Contributions and Evolutionary Potential of *ampC* Mutations to confer Aztreonam Resistance in *Pseudomonas aeruginosa*

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Abstract

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Aztreonam is a synthetic monobactam antibiotic that is increasingly used for chronic suppressive therapy in Cystic Fibrosis (CF) patients with *Pseudomonas aeruginosa* airway infection. *P. aeruginosa* can accumulate mutations in the chromosomal *ampC* β-lactamase which promote aztreonam resistance, but the extent to which spontaneous mutations can increase *ampC* activity against aztreonam remains incompletely explored. To address this question, here we both examined *ampC* mutations that occur in *P. aeruginosa* clinical isolates derived from CF patients treated with aztreonam and additionally performed *in vitro* evolution of *ampC* for enhanced aztreonam hydrolysis. First, to catalog spontaneous *ampC* mutations that confer increased aztreonam resistance in *P. aeruginosa*, we sequenced a collection of 60 pre- and post-treatment clonal isolate pairs showing elevated resistance levels and identified 14 isolates with treatment-associated mutations in that gene. The 12 alleles, containing 13 unique mutations, had a variable effect on the ability of *ampC* to confer elevated aztreonam resistance relative to the wild-type allele (range 8-fold decrease to 64-fold increase) when expressed either in *Escherichia coli* or a *P. aeruginosa* laboratory strain deficient for native *ampC*. The alleles conferred variable resistance to

ampicillin, ceftazidime, and cefpirome, but meropenem resistance was consistently unaffected. To more fully evaluate the potential of point mutations to increase *ampC* activity against aztreonam, we subsequently performed artificial evolution and aztreonam selection of plasmid-borne *ampC* expressed in *E. coli.* We recovered mutants showing up to 4,096-fold increases in aztreonam resistance (128 ug/mL) relative to wild-type *ampC,* with mainained or enhanced resistance provided to other β-lactam drugs. Collectively, these findings indicate that mutations in *ampC* which result in elevated aztreonam resistance are relatively frequent in *P. aeruginosa* isolates from CF patient lungs, and that the maximum potential for *ampC* to evolve activity against this synthetic antibiotic are greater than previously appreciated.

Introduction

Aztreonam is a fully synthetic monobactam antibiotic that inhibits penicillin binding protein 3 (PBP-3) of gram-negative aerobic pathogens, including *Pseudomonas aeruginosa*(1). Although aztreonam has been approved for medical use in injectable and intravenous formulations since 1986, inhaled aztreonam (AZLI) was approved as chronic suppressive therapy against *P. aeruginosa* airway infection in Cystic Fibrosis (CF) patients only in 2010 (2). Clinical trials have indicated that AZLI usage can significantly relieve respiratory symptoms, improve lung function, and extend periods between pulmonary exacerbations in CF patients with *P. aeruginosa* airway infection and moderate-to-severe pulmonary function (1, 3, 4). ALZI is now prescribed for nearly half of qualifying CF patients in the United States (5).

Multiple studies have found that *P. aeruginosa* isolates can evolve modest, seemingly transient increases in aztreonam resistance in response to AZLI treatment, in a dose-dependent fashion(6). Antibiotic resistance in the context of CF lung infections frequently arises as a result of spontaneous chromosomal mutations (7), and a variety of such mutations which confer enhanced aztreonam resistance have accordingly been identified. Changes effecting active drug efflux through overexpression of *mexAB-OprM* system (8, 9), alteration of *ftsI* (PBP-3) and subsequent disruption of drug binding (10), and a number of more poorly characterized causal mutations have previously been described (7). Notably, mutational overexpression (11, 12) or coding sequence alteration of *ampC* (7, 13)*,* the chromosomal Class C β-lactamase of *Enterobacteriaceae*, has been identified in *P. aeruginosa* isolates with elevated aztreonam resistance. Although aztreonam is known to be poorly hydrolyzed by *ampC* and also poorly induces *ampC* expression (14), mutations which increase this gene's activity against aztreonam are potentially concerning as resistance-causing alleles could be mobilized to plasmids and rapidly disseminated through a population (15). Nevertheless, the range of mutations arising in *ampC* following aztreonam therapy, and their potential impact on aztreonam resistance, have not yet been fully explored.

The goals of this study were two-fold. First, we sought to characterize the spectrum and functional effects of *ampC* mutations occurring in *P. aeruginosa* clinical isolates from CF patients treated with AZLI. Second, in order to more fully explore the evolutionary potential of *ampC* to hydrolyze this synthetic

antibiotic, we separately performed *in vitro* evolution studies to select *ampC* mutants for maximal activity against aztreonam.

Results

Prevalence of *ampC* mutations in patients treated with AZLI*.* We examined 64 pairs of pre- and post-treatment clinical isolates, collected during a clinical study of CF patients undergoing AZLI therapy (treatment arm *n*=52, control arm *n*=12), and which showed gains in aztreonam resistance over time. Relative to their paired pre-treatment isolate, a total of twelve *P. aeruginosa* clinical isolates demonstrated spontaneous point mutations in chromosomal *ampC* (Table 1), with all such instances identified from the treatment arm. We consequently estimate that the incidence of *ampC* mutation in *P. aeruginosa* lineages from patients treated with AZLI is 23%, and that selection for *ampC* mutation appears restricted to patients undergoing AZLI therapy.

Resistance profiles of *ampC* mutations in *E. coli* and *P. aeruginosa.* A total of 15 unique mutations were cataloged across 14 different alleles, where Allele 13 and Allele 14 were found in two independent isolates (designated Allele 1 through Allele 14, **Table 1**). Five mutations (p. T21A, p. G27D, p. R79Q, p. V356I, and p. G391A) have previously been reported from studies of β-lactam resistance in clinical *P. aeruginosa* isolates (13, 16, 17). The remaining ten mutations are newly reported here. Of these, the most prevalent was p. G242S, which was seen in 4 alleles, p. V239A, which occurred in 3 alleles, and p. L176R, found in 2 alleles.

To assess the activity of mutant *ampC* alleles against aztreonam, each was cloned into an expression vector and transformed in to *E. coli.* Minimum inhibitory concentrations (MICs) were assessed using aztreonam and other β-lactamases from different structural categories (**Table 2**)*.* Two alleles (Allele 8, and 9) conferred no measurable increase in aztreonam resistance, while the remaining ten provided 2- to 64-fold increases in MIC. Alleles conferred variable levels of resistance, either elevated or reduced, to the other β-lactam drugs tested. Five alleles increased resistance to aztreonam at the expense of ampicillin resistance, and nine alleles provided increased ceftazidime resistance concordant with gains in aztreonam resistance. The allele providing the highest levels of aztreonam resistance (Allele 6) also conferred the greatest levels of resistance to ampicillin, ceftazidime, and cefpirome. None of the alleles was able to

provide increased resistance to meropenem, remaining consistent with the lack of activity of wild-type *P. aeruginosa ampC* against that drug (18).

Similar resistance patterns were observed after expression vectors were transformed into a *P. aeruginosa* background from which chromosomal *ampC* had been ablated by transposon mutagenesis (19). Resistance levels for all drugs tested were higher than seen in *E. coli*, reflecting higher basal resistance levels in that organism. Interestingly, although the 2 alleles showing no increase in aztreonam activity when expressed in *E. coli* continued to exhibit equivalent or worse performance relative to wild type *ampC* when transferred to *P. aeruginosa*, all the remaining mutant alleles provided consistently enhanced resistance to ampicillin, ceftazidime, and cefpirome, which was not observed in *E. coli*. These findings suggest that higher resistance levels across multiple drugs can be achieved when expressing mutant *ampC* alleles in its species of origin. Conversely, increases in resistance to particular agents which were apparent in an *E. coli* background were not seen after transfer to *P. aeruginosa,* but this finding may reflect the decreased ability of 2-fold serial dilution MIC testing to resolve subtle differences between resistance phenotypes at high antibiotic concentrations.

***In vitro* selection of *ampC* for aztreonam resistance.** To evaluate the evolutionary potential of *ampC* to provide aztreonam resistance, we next performed artificial evolution of the gene using multiple cycles of mutagenic PCR, library cloning in *E. coli*, and selection of resultant populations using increasing concentrations of aztreonam. Bacterial growth at the highest drug concentration was harvested for plasmid and used as template for the next round of mutagenesis. Three separate evolutionary replicates were generated in parallel. Seven to nine rounds of selection were performed before evolved aztreonam resistance plateaued (**Fig.1**), after which single colonies were isolated from each replicate on aztreonam-containing media and subjected to formal MIC analysis. Isolates having different antibiotic resistance phenotypes originating from the same population were considered to potentially harbor different alleles, and the mutations in each were catalogued by sequencing.

Evolved populations proved largely homogeneous, with only two different alleles identified from Selection 1, two from Selection 2, and a single allele identified from the final selection. Artificially evolved alleles carried a range of 7- to 11-point mutations (**Table 3**) and conferred between 512- to 1,024-fold

increases in aztreonam resistance relative to the wild-type gene (**Table 4**). Evolved alleles maintained or demonstrated up to 16-fold increases in ampicillin resistance, 32- to 1,024-fold enhanced ceftazidime resistance and maintained or conferred up to 4-fold gains in cefpirome resistance (**Table 4**). As with alleles identified from clinical isolates, no increases in meropenem resistance were observed.

Discussion

Using a combination of clinical isolate analysis and experimental evolution, we have explored the mutations in *ampC* which render it capable of increasing *P. aeruginosa's* resistance to aztreonam. Broadly speaking, the *ampC* mutations could impact aztreonam resistance either through increased gene expression (11, 12) or by directly modifying the structure and function of the β-lactamase itself (7, 13).

Sequencing clinical isolates from patients treated with AZLI identified *de novo ampC* mutations in nearly a quarter of strains tested (14 mutant alleles out of 52 treatment arm isolates). These comprised 15 unique mutations (**Table 1**), only five of which have been previously reported in other work(7, 13, 17). These five known mutations, p. T21A, p. G27D, p. R79Q, p. V356I, and p. G391A, were identified from six separate isolates and are known to result in elevated *ampC* expression with consequent increases in MIC resulting from increased mass action (16, 17). It is likely that the p. P7S mutation newly reported in our study effects elevated resistance through the same mechanism, since, like p. T21A, it occurs in a 26 amino acid N-terminal signal peptide which is cleaved from the β-lactamase post-translationally during protein maturation (13).

Given their placement within the various functional domains of *ampC* (20), it is likely that the 9 variants newly reported in this study affect the substrate specificity or hydrolytic activity of the enzyme. Most central to hydrolysis, the catalytic residues of *ampC* are comprised of Ser90-Lys93-Tyr177 (21). The boundaries of the larger active site are defined by the Gln146 loop (residues 143 –154), Tyr177 loop (residues 176 – 179), Ω-loop (residues 238 – 252), R2-loop (residues 315 – 333), β11 (residues 338 – 346), and α11 (residues 373 – 390) (**Fig.2**) (20). Five of the ten newly described mutations identified in clinical isolates (p. L176R, p. V239A, p. G242S, p. D245G, and p. N347S) were located within the active site, while two others (p. Q174R and p. G302_P303insG) located in the immediate vicinity and could plausibly affect the conformation of the active site. Prior work has shown that the Ω-loop, in particular, accommodates the

bulky C7 side chain of β-lactams, and that greater catalytic abilities can be achieved following drug-specific mutations of that domain (20, 22). The insertion mutation in the R2 loop (p. G302_P303insG, Allele 5) are also consistent with reports that R2 loop alterations can result in conformational changes of *ampC* that increase β-lactam hydrolysis (23).

The results of *in vitro* evolution highlight similarities and differences with the results of clinical isolate sequencing. Artificially evolved alleles conferred greater levels of aztreonam resistance (512- to 1,024-fold increases) than observed in clinical isolates (2- to 12- fold increases) (**Tables 2 and 4**). Similarly, artificial selection resulted in a range of 7- to 11-point mutations, compared to a maximum of 3 mutations recovered from the in clinical isolates (**Tables 1 and 3**), indicating that these alleles were the product of considerably more selection than clinical strains. All 19 unique changes recovered from artificial selection were point mutations. Only four variants (p. T21A, p. V239A, p. N347K/S) were also seen in clinical isolates, indicating that they provide advantages both *in vivo* and *in vitro*. Two of the remaining 15 mutations that were only identified by *in vitro* evolution (p. S15A and p. T16A) occurred in the signal peptide region, thus making them likely to increase *ampC* expression. Four more mutations were within or adjacent to the *ampC* active site (L145R, p. Q146K, and p. Q152R in the Gln146 loop, and p. P180V that adjacent to the Tyr177 loop) suggesting that they altered the enzyme's substrate specificity: Q146K was present in all evolved alleles, suggesting that this change is of high importance. One additional mutation falling outside the active sites (p. A105T) was previously reported to increase *ampC* catalytic activity to particular drugs (16), but was not recovered from clinical isolates. The function of the remaining 8 mutations remain unclear, however, two changes (p. Q146K, and p. A256T) were universally found across all evolved alleles, suggesting high importance.

It is interesting to speculate why particular mutations were recovered only from artificial evolution, but not from clinical isolates. One possibility is that presence of these point mutations *in vivo* somehow compromises bacterial fitness. Consistent with this hypothesis, we noted that as *ampC* was subjected to artificial evolution, growth rates of bacteria carrying evolved alleles substantially decreased (not shown). Another possibility is that to achieve high level resistance multiple mutations need to occur simultaneously,

rather than as discrete stepwise changes. This situation is feasible by *in vitro* mutagenesis PCR, but rather unlikely *in vivo* bacterial evolution. Future work will explore these possibilities further.

In summary, our studies identify a spectrum of mutations which correlate with the ability of *ampC* to provide increased resistance to aztreonam. The major mechanisms appear to involve increased expression of *ampC* and alterations of the enzyme's active site, although a number of additional mutations without clear functional consequence have been recurrently identified. Mutations in *ampC* occur frequently in clinical isolates from patients treated with AZLI, and artificial evolution experiments indicate that *ampC* has a greater maximal potential to hydrolyze aztreonam than previously recognized. The possibility that *ampC*-mediated aztreonam resistance may limit the future application of aztreonam therapy should be carefully considered.

Material and Methods

Bacterial strains and growth conditions. *P. aeruginosa* strain MPAO1, Transposon mutant PW7954 (24) were a generous gift from Colin Manoil at the University of Washington. Electrocompetent *E. coli* DH5α and 10-β were purchased from New England Biolabs. *E. coli* λ-pir (25) was a generous gift from Pradeep Singh at the University of Washington. *P. aeruginosa* clinical isolates collected under the AIR-CF5 clinical trial (ClinicalTrials.gov Identifier: NCT01375036) were provided by Gilead Sciences, Inc. All strains were maintained at 37°C in Luria-Bertani (LB) under selection by the appropriate antibiotic, if indicated.

Genome sequencing and variant calling. Whole genome sequencing of *P. aeruginosa* clinical isolates was performed as described elsewhere (26, 27) (manuscript in preparation). Sequence reads were mapped to the PAO1 reference genome (Genbank Accession: AB198756), and variant calling performed as elsewhere (28).

Cloning of *ampC* alleles and MIC testing. We first replaced the *ampR* gene of *E. coli-Pseudomonas* shuttle vector pMMB190 (purchased from ATCC), with the gentamicin resistance cassette of pCN33 (28) to avoid analytic interference from β-lactamase activity of the vector's native selectable

marker. The gentamicin resistance cassette was amplified using primers F_Gibson_pMMB190_GM (5'-CCGGGGATCCATTTACCG-3', synthesized by IDT) and R_Gibson_pMMB190_GM (AGACGTCAGGTGGCACTTTTC-3') and introduced into pMMB190 by Gibson Assembly (29) to generate vector pMMB190_GM.

Wild-type or mutant *ampC* genes were PCR amplified from appropriate templates using primers F_Gibson_*ampC*_GM_pMMB190 (5'-GCTCCCGGGGCGGTTTCT-3') and R_Gibson_*ampC*_GM_pMMB190 (5'-CATAGCCAGGACCGGCGTC-3'). *ampC* amplicons were then inserted downstream of the *lac* promoter of pMMB190_GM by Gibson Assembly. Plasmids were transformed into *E. coli* DH5α by electroporation or into *P. aeruginosa* as described elsewhere (25).

Liquid MIC determination was performed according to CLSI guidelines (30), except that LB broth was used and induction with 3mM IPTG (ThermoScientific) was performed for pMMB190_GM-derived vectors.

***In vitro* selection for aztreonam resistance.** In order to support the construction of high-diversity mutant libraries, we constructed a small, high-efficiency cloning vector derived from pUC19 (obtained from NEB) and pMMB190_GM. The pUC19 origin of replication was amplified using primers pUC19_ori_expression_F (5'-GCGGTATCATTGCAGCACTGG-3') and pUC19_ori_expression_R (5'-TGAGCAAAAGGCCAGCAAAAG-3'). The *lacZ* promoter, multiple cloning site, and gentamicin resistance cassette of pMMB190_GM were PCR amplified using primers pMMB_MCS_F (5'-GCCGACATCATAACGGTTC-3') and pMMB_MCS_R (5'- TTTAAAAGACGTCAGGTGG-3'). These two products were Gibson assembled to produce vector pUC_MM.

Three replicates of artificial selection were performed independently, in parallel. Mutagenic PCR of *ampC* was performed using primer set as wild-type or mutant *ampC* amplification (F_Gibson_*ampC*_GM_pMMB190 and R_Gibson_*ampC*_GM_pMMB190) with the Diversify PCR Random Mutagenesis Kit (CloneTech) under conditions to target an average of 4.6 point-mutations per kb (3.9 mutations per gene copy). Products were digested with BamHI and EcoRI, ligated overnight into pUC_MM which had been similarly digested, CIP (NEB) dephosphorylated, and DNA cleaned-up (NEB). Ligations were then purified and transformed into electrocompetent *E. coli* 10-β. After 1 hr recovery in SOC medium

(NEB), 5 h of outgrowth was performed in 100 mL LB containing 10 ug/mL gentamycin. Libraries were plated before and after expansion for cell count estimation of effective library size. One million cells were added to 4 mL LB-aztreonam medium spanning four concentrations of antibiotic in increments of 2-fold serial dilutions. After 24 hours of incubation, we harvested cells from the highest concentration of antibiotic with visible growth. Incubation period was expanded to 36 hours after the resistance reached 256 µg/mL. Cryostocks were prepared from a fraction of the culture, while plasmid was extracted from the remainder and used as template for the following round of mutagenic PCR. This process was continued until the resistance reach 1,024 µg/mL or it shows plateau for three consecutive rounds for individual replicate.

***ampC* sequence confirmation.** Sanger sequencing of cloned *ampC* alleles was performed using primers F_Gibson_*ampC*_GM_pMMB190, R_Gibson_*ampC*_GM_pMMB190, ampC_SeqNested_F (5'-AGAAGGACCAGGCACAGATC –3') and ampC_SeqNested_R (5'- GAACACTTGCTGCTCCATGA -3').

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Table 1 Point mutations detected in clinical isolate *ampC* genes.

	Substitutions at position														Indel
Isolate	7	21	27	79	174	176	218	239	242	245	274	347	356	391	302_303
Wild type *ampC*	P	T	G	R	Q	L	V	V	G	D	P	N	V	G	
Allele 1 p. P7S, p. G242S	S								S						
Allele 2 p. G27D, p. R79Q, p. G242S			D	Q					S						
Allele 3 p. G242S									S						
Allele 4 p. R79Q, p. N347S				Q								S			
Allele 5 p. G302_P303insG															insG
Allele 6 p. R79Q, p. D245G				Q						G					
Allele 7 p. L176R, p. V239A						R		A							
Allele 8 p. T21A, p. Q174R, p. G391A		A			R									A	
Allele 9 p. L176R						R									
Allele 10 p. R79Q, p. V239A, p. P274L				Q				A			L				
Allele 11 p. V218M, p. V239A							M	A							
Allele 12 p. V356I													I		
Allele 13 p. G242S									S						
Allele 14 p. L176R						R									
Population mutation frequency	0.07	0.07	0.07	0.29	0.07	0.21	0.07	0.21	0.29	0.07	0.07	0.07	0.07	0.07	0.07

Table 2. β-lactam MICs of *ampC* alleles expressed in *E. coli* DH5α and *ΔampC P. aeruginosa* MPAO1.

		E. coli DH5 α					*ΔampC* MPAO1				
Isolate	Mutation	ATM[a]	AMP[b]	CAZ[c]	CPO[d]	MEM[e]	ATM	AMP	CAZ	CPO	MEM
Wild type	None	0.25	64	0.5	0.0156	0.0625	16	1024	16	8	4
Allele 1	p. P7S, p. G242S	1	32	2	0.0156	0.0625	128	2048	128	16	4
Allele 2	p. G27D, p. R79Q, p. G242S	0.5	32	0.5	0.0078	0.0625	64	2048	64	16	4
Allele 3	p. G242S	0.5	16	1	0.0156	0.0625	32	2048	64	16	4
Allele 4	p. R79Q, p. N347S	4	32	4	0.0156	0.0625	256	2048	256	32	4
Allele 5	p. G302_P303insG	0.5	32	2	0.0156	0.0625	32	2048	64	32	4
Allele 6	p. R79Q, p. D245G	16	256	16	0.0625	0.0625	256	2048	256	32	4
Allele 7	p. L176R, p. V239A	1	64	2	0.0156	0.0625	32	2048	64	16	4
Allele 8	p. T21A, p. Q174R, p. G391A	0.0625	2	0.125	0.0078	0.0625	2	32	2	1	4
Allele 9	p. L176R	0.25	64	0.5	0.0156	0.0625	16	1024	16	8	4
Allele 10	p. R79Q, p. V239A, p. P274L	0.5	64	1	0.0156	0.0625	32	2048	128	16	4
Allele 11	p. V218M, p. V239A	1	128	1	0.0156	0.0625	64	2048	64	16	4
Allele 12	p. V356I	0.5	64	1	0.0156	0.0625	32	1024	64	8	4

[a]Aztreonam
[b]Ampicillin
[c]Ceftazidime
[d]Cefpirome
[e]Meropenem

Table 3 Point mutations in *ampC* alleles from artificial aztreonam selection.

		Amino acid changes at position																	
Selection	Allele	15	16	21	49	105	114	145	146	152	159	180	189	197	239	256	320	321	347
Wild type		S	T	T	N	A	R	L	Q	Q	R	P	A	F	V	A	L	Q	N
1	A	A	A		Y		H		K	R				Y	A	T		L	K
1	B	A	A		Y	T	H	R	K	R					A	T		L	K
2	A					T	H		K	R	L		T		A	T	R		
2	B			A		T	H		K		L		T		A	T	R		
3	A					T	H	R	K			V				T			S
Overall mutation frequencies		0.5	0.5	0.1	0.5	0.7	1	0.5	1	0.6	0.2	0.3	0.2	0.3	0.7	1	0.2	0.5	K: 0.5 S: 0.3

Table 4. β-lactam MICs of *ampC* alleles from artificial aztreonam selection.

		E. coli 10-ß				
Selection	Alleles	ATM	AMP	CAZ	CPO	MEM
Wild type	N/A	0.125	32	0.25	0.031	0.062
1	A	64	256	256	0.125	0.062
1	B	64	32	8	0.062	0.062
2	A	64	128	32	0.031	0.125
2	B	128	512	64	0.062	0.062
3	A	64	64	16	0.031	0.125

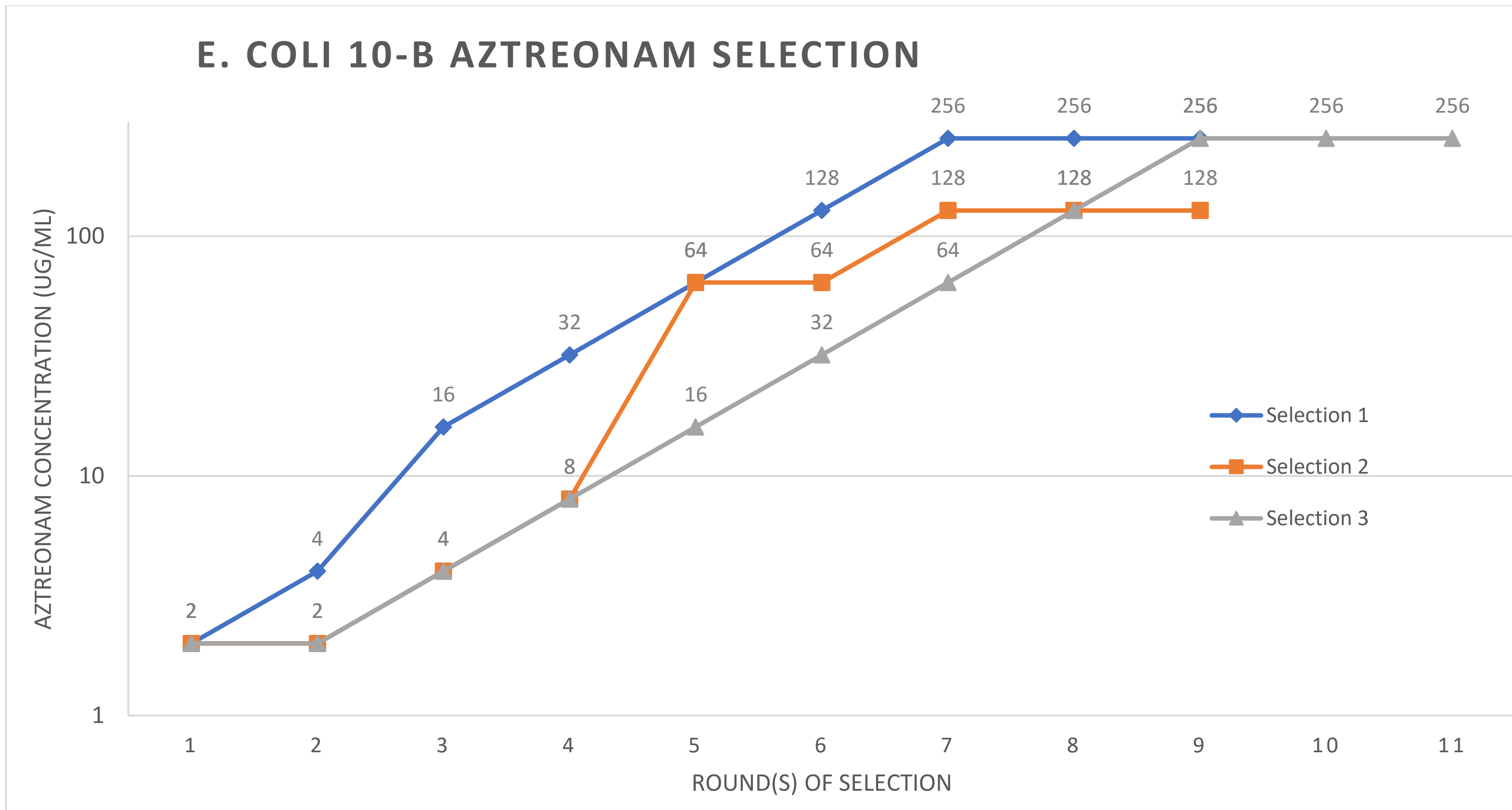

Fig. 1 Highest survival aztreonam concentration in *ampC* evolution. Results are depicted for three replicates selected in parallel. Note that graph indicates the highest concentration of aztreonam that enabled bacterial growth during selection of the evolved populations, rather the measured MIC of isolates from those populations.

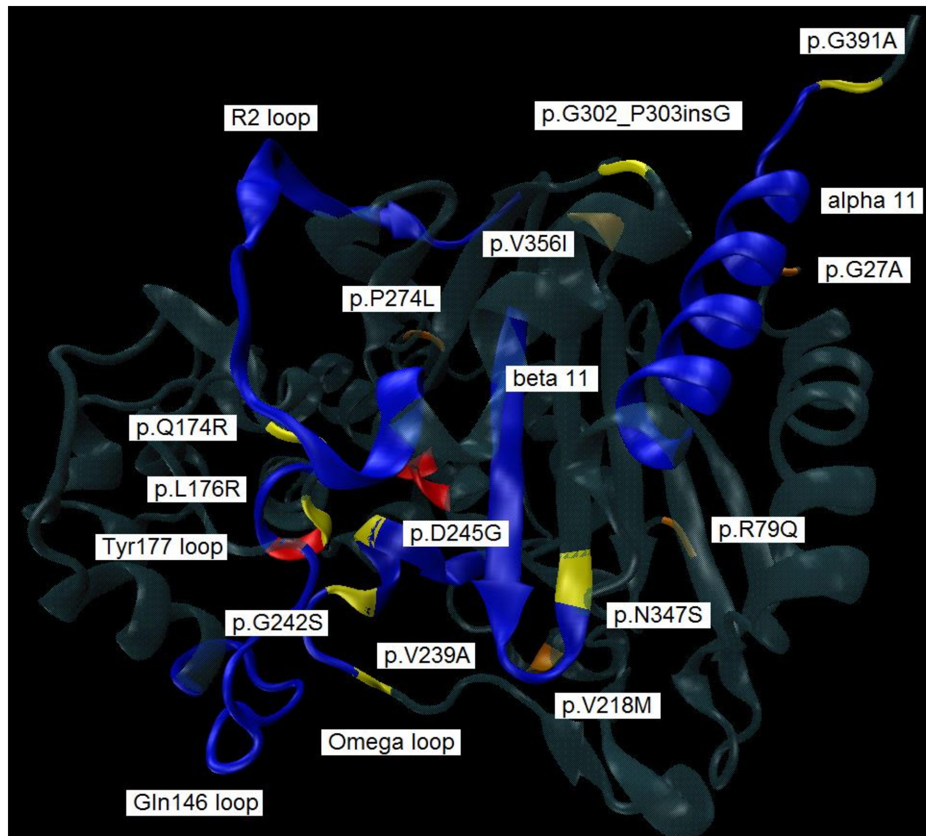

Fig. 2 Point mutations from clinical isolates in relation to *ampC* β-lactamase structure. Blue ribbons indicate boundaries of the active site, yellow highlighting corresponding point mutations that reside in active sites, orange residues are point mutations outside of active sites, the red is catalytic serine of *ampC* β-lactamase. Ten of the thirteen mutations recovered from clinical isolates overlap active sites.