ROLE OF THE CEPHALOSPORINASE GENE IN THE RESISTANCE OF THE CLINICALLY ISOLATED CEPHEM-RESISTANT *ESCHERICHIA COLI*

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A small number of highly cephem-resistant strains was found in extensive susceptibility testing of clinical isolates of *Escherichia coli* to the new cephalosporin derivatives. The cephem-resistance of these clinical isolates appeared to be due to the increased cephalosporinase activities. To clarify the mechanism of the resistance, we cloned the cephalosporinase genes from two typical cephem-resistant clinical isolates as well as from an *E. coli* K-12 strain. The following two lines of evidence indicated that the cephem-resistance resulted from hyper production of the cephalosporinase due to the up-mutation of the regulatory sequence of the cephalosporinase gene.

1) Reciprocal exchange of the regulatory sequence including a short segment of *N*-terminal coding sequence and the rest of the coding sequence between the cephalosporinase genes from *E. coli* K-12 and the cephem-resistant clinical isolate showed that the higher cephalosporinase activity was accompanied by the regulatory sequence of the cephalosporinase gene from the clinical isolate.

2) The promoter activities of the cephalosporinase genes were determined by cloning the regulatory sequences into a promoter analysis vector. The promoter activities of the cephalosporinase genes from the clinical isolates were 23~33-fold higher than that of the cephalosporinase gene from *E. coli* K-12.

A group of β-lactam antibiotics named the so-called third generation cephalosporins are characterized by their extremely high stability to both chromosomal and plasmid-coded β-lactamases.[1,2] It is this property of the third generation cephalosporins that extends the antibacterial spectrum to opportunistic pathogens such as *Enterobacter* and *Serratia*, which are highly resistant to the older β-lactam antibiotics. In contrast with the opportunistic pathogens, *Escherichia coli* is a typical Gram-negative strain which is susceptible to the older β-lactam antibiotics. However, the appearance of antibiotic resistant strains has been increasing among the clinical isolates of *E. coli* due to the spread of ampicillin-resistance plasmids which endow *E. coli* with high resistance to penicillins and moderate resistance to older cephalosporins.[3] The third generation cephalosporins exhibit the same antibacterial activity against ampicillin-resistant as against plasmid free strains, which is ten to several tens fold stronger than those of older β-lactam antibiotics. Our extensive survey on the susceptibility of *E. coli* to third generation cephalosporins has identified a small number of clinical isolates moderately resistant to these cephalosporins. Concern about the future trend of the cephalosporin-

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resistant strains of *E. coli* led us to investigate the mechanism of resistance of these clinical isolates. Preliminary studies showed that the cephalosporinase activity of the isolates was at least 10-fold greater than that of susceptible strains. This finding prompted us to characterize the cephalosporinase of the resistant isolates at both the enzyme and gene levels. In this paper, we report the molecular cloning of cephalosporinase genes of the cephalosporin-resistant isolates and identification of the element determining cephalosporin-resistance.

Materials and Methods

Bacterial Strains and Media

Bacterial strains used were *E. coli* K-12 derivatives HB101 (*F*$^-$, *hsdR*, *hsdS*, *recA*, *ara*, *proA*, *lacY*, *galK*, *rpsL*, *xyl*, *mel*, *supE*), MC1061 (*F*$^-$, *araD*, *Δ(ara*, *leu)*, *lacX*, *galU*, *galK*, *hsr*, *hsm*, *strA*), SF8 (*F*$^-$, *thy*, *recBC*, *lop11*, *lig*, *hsr*, *hsm*), W3160 and 10 strains of clinically isolated cephem-resistant *E. coli*. Plasmids pMB9 (*tet*) and pBR322 (*amp*, *tet*) were used as vector DNA for cloning experiments and R100 (*chl*, *str/spc*, *sul*, *tet*) was used as the source of chloramphenicol acetyltransferase gene. The media employed were L medium for transformation and Mueller-Hinton medium for antibiotic susceptibility testing except if otherwise specified.

Antibiotics

The antibiotics used in this study were cefazolin, ceftizoxime, benzylpenicillin (Fujisawa Pharmaceutical Co., Ltd., Japan), cephaloridine, cephalexin (Eli Lilly and Company, Indiana, U.S.A.), and ampicillin (Beecham Research Laboratories, England).

Antibiotic Susceptibility Testing

The MICs of the test antibiotics were determined by the agar dilution method. 100-fold dilutions of overnight cultures in Mueller-Hinton broth containing 10^4 colony forming units were inoculated with a multipoint replicating apparatus onto Mueller-Hinton agar plates containing serial 2-fold dilutions of an antibiotic. After incubation at 37°C for 18~20 hours, the lowest concentration that inhibited macroscopic colonial growth was regarded as the MIC.

Preparation of Cell Extracts and Enzyme Assays

Cells for enzyme assays were grown in Mueller-Hinton broth at 37°C. Where appropriate, tetracycline was added at a concentration of 20 μg/ml to the medium as a selective reagent for plasmid-harboring strains. Cells were harvested at an absorbance at 600 nm of 0.7, washed once with 1/15 M potassium phosphate buffer (pH 7), resuspended in one tenth volume of the buffer and disrupted in an ice-water bath by sonication at 20 kilocycles for 10 minutes. Supernatant obtained by centrifugation at 9,000×*g* for 20 minutes was used as the crude enzyme solution. β-Lactamase activity was determined by direct spectrophotometric assay using a Hitachi 220A spectrophotometer equipped with a thermostatted cell holder. The enzyme solution was mixed in a 1-cm quartz cuvette with 150 μg of cephaloridine as a substrate and 200 μmol of phosphate buffer (pH 7) to make a final volume of 3.0 ml and incubated at 37°C. The hydrolysis rate was followed by a decrease in absorption at 260 nm. Chloramphenicol acetyltransferase activity was determined by spectrophotometric assay according to the method of SHAW and BRODSKY.[4] The enzyme solution was mixed in a cuvette with 300 nmol each of chloramphenicol, acetyl CoA and 5,5'-dithiobis-2-nitrobenzoic acid and 300 μmol of Tris-HCl buffer (pH 7.8) to make a final volume of 3.0 ml and incubated at 37°C. The acetylation rate was followed by an increase in absorption at 412 nm. Protein concentrations were determined according to the method of LOWRY *et al.*[5]

Determination of Isoelectric Points of β-Lactamases

Analytical isoelectric focusing of β-lactamases was performed according to the method of OLSSON *et al.*[6] Crude β-lactamase preparations were applied on thin-layers of 5% polyacrylamide gel containing 2% Ampholine pH range 3.5~10. The gel was equilibrated by using LKB 2117 Multiphor. After the electrofocusing, β-lactamase activity was detected by staining with nitrocefin and the isoelec-

tric points were determined from the calibration curve made with pI markers.

Preparation of Bacterial and Plasmid DNA

Chromosomal DNA was prepared according to the method of HARRIS-WARRICK *et al.*[7] Large-scale isolation of plasmid DNA was performed by the cleared lysate method described by CLEWELL and HELINSKI.[8] Further purification was achieved in cesium chloride-ethidium bromide density gradients at 36,000 rpm for 40 hours at 15°C in the 50Ti rotor. For screening of the transformants, plasmid DNA was isolated by the alkaline extraction method described by BIRMBOIM and DOLY.[9]

Recombinant DNA Technique

Restriction endonucleases (Bethesda Research Laboratories, Maryland, U.S.A., and Takara Shuzo Co., Ltd., Japan) and T4 DNA ligase (New England Biolabs, Massachusetts, U.S.A.) were used as recommended by the suppliers. Agarose gel electrophoresis was performed as described by MANIATIS *et al.*[10] Transformation was performed by the RbCl-$CaCl_2$ method described by KUSHNER.[11]

Cloning of Cephalosporinase Genes

Plasmid vector pMB9 and chromosomal DNAs prepared from the clinical isolates of *E. coli* No. 253, No. 801 and *E. coli* SF8 were digested with *Eco*R I. The digestions were stopped by heating at 65°C for 15 minutes. One fourth microgram of the vector DNA and 1.5 μg of the chromosomal DNA were mixed, precipitated with ethanol and redissolved in 100 μl of the ligation mixture. The ligation reaction was carried out with T4 DNA ligase for 20 hours at 4°C and stopped by ethanol precipitation of DNA. After centrifugation, the ligated DNA was dried, dissolved in 10 mM Tris-1 mM EDTA (pH 8.0) buffer and used for transformation of *E. coli* MC1061 and HB101. Transformants were selected on L agar plates containing 20 μg of ampicillin and 20 μg of tetracycline per ml.

Construction of the Vectors for Analyzing Promoter Activity

The antibiotic resistance plasmid R100 and pBR322 were digested with restriction endonucleases *Sau*3A and *Bam*H I, respectively, and extracted with phenol. The digestion of R100 with *Sau*3A generates a DNA fragment containing a promoter-less chloramphenicol acetyltransferase gene (*cat*).[12] Mixtures were ligated with T4 DNA ligase and used for transformation. Transformants were selected for chloramphenicol- and ampicillin-resistance. A recombinant plasmid from the transformant was named pCF13 and found to possess an R100 derived *cat* gene lacking its own promoter but expressed by use of a promoter of the *tet* gene. The plasmid named pCF33 was constructed by deleting the *tet* promoter from pCF13 as described below (Fig. 5). pBR322 was cleaved with *Eco*R I, digested with SI nuclease and the residual sticky ends were filled in with Klenow fragment. *Hin*d III linkers were ligated to the DNA ends, digested with *Hin*d III and self ligation was performed with T4 DNA ligase. This procedure lead to the deletion of the *tet* promoter. A *tet* promoter lacking plasmid named pCF31 was obtained from a tetracycline-sensitive and ampicillin-resistant transformant. pCF31 and pCF13 DNAs were digested with *Hin*d III, *Sal* I and *Bam*H I and with *Hin*d III, *Sal* I and *Pst* I, respectively. Digested DNAs were combined and ligated with DNA ligase. Recombinant plasmid named pCF33 was selected from ampicillin-resistant transformants, where the *tet* structural gene in pCF31 was replaced by the *cat* structural gene in pCF13.

Results

Cloning of Cephalosporinase Genes from the Cephalosporin-resistant Clinical Isolates of *E. coli*

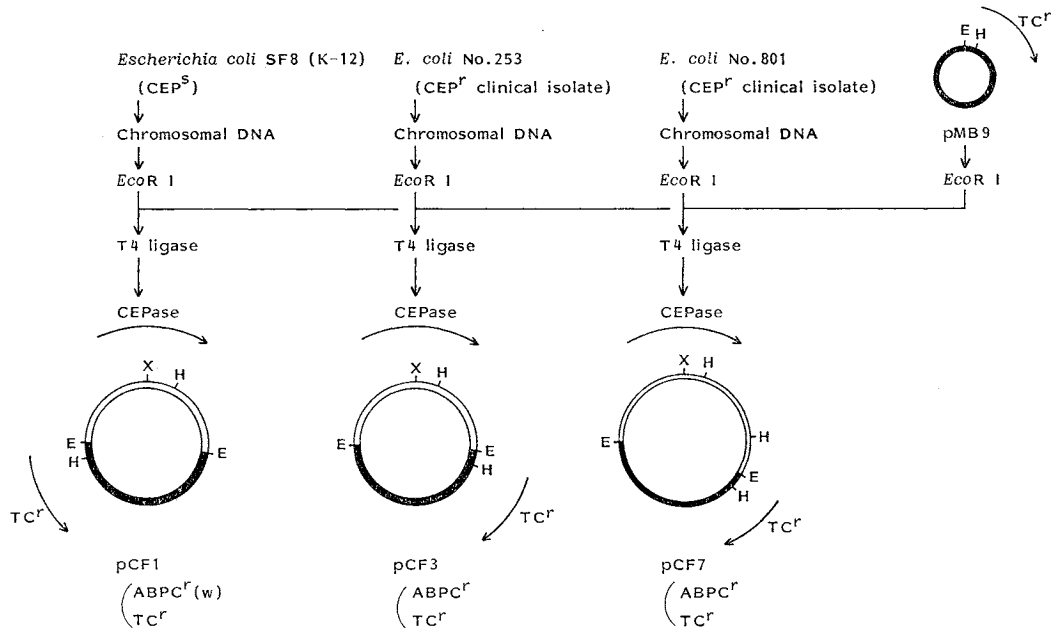
During a survey on the susceptibility of clinical isolates of *E. coli* to ceftizoxime, a representative of those cephalosporins, we found a small but discrete group of ceftizoxime-resistant strains. The susceptibility profile of the ceftizoxime-resistant strains was as follows; highly resistant to ampicillin (MICs range from 50 to 100 μg/ml), moderately resistant to cefazolin and cephaloridine (MICs: 6.25~100 μg/ml), and less moderately resistant to ceftizoxime (MICs: 1.56~12.5 μg/ml) (Table 1).

Table 1. β-Lactam antibiotic resistance of clinical isolates of *Escherichia coli*.

E. coli strain No.	MIC of antibiotic (μg/ml)			
	Ampicillin	Cephaloridine	Cefazolin	Ceftizoxime
36	50	12.5	12.5	1.56
253	400	50	100	6.25
327	400	50	50	3.13
551	100	50	50	6.25
621	100	25	25	3.13
625	100	25	25	3.13
801	400	50	100	12.5
818	100	25	25	1.56

Details of the susceptibility testing are described in the text.

Fig. 1. Cloning of cephalosporinase genes.
Details of the cloning method are described in the text.
CEP: Cephalosporin, TC: tetracycline, ABPC: ampicillin, ^{s}: susceptibility, ^{r}: resistance, X: *Xho* I, H: *Hind* III, E: *Eco*R I.



To clarify the role of cephalosporinase in this cephalosporin-resistance, we decided to clone the genes specifying cephalosporinase from two typical cephalosporin-resistant clinical isolates, *E. coli* No. 253 and No. 801 together with *E. coli* SF8, a derivative of the K-12 strain.

Chromosomal DNA of the above-mentioned strains was cleaved with *Eco*R I restriction enzyme and ligated with pMB9 vector at the *Eco*R I site. The resulting recombinant plasmids were used for transformation of *E. coli* HB101. Transformants which carry a plasmid bearing a cephalosporinase gene were screened by virtue of ampicillin-resistance. This yielded plasmids named pCF1, pCF3 and pCF7 whose inserted fragments were derived from *E. coli* SF8, No. 253 and No. 801, respectively (Fig. 1). The sizes of the inserted fragments were 5.8 kb for pCF1 and pCF3 and 6.5 kb for pCF7. Restriction endonuclease mapping showed that there was a segment in pCF1, whose cleavage map was the same as that of the *ampC* gene specifying chromosomal cephalosporinase[13,14] and that similar cleavage maps could also be found in pCF3 and pCF7 although a few restriction endonuclease cleavage

Fig. 2. Restriction endonuclease cleavage maps of pCF1, pCF3 and pCF7.

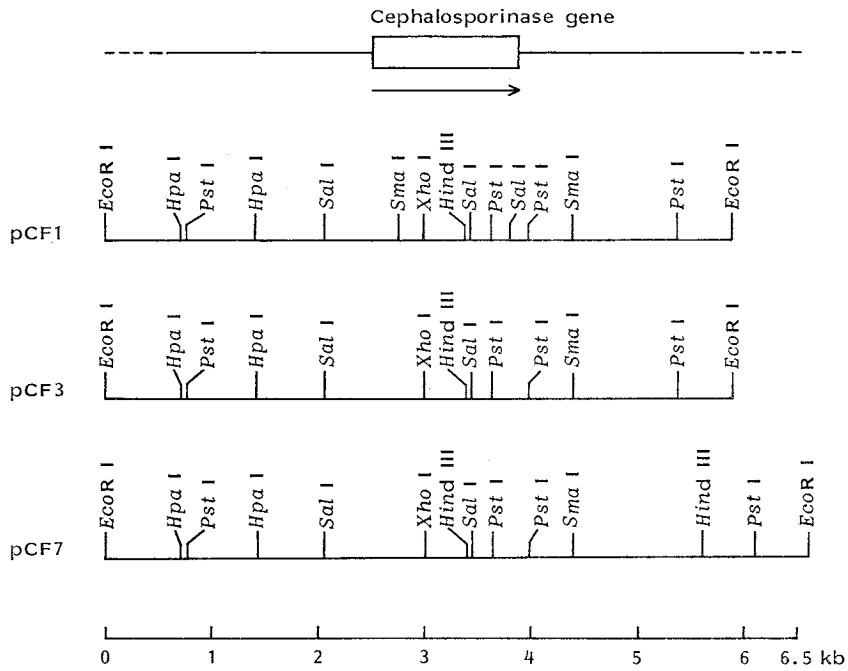


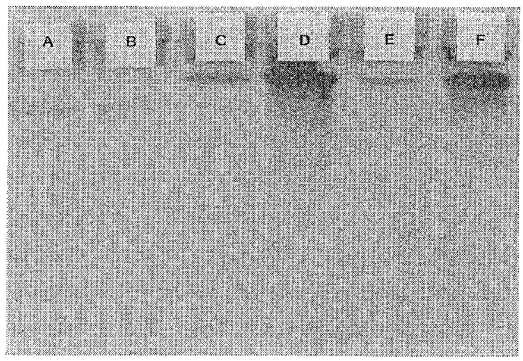
Table 2. Cephalosporinase activities of donor and transformant strains.

Escherichia coli strain	Specific activity of cephalosporinase[a]
SF8	<0.06
No. 253	14.8
No. 801	3.89
MC1061 (pCF1)	2.12 (1)[b]
MC1061 (pCF3)	148 (70)
MC1061 (pCF7)	81 (38)

[a] Specific activities were expressed as units per mg of protein. One unit of cephalosporinase was defined as the enzyme activity that hydrolyzes 1 μmol of cephaloridine per hour in 1/15 M phosphate buffer at 37°C.

[b] Relative specific activity.

Fig. 3. Isoelectric focusing of the cephalosporinases. Details of the method are described in the text. Applied samples were crude extracts from (A) *E. coli* SF8, (B) MC1061 (pCF1), (C) No. 253, (D) MC1061 (pCF3), (E) No. 801 and (F) MC1061 (pCF7).



sites were missing (Fig. 2).

Next, we characterized the cephalosporinases of the transformants. The specific activities of the cephalosporinases of the transformants were 10~20-fold higher than those of the corresponding donor strains (Table 2) although the substrate profiles of the cephalosporinases from the cephem-resistant clinical isolates and the corresponding transformants were similar to that of *E. coli* K-12 (Table 3). Accordingly, we confirmed by using isoelectric focusing that the gene products of the cloned fragments were identical to the chromosomal cephalosporinases of the corresponding donor strains. The isoelectric point (pI) values of the chromosomal cephalosporinases from the cephem-resistant clinical

Table 3. Substrate profiles of β-lactamases.

Escherichia coli strain	Relative rate of hydrolysis[a]				
	Cephaloridine	Cefazolin	Cephalexin	Ampicillin	Benzylpenicillin
SF8	100	65	75	ND	ND
MC1061 (pCF1)	100	72	49	1	100
No. 253	100	76	48	0.5	74
MC1061 (pCF3)	100	80	58	<0.6	74
No. 801	100	97	52	0.7	84
MC1061 (pCF7)	100	110	53	0.8	110

[a] The rate of hydrolysis of cephaloridine was taken as 100.
ND: Not determined.

isolates of *E. coli* were slightly higher than that of *E. coli* K-12. Transformants harboring pCF3 and pCF7 were shown to possess the cephalosporinases whose pI values were distinguishable from that of the cephalosporinase of the host strain and identical to those of the chromosomal cephalosporinases of the clinical isolates No. 253 and No. 801, respectively, while the pI value of the cephalosporinase from the transformant harboring pCF1 was shown to be the same as that of the chromosomal cephalosporinase of *E. coli* K-12 (Fig. 3).

Determinants Conferring Cephalosporin-resistance

The MIC values of cefazolin against cephem-resistant clinical isolates of *E. coli* were 8~64-fold higher than that against *E. coli* K-12, a susceptible strain. We found that the cephem-resistant clinical isolates possessed elevated cephalosporinase activities. However, it was unclear whether the cephalosporinase alone determined the cephem-resistance of the clinical isolates. Accordingly, we assessed the effect of cloned cephalosporinase genes on the susceptibility of *E. coli* K-12. The MICs of cefazolin against *E. coli* K-12 harboring pCF3 and pCF7 were 128- and 64-fold higher, respectively, than that against *E. coli* K-12 harboring pCF1 (Table 2). These values were in good agreement with the MICs of cefazolin against the cephem-resistant clinical isolates (Table 3). This indicates that the characteristics of the cephem-resistant strains were fully reproduced by the transformants carrying their cloned cephalosporinase genes. We concluded that the cephalosporinase was the essential factor that determines the cephem-resistance of the clinical isolates of *E. coli*. Next, we investigated whether a quantitative or qualitative change caused the elevation of the cephalosporinase activities of the cephem-resistant clinical isolates. This question could be answered by determining whether the structural or regulatory sequences of the cephalosporinase gene were resposible for the elevation of the cephalosporinase activities. We constructed chimeric cephalosporinase genes by a reciprocal exchange of a regulatory sequence of a cephalosporinase gene between pCF1 and pCF3 (Fig. 4). A *Xho* I site was chosen to divide the cephalosporinase gene into the regulatory sequence plus a small part of *N*-terminal structural sequence and the remainder of the structural gene. A chimeric plasmid named pCF8 which was composed of a regulatory sequence derived from the clinical isolate No. 253 and a structural sequence derived from the *E. coli* K-12 gene was shown to express the same cephalosporinase activity as pCF3 did (Table 4). Meanwhile, the cephalosporinase activity expressed by the other chimeric plasmid pCF9 remained at the level of the activity expressed by pCF1. This result suggested strongly that the elevation of cephalosporinase activity in the cephem-resistant clinical isolates resulted from the enhanced production of the cephalosporinase due to alteration of the re-

Fig. 4. Construction of chimeric cephalosporinase genes.
Details of the method are described in the text.
CEPase: Cephalosporinase, TC: tetracycline, ^{r}: resistance, X: *Xho* I, H: *Hin*d III, E: *Eco*R I, Hp: *Hpa* I.

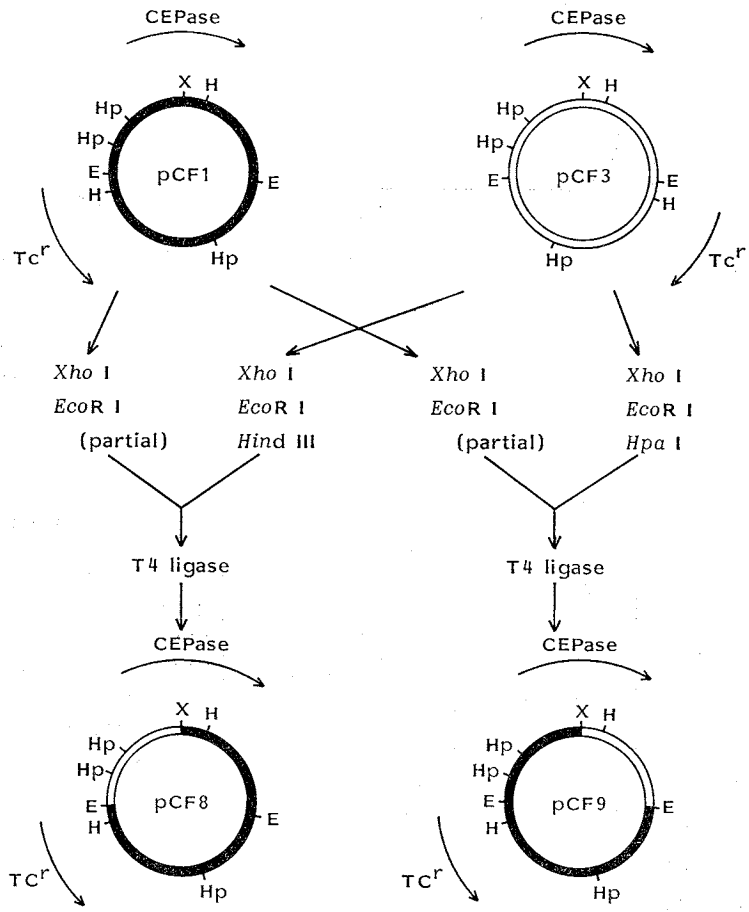


Table 4. Cephalosporinase activities of *Escherichia coli* strains carrying chimeric plasmids.

Plasmid	Specific activity of cephalosporinase (units per mg of protein)[a]
pCF1	2.01
pCF3	187
pCF8	145
pCF9	7.68

[a] One unit was defined as in Table 2.

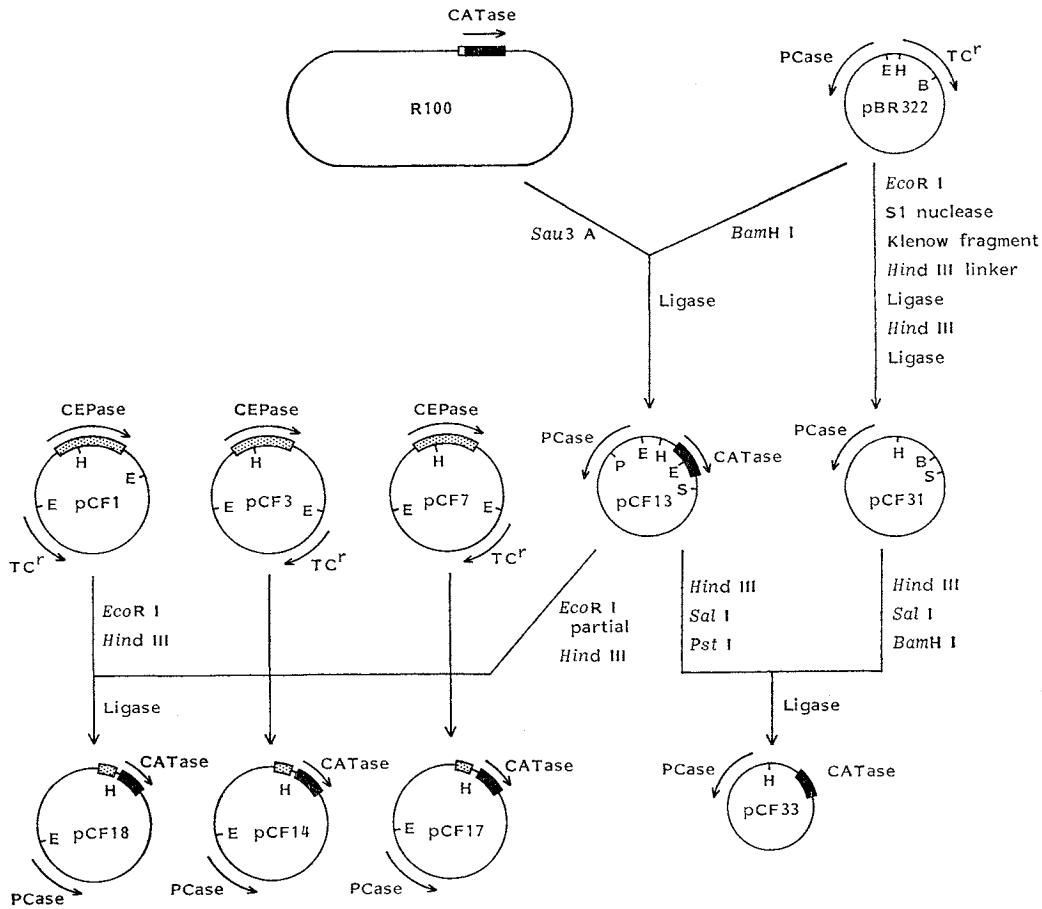
Table 5. Comparison of activities of the promoters.

Plasmid	Promoter	Promoter activity[a]
pCF18	*cep*K-12	1
pCF14	*cep*253	23~64
pCF17	*cep*801	33~68
pCF13	*tet*	17~25
pCF12	*cat*	36~73
pCF33	—	0.1

[a] Promoter activity was expressed as the ratio of chloramphenicol acetyltransferase activity to penicillinase activity in plasmid-carrying strains of *Escherichia coli*. The value of a strain carrying pCF18 was taken as 1.
—: Promoterless.

gulatory sequence. To ensure this claim, we developed the plasmids, pCF13 and pCF33, which enable us to estimate the strength of a promoter (Fig. 5). These plasmids were made from pBR322 and R100, the former of which provides the replication origin and penicillinase gene

Fig. 5. Construction of the vectors for analyzing promoter activity.
Details of the method are described in the text.
CEPase: Cephalosporinase, CATase: chloramphenicol acetyltransferase, PCase: penicillinase, TC: tetracycline, ^{r}: resistance, H: *Hin*d III, E: *Eco*R I, B: *Bam*H I, S: *Sau*3 A, P: *Pst* 1.



(*amp*) and the latter of which provides the chloramphenicol acetyltransferase gene (*cat*). The chloramphenicol acetyltransferase gene cleaved with *Sau*3A from R100 lacks its own promoter sequence. However, the *cat* gene in pCF13 was expressed by the promoter for the tetracycline resistance gene (*tet*) as an alternative for its own promoter whereas *cat* gene in pCF33 could not be expressed owing to the lack of the *tet* promoter. To compare the activity of the various promoters of the cephalosporinase genes of *E. coli*, we constructed pCF18, pCF14 and pCF17 by substituting the *tet* promoter in pCF13 with the DNA fragment containing the promoters of the cephalosporinase genes derived from *E. coli* K-12, No. 253 and No. 801, respectively. Promoter activity was estimated as the ratio of chloramphenicol acetyltransferase activity to penicillinase activity of a strain possessing the plasmid concerned. Division by penicillinase activity was introduced to cancel the effect of copy number and deletion of the plasmid. The results showed that the promoter activities of the cephalosporinase genes isolated from the cephem-resistant clinical isolates of *E. coli* were increased by more than 23~33-fold compared to that of the cephalosporinase gene of *E. coli* K-12 (Table 5). We concluded that the enhancement of cephalosporinase activity in the cephem-resistant clinical

isolates resulted from strengthened promoter activity of their cephalosporinase genes.

Discussion

We isolated and characterized the genes for the cephalosporinases of the cephem-resistant clinical isolates of *E. coli*. Our results indicated that the cephem-resistance of those clinical isolates of *E. coli* stems from increased expression of the chromosomal cephalosporinase gene. The promoter exchange experiments showed that this increased expression is due to up-promoter mutations. Similar ampicillin-resistant clinical isolates of *E. coli* were isolated and characterized by NORMARK *et al.*[15~17] They showed that these ampicillin-resistant clinical isolates of *E. coli* were classified into two groups. One arose from the double mutations which occurred both in the attenuator and promoter regions. The attenuator mutation resulted in a loss of the growth-rate-dependent control.[18] The other group achieved a higher level of expression of the β-lactamase by the mutation in the promoter region alone, which was assumed to be generated by spontaneous point mutations or horizontal chromosomal DNA transfer from *Shigella*. Both groups of the clinical isolates produced 24- to 48-fold increased levels of the chromosomal β-lactamase, which were similar to those of the isolates studied by us. It remains undetermined as to whether the mutational events in our isolates were similar or not to those studied by NORMARK. However, it may be pointed out that the expression of the β-lactamase of the clinical isolate No. 253 was 70-fold higher than that of *E. coli* K-12. This value is 2-fold higher than those for the isolates studied by NORMARK group, reflecting the MIC of ampicillin against No. 253 which is 2- to 4-fold higher than those against their isolates. These results suggested the possibility of some differences in the mutations of our strains and those studied by NORMARK.

Lastly, it should be noted that most of the cephem-resistant clinical isolates of *E. coli* are still within the efficacy range of the so-called third generation cephalosporins.

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