Cloning of chromosomal β -lactamase genes from *Yersinia enterocolitica*

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Two β -lactamase genes present in the chromosome of *Yersinia enterocolitica* have been cloned individually into the plasmid pACY184 and expressed in *Escherichia coli*. The gene for broad-spectrum β -lactamase I ('A') was cloned from a strain belonging to the O:3 serotype, and the gene for (cephalosporinase) β -lactomase II ('B') was cloned from a strain of the O:5b serotype. The properties of the β -lactamases expressed in *E. coli* are similar to those previously described in *Y. enterocolitica*.

Introduction

Chromosomal β -lactamase genes are common in Gram-negative bacteria. The enzymes encoded by these genes are usually specific for the hydrolysis of cephalosporins, are inducible and have an alkaline pI. High levels of enzymes of this type are associated with resistance to the newer cephalosporins. Chromosomal β -lactamase genes from *Escherichia coli*, *Citrobacter freundii*, *Shigella sonnei* (Bergström *et al.*, 1983), *Enterobacter cloacae* (Nicolas *et al.*, 1987) and *Klebsiella pneumoniae* (Arakawa *et al.*, 1986) have been cloned and are well characterized. Genes from *Serratia marcescens* (Joris *et al.*, 1986) and *Proteus vulgaris* (Maejima *et al.*, 1987) have been less well studied. The genes from *E. coli*, *C. freundii*, *S. sonnei* and *Ent. cloacae* are related and the β -lactamases they produce are considered as a homogeneous group and referred to as class C β -lactamases (Jaurin & Grundström, 1981).

Yersinia enterocolitica is also classified among the enterobacteria and is resistant to benzylpenicillin and cephalothin, and sometimes to ampicillin and carbenicillin, due to the production of chromosomal β -lactamases. Previous studies showed that strains of the O:3 serotype produce two different β -lactamases. One, referred to as the 'A' enzyme, is a broad-spectrum enzyme hydrolysing carbenicillin. Its molecular mass has been estimated to be 20 kDa and its pI to be 8.7. It is produced constitutively. The other, called the 'B' enzyme, is an inducible cephalosporinase of 34 kDa which focuses as two bands of pI 5.3 and 5.7. It has been reported also that

Y. enterocolitica strains of O:5b serotype only express the 'B' enzyme (Cornelis, 1981).

In this paper we report the cloning of the chromosomal genes coding for two β -lactamases in *Y. enterocolitica*, together with a preliminary analysis of the kinetic properties of the enzymes. The β -lactamases are referred to here as types I and II, rather than A and B, to avoid confusion with the sequence classes of β -lactamases.

Methods

Bacterial strains and plasmids. *Y. enterocolitica* strain Y56 (O:3) was isolated from the environment by L. Rodríguez Solórzano (Santander, Spain). Its serotype was kindly determined by H. H. Mollaret (Institute Pasteur, Paris, France). *Y. enterocolitica* IP97 (also from L. Rodríguez Solórzano) belongs to the O5:b serotype. As recipient for the cloning experiments we used *E. coli* HB101 (F⁻ hsdR hsdM recA13 proA2 leuB6 thi-1 ara-14 lacY1 galK2 xyl-5 mtl-1 supE44 rpsL20).

β -Lactamase activities and kinetic parameters were determined on enzymes isolated from *E. coli* K12 strain M11443 [Δ (frd-ampC) rpsL] containing the appropriate plasmids. The cloning vector used was the plasmid pACYC184 (Cm^r Tet^r Rep p15A) (Chang & Cohen, 1978).

Media and antibiotics. L-broth and L-agar (Maniatis *et al.*, 1982) were routinely used for bacterial growth. Ampicillin was added to the agar at 50 μ g ml⁻¹ and chloramphenicol at 25 μ g ml⁻¹.

DNA preparation and analysis. Chromosomal DNA was prepared as described by Silhavy *et al.* (1984). Plasmid DNA was purified by alkaline lysis followed by phenol extraction and ethanol precipitation. Agarose for gel electrophoresis was prepared in Tris/borate buffer, and gels were made essentially as described by Maniatis *et al.* (1982). Restriction enzymes were obtained from Boehringer Mannheim and used under the conditions recommended by the supplier. Restriction fragments were usually separated in horizontal 1% agarose gels. HindIII and EcoRI-HindIII fragments of λ DNA were used as molecular size standards.

Abbreviations: 6-APA, 6-aminopenicillanic acid; PADAC, pyridinium-2-azo-*p*-dimethylaniline cephalosporin.

Cloning procedure. Chromosomal DNA from *Y. enterocolitica* was partially digested with the restriction endonuclease *Sau*3AI. This DNA was ligated into the *Bam*HI site of the plasmid vector pACYC184 with T4 DNA ligase (New England Biolabs). Ligated DNA was introduced into competent cells of *E. coli* HB101 and recombinants were selected on agar containing ampicillin.

MIC determination. Minimal inhibitory concentrations were determined on Mueller–Hinton agar (Difco) containing twofold serial dilutions of the antibiotics. The inoculum size was 10^4 cells.

β -Lactamase assays. β -Lactamase activity in crude extracts was determined spectrophotometrically (Ross & O'Callaghan, 1975) at room temperature with 100 μ M-cephaloridine as substrate. To prepare cell-free extracts, 50 ml cultures were grown in LB to an OD₄₂₀ of 0.8 either in the presence (induced) or the absence (uninduced) of 125 μ g 6-aminopenicillanic acid (6-APA) ml⁻¹. Cells were resuspended in 1 ml 0.1 M-phosphate buffer pH 7.0 and disrupted by ultrasonic treatment for 1 min (three 20 s cycles with 30 s pauses) at 4 °C. Crude extracts were cleared of cell debris by centrifugation at 12000 *g* for 1 h at 4 °C. *K_m* and *V* values were calculated by a least-squares fit of a double reciprocal plot of initial velocities at eight different substrate concentrations. The following wavelengths were used for the enzyme assays: ampicillin, benzylpenicillin and carbenicillin, 235 nm; cefamandole, 272 nm; cefotaxime, 225 nm; cefoxitin, 260 nm; cephaloridine, 255 nm; cephalothin, 262 nm. Relative *V* and relative *V/K_m* values were used to compare enzyme activities, as recommended by Bush & Sykes (1986). Protein concentrations in the extracts were determined by the method of Bradford (1976). Osmotic fluids, prepared as described by Nossal & Heppel (1966), were used to determine β -lactamase molecular mass by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). Native electrophoresis on cellulose acetate using 50 mM-phosphate buffer (pH 7.0) followed by activity staining with pyridinium-2-azo-*p*-dimethylaniline cephalosporin (PADAC, Calbiochem) was also used to visualize β -lactamases (Jones *et al.*, 1982).

DNA hybridization. DNA restriction fragments to be used as probes were purified by electroelution from agarose gels. They were labelled with digoxigenin-modified dUTP using the Klenow fragment of *E. coli* DNA polymerase I in a random primed reaction (Boehringer Mannheim). DNA to be probed was fragmented and transferred to nitrocellulose as described by Southern (1975). Hybridization was carried out in plastic bags at 42 °C in the presence of 50% (v/v) formamide.

Membranes were washed twice for 5 min with 2 $\times$ SSC containing 0.1% SDS at room temperature and twice for 15 min in 0.1 $\times$ SSC, 0.1% SDS at 68 °C (1 $\times$ SSC is 0.15 M-NaCl, 0.015 M-trisodium citrate, pH 7.0). Hybridized filters were incubated with an anti-digoxigenin-DNA antibody tagged with alkaline phosphatase and then with the colour substrate 5-bromo-4-chloro-3-indolyl phosphate and nitrobluetetrazolium (Boehringer Mannheim Nonradioactive Labelling and Detection Kit).

Results

Cloning of the *blaA* gene, encoding β -lactamase type I

Chromosomal DNA from *Y. enterocolitica* strain Y56 (serotype O:3) was cloned in pACYC184. Colonies containing recombinant plasmids were selected initially on the basis of their Ap^r Cm^r Tc^s phenotype. One of these colonies, containing plasmid pSU601, encoded a β -

Fig. 1. Restriction map of the recombinant plasmids expressing β -lactamase type I and some of their derivatives. Thin lines represent the vector pACYC184 and thick lines the *Y. enterocolitica* Y56 chromosomal DNA insert. Restriction enzymes: E, *Eco*RI; h, *Hind*II; N, *Nru*I; b, *Bst*EII; c, *Cla*I; v, *Eco*RV; H, *Hind*III.

Fig. 2. Restriction maps of plasmid pSU602, expressing β -lactamase type II, and of its derivatives pSU622 and pSU623. Thin lines represent the vector pACYC184 and thick lines the *Y. enterocolitica* IP97 chromosomal DNA insert. Key to restriction enzymes as for Fig. 1, plus B, *Bam*HI.

lactamase. This plasmid has a 13 kb insert in the *Bam*HI site of pACYC184. The MICs of several β -lactam antibiotics for *E. coli* HB101 carrying this plasmid are shown in Table 1. Since this strain showed resistance towards cephalosporins and carbenicillin, it was assumed to contain the gene coding for the type I enzyme. A restriction map pSU601 is shown in Fig. 1. To localize the β -lactamase gene, pSU601 was partially digested with *Sau*3AI and subcloned into *Bam*HI-linearized pACYC184. This resulted in the isolation of plasmid pSU610, with a 3.3 kb insert and capable of producing a β -lactamase that was indistinguishable from that produced by pSU601.

Plasmid pSU610 DNA produced three *Nru*I fragments, of 4.4, 2.3 and 0.8 kb (Fig. 1). When these fragments were ligated and introduced into *E. coli* HB101, selecting for ampicillin resistance, plasmid pSU611, was isolated which contained only the 4.4 and 0.8 kb fragments. This indicated that the 2.3 kb *Nru*I fragment was not essential for β -lactamase production. A second type of recombinant was isolated on chloramphenicol agar and was susceptible to ampicillin. The plasmid from one such isolate, pSU612, was analysed and shown to lack just the 0.8 kb *Nru*I fragment. Taken together, the subclones indicate that the gene coding for β -lactamase type I is located on the 1.9 kb DNA

Table 1. MICs of representative β -lactam antibiotics for various strains

Abbreviations for antibiotics and limits between which MICs were determined: AP, ampicillin (1–1024 $\mu\text{g ml}^{-1}$); CB, carbenicillin (1–1024 $\mu\text{g ml}^{-1}$); CTN, cephalothin (1–1024 $\mu\text{g ml}^{-1}$); CFM, cefamandole (1–128 $\mu\text{g ml}^{-1}$); CFR, cephaloridine (1–256 $\mu\text{g ml}^{-1}$); CXT, ceftiofur (1–256 $\mu\text{g ml}^{-1}$); CTX, cefotaxime (0.03–32 $\mu\text{g ml}^{-1}$). The values reported are representative of three independent experiments.

Strain	MIC ($\mu\text{g ml}^{-1}$)						
	AP	CB	CTN	CFR	CFM	CXT	CTX
<i>E. coli</i> HB101	2	4	2	2	<1	2	<0.03
<i>Y. enterocolitica</i> Y56	16	256	128	8	4	2	0.06
<i>E. coli</i> HB101(pSU601)	256	>1024	128	16	32	2	0.12
<i>E. coli</i> HB101(pSU610)	1024	>1024	128	16	32	2	0.12
<i>E. coli</i> HB101(pSU611)	1024	>1024	128	16	32	2	0.25
<i>E. coli</i> HB101(pSU612)	2	4	2	2	<1	2	<0.03
<i>Y. enterocolitica</i> IP97	16	1	1024	64	4	16	0.12
<i>E. coli</i> HB101(pSU602)	256	8	512	128	8	64	0.50
<i>E. coli</i> HB101(pSU622)	512	16	1024	128	16	64	4.0
<i>E. coli</i> HB101(pSU623)	2	4	2	2	<1	2	<0.03

Table 2. β -Lactamase activity expressed from *Y. enterocolitica* Y56 and IP97 and from the cloned *bla* genes of *Y. enterocolitica* in *E. coli* K12 strain MI1443

Bacterial cells were grown to the end of the exponential phase, in either the presence (induced) or the absence (uninduced) of 6-APA (125 $\mu\text{g ml}^{-1}$), and crude extracts were prepared. The substrate was 100 μM -cephaloridine. The values given are representative of three independent experiments.

Strain	Enzyme	β -Lactamase activity [units (mg protein) $^{-1}$]		
		Uninduced (a)	Induced (b)	Ratio b/a
<i>Y. enterocolitica</i>				
Y56	I+II	0.036	0.038	1
IP97	II	0.017	0.152	8.9
<i>E. coli</i>				
MI1443	–	<0.001	<0.001	1
MI1443(pSU611)	I	0.071	0.078	1
MI1443(pSU602)	II	0.054	0.162	3
MI1443(pSU622)	II	1.44	1.55	1

fragment between the *Nru*I site at coordinate 4.4 and the *Bst*EII site at 6.3, of pSU601 (Fig. 1).

Cloning of the *blaB* gene, encoding β -lactamase type II

Chromosomal DNA from *Y. enterocolitica* strain IP97 (serotype O:56) was fragmented and cloned into pACYC184. Recombinants expressing β -lactamase were selected on ampicillin agar. Plasmid pSU602, containing a 2.7 kb insert, was isolated from one of these recombinants. A restriction map of this plasmid is shown in Fig. 2. Deletion of the *Eco*RI fragment from 0 to 3.5 kb resulted in plasmid pSU622, which still contained an active β -lactamase gene. Deletion of the 1.2 kb *Hind*III fragment from pSU602 gave plasmid pSU623, which no longer

conferred resistance to β -lactams. The MICs of different β -lactam antibiotics for *E. coli* HB101(pSU602), in particular its sensitivity to carbenicillin (Table 2), indicated that the β -lactamase encoded by this plasmid was the type II enzyme, the only type expressed by the strains of *Y. enterocolitica* belonging to the O:5b serotype.

Visualization of β -lactamases

The identity of the β -lactamase encoded by the recombinant plasmids was determined by electrophoresis of crude bacterial extracts on cellulose acetate and visualization of β -lactamases by staining with PADAC. This technique showed that *E. coli* MI1443 (pSU622) and *Y.*

Fig. 3. Cellulose acetate electrophoresis of crude extracts containing β -lactamases after staining with the chromogenic substrate PADAC (50 μ M). *Ent. cloacae ampC* β -lactamase cloned in plasmid pEC1C was included as a control. The direction of electrophoresis was from top (negative) to bottom (positive). The extracts were obtained from the following strains: A, *Y. enterocolitica* Y56; B, *Y. enterocolitica* IP97; C, *E. coli* MI1443(pSU611); D, *E. coli* MI1443(pSU622); E, *E. coli* MI1443(pEC1C) (Nicolas *et al.*, 1987).

enterocolitica IP97 expressed β -lactamases with the same mobility. Similarly, *E. coli* MI1443(pSU611) and *Y. enterocolitica* Y56 showed single bands of identical mobility under these conditions. The presumptive type I and II β -lactamases were easily differentiated from each other by using this native gel system (Fig. 3).

Properties of β -lactamases I and II

β -Lactamase I, expressed by *E. coli* MI1443(pSU611), showed an apparent molecular mass of 26 kDa in SDS-polyacrylamide gels. The specific activity of the enzyme was measured in crude bacterial extracts and the results in Table 2 indicate that the gene encoding this enzyme is not inducible either in *Y. enterocolitica* or in *E. coli* containing recombinant plasmids. β -Lactamase II showed an apparent size of 39 kDa in SDS-polyacrylamide gels. The specific activity measurements of this enzyme (Table 2) indicate that it is encoded by a gene that is inducible by 125 μ M-6-APA in *Y. enterocolitica* IP97 but not in *Y. enterocolitica* Y56. *E. coli* MI1443(pSU602) showed an intermediate, threefold increase in β -lactamase production in the presence of 6-APA. The plasmid of pSU622 produced a very high level of β -lactamase irrespective of the presence of 6-APA.

The kinetic parameters of β -lactamases I and II were measured (Table 3). β -Lactamase I from *E. coli*

Fig. 4. Southern blot analysis of *Y. enterocolitica* chromosomal DNA. (a) The agarose gel. (b) Results with the *blaB* gene probe; (c) results with the *blaA* gene probe. The same samples were loaded in the same lane in the three panels (λ DNA is only marked in panel a). DNA in lane 1, *Y. enterocolitica* Y56 *Hind*II digested; lane 2, *Y. enterocolitica* Y56 *Hind*III digested; lane 3, *Y. enterocolitica* IP97 *Hind*II digested; lane 4, *Y. enterocolitica* IP97 *Hind*III digested; lane 5, *E. coli* HB101 *Hind*III digested; lane 6, pSU611 *Nru*I digested; lane 7, pSU622 *Hind*III/*Bam*HI digested; lane 8, λ DNA *Eco*RI/*Hind*III digested.

Table 3 Kinetic parameters of hydrolysis of β -lactam antibiotics by *Y. enterocolitica* β -lactamases I and II from *E. coli* M11443(pSU611) and *E. coli* M11443(pSU622), respectively

V is expressed in μmol hydrolysed $(\text{mg protein})^{-1} \text{ min}^{-1}$. The relative V and relative V/K_m for each antibiotic were normalized with respect to cephaloridine. The results reported are representative of three independent experiments.

Antibiotic	β -Lactamase I				β -Lactamase II			
	V	Relative V	K_m (μM)	Relative V/K_m	V	Relative V	K_m (μM)	Relative V/K_m
Cephaloridine	0.65	100	793.6	100	2.29	100	90.9	100
Cephalothin	1.62	249	833.3	237.3	14.3	624.4	337.8	178.4
Cefamandole	0.52	80	333.3	190.6	ND			
Cefoxitin	ND				0.46	20.3	81.9	22.5
Cefotaxime	ND				ND			
Ampicillin	0.21	32.1	357.1	71.1	0.115	5.02	533.9	0.85
Carbenicillin	0.08	12.3	500	19.5	ND			
Benzylpenicillin	0.25	38.5	15.9	1926.5	1.21	52.8	7.14	677.8

ND, Not detected: hydrolysis rates were too low to be detected after 2 h of incubation.

M11443(pSU611) efficiently hydrolysed ampicillin and benzylpenicillin as well as cephalothin, cephaloridine and cefamandole. Carbenicillin was also hydrolysed, but less efficiently. β -Lactamase II, from *E. coli* M11443(pSU622) hydrolysed mainly cephalosporins and benzylpenicillin.

Southern blot analysis of *Y. enterocolitica* chromosomal DNA

Chromosomal DNA from *Y. enterocolitica* strains Y56 and IP97, belonging to the O:3 and O:5b serotypes, respectively, was digested with *Hind*III or *Hind*II, transferred to nitrocellulose and separately hybridized with probes presumed to contain the two cloned *bla* genes (Fig. 4). The probe for *blaA* was a 0.8 kb *Nru*I fragment prepared from pSU611 (Fig. 1), and that for *blaB* was the 0.9 kb *Hind*III/*Bam*HI fragment from pSU622 (Fig. 2). Both strains of *Y. enterocolitica* appeared identical when their chromosomes were hybridized with the *blaB* gene probe, with a 2.3 kb *Hind*III fragment and a 1.8 kb *Hind*II fragment present in both cases. However, when the two strains were probed for the *blaA* gene, the same *Hind*III fragment of about 10 kb was present in the two strains but they showed different *Hind*II fragments. The O:3 serotype strain (Y56) showed a 1.8 kb *Hind*II fragment (probably located between 4.1 and 5.9 kb in pSU601), whereas the size of the *Hind*II fragment from the O:5b strain (IP97) was about 7 kb (Fig. 4).

Discussion

The presence of two β -lactamases among *Y. enterocolitica* has been already reported. Here we describe the cloning

of the genes encoding two distinct β -lactamases from different strains of *Y. enterocolitica* and their expression in *E. coli*.

The characteristics of these enzymes, when expressed in *E. coli*, largely agree with those obtained previously. The gene encoding β -lactamase I was inducible in *Y. enterocolitica* IP97 and to a lesser extent also in *E. coli* M11443(pSU602). This result is consistent with those obtained for other chromosomal-mediated cephalosporinases (Nicolas *et al.*, 1987). However, the high level of β -lactamase II expression in *E. coli* strains containing pSU622 (see Table 2) suggests that this gene could be subject to negative regulation in the parental *Yersinia* strain. *Y. enterocolitica* Y56 is believed to contain active *blaA* and *blaB* genes (Cornelis, 1981). Southern hybridization experiments showed that there were sequences homologous to both *blaA* and *blaB* probes in the Y56 chromosome. However, there is circumstantial evidence from our results suggesting poor expression of the *blaB* gene from *Y. enterocolitica* Y56.

Similarly, it has been shown that strains belonging to the O:5b serotype only produced the type II β -lactamase (Cornelis, 1981). Again, the Southern hybridization showed that the chromosome of the O:5b strain IP97 hybridized with both *blaA* and *blaB* gene probes. However, the *Hind*II fragments from Y56 and IP97 hybridizing with the *blaA* probe were different. This difference, which relates to a *Hind*II site, could be involved in the already reported lack of *blaA* expression in strains of O:5b serotype.

In view of the above findings it is tempting to speculate that when two β -lactamase genes are simultaneously present in the chromosome of a particular bacterium, they evolve in such a way that only one is active (and the other perhaps finally lost). This could account, at least in part, for the distribution of

β -lactamase genes in the chromosomes of present-day enterobacteria.

The data obtained on these enzymes support the view that β -lactamase II is a cephalosporinase, probably related to the chromosomal enzymes of other Gram-negative organisms (molecular class C). The β -lactamase I could be similar to the chromosomal β -lactamase from *Klebsiella pneumoniae*, which confers resistance to carbenicillin and has been shown to be related to the plasmidic TEM-type enzyme (class A) (Arakawa *et al.*, 1986). The differences between these two genes were reinforced by our preliminary hybridization studies, which showed that the two β -lactamase genes do not share extensive sequence homology. We are now determining the nucleotide sequences of these genes, which should clarify the molecular classification of these two enzymes.

The cloning of these two β -lactamase genes from the *Y. enterocolitica* chromosome should be helpful in understanding the relationship among β -lactamase genes in Gram-negative bacteria. In addition they will be used to investigate the contribution of each enzyme to the resistance of *Y. enterocolitica* to β -lactams and may also be useful for studying gene expression in *Y. enterocolitica*.

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