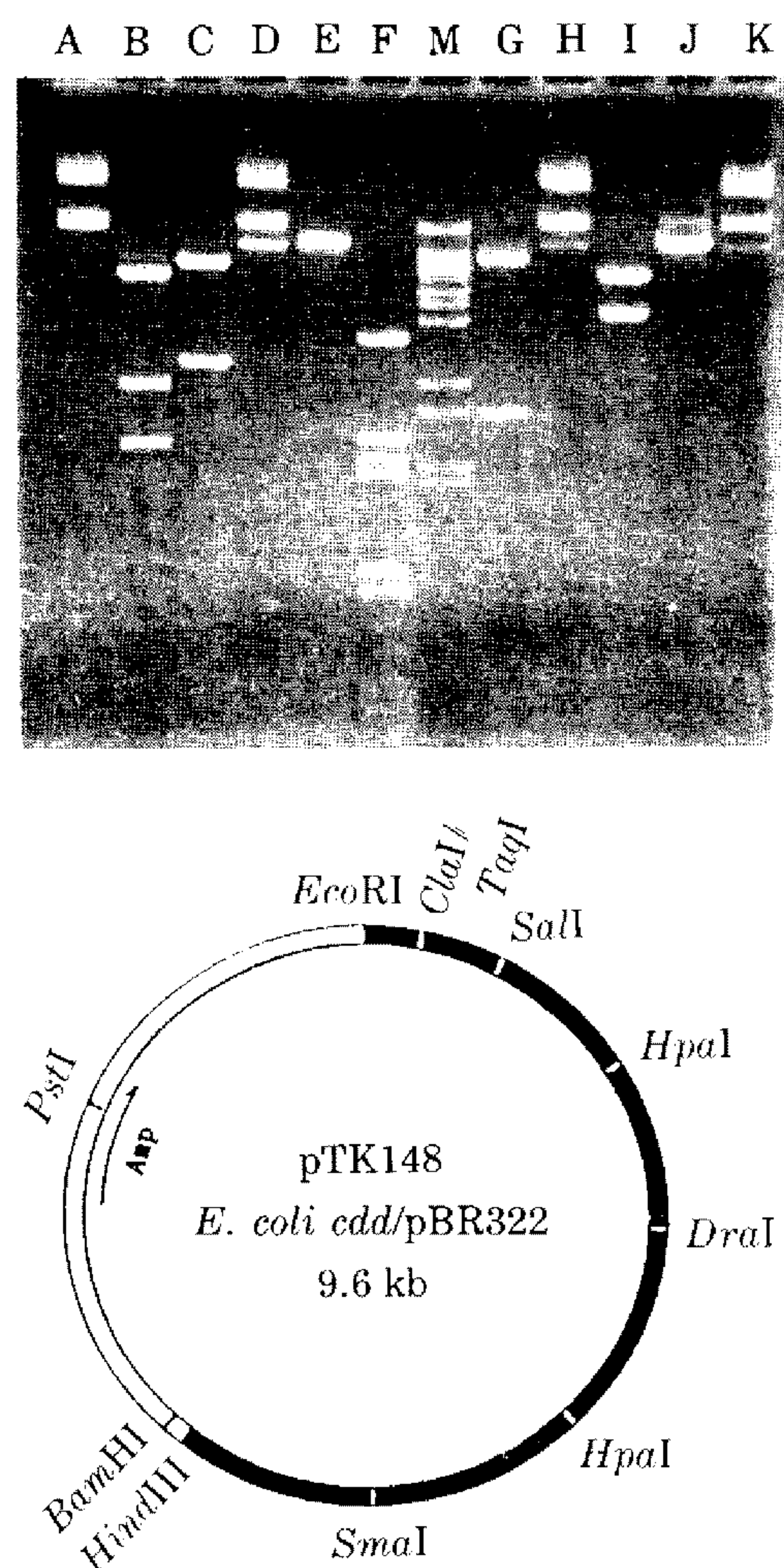


Fig. 5. Restriction patterns and structures of pTK-148. A: pTK-148 no cut B: pTK-148 *Acc*I C: pTK-148 *Ava*I D: pTK-148 *Bgl*II E: pTK-148 *Cla*I F: pTK-148 *Hind*II M: marker (λ-*Bst*EII) G: pTK-148 *Hpa*I H: pTK-148 *Sca*I I: pTK-148 *Sal*I J: pTK-148 *Sma*I K: pTK-148 *Xba*I

segment was about 27 kb (Fig. 3).

Subcloning of the *cdd* gene

A series of subcloning procedure were carried out using *E. coli* (*cdd*$^-$) as the host system (Fig. 4). The pTK111 was derived from pTK100 after deletion of *Eco*RI$_2$/*Eco*RI$_3$ fragment by selecting *cdd*$^+$ and ampicillin resistance. The pTK132 was constructed by *Taq*I (partial)-digested fragment insertion to the pBR322 cut with *Cla*I. And then, the pTK148 was drived from pTK 132 after deletion of *Hind*III$_1$/*Hind*III$_2$ fragment.

After mapping of the inserted fragment as shown in Fig. 5, subsequent deletion and religation were continued to localize the *cdd* gene in the insert. The *Eco*RI/*Hind*III fragment of pTK148 was transferred into the corresponding polylinkerr cloning sites of the pUC19. As shown in Fig. 6, when the *Eco*RI/*Hpa*I fragment from pTK603, *Hpa*I$_1$/*Hpa*I$_2$ from pTK601 and *Eco*RI/*Sal*I from pT605 were deleted, the *cdd* expression was blocked, while deletion of the *Sma*I /*Hind*III fragment in pTK600, *Hpa*I/*Bam*HI in pTK602 and *Dra*I/*Bam*HI in pTK605 did not affect the expression. Therefore, the *cdd* gene may be located in the 2.1 kb of *Sal*I/*Dra*I segment in the pTK509.

Expression of the cloned *cdd* gene in *E. coli*

The specific activity of the cytidine deaminase was determined from the crude extracts of strains carrying *E. coli cdd* gene in multicopy on various plasmids. From the results of Table 2, it can be seen that the

Table 2. Expression of *E. coli cdd* gene

Strains/plasmids	Relevant genotypes[b]	Specific activity (unit) nm/min/mg protein
E. coli[a] C-600	*cdd*$^+$ (wild type)	152
JF-611/pTK148 (pBR322)	*cdd* /p*cdd*$^+$ *E. coli*	56,507
JF-611/pTK605 (pUC19)	*cdd* /p*cdd*$^+$ *E. coli*	381,193
JF-611/pTK606 (pUC18)[c]	*cdd* /p*cdd*$^+$ *E. coli*	547,692

[a] Strains were grown in minimal medium containing 0.2% glycerol, 0.2% casamino acid, 40 μg/ml cytidine and 50 μg/ml ampicillin

[b] p*cdd*$^+$*E. coli* indicates the plasmid containing the *E. coli cdd* gene

[c] The *lac*Z promoter orintation of pUC18 is same as that of *E. coli cdd* gene

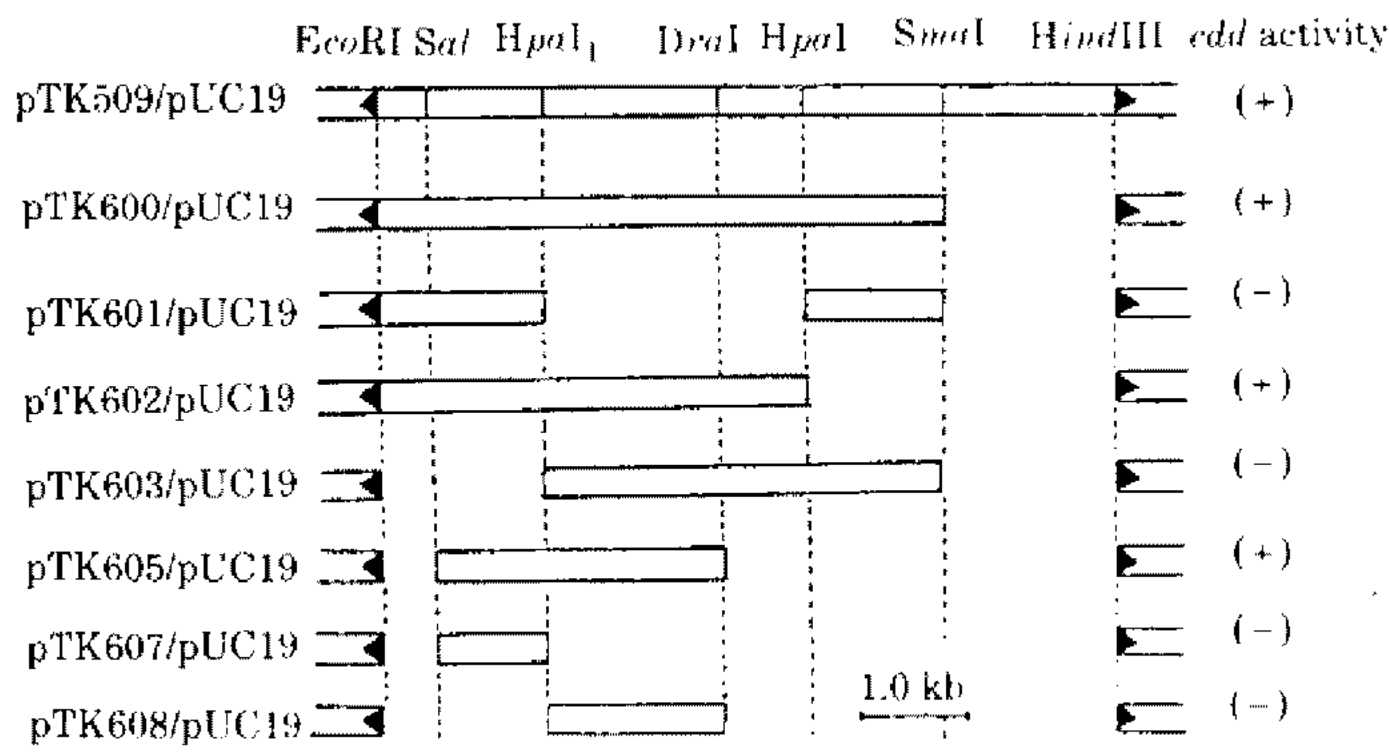


Fig. 6. Identification of *E. coli cdd* gene location by subcloning.
Filled bars represent the pUC18 and open bars represent the subcloned fragments. Each plasmid was tested for complementation of an *E. coli cdd* mutation and the results are indicated on the right.

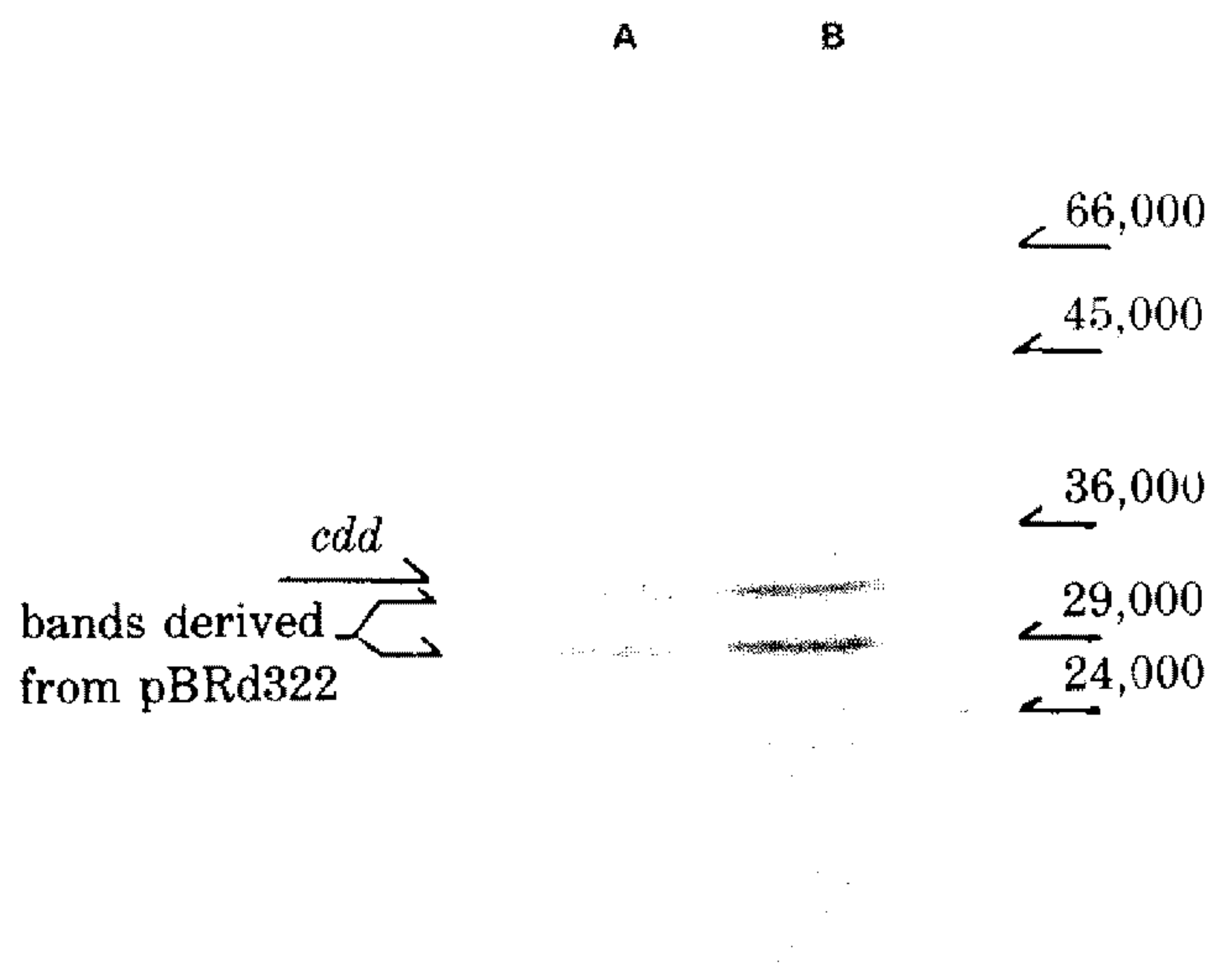


Fig. 7. Identification of polypeptide encoded by the *cdd* gene.
^{35}S-Methionine labeled polypeptides from the extracts of minicells harboring the plasmid pTK-148 and pBR322 were analysed by 12.5% SDS-polyacrylamide gel electroplhoresis and autoradiographed lane A: pTK-148 B: pBR322

cytidine deaminase activity was increased about 37 times by amplifying the *cdd* gene in pBR322 and it was 7 times more levels in the cell extracts carrying the *cdd* gene in the pUC plasmids compared to those carrying it in the pBR322 plasmid.

Molecular mass determination from the minicell experiment

In order to determine the molecular mass of the *cdd* gene product, the plasmid pTK148 and pBR322 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. 7, one polypeptide with a molecular mass of about 33,000 dalton appeared specifically in cells harboring the *cdd* complementing plasmid pTK148.

Acknowledgement

This work was supported by grants from the Korea Science and Engineering foundation.

요 약

*E. coli*의 cytidine deaminase(cytidine/2′-deoxycytidine aminohydrolase ; EC 3.5.1.5)를 코딩하는 *cdd* 유전자를 *E. coli cdd⁻pyr⁻* 결손 변이주를 cloning host로 하여 southern blotting과 colony hybridization을 통하여 클로닝하였다. *cdd* 유전자가 단편은, *cdd* 유전자의 transcription initiation 부위의 23개 nucleotide를 합성한 후 probe로 사용하여 Southern hybridization에 의해 회수된 *cdd* 유전자를 함유한 단편을 얻었으며, 이를 pBR322에 삽입한 후 형질전환하여 colony hybridization한 결과 *cdd*⁺ cell을 얻었다. 삽입된 DNA 단편의 size는 27kb이었으며 이를 결실 및 subcloning을 연속 수행한 결과 2.1kb의 *Sal*I/*Dra*I fragment(pTK605)에 *cdd* 유전자가 location되어 있음을 알게 되었다. Mini cell 실험결과 합성된 polypeptide는 약 33kDa이었으며, wild type의 cytidine deaminase의 활성이 pBR322에서 증폭시킴으로서 37배 정도 배가되었으며, pBR322에 비해 pUC vector계에서 다시 활성이 7배 정도 증가됨을 알 수 있었다.

References

1. Neuhard, J. and P. Nyggaard: *Escherichia coli* and *Salmonella typhimurium,* Cellular and Molecular Biology (Neihardt, F.C. ed) American Soc. for Microbiol., Washington, **1**, 445 (1987).
2. Neuhard, J.: *Acad.* Press, London 95 (1983).
3. Ashley, G.W. and P.A. Bartlett: *J. Biol. Chem.* **259,** 13615-13620 (1984).
4. Song, B.H. and J. Neuhard: *Mol. Gen. Gent.* **216,** 462 (1989).
5. Song, B.H., M.S. Yoon, K.H. Kim, J.S. Yeo and J. Neu

hard: *Kor. J. Appl. Microbiol. Bioeng.* **16**, 468-475 (1988).
6. Chang, J.S., B.H. Song, J.K. Kim and S.D. Hong: *Kor. J. Appl. Microbiol. Bioeng.* **17(4)**, 334-342 (1989).
7. Ashley, G.W. and P.A. Bartlett: *J. Biol. Chem.* **259(21)**, 13615 (1984).
8. Valentin-Hansen P.: London pp.273-288 Gene manipulation and expression (1985).
9. Gasse, F., M. Pascal and M. Chippaux: *Mol. Gen. Genet.* **124**, 253-257 ((1973).
10. Valentin-Hansen, P., B. Svenningsen, A. Munch Peterson and K. Hammer-Jespersen: *Mol. Gen. Genet.* **159**, 191 (1978).
11. Rodriguez, R.L. and R.C. Tait: Recombinant DNA Techniques (1979).
12. Birnboims, H.C. and J. Doly: *Nuecleic Acid Res.* **7**, 1513 (1979).
13. Southern, E.M.: *J. Mol. Biol.* **98**, 503 (1975).
14. Fraser, A.C. and R. Curtiss: *Curr. Top. Microbiol. Immunol.* **69**, 1 (1975).
15. Jensen, K.F., J.N. Larsen, L. Schack and A. Sivertsen: *Eur. J. Biochem.* **140**, 343 (1984).
16. Hammer-Jesperson, K., A. Munch-Ppetersen., P. Nygaard and M. Schwartz: *Eur. J. Biochem.* **19**, 533 (1971).
17. Lowry, O.N., N.J. Rosenbrough, A.L. Farr and R.J. Randall: *J. Bio. Chem.* **193**, 265 (1951).

(Received September 12, 1990)