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FEMS Microbiology Letters 165 (1998) 85–90

FEMS MICROBIOLOGY LETTERS

An extended-spectrum AmpC-type β-lactamase obtained by in vitro antibiotic selection

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Received 9 March 1998; revised 8 May 1998; accepted 25 May 1998

Abstract

A predictive approach was assayed to evaluate the possibility of mutant Amp-C β-lactamase emergence with increased substrate spectrum (including new C-3′ quaternary ammonium cephems). The *ampC* gene encoding the AmpC β-lactamase from *Enterobacter cloacae* was cloned and expressed in an AmpC-defective strain of *E. coli*. After the AmpC containing strain was challenged with cefpirome, an *ampC* variant encoding an enzyme with increased resistance to cefpirome and cefepime was selected. In addition, this variant conferred increased resistance to penicillins and third generation cephalosporins. The complete nucleotide sequence of the gene was determined. The deduced peptide sequence showed a single change with respect to the wild-type gene: valine to glutamic acid at position 318 of the native protein (298 of the mature enzyme). The potential emergence and spread of this type of AmpC variants among pathogens should be considered.

Keywords: Evolution of resistance; β-Lactamase; Cephem resistance; AmpC mutant

1. Introduction

β-Lactamase production is the main and widespread mechanism of resistance to β-lactam antibiotics among Gram-negative bacteria. These enzymes probably have a potential to evolve to enzymes able to hydrolyse almost any new β-lactam molecule. Among β-lactamases, two groups, chromosomally and plasmid-encoded enzymes have extensively been described [1]. Most members of *Enterobacteriaceae* produce Group I chromosomal cephalosporinases, their inducible expression being regulated according to particular genetic control mechanisms in several species [2]. Ambler Class A β-lactamases, which include plasmid-mediated TEM and SHV enzymes, are constitutively produced. Point mutations in the corresponding structural genes lead either to a widening of the hydrolytic spectrum to extended-spectrum β-lactams or to resistance to the action of β-lactamase inhibitors [1,3]. Recently, chromosomal *ampC* genes of *Enterobacteriaceae* and *Pseudomonas aeruginosa* have been found to be located on plasmids conferring the 'AmpC constitutive spectrum of

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0378-1097/98/$19.00
PII: S0378-1097(98)00239-0

resistance', including resistance to third-generation cephalosporins and cephamycins and susceptibility to cefpirome, cefepime and carbapenems [4–6]. Moreover, the AmpC-type β-lactamases are naturally resistant to the currently used β-lactamase inhibitors clavulanic acid, sulbactam, and tazobactam. Of particular concern, due to the epidemiogenic features of the genus, is the report of two isolates of *Salmonella* spp. harbouring plasmid-encoded AmpC β-lactamases [7,8]. In general, the risk of spreading of the AmpC resistance mechanism among enterobacterial isolates is worrying. An example of this situation may be represented by *Enterobacter* strains which, frequently isolated from seriously compromised patients, pose a challenge to the design of alternative therapeutic regimes.

The aim of this work was to predict and evaluate the possible appearance of plasmid-mediated *ampC* gene mutation(s) altering the amino acid AmpC structure, resulting in an eventual enlargement of the spectrum of β-lactam resistance, including resistance to the new C-3′ quaternary ammonium cephems.

2. Materials and methods

2.1. Construction of the ampC-plasmid-containing strain

A strain of *E. coli* harbouring a plasmid containing the *ampC*-MHN gene, was kindly provided by E. Collatz. Plasmid DNA was purified and used as template in a polymerase chain reaction (PCR). The gene encoding the chromosomal β-lactamase from *Enterobacter cloacae* was PCR amplified. Oligonucleotides E1 (5′-TCG**GAATTC**CGGAGGATTACTGATG-3′) and E2 (5′-TTA**GTCGAC**AATGTTTTACTGTAGCGC-3′) were used, respectively, as forward and reverse primers. Both primers contained a tail with the *Eco*RI (E1) and *Sal*I (E2) recognition sequences (in bold type). Since oligonucleotides E1 and E2 prime, respectively, at the origin and at the end of the structural gene, all regulatory signals from the original gene were eliminated by the cloning strategy. In addition, primer E1 contains a mismatched base (underlined) to generate a consensus *E. coli* ribosome binding site. The expected amplified product of ca. 1200 bp was verified by agarose gel electrophoresis. The *Eco*RI/*Sal*I-digested PCR product was ligated with the *Eco*RI/*Sal*I-digested vector pBGS18$^-$ [9], which confers resistance to kanamycin. In order to avoid interferences with the host-AmpC enzyme, the *Escherichia coli* K-12 strain MI1443 (*ampC* deleted mutant) [10] was used as recipient for the hybrid plasmid. The ligation mixture was introduced by transformation in $CaCl_2$ competent MI1443 cells. Transformants were selected onto agar plates containing kanamycin (30 μg ml^{-1}) and ampicillin (80 μg ml^{-1}). Several clones were selected, purified, and their plasmid content analysed. The presence of the desired hybrid plasmid was verified

Table 1
Resistance phenotype conferred by wild type and mutant *ampC* genes

	pBGS18	pBGMHN	pBGMHN-7.6
Amoxycillin	1	16	128
Amoxycillin/clavulanate	≤1/0.5	16/8	16/8
Piperacillin	1	2	8
Piperacillin/tazobactam	1/4	1/4	2/4
Ceftazidime	0.06	0.5	64
Cefotaxime	0.06	1	4
Cefpirome	0.015	0.015	1
Cefepime	0.015	0.015	4
Cefoxitin	1	32	8
Imipenem	0.06	0.06	0.06
Meropenem	0.03	0.03	0.03

MICs (in μg ml^{-1}) of several β-lactams against *E. coli* MI1443 harbouring pBGS18$^-$ control plasmid, hybrid plasmid pBGMHN and the evolved derivative after cefpirome challenge, pBGMHN-7.6. Proportion of amoxycillin and clavulanate was 2:1 in all the assays. Tazobactam was used at a fixed concentration of 4 μg ml^{-1}.

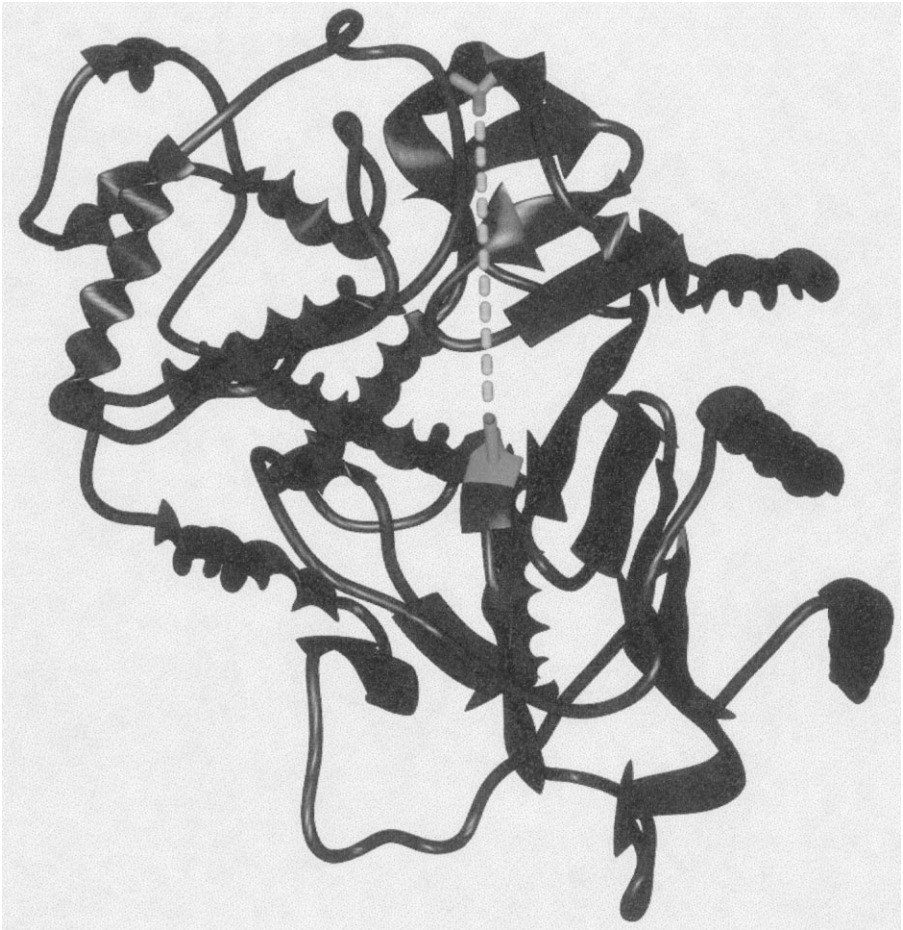

Fig. 1. Ribbon diagram of AmpC from *E. cloacae*, showing the relative positions of Val298 (at the top of the figure) and the catalytic Ser64 (at the center). Figure made with MidasPlus [18].

by restriction analysis, PCR, and by sequencing the whole cloned *ampC* gene. The nucleotide sequence has been deposited at the EMBL Nucleotide Sequence Database under the accession number AJ005633.

2.2. Selection of the cefpirome-resistant variant

To obtain molecular variants with increased activity against new cephalosporins, the strain MI1443 (pBGMHN) was submitted to step-wise selection experiments using cefpirome as selecting antibiotic. The experiment was performed by a broth macrodilution method with daily transfer of an adjusted $1–5\times10^5$ CFU ml^{-1} inoculum to fresh media containing two-fold serial dilutions of cefpirome. Serial transfer was continued until no visible growth was observed. Plasmid DNA was extracted from an aliquot of the last tube that exhibited turbidity. The DNA was introduced by transformation in the strain MI1443 and transformants were isolated on LB agar plates containing 30 μg ml^{-1} of kanamycin (ampicillin was avoided to overcome the possible counter-selection of mutants). Several colonies were selected and a first screening of antibiotic susceptibility to amoxicyllin (AMX), amoxycillin-clavulanate (AMC), piperacillin (PIP), piperacillin-tazobactam (PTZ), cefotaxime (CTX), ceftazidime (CAZ), cefepime (FEP), cefpirome (CPR), cefoxitin (FOX), imipenem (IMP) and meropenem (MEM) was per-

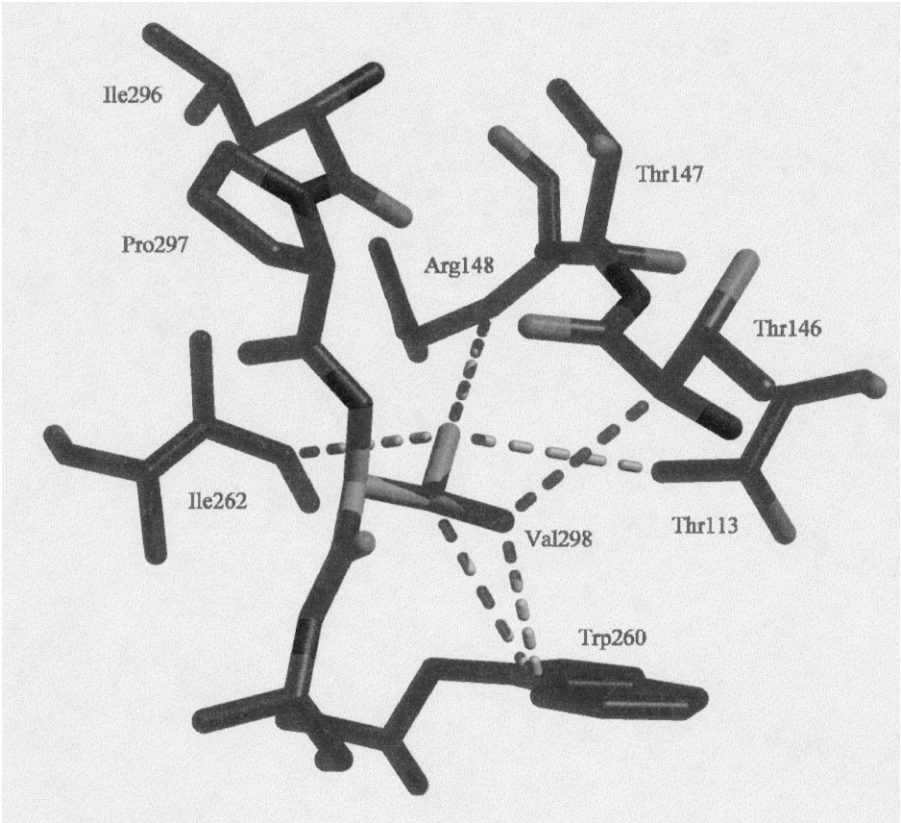

Fig. 2. The residue environment of Val298. Dashed lines indicated van der Walls contacts. Figure made with MidasPlus [18].

formed by the E-test (data not shown). One clone, 7.6, was chosen for further study as it presented the higher levels of resistance to the antibiotics tested.

3. Results and discussion

3.1. Phenotypic characterisation of the mutant

Table 1 shows the resistance phenotypes (obtained by agar dilution according to the NCCLS guidelines) of the strain MI1443 containing the vector pBGS-18$^-$ (lacking the *ampC* gene), the wild-type plasmid pBGMHN, or the mutant pBGMHN-7.6. The mutant AmpC enzyme was able to confer resistance to cefepime and cefpirome and also had increased MICs to cefotaxime and ceftazidime. Interestingly, the MIC to cefoxitin was 4-fold decreased. Activity on carbapenems, imipenem and meropenem remained unchanged.

3.2. Genotypic characterisation of the mutant

The increased resistance conferred by the plasmid pBGMHN-7.6 could be theoretically due to mutations in the promoter region, mutations on the structural *ampC* gene or mutations that increase the plasmid copy number. To verify that this resistance is actually due to mutations on the structural *ampC* gene, the *ampC* region from this less susceptible variant was amplified by the same PCR procedure and again cloned in pBGS18$^-$, with identical strategy as before. The *ampC*-containing fragment from clone 7.6 was able to confer the same increased resistance to cefepime and cefpirome as the original mutant strain. This indicated that mutation(s) responsible for cephalosporins resistance was/were in the structural *ampC* gene. The plasmid harbouring the mutant *ampC* gene was named pBGMHN-7.6. To characterise the mutation(s) responsible for increased resistance, the whole *ampC*-7.6 structural gene was

sequenced using the reverse and universal primers and *ampC* internal oligonucleotides. The obtained sequence was compared with the wild type obtained before the cefpirome challenge. This comparison revealed a single base replacement at codon 318 of the native protein which corresponds to amino acid position 298 in the mature protein. The amino acid change was Val^{318} (GTG) to Glu (GAG).

3.3. Structural analysis

Val^{298} is located on a short segment of β-strand on an outer edge of the AmpC structure. The residue itself is distant from the catalytic site, being 18.1 Å from the Oγ of the catalytic Ser^{64} (Fig. 1). The residue points towards the centre of a small hydrophobic cluster in AmpC, making van der Waals and 'hydrophobic' contacts with the side chain of residue Thr^{113}, the Cβ of Arg^{148}, the main chain Cα of Thr^{146} and Thr^{147}, and the side chain of Trp^{260} and Ile^{262} (Fig. 2).

The substitution of Val^{298} by a Glu is unlikely to have a direct effect on ligand recognition, as for instance by polar or non-polar bonds with the substrate, or even by interacting with substrate binding residues. This is because of the long distance from the Val^{298} to the catalytic site. Of course, a long-range electrostatic effect of the glutamic acid cannot be ruled out, but even this seems unlikely. Instead, the interaction is probably indirect. At present, the form of this indirect interaction can only be a matter of speculation. Still, several interesting points may be noted. Val^{298} is at the centre of a small hydrophobic patch and is largely buried from solvent. The substitution of glutamic acid for the valine at this position should disrupt the local structure nucleated by this mini-hydrophobic 'core' [11]. This disruption may increase the overall flexibility of the enzyme, broadening its spectrum of action including traditionally resistant substrates. At the same time, the substitution, coming well away from the central hydrophobic core of AmpC, is probably not so disruptive as to unfold the enzyme. This would explain why AmpC enzymes do not have a Glu at position 298. Small hydrophobic residues like valine are highly conserved here, and indeed the nature of the surrounding residues (Fig. 2) is also highly conserved. Replacement of the valine would thus be costly as far as the stability of the enzyme is concerned. Thus, only under the pressure of antibiotic action would this substitution arise. It may be interesting to undertake further enzymatic and stability studies to test this hypothesis [12]. Overall, the long distance of this substitution from the catalytic site, and its residue environment, resemble resistance mutations in HIV-1 protease that have been observed to arise during the course of protease-inhibitor treatment against AIDS [13].

3.4. Concluding remarks

Evolution of β-lactamases represents a paramount example of adaptive behaviour by means of amino acid changes yielding more efficacious enzymes. The objective of this work was to obtain some experimentally-based prediction on the possible emergence of 'extended spectrum' AmpC β-lactamases, considering the increasing use of antibiotics active on strains harbouring these enzymes. Resistance mediated by AmpC enzymes is dependent on the modification of genes that control the amount of enzyme produced [14]. In this work we present an AmpC molecular variant obtained in vitro after a challenge with cefpirome as selecting agent, that confers considerable levels of resistance to both cefepime and cefpirome (64 and 256 times increase in MIC in comparison with the control strain harbouring the wild-type hybrid plasmid).

Because of the higher activity of the new C-3′ quaternary ammonium cephalosporin compounds, cefepime and cefpirome, on AmpC derepressed mutants compared with ceftazidime or cefotaxime, they have been considered poor selectors for resistance in the hospital setting [15–17]. This paper opens the possibility that these antibiotics may select AmpC variants with cross-resistance to all available cephalosporins. Interestingly, cephamycins (as cefoxitin), classically considered susceptible to AmpC, decrease their susceptibility in the 7.6 mutant, reducing the MIC from 32 to 8 μg ml^{-1}.

The possible emergence of isolates harbouring a resistance mechanism like this type of 'extended-spectrum chromosomal enzymes' in the clinical setting that could be selected during prolonged treatments with the novel C-3′ quaternary ammonium compounds remains to be evaluated.

Acknowledgments

The authors wish to thank E. Collatz who kindly provided the cloned *ampC*-MHN gene and L. de Rafael for English corrections. This study was supported in part by a grant from Eli Lilly.

References

[1] Bush, K., Jacoby, G.A. and Medeiros, A.A. (1995) A functional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob. Agents Chemother. 39, 1211–1233.

[2] Tuomanen, E., Lindquist, S., Sande, S., Galleni, M., Light, K., Gage, D. and Normark, S. (1991) Coordinate regulation of β-lactamase induction and peptidoglycan composition by the *amp* operon. Science 251, 201–204.

[3] Knox, J.R. (1995) Extended-spectrum and inhibitor-resistant TEM-type β-lactamases: mutations, specificity, and three-dimensional structure. Antimicrob. Agents Chemother. 39, 2593–2601.

[4] Galleni, M., Lindberg, F., Normark, S., Cole, S., Honoré, N., Joris, B. and Frère, J.M. (1988) Sequence and comparative analysis of three *Enterobacter cloacae ampC* β-lactamase genes and their products. Biochem. J. 250, 753–760.

[5] González-Leiza, M., Pérez-Díaz, J.C., Ayala, J., Casellas, J.M., Martínez-Beltrán, J., Bush, K. and Baquero, F. (1994) Gene sequence and biochemical characterization of FOX-1 from *Klebsiella pneumoniae*, a new AmpC-type plasmid-mediated β-lactamase with two molecular variants. Antimicrob. Agents Chemother. 38, 2150–2157.

[6] Horii, T., Arakawa, Y., Ohta, M., Sugiyama, T., Wacharotayankun, R., Ito, H. and Kato, N. (1994) Characterization of a plasmid-borne and constitutively expressed bla_{MOX-1} gene encoding AmpC type-β-lactamase. Gene 139, 93–98.

[7] Gaillot, O., Clément, C., Simonet, M. and Philippon, A. (1997) Novel transferable β-lactam resistance with cephalosporinase characteristics in *Salmonella enteritidis*. J. Antimicrob. Chemother. 39, 85–87.

[8] Koeck, J.L., Arlet, G., Philippon, A., Basmaciogullari, S., Thien, H.V., Buisson, Y. and Cavallo, J.D. (1997) A plasmid-mediated CMY-2 β-lactamase from an algerian clinical isolate of *Salmonella senftenberg*. FEMS Microbiol. Lett. 152, 255–260.

[9] Spratt, B.G., Hedge, P.I., Heesen, S., Edelman, A. and Broome-Smith, J.K. (1986) Kanamycin-resistant vectors that are analogs of plasmids pUC8, pUC9, pEMBL8, and pEMBL9. Gene 41, 337–342.

[10] Condon, C. and Weiner, J.H. (1988) Fumarate reductase of *Escherichia coli*: an investigation of function and assembly using in vivo complementation. Mol. Microbiol. 2, 43–52.

[11] Dao-pin, S., Anderson, D.E., Baase, W.A., Dahlquist, F.W. and Matthews, B.W. (1991) Structural and thermodynamic consequences of burying a charged residue within the hydrophobic core of T4 lysozyme. Biochemistry 30, 11521–11529.

[12] Shoichet, B.K., Baase, W.A., Kuroki, R. and Matthews, B.W. (1995) A relationship between protein stability and protein function. Proc. Natl. Acad. Sci USA 92, 452–456.

[13] Rose, R.B., Craik, C.S. and Stroud, R.M. (19??) Domain flexibility in retroviral proteases: structural implications for drug resistant mutations. Biochemistry 37, 2607–2621.

[14] Lindberg, F., Lindquist, S. and Normark, S. (1986) Induction of chromosomal β-lactamase expression in enterobacteria. J. Antimicrob. Chemother. 18, Suppl. C, 43–50.

[15] Garau, J., Wilson, W., Wood, M. and Carlet, J. (1997) Fourth-generation cephalosporins: a review of in vitro activity, pharmacokinetics, pharmacodynamics and clinical utility. Clin. Microbiol. Infect. 13, Suppl. 1, 87–101.

[16] Sanders, W.E. Jr., Tenney, J.H. and Kessler, R.E. (1996) Efficacy of cefepime in the treatment of infections due to multiply resistant *Enterobacter* species. Clin. Infect. Dis. 23, 454–461.

[17] Segreti, J. and Levin, S. (1996) Bacteriologic and clinical applications of a new-spectrum parenteral cephalosporin. Am. J. Med. 100, 45S–51S.

[18] Ferrin, T.E., Huang, C.C., Jarvis, L.E. and Langridge, R. (1988) The MIDAS display system. J. Mol. Graph. 6, 13–27.