IJMR

International Journal of Microbiology Research
ISSN: 0975-5276 & E-ISSN: 0975-9174, Volume 7, Issue 6, 2015, pp.-710-716.
Available online at http://www.bioinfopublication.org/jouarchive.php?opt=&jouid=BPJ0000234

THE EFFECT ON PEPTIDOGLYCAN COMPOSITION OF UNCHARACTERIZED PAE-AMPC MUTANTS PROBES ITS FUNCTIONALITY AS DD-PEPTIDASE

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Received: October 29, 2015; Revised: November 09, 2015; Accepted: November 19, 2015

Abstract- Introduction: It has been largely hypothesized, but never probed, that PBPs and β-lactamases come from an ancient common ancestor, although, there are examples of cross enzymatic reactions (DD-peptidase and β-lactamase) for both types of enzymes. This work aimed to characterize the effect of point mutations [R2→G (AmpC-F2), P243→L (AmpC-F4:C3) and I51→T (AmpC-F4:C6)] on β-lactamase activity of AmpC (Pae-AmpC) from Pseudomonas aeruginosa PAO1 strain; also to track the effect of AmpC activity on peptidoglycan composition, as a consequence of DD-peptidase activities . So, periplasmic and cytoplasmic forms of these Pae-AmpC mutants and the wild type Pae-AmpC were cloned, purified by Ni-affinity chromatography, and then tested for their β-lactamase activities and their effect on PG composition from wild type and mutants of E. coli and P. aeruginosa. *In vitro* assay for β-lactamase activities showed that both point mutations P243→L and I51→T caused 5-fold decrease, while R2→G change caused 2.5-fold decrease in β-lactamase activity when compared with AmpC-F4. On the other hand the cytoplasmic form (AmpC-F3) displayed 8-fold increase in β-lactamase activity. Moreover, AmpC-F3 displayed a secondary DD-endopeptidase/DD-carboxypeptidase on the whole PG in vitro, and DD-endopeptidase activity on individual purified muropeptides. Data obtained from HPLC analysis of PG composition support previous suggestions that AmpC can elicit DD-carboxypeptidase or DD-endopeptidase activity most probably due to structural similarities of the active site with DD-peptidase enzymes having these activities (LMM-PBPs).

Keywords: - AmpC beta-lactamase, Endopeptidase, Carboxypepetidase, DD-peptidases, Low-molecular-mass penicillin-binding proteins, Peptidoglycan, *Pseudomonas aeruginosa*

Citation: Ropy A. and Ayala J.A., (2015) The Effect on Peptidoglycan Composition of Uncharacterized Pae-Ampc Mutants Probes Its Functionality as DD-Peptidase. International Journal of Microbiology Research, ISSN: 0975-5276 & E-ISSN: 0975-9174, Volume 7, Issue 6, pp.-710-716.

Introduction

AmpC enzymes belong to group 1 class C serine β-lactamases, which hydrolyze cephamycins, and are more active on cephalosporins than bezylpenicillin. They showed low affinities to cefepime, cefpirome and carbapenems. Also, they are not inhibited by clavulanic acid but inhibited by cloxacillin, oxacillin, and aztreonam [1,2]. AmpC overproduction confers resistance to most penicillins, β-lactamase inhibitor/β-lactam combinations, and resistance to many broad-spectrum cephalosporins (e.g. cefotaxime, ceftazidime, and ceftriaxone) with reduced susceptibilities to carbapenem, cefepime and cefpirome [2-4]. AmpC can be induced by certain β-lactams (e.g. imipenem and cefoxitin) in many bacteria (e.g. *P. aeruginosa*), but it cannot be in some others (e.g. *E. coli*). In *P. aeruginosa*, *ampC* is chromosomally encoded, expressed in low basal amount, and can be induced by β-lactam challenge. AmpC expression is regulated mainly by many enzymes; AmpG permeases, AmpD amidases, AmpR (LysR super family) and NagZ. Moreover, two different types of muropeptides derived from PG biosynthesis and recycling pathways have the final word on *ampC* expression through binding to AmpR, which undergoes conformational changes and enables turning on/off AmpC overproduction. The first type is suppressing muropeptide, UDP-MurNAc-pentapeptide, which sets *ampC* expression at basal level during normal growth conditions. While the second is inducing muropeptide, 1,6-anhydro muropeptide, which takes the lead in AmpR binding during β-lactam exposure, and so they are able to trigger AmpC overproduction until β-lactam elimination [5-7]. AmpC enzymes were reported principally as chromosomally encoded in many members of Proteobacteria. However, later on, plasmid-mediated AmpC enzymes (e.g. ACT-1, CMY-2, FOX-1 and MIR-1) have emerged. Extended-spectrum AmpC β-lactamases (ESACs) like GC1 in *E. cloacae* and plasmid-mediated CMY-10, CMY-19, CMY-37 enzymes are categorized as subgroup 1e [1,2]. The 3-D structure of *E. coli* AmpC showed that the active site residues are Ser64* (catalytic residue), Lys67, Gln120, Tyr150, Asn152, Lys315, Thr316 and Ala318 (Ser318 in some other types). These residues are conserved in all class C β-lactamases [2,8]. Recently, 3-D structure of *P. aeruginosa* AmpC was developed and showed active site similarity to the *E. coli* AmpC, except for having Ser318 [9]. ESACs differ from wild-type AmpC by amino acid substitutions or insertions in some regions in the vicinity of the active site (e.g. the Ω-loop, the H-10 helix, the H-2 helix and the C-terminal end of the protein),which improve affinities and reactions with more β-lactam substrates like broad spectrum cephalosporins (e.g. cefotaxime, ceftazidime) [4].

DD-endopeptidase and DD-carboxypeptidase activities are properties of some periplasmic enzymes called low molecular mass penicillin-binding proteins (LMM-PBPs, class C PBPs), which participate in PG maturation and recycling, and cell separation. LMM-PBPs were sub-divided into 4 sub-groups [type-4 (e.g. Eco-PBP4), type-5 (e.g. Eco-PBP5), type-7 (e.g. Eco-PBP7) and type-AmpH (e.g. Eco-AmpH)] referring to *E. coli* (Eco) type concerning structural and functional similarities [10].Concerning their structural similarity, it was found that both class C β-lactamases and class C LMM-PBPs type-AmpH (e.g. R61 DD-peptidase) have a close similarity in their general structure and conserved motifs near the active site serine [2,10]. This suggests that both of them have a common ancestor, and implies that class C β-lactamases may have a secondary peptidase activity [11-14]. The active site of these proteins (PBPs and serine β-lactamases) contains nine highly conserved residues; the catalytic serine is located at the beginning of α2 helix and followed by a lysine to form the S*XXK sequence; a second sequence, SxN, is located in a loop between helix α4 and α5; four conserved residues form the KTG(T/S) form the third sequence; and a ninth residue, a glycine (G) at the back portion of the active site, is also strictly conserved [10]. Recently, Activity of some LMM-PBPs was related to the intrinsic bacterial resistance and AmpC overproduction as reported for increased bacterial

resistance and AmpC derepression beyond PBP4 inactivation in *P. aeruginosa* and *Aeromonas spp.* [15-17].

PG is a heterogeneous elastic biopolymer of muropeptides, which are linked together by β-1,4-bonds to form a long glycan strands cross-linked by bridges between its peptide stems. Each muropeptide subunit is composed of disaccharide derivatives of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) in addition to a peptide stem of di-, tri-, tetra- or pentapeptides. Moreover, the glycan chains of muropeptides are crosslinked together frequently by 3-4 cross-links, which can be either direct as in most Gram-negative bacteria. The peptide stem in PG of *E. coli* and most Gram-negative bacteria is composed of amino acids L-Ala, D-Glu, Dap, D-Ala and D-Ala which occupy positions of numbers 1, 2, 3, 4 and 5, respectively, where amino acid number 1 (L-Ala) is linked to the D-lactyl group of MurNAc [18-21].

The functional characterization of Pae-AmpC from *P. aeruginosa* has a significant importance, because AmpC overproduction and emergence of AmpC mutants (e.g. ESACs) are considered as one of the basic resistance tools used by this clinically problematic microorganism to overcome the inhibitory effect of most of β-lactam antibiotics [1,2,5]. AmpC enzymes are historically known as β-lactamases, but they were thought to have a DD-peptidase activity due to structural similarities with LMM-PBPs (e.g. Eco-AmpH), that have these activities [10-14,22]. Considering above points, this study is concerned with studying the effect of some spontaneous uncharacterized mutations on Pae-AmpC β-lactamase activity, and to track DD-peptidase activities beyond the *in vivo* expression and the *in vitro* reactions of various Pae-AmpC forms (wild type and mutants) with muropeptides and whole PG, in *E. coli* and *P. aeruginosa*.

Material and methods

DNA purification, sequencing and electrophoresis

DNA purification was achieved by following the manufacturer's instructions using the kits (Promega); Wizard® genomic DNA purification kit, Wizard® Plus SV minipreps DNA purification system, and Wizard® SV gel and PCR clean-up system. Sequencing of PCR products and cloned genes was done at Parque Científico de Madrid, Spain. For DNA agarose electrophoresis, we used 1x TAE electrophoresis buffer, 0.5 µg/ml ethidium bromide and 0.8-1.0 g% Agarose gel were used [23]. The used units were Mini-sub® cell GT (BIO-RAD) and sub-cell GT WIDE MINI (BIO-RAD).

Outline of the cloned Pae-ampC forms

The different Pae-*ampC* forms are outlined in [Table-1]. *ampC*-F2 is an *ampC* mutant with $G^{4}\rightarrow C$ single nucleotide mutation, which corresponds to $R^{2}\rightarrow G$ amino acid mutation in the signal peptide. *ampC*-F3 is the mature form of Pae-*ampC* with an insertion of ATG as initiation codon (coding for methionine before A^{26}). Also, *ampC*-F4, *ampC*-F4:C3 and *ampC*-F4:C6 are designed *ampC* forms with insertion of two codons (ATG GCC) before the starting codon of wild type *ampC* sequence inserting the two amino acids M A to the AmpC peptide (before M^{1}). Moreover, *ampC*-F4:C3 has $T^{728}\rightarrow C$ single nucleotide mutation (we called it C3 mutation) which corresponds to single amino acid change ($P^{243}\rightarrow L$); *ampC*-F4:C6 has $C^{152}\rightarrow T$ single nucleotide mutation (we called it C6 mutation) which corresponds to single amino acid change $I^{51}\rightarrow T$. Both of C3 and C6 were developed spontaneously in the lab during the cloning procedure, and it was interesting to continue characterization of their effect on PG composition after we found they affected largely the β-lactamase activity. All these *ampC* forms have a sequence for poly-His tag at C-terminal. All AmpC forms were expected to be periplasmic, except AmpC-F2 having a charge defect in the signal peptide, and AmpC-F3 that was produced as cytoplasmic form because they do not have the signal peptides.

Table-1 *Mutations and changes in the main sequence of the cloned Pae ampC constructs*

Pae-*ampC* form/ name[a]	Nucleotide change[b]	Amino acid change[c]
***ampC*-F4**	ATG GCC insertion before A^{1}	M A insertion before M^{1}
***ampC*-F4:C3**	$T^{728}\rightarrow C$ mutation, and ATG GCC insertion before A^{1}	$P^{243}\rightarrow L$ mutation, and M A insertion before M^{1}
***ampC*-F4:C6**	$C^{152}\rightarrow T$ mutation, and ATG GCC insertion before A^{1}	$I^{51}\rightarrow T$ mutation, and M A insertion before M^{1}
***ampC*-F2**	$G^{4}\rightarrow C$	$R^{2}\rightarrow G$
***ampC*-F3**	ATG insertion as a start codon	M insertion before A^{26}

[a]The cloned Pae-ampC forms from PAO1 were named referring to their corresponding forward primers (F2→F4), and the presence of C3 (i.e. T728→C) or C6 (i.e. C152→T) mutations[b]. Nucleotide changes[b] and its corresponding amino acid changes[c] within different ampC constructs are shown compared to the wild type PAO1ampC (PA4110).

ampC cloning and expression using pET28b plasmid

PCR amplifications of various forms of Pae-*ampC,* PA4110, [Tables-1,1S] were cloned in pET28b plasmid using restriction sites of EcoRI and NcoI (Fermentas life sciences), and ligation by T4 DNA ligase (Promega). All the developed AmpC proteins were designed to have C-terminal His-tag. DH5α was used for cloning while *E. coli* BL21(DE3) and DV900(DE3) were transformed, by heat-shock, with recombinant plasmids (e.g. pET-F2, -F3, -F4, -F4:C3 and -F4:C6) for AmpC over-expression and characterization. All of these recombinant vectors were confirmed by DNA sequencing. For AmpC over expression, the inoculation was performed at OD=0.3 in LB media containing a 30 µg/ml kanamycin and 0.1-1.0 mM IPTG over different incubation periods (indicated in results) at 37°C and 180 rpm.

ampC cloning and expression using pUCP24 plasmid

The pUCP24 plasmid, used for *ampC* cloning and expression in *P. aeruginosa*, has a gentamycin resistance marker (*aacC1*) and multiple cloning sites as those present in the plasmid pUCP18 [24]. PCR of *ampC*-F3 and *ampC*-F4 were achieved using pET-F4 and pET-F3 as a templates and primers indicated in [Table-1S] to generate pUCP-F3 and pUCP-F4, respectively. The ligation products were used to transform, by heat-shock, DH5α which were grown in LB plates containing 10 µg/ml gentamycin, 10 mg/ml X-Gal (Sigma) and 0.5 mM IPTG and incubated overnight at 37°C. The verified recombinant plasmids were used to transform, by electroporation, some previously constructed *P. aeruginosa* mutants, PAOΔ*ampC*, PAOΔ*dacB*Δ*pbpG*Δ*ampC*, PAOΔ*dacB*Δ*dacC*Δ*ampC* and PAOΔ*dacB*Δ*dacC*Δ*pbpG*Δ*ampC* [25]. AmpC expression in these *P. aeruginosa* transformants was achieved by inoculating LB media, at OD=0.3, containing a 10 µg/ml gentamycin and 0.1-1.0 mM IPTG for 3 hours at 37°C with agitation.

SDS-PAGE electrophoresis and western blot

The buffer Tris/Glycine/SDS and 8-10% acrylamide gels were prepared as previously described [26]. The unit of MINI PROTEAN® TETRA CELL (BIO-RAD), pre-stained molecular markers (SeeBlue® Plus-2, Invitroen) and Coomassie blue G-250 staining were utilized. Estimation of protein concentration was achieved using BIO-RAD™ DC protein assay, by following the provider´s instructions. A standard curve was developed for each assay using BSA (Sigma). After electrophoresis run is complete, one gel was used for transfer proteins into Immobilon-P transfer membrane (Millipore Co.) using the system CriterionTM blotter (BIO-RAD) under a previously described conditions [22].

β-lactamase activity assay

For each assay, 2 µl sample was mixed with 988 µl PBPs (1x, pH 7.5) and 10 µl nitrocefin stock solution (500 µg/ml, Oxoid, Cambridge, United Kingdom), then it was incubated in a dark place at 23°C for 15 min then centrifuged at 14000 rpm for 2 min. The supernatant absorbance was measured at 486 nm on U-2000 spectrophotometer (HITACHI). One milli unit β-lactamase activity is defined as 1

Table-1S *AmpC forms, corresponding primers, and expression plasmids.*

AmpC form	Primers[a]	Constructed plasmids[b]
AmpC-F4	Fw: TTTCCATGGCCATGCGCGATACCAGATTCC Rv: TTTGAATTCCGCTTCAGCGGCACCTTGC	pET-F4
	Fw: TAATACGACTCACTATAG (T7Fw) Rv: GCATGCT TGTTAGCAGCCGGATCTCAG	pUCP-F4
AmpC-F4:C3	Fw: TTTCCATGGCCATGCGCGATACCAGATTCC Rv: TTTGAATTCCGCTTCAGCGGCACCTTGC	pET-F4:C3
AmpC-F4:C6	Fw: TTTCCATGGCCATGCGCGATACCAGATTCC Rv: TTTGAATTCCGCTTCAGCGGCACCTTGC	pET-F4:C6
AmpC-F2	Fw: TTTCCATGGGCGATACCAGATTCCCCT Rv: TTTGAATTCCGCTTCAGCGGCACCTTGC	pET-F2
AmpC-F3	Fw: TTTCCATGGCCGGCGAGGCCCCGG Rv: TTTGAATTCCGCTTCAGCGGCACCTTGC	pET-F3
	Fw: TAATACGACTCACTATAG (T7Fw) Rv: GCATGCT TGTTAGCAGCCGGATCTCAG	pUCP-F3

[a]Fw: forward; Rv: reverse. Underlined sequences are restriction sites for NcoI (CCATGG), EcoRI (GAATTC) and PaeI (GCATGC). [b]Recombinant plasmids constructed from cloning of PCR ampC amplifications, via the indicated restriction sites, into pET28b plasmid (eg. pET-F2, -F3, -F4, -F4:C3 and -F4:C6) for over expression in *E. coli*, and pUCP24 plasmid (eg. pUCP-F3 and -F4) for over expression in *P. aeruginosa*.

nanomole of nitrocefin hydrolyzed per min per microgram of protein. It was modified from a previous method [27]. Values of V_{max} and K_m of purified AmpC forms were identified (using nitrocefin as a substrate) from a plot of the equation of Lineweaver-Burk double reciprocal plot, which was driven from the Michaelis-Menten equation [28].

Cell fractionation for protein localization

Fresh LB cultures of Bl21(DE3), DV900(DE3) and PAO1 strains, harboring inducible *ampC* vector, were induced at OD=0.3 with IPTG for 1-3 h at 37°C and 180 rpm agitation. The cells were collected by centrifugation at 5000 rpm for 15 min at 4°C, re-suspended in ice-cold 1x PBS, pH 7.5, and then lysed by sonication on ice. A small portion of the total sonicate was left on ice (total sonicate), while the other portion was centrifuged at 85000 rpm for 40 min at 4°C. The cell extract (supernatant) was left on ice, while the cell pellet (membranes) was re-suspended in a proper volume of ice-cold 1x PBS, pH 7.5. Ice-reserved cell fractions were checked for AmpC presence by SDS-PAGE, western blot and β-lactamase activity, and then stored at -20°C.

AmpC purification

For AmpC overproduction, Bl21(DE3) transformants with pET28b recombinant vectors were grown in a 30 L fermenter (Biostat UD30, B. Braun Biotech) in LB medium supplemented with 30 µg/ml kanamycin at 37°C and induced at OD_{600} ~ 0.3 with 1 mM IPTG for 3 h in case of AmpC-F3,or induced with 0.1 mM IPTG for 1 h in case of AmpC-F2, AmpC-F4, AmpC-F4:C3 and AmpC-F4:C6. After that, cells were collected, re-suspended in 1x phosphate buffer (43 mM Na_2HPO_4 and 14 mM KH_2PO_4, pH 7.5), broken within French pressure cell (American Instrument co, Urbana, III) at 20000 psi, and further centrifuged at 50000 rpm for 30 min at 4°C. Both of supernatant and pellet were either stored at -20°C or used in the next step of purification. AmpC-F3 was purified from supernatant, which passed twice through the Ni-NTA column (QIAGEN GmbH) equilibrated with 1x phosphate buffer (10 mM imidazole, pH 7.5). Unbound proteins were washed away with 1x phosphate buffer (50 mM imidazole, pH 7.5). Bound AmpC was then eluted with 250 mM imidazole (Merck, Germany) in phosphate buffer, pH 7.5. Highly purified fractions were mixed and concentrated using Amicon® Ultra Centrifugal Filters (Ultracel®-30K; Millipore Ireland Ltd) on Megafuge 2.0 R Heraeus (SEPATECH) at 4° C. The concentrated AmpC-F3 was stored at -20°C. The majority of AmpC-F2, -F4, -F4:C3 and -F4:C6 proteins were found insoluble in the membrane pellet fraction. So, they were extracted from the pellet fraction in phosphate buffer containing 3 M guanidine HCl (Gn-HCl; Sigma) at pH 7.5, and centrifuged at 50000 rpm for 15 min at 4°C. The extract was passed directly through Ni-NTA column equilibrated with 1x phosphate buffer (3 M imidazole, pH 7.5). Unbound proteins were washed away by 1x phosphate buffer (3 M Gn-HCl, 20 mM imidazole, pH 7.5), while bound AmpC was then eluted by 250 mM imidazole (in the equilibration buffer) and dialyzed against 20 mM Tris HCl, pH 7.5, as mentioned above. The peptide sequence of AmpC precursor and mature forms were identified by MALDI-TOF analysis by the unit of proteomics at the Center of Molecular Biology "Severo Ochoa" (CBMSO). The purified AmpC proteins were used for characterization of their β-lactamase activities on nitrocefin and for *in vitro* reactions with purified PG and individual muropeptides as described later.

Peptidoglycan manipulation

For PG preparation from transformants of *E coli* and *P. aeruginosa*, overnight culture of one colony was 1:100 diluted in fresh LB media with a specific antibiotic (30 µg/ml kanamycin for pET28b transformants, and 10 µg/ml gentamycin for pUCP24 transformants),and left growing at 37°C and 185 rpm agitation until the exponential phase (OD_{600}- 0.75-0.8) was achieved. After that, the cells were collected by centrifugation at 5000 rpm/min at 4°C and re-suspended in 1x PBS buffer, pH 7.5. The cell suspension was added drop by drop to an equal volume of boiling 6% SDS (Merck, Germany) solution with strong stirring. The final cell-SDS suspension was left under boiling conditions for 4 hours (case of *E. coli*) or 12 hours (case of *P. aeruginosa*) with stirring. For the next steps in PG preparation (PG washing and treatment with α-amylase, pronase E, muramidase, and NaBH4) and HPLC analysis, please follow precisely the method used in our recent publication [22,25]. Individual muropeptides were collected from the HPLC elution either for *in vitro* reactions or for MALDI-TOF analysis.

In vitro assays, using the whole PG and purified individual muropeptides, were done in 250 µl of 20 mM Tris-HCl, pH 7.5, to confirm that Pae-AmpC can elicit DD-peptidase activity. *In vitro* reactions of whole PG contained AmpC-F4 (2 µg/µl), AmpC-F2 (2 µg /µl), AmpC-F4:C3 (2 µg /µl), AmpC-F4:C6 (2 µg /µl) or AmpC-F3 (0.4 µg /µl) with about 160 µg of *E. coli* DV900 PG or*E. coli* CS109 were left at 37°C for 42 h. Then, reactions containing PG were boiled for 15 min, digested with muramidase, reduced as described above and analyzed by HPLC. For *in vitro* reactions with individual muropeptides, it contained 0.4 µg/µl AmpC-F3 with each of D44 (5 ng/ µl) and D45 (10 ng/ µl). After incubation at 42°C for 24 h, the reactions were boiled for 5 min, centrifuged at 14000 rpm for 10 min and subjected to HPLC analysis.

Results

Protein analysis of purified Pae-AmpC forms

Protein analysis by SDS-PAGE and western blot showed that AmpC-F4, AmpC-F4:C3, AmpC-F4:C6, AmpC-F2 and AmpC-F3 were highly purified and displayed that all of AmpC-F4, AmpC-F4:C3 and AmpC-F4:C6 have two bands, where the upper corresponds to the precursor form and the lower corresponds to the mature form. AmpC-F2 showed mostly one band of the precursor form; AmpC-F3 displayed one band of the mature form [Fig-1]. Both precursor and mature forms were verified by MALDI-TOF analysis. All of AmpC-F2, AmpC-F4, AmpC-F4:C3 and AmpC-F4:C6 are periplasmic forms except AmpC-F3 is a cytoplasmic form. Among the produced periplasmic forms, AmpC-F4 was used as a control for wild type activity because it has no mutations in the main peptide sequence of Pae-AmpC.

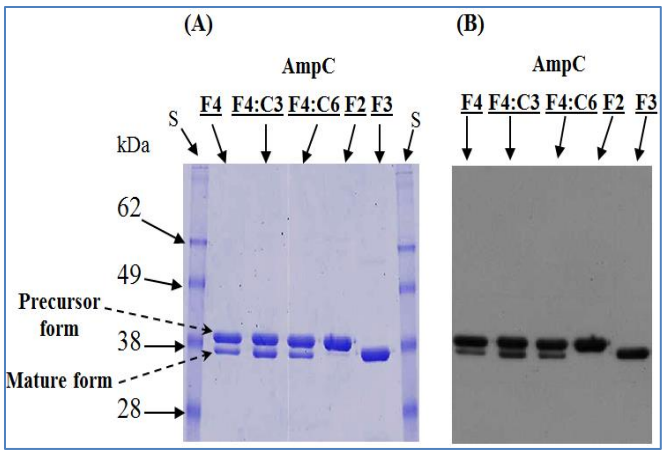

Fig-1 Analysis of purified AmpC forms by SDS-PAGE and western blot.

Purified AmpC-F4, AmpC-F4:C3, AmpC-F4:C6, AmpC-F2 and AmpC-F3 were analyzed by SDS-PAGE (A) and western blot (B); where the Purified AmpC-F4, AmpC-F4:C3 and AmpC-F4:C6 displayed two bands corresponding to precursor form (upper band) and mature form (lower band); AmpC-F2 displayed mostly the precursor form while AmpC-F3 displayed only the mature form. S: standard protein molecular mass markers. kDa. kiloDaltons. Dashed arrows refer to protein bands of precursor and mature forms of AmpC forms.

From expression profiles detected by SDS-PAGE and western blot (not shown), we found that All of AmpC-F2, AmpC-F4, AmpC-F4:C3 and AmpC-F4:C6 were produced in large amounts, that can be detected by western blot and SDS-PAGE. While AmpC-F3 was detected by western blot only, due to low production. Detection of AmpC proteins in cellular fractions of Bl21(DE3)/pET-*ampC* by SDS-PAGE and western blot (not shown) displayed that under induction conditions the majority of AmpC-F2 was produced as a precursor form and was present in membrane fraction. Also, AmpC-F4:C3 and AmpC-F4:C6 (precursor and mature forms) were found only in the membrane fraction. Most of mature form AmpC-F4 was found in the cell extract, while the majority of its precursor form was found in the membrane fraction. As expected, the mature form AmpC-F3 was found soluble in the extract fraction. Equivalent expression results were obtained from cellular fractions of DV900 (DE3)/pET-*ampC* under IPTG induction, with the exception of expression AmpC-F4 whose precursor and mature forms were found mostly in the membrane fractions. On the other hand, expression profiles of AmpC-F4 and AmpC-F3 in *P. aeruginosa* (*ampC* mutants) were similar using non-induction and IPTG induction conditions. They were detected only as low amounts by western blot (not shown), which indicate that IPTG induction did not help in AmpC production from pUCP-F3 and pUCP-F4. Also, blots showed that there was a basal production of AmpC-F3 and AmpC-F4.

Point mutations $P^{243}\rightarrow L$ and $I^{51}\rightarrow T$ caused a large decrease in β-lactamase activity of Pae-AmpC

Characterization of β-lactamase activities of purified AmpC forms showed that AmpC-F2 had a 2.5-fold decrease in Vmax; AmpC-F4:C3 and AmpC-F4:C3 had a 5-fold decrease in Vmax; however, AmpC-F3 displayed 8-fold increase in Vmax when all are compared to AmpC-F4. Also, AmpC-F4, AmpC-F4:C3, AmpC-F4:C6, AmpC-F2 and AmpC-F3 had Km values 11.3, 13.5, 16.8, 10.5 and 10, respectively, for nitrocefin [Table-2].

Cellular fractions of Bl21(DE3)/pET-*ampC* and DV900(DE3)/pET-*ampC* were tested for β-lactamase activity [Table-3], where fractions of Bl21(DE3)/pET-F4 showed the highest activity in fractions of total sonicate (526.4 µmole/min/UOD) and cell extract (329 µmole/min/UOD).Cellular fractions (total sonicate) of Bl21(DE3)/pET-F2 displayed a medium β-lactamase activity (4.6-fold decrease). While cellular fractions of Bl21(DE3)/pET-F3 (18.7-fold decrease), Bl21(DE3)/pET-F4:C3 (44-fold decrease) and Bl21(DE3)/pET-F4:C6 (16.4-fold decrease) displayed lower β-lactamase activities. Concerning transformants of DV900(DE3), cellular fractions (total sonicate) of DV900(DE3)/pET-F4 (196 µmole/min/UOD)and DV900(DE3)/pET-F2 (1.3-fold increase) showed high β-lactamase activities; cellular fractions of DV900 (DE3)/pET-F3 (7.4-fold decrease) displayed a low β-lactamase activities; while cellular fractions of DV900(DE3)/pET-F4:C3 (56-fold decrease) and DV900 (DE3)/pET-F4:C6 (89.1-fold decrease) showed very low β-lactamase activities.

Table-2 *Catalytic parameters on model β-lactam of purified AmpC forms.*

Purified AmpC	V_{max}	K_m (µM)	M.M. (kDa)	pI
AmpC-F4	12.5	11.3	45.7	8.3
AmpC-F4:C3	2.5	13.5	45.7	8.3
AmpC-F4:C6	2.5	16.8	45.7	8.3
AmpC-F2	5	10.5	45.4	7.8
AmpC-F3	100	10	43	7.9

Vmax is the maximum activity expressed in µmol/min/mg for reactions of different AmpC forms with nitrocefin (5 ng/µl) at 23 °C for 15 min. Vmax and Km were calculated from the β-lactamase activity of different forms of Pae-AmpC on nitrocefin as described in Methods. M.M.: molecular mass; pI: theoretical isoelectric point (online ExPASy tools).

β-lactamase activity assays for cellular fractions (total sonicate) of transformants (pUCP-F3 and pUCP-F4) of PAO1 *ampC* mutants (mentioned in methods) showed that activity of AmpC-F4 (~ 673.3 µmole/min/mg) was also very high when compared with AmpC-F3 (~25-fold decrease) in all transformants. The values of β-lactamase activities were very close for cellular fractions with 1 mM IPTG induction (for 3 h, at 37°C) compared with those without induction which are in perfect accordance with data obtained from their production detected by western blot(not shown).

Effect of purified Pae-AmpC on whole PG composition (*in vitro*)

In vitro activity of purified AmpC forms on PG of *E. coli* CS109 and DV900 was followed. We found that incubation of (0.4-2 µg/µl) AmpC-F4, AmpC-F2, AmpC-F3, AmpC-F4:C3 and AmpC-F4:C6 with the whole PG of each of CS109 and DV900 at 37°C for up to 24 hours produced no significant structural change. While extending incubation (42 h) time at 37°C produced some interesting changes in PG composition especially with AmpC-F3; indicating that AmpC may elicit a secondary DD-peptidase activity under some conditions [Table-4]. In case of incubation with PG of the wild type *E. coli* CS109 for 42 hours, there was a decrease in anhydromuropeptides, which was correlated with the decrease in crosslinking, and produced an increase in monomers and a decrease in dimers and trimers only with AmpC-F3. Moreover, there were a decrease in D44 and an increase in M4 beside the decrease in the crosslinking degree, which indicates that there was some DD-endopeptidase activity [Table-4]. In case of incubation of different AmpC forms with PG of DV900 for 42 hours, there was also an increase in monomers and decrease in dimers and trimers only with AmpC-F3. Again, analysis of individual muropeptides after treatment with AmpC-F3 of PG of DV900 showed a large increase in M4 and D44 and a decrease in M5, D45, T445, D45N and T445N [Table-4]. This indicates that AmpC-F3 acted as DD-carboxypeptidase and DD-endopeptidase on the whole PG of *E. coli* DV900.The fact that in an old preparation of AmpC-F3 the decrease of M5 is higher and the decrease of D45 was smaller, and the increase of M4 and D44 was also smaller,

when compared with the fresh preparation of AmpC-F3, indicates that freezing may favor the DD-peptidase activity of the sample.

Effect of purified Pae-AmpC on individual purified muropeptides (*in vitro*)

Trying to reinforce or confirm data on isolated whole PG *in vitro*, described in the previous paragraph, we performed analysis with individual purified muropeptides. The reaction involved incubation of (0.4-2 µg/µl) AmpC-F4, AmpC-2, AmpC-F3, AmpC-F4:C3 and AmpC-F4:C6 with each of the individual purified muropeptides (M4, M5, D44 and D45) within different conditions [temperature (37 and 42°C) and +/- 50 mM NaCl addition, various AmpC concentrations], and was continued as described in methods. The results showed that a concentration of up to 2 µg/µl of AmpC-F4, AmpC-F2, AmpC-F4:C3 and AmpC-F4:C6, had no activity on muropeptides (not shown); while 0.4 µg/µl AmpC-F3 displayed DD-endopeptidase activity on D44 and D45 at 42°C for 24 hours however there was no activity on M4 (4ng/µl) and M5 (16 ng/µl), (Fig. 2). These data confirm the DD-endopeptidase activity of AmpC-F3 on isolated muropeptides, but it was not detected, the DD-carboxypeptidase activity, on the purified monomers muropeptide (M4, M5). As this last activity was clearly seen on whole isolated PG of DV900, it may indicate that particular conformations of the muropeptides on the whole structure are required for bringing out that activity.

Table-3 *β-lactamase activities for cellular fractions of E. coli BL21(DE3) and DV900(DE3)transformants*

***E. coli* Strain**	**Recombinant vectors**	**IPTG mM**	**µmole/min/UOD[a]**		
			Total sonicate fraction	**Cell extract fraction**	**Cell membrane fraction**
BL21(DE3)	pET-F4	0	27.1	25.8	11
		0.1	526.4	329.0	153
	pET-F4:C3	0	1.3	0.7	0.5
		0.1	10.5	3.4	2.5
	pET-F4:C6	0	0.8	0.4	0.3
		0.1	32.1	28.4	5
	pET-F2	0	6.5	5	1.3
		0.1	113.4	36.1	38
	pET-F3	0	1.2	0.8	0.1
		1	28.2	24.4	1
DV900(DE3)	pET-F4	0	9.5	6.3	2.6
		0.1	196	75.4	128.2
	pET-F4:C3	0	0.53	0.32	0.16
		0.1	3.5	1.4	2
	pET-F4:C6	0	0.13	0.12	0
		0.1	2.2	0.74	1.5
	pET-F2	0	63.3	52.8	4.2
		0.1	260.3	86.8	181
	pET-F3	0	1.1	0.9	0
		0.1	26.4	23.8	0
		1	46.9	42	0

[a]β-lactamase Activity expressed in µmole/min/UOD detected at 486 nm after incubation of cellular fractions (total sonicate, cell extract and cell membrane) with nitrocefin (5 ng/µl) at 23 °C for 15 min. Expression of ampC forms in *E. coli* BL21(DE3) and DV900(DE3) using pET28b recombinant vectors was done under non-induction and induction conditions with 0.1 or 1 mM IPTG for 1 h at 37 °C. UOD stands for Unit of Optical Density

Discussion

Mutations, sub-cellular localization and solubility can largely affect AmpC β-lactamase activity

In *E. coli* BL21(DE3)/pET-*ampC*, AmpC-F4 showed the highest β-lactamase activity in cellular fractions of *E. coli* Bl21(DE3)/pET-F4, because it had the majority of its mature form soluble. On the other side, among purified AmpC forms, the mature form AmpC-F3, showed the highest activity on nitrocefin; however, it showed a very low activity *in vivo* which may be due to its low expression or degradation as displayed by HPLC analysis (Fig. 1). AmpC-F2 showed a moderate β-lactamase activity, which may be due to the very low production of the mature form. The two mutants, AmpC-F4:C3 and AmpC-F4:C6, showed a very low β-lactamase activity *in vivo* and *in vitro*; indicating that these two mutations affected largely the β-lactamase activity of Pae-AmpC. Although both of AmpC-F2 and AmpC-F4 have some modification in the signal peptide, both of them would be proficient to produce the wild type of the mature AmpC form by elimination of the signal peptide. By following the β-lactamase activity in cellular fractions of *E. coli* DV900(DE3)/pET-*ampC*, we found that AmpC-F3, AmpC-F4:C3 and AmpC-F4:C6 showed the same behavior as encountered in Bl21(DE3),while AmpC-F2 showed higher activity and AmpC-F4 produced lower activity, which could be due to the low production of the soluble mature form. As described before, AmpC-F2, AmpC-F4:C3 and AmpC-F4:C6 have single amino acid mutations $R^{2}\rightarrow G$, $P^{243}\rightarrow L$ and $I^{51}\rightarrow T$, respectively. These mutations could affect the general protein folding and in turn AmpC activity as demonstrated by low β-lactamase activity of AmpC-F4:C3 and AmpC-F4:C6 and the intermediate activity of AmpC-F2. In Pseudomonas transformants of pUCP-F3 and pUCP-F4, the β-lactamase activities of AmpC-F4 were ~ 20-times higher than that of AmpC-F3, which may be due to the low expression, instability or the misfolding of

Table-4 *HPLC analysis for peptidoglycan from E. coli DV900 and CS109 after in vitro incubation with purified AmpC forms.*

Reaction with whole PG[a]	Muropeptides (% Molar)[b]												Crosslink	D-D/T	Length
	Mono	**Di**	**Tri**	**Gly**	**D-D**	**Lpp**	**Anhy**	**Penta**	**M4**	**M5**	**D44**	**D45**			
DV900-CTRL	60	35.9	4.1	5.3	0.9	1.5	4	85	3.3	29.7	4	32.3	44.1	1.9	25.1
AmpC-F4 + DV900	59.5 (99.2)	36.2 (101)	4.3 (105)	5.6 (106)	0.9 (100)	0.9 (60)	4.1 (103)	85 (100)	3.4 (103)	28.8 (97)	4.1 (103)	31.8 (99)	44.8 (106)	2.1 (111)	24.5 (98)
AmpC-F4:C3 + DV900	60 (100)	36 (100)	4.1 (100)	5.6 (106)	0.9 (100)	0.7 (47)	3.9 (98)	85.3 (100)	3.5 (106)	29.1 (98)	3.9 (98)	31.9 (99)	44.1 (100)	2 (105)	25.9 (103)
AmpC-F4:C6 + DV900	60.4 (101)	35.8 (100)	3.8 (93)	5.5 (104)	0.9 (100)	1.1 (73)	3.7 (93)	85.2 (100)	3.4 (103)	28.8 (97)	3.8 (95)	31.7 (98)	43.4 (98)	2 (105)	26.9 (107)
AmpC-F2 + DV900	59.5 (99.2)	36.6 (102)	4 (98)	5.6 (106)	0.6 (67)	0.7 (47)	3.8 (95)	86.4 (102)	3 (91)	30.5 (103)	4.3 (108)	33.6 (104)	44.5 (101)	1.5 (79)	26.5 (106)
AmpC-F3 + DV900	68.6 (114)	28.8 (80)	2.5 (61)	5 (94)	0.8 (89)	1 (67)	1.3 (33)	67.1 (79)	15.4 (467)	25.6 (86)	5.9 (148)	27.4 (85)	33.9 (77)	2.4 (126)	74.2 (296)
CS109-CTRL	60.3	37	2.7	7.7	4.4	16.6	5.5	-	22.1	-	18.5	-	42.4	10.3	18.3
AmpC-F3 + CS109	71 (118)	27.9 (75)	1.1 (41)	6.9 (90)	3.5 (80)	14.9 (90)	3.1 (56)	-	31 (140)	-	15.5 (84)	-	30.1 (71)	11.6 (113)	32.7 (179)

[a]In vitro reactions contained AmpC-F4, -F2, -F4:C3, -F4:C6 or -F3 with whole PG of and *E. coli* DV900 and *E. coli* CS109 (the last two rows). [b]Relative abundance in % molar of different types of muropeptides. Values in brackets represent percentage of the values obtained for each raction related to either DV900-CTRL or CS109-CTRL which are negative controls for DV900 PG and CS109 PG, respectively, without AmpC treatment. Mono: monomeric muropeptides (e.g. M4, M5); Di: dimeric muropeptides (e.g. D44, D45); Tri: trimeric muropeptides (e.g. T444); Lpp: Muropeptides bound to Braun's Lipoproteins (e.g. M3L); Anhy: anhydromuropeptides (e.g. D44N). Crosslink: degree of crosslinking in percentage. D-D: total muropeptides that have Dap-Dap cross-linking (e.g. D34D); D-D/T: ratio of Dap-Dap crosslinking to the total crosslinking. Length: measurement for PG length. PG was prepared as described in methods. M4: disaccharide tetrapeptide; M5: disaccharide pentapeptide; M3L: disaccharide tripeptide bound to Braun's lipoprotein; D44: crosslinked-dimer of disaccharide tetrapeptide-disaccharide tetrapeptide; D34D: Dap-Dap crosslinked-dimer of disaccharide tripeptide-disaccharide tetrapeptide; D45: cross-linked-dimer of disaccharide tetrapeptide-disaccharide pentapeptide; T444: crosslinked-trimer of disaccharide tetrapeptide-disaccharide tetrapeptide- disaccharide tetrapeptide; D44N has the same structure of muropeptide D44 but with anhydro-N-acetylmuramic acid instead of N-acetylmuramic acid.

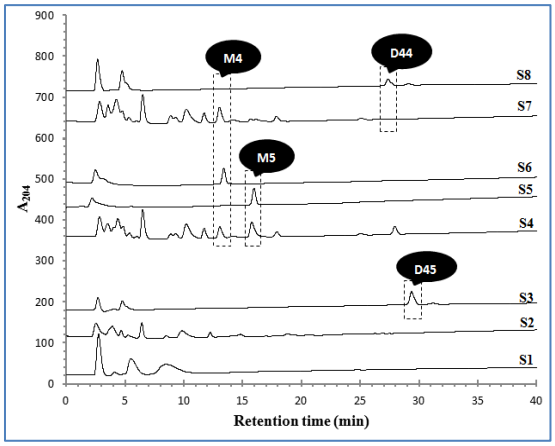

Fig-2 Effect of AmpC-F3 activity on individual muropeptides (in vitro).
HPLC chromatograms for in vitro reactions of AmpC-F3 with individual muropeptides whose positions are surrounded by dotted rectangles. S1: control for purified enzyme before incubation; S2: control for purified enzyme after incubation without substrates; S3: control for D45 muropeptide; S4: reaction of AmpC-F3 with D45; S5: control for M5 muropeptide; S6: control for M4 muropeptide; S7: reaction of AmpC-F3 with D44; S8: control for D44 muropeptide.

AmpC-F3 *in vivo*. Although AmpC-F4 was detected by SDS-PAGE and western blot at a very high level in *E. coli* Bl21(DE3) and very low level in all PAO1 strains, cellular fractions in both cases displayed high β-lactamase activities which may be explained by more active and higher AmpC folding in homologous PAO1 strains rather than in heterologous Bl21(DE3).

On line databases [29-31] showed that there is a total number of 179 SNPs, which include 80 silent SNPs and 99 missense SNPs, along the total length of Pae-*ampC* (PA4110) gene, representing about 15 SNPs, involving 8 silent SNPs and 7 missense SNPs per gene length unit. These values are largely about the mean value for Pseudomonas SNPs. Also, it was found that SNPs changes did not take place within the active site residues, and neither in amino acids (e.g. N70 and R76) responsible for interaction with C3 and C4 of β-lactam nucleus.

It was reported in some *P. aeruginosa* isolates that some amino acid mutations (e.g. A97V and T105A) in the region close to the active site Ser64 in some clinical *ampC* variants (e.g. PDC-2 variant with G27D, A97V, T105A, and V205L substitutions) enhanced the hydrolytic activity of AmpC into an extended spectrum AmpC cephalosporinases activity, ESAC [4]. Likely, it was described recently that some AmpC mutations (e.g. P154L and F121L) reported in some *P. aeruginosa* strains (e.g. PDC-73, -81, or -82)increased affinities of AmpC active site to many poor substrates, and hence stimulate resistance to antipseudomonal penicillins and cephalosporins [32]. On the other hand, mutations in active site residues results in decreased β-lactamase activity [2]. Similarly, K67R mutant displayed lower β-lactamase activity and minimal conformational changes compared to the wild type AmpC [33]. Conversely, all these missense SNPs must indicate adaptations to change the global structure to adapt the active site to new substrates, without changes on the essential and well conserved catalytic residues[32].

AmpC displayed a secondary DD-peptidase activity

It was previously noticed that AmpC from *Salmonella* may develop a secondary DD-carboxypeptidase activity [34]. Our data proved basically previous

suggestions [10-14] that AmpC can produce DD-peptidase activity (DD-endopeptidase and/or DD-carboxypeptidase) which can be inferred or clearly observed from the following observations on the HPLC analysis of PG composition and muropeptides: 1) The DD-endopeptidase activity could be deduced from the increase in monomers, anhydromuropeptides and pentapeptides in parallel with the decrease in dimers upon AmpC expression in Bl21(DE3)/pET-*ampCin vivo* with most of AmpC forms, and also, from *in vitro* analysis with whole PG of CS109 and DV900 with AmpC-F3 form only. 2) The DD-carboxypeptidase activity could be inferred from the increase in M4 and decrease in M5, D44, D45, T445, D45N and T445N beyond incubation of purified AmpC-F3 with the whole DV900-PG. 3) the endopeptidase activity was verified by the hydrolysis of individual dimeric muropeptides, D44 and D45, after incubation with AmpC-F3. Our data, also showed that the produced mature form (AmpC-F3) had the highest β-lactamase activity *in vitro* (V_{max}=100 µM), which was very high (8-fold or more) compared to the other AmpC forms. Also, it was the only AmpC form having DD-peptidase activity *in vitro* and it had inferred DD-Carboxypeptidase and DD-Endopeptidase *in vitro* higher than the other forms. These data indicates that the active site in AmpC-F3 has high accommodation, folding and flexibility permitting higher binding affinity and faster reaction with its β-lactam substrates, and also, with some muropeptides which are substrates for LMM-PBPs (DD-peptidases), which have a close structural similarity with AmpC β-lactamases [10, 11]. On the other hand, orthologous of Eco-PBP5 are known as been the main DD-Carboxypeptidases of the different bacteria, and it was reported that the mature form of Pae-PBP5 (PAO sPBP5) was produced and characterized having both a DD-Carboxypeptidase and wide spectrum β-lactamase activity [35]. That result is in good accordance with our data, and allows highlighting two things; firstly, the production of the soluble mature form can display higher activity to its common substrates and can increase the affinity to other secondary or low-affinity substrates. Furthermore, these findings may explain that both of class C serine β-lactamases and DD-peptidases have a common ancestor and how β-lactamases were evolved from the DD-peptidases [11]. Our data highlighted the other side of AmpC activity and the physiological function concerning its ancestral DD-peptidases (PBPs) which were clustered together in one grouped (COG1680) by phylogenetic classification of proteins encoded in complete genomes [36].

Acknowledgments

This work was supported by grantsBFU2009-09200 from the Ministerio de Economía y Competitividad of Spain and 223431 DIVINOCELL from the European Union. Predoctoral grant JAE/Predoc from the Consejo Superior de Investigaciones Cientificas to A.R. is acknowledged.

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