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Cloning and Overexpression of the *Cdd* Gene Encoding Cytidine Deaminase from *Salmonella typhimurium*

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Abstract - The *Salmonella typhimurium cdd* gene encoding cytidine deaminase (cytidine/2'-deoxycytidine aminohydrolase; EC 3.5.4.5.) was isolated through shotgun cloning by complementation of the *E. coli cdd* mutation. By subsequent deletion and subcloning from the original 3.7 Kb of EcoRI insert (pSAM1), the precise region of the *cdd* structural gene is located around the BglII site in the middle part of 1.7 Kb of NruI/PvuI segment. The 1.7 Kb containing *cdd* gene was subcloned to the pUC18 vector and the nucleotide sequence of the *cdd* gene was determined. When the putative ribosome-binding site (Shine-Dalgarno sequence) and initiation codon were predicted to be GAGG at the position 459 and ATG at the position 470, respectively, there was an open reading frame of 885 nucleotides, encoding an 294 amino acid protein. The *cdd* gene expression in *E. coli* JF611/pSAM1 was amplified about 50 fold compared to that of the wild type. The *cdd* gene expression was maintained in the stationary phase after reaching the peak in the late logarithmic phase.

Key words : *Salmonella typhimurium, cdd* gene, cloning, sequencing, gene expression

INTRODUCTION

The chemical and biological factors of environment act on a living organism. Especially, the contaminated environment is harmful to the human and warm-blooded animals. Enteric bacteria such as *Salmonella*, *Shigella* and *Escherichia*, all potential pathogens, can spoil meat product. *Salmonella typhimurium* is the most cause of salmonellosis in human. The researches of *Salmonella typhimurium* in company with *Escherichia coli* have been advanced in genetic, biochemical, enzymatic, and sanitary parts (Frederick 1987).

The cytidine/deoxycytidine deaminase (cytidine/2'-deoxycytidine aminohydrolase; EC 3.5.4.5.) encoded by the *cdd* gene converts cytidine and deoxycytidine to their corresponding nucleosides, uridine and deoxyuridine respectively, in the pyrimidine salvage pathways of *Salmonella typhimurium* and *Escherichia coli* (O'donovan and Neuhard 1970). The nucleotide sequence of the enzyme was clarified various biological species : *Arabidopsis thaliana* (Vincenzetti *et al.* 1999) *Bacillus subtilis* (Song *et al.* 1989) Human (Demontis *et al.* 1998). The putative *cdd* gene is contained in whole genome sequence of *E. coli* (Acession No. M60916), *Haemophilus influenzae* (Accession No. U32814), *Caenorhaditis elegans* (Acession No. U64861), *Saccaromyces cerevisiae* (Acession No. U20865) *Mycoplasma pneumoniae* (Acession No. AE000011). The complete full length sequence of *S. typhimurium* LT2 genome was clarified in 2001 (McClelland *et al.*). Among them, the *Salmonella cdd* gene could be deduced from sequences of other biological species by using BLAST program of NCBI.

The purpose of this experiment is to find whether

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cytidine deaminase comes from the sequenced *cdd* gene or not in *S. typhimurium*. The *cdd* gene of *S. typhimurium* was cloned into *E. coli* and expressed it's enzyme.

MATERIALS AND METHODS

1. Strains

A *S. typhimurium* KP1001 was used as a donor strain of the *cdd* gene. A *E. coli* JF611 as a strain of *cdd*$^-$, *pyr*$^-$ was used as a cloning host. The *E. coli* K12 derivatives with their genotypes as well as plasmid vectors are listed in Table 1.

Table 1. Bacterial strains and plasmid vectors

	Phenotype or Genotype	Source
Bacterial strain		
S. typhimurium KP 1001	wild type	J. L. Ingraham
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* K2, *lac* Y1, *rps* L	J. Friesen
E. coli JM109	*rec* A1, *end* A1, *gyr* A96, *thi*, *hsd* R17, *sup* E44, *rel* A1, Δ(*lac-pro* AB) [F'*tra* D36, *pro* AB, *lac* 1, Z ΔM15]	
Plasmid		
pUC18	Ap^r	P. Valentin-hansen
pSAM1	3.7 Kb *cdd*/pUC18	this work
pSAM10	2.6 Kb *cdd*/pUC18	〃
pSAM11	2.7 Kb *cdd*/pUC18	〃
pSAM21	1.7 Kb *cdd*/pUC18	〃

2. Media and reagents

E. coli and *S. typhimurium* strains were grown in a L broth consisting of 1% tryptone, 0.5% yeast extract and 0.5% NaCl. For enzyme assay and transformants isolation, *E. coli* and *S. typhimurium* strains were cultured in AB minimal medium consisting of 0.2% $(NH_4)_2SO_4$, 0.75% Na_2HPO_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, antibiotics, and glucose (0.2%) or glycerol (0.2%) as a carbon source (Chang *et al.* 1989). For the selection of *cdd* positive cells, cytidine and deoxycytidine (40 μg ml^{-1}) and ampicillin (50 μg ml^{-1}) were added to the minimal medium (Song and Neuhard 1989). For detection of the transformant, agar plates of L broth were supplemented with 0.1 mM of IPTG (isopropyl-β-D-thiogalactopyranoside) and 0.004% of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) per plate.

3. DNA techniques

S. typhimurium chromosomal DNA was isolated by the standard method (Maniatis *et al.* 1989). Isolation of plasmids was carried out by a modified alkaline lysis procedure (Birnboim and Doly 1979). *E. coli* cells were transformed by the $CaCl_2$ method. The transformants were selected by colonization with the complementation of *cdd* mutation in the host. DNA was analyzed by agarose gel electrophoresis according to the standard method (Maniatis *et al.* 1989). In order to sequencing of the fragment of *cdd* gene, pUC vector forward primer, pUC reverse primer and four degenerate primers (pri1 CCATTTTCCCGCCATGCT; pri2 ATACGGCCATCTTTACA; pri3 GCAGCCAATAAGAGCCAT; pri4 ACTATGCGGATATTCAGCG) were designed and primers chemically synthesized (Bioneer, Chungbuk, Korea). The nucleotide sequence of the 1.7 kb-EcoRI/HindIII fragment was determined by the macrogen coperation (Seoul, Korea) The sequence alignment and ORF searching were analyzed by BLASTP or ORF seach of the National Center for Biotechnology Information protein database.

4. Enzyme activity assay

Assay mixtures present in a volume of 360 μl : 250 μl of Tris-Mg (2.35 ml of 100 mM Tris-Cl at pH 7.0 and 50 μl of 0.2 M of $MgCl_2$), 10 μl of 0.05 M cytidine and 100 μl of appropriate diluted enzyme. At 4, 8, and 12 min, 100 μl samples were pipetted into 0.9 ml of 0.5 N perchloric acid. The samples were centrifuged and the absorbance of the supernatant was measured at 290 nm. For the calculation of the enzyme activity, the difference in a molar extinction of 10.1×10^3 between cytidine and uridine was used. One unit is defined as the amount of enzyme which will deaminate 1 μmole of cytidine per min at 37°C. Protein determination was performed using bovine serum albumin as a standard (Lowry *et al.* 1951).

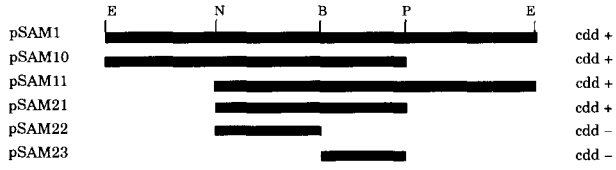

Fig. 1. Identification of *S. typhimurium cdd* gene location. N, NruI; E, EcoRI; P, PvuI; B, BglII.

RESULTS AND DISCUSSION

1. Cloning of the *cdd* gene into pUC18

S. typhimurium chromosomal DNA was digested with restriction endonuclease EcoRI. The isolated 3-10 Kb fragments from electroelution in agarose gel were inserted into the same site of pUC18, and from the transformation with ligated plasmids cdd^+ colonies were selected by shotgun complementation of the *cdd* mutation of *E. coli* JF611. Transformants of cdd^+ cells were selected by ampicillin resistance and cdd^+ in minimal medium was supplemented with deoxycytidine as a sole pyrimidine source and ampicillin. From one of the two transformants from the selection plates, named *E. coli* KM401, the pSAM1 was extracted as a hybrid plasmid. The pSAM1 contained a 3.7 Kb passenger DNA fragment containing the *cdd* gene in the pUC18. Subsequential deletion and religation were continued to localize the *cdd* gene in the insert, and to minimize the size of the exogenote (Fig. 1). The deleted plasmid, pSAM10 and pSAM11 were derived from the pSAM1 by PvuI and NruI sites deletions respectively, through selection of ampicillin resistancy and cdd^+. To find out the precise location of *cdd* in the inserted DNA fragment, the pSAM22 and pSAM23 were constructed by insertion of 1.0 Kb of NruI/BglII and 0.7 Kb of BglII/PvuI fragment from 1.7 Kb NruI/PvuI into pUC18 and tested for the *cdd* expression. However, both fragments did not expressed the *cdd* activity independently. It can be inferred that the protein coding region of the *cdd* gene is contained to the 1.7 Kb NruI/PvuI fragment of pSAM10 and 11.

2. DNA sequence and deduced amino acid sequence of the cytidine deaminase gene

The 1.7 kb containing *cdd* gene was subclonded to pUC18 and the nucleotide sequence was determined. The nucleotide sequence of the 1770 nucleotide including the unique ORF and adjacent sequences are shown in Fig. 2. There was an open reading frame of 885 nucleotides encoding an 294 amino acid protein. The putative ribosome-binding site (Shine-Dalgarno sequence) and initiation codon were predicted to be GAGG at the position 459 and ATG at the position 470, respectively. The sequence of the *cdd* gene is exactly identical with the predicted *cdd* gene of *S. typhimurium* LT2 (Acession No. AE006468) in the gene bank. The *cdd* gene of *S. typhimurium* was compared with the genome of the various species by using BLAST through a server system supported by NCBI. The predicted amino acid sequence based on the DNA nucleotide sequence showed significant homology to the sequence of some kind of cytidine deaminase genes, including the cytidine deaminase gene of *E. coli* (82%, 453/550), cytidine deaminase gene of

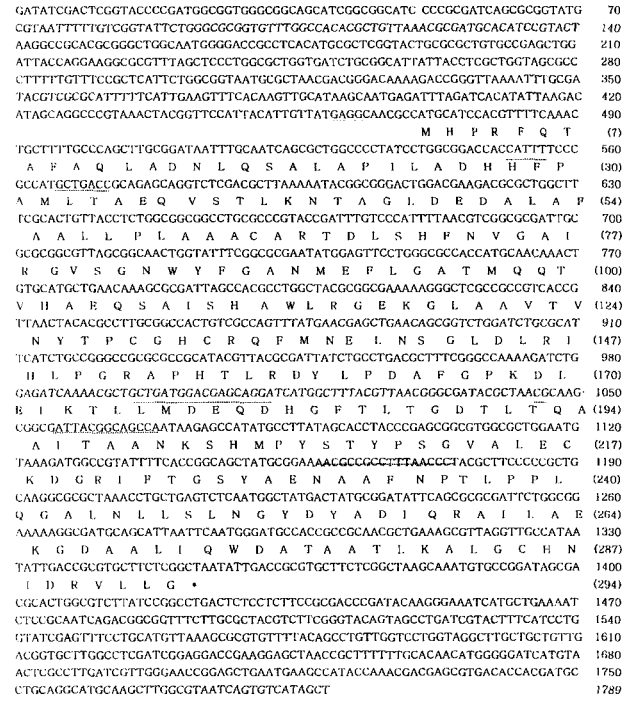

Fig. 2. Nucleotide and deduced amino acid sequence of the *cdd* gene. The deduced amino sequence is shown below the nucleotide sequence in single letter code. An asterisk indicates the stop codon, and the putative ribosome binding sites (Shine-Dalgarno sequence) is underlined. The oligonucleotide primers used for sequencing are indicated by the dotted lines.

Table 2. Expression of the *S. typhimurium cdd* gene encoding cytidine deaminase in *E. coli*

Strain/Plasmid		Relevant genotype	Specific[1] activity
S. typhimurium		*cdd*$^+$ (wild type)	27
E. coli	JF611/pSAM1	*cdd*/p*cdd*$^+{}_{st}$[2]	1625
	JF611/pSAM10	*cdd*/p*cdd*	1187
	JF611/pSAM11	*cdd*/p*cdd*$^+{}_{st}$	1006
	JF611/pSAM21	*cdd*/p*cdd*$^+{}_{st}$	1224

[1] nmol/min/mg protain
[2] cdd$^+{}_{st}$ indicates the wild type *S. typhimurium cdd* gene.

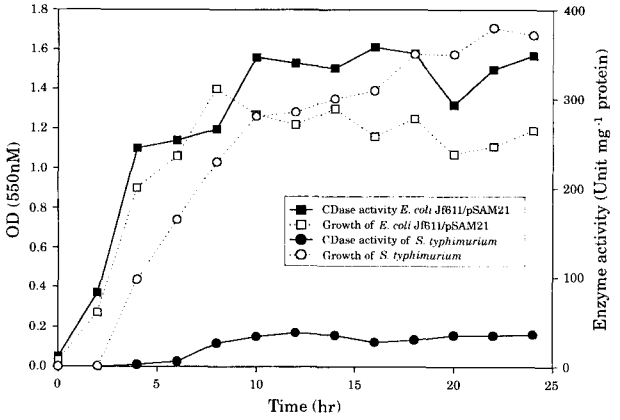

Fig. 3. Growth and enzyme production by *S. typhimurium* wild type and *E. coli* JF611/pSAM21.

Shigella flexner (82%, 451/550). However, there is no resonable identical homology between registered whole genome sequence of the various organisms. This sequence diversity of the *cdd* gene suggests two possibilities. One is that the cytidine deaminase subunit is not involved in the catalytic reaction of the holoenzyme. The other one is that the cytidine deaminase subunit does some kinds of species specific function. Further biochemical and enzymatic characterization of this subunit is required to prove these possibilities.

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

The specific activity of cytidine deaminase in crude extracts of *E. coli* carrying the *S. typhimurium cdd* gene in multicopy on pUC18 was determined. The activity level in *S. typhimurium* as a reference is given. From the results of Table 2, the *cdd* gene expression in *E. coli* JF611 harboring pSAM1 or pSAM21 was amplilfied about 50 fold compared to that in the wild type. Fig. 3 shows the growth pattern and cytidine deaminase production of *S. typhimurium* wild type compared with *E. coli* JF611 harboring pSAM21. Although the growth of *S. typhimurium* was better than that of *E. coli* JF611/pSAM21, the enzyme production in *E. coli* JF611/pSAM21, was approximately 50 fold above than that in *S. typhimurium* wild type. The maximum specific activity of cytidine deaminase in *E. coli* JF611/pSAM21 was found in the cell lysates after culturing 12 hrs.

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