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Excretion of the Penicillinase of an Alkalophilic *Bacillus* sp. Through the *Escherichia coli* Outer Membrane

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Two plasmids containing the penicillinase gene of alkalophilic *Bacillus* sp. strain 170, pEAP1 and pEAP2, were constructed. Most of the penicillinase produced by *Escherichia coli*, which carried these plasmids, was found in the culture medium. This excretion is caused by the cloned DNA fragment which contains some component that changes the outer membrane of *E. coli*.

Recently, the gene for the penicillinase of *Bacillus licheniformis* 749/C was cloned in *Escherichia coli* (1, 4, 6), and it was found that 26 amino acids corresponded to the signal peptide which was important for secretion (2, 11).

In our laboratory, many alkalophilic bacteria which grow well at the high pH range (10.0 to 11.0) have been isolated (5). One of them, alkalophilic *Bacillus* sp. strain 170, produced penicillinase in an alkaline medium (17). The penicillinase gene of alkalophilic *Bacillus* sp. strain 170 DNA was cloned by using pMB9 in *E. coli*. This paper deals with an interesting observation which is indicated by the fact that most of the plasmid-borne penicillinase in *E. coli* was found in the culture medium.

E. coli K-12 strain HB101 (*pro leuB* B1 *lacY hsdR hsdM ara-14 galKZ xyl-5 mtl-1 supE44* F^- endoI$^-$ *recA* Str^r), *E. coli* C600 (*hsdR hsdM trpB leuB thr* B1), pBR322, and pMB9 were used.

Alkalophilic *Bacillus* sp. strain 170 was grown aerobically to the early stationary phase at 37°C in alkaline medium (17). Bacterial chromosomal DNA was purified by the method of Saito and Miura (13). The DNAs were digested with *Hin*dIII or *Eco*RI at 37°C for 1 h (pMB9) or for 4 h (chromosomal DNA). After the digestion, 1 μg of plasmid and 3 μg of bacterial chromosomal DNA were mixed and ligated with the T4 ligase overnight at room temperature.

The recombinant DNAs were introduced to *E. coli* C600 (9), and transformants having *Eco*RI or *Hin*dIII fragments were selected on LB-agar plates containing 20 μg of ampicillin per ml with or without 50 μg of tetracycline per ml. Penicillinase activity was detected by using the polyvinyl alcohol plate method (15). The yield of penicillinase-positive (penicillinase$^+$) transformants was 7×10^{-5}. A plasmid, pEAP1, was obtained from the transformant (Tc^r Ap^r penicillinase$^+$) which contained a 4.5-kilobase-pair (kb) *Eco*RI fragment of alkalophilic *Bacillus* sp. strain 170 DNA. Another plasmid, pEAP2, was isolated from the transformant (Ap^r penicillinase$^+$) containing a 2.4-kb *Hin*dIII fragment. Both plasmids could transform *E. coli* HB101 and yielded Ap^r penicillinase$^+$ transformants at a high frequency.

The cleavage maps of pEAP1 and pEAP2 are shown in Fig. 1. Neither the *Eco*RI fragment (4.5 kb) nor the *Hin*dIII fragment (2.4 kb) were digested by *Pst*I, *Bam*HI, *Sal*I, and *Sma*I. The 2.4-kb *Hin*dIII fragment which contained the penicillinase gene was located in the middle of the 4.5-kb *Eco*RI fragment.

By using the genomic hybridization method (12, 16), we found that radioactively labeled pEAP2 hybridized to the 4.5-kb *Eco*RI fragment of pEAP1 and the 2.4-kb *Hin*dIII DNA fragment from alkalophilic *Bacillus* sp. strain 170 on nitrocellulose filters. No sequences complementary to pEAP2 were detected in the *E. coli* DNA fragment.

The microorganisms were aerobically grown in LB-broth for 20 h at 37°C. The penicillinase activities (14) in extracellular, periplasmic, and cellular fractions (3) were assayed. Most of the penicillinase activity produced by *E. coli* HB101 (pEAP1 or pEAP2) and *E. coli* C600 (pEAP2) was found in the culture medium (Table 1). Less than 15% of the total activity was observed in the periplasmic and cellular fractions. But, almost all of the β-lactamase produced by *E. coli* HB101 (pBR322) was trapped in the periplasmic space.

The molecular weight of pEAP2-encoded penicillinase purified by carboxymethyl cellulose chromatography was 24,000 (Fig. 2). A 24,000-dalton protein in the culture broths of *E. coli* (pEAP1) and alkalophilic *Bacillus* sp. strain 170 reacted with the rabbit antiserum prepared against pEAP2-encoded penicillinase (18). The pEAP2-borne penicillinase had the following properties: The optimal pH was 6.0, and the

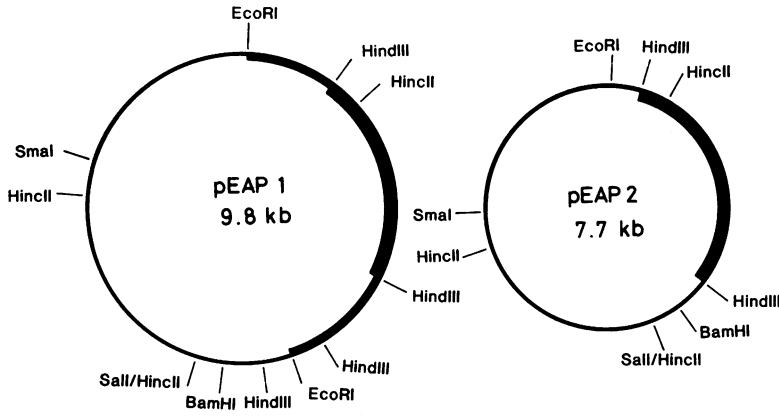

FIG. 1. Physical maps of plasmids pEAP1 and pEAP2. The thin lines represent the pMB9 DNA. The thick lines represent the penicillinase gene from alkalophilic *Bacillus* sp. strain 170 DNA.

stable pH range was broad, ranging from about 7.0 to 10.0. No significant differences were observed between *Bacillus* sp. strain 170 penicillinase and *E. coli* HB101 (pEAP2) penicillinase.

Distribution of β-galactosidase and alkaline phosphatase activities in *E. coli* HB101, which carried the plasmid, were also studied. Essentially, neither protein nor enzymatic activities was detected in the culture broths of *E. coli* HB101 or *E. coli* HB101 (pMB9) (Table 2). On the other hand, for *E. coli* HB101 (pEAP2), it is striking that 21% of the total protein, 58% of alkaline phosphatase, and 83% of penicillinase were found in the culture broth. About 87% of the β-galactosidase activity was, however, detected in the cellular fraction and not in the periplasmic fraction. These results suggest that

TABLE 1. Distribution of penicillinase in *E. coli*

Strain[a]	Penicillinase activity[b] in the following fractions:			Viable counts ($\times 10^9$)[c]
	Extracellular	Periplasmic	Cellular	
Alkalophilic *Bacillus* sp. strain 170	29.6		0.0	
E. coli HB101 (pEAP1)	6.5	0.3	0.4	2.2
E. coli HB101 (pEAP2)	16.7	0.3	1.7	1.8
E. coli C600 (pEAP1)	16.5	0.1	3.0	1.7
E. coli HB101 (pBR322)	4.0	21.7	6.4	

[a] *E. coli* strains were aerobically grown in the LB-broth [tryptone (Difco Laboratories), 10 g; yeast extract (Difco), 5 g; NaCl, 10 g; glucose, 1 g; and glycerol, 2 g; all in 1 liter of water] for 20 h at 37°C. Alkalophilic *Bacillus* sp. strain 170 was cultured in the alkaline medium (glycerol, 2 g; yeast extract (Difco), 5 g; polypeptone, 5 g; K_2HPO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.02 g; and $NaHCO_3$, 10 g; all in 1 liter of water) with continuous shaking for 13 h at 37°C, because under this condition, the best production of penicillinase was observed.

[b] Values represent units of penicillinase activity per milliliter of culture.

[c] Viable counts represent viable cells per milliliter of culture.

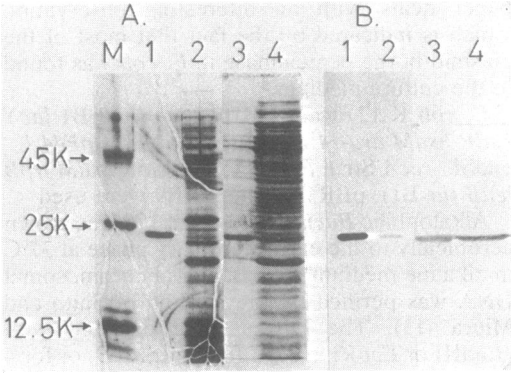

FIG. 2. Immunological blot identification on nitrocellulose paper of penicillinase produced in *E. coli* and alkalophilic *Bacillus* sp. strain 170. (A) Gel electrophoresis of bacterial proteins. Samples were electrophoresed on a 12% polyacrylamide gel as described previously (8). Lane M, Cytochrome C (molecular weight, 12,500), chymotrypsinogen A (molecular weight, 25,000), and albumin (molecular weight, 45,000) were used as molecular size markers. Lanes 1 and 3, Penicillinase (2 μg) purified from the culture of *E. coli* HB101 (pEAP2); lane 2, proteins (15 μg) from the culture of alkalophilic *Bacillus* sp. strain 170; lane 4, proteins (30 μg) from the culture of *E. coli* HB101 (pEAP1). (B) Immunological detection of penicillinase protein. An immunological assay kit (Bio-Rad Laboratories) was used. Lanes 1 through 4 are as described in (A).

TABLE 2. Distribution of enzymes in *E. coli* HB101[a] carrying plasmids

Plasmids and enzymes	Activity in the following fractions:		
	Extracellular (%)	Periplasmic (%)	Cellular (%)
None			
Protein[b]	0.04 (4)	0.07 (6)	0.97 (90)
APase[c]	0.02 (2)	1.05 (79)	0.26 (20)
β-gal[c]	0.03 (2)	0.00 (0)	1.20 (98)
pMB9			
Protein[b]	0.01 (1)	0.03 (4)	0.75 (95)
APase[c]	0.01 (2)	0.32 (67)	0.15 (31)
β-gal[c]	0.00 (0)	0.00 (0)	0.80 (100)
pEAP2			
Protein[b]	0.18 (21)	0.12 (14)	0.55 (65)
APase[c]	0.29 (58)	0.15 (30)	0.06 (12)
β-gal[c]	0.09 (10)	0.03 (3)	0.78 (87)
PCase[d]	10.70 (83)	0.40 (3)	1.80 (14)

[a] *E. coli* strains were aerobically grown in LB-broth for 20 h at 37°C.

[b] Protein concentration is expressed as milligrams in 1 ml of broth.

[c] Enzymatic activities of alkaline phosphatase (APase) and β-galactosidase (β-gal) are expressed as absorbance at 420 nm (7, 10).

[d] Penicillinase (PCase) activity is expressed as units per milliliter of broth.

the outer membrane of *E. coli* was changed by the introduction of pEAP2 into the cells, because a periplasmic enzyme, alkaline phosphatase, was released from the periplasmic space, and a typical cellular enzyme, β-galactosidase, was not secreted into the periplasm.

Two possibilities are considered at this point: (i) The plasmid-encoded penicillinase affects the outer membrane directly or indirectly and is excreted into the medium. (ii) The unknown gene product(s) from the 2.4-kb fragment affects the outer membrane, and proteins including penicillinase are excreted. Although there is no crucial experiment to verify this, it is clear that the cloned DNA fragment contains some component that changes the outer membrane of *E. coli*.

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