Increased resistance to ceftazidime in a novel CMY-54 AmpC-type enzyme with an insertion in the Omega loop.

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RUNNING TITLE: CMY-54 β-lactamase

ABSTRACT

During a Spanish surveillance study, a natural variant of a CMY-type β-lactamase with a GluLeu$^{217-218}$ insertion in the omega loop relative to CMY-2 (and designated CMY-54) was found to confer more resistance to ceftazidime and cefotaxime in a clinical strain of *Escherichia coli*. The aim of this study was to characterize CMY-54 by genetic, microbiological and biochemical analysis.

The *bla*$_{CMY-54}$ gene is encoded by a plasmid of around 100 kb that hybridized with K and FIB probes. The genetic context of *bla*$_{CMY-54}$ and *bla*$_{CMY-2}$ genes was very similar. The MICs of the two CMY-type genes expressed under isogenic conditions in *E. coli* showed a clear increase in resistance to cefotaxime, ceftazidime y aztreonam in CMY-54 relative to CMY-2, in contrast to a slight increase in resistance to cefoxitin in CMY-2 relative to CMY-54. The catalytic efficiencies of the pure CMY-2 and CMY-54 proteins were correlated with the microbiological parameters. The ratio of MICs between aztreonam, ampicillin and ceftazidime in combination with avibactam were two fold higher in CMY-2 compared to CMY-54 and correlates with a four times decrease in IC_{50} for avibactam in CMY-54 compared to CMY-2. The CMY-2 protein is more stable than CMY-54 to thermal denaturation.

In summary, the GluLeu$^{217-218}$ insertion observed in CMY-54 compared to CMY-2 produce a β-lactamase with better catalytic efficacy for oximino-cephalosporins and lower inhibition by avibactam, all together producing a potentially troublesome enzyme.

Keywords: CMY-54, CMY-2, insertion, omega loop, ceftazidime, avibactam

INTRODUCTION

Plasmid mediated AmpC β-lactamases are clinically important cephalosporinases, particularly in *Enterobacteriaceae*, and transmission of plasmids carrying AmpC genes has been detected in bacteria such as *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis* (1,2). At present, none of the classical available β-lactamase inhibitors (clavulanic acid, sulbactam and tazobactam) inactivate high-level class C producers, although tazobactam shows inhibitory activity in some species, such as *Morganella morganii* (3-5). Avibactam has been recently approved for commercial use and it is a good inhibitor of AmpC-type β-lactamases (6). Boronic acids are promising new candidates as AmpC β-lactamase inhibitors (7).

The CMY-type enzymes, which were first described in 1989, are plasmid AmpC β-lactamases. The dissemination of plasmid-mediated CMY β-lactamases in *Enterobacteriaceae* has become an important public health concern (8). At least 131 variants have been identified (http://www.lahey.org/Studies). The $bla_{\text{CMY-2}}$ gene and its minor variants are prevalent worldwide and are commonly associated with different incompatibility groups, mainly IncA/C and IncI1 and rarely IncF, K and ColE plasmids (9,10). The plasmid-encoded $bla_{\text{CMY-2}}$ has frequently been observed in a transposon-like element consisting of ISEcp1-$bla_{\text{CMY-2}}$-*blc*-*sug*E-*enc*R, and horizontal gene transfer has been demonstrated (11,12).

Extensive kinetic and structural studies have been carried out with these enzymes in the last three decades. New extended-spectrum CMY-type enzymes capable of hydrolyzing cefepime and cephalosporins with large side chains are emerging (2,13). These enzymes differ from the typical CMY-type β-lactamases as a result of amino acid insertions, deletions and substitutions(2,13,14). The three regions involved in these modifications are the omega-loop, the R2 loop and the H-10 helix (2,13,14). A structural alteration of Y-X-N loop has

72 recently been shown to be responsible for extension of the hydrolysis spectrum in CMY-2
73 enzymes (15).

74 In the present study, we compared the genetic context and location of *bla*$_{CMY-2}$ and *bla*$_{CMY-54}$
75 genes in *Enterobacteriaceae* clinical strains isolated in Spain. We also provide the first
76 detailed kinetic characterization of the CMY-54 protein, which differ in the GluLeu$^{217-218}$
77 insertion compared to CMY-2. This mutation, which results from a 6 base duplication, is
78 significantly improving ceftazidime hydrolysis and produce an enzyme with higher IC_{50} for
79 avibactam compared to CMY-2

80 **MATERIALS AND METHODS**

81 **Antibiotics and other chemicals**

82 Ampicillin, cephalothin, cefoxitin, ceftazidime and cefotaxime, cefepime, aztreonam,
83 imipenem and the inhibitors clavulanic acid, tazobactam and sulbactam,were purchased
84 from Sigma Chemical Co (St. Louis, MO).Nitrocefin was obtained from Unipath Oxoid
85 (Basingtoke, Hants, United Kingdom), IPTG (isopropyl-β-D-thiogalactopyranoside) was
86 purchased from Roche (Basel, Switzerland), and avibactam was a gift from Astrazeneca
87 (London, United Kingdom).

88 **Bacterial strains**

89 *E. coli* clinical strain 63024 was used to clone the *bla*$_{CMY-2}$ gene and for MIC analysis. The
90 strain was isolated in the CHUA Coruña , A Coruña, Spain.

91 *E. coli* clinical strain 71041 was used to clone the *bla*$_{CMY-54}$ gene and for MIC analysis. It
92 was isolated in the Hospital Universitario Central de Asturias, Oviedo, Spain. Both strains
93 were isolated during the course of a nationwide multicentre study .

E. coli TG1 (*sup*E *hsd*Δ5 *thi* Δ(*lac-pro*AB) F´[*tra*D36 *pro* AB^{+}*lac*I^{q} ΔM15]) was used as the recipient in cloning experiments and for MIC analysis.

E. coli BL21 (*hsd*S *gal* [*λ*c*Its*857 *ind*1 *Sam*7 *nin*5 *lac* UV5-T7 gene1]) was used in expression experiments.

In vitro susceptibility testing

The MIC of antibiotic indicated in Table 1 were determined by microdilution, according to CLSI methodology, and confirmed by Etest (Biomérieux; Marcy l'Etoile, France), according to the manufacturer's recommendations (16).

Genetic context of the *bla*$_{CMY}$ genes in *Enterobacteriaceae* isolates

The plasmids were characterized by pulsed-field gel electrophoresis (PFGE) after S1 nuclease digestion of whole-genome DNA (S1-PFGE) and PCR-based replicon typing (PBRT), as previously described (17). The S1-PFGE-I gel was transferred and hybridized with CIT, and I1, FIA, FIB, K, F, B/O incompatibility groups (the amplicons obtained in PBRT) probes. PFGE was carried out after I-CeuI digestion of whole genome DNA to determine whether the *bla*$_{CMY}$ genes were located in the chromosome (18). The PFGE-I-*Ceu*I gel was transferred and hybridized with 16rRNA and CIT probes.

The genetic context of *bla*CMY-54 genes was determined by PCR followed by sequencing, according to previously described structures (19). Sequencing reactions were performed with the BigDye Terminator kit (PE Applied Biosystems, Foster City, CA), and sequences were analyzed in an ABI Prism 3100 DNA sequencer (PE Applied Biosystems). The resulting sequences were then compared with those available in the GenBank database (www.ncbi.nih.gov/BLAST**)**

Cloning and DNA analysis

PCR techniques were used to obtain the bla_{CMY-2} and bla_{CMY-54} genes from *E. coli* 63024, and *E. coli* 71041 respectively, and the genes were then cloned in plasmid pBGS18 harbouring an external promoter, pBGS18-pCTX (20). The following primers were used to clone the CMY genes: CMY-pBGS18 fw 5′-AAAAGGTACCATGATGAAAAAATCGTTATGCTGC (forward), and CMY-pBGS18 rv 5′- AAAAGAATTC TTATTGCAGCTTTTCAAGAATGC (reverse), which introduced the restriction sites *Kpn*I and *Eco*RI, respectively. For microbiological analysis, all constructs were transformed in *E.coli* TG1.

Purification of CMY-type enzymes

To purify the CMY-2 and CMY-54 proteins, the corresponding genes were cloned in the pGEX-6P-1 vector with the following primers: CMY-pGEX fw 5′-AAAAGGATCCAAAACAGAACAACAGATT (forward) and CMY-pGEX rv 5′-AAAAGAATTCTTATTGCAGCTTTTCAAG (reverse), which generated the restriction sites *Bam*HI and *Eco*RI respectively. The constructs were transformed in *E. coli* BL21 to induce fusion between glutathione *S*-transferase (GST) and the CMY enzymes without the signal peptide. The β-lactamases were purified to homogeneity, and the GST was removed from the CMY enzymes, following the manufacturer's instructions for the GST gene fusion system (Amersham Pharmacia Biotech Europe GmbH, Germany).

Determination of kinetic parameters

In order to monitor hydrolysis of antibiotics by CMY β-lactamases, the variation in absorbance resulting from opening of the β-lactam ring was recorded under the following conditions. The antibiotic extinction coefficients for nitrocefin, cefotaxime, ceftazidime, cefoxitin and cephalothin were +15,000, -7500, -9000, -7700 and -6500 $M^{-1}cm^{-1}$, respectively, at wavelengths of 260 nm (for cefotaxime, ceftazidime, cefoxitin and

cephalothin), and 482 nm (nitrocefin). The antibiotics were dissolved in PBS supplemented with 20µg BSA/ml, and the tests were repeated three times at 25ºC. The kinetic parameters for nitrocefin were determined by measuring the initial hydrolysis rates and applying the Hanes-Woolf linearization of the Henri-Michaelis-Menten equation. For the other antibiotics, the *Km* value was measured as *Ki* in a competition experiment, with nitrocefin as the reporter substrate. The k_{cat} values were obtained by monitoring hydrolysis of the antibiotic at a concentration >10 times the *Km.* For ceftazidime, the *Km* value was too high and the k_{cat}/Km ratio was determined on the basis of the first order reaction time hydrolysis. IC_{50} values for avibactam, tazobactam, sulbactam and clavulanic acid were determined according to previous methods, with a ten minutes incubation of enzyme-inhibitor (21).

Stability experiments

Pure protein samples of the three CMY-type β -lactamases were incubated at 50 ºC. The residual activity against nitrocefin was measured at 10 minute intervals, for 40 minutes (20). Experiments were performed in triplicate and the data were calculated as the mean values of three independent assays.

Molecular modelling

The X-ray structure of CMY-2 (PDB code 1ZC2) was used as a template for the modelling of the CMY-54 protein using the homology modelling protocol of the YASARA software (22). Because of the very high similarity with the model, the overall quality Z-score is ranked as optimal (0.09), as well as the dihedrals (1.625) and 1D packing (0.414) Z-scores. The 3D packing Z-score is considered good (-0,660). The figure displaying this structure was obtained using the software program Pymol (The PyMOL Molecular Graphics System, Version 1.7.4.3 Enhanced for Mac OS X, Schrödinger, LLC.).

RESULTS AND DISCUSSION

During a national multicentre survey of plasmid mediated AmpC β-lactamases, the *bla*$_{CMY-2}$ and *bla*$_{CMY-54}$ genes were located in two different strains of *Enterobacteriaceae*: *E. coli* 63024 and *E. coli* 71041 (23). Plasmid characterization of *E. coli* 63024 and *E. coli* 71041 by PFGE (M&M) revealed that the *bla*$_{CMY-54}$ gene is encoded in a plasmid of around 100 kb that hybridized with K and FIB probes. Although a certain degree of variability was detected, in accordance with previously reported data, the genetic structures harbouring the *bla*$_{CMY2}$ and *bla*$_{CMY54}$ genes studied were highly conserved, with the exception of the the 3'end of bla$_{CMY}$ gene (Figure 1) (24,25).

For comparative microbiological analysis, CMY-2 and CMY-54 genes were cloned under isogenic conditions in *E. coli* TG1, and the MICs of a large number of antibiotics were calculated for all bacterial strains (after cloning and transformation) analyzed in this study, including the original clinical bacterial isolates (Table 1). The results revealed slight differences between the isogenic bacterial isolates expressing CMY-type proteins, except for cefotaxime, ceftazidime and aztreonam. The CMY-54 transformants were more resistant to ceftazidime than the CMY-2 transformants, yielding a 4-fold increase in MIC (Table 1) There was also a 8-fold increase in the MIC of cefotaxime in CMY-54 relative to CMY-2.Interestingly, the cefoxitin MIC was two fold higher for CMY-2 than for CMY-54. Microbiological analysis of the efficiency of classical inhibitors showed that although tazobactam and sulbactam exert some inhibitory activity against CMY transformants, relative to the null effect of clavulanic acid, there was no noticeable difference between the two CMY types. However the new β-lactamase inhibitor avibactam is a good inhibitor for CMY-2 β-lactamase. The ratio of MICs between aztreonam, ampicillin and ceftazidime in combination with avibactam were two fold higher in CMY-2 compared to CMY-54 .

190 The kinetic parameters of the purified CMY-2 and CMY-54 β-lactamases were determined
191 for nitrocefin, cephalothin, cefotaxime, ceftazidime and cefoxitin. The results revealed only
192 small differences between the two proteins in relation to nitrocefin, although the catalytic
193 efficiency against cephalotin and cefoxitin was around three times higher in CMY-2 than in
194 CMY-54. The catalytic efficiency against cefotaxime was around two to three times lower
195 for CMY-2 than for CMY-54, and the catalytic efficiency against ceftazidime was much
196 higher (23 times) in CMY-54 than in CMY-2. There is therefore a significant correlation
197 between enzymatic and microbiological data. The kinetic parameters for aztreonam were not
198 calculated because this antibiotic was not hydrolyzed. Aztreonam has been considered to
199 inactivate different class C β-lactamases (26).

200 The IC_{50} values revealed that avibactam is the best inhibitor for CMYs β-lactamases,
201 particulary for CMY-2 (18 nM) a four times better than for CMY-54. This is important,
202 considering than CMY-54 is also a better hidrolyzing enzyme for oximino-cephalosporins.
203 However the classical inhibitors clavulanic acid, sulbactam and tazobactam are poor
204 inhibitors of the both CMY β -lactamases studied in this work.

205 The stability of these CMY-type enzymes was assessed in temperature inactivation studies,
206 which revealed that the CMY-2 protein was significantly more stable than CMY-54. After
207 incubation for 20 minutes at 50ºC, CMY-2 retained 70% of residual activity on nitrocefin
208 compared to complete inactivation of CMY-54 (Figure 2).

209 CMY-54 was found to have a single mutation, relative to CMY-2, $GluLeu^{217-218}$ insertion in
210 the omega loop (Figure 3). Different single mutations have been identified in the omega
211 loop of CMY-type enzymes and are involved in a large increase in the efficacy of hydrolysis
212 against ceftazidime according to kinetic data (27,28). A 3-aa insertion in the omega loop in
213 *Enterobacter cloacae* GC1 AmpC produced an improved enzyme against the same

antibiotic (29,30). Morever, a 3-aa deletion in the R2 loop in CMY-10 also leads to increased catalytic efficiency against ceftazidime (31).

The model of CMY-54 only significantly differs from CMY-2 between P213 and D217 with the disruption of the hydrogen bond between Q215 and K128 and the modification of the hydrophobic cluster in the Q215 and L216 region (Figure 4). The model doesn't predict the loosening observed in the GC1 structure, witch has a 3 amino acid insertion in the Ω-loop compared to P99, leading to a wider active site and increased hydrolysis of ceftazidime and aztreonam similar to CMY-54 (29,30). The important decrease in the CMY-54 stability is however an indication that its Ω-loop, which makes a link between the α and α/β domains must be destabilized and more flexible than in CMY-2 providing more space for the accommodation of the bulky side chain of ceftazidime, cefotaxime and aztreonam like in GC1, CMY-30 (V211G mutation) and CMY-32 (G214E) (27,28).

The reduced inhibition of CMY-54 by avibactam highlights another potential explanation. Indeed, in this case no bulky side chain has to be accommodated and none of the residues displaying different conformation in the CMY-54 model are involved in avibactam binding in the PDC-1:avibactam complex (PDB ID 4HEF) or in the molecular dynamic simulations done with CMY-2 (6,32). Therefore, the lower inhibition of CMY-54 is likely due to a decreased stability of the acyl enzyme that was observed for CMY-2 (6). This effect could result from the increased Ω-loop flexibility that induces a greater overall enzyme plasticity, with a downside lower stability, favorable for the deacylation and recyclization characteristic of avibactam. In a similar way, this increased enzyme flexibility could also contribute to the improved deacylation of ceftazidime, cefotaxime and aztreonam.

In summary, we report here the first detailed kinetic characterization of the CMY-54 enzyme and identify the $GluLeu^{217\text{-}218}$ insertion, located in the omega loop, as important for the hydrolysis of extended spectrum cephalosporins, particularly ceftazidime. We also report a

better inhibition profile of avibactam for CMY-2 β-lactamase than for CMY-54. All together this makes CMY-54 a potentially problematic β-lactamase.

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TRANSPARENCY DECLARATIONS

None to declare

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Table 1. MICs of several antibiotics for CMY-2 and CMY-54 β-lactamases in different bacterial strains.

[a]Clinical isolates harbouring the β–lactamase gene are shown in brackets.
[b]pBGS18- pCTX plasmid expressing the indicated β-lactamases under the control of an external promoter
[c]Ampicillin was tested, when indicated, with fixed concentrations of clavulanic acid, sulbactam, tazobactam and avibactam (4 mg/L).

Antibiotic[c]	*E. coli* TG1 pBGS18-pCTX	*E. coli* 63024 [a] (CMY-2)	*E. coli* 71041[a] (CMY-54)	*E. coli* TG1 pBGS18- pCTX[b] (CMY-2)	*E. coli* TG1 pBGS18- pCTX (CMY-54)
Ampicillin	2	>2056	2056	1028	1028
Ampicillin-clavulanate	2	1028	1028	1028	1028
Ampicillin-sulbactam	2	>2056	256	64	64
Ampicillin-tazobactam	2	512	128	64	64
Ampicillin-avibactam	2	32	16	8	32
Cephalothin	4	512	256	512	512
Cefoxitin	1	64	32	128	64
Ceftazidime	0.12	16	64	64	256
Ceftazidime-avibactam	<0,06	0.25	0.5	<0,06	1
Cefotaxime	0.06	2	16	16	128
Cefepime	<0.12	0.12	0.25	0.12	0.25
Aztreonam	0.12	8	32	16	64
Azteronam-avibactam	<0,06	0,5	2	0,25	2
Imipenem	0.12	0.25	0.25	0.25	0.25

373 **Table 2**. Kinetic data for the pure CMY- 2 and CMY-54 β-lactamases. Data are mean values ± standard deviation (where applicable).

	Kinetic parameters	**CMY-2**	**CMY-54**
NITROCEFIN	*Km(* µM)	17±5	5.2±0.04
	k_{cat}(s^{-1})	609±43	125.5±2.5
	k_{cat} /*Km(* μM^{-1} s^{-1})	**35.8**	**23.85**
CEPHALOTHIN	*Km(* uM)	5.18±1.3	0.58±0.098
	k_{cat}(s^{-1})	591.39±76	19.96±5.54
	k_{cat} /*Km(* μM^{-1} s^{-1})	**114.16**	**34.41**
CEFOTAXIME	*Km(* uM)	2.23±0.32	0.117±0.06
	k_{cat}(s^{-1})	1.99±0.4	0.33±0.043
	k_{cat} /*Km(* μM^{-1} s^{-1})	**0.893**	**2.82**
CEFTAZIDIME	*Km(* uM)	28.546±6.8	>1000
	k_{cat}(s^{-1})	0.00882±0.0019	ND
	k_{cat} /*Km(* μM^{-1} s^{-1})	**$3.08\ 10^{-4}$**	**$6.56\ 10^{-3}$**
CEFOXITIN	*Km(* uM)	0.23±0.021	0.0606±0.0378
	k_{cat}(s^{-1})	0.4049±0.083	0.036±0.004
	k_{cat} /*Km(* μM^{-1} s^{-1})	**1.76**	**0.59**
CLAVULANIC ACID	IC_{50} (µM)	40.5 ± 11,9	158 ± 95
SULBACTAM	IC_{50} (µM)	5.91 ± 1.94	0.848 ± 0.265
TAZOBACTAM	IC_{50} (µM)	1.53 ± 0.46	0.304 ± 0.032
AVIBACTAM	IC_{50} (µM)	0.018 ± 0.001	0.075 ± 0.015

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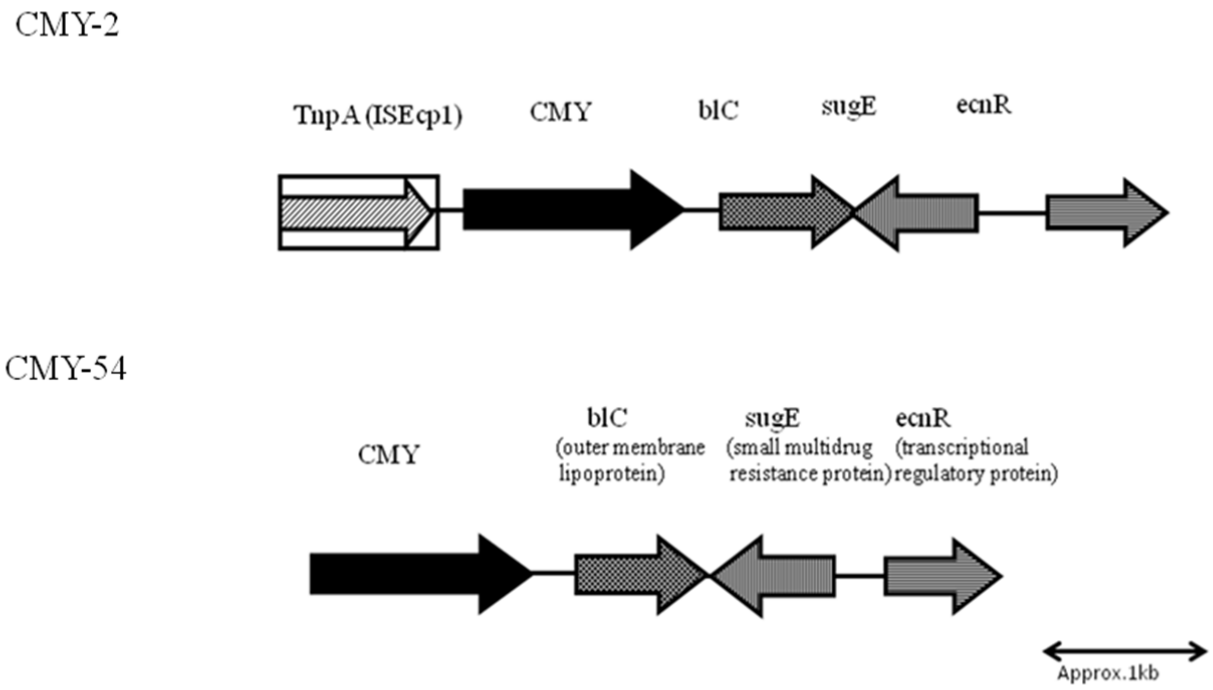

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379 **Figure 1**. Representation of the genetic context of *bla*$_{\text{CMY-54}}$ and *bla*$_{\text{CMY-2}}$ structures detected in the same multicentre study.

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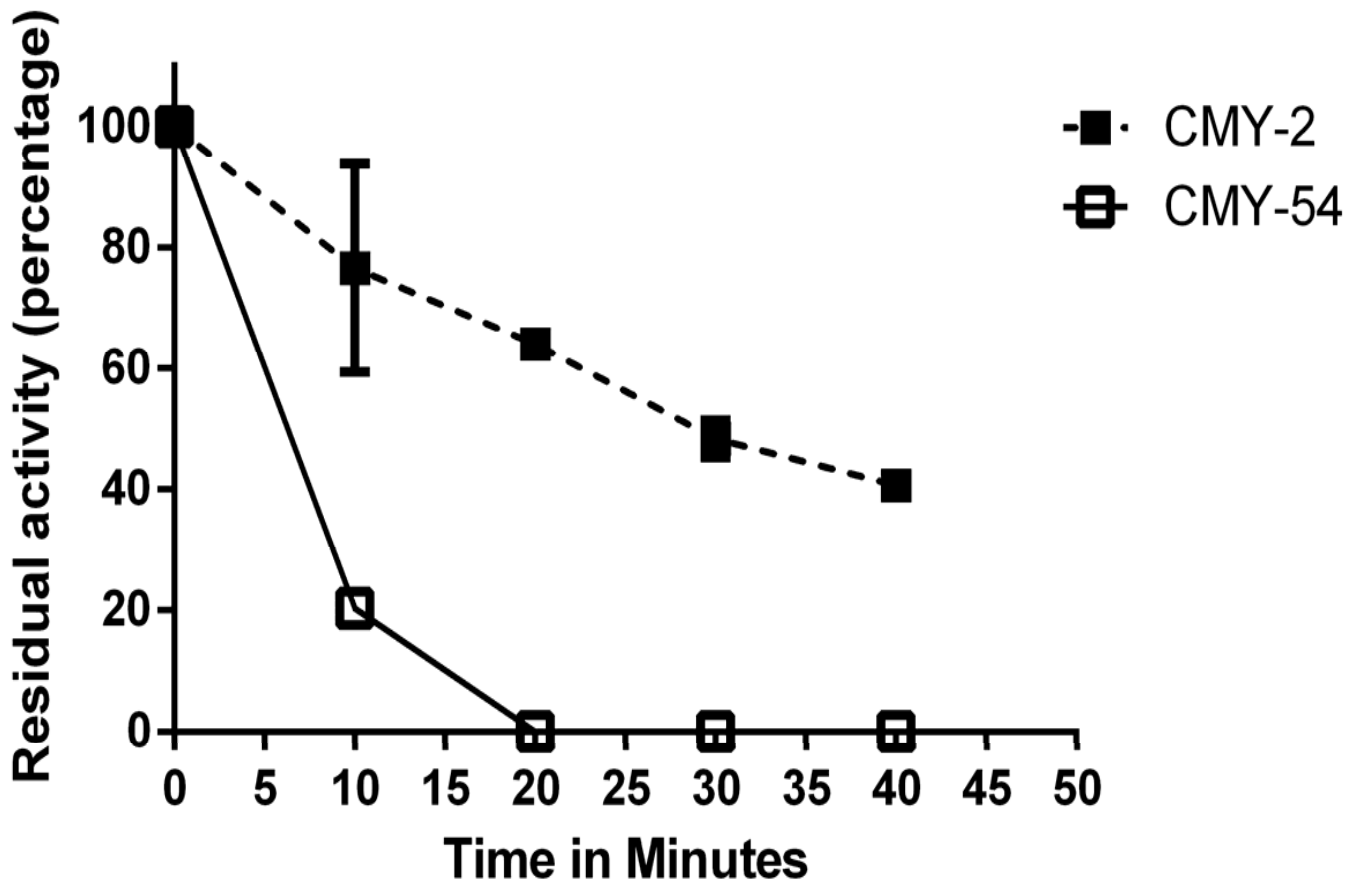


Figure 2. Percentage of residual activity of CMY-type enzymes after denaturation experiments at 50ºC.

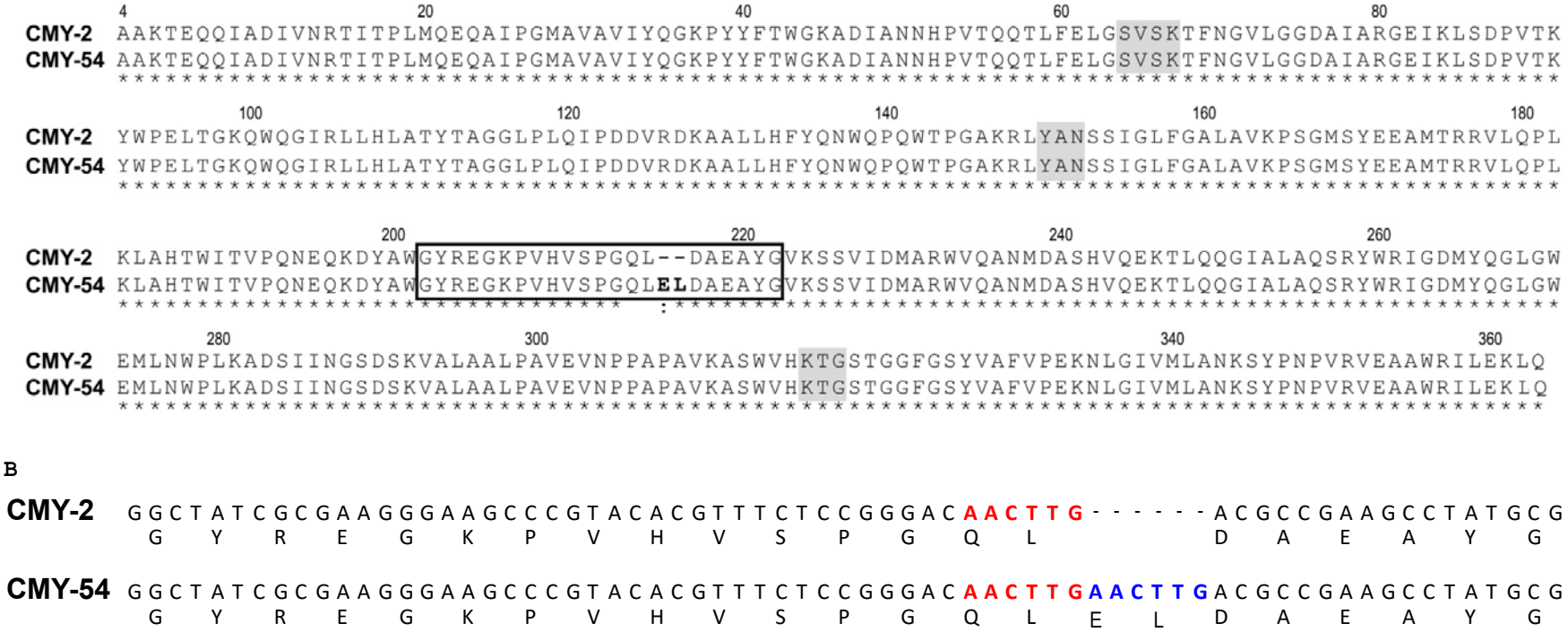
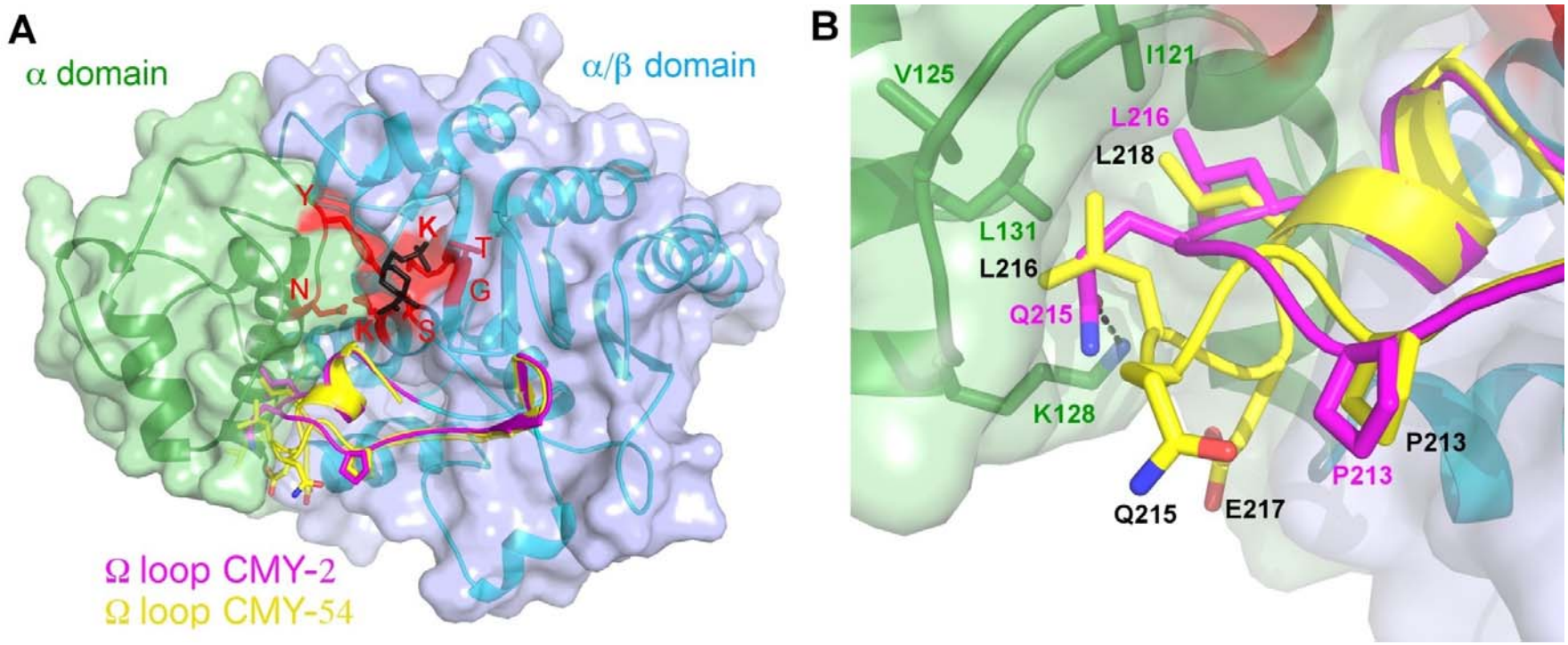


Figure 3. A. Amino acid sequence alignment of the CMY β-lactamase family (CMY-2, X91840; CMY-54, HM544039). Asterisks indicate strictly conserved amino acids. The GluLeu$^{217\text{-}218}$ insertion is displayed in bold. The three classical conserved motifs are highlighted in grey. The alignment was performed with the CLUSTAL W program of the EMBL-EBL (33)
B. DNA sequence alignment of the Ω-loop region boxed in panel A with the 6 base repeat highlighted in red and blue.



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412 **Figure 4**. A. Representation of the CMY-2 structure. The α/β and α domains are shown as cartoon and surface in cyan and green respectively,
413 amino acids of the 3 conserved motifs are displayed as red sticks. The CMY-2 Ω loop is shown in magenta and the Ω loop of the superposed
414 CMY-54 model in yellow. An avibactam molecule from the superposed PDC-1:avibactam complex is shown as black sticks. B. Close up view of
415 the differences between the CMY-2 and CMY-54 Ω loops with the same color code. The hydrogen bond between Q215 and K128 in CMY-2
416 appears as a black dash line.

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