Nucleotide Sequence of the $\beta$-Lactamase Gene of Alkalophilic Bacillus sp. Strain 170

By CHIAKI KATO,* TOSHIAKI KUDO, KAZUHO WATANABE AND KOKI HORIKOSHI

The Riken Institute, Wako-shi, Saitama-ken 351-01, Japan

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A gene for the $\beta$-lactamase from alkalophilic Bacillus sp. strain 170 was cloned in a functional state on a 1.0 kb DNA fragment and its nucleotide sequence was determined. The coding sequence showed an open reading frame of 257 amino acids, which represents the $\beta$-lactamase precursor protein. It is considered that the signal peptide consisted of 30 amino acids including 12 hydrophobic amino acids.

INTRODUCTION

There have been several reports of the cloning of genes for extracellular enzymes from Bacillus strains. These include $\beta$-lactamases from B. licheniformis and B. cereus (Neugebauer et al., 1981; Sloma & Gross, 1983). Each gene in which the nucleotide sequence has been determined has a region coding for a signal peptide at the N-terminus. The signal peptide directs the protein through the cell membrane and is cleaved during the translocation process.

Recently, several genes of alkalophilic Bacillus sp. strains have been cloned in Escherichia coli or in B. subtilis (Kudo et al., 1983, 1985; Kato et al., 1984; Sashihara et al., 1984). $\beta$-Lactamase (penicillinase) is one of the secretory proteins produced by alkalophilic Bacillus sp. strain 170 (Sunaga et al., 1976). The DNA fragment containing the $\beta$-lactamase gene of this strain has been introduced into E. coli and has been shown to be strongly expressed (Kudo et al., 1983).

The nucleotide sequence of the $\beta$-lactamase gene is reported in this paper.

METHODS

Bacterial strains and plasmids. Alkalophilic Bacillus sp. strain 170 (FERM 3221), a producer of a $\beta$-lactamase in alkaline media, was isolated from soil (Sunaga et al., 1976). E. coli K12 strain HB101 Pro– leuB B, lacY hsdR– hsdM– ala–14 galK2 xyl–5 mtl–1 supE44 F− endoI− recA Str (Goldfarb et al., 1982) was used for transformation, and E. coli CSR603 thr–1 leuB6 proA2 phe–1 recA1 argE3 thi–1 uraA ara–14 lacY1 galK2 xyl–5 mtl–1 rpsL3I tsx–33 supE44 λ− F− (Sancar & Rupert, 1978) was used for the maxicell experiment.

Plasmids used were pBR325, pMB9 and pEAP2 (Kudo et al., 1983).

DNA preparation. Plasmid DNA was isolated from 1 litre of culture by the cleared lysate procedure (Boliver et al., 1977) and purified on a Bio-Gel A50 column (Blin & Stafford, 1976). Slab gel electrophoresis and electrophoresion conditions were as described by Lawn et al. (1981).

Enzymes. RNAase and lysozyme were purchased from Sigma. Restriction endonucleases, DNA polymerase I (Klenow fragment) and T4 DNA ligase were obtained from BRL, and used according to the methods suggested by the manufacturer.

Transformation. E. coli transformations were done by the calcium shock procedure (Hershfield et al., 1974). Transformants were selected on LB plates supplemented with antibiotics at the appropriate concentration (10 μg ampicillin ml$^{-1}$ and/or 50 μg tetracycline ml$^{-1}$).

Purification of $\beta$-lactamase and enzyme assay. Extracellular $\beta$-lactamase was purified from the culture filtrate of E. coli HB101 carrying pEAP2 as previously described (Kato et al., 1983). $\beta$-Lactamase was assayed by the method of Kudo et al. (1983).
Maxicells. For the identification of plasmid-encoded polypeptide, a modification of the maxicell method described by Sancar et al. (1979) was used. After UV irradiation, the cells were incubated at 37 °C with shaking in the presence of D-cycloserine (100 μg ml⁻¹) for 12-16 h. Labelling with L-[¹⁵S]methionine (5 μCi ml⁻¹; New England Nuclear) proceeded for 1 h, after which the cells were washed with Hershey salts (NaCl 5.4 g, KCl 3.0 g, NH₄Cl 1.1 g, CaCl₂ 2H₂O 15 mg, MgCl₂ 6H₂O 0.2 g, FeCl₃ 6H₂O 0.2 mg, KH₂PO₄ 87 mg and Trizma base 12.1 g per litre of water, pH 7.4), suspended in the lysis buffer and loaded directly onto the gel.

Amino acid analysis and sequencing. Amino acid analyses were done on a JEOL JLC-6AH amino acid analyser after hydrolysis of the protein samples in 6 M-HCl at 110 °C for 24 h. For the determination of methionine and cysteine, cyanogen bromide degradation and performic acid oxidation were used. The N-terminal amino acid sequence was determined by a Beckman Protein/Peptide Sequencer 890-M (Edman & Henschen, 1975). Amino acid phenylthiohydantoins were identified by a Hitachi 655 high pressure liquid chromatography system.

DNA sequencing. DNA sequencing was done by the dideoxy chain termination method (Sanger et al., 1977) using a long sequencing gel (80 × 20 cm) if necessary. Specific restriction fragments were cloned into the mp8 or mp9 M13 vectors for dideoxy sequencing (Messing, 1981) by using [γ-³²P]dATPdS (Amersham). The sequencing was done in both directions.

RESULTS

DNA sequencing

The gene for the β-lactamase of alkalophilic Bacillus sp. strain 170 was cloned in E. coli with pMB9. The plasmid pEAP2 thus constructed contained a 2.4 kb HindIII fragment (Kudo et al., 1983). The β-lactamase gene in the 2.4 kb HindIII fragment from pEAP2 was recloned, and was found to be located in the 1.0 kb DraI fragment. The restriction endonuclease map of the 1.0 kb DraI fragment from pEAP2 is shown in Fig. 1, and the nucleotide sequence of the β-lactamase gene in Fig. 2. Analysis of the sequence showed that there was a single open reading frame of 771 bp, from which a protein of molecular mass 27 kDal could be translated.

 Determination of amino acid sequences

Amino acid compositions of the extracellular form of β-lactamase are presented in Table 1. The values obtained by analysis of amino acids were mostly consistent with those calculated from the DNA sequence. Edman degradation showed that the sequence of N-terminal amino acids of the extracellular β-lactamase was NH₂Ser-Gln-Lys-Val-Glu-Gln-Ile-Val-Ile-Lys-Asn-
8-lactamase gene from alkalophilic Bacillus sp.

**Fig. 2.** Complete nucleotide sequence of the alkalophilic Bacillus sp. strain 170 \(\beta\)-lactamase gene and the primary structure of its product. The 18 amino acids corresponding to the determined amino terminus of the extracellular \(\beta\)-lactamase are underlined. The amino acids are numbered taking the N-terminal amino acid of the mature protein as (1). The putative terminator sequence is underlined. The Shine-Dalgarno (SD) sequence is indicated by broken lines.

Glu-Thr-Gly-Thr-Ile-Ser-Ile. This amino acid sequence is identical to that deduced from the DNA sequence. Therefore, 30 amino acid residues (residues -30 to -1) are considered to represent the signal peptide which is removed during secretion of the \(\beta\)-lactamase.

**Analysis of the gene products**

The maxicell strain *E. coli* CSR603 carrying pBR325, pMB9 or pEAP2 was irradiated with UV light to destroy the chromosomal DNA. Proteins encoded by the plasmids in these strains were analysed on SDS-polyacrylamide gels after incorporation of \(\text{L-[\text{\textsuperscript{35}}S]}\) methionine (Fig. 3). No incorporation of label into protein occurred in the parent strain CSR603 after irradiation. The strain carrying pBR325 produced proteins of molecular mass 35 kDal, 28 kDal and 23 kDal,
corresponding to the products of the Tc', Ap' and Cm' genes, respectively. A Tc' gene product was also detected in the strain carrying pMB9. Cells carrying pEAP2, which contained the insert in the Tc' gene of pMB9, lacked the 35 kDal protein, and bands of about 27 kDal and 24 kDal, which corresponded to the precursor and extracellular β-lactamase, were observed.

**DISCUSSION**

We have reported the sequencing of the gene for β-lactamase from alkalophilic Bacillus sp. strain 170. The identification of this gene as that for β-lactamase is unambiguous because (1) the cloned DNA can transform a Pen' form to Pen' (Kudo et al., 1983) and (2) it contains a codon
sequence of 18 amino acids deduced to be identical to the determined N-terminal sequence of the mature β-lactamase.

The molecular mass of the protein derived from the 771 bp open reading frame beginning with the first ATG coincided with the molecular mass of the precursor β-lactamase. The precursor and mature β-lactamase possess three and two methionine residues respectively. Considering this, the ratio of precursor to mature β-lactamase was about 1 in this maxicell system (Fig. 3). The precursor and mature β-lactamase were observed in this system, suggesting that the enzyme was processed by an E. coli processing system.

The precursor β-lactamase possesses an extra, 30 amino acid, peptide, corresponding to the difference in molecular weight between the precursor and mature β-lactamase. This initial peptide has a sequence that has similar properties to other identified signal peptides (Kreil, 1981). It has a segment that is basic, with lysine residues at −29 and −28, followed by a series of hydrophobic amino acids. The residue at the cleavage site (residue −1) is alanine. The signal sequence of the β-lactamase was compared with those of β-lactamases from other Bacillus strains (Fig. 4). A proportion of the β-lactamases from Gram-positive organisms are membrane-bound (Nielsen & Lampen, 1982). In B. licheniformis this binding is achieved through a glyceride thioether modification of the cysteine in the signal peptide sequence (Lai et al., 1981). A cysteine residue was also observed in the signal sequence of the alkalophilic Bacillus sp. strain 170 β-lactamase. The amino acid sequence around the cysteine of the β-lactamase is Val-Gly-Leu-Cys-Val. This sequence is similar to that reported for B. cereus β-lactamase I (Ile-Gly-Ile-Cys-Val) but different from that reported for B. licheniformis β-lactamase (Leu-Ala-Gly-Cys-Ala).

From the DNA sequence analysis, the β-lactamase gene of alkalophilic Bacillus sp. strain 170 had no homology with that of B. licheniformis, but had partial homology with that of B. cereus in
A.

\[ 2\text{-TGA}\cdots\text{AA}\text{GATGCTGTTAAAAGATCGATATGTGTAAGTTTACTAGGA-51} \]

\[ 8\text{-TGA}\cdots\text{AA}\text{GATGCTGTTAAAAGATCGATATGTGTAAGTTTACTAGGA-51} \]

B.

\[ 286\text{-GA}\cdots\text{ACT}\cdots\text{AATAGAAATGCGTAAGGATGCTAAATATATGTAAGTTTACTAGGA-51} \]

\[ 397\text{-GATACCTGGAATGGAACAGAATGCGTAAGGATGCTAAATATATGTAAGTTTACTAGGA-51} \]

C.

\[ 538\text{-AC}\cdots\text{AGA}\cdots\text{ACTGCGTTAAAAGATCGATATGTGTAAGTTTACTAGGA-51} \]

\[ 781\text{-ACAAGAA-ATGATATTGCTGTC-}\cdots\text{GGCGTAGGCTGAATATATGTAAGTTTACTAGGA-51} \]

Fig. 5. DNA sequence comparison between the alkalophilic Bacillus sp. strain 170 β-lactamase gene [I] and the B. cereus β-lactamase I gene [II]. A, B, and C show three regions containing partial DNA homology. Homologous nucleotides are connected with ↔. The nucleotides are numbered taking the first nucleotide of the initiation codon as (1). The sequences of the B. cereus gene are taken from Sloma & Gross (1983).

A.

\[ (103) \text{IleLysAlaHisSerThrAlaLeuThrAlaGluLeuAla}^{(116)} \text{Lys} \]

\[ (111) \text{IleLysAlaHisSerThrAlaLeuThrAlaGluLeuAla} \]

B.

\[ (135) \text{PheGlyAsnThrLysValGluThrPheTyrProGlyLysGlyHis}^{(149)} \]

\[ (141) \text{PheGlyAsnMetLysValGluThrPheTyrProGlyLysGlyHis} \]

C.

\[ (163) \text{IleLeuAlaGlyGlyCysLeuValLysSerAlaGluLeuAla}^{(177)} \text{Asn} \]

\[ (177) \text{IleLeuValGlyGlyCysLeuValLysSerThrSerAlaLysAsp} \]

D.

\[ (202) \text{Asn}^{(216)} \text{IleAsnLeuValValProGlyHisGlyLysValGlyAspLys} \]

\[ (216) \text{Asn}^{(216)} \text{IleAsnLeuValValProGlyHisGlyLysValGlyAspLys} \]

Fig. 6. Amino acid sequence homology between the sequence around the histidine and cysteine residues of the alkalophilic Bacillus sp. strain 170 β-lactamase [I] and the B. cereus β-lactamase II (class B enzyme) [II]. A, B, C and D show four regions of amino acid sequences around the histidine and cysteine residues. Homologous amino acids are connected with ↔. The amino acids are numbered taking the N-terminal amino acid of the mature protein as (1). The amino acid sequences of B. cereus β-lactamase II are taken from Ambler (1980).

three regions (Fig. 5). One of these was found in the signal peptide region of the gene (Fig. 5 A). The β-lactamase of alkalophilic Bacillus sp. strain 170 looks like a class B enzyme according to the classification system proposed by Ambler (1980), because strong homology was observed between the sequences around the histidine and cysteine residues of B. cereus β-lactamase II (Ambler, 1980) and the amino acid sequence of β-lactamase of alkalophilic Bacillus sp. strain 170 (Fig. 6). These results support the idea that alkalophilic Bacillus sp. strain 170 is closely related to B. cereus.
An examination of the sequences preceding and following the coding regions shows several features of interest. The initial methionine is preceded by a possible ribosome binding site sequence (near nucleotide -10) which has homology with B. subtilis 16S rRNA (Fig. 2).

Following the termination codon of β-lactamase there is one inverted repeat structure (nucleotides 792–823) which could play a role in transcription termination. This sequence is followed by a T-rich region which is similar to termination structures seen in E. coli (Rosenberg & Court, 1979).

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**REFERENCES**


