INDUCTION OF β-LACTAMASE AND PENICILLIN-BINDING PROTEINS IN *ESCHERICHIA COLI* BY INTRODUCTION OF *STREPTOMYCES* DNA

Hisayoshi Nakazawa, Michiko M. Nakano and Hiroshi Ogawara*

Department of Biochemistry, Meiji College of Pharmacy,
Nozawa 1-35-23, Setagaya-ku, Tokyo 154, Japan

(Received for publication June 29, 1983)

Introduction of hybrid plasmids, which were constructed by ligation of pCR1 or pMN1 vector plasmid and *Sal*I restriction endonuclease cleaved segments of *Streptomyces cacaoi* chromosome, resulted in the production of new β-lactamase and penicillin-binding protein in *Escherichia coli*. The β-lactamase and penicillin-binding protein were not from *S. cacaoi* but rather induced by the plasmids. Close relationship was observed between plasmids and penicillin-binding proteins but not with β-lactamase.

Streptomyces cacaoi strain KCC S-0352 produces an acidic β-lactamase constitutively and extracellularly[1], and this enzyme was purified and characterized[2]. In addition, penicillin-binding proteins (PBP) of this strain were examined in detail[3~7]. In order to clarify further the relationships between β-lactamase, PBP and β-lactams in *Streptomyces*, we tried to clone the β-lactamase gene of *S. cacaoi.*

In this paper, we describe the induction of β-lactamase and PBP in *Escherichia coli* in the process of transformation with chromosomal DNA of *S. cacaoi.*

Materials and Methods

Enzymes

Restriction endonucleases and T4 DNA ligase were purchased from Takara Shuzo Co., Ltd.

Medium

Antibiotic medium 3 and Nutrient broth were obtained from Difco Laboratories. L-Broth consisted of 1% Polypeptone, 0.5% yeast extract, 0.1% glucose and 0.5% NaCl (pH 7.3).

Chemicals

Agarose (Type II: Medium EEO) was purchased from Sigma Chemical Co., polyvinyl alcohol (p.d. 500) from Nakarai Chemicals Ltd., and carrier ampholite from LKB produkter AB (Ampholine pH 3.5~5, pH 5~7, pH 7~9). [^{14}C]Benzylpenicillin potassium (50 mCi/mmol) and deoxyadenosine 5′-[α-^{32}P]-triphosphate triethylammonium salt (3,000 mCi/mmol) were obtained from the Radiochemical Centre, Amersham. Nitrocellulose filter (BA 85, 0.45 μm pore diameter) was obtained from Scheicher and Schüll. Nitrocefin was a kind gift of Dr. M. Matthew of Glaxo Research Ltd. Other chemicals and antibiotics were commercially available.

Bacterial Strains and Plasmids

E. coli C600 (*thi thr leu lac* r^-m^-) and a kanamycin-resistant plasmid pCR1[8] were provided by Dr. M. Yoshikawa. pSL1 was isolated from *Streptomyces lavendulae* KCC S-0985 as described previously[9,10]. A chimeric plasmid pMN1[11] (Km^r) was constructed between pCR1 and pSL1. *S. cacaoi* KCC S-0352 was provided by Dr. A. Seino of Kaken Chemical Co.

Preparation of DNA and Transformation

For preparation of plasmids, *E. coli* C600 cells harboring plasmids were grown to exponential phase in L-broth or antibiotic medium 3 in the presence of chloramphenicol (170 μg/ml) according to Cle-

WELL[12]. The plasmid DNAs were isolated as described by GUERRY *et al.*[13] and purified by vertical dye-buoyant density gradients[14]. Recombinant DNAs were screened by the method of KLEIN[15]. Chromosomal DNA segments from *S. cacaoi* were cloned into vector plasmids pCR1 and pMN1 by the *Sal*I-ligase method[16]. Transformation was performed according to NORGARD *et al.*[17] Transformants were selected first as follows: After uptake of DNA the cells were diluted 10 times into L-broth or antibiotic medium 3 and incubated for 1 hour. Then the cells were collected by centrifugation, washed with 0.8% NaCl solution (PSS), and suspended with PSS.

Transformed cells were incubated on agar plates of antibiotic medium 3 supplemented with 50 μg/ml of kanamycin and 20 μg/ml of ampicillin. β-Lactamase-positive clones were detected on polyvinyl alcohol plate[18] or by nitrocefin[19].

Assay Methods

β-Lactamase activities were determined iodometrically in the supernatant of the cultured broth or after destruction of cells by sonication[8]. Isoelectric points[1] were measured on a sheet of polyacrylamide gel. Preparation of membrane fractions and binding of [^{14}C]benzylpenicillin to PBPs were described before[8].

Results and Discussions

When hybrid plasmids constructed by the *Sal*I-ligase method were transformed into *E. coli*, 3 clones were detected on plates containing 20 μg/ml ampicillin and 50 μg/ml kanamycin. These strains produced β-lactamase and were designated as RCA2, RMA13 and RMA27. RCA2 was obtained after transformation with pCR1 as a vector plasmid and the other two strains with pMN1. We mainly used RCA2 and RMA27 in further experiments.

The electrophoretic patterns of the isolated plasmid DNAs are shown in Fig. 1. The molecular sizes of the plasmids from RCA2 and RMA27 were 10.5 and 10.9 megadaltons (Md), respectively, showing that 1.8 and 0.9 Md DNA segments were inserted in the plasmids from RCA2 and RMA27. Whether those segments came from chromosomal DNA of *S. cacaoi* were tested by SOUTHERN hybridization[20,21]. ^{32}P-Labeled RMA27 plasmid probe could hybridize with total DNA of *S. cacaoi* (Fig. 2, lane 2~5), but not chromosomal DNA of *E. coli* C600 (lane 10) nor *Hin*dIII fragments of bacteriophage λ (lane 1). As hybridization to restriction enzyme cleaved total DNA of *S. cacaoi* generated discrete bands (lane 3~5), a possibility of non-specific hybridization could be excluded. Also the ^{32}P-labeled RCA2 plasmid probe showed similar autoradiographic patterns (data not shown). These results indi-

Table 1. β-Lactamase activities of *E. coli* with or without plasmid.

Strain	Plasmid	Enzyme activity (OD 620 nm)		
		Substrate		(%)*
		Benzylpenicillin	Methicillin	
C600	−	ND**	ND	ND
RCA2	+	0.38	0.18	47
RMA13	+	0.78	0.12	15
RMA27	+	1.23	0.29	23
C600A	pCR1	ND	ND	ND
C600I3	pMN1	ND	ND	ND
S-0352***	−			73

* Rate of hydrolysis of methicillin was expressed as a percentage of hydrolysis of benzylpenicillin (penicillin G).

** Not detected (less than 0.06).

*** *S. cacaoi*, S-0352, the data was cited from reference 2.

Fig. 1. Agarose gel electrophoresis of isolated hybrid plasmids.

1. pCR1, 2. RCA2 plasmid, 3. pMN1, 4. RMA-27 plasmid.

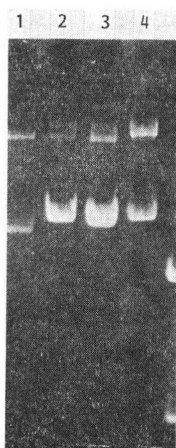


Table 2. Minimum inhibitory concentrations (μg/ml) of antibiotics against *E. coli* strains.

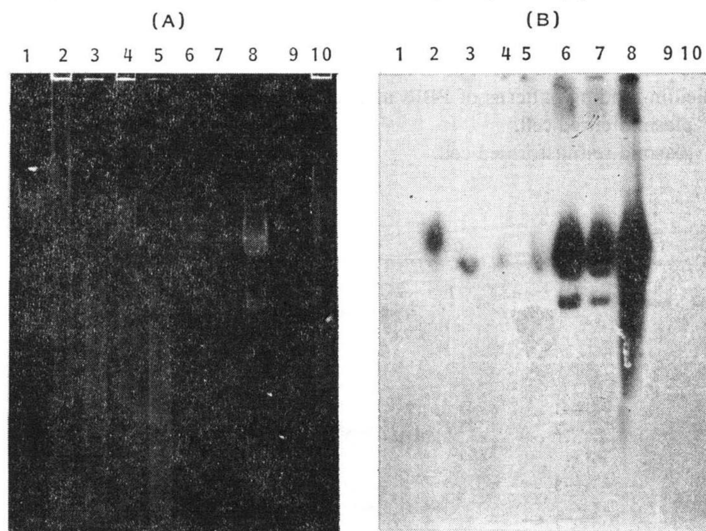
Antibiotics	Strain		
	C600	RCA2	RMA27
Kanamycin	0.78	100∼200	200
Ampicillin	1.56	200	200
Methicillin	1.56	100∼200	200
Mecillinam	1.56	3.12	3.12
Cephamycin C	6.25	6.25	6.25
Cefotiam	0.39	0.78	0.39
Cefazolin	0.78	0.78	1.56
Y-G19Z-GG	0.39	1.56	1.56
Latamoxef	0.39	0.39	0.78
Streptomycin	3.12	1.56	3.12
Tetracycline	1.56	1.56	1.56
Chloramphenicol	0.78	0.78	0.78

Fig. 2. Analysis of DNA by SOUTHERN hybridization.

One μg of the plasmid DNAs or 2 μg of *S. cacaoi* or *E. coli* C600 chromosomal DNAs were applied onto 0.7% agarose gels and stained with ethidium bromide (A).

1. λ-*Hin*dIII fragments, 2∼5. *S. cacaoi* chromosomal DNA (3. *Kpn*I digested, 4. *Bgl*II digested, 5. *Sal*I digested,) 6. RCA2 plasmid, 7. RMA27 plasmid (probe), 8. pMN1, 9. No sample, 10. *E. coli* C600 chromosomal DNA.

DNA from agarose gels was transferred to nitrocellulose papers and hybridized to ^{32}P-labeled RMA27 plasmid which was nick-translated with [α-^{32}P]dATP (B).



cate that the inserts in the hybrid plasmids derive from chromosomal DNA of *S. cacaoi* but not from *E. coli*.

Table 1 shows β-lactamase activities of cultured supernatant of *E. coli*. Negligible amount of enzyme activity was detected in the host strain C600. On the other hand, three transformants, RCA2, RMA13 and RMA27 showed much higher β-lactamase activity. This activity was due to the introduction of DNA of *S. cacaoi* because β-lactamase activity was not detected in the strains carrying only vec-

tor plasmids such as pCR1 and pMN1 (Table 1).

This was confirmed by the results that retransformation of *E. coli* C600 with the hybrid plasmids resulted in the production of similar amounts of β-lactamase (data not shown). However, to our great surprise, the substrate specificity was different from that of *S. cacaoi* enzyme[2,22] as shown by a different hydrolysis rate of methicillin to benzylpenicillin (Table 1). In addition, isoelectric points of the β-lactamases from RCA2, RMA13 or RMA27 were about 9.8 in contrast to 4.7 of *S. cacaoi* enzyme[2]. Therefore it was suggested that introduction of hybrid plasmids such as RCA2, RMA13 and RMA27 plasmids induced β-lactamase production in *E. coli*.

MIC values of various antibiotics are shown in Table 2. Although MIC values of kanamycin, ampicillin and methicillin increased very much, those of other antibiotics did not change significantly. This indicated that resistance to ampicillin was not due to the change of the permeability.

Therefore we analyzed the PBP patterns of the strains. A new PBP (PBP 3*, molecular weight, MW 55,000) appeared in RCA2, RMA13 and RMA27 strains (Fig. 3A). Its molecular weight was similar to that of PBP 4 in *S. cacaoi*. However the affinity patterns of various β-lactams to PBPs were different between them (data not shown). Thus, PBP 3* was not coded by the plasmids but rather induced by it.

In order to examine correlation between the plasmids and induction of β-lactamase and PBP, we tried to eliminate the plasmids with SDS[23]. No progeny could be obtained which showed no β-lactamase production. Further, 10 to 15% of the progeny produced a small amount of β-lactamase, although no plasmid could be detected (42 among 402 in the case of RCA2, and 48 among 320 in the case of

Fig. 3. Penicillin-binding profile.

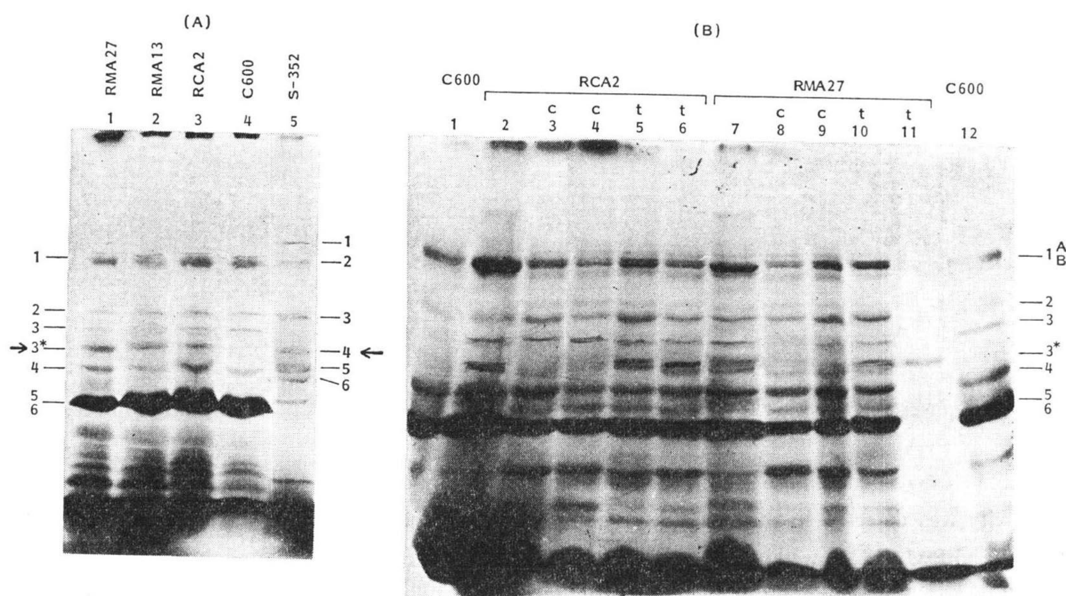
(A) Penicillin-binding patterns of transformants. PBPs were prepared from the membrane of the strains as indicated at the top.

PBP 3* of *E. coli* and PBP 4 of *S. cacaoi* are shown by arrows.

(B) Penicillin-binding patterns of PBPs after curing and retransformation

c: plasmid cured cell.

t: plasmid retransformed cell.



RMA27). In contrast to the case of β-lactamase, PBP 3* was eliminated by curing of the plasmids (Fig. 3B). Retransformation of the cured *E. coli* with RCA2 or RMA27 plasmid gave again PBP 3*. Especially in number 11 in Fig. 3B, almost all the PBP was PBP 3*. It was concluded therefore, that the DNA fragments from *S. cacaoi* play an important role in the induction of regulation of gene expression related to β-lactamase and especially PBP. This makes *E. coli* resistant to ampicillin and methicillin. A similar phenomenon was observed by OKANISHI (personal communication) where introduction of a *Streptomyces* plasmid DNA into *E. coli* led to the production of a new antibiotics related to tetracycline and named echomycin.

Acknowledgments

We thank Miss R. KANAI for helpful technical assistance. This work was supported in part by the Institute of Microbial Chemistry and Grants-in-Aids from The Ministry of Education, Science and Culture in Japan.

References

1) OGAWARA, H.; S. HORIKAWA, S. SHIMADA-MIYOSHI & K. YASUZAWA: Production and property of beta-lactamase in *Streptomyces*: Comparison of the strains isolated newly and thirty years ago. Antimicrob. Agents Chemother. 13: 865~870, 1978
2) OGAWARA, H.; A. MANTOKU & S. SHIMADA: β-Lactamase from *Streptomyces cacaoi*. J. Biol. Chem. 256: 2649~2655, 1981
3) OGAWARA, H. & S. HORIKAWA: Penicillin-binding proteins of *Streptomyces cacaoi*, *Streptomyces olivaceus* and *Streptomyces clavuligerus*. Antimicrob. Agents Chemother. 17: 1~7, 1980
4) OGAWARA, H. & S. HORIKAWA: Penicillin-binding proteins and antibacterial activities of β-lactams. J. Antibiotics 33: 620~624, 1980
5) HORIKAWA, S.; H. NAKAZAWA & H. OGAWARA: Penicillin-binding proteins in *Streptomyces cacaoi* and *Streptomyces clavuligerus*. Kinetics of [^{14}C]benzylpenicillin binding, temperature sensitivity and release of [^{14}C]benzylpenicillin from complex. J. Antibiotics 33: 1364~1368, 1980
6) NAKAZAWA, H.; S. HORIKAWA & H. OGAWARA: Penicillin-binding proteins in *Streptomyces* strains. J. Antibiotics 34: 1070~1072, 1981
7) NAKAZAWA, H. & H. OGAWARA: Mechanisms of penicillin-resistance in penicillin resistant mutants. J. Antibiotics 35: 1683~1691, 1982
8) COVEY, C.; D. RICHARDSON & J. CARBON: A method for the deletion of restriction sites in bacterial plasmid deoxyribonucleic acid. Molec. Gen. Genet. 145: 155~158, 1976
9) NAKANO, M. M.; K. OZAWA & H. OGAWARA: Isolation and characterization of a plasmid pSL1 from *Streptomyces lavendulae*. FEMS Microbiol. Lett. 9: 111~113, 1980
10) NAKANO, M. M.; Y. SHINDOH & H. OGAWARA: Further characterization of the pSL1 plasmid from *Streptomyces lavendulae*. FEMS Microbiol. Lett. 13: 279~281, 1982
11) NAKANO, M. M. & H. OGAWARA: Construction of *Streptomyces* and *Escherichia coli* composite plasmid *in vitro*. Seikagaku 58: 814, 1981
12) CLEWELL, D. B.: Nature of ColEl plasmid replication in *Escherichia coli* in the presence of chloramphenicol. J. Bacteriol. 110: 667~676, 1972
13) GUERRY, P.; D. J. LEBLANC & S. FALKOW: General method for the isolation of plasmid deoxyribonucleic acid. J. Bacteriol. 116: 1064~1066, 1973
14) STOUGAARD, P. & S. MOLIN: Vertical dye-buoyant density gradients for rapid analysis and preparation of plasmid DNA. Anal. Biochem. 118: 191~193, 1981
15) KLEIN, R. D.; E. SELSING & R. D. WELLS: A rapid microscale technique for isolation of recombinant plasmid DNA suitable for restriction enzyme analysis. Plasmid 3: 88~91, 1980
16) HORINOUCHI, S.; T. UOZUMI & T. BEPPU: Cloning of *Streptomyces* DNA into *Escherichia coli*: Absence of heterospecific gene expression of *Streptomyces* genes in *E. coli*. Agric. Biol. Chem. 44: 367~381, 1980
17) NORGARD, M. V.; K. KEEM & J. MONAHAN: Factors affecting the transformation of *Escherichia coli* strain χ1776 by pBR322 plasmid DNA. Gene 3: 279~292, 1978
18) SHERRATT, D. & T. COLLINS: Analysis by transformation of penicillinase system in *Bacillus licheniformis*. J. Gen. Microbiol. 76: 217~230, 1973

19) O'CALLAGHAN, C. H.; A. MORRIS, M. KIRBY & A. H. SHINGER: Novel method for detection of β-lactamase by using a chromogenic cephalosporin substrate. Antimicrob. Agents Chemother. 1: 283~288, 1972
20) SOUTHERN, E. M.: Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98: 503~517, 1975
21) RIGBY, P. W. J.; M. DIECKMAN, C. RHODES & P. BERG: Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. 113: 237~251, 1977
22) OGAWARA, H.: Antibiotic resistance in pathogenic and producing bacteria, with special reference to β-lactam antibiotics. Microbiol. Rev. 45: 591~619, 1981
23) TOMOEDA, M.; M. INUZUKA, Z. KUBO & S. NAKAMURA: Effective elimination of drug resistance and sex factors in *Escherichia coli* by sodium dodecyl sulfate. J. Bacteriol. 95: 1078~1089, 1968
24) BERGSTRÖM, S.; O. OLSSON & S. NORMARK: Common evolutionary origin of chromosomal β-lactamase genes in enterobacteria. J. Bacteriol. 150: 528~534, 1982