Nucleotide Sequence of the β -Lactamase Gene of Alkalophilic *Bacillus* sp. Strain 170

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A gene for the β -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 β -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 β -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). β -Lactamase (penicillinase) is one of the secretory proteins produced by alkalophilic *Bacillus* sp. strain 170 (Sunaga *et al.*, 1976). The DNA fragment containing the β -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 β -lactamase gene is reported in this paper.

METHODS

Bacterial strains and plasmids. Alkalophilic *Bacillus* sp. strain 170 (FERM 3221), a producer of a β -lactamase in alkaline media, was isolated from soil (Sunaga *et al.*, 1976). *E. coli* K12 strain HB101 Pro⁻ leuB⁻ lacY⁻ hsdR⁻ hsdM⁻ ala-14 galK2 xyl-5 mtl-1 supE44 F⁻ endoI⁻ recA Str^r (Goldfarb *et al.*, 1982) was used for transformation, and *E. coli* CSR603 thr-1 leuB6 proA2 phr-1 recA1 argE3 thi-1 uvrA6 ara-14 lacY1 galK2 xyl-5 mtl-1 rpsL31 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 electroelution 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 μ g ampicillin ml⁻¹ and/or 50 μ g tetracycline ml⁻¹).

Purification of β -lactamase and enzyme assay. Extracellular β -lactamase was purified from the culture filtrate of *E. coli* HB101 carrying pEAP2 as previously described (Kato *et al.*, 1983). β -Lactamase was assayed by the method of Kudo *et al.* (1983).

Fig. 1. Restriction map of the region surrounding the β -lactamase gene, and sequencing strategy. The cloned insert of pEAP2 is indicated as the 2.4 kb *Hind*III fragment. The filled box indicates the coding sequence for the mature protein, and the open box indicates the coding sequence of the signal peptide. The horizontal arrows indicate the sequencing strategy.

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 μ g ml⁻¹) for 12–16 h. Labelling with L-[³⁵S]methionine (5 μ Ci 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 phenylthiohydantoin 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 [α -³⁵S]dATP α S (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 *Hind*III fragment (Kudo *et al.*, 1983). The β -lactamase gene in the 2.4 kb *Hind*III fragment from pEAP2 was recloned, and was found to be located in the 1.0 kb *Dra*I fragment. The restriction endonuclease map of the 1.0 kb *Dra*I 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 kDa 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-

-134 DraI
 TTTAAAGCGTACAAAATTTTGTACGCTTTTTTGTAAATTACATAAAAGTATGCAAATGA
 -75
 AGATGGAACAAACATTTGAGATGAATTGTCTAATATAGGTAATACTATTTAGCTTGAAAGAAAGGGTTGATAAAC
 SD 75
 1
 ATGAAAAAGAATACGTTGTAAAAAGTAGGATTATGTGTAAGTTTACTAGGAACAACTCAATTTGTAGCACGATT
 MetLysLysAsnThrLeuLeuLysValGlyLeuCysValSerLeuLeuGlyThrThrGlnPheValSerThrIle
 (-30)
 150
 TCTTCTGTACAAGCATCACAAAAGGTAGAGCAAATAGTAATCAAAAATGAGACGGGAACCATTTCAATATCTCAG
 SerSerValGlnAlaSerGlnLysValGluGlnIleValIleLysAsnGluThrGlyThrIleSerIleSerGln
 (1)
 225
 TTAACAAGAATGTATGGGTTTCATACGGAGTTAGGTTATTTTAATGGAGAAGCAGTTTCCTTCGAACGGTCTAGTT
 LeuAsnLysAsnValTrpValHisThrGluLeuGlyTyrPheAsnGlyGluAlaValProSerAsnGlyLeuVal
 300
 CTTAATACTTCTAAAGGGCTAGTACTTGTGATTCTTCTGGGATAACAAATTAACGAAGGAATAATAGAAATG
 LeuAsnThrSerLysGlyLeuValLeuValAspSerSerTrpAspAsnLysLeuThrLysGluLeuIleGluMet
 375
 GTAGAAAAGAAATTCAGAAGCGCGTAACAGATGTCATTATTACACATGCGCACGCTGATCGAATTGGCGGAATA
 ValGluLysLysPheGlnLysArgValThrAspValIleIleThrHisAlaHisAlaAspArgIleGlyGlyIle
 450
 ACAGCGTTGAAAGAAAGAGGCATTAAAGCGCATAGTACAGCATTAAACGCAGAAGTACGAAAGAAAGTGGATAT
 ThrAlaLeuLysGluArgGlyIleLysAlaHisSerThrAlaLeuThrAlaGluLeuAlaLysLysSerGlyTyr
 525
 GAAGAGCCACTTGGAGATTTACAAACAGTTACGAATTTGAAGTTTGGCAATACAAAAGTAGAAACGTTCTATCCA
 GluGluProLeuGlyAspLeuGlnThrValThrAsnLeuLysPheGlyAsnThrLysValGluThrPheTyrPro
 600
 GGGAAAGGACATACAGAAGATAATATTGTTGTTTGGTTGCCACAATATCAAATTTTAGCTGGAGGCTGTTTAGTA
 GlyLysGlyHisThrGluAspAsnIleValValTrpLeuProGlnTyrGlnIleLeuAlaGlyGlyCysLeuVal
 675
 AAATCTGCGGAAGCTAAAAATTTAGGAAATGTTGCGGATGCGTACGTAAATGAATGGTCCACATCGATTGAGAAT
 LysSerAlaGluAlaLysAsnLeuGlyAsnValAlaAspAlaTyrValAsnGluTrpSerThrSerIleGluAsn
 750
 ATGCTGAAGCGATATAGAAATATAAATTTGGTAGTACCTGGTCACGGGAAAGTAGGAGACAAGGGATTACTTTTA
 MetLeuLysArgTyrArgAsnIleAsnLeuValValProGlyHisGlyLysValGlyAspLysGlyLeuLeuLeu
 825
 CATACATTGGATTTATTAATAAATAAGAAATTTAGTAAATACAAAAGAGAGAGAAATAATTTCTCCTCTCTTTCT
 HisThrLeuAspLeuLeuLys***
 (227)
 842
 TTTCAACTATATTTAAA
DraI

Fig. 2. Complete nucleotide sequence of the alkalophilic *Bacillus* sp. strain 170 β -lactamase gene and the primary structure of its product. The 18 amino acids corresponding to the determined amino terminus of the extracellular β -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 β -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 L-[³⁵S]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 kDa, 28 kDa and 23 kDa,

Fig. 3. Autoradiogram of [^{35}S]methionine-labelled proteins made in *E. coli* CSR603 (lane 1), and *E. coli* CSR603 carrying pBR325 (lane 2), pEAP2 (lane 3) and pMB9 (lane 4). Tc^r (35 kDal), Ap^r (28 kDal) and Cm^r (23 kDal) gene products from pBR325 were used as the molecular mass markers.

Table 1. *Amino acid composition of mature β -lactamase*

Amino acid	Amino acid analysis	From DNA sequence	Amino acid	Amino acid analysis	From DNA sequence
Lys	22	22	Ala	14	13
His	7	7	Cys	1	1
Arg	5	5	Val	20	21
Asx*	26	25	Met	2	2
Thr	18	17	Ile	13	14
Ser	12	12	Leu	26	26
Glx*	24	23	Tyr	5	6
Pro	3	5	Phe	4	4
Gly	20	20	Trp	ND	4

* Asx represents Asp and Asn; Glx represents Glu and Gln.
ND, Not determined.

corresponding to the products of the Tc^r, Ap^r and Cm^r genes, respectively. A Tc^r gene product was also detected in the strain carrying pMB9. Cells carrying pEAP2, which contained the insert in the Tc^r 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^s form to Pen^r (Kudo *et al.*, 1983) and (2) it contains a codon

Fig. 4. Comparison of the signal sequences of β -lactamase in *Bacillus* strains. A, *B. licheniformis* β -lactamase (Kroyer & Chang, 1981); B, *B. cereus* β -lactamase I (Mezes *et al.*, 1983); C, alkalophilic *Bacillus* sp. strain 170 β -lactamase. ♦, Cutting site of signal peptide (ExoL and ExoS indicate the processing sites for the primary and secondary exo- β -lactamase cleavage products, respectively) (Simons *et al.*, 1978; Izui *et al.*, 1980); M♦, cutting site that yields the membrane bound form of the enzyme; v, possible cutting sites of the signal peptide. Hydrophilic and basic amino acids near the N-terminal of the signal peptide are underlined. The pentapeptide around the cysteine residue is boxed (see discussion). The amino acids are numbered taking the N-terminal amino acid of the signal peptide as (1).

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

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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