Mutations at Arg220 and Thr237 in PER-2 β-lactamase: impact on conformation, activity and susceptibility to inhibitors.

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Running head: Impact of mutations in the active site of PER-2

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Abstract:

PER-2 accounts for up to 10% of oxyimino-cephalosporin resistance in *Klebsiella pneumoniae* and *Escherichia coli* in Argentina, and hydrolyzes both cefotaxime and ceftazidime with high catalytic efficiencies (k_{cat}/K_m). Through crystallographic analyses, we recently proposed the existence of a hydrogen-bond network connecting Ser70-Gln69-oxyanion water-Thr237-Arg220 that might be important for the activity and inhibition of the enzyme. Mutations at Arg244 in most class A β-lactamases (as TEM and SHV) reduce susceptibility to mechanism-based inactivators, and Arg220 in PER β-lactamases is equivalent to Arg244. Alterations in the hydrogen bond network of the active site in PER-2, through modifications in key residues such as Arg220 and (to a much lesser extent) Thr237, dramatically impact the overall susceptibility to inactivation, with up to ~300 and 500-fold reduction in the k_{inact}/K_I values for clavulanic acid and tazobactam, respectively. Hydrolysis on cephalosporins and aztreonam was also affected although in different extents compared to wild-type PER-2; for cefepime, only Arg220Gly mutation resulted in a strong reduction in the catalytic efficiency. Mutations at Arg220 entail modifications in the catalytic activity of PER-2, and probably local perturbations in the protein, but not global conformational changes. Therefore, the apparent structural stability of the mutants suggests that these enzymes could be possibly selected *in vivo*.

Introduction:

Since the description of the first two acquired narrow spectrum β-lactamases (TEM-1, and SHV-1), clinically relevant β-lactamases evolved following two main paths, contributing to the alarming resistance levels found today (1). One of them involves accumulation of few mutations in key positions directly affecting the enzyme's activity (or inhibition) profile. By this mechanism, some β-lactamases evolved from a broad to an extended-spectrum profile, resulting in enzymes like the first known extended-spectrum β-lactamases (ESBLs) and inhibitor-resistant (IR) enzymes (2-5). The second path is relevant for some ESBLs and involves direct dissemination of originally chromosomal genes residing in environmental or non-pathogenic species to pathogens (6, 7).

Plasmid-borne PER extended-spectrum β-lactamases ("*P*seudomonas Extended Resistance" (8)) have been detected in few locations around the world. In South American countries like Argentina and Uruguay, PER-2 is still the only variant detected and may account for up to 10% and 5% of oxyimino-cephalosporin resistance in *Klebsiella pneumoniae* and *Escherichia coli*, respectively (1, 9, 10), as the second most prevalent ESBL family after the pandemic CTX-M derivatives.

PER-2 has 86% amino acid sequence identity with PER-1. Both hydrolyze oxyimino-cephalosporins with high catalytic efficiencies (k_{cat}/K_m) for cefotaxime and ceftazidime, even if PER-2 is *ca.* 20-fold more efficient as compared to PER-1 on the latter; they are also strongly inhibited by mechanism-based inhibitors such as clavulanate and tazobactam (11, 12).

Recently, we solved the crystallographic structure of PER-2 at 2.2 Å (13). When compared to other class A β-lactamases, PER-2 has an inverted Ω loop due to a *trans* bond between

64 residues 166-167, and an expanded β3-β4 loop, creating an enlarged active site cavity that
65 allows for an efficient oxyimino-cephalosporins hydrolysis. We also provided structural
66 evidence for the existence of a hydrogen-bond network connecting Ser70-Gln69-oxyanion
67 water-Thr237-Arg220 that might be important for the activity and inhibition of the enzyme
68 (13).
69 It has been hypothesized that mutations in either Arg220 (the counterpart of Arg244 in
70 TEM/SHV variants) or Thr237 would probably result in modifications in the kinetic behavior;
71 the same role has also been suggested for Arg220 in PER-1 and other β-lactamases with an
72 arginine at this position, such as KPC-2 (12-14). Noteworthy, Arg220 in PER β-lactamases is
73 replaced by Arg276 in the CTX-M enzymes (15), and Arg244 in most of the other class A β-
74 lactamases, especially TEM and SHV. Nevertheless, only mutations at Arg244 have proved to
75 negatively impact inhibition by mechanism-based inactivators, selecting "inhibitor resistant"
76 β-lactamases (16-18). Noteworthy, in all these arginine residues, the positively charged
77 guanidinium group shares the same spatial position in the structure.
78 As no variants of PER β-lactamases with decreased susceptibility to mechanism-based
79 inhibitors have been described among clinical isolates so far, the rationale of this study was
80 to evaluate if mutations at Arg220 and Thr237 in PER-2 β-lactamase are able to modify the
81 response to mechanism-based inhibitors, and their impact on the activity towards different
82 substrates, so as to anticipate their possible *in vivo* selection.

Materials and Methods:

Strains and plasmids:

E. coli TC9 is a transconjugant clone harboring the pCf587 plasmid, used as the source of bla_{PER-2} gene (11). *E. coli* Top10F' (Invitrogen, USA) and *E. coli* BL21(DE3) (Novagen, USA) were hosts for transformation experiments. Plasmid vectors pTZ57R/T (Thermo Scientific, USA), pJET1.2/blunt vector (Thermo Scientific, USA) and pK19 vector (19) were used for routine cloning experiments. Kanamycin resistant pET28a(+) (Novagen, Germany) were used as overexpression vector.

Antibiotic susceptibility:

Minimum inhibitory concentration (MIC) of β-lactams were evaluated on *E. coli* Top10F' clones producing PER-2 variants by the agar dilution method according to the Clinical and Laboratory Standars Institute (CLSI) recommendations (20). For β-lactams/β-lactamase inhibitors combinations, empirical concentrations of inhibitors were tested in order to assess their ability to protect the partner β-lactam.

Molecular biology techniques:

Plasmid DNA (pCf587) was purified using the GeneJET Plasmid Miniprep Kit (Thermo Scientific, USA). The bla_{PER-2} gene and the two upstream promoter regions were amplified by PCR from plasmid pCf587, using 0.6 U *Pfu* DNA Polymerase (Thermo Scientific, USA) and 1 µM PER2-BamF4 (5'-TCATGTGAGTTTGGATCCCAAGTG-3') and PER2-KpnR (5'-GAAGCGACGGTACCTAATAACTG-3') primers, containing the *Bam*HI and *Kpn*I restriction sites, respectively (underlined in the sequences). The PCR product was first ligated in a pTZ57R/T

vector (Thermo Scientific, USA); the insert was sequenced for verification of the identity of the *bla*$_{PER-2}$ gene and generated restriction sites, as well as the absence of aberrant nucleotides. The resulting recombinant plasmid (pTZ/PER2-BK) was then digested with *Bam*HI and *Kpn*I, and the released insert was subsequently purified and cloned in the *Bam*HI-*Kpn*I sites of a pK19 vector for yielding plasmid pK19/PER-2 which was used as a template to obtain mutants at Arg220 and Thr237 by a two-stage procedure based on the QuikChange site-directed mutagenesis protocol as described by Wang and Malcolm (21). Generated mutants and primers used for generating the amino acid substitutions are detailed in Table 1. The new recombinant plasmids harboring the different mutations were used to transform *E. coli* Top10F' competent cells for sequencing and antimicrobial susceptibility evaluation. After the presence of the mutations were verified by DNA sequencing, the wild-type *bla*$_{PER-2}$ gene and the different allelic variants were amplified by PCR using 0.6 U *Pfu* DNA Polymerase (Thermo Scientific, USA) and 1 µM PER2-NdeI (5'-AGTTCATTTCATATGTCAGCCCAATC-3') and PER2-SacR1 (5'-CTTTAAGAGCTCGCTTAGATAGTG-3') primers, containing the *Nde*I and *Sac*I restriction sites, respectively (underlined in the sequences). PCR products were cloned in the *Nde*I-*Sac*I sites of a pET28a(+) vector, and transformed into chemically competent *E. coli* BL21(DE3) cells upon selection in LB agar plates supplemented with 30 µg/mL kanamycin. Final recombinant plasmids were sequenced at Macrogen Inc. (South Korea) for nucleotide sequence verification. Nucleotide and amino acid sequence analysis was performed by NCBI (http://www.ncbi.nlm.nih.gov/) and ExPASy (http://www.expasy.org/) bioinformatics tools.

Enzyme production and purification:

Overnight cultures were diluted (1/20) in 300 mL LB containing 30 µg/mL kanamycin and grown at 37°C until ca. 0.8 OD units (λ = 600 nm). In order to induce β-lactamase expression, 1 mM IPTG was added and cultures were incubated at 18°C overnight. After centrifugation at 8,000 rpm (4°C) in a Sorvall RC-5C, cells were resuspended in buffer A (20 mM sodium phosphate buffer, pH 8.0; 0.5 M sodium chloride) and crude extracts were obtained by sonication. After clarification by centrifugation at 12,000 rpm (4ºC), supernatants containing the respective fusion proteins were filtrated by 0.45 and 0.22 µm pore size membranes, and loaded onto 1-mL HisTrap HP affinity columns (GE Healthcare Life Sciences, USA) equilibrated with buffer A; the columns were extensively washed to remove unbound proteins, and β-lactamases were eluted with a step gradient with 125 and 300 mM imidazole in buffer A (1 ml/min flow rate). Active eluted fractions were dialyzed overnight against 20 mM Tris-HCl (pH 8.0), and then against 1X PBS (pH 7.4). The HisTag was eliminated by thrombin digestion (16 h at 22°C), using 1U thrombin per mg protein for complete proteolysis, and removed by affinity chromatography in 1-mL HisTrap HP columns (GE Healthcare Life Sciences, USA). Proteins purity was estimated as >95% by analysis on 12% SDS-PAGE gels and Coomassie blue staining, and protein concentration was determined by measuring the absorbance at 280 nm, according to Lambert-Beer law (molar extinction coefficient for PER-2 = 33,700 M^{-1} cm^{-1}) (22).

Steady-state kinetics:

Steady-state kinetic parameters were determined using a T80 UV/vis spectrophotometer (PG Instruments Ltd., UK). Each reaction was performed in a total volume of 500 µL at 22-24°C in 1X PBS buffer. The steady-state kinetic parameters K_m and V_{max} were obtained by measuring initial-rates (v_0) as described previously (23), with non-linear least squares fit of

the data (Henri Michaelis–Menten equation; Eq. 1) using GraphPad Prism 5.03 for Windows (GraphPad Software, San Diego, CA, USA):

$$v = \frac{V_{max} \times [S]}{K_m + [S]} \qquad \text{(Eq. 1)}$$

For low K_m values, the k_{cat} values were derived by evaluation of the complete hydrolysis time courses as described by De Meester et al (24). For competitive inhibitors, or poor substrates, inhibition constant K_I (as K_I obs) was determined by monitoring the residual activity of the enzyme in the presence of various concentrations of the drug and 100 µM nitrocefin as the reporter substrate (11). For irreversible inhibitors, the rate constant of inactivation, k_{inact}, was measured directly by time-dependent inactivation of PER-2 in the presence of the inhibitor, using a fixed concentration of enzyme and 100 µM nitrocefin as a reporter substrate, and increasing concentrations of the inhibitor. The observed rate constants for inactivation (k_{obs}) were determined by nonlinear least-squares fit of the data using OriginPro 8.0 (Northampton, MA, USA), using the Eq. 2, as described elsewhere (25):

$$A = A_0 + v_f \times t + (v_0 - v_f) \times (1 - e^{-k_{obs} \times t}) / k_{obs} \qquad \text{(Eq. 2)}$$

The k_{obs} values were plotted against the inhibitor concentration, and inactivation constant, k_{inact} and $K_{I\ obs}$ were obtained by nonlinear fitting of the Eq. 3, using GraphPad Prism:

$$k_{obs} = \frac{k_{inact} \times [I]}{K_{I\ obs} + [I]} \qquad \text{(Eq. 3)}$$

In cases in which Eq. 3 brings a linear fitting (instead of a nonlinear fit), it was assumed that K_I values were much higher than the [*I*] range, for which the resulting slope is k_{inact}/K_I.

Finally, K_I was obtained from $K_{I\ obs}$, taking into account the substrate K_m using the Eq. 4:

$$K_I = \frac{K_{I\ obs}}{(1 + [S])/K_{m(S)}} \qquad \text{(Eq. 4)}$$

The following extinction coefficients and wavelengths were used: ampicillin ($\Delta\varepsilon_{235}$ = −820 M^{-1} cm^{-1}), cephalothin ($\Delta\varepsilon_{273}$ = −6,300 M^{-1} cm^{-1}), ceftazidime ($\Delta\varepsilon_{260}$ = −9,000 M^{-1} cm^{-1}), cefotaxime ($\Delta\varepsilon_{260}$ = −7,500 M^{-1} cm^{-1}), cefepime ($\Delta\varepsilon_{260}$ = −10,000 M^{-1} cm^{-1}), aztreonam ($\Delta\varepsilon_{318}$ = −750 M^{-1} cm^{-1}), and nitrocefin ($\Delta\varepsilon_{482}$ = +15,000 M^{-1} cm^{-1}).

Circular dichroism:

Spectra were recorded on a Jasco J-810 spectropolarimeter 810 (JASCO, Easton, MD). Data in the near UV (250−320 nm) or in the far UV (195−250 nm) regions were collected at 25°C using 10 or 1 mm path length cuvettes, respectively. A scan speed of 20 nm/min with a time constant of 1 s was used for all proteins. Each spectrum was measured at least three times, and the data were averaged to minimize noise. Molar ellipticity was calculated as described elsewhere (26), using a mean residue weight value of 107.

Fluorescence measurements:

Fluorescence measurements were performed at 25°C in a Jasco FP-6500 spectrofluorimeter equipped with a thermostatized cell. A 3 mm path cuvette sealed with a Teflon cap was used. The excitation wavelength was 295 nm, and emission was collected in the range 310−410 nm. The excitation and emission monochromator slit widths were both set at 3 nm.

Chemical denaturation:

Conformational transitions were monitored as a function of denaturant concentration by measuring the change in the intrinsic fluorescence intensity of the proteins. Individual samples of protein (~ 10 µM final concentration) ranging in denaturant concentration from

199 0 to 3 M guanidinium chloride (GdmCl) were prepared by dilution of a fixed volume of a
200 stock solution of protein in mixtures of 1X PBS buffer and 5 M GdmCl. Samples were
201 analyzed after incubation for at least 1 h to ensure that the equilibrium had been reached.
202 Data did not fit well to a two-state unfolding transition. Therefore, it was fitted to a three-
203 state model with two sequential transitions and one partially folded state I. Since the three-
204 state model may be an oversimplification and more complex equilibria may occur, the
205 calculated curves are discussed only to illustrate general differences between the variants
206 and are not to be taken as supportive of a particular unfolding mechanism, as proposed by
207 Santos *et al* for *B. licheniformis* exo-small β-lactamase (27).

208 **Results and Discussion:**

209

210 **Mutations at Arg220 and Thr237 have different effects in the inhibition efficiency of**
211 **mechanism-based inhibitors:**

212 Mutations at Arg220 resulted in a marked increase in the K_I values for the inhibitors, which
213 led to a dramatic decrease in the inactivation efficiencies (expressed as k_{inact}/K_I; Table 2). In
214 the Arg220Gly variant, this resulted in a ~300 and 500-fold reduction in the k_{inact}/K_I values
215 for clavulanic acid and tazobactam, respectively. In other mutants, a maximum of 6.5% and
216 2.4% relative k_{inact}/K_I (both values corresponding to Arg220Ser mutant) was observed for
217 clavulanic acid and tazobactam, respectively, compared to wild type PER-2, which clearly
218 indicates that the substitution of this arginine residue at position 220 leads to major impact
219 in the inhibition efficiency of mechanism-based inhibitors. These results reinforce the
220 previous hypothesis about the essential role of Arg220 in the activity of PER-2 (13).
221 This behavior suggests that mutations at Arg220, resulting in a modification or suppression
222 of the positive charge conferred by the arginine residue, entail a negative impact in the
223 accommodation of the inhibitor within the active site and the proper formation of the
224 Michaelis complex. These changes are expected to have different consequences in the
225 hydrogen-bond network integrity, depending on the specific amino acid that replaces the
226 arginine residue. In fact, substitution of Arg220 by lysine in KPC-2 has been demonstrated to
227 result in an "inhibitor susceptible" variant (probably by reinforcing the positive
228 environment), while the Arg220Met mutation reduced the inhibition efficiency on KPC-2
229 (14).
230 As shown in Table 2, the Thr237Ala mutation resulted in only minor (but still detectable)
231 differences in inhibition by both inhibitors as compared with wild-type PER-2.

Hydrolysis of several β-lactams is also affected by mutations at Arg220 and Thr237:

Modifications in Arg220 of PER-2 not only affect the susceptibility to inhibitors but also seem to impact on the catalytic behavior towards several antibiotics, as observed in Table 3. For all the Arg220 mutants, the K_m values towards ampicillin were reduced up to 7-fold, being the K_m for the Arg220Gly variant the most affected. In some cases the k_{cat} constants somewhat compensated this decrease in the affinity, resulting in k_{cat}/K_m of ampicillin that were almost invariable for some mutants, except for Arg220Gly. The Thr237Ala mutation resulted in higher k_{cat}/K_m for ampicillin, compared to both the wild-type PER-2 and Arg220 mutants.

For cephalothin, cefotaxime, and ceftazidime, higher K_m values with a concomitant reduction in the turnover rates (smaller k_{cat}) in all Arg220 mutants were observed, yielding relative k_{cat}/K_m values that were slightly weaker compared to the wild-type PER-2. Moreover, for ceftazidime, only k_{cat}/K_m values could be determined, resulting in K_m values within the millimolar range and unreachable maximum velocities under the experimental conditions (data not shown). Aztreonam hydrolysis was considerably affected in all Arg220 mutants, where only K_m values were substantially modified (up to 145-fold increase in K_m values) while keeping almost invariable k_{cat} values, resulting in relative catalytic efficiencies as low as 3%).

Cefepime behaved in a different way, with smaller reductions in k_{cat}/K_m values in comparison to the wild-type PER-2, and compared to other oxyimino-β-lactams such as cefotaxime, ceftazidime and aztreonam; only the Arg220Gly mutant displayed a strong reduction in the catalytic efficiency. In silico models of PER-2 with cefepime (not shown) shown unfavorable interactions between Arg220 and the quaternary ammonium of

256 cefepime at C3, not present in cefotaxime, that could explain the different catalytic
257 properties of PER-2 over cefotaxime and cefepime, at least in the Arg220Gly mutant. The β-
258 lactamase stability of cefepime and other fourth generation cephalosporins is possibly
259 related to a reaction scheme involving a branched pathway with a relatively stable modified
260 acyl-enzyme dependent on the nature of the C3 leaving group (28). In PER-2, both electron
261 withdrawing properties and interaction between Arg220 and C3-ammonium could therefore
262 induce different catalytic properties over cefotaxime and cefepime. In PER-1, the Arg220Leu
263 mutation resulted in an opposite behavior: a general increase in apparent affinity of
264 cefotaxime, ceftazidime and aztreonam was observed, and an increase in k_{cat}/K_m was also
265 noticed (12).
266 The Thr237Ala substitution resulted in an enzyme with either lower turnover values
267 (cephalothin and cefotaxime), or higher K_m constants (cefepime and aztreonam), giving
268 lower catalytic efficiency values towards the cephalosporins and aztreonam, compared to
269 the wild-type PER-2. Nevertheless, the k_{cat}/K_m values remained much higher than those for
270 Arg220-harboring mutants, which is in agreement with our previous observations suggesting
271 a minor role of Thr237 in the overall stabilization of the active site coordination (13). In PER-
272 1, the Thr237Ala modification yielded an enzyme with increased k_{cat}/K_m ratios for
273 cefotaxime, ceftazidime and aztreonam (12), being this behavior opposite to what we
274 observed in PER-2.
275 It is noteworthy that the catalytic efficiency of mutants in both Arg220 and Thr237
276 diminished in the same proportion towards cefotaxime and ceftazidime, compared to wild-
277 type PER-2.
278

Mutations at Arg220 and Thr237 do not seem to disturb the PER-2 secondary and tertiary structures globally:

According to the CD and fluorescence spectra (Fig. S1) we propose that both wild-type PER-2 and derived mutants in Arg220 and Thr237 present equivalent secondary and tertiary structures. Therefore, mutations in either position might only lead to subtle local rearrangements that do not alter the overall conformation of the variants. Moreover, chemical induced equilibrium unfolding transitions reveal that all proteins follow a similar mechanism involving, at least, the existence on an intermediate species (Fig. S1). Although quantitative differences in the thermodynamic parameters are obtained (Table S1), the main conclusion of this approach is that all protein variants have similar stability than the wild-type protein and therefore they should be considered as stable and well folded entities.

Mutations at Arg220 and Thr237 have different outcomes in the phenotypic behavior:

The MIC values obtained for *E. coli* Top10F' clones producing wild-type PER-2 and the derived mutants are shown in Table 4.

Overall, clavulanic acid restored partner ampicillin activity in most variants to the levels found in the recipient strain only when 10 µg/mL was used, due to the inherently high level resistance conferred to this drug. The exception was the *E. coli* producing the Arg220Gly, both in presence of 1 and 10 µg/mL clavulanic acid. For the other mutants in Arg220, the amino acid substitutions did not impact final phenotypic behavior in the presence of clavulanate, in opposition to what could be expected considering the previous findings on mutations at Arg244 in TEM β-lactamases (16). This correlated to the observed kinetic behavior of the different variants, provided that a strong reduction in the inhibition

efficiency is necessary to have a phenotypic impact in the susceptibility, and only the Arg220Gly mutation, resulting in up to a 300-fold decrease in the k_{inact}/K_I value, was able to give a marked increase in the MIC of ampicillin/clavulanate (Table 2).

The MIC of cephalothin for the *E. coli* clone producing the Arg220Gly variant was reduced in at least 6 serial dilutions. The MICs of cefotaxime were reduced up to 8 serial dilutions in clones producing all the tested Arg220 variants, compared to the wild-type PER-2. For ceftazidime, even when the MICs were reduced, these values were still higher than those for other oxyimino-cephalosporins. For cefepime, the MICs were reduced at least 3 serial dilutions in *E. coli* clones producing all PER-2 mutants. For aztreonam, an up to a 5-dilutions decrease in the MIC was observed (Arg220Thr variant).

On the other hand, replacing Thr237 by alanine seemed to have minor impact in the observed MICs of *E. coli* clones, which were similar to the *E. coli* clone producing wild-type PER-2, and only cefotaxime MIC was reduced in 3 serial dilutions compared to the wild-type producer.

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318 **Conclusions:**

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320 Alterations in the hydrogen bonding network of the active site in PER-2, through
321 modifications in key residues as Arg220 and (to a much lesser extent) Thr237, not only
322 impact the overall susceptibility for inactivation by mechanism-based inhibitors, but also on
323 cephalosporin hydrolysis.

324 Mutations at Arg220 entail modifications in the catalytic activity of PER-2, and probably local
325 perturbations in the protein, but not global conformational changes. Therefore, the
326 apparent structural stability of the mutants suggests that these enzymes might be selected
327 *in vivo* if the use of β-lactams/β-lactamase inhibitors is intensified, and dissemination of
328 bla_{PER-2} genes is associated to more prevalent plasmid types. This would eventually ensure
329 the accumulation of naturally occurring mutations that result in successful amino acid
330 substitutions.

331 Our findings also reinforce the constant need for, at least, an active surveillance in those
332 countries where PER-2 β-lactamases are still detected, as well as in others in which PER
333 enzymes are not common. This is especially important during molecular screening of bla_{PER-2}
334 genes, as its presence could be underestimated due to the use of improper primers for its
335 detection (original primers used for detecting bla_{PER-1} do not detect bla_{PER-2} properly), or
336 because the enzyme may be occasionally confused for other ESBLs with similar phenotypic
337 behaviors.

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435 **Table 1.** List of oligonucleotide primers used for generating the *bla*$_{PER-2}$ allelic variants, and recombinant plasmids encoding for the Arg220 and

436 Thr237 PER-2 mutants.

Primer name	Primer sequence (5'-3')	Recombinant plasmids
PER2-RxL-Fw	GAAACCACCACAGG**G**CC**C**CAGC**T**GTTAAAAGGCTTGTTACCTGC	pK19/PER-2 Arg220Leu
PER2-RxL-Rv	GCAGGTAACAAGCCTTTTAAC**A**GCTG**G**GG**C**CCTGTGGTGGTTTC	
PER2-RxS-Fw	GAAACCACCACAGG**G**CC**C**CAG**A**G**C**TTAAAAGGCTTGTTACCTGC	pK19/PER-2 Arg220Ser
PER2-RxS-Rv	GCAGGTAACAAGCCTTTTAA**G**C**T**CTG**G**GG**C**CCTGTGGTGGTTTC	
PER2-RxG-Fw	GAAACCACCACAGG**G**CC**C**CAG**G**G**C**TTAAAAGGCTTGTTACCTGC	pK19/PER-2 Arg220Gly
PER2-RxG-Rv	GCAGGTAACAAGCCTTTTAA**G**C**C**CTG**G**GG**C**CCTGTGGTGGTTTC	
PER2-RxH-Fw	GAAACCACCACAGG**G**CC**C**CAGC**AT**TTAAAAGGCTTGTTACCTGC	pK19/PER-2 Arg220His
PER2-RxH-Rv	GCAGGTAACAAGCCTTTTAA**AT**GCTG**G**GG**C**CCTGTGGTGGTTTC	
PER2-RxT-Fw	GAAACCACCACAGG**G**CC**C**CAG**AC**GTTAAAAGGCTTGTTACCTGC	pK19/PER-2 Arg220Thr
PER2-RxT-Rv	GCAGGTAACAAGCCTTTTAAC**GT**CTG**G**GG**C**CCTGTGGTGGTTTC	
PER2-RxC-Fw	GAAACCACCACAGG**G**CC**C**CAG**T**G**C**TTAAAAGGCTTGTTACCTGC	pK19/PER-2 Arg220Cys
PER2-RxC-Rv	GCAGGTAACAAGCCTTTTAA**G**C**A**CTG**G**GG**C**CCTGTGGTGGTTTC	

PER2-T237A-Fw	TAAAACCGGT**G**CTTCGGGCGTCAGAGCAGGAAAAACTGC	pK19/PER-2 Thr237Ala
PER2-T237A-Rv	CTCTGACGCCCGAAG**C**ACCGGTTTTATGCGCCACTATA	

437 Nucleotides that differ from the original sequence are shown in bold. Restriction sites for *Apa*I enzyme generated by a silent mutation are

438 underlined. Fw: forward primer, Rv: reverse primer.

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441 **Table 2.** Inhibition parameters of PER-2 and derived mutants in Arg220 and Thr237

	Clavulanic acid				Tazobactam			
	k_{inact} (sec^{-1})	K_I (μM)	k_{inact}/K_I ($\mu M^{-1}. sec^{-1}$)	*Relative* k_{inact}/K_I (%)[a]	k_{inact} (sec^{-1})	K_I (μM)	k_{inact}/K_I ($\mu M^{-1}. sec^{-1}$)	*Relative* k_{inact}/K_I (%)[a]
PER-2 wt	0.031 ± 0.001	0.064 ± 0.007	0.48 ± 0.07	100	0.047 ± 0.002	0.18 ± 0.02	0.26 ± 0.04	100
Arg220Leu	0.045 ± 0.001	4.1 ± 0.2	0.0109 ± 0.0008	2.3	0.016 ± 0.001	10 ± 1	0.0016 ± 0.0004	0.6
Arg220Ser	0.055 ± 0.002	1.8 ± 0.1	0.031 ± 0.003	6.5	0.0172 ± 0.0005	2.8 ± 0.2	0.0062 ± 0.0007	2.4
Arg220Gly	0.11 ± 0.01	80 ± 10	0.0014 ± 0.0003	0.3	0.0157 ± 0.0006	31 ± 3	0.00050 ± 0.00007	0.2
Arg220His	0.032 ± 0.002	3.3 ± 0.5	0.010 ± 0.002	2.0	0.0150 ± 0.0005	5.1 ± 0.6	0.0029 ± 0.0004	1.1
Arg220Thr	ND	ND	0.0068 ± 0.0002	1.4	0.022 ± 0.001	11 ± 1	0.0019 ± 0.0004	0.7

Arg220Cys	0.31 ± 0.04	19 ± 3	0.016 ± 0.005	3.3	0.0187 ± 0.0004	6.0 ± 0.4	0.0031 ± 0.0002	1.2
Thr237Ala	0.027 ± 0.001	0.070 ± 0.009	0.38 ± 0.07	79.8	0.022 ± 0.002	0.27 ± 0.06	0.08 ± 0.02	30.6

[a] Relative to wild-type PER-2

ND: not determined

Table 3. Steady-state kinetic parameters of PER-2 and derived mutants in Arg220 and Thr237.

	Ampicillin			Cephalothin			Cefotaxime			Ceftazidime			Cefepime			Aztreonam [a]		
	k_{cat} (sec^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.sec^{-1}$)	k_{cat} (sec^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.sec^{-1}$)	k_{cat} (sec^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.sec^{-1}$)	k_{cat} (sec^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.sec^{-1}$)	k_{cat} (sec^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.sec^{-1}$)	k_{cat} (sec^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.sec^{-1}$)
PER-2 wt	53.0 ± 0.5	18 ± 1	3.0 ± 0.2	49.0 ± 0.8	12.7 ± 0.7	3.9 ± 0.3	92 ± 3	51 ± 3	1.8 ± 0.1	75 ± 1	105 ± 4	0.71 ± 0.04	18.6 ± 0.6	40 ± 2	0.47 ± 0.05	1.0 ± 0.1	2.4 ± 0.1	0.40 ± 0.07
Arg220Leu	105 ± 3	108 ± 10	1.0 ± 0.1	27.8 ± 0.8	58 ± 3	0.48 ± 0.04	12.3 ± 0.6	129 ± 11	0.10 ± 0.01	ND	ND	0.0266 ± 0.0003	ND	ND	0.130 ± 0.003	1.16 ± 0.04	136 ± 2	0.0085 ± 0.0005
Arg220Ser	72 ± 2	81 ± 8	0.9 ± 0.1	22.6 ± 0.5	43 ± 2	0.53 ± 0.04	21.0 ± 0.9	118 ± 9	0.18 ± 0.02	ND	ND	0.0719 ± 0.0006	27 ± 1	103 ± 10	0.26 ± 0.05	0.75 ± 0.04	73 ± 3	0.010 ± 0.001
Arg220Gly	64 ± 1	125 ± 5	0.51 ± 0.03	20.4 ± 0.5	510 ± 22	0.040 ± 0.003	9.2 ± 0.6	543 ± 49	0.017 ± 0.003	ND	ND	0.0655 ± 0.0008	ND	ND	0.060 ± 0.002	1.12 ± 0.06	350 ± 11	0.0032 ± 0.0003
Arg220His	72 ± 2	60 ± 6	1.2 ± 0.1	14.2 ± 0.4	82 ± 5	0.17 ± 0.01	32 ± 1	263 ± 26	0.12 ± 0.02	ND	ND	0.041 ± 0.001	21 ± 2	164 ± 30	0.13 ± 0.04	0.80 ± 0.06	148 ± 7	0.0054 ± 0.0007
Arg220Thr	100 ± 3	86 ± 8	1.2 ±	31 ± 1	63 ± 4	0.50 ±	13.3 ±	104 ±	0.13 ±	ND	ND	0.094	18 ± 1	80 ± 9	0.22 ±	1.05 ±	181 ±	0.0058

			0.2			0.05	0.3	5	0.01			± 0.001			0.04	0.06	2	± 0.0004
Arg220Cys	124 ± 3	62 ± 6	2.0 ± 0.2	17.5 ± 0.5	40 ± 3	0.44 ± 0.05	21,2 ± 0,9	139 ± 11	0.15 ± 0.02	ND	ND	0.0647 ± 0.0008	63 ± 6	268 ± 33	0.24 ± 0.05	0.93 ± 0.05	234 ± 5	0.0039 ± 0.0003
Thr237Ala	133 ± 3	30 ± 3	4.4 ± 0.6	13.6 ± 0.4	6.8 ± 0.8	2.0 ± 0.3	9,9 ± 0,2	13.1 ± 0.7	0.76 ± 0.06	ND	ND	0.351 ± 0.003	117 ± 11	326 ± 43	0.36 ± 0.08	2.545 ± 0.006	13.4 ± 0.3	0.190 ± 0.004

[a] K_m determined as K_i

ND: not determined

448 **Table 4.** Minimum inhibitory concentrations (MIC) of selected antibiotics on recombinant clones

E. coli Top10F' derivative harboring plasmid:	MIC (µg/mL)							
	AMP	AMP CL 1 [a]	AMP CL 10 [a]	CEF	CTX	CAZ	FEP	AZT
No plasmid	4	4	2	8	<0.03	0.125	0.03	0.125
pK19	4	4	2	8	<0.03	0.25	0.03	0.25
pK19/PER-2 (WT)	>1,024	16	4	>1,024	128	>1,024	64	>256
pK19/PER-2 Arg220Leu	1,024	16	4	64	0.5	128	8	16
pK19/PER-2 Arg220Ser	>1,024	16	4	256	2	512	8	32
pK19/PER-2 Arg220Gly	>1,024	128	16	16	0.5	128	8	32
pK19/PER-2 Arg220His	1,024	16	4	64	1	256	8	16
pK19/PER-2 Arg220Thr	1,024	16	4	32	0.5	64	4	8
pK19/PER-2 Arg220Cys	512	16	4	64	2	64	2	16
pK19/PER-2 Thr237Ala	>1,024	16	8	512	16	>1,024	32	>256

449 AMP, ampicillin; CEF, cephalothin; CAZ, ceftazidime; CTX, cetotaxime; FEP, cefepime; AZT, aztreonam; CL, clavulanic acid

[a] Numbers correspond to fix concentration of inhibitor expressed in µg/mL.