β -Lactamase of *Lysobacter enzymogenes*: cloning, characterization and expression of the gene and comparison of the enzyme to other lactamases

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The gene for the periplasmic β -lactamase of *Lysobacter enzymogenes* was isolated as part of a 1017 bp *EcoRI* fragment and the nucleotide sequence of the gene was determined. It has a G + C content of 71.5% and encodes a 27 amino acid signal sequence and the mature β -lactamase of 276 amino acids which has a mass of 29 146 Da. The enzyme appears to be unique to *L. enzymogenes* but its amino acid sequence shows a high degree of homology with the amino acid sequences of the lactamase from *Citrobacter diversus* and other Class A β -lactamases. The β -lactamase gene of *L. enzymogenes* was expressed in *Escherichia coli* using pUC118 as the vector. The production of active β -lactamase was highest after the active growth phase of the expression host and reached levels which were about three times higher than those obtained with *L. enzymogenes*.

Introduction

Lysobacter enzymogenes produces a number of extra-cellular enzymes. Some are secreted into the medium and others are localized in the cell envelope. One of the cell-associated enzymes is an inducible, periplasmic β -lactamase (von Tigerstrom & Boras, 1990). This enzyme was found to have a molecular mass of about 28 kDa and a high isoelectric point, and its activity was inhibited by *p*-chloromercuribenzoate (*p*CMB), *p*-chloromercuribenzenesulphonic acid (*p*CMBS) and clavulanic acid. Based primarily on its substrate specificity, it was thought initially to belong to Group 2B, perhaps being similar in its properties to the β -lactamase of *Pseudomonas cepacia* (Bush, 1989).

A large number of chromosome- and plasmid-encoded β -lactamases have been reported from Gram-positive and Gram-negative bacteria and various schemes have been used to classify these enzymes (Ambler, 1980; Bush, 1989). They are produced by both pathogenic and non-

pathogenic organisms and have received more attention than most other groups of enzymes. One reason for this is their clinical importance, since they are often responsible for the resistance of bacteria to β -lactam antibiotics. In addition, the large amount of information which has been obtained about β -lactamases makes studies of the structure–function relationship and the molecular evolution of the enzymes possible. The classification scheme proposed by Ambler (1980) is based on the primary structures of β -lactamases. The amino acid sequences of more than 20 β -lactamases which belong to Class A according to this scheme are now known, most having been deduced from the DNA sequence of their respective genes. They have been used in comparative studies (Forsman *et al.*, 1990; Pastor *et al.*, 1990; Boissinot & Levesque, 1990; Huletsky *et al.*, 1990; Ambler *et al.*, 1991; Kirby, 1992) and, to standardize further comparisons, a consensus sequence has been proposed for enzymes of this Class (Ambler *et al.*, 1991).

L. enzymogenes is a non-pathogenic soil bacterium that probably does not encounter high enough concentrations of β -lactams in nature to achieve a significant induction of lactamase production. Thus, the physiological role of the enzyme for this organism is not clear. However, we thought it was important to describe the enzyme in detail to compare it to other lactamases and trace its origin. Furthermore, a comparison of the β -lactamase gene with the genes of the α -lytic protease (Epstein & Wensink, 1987; Silen *et al.*, 1989) and the

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Abbreviations: PADAC, 7-(thienyl-2-acetamido)-3-[4-*N,N*-dimethylaminophenylazo]-pyridinium methyl]-3-cephem-4-carboxylic acid; *p*CMB, *p*-chloromercuribenzoate; *p*CMBS, *p*-chloromercuribenzenesulphonic acid.

The nucleotide sequence data reported in this paper have been submitted to GenBank and assigned the accession number M97392.

secreted phosphatase (Au *et al.*, 1991) of *L. enzymogenes* should allow us to gain more insight into the secretion and the targeting of extracellular enzymes by this organism.

In this paper we report the cloning and characteristics of the *L. enzymogenes* β -lactamase gene. Most of the primary amino acid sequence of the enzyme was deduced from the nucleotide sequence. It belongs to Class A and a comparison of the enzyme with other β -lactamases indicates that it has a unique primary structure. The cloned gene was expressed in *Escherichia coli*.

Methods

Bacterial strains and plasmids. *Lysobacter enzymogenes* ATCC 29487 was used for the preparation of genomic DNA and for the production of β -lactamase. *Escherichia coli* LE392 (Murray *et al.*, 1977) was used to establish the recombinant λ phage library and *E. coli* MV1193 (Vieira & Messing, 1987) for the isolation of recombinant plasmids and the generation of single-stranded plasmid DNA. λ -DASH DNA (Sorge, 1988) was used to prepare the library of *L. enzymogenes* DNA, and pUC118 for subcloning restriction fragments and, together with phage M13K07, to generate single-stranded (ss) DNA (Vieira & Messing, 1987). pUC118 was also used to express the *L. enzymogenes* β -lactamase gene in *E. coli* MV1193.

Reagents and enzymes. The sources of restriction endonucleases, T4 DNA ligase, polynucleotide kinase, the large fragment of *E. coli* DNA polymerase, T7 DNA polymerase, [α - 32 P]dATP and [γ - 32 P]ATP and other reagents and chemicals have been reported previously (Au *et al.*, 1991; von Tigerstrom & Boras, 1990).

Culture conditions and β -lactamase assay. The maintenance of *L. enzymogenes* (von Tigerstrom & Stelmaschuk, 1987) and the conditions used to grow the organism for the production of β -lactamase (von Tigerstrom & Boras, 1990) have been described previously. Stock cultures of transformed *E. coli* cells were kept at -70°C in 0.8% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.25% (w/v) NaCl, 10% (w/v) glycerol. Genomic DNA was prepared from cells grown in 3% (w/v) trypticase soy broth. Culture conditions for *E. coli* LE392 and MV1193 have been described previously (Au *et al.*, 1991). For the production of β -lactamase by *E. coli* MV1193 transformed with pUC118 or with pGB-2a, the cells were grown aerobically at 37°C for 17 h in $2 \times \text{YT}$ [1.6% (w/v) tryptone, 1.0% (w/v) yeast extract, 0.5% (w/v) NaCl] containing 100 μg ampicillin ml^{-1} and 0.5 mM-IPTG. The cells were suspended in 50 mM-Tris/HCl, pH 7.5, disrupted in a French pressure cell and centrifuged at 3000 g for 15 min to prepare the cell-free extract. The β -lactamase assays were carried out at 22°C , pH 7.5, with 7-thienyl-2-acetamido-3-[4-*N,N*-dimethyl-aminophenylazo]-pyridinium methyl]-3-cephem-4-carboxylic acid (PADAC) as the substrate (von Tigerstrom & Boras, 1990). The cell-free extract was diluted, usually 100-fold, using 50 mM-Tris/HCl, 0.2 mg bovine serum albumin ml^{-1} , pH 7.5. The effect of pCMBS on β -lactamase activity was determined by incubating the diluted enzyme with and without 0.10 mM-pCMBS for 10 min at 22°C immediately before the assay. The pCMBS-sensitive activity was determined by subtracting the activity obtained in the presence of pCMBS from the activity obtained without pCMBS.

Oligonucleotide probes and primers. Oligonucleotide probes based on the N-terminal amino acid sequence of the *Lysobacter* β -lactamase (von Tigerstrom & Boras, 1990) and 17mer oligonucleotide primers for the sequencing reactions were synthesized in our department (Au *et al.*, 1991).

EcoRI fragments, obtained by digestion of pGB-2a and isolation by agarose gel electrophoresis were labelled by the random primer method (Feinberg & Vogelstein, 1983, 1984) for use as hybridization probes of the restriction digests of *L. enzymogenes* DNA.

Recombinant methods. The procedures used for the isolation and sequencing of DNA and related methods were described by Sambrook *et al.* (1989) and Au *et al.* (1991). From the genomic library a *Bam*HI fragment of about 12 kb was cloned into pUC118. It was called pGB-1 and was used to generate pGB-2a and pGB-2b, subclones of approximately 1 kb *EcoRI* fragments oriented in both directions in pUC118. A further subclone of a *SphI* fragment of about 1.2 kb was called pGB-3.

Other methods. The adsorption of the *Lysobacter* β -lactamase to CM-Sephadex, its subsequent elution, and the electrophoresis of the native β -lactamase and activity staining with PADAC have been described previously (von Tigerstrom & Boras, 1990). The β -lactamase produced by *E. coli* MV1193 (pGB-2a) was purified from cell-free extracts by the published method (von Tigerstrom & Boras, 1990). In addition, SDS-PAGE was used as a final purification step before the N-terminal amino acid sequence of the enzyme was obtained by the Tripartite Microanalytical Centre, University of Victoria, Victoria, BC, Canada. The osmotic shocking procedure was carried out according to the method of Neu & Heppel (1965) at pH 8.0 and also at pH 11.0, in which case the shock fluid was adjusted to pH 8.0 immediately after centrifugation. The β -lactamase sequence homology searches were made using FASTA Serve Search Results (Pearson & Lipman, 1988) from the GenBank Retrieval System. The amino acid sequence of the *L. enzymogenes* β -lactamase, deduced from the nucleotide sequence of its gene, was compared pair-wise to other β -lactamase sequences using the Lipman-Pearson Align program of DNASTAR.

Results and Discussion

Isolation of the β -lactamase gene

L. enzymogenes was grown and its DNA was isolated as described in Methods. The DNA was subjected to a partial digestion with endonuclease *MboI*. DNA fragments of about 20 kb were isolated on a sucrose gradient and cloned into λ -DASH to obtain the genomic library for *L. enzymogenes*.

The N-terminal amino acid sequence of the β -lactamase has been reported previously (von Tigerstrom & Boras, 1990). The information was used to synthesize two oligonucleotide probes as shown in Table 1. Based on the information we had obtained from the secreted phosphatase of *L. enzymogenes* (Au *et al.*, 1991), we used G or C nucleotides in the third position of nearly all codons. Probe 1, based on the codons for amino acids 3–13 was used to clone and sequence approximately 1 kb of a 2.1 kb *SalI* fragment. However, the nucleotide sequence obtained did not encode the expected amino acid sequence, nor was it related to any known β -lactamase gene. Further work was carried out using probe 2, based on the codons for amino acids 11–20. With this probe we isolated a clone called LEM-2 (*L. enzymogenes*, *MboI*, probe 2) from the genomic library. We selected a *Bam*HI fragment of about 12 kb from a

Table 1. *N*-Terminal amino acid sequence of β -lactamase from *L. enzymogenes* and constructed oligonucleotide probes

Position	Amino acid	Codons	
		Probe 1	Probe 2
1	Leu		
2	Ser		
3	Pro	CCG/C	
4	Lys	AAG	
5	Pro	CCG/C	
6	Ala	GCG/C	
7	Ala	GCG/C	
8	Ser	TCG	
9	Ala	GCG/C	
10	Asp	GAC	
11	Ala	GCG/C	GCG/C
12	Ala	GC	GCG/C
13	Leu		CTG/C
14	Arg		CGG/C
15	Ala		GCG/C
16	Arg		CGG/C
17	Leu		CTG/C
18	Ala		GCG/C
19	Glu		GAA/G
20	Leu		CT

number of restriction digests of LEM-2 and cloned it into pUC118 to obtain pGB-1. This, in turn, was used to generate subclones in pUC118 with the β -lactamase gene in both directions (pGB-2a and pGB-2b), using approximately 1 kb *EcoRI* fragments and also to generate a subclone (pGB-3) using a *SphI* fragment of similar size. Fig. 1 shows restriction digests of *L. enzymogenes* DNA hybridized with a probe for the *EcoRI* fragment containing the β -lactamase gene of *L. enzymogenes*. The probe hybridized strongly to one *BamHI* fragment of 12 kb, to one *EcoRI* fragment of about 1.0 kb and to two *SphI* fragments of similar size. The presence of two bands is in agreement with the fact that the β -lactamase gene has a *SphI* site, as indicated below.

Sequencing of the lactamase gene

ssDNA was generated from the cloned fragments and the DNA sequencing was carried out as described in Methods. The strands of the β -lactamase gene were sequenced using mainly pGB-2a and pGB-2b as indicated in Fig. 2. ssDNA generated from pGB-2a was used to sequence the coding strand (right to left) and ssDNA generated from pGB-2b was used to sequence the non-coding strand (left to right). Some of the unique restriction sites are indicated. A 1017 bp *EcoRI* fragment contained the whole β -lactamase gene. The *SphI* fragment (pGB-3), however, was lacking about 35% of the gene corresponding to the C-terminal end of the enzyme.

Fig. 1. Hybridization of the gene probe for *L. enzymogenes* β -lactamase. Lanes 1 to 5 are ethidium bromide-stained agarose gels of λ DNA molecular mass standards digested with *PstI* (1) and *HindIII* (5) and *L. enzymogenes* genomic DNA digested with *SphI* (2), *EcoRI* (3) and *BamHI* (4). Lanes 6, 7 and 8 are autoradiographs of Southern transfers of lanes 2, 3 and 4, respectively, which were hybridized with 32 P-labelled random primer probes.

Fig. 2. Sequencing strategy of the *L. enzymogenes* β -lactamase gene. The arrows indicate the direct of sequencing. Some key restriction sites are indicated and ATG and CTG indicate the start of the signal sequence (black) and of the mature β -lactamase (stippled), respectively.

The nucleotide sequence of 1190 bp containing the β -lactamase gene and the deduced amino acid sequence of the β -lactamase are shown in Fig. 3. The nucleotide sequence from 234 to 1143 encodes a 27 amino acid signal sequence and the mature β -lactamase of 276 amino acids. The calculated mass of the β -lactamase is

AGTTCGTGTCGAGCGCAGTGTGCGGGTCTGTCGCGCTCTACAGGAGAACGCGAAGCAGC	60
GCGCAGTCCGATTGCGGGTTCATCTGATCCGCCAGCCATCGGACCGTTTGGCAAAC	120
EcoRI	
GCGCGCTGCAACCCGCGCGCTGAAGGATTCTTTTCCAGCGCGGAGATTCTCGTTT	180
GCGCGCTCCGCGGGCGCTGCGCAATATCGGGTTCGCGCGGAGGAGTCCCGCATGCAA	240
FM Q (-27)	
CGACGCGCTTCTCTCAATGCACCGGCGCTTGTCTGCTGCGCGGGCGCGCTGCGCAGT	300
R R A F L Q C T G S L L L A G G A V A S	(-20)
TTCTGGCGCGCGCGCTGAAGCCTAAGCGCGCGCATCCGCCGATGCGCGCTGCGCGCG	360
F G A A A L S P K F A A S A D A A L R A	(+1)
CGCTGGCGCAACTGGAAGCGCAGCGCGGGCGCGCTGGCGCTGTCGCGCTGCGACCC	420
R L A E L E K R S G G R L G V S A L D T	(+20)
GCGCAGCGCGCGCGCTTCGATGTGCGCGCGCAGCAGCGCTTCGCCATGTGCGCAGCCTTC	480
A D G R R E D V R G D E R F A M C S T F	(+40)
AAGTCTCTGCTGCGCGCGCGTCTGCTGCGCAAGCGCGATCTGGCGCAACTCGACATGCAG	540
K F L L A A A V L R K A D L G E L D M Q	(+60)
CAACGCTGCGCGCTGCGAAGTGGCGCTGATCTGCGATTCCCGCATCACCAGCGCGATC	600
Q R L A V P K S A L I S H S P I T T P I	(+80)
GCGAAGCGCGCGCGCAGCGCGCGCTGCGCGCGCTGTCGCGAGCGGACCATGACCATCAGC	660
A E A G G S A S L A Q L C E A T M T I S	(+100)
GACAACGCGCGCGCGCAACCTGCTGCTGCGCGCTGCTGCGCAATCGCGCGCGCTGACCGG	720
D N A A A N L L L P L V G N P A G L T R	(+120)
TTCTGCGCGCGCATGCGCGCAAGGTCAACGCTGCGAAGCGCGAGCGCGAGCTCAAC	780
F L R G I G D K V T R L D R N E P E L N	(+140)
Sph I	
ACCGCGCTGCGCGCGATCGCGCGCAGCAACCTGCGCGCAACGCGATGCTGGCGAGCATG	840
T A L P G D P R D T T S P N A M L A S M	(+160)
CGCAGCTGCTGCTGCGGAGCGCGCTGAGCGCGCGCTGCGCGCGCGCGAGCTGCGATTGG	900
R T L L L G D A L S A A S R R Q L T D W	(+180)
CTGATGCGCAACAGCGCGCGCAGCGCGCGCTGCGCGCGCGCGCTGCGAAGAGCTGCGCG	960
L I A N Q T G D K R L R A G L P K S W R	(+200)
ATCGCGGACAGCGCGCGCGCGCAACCGCAGCAGCAACGACATCGCCATTATCTGCGCG	1020
I G D K T G S G N G T T N D I A I I W F	(+220)
CAGGGCGCGAAGCGCGGTTCATTACGACCTATCTGACCCAGCGCGCGCGAGGAGCGAT	1080
Q G R K P V F I T T Y L T Q A Q G E D D	(+240)
GCGGAGCGCGCATCTGCGCGCAGCGCGCGCGCGCTGCGCGCAGCACTGGATGCGCGCG	1140
A R S A I L A D A A R A L A Q H W I G A	(+260)
EcoRI	
GCGTACGCGCGCGCGCGCAGCAGGAATCTTTGCTGGCGCGAGAAATTC	1200
A Z	

Fig. 3. Nucleotide sequence and deduced amino acid sequence of the *L. enzymogenes* β -lactamase. The two *EcoRI* sites are indicated. The probable ribosome binding site is underlined. Amino acid sequence -27 to -1 represents the signal sequence and amino acid +1 represents the N terminus of the mature β -lactamase.

29146 Da, in good agreement with the value of 28 kDa determined by SDS-PAGE (von Tigerstrom & Boras, 1990). The deduced amino acid sequence of the N-terminal end also agrees perfectly with the sequence obtained from the isolated enzyme. The G+C content of the gene is 71.5%, reflecting the high G+C content of *L. enzymogenes* DNA (Christensen & Cook, 1978). A possible ribosome binding site is located between nucleotides 222 and 228. The two *EcoRI* restriction sites at positions 168 and 1185, and the *SphI* site at nucleotide 840 are indicated in Fig. 3.

Cons	-SAA-AA-G- AAVPSIAAAG -APGSNPA-- KE-KALEKQF	40
<i>L.e.</i>	LSPK PAASADAALR ARLAELEKRS	
<i>C.d.</i>	RTQGEPTQVQ QKLAALAEKQF	
Cons	DARLGVYA-D TGTGRTV-AY RADERFPMAS TFKALA--AV	80
<i>L.e.</i>	GGRLGVSAID TADGRRFDV- RGDERFAMCS TFKFLAAAV	
<i>C.d.</i>	GGRLGV-ALI NTADRSQILY RGDERFAMCS TSKTMVAADV	
Cons	L-Q-----E- L--RITYTK- DLVDYSPVTE KHVDTGMTL-	120
<i>L.e.</i>	LRKADLGELD MQORLAVPKS ALISHSPITT AEAGGSASIA	
<i>C.d.</i>	LKQSETQHDI LQKQMKVICKA DLTNWNPVTE KYVDKEMTIL	
Cons	ELCDAAV-YS DNTAANLLLR ELGGPKGVTA FLRSLGD-VT	160
<i>L.e.</i>	QLCEATMTIS DNAANLLLP LVGNPAGLTR FLRIGIDKVT	
<i>C.d.</i>	ELSAATLQYS DNTAMNKLE MLGGSNVTA FARSIGDTTF	
Cons	RLDWEPELN EAEPGDKRDT TTPRAIARTL R-LLLGDALS	200
<i>L.e.</i>	RLDREPELN TALPGDPRDT TSPNAMLASM RTLLLDALS	
<i>C.d.</i>	RLDREPLEN TAIPGDERDT TCPLAMAKSL HKLTLDGALA	
Cons	AE-RKQLTDW MLGNTTGDAL IRAGLPADWV VADKTGAG-S	240
<i>L.e.</i>	AASRRQLTDW LIANQTGDKR LRAGLPKSWR IGDKTGSG--	
<i>C.d.</i>	GAQRAQLVEW LKGNITGGQS IRAGLPEGWV VGDKTGAG-D	
Cons	YGTRNDIAVV WP-PGRAPIV LAISTKD-- DAE-DN-LIA	280
<i>L.e.</i>	NGTTNDIALI WP-QGRKPVF ITTYLTQAGG EDDARSAILA	
<i>C.d.</i>	YGTINDIAVI WPE-DRAPLI LVTYFTQPPQ DAKGRKDILA	
Cons	EAAKVVAEAL -S--K	295
<i>L.e.</i>	DAARALAQHW IGAA	
<i>C.d.</i>	AAAKIVTEGL	

Fig. 4. Comparison of the amino acid sequence of *L. enzymogenes* β -lactamase (*L.e.*) to the sequences of the Class A β -lactamase consensus sequence (Cons) (Ambler *et al.*, 1991) and the β -lactamase of *C. diversus* (*C.d.*). The comparisons were made as described in the methods. Horizontal lines within the sequence represent apparent deletions, vertical lines indicate sequence identities. Amino acids conserved in Class A β -lactamases (Ambler *et al.*, 1991) are underlined in the consensus sequence. The only exception (position 144 in the *Lysobacter* β -lactamase) is double-underlined. The proposed active site is overlined. A two amino acid insertion in the *Lysobacter* β -lactamase after position 110 is represented by PI. The numbering is that proposed for the consensus sequence (Ambler *et al.*, 1991) and, therefore, is different from that in Fig. 3.

Comparison of the *L. enzymogenes* β -lactamase to other β -lactamases

The amino acid sequence of the *L. enzymogenes* β -lactamase was compared to the sequences of other β -lactamases and it became clear that it was most similar to enzymes of Class A (Ambler, 1980). The enzyme appeared to be most closely related to the β -lactamases

Table 2. Comparison of *Lysobacter* β -lactamase to other Class A β -lactamases

Organism	Similarity index	Cysteine in active site	Reference
<i>Lysobacter enzymogenes</i>	100.0	+	This study
<i>Citrobacter diversus</i>	48.1	+	Perilli <i>et al.</i> (1991)
<i>Klebsiella oxytoca</i> E23004	47.7	+	Arakawa <i>et al.</i> (1989)
<i>Streptomyces albus</i>	46.8	+	Dehottay <i>et al.</i> (1987)
<i>Yersinia enterocolitica</i>	46.7	+	GenBank (X57079)
Consensus β -lactamase	45.4	—	Ambler <i>et al.</i> (1991)
<i>Escherichia coli</i> (PIT-2)	41.6	—	Barthelemy <i>et al.</i> (1988).
<i>Streptomyces fradiae</i>	41.1	—	Forsman <i>et al.</i> (1990)
<i>Klebsiella</i> sp.	40.9	—	Mercier & Levesque (1990)
<i>Escherichia coli</i> (TEM)	40.8	—	Sutcliffe (1978)
<i>Klebsiella pneumoniae</i>	40.6	—	Arakawa <i>et al.</i> (1986)
<i>Enterobacter cloacae</i>	40.5	—	Shlaes <i>et al.</i> (1990)
<i>Streptomyces aureofaciens</i>	40.4	+	GenBank (X13597)
<i>Streptomyces badius</i>	40.2	—	Forsman <i>et al.</i> (1990)
<i>Rhodopseudomonas capsulata</i>	39.0	—	Campbell <i>et al.</i> (1989)
<i>Streptomyces cacaеoi</i>	38.3	—	Forsman <i>et al.</i> (1989)
<i>Bacillus cereus</i> (PSE-4)	37.8	—	Sloma & Gross (1983)
<i>Pseudomonas aeruginosa</i>	37.7	—	Boissinot & Levesque (1990)
<i>Bacillus cereus</i> (III)	37.1	—	Hussain <i>et al.</i> (1987)
<i>Bacillus cereus</i> 5/B	36.6	—	Wang <i>et al.</i> (1985)
<i>Bacillus licheniformis</i>	35.8	—	Neugebauer <i>et al.</i> (1981)
<i>Streptomyces cacaеoi</i> Ulg	35.6	—	Lenzini <i>et al.</i> (1988)
<i>Staphylococcus aureus</i>	28.7	—	Chan (1986)
<i>Enterococcus faecalis</i>	28.1	—	GenBank (M60253)

of *Citrobacter diversus* (Perilli *et al.*, 1991) and *Klebsiella oxytoca* E 23004 (Arakawa *et al.*, 1989). However, in contrast to the high G+C content of the gene for the *Lysobacter* β -lactamase, the genes of the enzymes from these organisms have a G+C content of only 54% (Perilli *et al.*, 1991) and 58% (Arakawa *et al.*, 1989), respectively. Recently Ambler *et al.* (1991) analysed 20 Class A β -lactamases and proposed a consensus sequence to allow for easier comparison. In Fig. 4 the sequence of the mature *Lysobacter* β -lactamase is compared to this consensus sequence and to the *C. diversus* β -lactamase. The numbering of the sequences is according to that proposed for the consensus sequence (Ambler *et al.*, 1991). Thus, the N- and C-termini of the *Lysobacter* enzyme are Leu (ABL 17) and Ala (ABL 294), respectively. Compared to the consensus sequence there are two single amino acid deletions (ABL 60 and 253), one two-amino acid deletion (ABL 239 and 240) and a two-amino acid insertion (Pro and Ile) after ABL 110. We feel that this insertion does not warrant a change in the numbering of the consensus sequence at this time. A revision of the consensus sequence might be necessary in the future if more insertions are found in other β -lactamases. In the consensus sequence there are 22 conserved amino acids (underlined in Fig. 4). All but one (ABL 184) of these positions are also conserved in the *Lysobacter* β -lactamase. The proposed active site of the Class A β -lactamases around the serine at ABL 70, with the three conserved amino acids, is overlined in Fig. 4.

The lactamase of *L. enzymogenes* has a cysteine at ABL 69, in agreement with the inhibition of the enzyme by pCMB and pCMBS reported previously (von Tigerstrom & Boras, 1990).

A further comparison of the *Lysobacter* enzyme to other Class A β -lactamases and the consensus β -lactamase was made as described in Methods. Table 2 lists the similarity indices that we obtained. The indices are approximately equal to the percentage of identical amino acids in the sequence and range from 28.1 to 48.1% for the enzymes from *Enterococcus faecalis* and *C. diversus*, respectively, with a similarity index of 45.4 for the consensus β -lactamase.

Expression of the β -lactamase gene

The cloned *EcoRI* fragment in pUC118, described above as pGB-2a, was used for the expression of the β -lactamase gene in *E. coli* since the β -lactamase gene was inserted in the correct orientation behind the *lac* promoter. *E. coli* MV1193 was transformed with pGB-2a. The isolates were grown as described in Methods and cell-free extracts were prepared from their cultures for the determination of β -lactamase activities. The β -lactamase of *L. enzymogenes* is inhibited by pCMBS (von Tigerstrom & Boras, 1990), whereas the TEM β -lactamase encoded by pUC118 is not (Bush, 1989). We confirmed this in our study. Thus, assays with and without pCMBS served as a means to detect *Lysobacter*

Table 3. *Production of Lysobacter β -lactamase*
 Values given are from a representative experiment. Values in brackets indicate $\mu\text{mol min}^{-1}$ (OD_{600} unit) $^{-1}$.

Organism and plasmid	β -Lactamase activity [$\mu\text{mol min}^{-1}$ (ml culture) $^{-1}$]		
	–pCMBS	+pCMBS	pCMBS-sensitive
<i>L. enzymogenes</i>	0.29 (0.049)	0.03 (0.0049)	0.26 (0.044)
<i>E. coli</i> MV1193 (pUC118)*	0.17 (0.020)	0.17 (0.020)	0.00
<i>E. coli</i> MV1193 (pGB-2a)*	1.99 (0.29)	0.28 (0.041)	1.71 (0.25)

* Values from the early stationary phase of growth are used.

β -lactamase activity. Four of 16 isolates seemed to produce significant amounts of the *Lysobacter* enzyme. One was selected for a more detailed analysis. Table 3 shows the β -lactamase activities in a representative experiment in the presence and absence of pCMBS in cell-free extracts of *L. enzymogenes* and of *E. coli* MV1193, transformed with pUC118 or pGB-2a. The activity is highest from pGB-2a and, more significantly, it is inhibited by pCMBS nearly as much as the β -lactamase from *L. enzymogenes*. The enzyme activity from pGB-2a also was adsorbed to CM-Sephadex at pH 7.5, whereas the lactamase encoded by pUC118 was not. This is in agreement with the different isoelectric points of about 9.6 for the *L. enzymogenes* β -lactamase (von Tigerstrom & Boras, 1990) and 5.4 for the TEM β -lactamase (Bush, 1989). Furthermore, the β -lactamase activity from pGB-2a was increased by a factor of six in the presence of IPTG, in agreement with the position of the lactamase gene in pUC118.

Determining β -lactamase activities during the growth of *E. coli* MV1193 (pGB-2a), we found that *Lysobacter* β -lactamase was produced mainly after the exponential growth phase. Mean values $\pm$ SD were calculated from six determinations. Stationary phase cultures had β -lactamase activities of $2.2 \pm 1.3 \mu\text{mol min}^{-1} \text{ml}^{-1}$, of which $1.6 \pm 0.35 \mu\text{mol min}^{-1} \text{ml}^{-1}$ or $71 \pm 16\%$ was pCMBS-sensitive. In exponentially growing cells only $11.3 \pm 7\%$ of the activity was pCMBS-sensitive. Thus cultures in the early stationary phase were used for the characterization of the expressed *Lysobacter* β -lactamase.

In a separate experiment we attempted to determine the location of the *Lysobacter* β -lactamase in *E. coli* MV1193 (i.e. the pCMBS-sensitive activity). A culture grown to just past the exponential growth phase ($\text{OD}_{600} = 5.4$ and 60% inhibition of β -lactamase with pCMBS) was used to prepare shock fluids and shocked cells at pH 8 and pH 11. Essentially all of the pCMBS-resistant β -lactamase was released into the shock fluid at both pH values. Little or no pCMBS-sensitive activity was released from the cells at pH 8 but 32% of the β -lactamase was released at pH 11. We believe that

both enzymes are located in the periplasm but that the *Lysobacter* β -lactamase, due to its high isoelectric point, is held more tightly in the periplasm by ionic interaction with negatively-charged cell envelope components than the TEM β -lactamase. We also observed that *E. coli* MV1193 transformed with pGB-2a always releases some pCMBS-resistant β -lactamase but no pCMBS-sensitive β -lactamase into the culture medium.

PAGE and N-terminal sequencing

PAGE at low pH was carried out using the following enzyme preparations: (i) β -lactamase from the concentrated shock fluid of *L. enzymogenes* (von Tigerstrom & Boras, 1990); (ii) pCMBS-sensitive β -lactamase from *E. coli* MV1193 transformed with pGB-2a, partially purified from cell-free extract by CM-Sephadex chromatography; (iii) partially purified TEM β -lactamase from *E. coli* MV1193 transformed with pUC118. The electrophoretic mobilities relative to methyl green were 0.58, 0.58 and 0.52, respectively. In addition, the β -lactamase expressed from pGB-2a was purified as described in Methods to determine the N-terminal amino acid sequence of the protein. A sequence of 19 residues was obtained and all but residue 16 (asp) agreed with the N-terminal sequence of the mature β -lactamase (von Tigerstrom & Boras, 1990) and with the sequence shown in Fig. 3, which has only a 10% identity with the N-terminal sequence of the TEM β -lactamase.

Concluding remarks

The gene of the β -lactamase of *L. enzymogenes* was isolated and the DNA sequence was determined. This appears to be the first characterization of a β -lactamase gene from a gliding bacterium. The gene was found to contain 909 bp, with a G+C content of 71.5%, located in a 1017 bp *EcoRI* fragment of the genomic DNA. The gene encodes the pre- β -lactamase comprising the signal sequence of 27 amino acids and the mature enzyme of 276 amino acids. Comparing the amino acid sequences

of the *Lysobacter* β -lactamase to the sequence of the Class A consensus β -lactamase (Ambler *et al.*, 1991), a similarity index of 45.4 was obtained. Thus, the enzyme is a typical Class A β -lactamase. Based on the similarity indices, the enzyme is most closely related to the β -lactamases of *C. diversus*, *K. oxytoca*, *S. albus* and *Y. enterocolitica* (similarity indices of 48.1 to 46.7). The *Lysobacter* enzyme also shares the presence of a cysteine at ABL 69 with the β -lactamases of these four organisms and the β -lactamase of *S. aureofaciens* (similarity index of 40.4). We believe that this cysteine residue in the *Lysobacter* β -lactamase is the site of pCMBS inhibition.

The origin of the β -lactamase of *L. enzymogenes* is not clear. Since *L. enzymogenes* is a typical soil organism, the enzyme probably has not arisen from the over-use of β -lactam antibiotics during the last 50 years. Compared to other Class A β -lactamases, the *Lysobacter* enzyme is unique in that it has two additional amino acids after ABL 110 and a two amino acid deletion at ABL 239 and 240. This and the similarity of the G + C content of the β -lactamase gene (71.5%) to the G + C content of the *L. enzymogenes* genomic DNA (69%) (Christensen & Cook, 1978) may indicate that the enzyme has evolved for some time in *L. enzymogenes*. Alternatively, it may have been acquired recently from another soil organism with a high G + C content, such as a *Streptomyces* or *Pseudomonas* species. It is unlikely that β -lactamase was acquired from organisms such as *C. diversus* and *K. oxytoca*, since the genes of their β -lactamases have a much lower G + C content.

The plasmid pGB-2a was used for the sequencing and the expression of the *Lysobacter* β -lactamase gene in *E. coli*. Since this plasmid also encodes the TEM β -lactamase of pUC118, we had to show specifically that the β -lactamase genes of *L. enzymogenes* was expressed. This was possible by selective inhibition of the *Lysobacter* enzyme with pCMBS, selective adsorption to CM-Sephadex, separation of the TEM and *Lysobacter* β -lactamases by PAGE, and determination of the N-terminal amino acid sequence of the enzyme produced by the cloned gene. The results clearly show that the β -lactamase of *L. enzymogenes* was expressed by *E. coli* and that the enzyme was processed to yield the mature β -lactamase.

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