Sequence and comparative analysis of three *Enterobacter cloacae* *ampC* β -lactamase genes and their products

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The sequences of three *Enterobacter cloacae* *ampC* β -lactamase genes have been determined. The deduced amino acid sequences are very similar: out of a total of 361 residues, only eight positions were found to be variable, and several mutations yielded residues with very similar properties. The kinetic properties of two of the enzymes were not significantly different. The three enzymes also exhibited a high degree of homology (> 70%) with the *ampC* β -lactamases of *Escherichia coli* K12 and *Citrobacter freundii*, confirming the homogeneity of class-C β -lactamases.

INTRODUCTION

Various Gram-negative bacteria, mainly *Enterobacter cloacae*, *Citrobacter freundii* and *Pseudomonas* sp., mutate with high frequency to high resistance to most third-generation cephalosporins. This resistance is based on the overproduction of a class-C β -lactamase, an enzyme encoded by the chromosomal *ampC* gene, which is present in most enterobacteria.

Class-C β -lactamases are active-site-serine enzymes, and hydrolysis proceeds via the formation of an acyl-enzyme intermediate (Knott-Hunziker *et al.*, 1982; Joris *et al.*, 1984).

The *ampC* genes from *Escherichia coli* K12 and *C. freundii* OS60 have been cloned and sequenced (Jaurin & Grundström, 1981; Lindberg & Normark, 1986), whereas the *ampC* genes from *E. cloacae* strains P99 and MHN1 have been cloned (Lindberg & Normark, 1987; Nicolas *et al.*, 1987). Of all *ampC* β -lactamases, that produced by *E. cloacae* P99 has been the most studied. Moreover, it has been crystallized, and structural investigations have been commenced (Charlier *et al.*, 1983). Recently, the β -lactamase produced by another *Enterobacter cloacae* strain, Q908R, was purified and crystallized (O. Dideberg & P. Charlier, personal communication). This enzyme is also produced in large amounts, and its gene has been cloned in the present study. The principal goals of the present work were to compare the P99 and Q908R enzymes biochemically and to determine the sequences of the three *E. cloacae* *ampC* genes. Hereafter the enzymes are given the names of the strains which produce them.

MATERIALS AND METHODS

Bacterial strains and plasmids

Enterobacter cloacae strains P99 and Q908R were gifts from Dr. W. Zimmermann (Ciba-Geigy, Basle, Switzer-

land). *E. coli* FL01 (Lindberg & Normark, 1987) was used for plasmid propagation and in subcloning experiments. *E. coli* SN0302 (Lindberg & Normark, 1987) was used in the cloning of the *E. cloacae* Q908R *ampC* gene. Plasmids pNU359 and pNU354 were used as sources for *E. cloacae* P99 *ampC* DNA and have been described previously (Lindberg & Normark, 1987). Fragments for DNA sequencing of the *E. cloacae* MHN1 *ampC* gene were obtained from pEC1B (Nicolas *et al.*, 1987; Honoré *et al.*, 1986).

Media chemicals and enzymes

Enterobacter cloacae (P99 and Q908R) was grown and the β -lactamases purified as previously described (Joris *et al.*, 1985). The affinity procedure of Cartwright & Waley (1984) was also used. *E. coli* was routinely grown in L-broth (Bertani, 1951) supplemented with medium E (Vogel & Bonner, 1956) and 0.2% glucose. For phage preparation, JM103 was grown in 2YT (Miller, 1972) medium. When necessary, media were supplemented with tetracycline (20 μ g/ml), chloramphenicol (20 μ g/ml), or β -lactam antibiotics at various concentrations. Restriction endonucleases, phage-T₄ DNA ligase, phage-T₄ polymerase and the Klenow fragment of *E. coli* DNA polymerase I were purchased from New England Biolabs, Boehringer Mannheim G.m.b.H., Pharmacia Molecular Biologicals or Amersham International. [³⁵S] α dATP was purchased from Amersham International. dNTPs and ddNTPs were obtained from Pharmacia Molecular Biologicals. All other chemicals were of the highest grade commercially available.

DNA sequencing

The approach taken for DNA sequencing of the *E. cloacae* *ampC* genes was to create a random library of subfragments from a suitable restriction fragment in phage M13mp8. The fragment was purified from a 5%-(w/v)-polyacrylamide gel by electroelution followed by recircularization with phage-T₄ DNA ligase. The re-

Abbreviations used: bp, base-pair(s); f.p.l.c., fast protein liquid chromatography.

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circularized fragment (10 μ g) was dissolved in 250 μ l of 10 mM-Tris/HCl/1 mM-EDTA, pH 8.0, and randomly fragmented by sonication (25 s at setting 4 on a Branson sonifier) to give a size distribution with a peak around 400 bp. After phenol extraction and ethanol precipitation, the fragments were repaired by using phage-T₄ polymerase in the presence of 250 μ M-dNTPs, whereafter they were applied to a 5%-polyacrylamide gel. Fragments ranging from 350 to 600 bp in size were cut out from the gel, extracted by electroelution and cloned into endonuclease-SmaI-digested M13mp8. Single-stranded preparations of recombinant phage were sequenced by using the dideoxy chain-termination method of Sanger *et al.* (1977), using deoxyadenosine 5'- α -[³⁵S]thio-triphosphate. The reactions were run on denaturing 7%-polyacrylamide/7 M-urea gels without temperature control. Compressions were resolved by running the gel at a high temperature (75 °C) or by applying the samples to gels containing 20% formamide.

Computer analysis

The GENEUS sequence-analysis system as well as the programs developed by Staden (see Harr *et al.*, 1986) were utilized to assemble and analyse the sequence.

Kinetic analysis

The kinetic parameters for nitrocefin and cephalothin were obtained by analysing complete time courses as described by De Meester *et al.* (1987). The buffer was 50 mM-Hepes, pH 8.2, containing 0.2 M-NaCl and bovine serum albumin (0.2 mg·ml⁻¹). For benzylpenicillin, the value of k_{cat} was measured at a substrate concentration much higher than the K_m (100 μ M) and that of K_m by substrate competition with 100 μ M-nitrocefin.

Amino acid analysis

Amino acid analysis was performed on a Dionex D-300 automatic analyser.

N- and C-terminal sequences

The N-terminal residues of the Q908R β -lactamase were determined by the method of Wittmann-Liebold & Lehmann (1980). The N-terminal residues of the tryptic and CNBr peptides were determined with dansyl chloride as described by Gray (1972). C-Terminal sequences were determined by digestion of the SDS-denatured protein with carboxypeptidase Y. The protein (1.6 mg), dissolved in 4.5 ml of 100 mM-sodium phosphate buffer, pH 7.5, was denatured by the addition of 0.1% SDS (final concn.). After addition of norleucine (as an internal standard) and of carboxypeptidase Y (0.02 mg), the mixture was incubated at 37 °C, and samples were withdrawn after 0, 2, 5, 15, 30 and 60 min. The reaction was stopped by addition of 0.5 ml of acetone and 10 μ l of 1 M-HCl. After centrifugation, each supernatant was divided in two portions, which were evaporated to dryness. One dry residue was directly redissolved in citrate buffer, pH 2.2, and submitted to amino acid analysis. The second was similarly analysed after a 24 h hydrolysis at 120 °C in azeotropic HCl. This double analysis was performed because, on the basis of the gene sequence, the expected C-terminal residue was glutamine for both P99 and Q908R β -lactamases and, in our analytical system, glutamine was eluted in the same position as serine. After acid hydrolysis, glutamic acid was easily distinguished from serine.

Peptide maps of P99 and Q908R β -lactamases

The freeze-dried proteins (3 mg) were dissolved in 0.5 ml of 10 mM-sodium phosphate buffer, containing 4 M-urea, and incubated at 37 °C for 30 min. Trypsin (30 μ g) was added, and the samples were further incubated for 4 h at 37 °C. The reaction was stopped by addition of 30 μ l of 86% (v/v) H₃PO₄. A 15 μ l aliquot was analysed with the help of a Pharmacia f.p.l.c. apparatus equipped with a reverse-phase Pro-RPC column (0.5 cm $\times$ 10 cm). Buffers contained 1% H₃PO₄, pH 2.2, in (100%) water (eluent A) or in water/acetonitrile (3:2, v/v) (eluent B). The gradient went from 0 to 100% of B in 20 min (flow rate 0.3 ml·min⁻¹). Peptides were detected at 214 nm.

Isolation of tryptic peptides

The P99 β -lactamase was digested by trypsin as described above. Peptides were purified by chromatography on Sephadex G-25, Sephadex G-50 and on an f.p.l.c. reverse-phase Pep-RPC column (0.5 cm $\times$ 5 cm). In this case the buffers contained 0.1% trifluoroacetic acid in water (eluent A) or in water/acetonitrile (3:2, v/v) (eluent B). The gradient went from 0 to 100% of B in 20 min (flow rate 0.5 ml·min⁻¹).

Isolation of CNBr peptides

The freeze-dried protein (13 mg) was dissolved in 7 ml of 70% formic acid, and 40 mg of CNBr were added. The reaction was performed at 20 °C under N₂ and in the dark. After 24 h, the sample was freeze-dried. The residue was dissolved in 5% (v/v) formic acid. Peptides were purified by chromatography on Sephadex G-50 and reverse-phase f.p.l.c. on the Pep-RPC column as described above.

RESULTS

Kinetic parameters

Table 1 compares some kinetic parameters of the P99 and Q908R β -lactamases. The two enzymes appeared to exhibit similar, if not identical, properties. Moreover, they could not be distinguished on the basis of their behaviour during the purification procedure (molecular-sieve and ion-exchange chromatography) or of their electrophoretic mobilities in the presence of SDS. It appeared that the easiest way to verify a possible identity between the two proteins was to obtain peptide maps after trypsin digestion.

Peptide maps

On the basis of the lysine and arginine content of the protein (Joris *et al.*, 1985), 37 tryptic peptides were expected. The technique described above revealed 20 peptides. It was not impossible that some very hydrophilic peptides were eluted in the void volume with the urea peak. Of those 20 peptides, three were in clearly different positions when the P99 and Q908R enzymes were compared. This result was verified by digesting an equimolar mixture of the two enzymes and submitting the peptide mixture to chromatographic analysis; this experiment confirmed the differences observed with the individual proteins. While this work was in progress, crystals of the Q908R β -lactamase were obtained which were different from those obtained under the same conditions with the P99 enzyme (O. Dideberg & P. Charlier, personal communication). For these reasons,

Table 1. Kinetic parameters for the P99 and Q908R β -lactamases

Substrate and concn.	Enzyme . . . Parameter . . .	P99			Q908R		
		Amount of enzyme	K_m (μ M)	k_{cat} (s^{-1})	Amount of enzyme	K_m (μ M)	k_{cat} (s^{-1})
Nitrocefin (50–150 μ M)		10–30 ng	25 $\pm$ 2	780 $\pm$ 30	10–30 ng	23 $\pm$ 2	780 $\pm$ 30
Cephalothin (50–100 μ M)		30–50 ng	11 $\pm$ 1	200 $\pm$ 10	30–50 ng	10 $\pm$ 1	180 $\pm$ 10
Benzylpenicillin (1 mM)		10 μ g	–	14 $\pm$ 1.5	10 μ g	–	18 $\pm$ 0.7
(1–5 μ M)		5 ng	0.6 $\pm$ 0.1	–	5 ng	0.5 $\pm$ 0.1	–

it was decided to clone and sequence the Q908R β -lactamase gene and to compare its sequence with those of the *ampC* genes from strains P99 and MHN1, which had been cloned previously (Lindberg & Normark, 1987; Nicolas *et al.*, 1987).

Cloning of the *E. cloacae* Q908R *ampC* β -lactamase gene

The cloning strategy successfully employed for the *ampC* gene of *E. cloacae* strain P99 was again adopted. An *EcoRI* digest of Q908R chromosomal DNA was ligated to pACYC184 digested with the same enzyme, and the ligation mixture was transformed into *E. coli* FL01 selecting for resistance to tetracycline and ampicillin (4 μ g/ml). When the *E. cloacae* P99 *ampC* gene, together with its regulator *ampR*, is cloned into an *E. coli* *ampD* mutant, β -lactamase expression is increased more than 20-fold. Since *ampR* should be present in the Q908R *ampC* clones, mini-plasmid preparations from the resistant colonies were used to transform *E. coli* SN0302 (*ampD2*) to tetracycline resistance (Lindberg & Normark, 1987). Subsequently the transformants were screened for resistance to more than 100 μ g of ampicillin/ml. One of

the clones meeting these criteria was shown to inducibly express β -lactamase and to hybridize a P99 *ampC* probe (results not shown). Plasmid pNU368 was obtained by subcloning a *SalI* *Bam*HI digest of the original clone into pUC8. As is apparent from Fig. 1, the restriction maps of the *ampR-ampC* insert from P99 (pNU359), MHN1 (pEC1B) and Q908R (pNU368) were very similar.

Nucleotide sequencing of the three *E. cloacae* *ampC* genes

Virtually the entire *ampC* gene of *E. cloacae* P99 was isolated from pNU354 as one *SphI* fragment and one *SphI/XhoI* fragment. A *XhoI/Bam*HI fragment, containing the last 60 nucleotide pairs of the gene, was also isolated from pNU359. These fragments were isolated, recircularized and used to create a library of M13mp8 clones containing subfragments of this DNA. In all, 50 such random clones were sequenced, giving a theoretical coverage of both strands with 95 % probability. By using conventional cloning of restriction fragments, the gaps in the sequence were filled in and the sequence was extended to also cover the end of the *ampC* gene. As a result, every nucleotide was sequenced on average seven times, and at

Fig. 1. Restriction map of the *E. cloacae* *ampR* and *ampC* genes

Fig. 3. Time course of the carboxypeptidase Y degradation of the denatured P99 β -lactamase

The first-released residues were unambiguously Gln, Leu, Ala and Glu in that order. After 5 min, some Gly was also present, but the amount did not increase with time and probably represented an impurity.

The following amino acids could not be positioned: Glu, Tyr, Ile, Val, His and an additional Ala. The observed results clearly corresponded in both cases to the amino acid sequence deduced from the gene sequence (Fig. 2) just before the TAA stop codon at positions 1187–1189.

Analysis of some tryptic and CNBr peptides

Four tryptic and four CNBr peptides were obtained with a suitable degree of purity. Their compositions presented in Table 2 allowed their positioning in the sequence.

DISCUSSION

The determination of the nucleotide sequence of the genes coding for the three *E. cloacae* β -lactamases indicates that the three proteins are nearly identical. The mature P99 and Q908R proteins also exhibit the same *N*-terminal sequences. It seems very likely that the mature MHN1 enzyme starts with the related H-Ala-Pro-Val sequence. When the mature proteins were compared, only four, six and six differences respectively (out of a total of 361 residues) were found for the pairs P99–Q908R, P99–MHN1 and Q908R–MHN1. These differences are found in only eight positions. Various chemical

properties of the P99 β -lactamase had been reported previously (Joris *et al.*, 1985). The published amino acid composition contained only one major error (Table 2): the number of Met residues was strongly underestimated (two or three instead of 12). In contrast, the estimation of the number of Trp residues (9.2 ± 0.8 instead of 11) was quite good. Of the various methods tested in that previous work, *N*-bromosuccinimide titration and direct estimation on the basis of A_{280} yielded the best results (9.8 residues, i.e. only a 10% error). Few mistakes were made in the determinations of the *N*-terminal sequence of the protein and of the active-site tryptic peptide when performed on an automatic gas-phase sequencer. Only Ala-29 was mistakenly identified as a Glu residue. Residues 39 (His) and 44 (Gly) had been tentatively identified as Arg and Lys respectively, but the evidence had been acknowledged as weak. On the other hand, the published *C*-terminal sequence, -Glu-Leu-Ala-OH, did not correspond to the nucleotides preceding the stop codon. Careful re-examination of the chromatograms and of the experimental conditions indicated that the digestion (β -lactamase/carboxypeptidase 100:1, w/w) had probably been too long and that the liberated Ala represented more than one residue. In the present work, the experiments were performed with the same β -lactamase/peptidase ratio, but samples were taken after much shorter digestion times and the sequence thus found was in agreement with that of the nucleotides.

The number of CNBr and tryptic peptides that could be completely purified in one f.p.l.c. run was disappointingly small. It had been hoped that such a method would allow the few residues which might differ in very similar enzymes, such as P99 and Q908R, to be directly identified. Unfortunately, the technique utilized in the present work did not allow this goal to be achieved.

Fig. 4 and Table 3 show that the three *Enterobacter* β -lactamases exhibit a high degree of homology with the chromosome-encoded β -lactamases of *E. coli* K12 and *Citrobacter freundii*; the sequences of the mature proteins can easily be aligned with the introduction of only one deletion, the *N*-terminus of the K12 enzyme being three residues shorter. This difference represents a real deletion and not a different specificity of the signal peptidase, since the signal peptides were also easily aligned (Fig. 5) and exhibit the same *C*-terminal alanine in all cases. There is, however, no deletion within the mature proteins. Comparison of the signal peptides shows only one additional deletion in the *E. coli* K12 pre-protein. Generally, however, the signal peptides exhibit a somewhat lower degree of homology than the mature proteins. This is also reflected in the number of residues which are conserved in all five signal peptides (8/20, i.e. 40%, against 63% for the mature proteins). However, several of the substitutions are highly conservative (positions 3, 5, 6, 12, 14, 19) and, as expected, after the basic sequences near the *N*-terminus, the signal peptides become quite hydrophobic. Structure predictions indicate a clear possibility of helix formation starting at residue 9.

As noted before, the residues immediately surrounding the active-site serine residue (64) are highly conserved. Between Val-54 and Gly-74, 16 residues (76%) are common to the five proteins, and, of the five substitutions observed, two are highly conservative (positions 62 and 65).

Only a limited amount of information is available

Table 2. Comparison of the published amino acid composition with that derived from the sequence and composition and *N*-terminal residues of purified peptides

Amino acid	No. of residues in protein		Composition (residues/molecule)							
			Tryptic peptides				CNBr peptides			
	Published values	From sequence	T1	T2	T3	T4	CB1	CB2	CB3	CB4
Lys	23	19	0.73 (1)*	0.82 (1)		0.73 (1)	1.4 (1)	3.6 (3-4)	0.21 (0)	1 (1)
His	6/7	5	0.2 (0)				0.1 (0)	0.5 (0-1)		0.27 (0)
Arg	13	11			1.05 (1)	0.78 (1)				
Asx	33	28	1.02 (1)			0.93 (1)	2.9 (3)	5.38 (5)	0.30 (0)	0.88 (1)
Thr	26	23	0.64 (1)	0.78 (1)		1.99 (2)	1 (1)	3.4 (3)		
Ser	18	18	0.75 (1)	0.85 (1)				4.17 (4)		
Glx	39	35	1.13 (1)	0.89 (1)	0.2 (0)	3.09 (3)	2.2 (2)	5.38 (5)	1.7 (2)	
Pro	21	27	1.36 (1)	0.63 (1)		1.9 (2)		7.34 (7)		
Gly	34	30	1.47 (1-2)	0.3 (0)		2.75 (3)	1.1 (1)	5.67 (6)	1.82 (2)	
Ala	46	44	1.89 (2)				2.1 (2)	7.6 (7-8)	0.28 (0)	1.17 (1)
Val	30	31	0.9 (1)	1.27 (1)			1.9 (2)	10.1 (10)	0.3 (0)	1.1 (1)
Met or Hse	2/3	12	0.49 (1)				1 (1)	1 (1)	1 (1)	1 (1)
Ile	14	15						2.18 (2)		
Leu	32	29	0.82 (1)				1 (1)	3.6 (4)	0.75 (1)	
Tyr	13	16	0.43 (0-1)		0.92(1)	0.69 (1)	0.9 (1)	1.60 (2)	0.8 (1)	
Phe	8	7				0.78 (1)		1.7 (2)		
Trp	9/10	11	ND§	ND	ND	ND	ND	ND	ND	ND
Total...	367/370	361	12-14	6	2†	15‡	15	61-64‡	8	4†
<i>N</i> -Term- inus...	Thr	Thr	Val	Thr	Tyr	Phe	Leu	Leu	Tyr	Ala
Position in sequence...			211-224	1-6	259-261	134-148	216-231	274-338	266-273	231-235

* Values in parentheses represent the rounded-up number of residues in the peptide.

† One Trp present in the sequence.

‡ Two Trp present in the sequence (but excess of Gly found in the composition of peptide T₄).

§ ND, not determined.

about the *Pseudomonas aeruginosa* class-C β -lactamase. The 33 *N*-terminal residues (Emanuel *et al.*, 1985) can be aligned with residues 1-33, and the active-site peptide (Knott-Hunziker *et al.*, 1982) with residues 53-67 of the three *E. cloacae* and of the *C. freundii* enzymes. Although the area around the active-site Ser-64 residue is again very strongly conserved, the *N*-terminal portion exhibits a much higher degree of heterogeneity (only about 30% identity or less with any of the other five enzymes). On this basis, the *Pseudomonas* enzyme appears to be a further relative of the family. It should be remembered that, in contrast with the three other genera, *Pseudomonas* is not a member of the Enterobacteriaceae.

As outlined above, the amino acid sequence of the three *Enterobacter* enzymes are extremely similar. At the DNA level, a pairwise comparison yields the following results (including the signal peptides): P99-Q908R: seven differences, of which four result in a change of amino acid; P99-MNH1: 18 differences, of which seven result in a change of amino acid; and MNH1-Q908R: 17 differences (six different amino acids). Indeed, despite the isolation of these three *E. cloacae* strains in different countries and at different times, very little DNA sequence divergence has occurred. The very similar enzymic activities of P99 and Q908R indicate that the four mutations are unlikely to affect the active and substrate-binding sites. Surprisingly, however, Q908R crystallized in a completely different symmetry, and it could be

hypothesized that the substitutions mainly affect the surface of the enzyme, thus altering the intermolecular interactions. However, the isoleucine $\rightarrow$ valine and leucine $\rightarrow$ valine transitions at positions 16 and 132 respectively are unlikely to result in very drastic changes of properties, even if those residues are exposed.

Although little sequence homology was found between the DD-peptidase of *Streptomyces* R61 and class-A β -lactamase, striking similarities were observed in the three-dimensional structures (Samraoui *et al.*, 1986; Kelly *et al.*, 1986). Since class-C β -lactamases exhibit a significant sequence homology with the peptidase (B. Joris, unpublished work), it is quite likely that the three-dimensional structures of the DD-peptidase and of the *Enterobacter cloacae* enzymes will be very similar. The cloning and sequencing of the genes open the door to site-directed mutagenesis which, combined with the crystallographic data, will help to explain the mode of action of these enzymes.

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Fig. 4. Amino acid sequence of *ampC* β -lactamases in standard one-letter code

Shown is the sequence of the mature *E. cloacae* P99 β -lactamase, as inferred from partial protein sequence (Joris *et al.*, 1985) and the gene sequence. The double underlining indicates errors in the partial protein sequence. The sequence of the *E. cloacae* Q908R (908) and MHN1 (MHN) is shown only when different from that of P99. Also the sequence of the *E. coli* K12 (K12) (Jaurin & Grünström, 1981) and *C. freundii* (CF) (Lindberg & Normark, 1986) β -lactamases are shown in a similar manner. For the *Pseudomonas aeruginosa* 18S β -lactamase, only partial sequence data are available, corresponding to residues 1–33 and 53–67 of the P99 enzyme (Knott-Hunziker *et al.*, 1982; Emanuel *et al.*, 1985). The wavy line corresponds to portions which have not been sequenced. Dashes indicate a deletion (in the *E. coli* K12 enzyme) as discussed in the text. The position of the active-site serine residue is marked with an asterisk.

Table 3. Homology between the mature class-C β -lactamases and between their signal peptides

Values in square brackets were computed on the basis of the incomplete sequence of the Q908R signal peptide. Homology is shown as a percentage.

		Mature protein						
		P99	Q908R	MHN1	CF	K12		
Signal peptide	P99						P99	
	Q908R	[100]					Q908R	
	MHN 1	90	[93]				MHN1	
	CF	65	[79]	70		77	CF	
	K12	50	[50]	55	65		K12	
		P99	Q908R	MHN1	CF	K12		
		Signal peptide						

	1	5	10	15	20															
K12	M	F	K	T	T	L	C	-	A	L	L	I	T	A	S	C	S	T	F	A
CF	M	M	K	K	S	I	C	C	A	L	L	L	T	A	S	F	S	T	F	A
MHN1	M	M	K	K	S	L	C	C	A	L	L	L	G	L	S	C	S	A	L	A
P99	M	M	R	K	S	L	C	C	A	L	L	L	G	I	S	C	S	A	L	A
Q908R							C	C	A	L	L	L	G	I	S	C	S	A	L	A

Fig. 5. Comparison of the sequences of the signal peptides of the five known class-C β -lactamases

Abbreviations and symbols are as in Fig. 4.

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