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Insights into substrate recognition and export tunnel preferences in the efflux transporter AcrB

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Abstract

In *Escherichia coli* AcrB is a major multidrug exporter, which confers the bacterium resistance to many antibiotics with diverse structural and chemical properties. Studies have identified three possible tunnels (or channels) within AcrB that different substrates use before reaching the distal pocket, from which they are subsequently extruded. Recently, we reported that mutations in the AcrB gate loop may affect the conformational change kinetics involved in substrate export rather than directly affecting molecular interactions with this loop, and we highlighted the distinct export tunnel preferences between erythromycin and doxorubicin. To further understand the gate loop's role in AcrB's export activity and the rationale behind substrate preferences among the three possible export tunnels, namely, tunnel 1, 2, and 3, we investigated the structural and functional effects of several single and multiple mutations in the gate loop of AcrB. Our findings indicate that all three tunnels are energetically favourable for the substrates studied, with the majority forming more hydrogen bonds in any tunnel compared to the distal pocket. Moreover, our experimental and computational data revealed that some substrates with high molecular similarity exhibited different export tunnel preferences, as strongly suggested by their MIC values. To explain this unexpected outcome, we propose a generalised explanation that the conformational change kinetics in AcrB is substrate dependent.

Keywords: Bacterial efflux pumps - AcrB-substrate interactions - Conformational change kinetics - Substrate recognition - X-ray crystallography

Statement: This study provides new insights into the importance of molecular dynamics in AcrB's drug export activity. By elucidating the effects of specific mutations on the export of several antibiotics, we highlight the significance of conformational change kinetics in AcrB protomers. Our findings enhance the understanding of AcrB's multidrug efflux mechanism, potentially paving the way for new strategies to develop AcrB inhibitors.

1. Introduction

AcrB is the primary efflux transporter in *Escherichia coli*. It is a homotrimeric integral membrane protein with an exceptionally broad substrate specificity, capable of exporting structurally diverse and unrelated compounds such as antibiotics, detergents, dyes, organic solvents, and biofuels (Ma et al., 1996; White et al., 1997; Tsukagoshi and Aono, 2000; Yu et al., 2003; Nikaido, 2009; Nikaido and Takatsuka, 2009; Foo and Leong, 2013). As a component of the tripartite efflux pump AcrAB-TolC, along with its many homologues, it is constitutively expressed in many pathogenic Gram-negative bacteria (Johnson et al., 2020; Li et al., 2020). Over the past two decades, our understanding of AcrB's molecular export mechanism has steadily advanced, since early crystal structures determination of its resting and active states (Thanassi et al., 1997; Murakami et al., 2006; Seeger et al., 2006; Nikaido, 2011). The functional rotating mechanism was proposed to explain how AcrB exported its substrates, based on three distinct conformation of each of its protomers, namely, access/loose, binding/tight, and extrusion/open states (Murakami et al., 2006; Seeger et al., 2006). The reporting of few crystal structures of AcrB with few substrates, such as Minocycline (MIY), Erythromycin (ERY), and Doxorubicin (DOX), revealed further details on the functional rotating mechanism, linking the two tunnels (or channels), namely, tunnel-1 (located next to the vestibule at each protomer) and tunnel-2 (access or proximal pocket), to the deep binding pocket (distal pocket), along with highlighting the importance of the gate loop, in tunnel-2, separating the proximal and distal pockets (Murakami et al., 2006; Nakashima et al., 2011; Eicher et al., 2012). This gate loop is highly conserved across many RND efflux transporters from various bacteria (**Figure 1A**). However, in some members, such as AcrD, AmrB, or SmeB, as well as others not shown, the lack of conservation at positions 615 and 617, and possibly 620, may suggest differences in the gate loop's role in their export activity (e.g. substrate specificity, export rate, etc). Further details on this gate loop can be found in our previous study (Ababou, 2018).

Further AcrB's studies, inspired from previous reports (Husain and Nikaido, 2010; Husain et al., 2011), have shown the existence of a third tunnel, tunnel-3, which is open to the central cavity within the AcrB trimer, and connected directly to the distal pocket without passing by the gate loop (Nakashima et al., 2011; Zwama et al., 2018). These tunnels have been shown, to some extent, to have some specificity for AcrB substrates suggesting that substrates with similar molecular properties should be exported through the same export tunnels. Importantly, mutations that are far from the substrate's preferred tunnels are unlikely to impact the drug susceptibility assay for that substrate.

However, several substrates have been reported to have conflicting tunnels preferences for their export route, such as Benzalkonium (BZK) and Rhodamin 6G (R6G) that were reported to have preference to tunnel-3 (Zwama et al., 2018), while other reports suggesting tunnel-2 as their preferred tunnel (Cha et al., 2014; Muller et

al., 2017). Structural and functional investigation of AcrB in the presence of Fusidic acid (FSA) resulted in the suggestion of a new tunnel located between the transmembrane helices TM1 and TM2, which may be putatively specific for FSA and carboxylated drugs in general (Oswald et al., 2016; Tam et al., 2020). Subsequently, it has been reported, by the same group, that FSA to be shown in the tunnel-1 in AcrB-FSA complex structure, suggesting its export route may also include preference to tunnel-1 (Tam et al., 2021). Several reports have shown that ERY prefers tunnel-2 as part of its export route (Bohnert et al., 2008; Wehmeier et al., 2009; Nakashima et al., 2011; Eicher et al., 2012; Ababou and Koronakis, 2016). AcrB mutation E673G increased ERY resistance 2 fold, while structurally no contact is detected between E673 and ERY (Kobayashi et al., 2014). While Q569R mutation has no effect on the resistance of ERY, as Q569 is located at the entrance to the proximal pocket (shortest contact ~ 10.0 Å), the double mutant Q569R-E673G showed also no change in ERY resistance (Kobayashi et al., 2014). AcrB double mutant I626R-E673G which decreased ERY resistance 8 fold while I626R alone decreased ERY resistance 4 fold (Kobayashi et al., 2014). AcrB quadruple mutant A33W-T37W-A100W-N298W, part of tunnel-3, resulted in 4 fold increase susceptibility for ERY (Zwama et al., 2018). Recently, AcrB R717Q and R717L mutations reported to decrease macrolide and fluoroquinolone susceptibility but increased the susceptibility for Novobiocin and Cloxacillin (Zwama and Nishino, 2022). These experimental observations clearly suggest that our understanding of substrate's preference export route in AcrB is still incomplete, and possibly still superficial. Beyond the obvious experimental difficulties and the complexity to investigate such large efflux transporter, the major contributors to such incomplete understanding of AcrB export activity at the molecular level are due to:

- (1) Lack of solved AcrB-substrate complex structures
- (2) Lack of discovery of intermediate structure forms of these complexes
- (3) Lack of experimental data on the molecular dynamics of these complexes
- (4) Lack of molecular kinetics aspects of these complexes (i.e. substrate binding, protomer conformational changes).

To better understand AcrB's substrate export tunnel preferences, we conducted structural and functional studies on various AcrB gate loop mutants, including single mutants (F615A, F617A, R620A, R620K, R620E), double mutants (F615A-F617A, F615A-R620A, F617A-R620A), a triple mutant (F615A-F617A-R620A), and a deletion mutant (F615G- Δ [G616-R620]). Using several antibiotics, well-documented in the literature as AcrB substrates, we demonstrate that (1) most AcrB substrates are primarily directed toward the export tunnels through hydrogen bond formation; (2) most of these substrates form significantly fewer hydrogen bonds within the distal pocket, facilitating their transport towards extrusion; and (3) substrates with high molecular similarity can exhibit substantial differences in drug susceptibility assays, ranging from a significant effect to no effect at all from the gate loop mutation. To explain these seemingly contradictory outcomes, we propose that substrate-

dependent conformational change kinetics, may generally explain how AcrB's export activity occurs.

2. Results

2.1. Structures of the asymmetric trimers of AcrB mutants

Although we have co-crystallised all the gate loop mutants with several antibiotics, the X-ray diffraction data resulted in electron density maps that either have weak or no electron density to be assigned for those antibiotics within their reported binding sites or elsewhere in the structure (Nakashima et al., 2011; Eicher et al., 2012). Furthermore, the high similarity of the refined structures of R620A (RA), R620K (RK), and F615A-F617A (AA) mutants, in P2₁ space group, with the structure of AcrB (Ababou and Koronakis, 2016), and the limited structural information of these structures, we did not pursue further the structures of the other mutants. Indeed, comparison of RA, RK, and AA mutants' structures with our previously reported structure of AcrB (Ababou and Koronakis, 2016), shows that overall, these mutants structures have high structural similarities as reflected by their low RMSD's (between 0.60 and 1.42 Å) (**Figure 1** and **Table S2**). However, slight structural differences may occur around the mutation positions, as shown in **Figure 1D** and **1E**, where the highest changes was 3.8 Å between C α of F617 in AcrB and A617 in AA. Detailed data collection and structures statistics are summarized in **Table S1**. Note for the refined structures one detergent molecule (Dodecyl- β -D-maltoside, DDM) per monomer was found in the mutants' structures (**Figure 1F**), as previously reported (Ababou, 2018). However, we omitted the metal Ni in our refinement, even though the electron densities are clearly showing the presence of such metal (**Figure S1**), as previously reported and discussed in detail (Ababou, 2018).

2.2. Gate loop mutations effect on the solution fold and stability of AcrB

Prior to perform any functional assay, we have checked whether the mutations affect the fold and stability of our mutants in solution. Consequently, we have used CD spectroscopy to collect the far-UV CD spectra and the thermal unfolding data of AcrB and the mutants. The far-UV CD spectra of AcrB and mutants show no substantial differences (**Figure S2**), suggesting that the mutants kept conserved secondary structures content and most probably overall similar structures to the WT in solution, as reported by our crystal structures of some of these mutants (**Figure 1**). The thermal denaturation results of the gate loop mutants revealed small shift in their melting curves as compared to the WT (**Figure 1G**). A minor stability of the WT over the mutants is observed as shown by the T_m difference of less than 3.5 °C, except for the mutant AA the T_m was 4.1 °C (**Table S3**). Such relatively small difference in T_m suggests an overall similar stability between the WT and the mutants, and in particular at physiological temperature (**Figure 1G**). Hence, these results demonstrate that AcrB's gate loop mutations do not affect the fold and the overall

stability of AcrB in solution, as previously reported (Ababou and Koronakis, 2016; Ababou, 2018).

2.3. Antibiotics susceptibilities of AcrB and mutants

Previously, we have shown the involvement of the gate loop in two mutants, AAA and Δ Loop, and two antibiotics ERY and DOX. To further investigate the importance of gate loop in the export activity of AcrB, in this work we have performed drug susceptibility assay for nine new mutants of the gate loop, along with F615A-F617A-R620A (AAA) and F615G- Δ (G616-R620) (Δ Loop), using twenty antibiotics (**Figure S3**). In the absence of antibiotics, except for cells selection using kanamycin, the cell growth of AcrB and all mutants were similar (**Figure S4**), indicating the absence of any effect of these mutations on either the growth or survival of the cells. **Table S4** shows the effect of the *acrB* deficient *E. coli* (Δ *acrB*) and AcrB's single or combined mutations on the minimum inhibitory concentration (MIC) values for the 20 antibiotics. The MIC values of 12 and 26 μ g/mL for Rifampicin (RIF) and Carbonyl cyanide 3-chlorophenylhydrazone (CCP), respectively, were constant for Δ *acrB*, AcrB, and mutants (**Table S4**). Clearly, our results show that these antibiotics are not substrates of AcrB, as reported for RIF (Ohene-Agyei et al., 2014). In fact, similar MIC values of RIF ranging from 8 to 16 μ g/mL were reported (Schuster et al., 2016; Zwama et al., 2018); however, whether RIF is a substrate of AcrB is still unclear, in particular when the structure of AcrB binding to RIF was reported (Nakashima et al., 2011). CCP is rather well known to block efflux pumps by collapsing the proton motive force that energises them (Mallea et al., 1998; Nikaido and Pages, 2012). Although Phosphomycin (PPM) is not known to be a substrate of AcrB, we found a small but reproducible MIC ratio of AcrB to Δ *acrB* of 2. Consequently, RIF and CCP will not be included in any further investigations in this work.

For better analysis of the mutations' effect on AcrB's MIC for the antibiotics, we have calculated the MIC ratio of AcrB to the mutant (R), as shown in **Table 1**. For 6 antibiotics, DOX, Clindamycin (CLD), Tetracycline (TRC), Acriflavine (ACR), Nalidixic acid (NLD), and PPM, all the mutations have no effect on the susceptibility to these antibiotics ($R = 1$), while for Spiramycin (SPR) and Ciprofloxacin (CIP) the R value was also 1, except for only two mutants was 2, R620E (RE) and Δ Loop, and R620D (RD) and RE, respectively. For 7 antibiotics, MIY, R6G, Moxifloxacin (MXF), Ofloxacin (OFL), Lomefloxacin (LMF), BZK, and PPM, all the mutations have increased susceptibility to these antibiotics ($2 < R < 16$). The highest susceptibility increase was for MIY with $R = 16$ for Δ Loop and $R = 8$ for the rest of the mutants. The lowest susceptibility increase was for OFL at $R = 2$ for all the mutants. For ERY and Novobiocin (NOV), all the mutations have increased susceptibility to these antibiotics ($2 < R < 8$), except of RA and RK where no susceptibility change was occurred ($R = 1$). For FSA, the mutants AA, AAA, and Δ Loop have increased susceptibility ($R = 8$) and no change for the rest of the mutants ($R = 1$). Instead for

Chloramphenicol (CLP), all R620 single mutants and Δ Loop have $R = 2$ or 4 , and the rest of mutants have no change in their susceptibility ($R = 1$). These results show that AcrB's gate loop is directly involved in the export of nearly 8 antibiotics and potentially not at all for the other 10 antibiotics.

2.4. Prediction and analysis of antibiotics binding to AcrB and mutants

We performed structure prediction calculations for the binding of the antibiotics to AcrB and mutants to the proximal (i.e. tunnel-2) and distal pockets, and the tunnel-1 and tunnel-3 sites, using molecular docking with their access and binding protomers (**Figure 2A**). All the antibiotics were docked with free rotation at all their rotatable bonds for best estimation of their binding mode within each binding site (**Figure 2B**, **Figure 3A**, **Figure S5**, **Figure S6**). The estimated binding free energies for the antibiotics in either proximal pocket or distal pocket are reported in **Table S5** and **Table S6**, and their energies difference are shown in **Table S7**. Clearly, antibiotics binding to the distal pocket is favoured than the proximal pocket, in almost all cases, with the exceptions of CLD and SPR with relatively small differences, for 4 and 1 mutants, respectively (**Table S7**). Similarly, the binding to the distal pocket in AcrB is favoured than either tunnel-1 or tunnel-3 for all antibiotics (**Table S8**). However, while comparison between the proximal pocket and tunnel-1 or -3 shows most of antibiotics slightly favoured the proximal pocket than the tunnels, ACR and NLD favours the tunnels instead. Comparison between the tunnels clearly shows that most of the antibiotics slightly favoured tunnel-1 over tunnel-3 (**Table S8**). Interestingly, while our docking for the distal pocket included all the area between the domains PC1 and PC2 (**Figure 2A**), all the antibiotics revealed an overall well localised binding site in this pocket as represented by a small antibiotic NLD (29 atoms), except for the smallest one PPM (15 atoms), as shown in **Figure 3B** (See also **Figure S7** and **S8**). This exception maybe due to the high number of hydrogen bonds occurred for PPM binding site when compared to most of other antibiotics (**Table S10**). Importantly, the estimated binding free energy for all the antibiotics binding to proximal pocket, distal pocket, tunnel-1, and tunnel-3 are favourable (**Table S5**, **Table S6**, **Table S8**).

To assess further the difference in the binding of these antibiotics, we inspected the number of hydrogen bonds and atomic contacts between the antibiotics and AcrB and mutants (**Table 2-4**, **Table S9-13**). Although the distal pocket was energetically favoured than the proximal pocket, the number of antibiotics with higher number of hydrogen bonds in the proximal pocket was greater than those in the distal pocket in AcrB and mutants with a ratio between 2 and 5 (**Table 2**). The same applies to the tunnel-1 and -3 in AcrB (**Table 4A**), while energetically the distal pocket is favoured than either of the tunnels (**Table S8**). Note that for few antibiotics the number of hydrogen bonds was equal in the proximal and distal pockets. However, close inspection of the number of atomic contacts reveals, in AcrB and most of the mutants, higher number of antibiotics with higher number of atomic contacts in distal

pocket than in the proximal pocket (**Table 3**). Similar trend is observed for tunnel-1 and 3-, as most of antibiotics have higher number of contacts in distal pocket (**Table 4B**).

2.5. Antibiotics' molecular characteristics versus MIC ratio analysis

To assess the presence of any correlation between the antibiotics' MIC ratio (R) for the mutants with their chemical and structural proprieties and ultimately with the antibiotics export route preferences, namely, tunnel-1, -2, or -3, we have performed PCA analysis using the antibiotics MIC ratio, molecular mass, geometry and chemical descriptors, and functional groups. To be as exhaustive as possible we have used 33 parameters to describe each antibiotic, excluding MIC ratio (**Table S14-S16**). PCA analysis shows some putative clustering of some antibiotics, such as CLD, MXF, OFL, LMF, CIP, NLD, and ACR; however, close inspection reveals that their MIC ratio are different (R of 1, 2 or 4, **Figure 4, Figure S9A-C**). Furthermore, some antibiotics with R = 1 are far away from this cluster (ex: SPR or DOX). This shows that the MIC ratio may not have any direct or simple correlation with the antibiotic molecular characteristics, as identified by these 33 parameters. Nevertheless, we have searched for the minimum parameters that could reproduce similar PCA analysis outcomes. We found that 5 parameters reproduce similar clustering and/or trend of the PCA results using all 33 parameters, for F617A, RA, RK, RD, and RE, and with an overall pseudo-rotation of 180 degrees of the original plot for the other mutants. Those parameters were the topological polar surface area (TPSA), LogP, the number of rotatable bonds (Rotatables), the number of hydrogen bond acceptors (HB Acceptor), and the number of hydrogen bond donors (HB Donor) (**Figure 4**).

Although nearly 8 antibiotics have their R equal to 1, our expectation using PCA analysis was to identify the overall common molecular characteristics that may explain such R value. The R value of 1 means that the gate loop has no effect on the antibiotic export and possibly the antibiotic prefers tunnel-1 and/or tunnel-3 as its preferred export tunnel(s) instead of tunnel-2, where the gate loop is located. Since PCA analysis failed in that context, we have performed molecular similarity calculations of these antibiotics, as summarised in **Table 5**. Surprisingly, TRC and MIY are highly similar, with a value of 0.86, and yet R values of these antibiotics are 1 and 8 (or 16), respectively. Similarly, CIP has an R = 1 (excluding mutants RD and RE) and has high molecular similarity to MXF, LMF, and OFL, values between 0.77 and 0.89, but their R values are between 2 and 4. Clearly, molecular similarity for these antibiotics cannot explain the substantial difference in their R values.

3. Discussion

Over the past two decades, a substantial amount of structural and functional information on AcrB export activity has been reported. This has led to widely

accepted insights and hypotheses regarding the resting state and active state structures (Murakami et al., 2002; Murakami et al., 2006; Seeger et al., 2006; Nikaido, 2011), the functional rotation mechanism of AcrB (Murakami et al., 2006; Nakashima et al., 2011; Eicher et al., 2012), and the three possible export tunnels for substrates, known as channel or tunnel-1, -2 and -3 (Nakashima et al., 2011; Yamaguchi et al., 2015; Zwama et al., 2018). However, the driving forces behind its substrates' specificity and export tunnels preferences remain to be fully understood. Consequently, in this work, we used several gate-loop mutants of AcrB to further investigate their effect on AcrB's structure and drug susceptibility, as well as to elucidate the export tunnel preferences of several antibiotics.

Our recent crystal structures of AcrB and mutants AAA and Δ Loop, and the present mutants RA, RK, and AA, clearly show that the overall structures of the gate-loop mutants are largely similar to the wild type AcrB (Ababou and Koronakis, 2016; Ababou, 2018). We anticipate that this similarity extends to all other mutants, as they involve either single or double mutations and exhibit a comparable fold and stability, as confirmed by their CD spectra and thermal unfolding data (**Figure S2, Figure 1F, Table S3**). However, drug susceptibility assays for several antibiotics revealed varying effects between AcrB and these mutants, ranging from significant differences to no effect, as indicated by their MIC values (**Table 1, Table S4**).

To address the lack of complex structures of AcrB with its substrates, we used molecular modelling to investigate the molecular interactions between AcrB, and its mutants, and its substrates used in this work. Binding of all antibiotics to proximal pocket, distal pocket, tunnel-1, and tunnel-3 were energetically favourable (**Table S5-S8**). Almost all the substrates were energetically favoured in the distal pocket compared to the proximal and the other tunnels (**Table S7, Table S8**). This outcome is expected, as substrates must pass through the distal pocket to be extruded, despite differences in their molecular and chemical properties. Notably, while this trend correlates with the higher number of contacts in the distal pocket compared to the proximal pocket and other tunnels (**Table 3, Table 4B, Table S11-13**), the opposite was observed when comparing the number of hydrogen bonds (**Table 2, Table 4A, Table S9-S10**). This suggests that hydrogen bond formation is a key driving force in directing the substrates toward specific tunnels; however, for the substrates like BZK, which lack the ability to form hydrogen bonds, other driving forces are likely at play.

Assuming the MIC ratio, R, of 1 means that the substrate export does not involve tunnel-2, where the gate loop is localised, then the substrate must have preference to tunnel-1 or -3. Interestingly, based solely on the number of hydrogen bonds in tunnel-1 and -3 compared to proximal pocket, a clear preference trend emerges for such substrates. For example, DOX and TRC exhibit a higher hydrogen bonds number in tunnel-1 and -3 compared to the proximal pocket (**Table 4 and Table S9**), with higher

number in tunnel-1 compared to tunnel-3. This suggests that these substrates may favour tunnel-1 over tunnel-3, as previously proposed for DOX (Ababou, 2018). However, this trend does not hold for CLD, ACR, NLD, PPM, SPR (excluding R=2 with RE and Δ Loop), and CIP (excluding R=2 with RD and RE), as these antibiotics have fewer or similar number of hydrogen bonds in tunnels-1 and/or tunnel-3 compared to the proximal pocket. One possible explanation for this could be related to their size or molecular weight, as previously suggested in studies on the involvement of the gate loop in AcrB (Nakashima et al., 2011; Cha et al., 2014). Specifically, ACR (32 atoms), NLD (29 atoms), CLD (60 atoms), PPM (15 atoms), and CIP (42 atoms) might still prefer tunnel 2 without the involvement of the gate loop, or they may have no particular preference among the tunnels. However, this explanation does not apply to SPR (135 atoms), the largest antibiotic used in this study. Therefore, both explanations have limitations.

To better analyse the relationship between R value and these substrates' molecular and chemical properties, along with their functional groups, we conducted a PCA analysis (**Figure 4**). Surprisingly, no clear clustering of substrates with R value of 1 was observed. Interestingly, the PCA analysis using 5 parameters (TPSA, LogP, Rotatables, HB Acceptor, and HB Donnor), revealed that hydrophobicity, polar surface, flexibility, and hydrogen bonds formation are the most significant molecular and chemical properties for these substrates (**Figure 4, Figure S9A-C, Table S14-S15**). To further investigate and identify any common molecular or chemical properties among these substrates, we performed molecular similarity calculations, (**Table 5**). Using a similarity cutoff of 0.4 and above, we found ACR, NLD and CIP have molecular similarities values between 0.41 and 0.58. This may provide a promising explanation for why these substrates share an R of 1. However, DOX, CLD, TRC and SPR have low molecular similarities values (0.14-0.19), indicating that substrates with very low molecular similarity may still have similar export tunnel preferences. On the other hand, NLD shows substantial molecular similarity with MXF, LMF, and OFL (~ 0.5 -0.6), yet the latter have $R \geq 2$, indicating a preference for tunnel-2. Interestingly, CIP has $R=1$ (ignoring R of 2 with RD and RE) with high molecular similarity to MXF, LMF, OFL (~ 0.8 -0.9), yet the latter have $R \geq 2$. Strikingly, MIC values of MIY and TRC for AcrB were 3.7 μ g/mL and 1.8 μ g/mL, respectively (**Table S4**) - a small difference - but despite their high molecular similarity (~ 0.86), MIY has $R \geq 8$, indicating that MIY favours tunnel 2.

It is important to note that, in principle, substrates with high molecular similarities are likely to have similar preferences for their export tunnels, as reported by others (Murakami et al., 2006; Husain and Nikaido, 2010; Husain et al., 2011; Nakashima et al., 2011; Eicher et al., 2012; Oswald et al., 2016; Zwama et al., 2018; Tam et al., 2020). However, our data contradict, somehow, this hypothesis, as observed for TRC and MIY, for example, when their only molecular difference is the exchange of a H atom, -OH group, and -CH₃ group in TRC by -N(CH₃)₂ group, H atom, and H atom in MIY, respectively (**Figure S10**). This limited molecular change did not affect

dramatically either the molecular or the chemical proprieties of these two substrates (**Table S14-S16**). Therefore, while a change in tunnel preference for TRC is plausible - given the higher number of hydrogen bonds in tunnels 1 and 3 compared to the proximal pocket - it is difficult to dismiss the possibility that the MIC values may not accurately reflect TRC's true export tunnel preference. For clarity, we cannot eliminate the possibility of TRC to be exported through tunnel-2 like MIY, passing by the gate loop, and yet no detectable effect of the gate loop mutations on the MIC values of TRC.

Recently, it was reported that BZK, R6G, and ACR have preference to tunnel-3. The rationale for this was the planar aromatic cation nature of these substrates (Zwama et al., 2018). While this may be in line with our inferences about ACR, our results for BZK and R6G contradict that, since these substrates have R values of 2-8 and 2-16, respectively (**Table 2**). Note that for R6G other reports agree with our results (Cha et al., 2014; Muller et al., 2017). In addition, FSA has R values of 1 for all mutants except AA, AAA and Δ Loop with values of 8 (**Table 1**). Clearly, this suggests that FSA export tunnel is not unique but rather can be either tunnel-1, based on number of hydrogen bonds compared to proximal pocket (**Table 4, Table S9**), or tunnel-2. Recently, it was reported that FSA prefers an export tunnel at the groove between transmembrane helices TM1 and TM2 (Tam et al., 2020). However, recent experimental data, from the same group, clearly suggest that FSA can also use tunnel-1 (Tam et al., 2021), which is in line with our present results.

Mutations in AcrB located in different tunnels or putative grooves can indeed help highlight the preferred export tunnels for various substrates, but this should not be considered a rule for determining their preferences. In fact, it is challenging to reconcile conflicting data that show substrates entering different tunnels, especially when they are the same (e.g., FSA, DOX) (Eicher et al., 2012; Ababou, 2018; Tam et al., 2020; Tam et al., 2021) or highly similar to each other, as in our case of TRC and MIY. There are two main plausible explanations for this discrepancy.

The first explanation suggests that AcrB substrates may be promiscuous in their choice of export tunnel, based on accessibility and/or opportunity, and possibly in concentration dependant manner. Indeed, several studies have shown that substrates like BZK, ERY, DOX, R6G, FSA, and NOV can enter different tunnels (Nakashima et al., 2011; Eicher et al., 2012; Zwama et al., 2018; Tam et al., 2021). Furthermore, our computational analysis of AcrB-substrate interactions revealed that all these substrates were energetically favourable in all three tunnels and were more energetically favoured in the distal pocket as compared to all other tunnels entrances (**Table S5-S8**). Except for BZK, the antibiotics generally formed hydrogen bonds in all tunnels, suggesting that these substrates can be directed to any of AcrB's tunnels and become transiently stabilised prior to further movement towards the distal pocket, where the number of hydrogen bonds decreases (**Table 4, Table S9-10**).

The second explanation considers the implication of the molecular dynamics AcrB, because for any substrate export, there are large conformational changes between the AcrB's protomers (Access/Loose (L), Tight/Binding (T), and Extrusion/Open (O)). Strong evidence for this, will be the number of mutations reported to affect substantially the MIC values of several substrates, when structurally the mutations and the substrates have no contact. We reported the X-ray structures of the resting and active states of AAA and Δ Loop mutants to be identical to AcrB, but their MIC values were largely different for ERY. We demonstrated that the main difference between these mutants and AcrB, leading to different export activity of ERY, was in their molecular dynamics behaviour, as shown by our detailed MD simulation investigation (Ababou and Koronakis, 2016; Ababou, 2018). Mutation E673G was reported to increase ERY resistance 2-fold, while structurally no contact is detected between E673 and ERY. While Q569R mutation has no effect on the resistance of ERY, as Q569 is located at the entrance to the proximal pocket (shortest contact is ~ 10.0 Å), the double mutant Q569R-E673G also showed no change in ERY resistance, which cancelled the effect of E673G (Kobayashi et al., 2014). Mutations V127A and Y49S increased ERY susceptibility by 2 to 4-fold, when the shortest distance between V127 or Y49 and ERY is ~ 16.3 Å for both residues, and also D153E, which is located outside the export tunnel-2 (shortest contact is ~ 24.0 Å), increased ERY susceptibility by 2 to 4-fold (Soparkar et al., 2015). AcrB quadruple mutant A33W-T37W-A100W-N298W, part of tunnel-3, resulted in 4-fold increase susceptibility for ERY, which prefers most exclusively tunnel-2 (Zwama et al., 2018). In addition, recently R717Q and R717L mutations reported to decrease macrolide, and fluoroquinolone, susceptibility but increased the susceptibility for Novobiocin and Cloxacillin (Zwama and Nishino, 2022). Several mutations were reported to improve AcrB efflux rate of biofuels (Foo and Leong, 2013). Mutation Q737L significantly increased the export rate of α -pinene by 400% and for n-octane by 51%, while Q737 is in the docking domain at ~ 47 Å from F617 in the gate loop (C β -C β distance). Furthermore, mutation M987T decreased the export rate of α -pinene and n-octane by $\sim 25\%$, while M987 is in the membrane domain at ~ 41 Å from F617 (Foo and Leong, 2013). If the mutations described above, without direct contact with the substrate, can lead to an increase or decrease in drug susceptibility and/or efflux rate, then the most plausible explanation is a change in the molecular dynamics of AcrB. Thus, these mutations likely affect the conformational change kinetics of AcrB's protomers (slow, intermediate, or fast), which in some cases can be reflected in MIC values, as we have previously reported (Ababou, 2018). Indeed, we have suggested that AcrB mutations, AAA and Δ Loop, may have affected the conformational change kinetics to explain the discrepancy between our structural and functional data, as inferred from the difference in molecular dynamics between AcrB and its mutants (Ababou, 2018). This means, the interpretation of MIC values can be correlated to the time required to reach and populate each conformation adopted by the protomers (i.e. AcrB/fast, AAA/medium, Δ Loop/slow). For clarity, this is not about export rate/velocity but the rate of conformational change between L, T, and O conformations; however, the

overall speed at which AcrB exports its substrates can be in part proportional to its conformational change kinetics, but undoubtedly other molecular and environmental factors can affect substantially the export velocity beyond AcrB's conformational change kinetics.

All reported AcrB structures determination, using X-ray crystallography, present only two conformational states, namely, the symmetric trimer, known as the resting state with all protomers adopting the same conformation (LLL) (Murakami et al., 2002; Das et al., 2007; Veesler et al., 2008), and the asymmetric trimer, known as the active state with each protomer in different conformation (LTO) (Murakami et al., 2006; Seeger et al., 2006; Nakashima et al., 2011). Also, it was not easy to observe experimentally any intermediate structural forms of any other RND efflux transporter beyond their LLL and LTO conformational states. However, recently several Cryo-EM structures of RND efflux transporters reported intermediate conformational states of their trimer complex. CmeB from *Campylobacter jejuni*, has been reported in two forms in which all the protomers adopt O conformation (OOO) and another form with one protomer adopted an L like conformation (L*), called resting conformation, (L*OO) (Su et al., 2017). AdeB from *Acinetobacter baumannii*, has been reported in three forms, in substrate free state, consisting of form I OOO with the protomers in close contact in inner membrane part, form II OOO with one protomer is moved away from the rest in the inner membrane part, and form III OOO with all protomers moved away from each other in the inner membrane part. In the presence of ethidium (Et), AdeB was reported to adopt three forms consisting of form Et-I OOT. form Et-II in L*TO, and form Et-III LTO (Morgan et al., 2021). Recently, the structure of AcrD from *E. coli*, in the absence and presence of its substrate, gentamicin, revealed a trimer structure adopting the conformational state L*TO (Zhang et al., 2023). In addition, the heavy-metal RND efflux transporter CusA was reported to adopt different conformational states of its trimer form, namely, OOT, OTT, OOO, and TTT, suggesting the protomers function independently (Moseng et al., 2021). These findings, by Cryo-EM technique, clearly suggest the possibility for RND efflux transporters to adopt different intermediate conformational states, with the possibility that the protomers to function independently of each other, during their export activity. Importantly, these cryo-EM structures revealed that conformational change kinetics is a crucial factor in these intermediate forms, and ultimately in the export activity of these RND efflux transporters. Furthermore, recently experimental evidence on the change in molecular dynamics of AcrB has been reported to modulate the binding and efflux of substrates in the presence of an inhibitor, which by analogy can be considered as a mutation that increases the antibiotic susceptibility of AcrB (Reading et al., 2020). Consequently, our conformational change kinetics explanation for AcrB's gate loop mutations effect on its export activity, may be strengthened further because what we did predicted to be possible for AcrB, has been corroborated experimentally, and importantly it can be observed for other RND efflux transporters, as shown above.

As discussed earlier, two primary explanations are suggested: the accessibility and opportunity for the substrate to use any export tunnel, and the conformational change kinetics of AcrB's protomers. TRC and MIY have comparable MIC values for AcrB and have high molecular similarity, yet their R values differ significantly with 1 and 8 (or 16), respectively. If both substrates were expected to use the same tunnel-2, this discrepancy in R values for TRC suggests that TRC might prefer tunnel-1 and/or tunnel-3. While conformational change kinetics is most favourable explanation for this striking R difference between MIY and TRC, based on recent evidence and inferences (Su et al., 2017; Ababou, 2018; Reading et al., 2020; Morgan et al., 2021; Moseng et al., 2021), it is crucial to find an explanation that integrates both conformational change kinetics and substrate effects and form a general explanation instead. Thus, considering the case of MIY and TRC, as well as and the efflux rate changes for biofuels (Foo and Leong, 2013), it is plausible that the conformational change kinetics may be substrate dependent. This suggests a more general explanation where the substrate, and possibly its chosen export tunnel, can influence the conformational change kinetics, as illustrated in **Figure 5**. This generalised explanation is further supported by our PCA analysis, based on those 5 minimum parameters of TPSA, LogP, Rotatables, HB Acceptor, and HB Donor (**Figure 4, Figure S9A-C**). The substrate's flexibility, hydrogen bond formation, and hydrophobicity will likely impact the molecular dynamics of AcrB's protomers, thereby affecting their conformational change kinetics.

4. Conclusion

Our work demonstrates that investigating AcrB's gate loop mutants has provided crucial insights into AcrB export activity at the molecular level. Molecular modelling and computational analysis of interactions between antibiotics and AcrB revealed that all substrates exhibit favourable binding free energies across all three tunnels. With few exceptions, hydrogen bonds appear to be the primary driving force directing substrates toward any of the three tunnels, supporting the idea of substrate promiscuity. Additionally, we found that, energetically, the distal pocket is more favourable than any of the tunnels, despite generally having fewer hydrogen bonds. This suggests a sequential mechanism for the export of most AcrB substrates: (1) the substrate is primarily captured and directed by hydrogen bond formation and is transiently stabilized; (2) as the protomer changes conformation from access/loose to binding/tight, the substrate is pushed through the tunnel into the distal pocket, where it forms more atomic contacts (highly favourable energetically) but fewer or no hydrogen bonds, allowing loose binding within this pocket to facilitate substrate movement toward extrusion; and (3) when the protomer changes conformation from binding/tight to extrusion/open, the substrate is easily pushed from the distal pocket into the extrusion channel.

Our MIC R values highlight the substrates' preferences for one of the three tunnels. Interestingly, while R values of 1 would typically indicate that an antibiotic may prefer

tunnels other than tunnel-2 (which contains the gate loop mutations), this simple explanation was challenged by MIY and TRC. These two substrates have high molecular similarity and comparable MIC values for AcrB. To explain these experimental observations, we propose two possible explanations: (1) substrate promiscuity in choosing their export tunnel, likely based on accessibility and/or opportunity, possibly in a concentration-dependent manner; and (2) generalized conformational change kinetics, where the kinetics of protomer conformational changes are suggested to be substrate-dependent. This explanation is supported by our MIY and TRC data, PCA analysis, and is corroborated to some extent by recent reports on the cryo-EM structures of intermediates in a few RND efflux transporters, as well as experimental evidence on the impact of molecular dynamics on AcrB's export activity.

5. Materials and Methods

5.1. Expression and purification of AcrB mutants

All AcrB's gate loop mutants were prepared, expressed, and purified as previously reported (Ababou, 2018). In addition to our previous mutants, AAA, and Δ Loop, in this work we have prepared single and double mutation of the bulky residues in the gate loop, namely, F615, F617, and R620. The mutants are F615A, F617A, RA, RK, RD, RE, AA, F615A-R620A (F615A-RA), and F617A-R620A (F617A-RA).

5.4. Structural stability of AcrB mutants

CD spectra and thermal unfolding data were collected as previously reported (Ababou, 2018). The protein concentration was held constant at 5-10 μ M. All CD measurements were corrected by subtracting the buffer spectra. The unfolding experiments were fitted as previously reported (Ababou, 2018).

5.2. Crystallization, data collection, and structure refinement of AcrB mutants

All AcrB mutants were crystallised as previously reported (Ababou and Koronakis, 2016). X-ray diffraction data were collected at Diamond Light Source (Didcot, UK). X-ray data sets were indexed and integrated using iMosflm (Battye et al., 2011) and scaled using Scala or Aimless in the CCP4 suite (Winn et al., 2011). The structures were solved by molecular replacement using Phaser (McCoy et al., 2007) or Molrep (Vagin and Teplyakov, 2010). Structures were solved using PDB file 3D9B (Veesler et al., 2008) containing residues 1-1033. All Structures refinement were performed as previously reported (Ababou and Koronakis, 2016). The structures were completed with iterative rounds of manual model-building with Coot (Emsley et al., 2010) and refinement in Phenix (Adams et al., 2010). Figures were prepared using PyMol (www.pymol.org). The atomic coordinates and structure factors of AA, RA, and RK

structures in space group P21 have been deposited to the Protein Data Bank, and released with the accession code 7O3L, 7O3M, and 7O3N, respectively.

5.5. Antibiotic susceptibility assay

Functional data were performed using *E. coli* strain JW0451-2 (Δ acrB) cells complemented with plasmid encoding the AcrB gate loop mutants as previously reported (Ababou, 2018). Cells adjusted OD at 600 nm of ~ 0.05 were transferred to 100 μ l 2TY medium containing carbenicillin, for the drug susceptibility test, using serial dilution in 96 well plate for a starting concentration of SPR at 1686.1 μ g/mL, RIF at 49.4 μ g/mL, ERY at 1467.9 μ g/mL, NOV at 1269.2 μ g/mL, DOX at 870.0 μ g/mL, FSA at 129.2 μ g/mL, MIY at 29.6 μ g/mL, TRC at 28.9 μ g/mL, R6G at 958.0 μ g/mL, CLD at 461.4 μ g/mL, MXF at 26.3 μ g/mL, LMF at 23.3 μ g/mL, OFL at 10.8 μ g/mL, CIP at 19.9 μ g/mL, CLP at 19.4 μ g/mL, BZK at 71.0 μ g/mL, ACR at 541.9 μ g/mL, NLD at 63.6 μ g/mL, CCP at 409.2 μ g/mL, PPM at 45.5 μ g/mL. The bacterial growth was measured using 96-well flat-bottomed plates (Nunc, Thermo Scientific), and a Synergy HTX Multi-Mode microplate reader (BioTek Instruments). The experiments were performed in triplicate, except for RIF in 9 duplicates, and CCP and PPM in 5 duplicates.

5.6. Molecular docking and structural analysis

The unsolved AcrB mutant structures (F615A, F617A, RD, RE, F615A-RA, F617A-RA) were modelled by mutating the residue(s) of interest in each monomer of trimer-1 of AcrB structure (Ababou, 2018). The modelled structures and the crystal structures of AcrB and its mutants were energy minimized prior to the molecular docking with the antibiotics, using Amber14 package (Case et al., 2014). Antibiotic structures were optimised, and their CM2 partial charges were calculated using the semi-empirical PM3 Hamiltonian as implemented in DivCon program (Dixon and Merz, 1997; Gogonea and Merz, 1999). The molecular docking calculations were performed using AutoDock Vina (Trott and Olson, 2010). AcrB and its mutants and the antibiotics were prepared for docking using AutoDock Tools (Morris et al., 2009), where the calculated partial charges of the antibiotics were used instead of those assigned by AutoDock Tools. The molecular docking calculations were performed for the following docking sites: proximal pocket in access/loose monomer, distal pocket in the tight/binding monomer, binding pockets in tunnel-1 and tunnel-3 in access/loose monomer. To avoid any potential bias in our docking calculations, the 4docking search space was set to 22 Å $\times$ 22 Å $\times$ 22 Å which is larger than all the actual docking sites. For extensive sampling of docking positions, we set the exhaustiveness parameter to 80.

All calculated docking structures were analysed for close contacts and hydrogen bonds using in-house scripts and codes in Fortran 90. Only the best docking position

for each antibiotic was considered based on its estimated binding energy. To consider the subtle difference in the structures and the molecular dynamics of the docking sites, which may lead to the presence or absence of a hydrogen bond we have changed slightly the cut-off distance for hydrogen bonds from 2.40 to 2.55 Å. Also, the atomic contacts were determined using a distance cut-off of 3.80 Å, excluding hydrogen atoms.

5. 7. Antibiotics molecular, similarity, clustering, and PCA analysis

The chemical and geometrical descriptors for the antibiotics were produced using ChemAxon (www.chemaxon.com), and the cheminformatics toolkit ChemmineR package in R program (Cao et al., 2008). Antibiotics structural similarity search and clustering were performed using LiSiCA software (Lesnik et al., 2015). Principal component analysis (PCA) was performed using R program.

Supplementary material description

Additional supporting information, tables and figures in order of appearance in the manuscript, can be found in SuppMat_Ababou.pdf. Full RND efflux transporters sequences alignment in AcrB-RND-Align.pdf. Full validation report for the structures of AA, RA, and RK are in Val-report-full-AA.pdf, Val-report-full-RA.pdf, and Val-report-full-RK.pdf, respectively.

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Figure Legends:

Figure 1. Gate loop in AcrB and RND efflux transporters, and crystal structure and thermal stability of AcrB and gate loop mutants. (A) Sequence alignment of AcrB and other RND efflux transporters. Alignment was performed over the whole sequences, using ESPript 3.0 (Robert and Gouet, 2014) (Supplementary Materials), but for clarity only the region covering the gate loop is shown. Sequences are from *Escherichia coli* (ecAcrB, ecAcrD, ecAcrF, ecMdtF), *Salmonella Typhimurium* (stAcrB), *Yersinia enterocolitica* (yeAcrB), *Klebsiella pneumoniae* (kpAcrB), *Vibrio parahaemolyticus* (vpVmeB), *Acinetobacter baumannii* (abAdeJ, abAdeB), *Pseudomonas aeruginosa* (paMexB, paMexD), *Agrobacterium tumefaciens* (atAmeB), *Burkholderia pseudomallei* (bpAmrB), *Neisseria gonorrhoeae* (ngMtrD), *Stenotrophomonas maltophilia* (smSmeB). Strictly conserved residues are boxed in white on a red background and highly conserved residues are boxed in red on a white background. The green bar locates the 11 residues in the gate loop and AcrB's residues used for mutations or deletion, where the numbering is based on AcrB sequence. (B) Structural comparison between AcrB (green, pdb code 4ZIT) and its mutants, RA (blue, pdb code 7O3M), RK (magenta, pdb code 7O3N), and AA (yellow, pdb code 7O3L), in P2₁ space group (for clarity, only access monomer A is shown). (C) A close-up view, from (B), at the export channel (proximal pocket, gate loop, and distal pocket), depicting the overall structural conservation. (D) A close-up view, from (B), showing the superposition of the gate loops with residues at positions 615, 617, and 620. (E) Different view from (D) showing the differences between the gate loop in AcrB and the mutants. (F) 2Fo - Fc electron density map of DDM molecule binding to monomer A (access monomer in trimer-1) and contoured at 1.0 σ . (G) Thermal denaturation plot of the apparent fraction of unfolded protein (F_{app}) as a function of temperature.

Figure 2. AcrB in complex with antibiotics in tunnel-2. (A) AcrB monomer depicting the localisation and the pathway of each tunnel. Tunnel-1 (green), tunnel-2 (magenta), and tunnel-3 (cyan). The gate loop is coloured in magenta, as part of tunnel-2. (B) Antibiotics binding to the proximal and distal pockets, in AcrB and its representative gate loop mutants (AAA, Δ Loop), as predicted by molecular docking. For better visual inspection, the antibiotics were clustered into 4 groups based on their molecular weight. G1 for SPR, ERY, NOV, DOX, and FSA (842-516 g/mol). G2 for MIY, TRC, R6G, CLD, and MXF (458-401 g/mol). G3 for OFL, LMF, CIP, and CLP (362-323 g/mol). G4 for BZK, ACR, NLD, and PPM (284-138 g/mol). Mainly all the antibiotics populate similar binding region in either binding pocket, despite their size difference or the deletion of the gate loop.

Figure 3. AcrB in complex with antibiotics. (A) Antibiotics binding to the tunnel-1 and -3 in AcrB, as predicted by molecular docking. For better visual inspection, the antibiotics were clustered into 4 groups based on their molecular weight. G1 for SPR, ERY, NOV, DOX, and FSA (842-516 g/mol). G2 for MIY, TRC, R6G, CLD, and MXF (458-401 g/mol). G3 for OFL, LMF, CIP, and CLP (362-323 g/mol). G4 for BZK, ACR, NLD, and PPM (284-138 g/mol). (B) All 9 binding modes of the antibiotic NLD and PPM within the large binding site of the distal pocket, in AcrB and its representative gate loop mutants (AAA, Δ Loop), as predicted by molecular docking.

Figure 4. Principal component analysis (PCA) of representative mutants (F615A, AAA, Δ Loop), using 33 and 5 parameters (see details in Results).

Figure 5. Depiction of AcrB mutations effect on its conformational change kinetics between resting state (Access/Loose (L) – LLL trimer) and active state (Access/Loose (L), Binding/Tight (T), Extrusion/Open (O) – LTO timer). (A) Substrate independent conformational change kinetics pathway, where the trimer's unknown conformational state is denoted as XYZ. (B) Substrate dependent conformational change kinetics pathways, where the trimer's unknown conformational state is as denoted as $(XYZ)^S$, and S denotes the substrate.

Table 1. Antibiotics susceptibilities of AcrB and gate loop mutants (MIC ratio, R, of AcrB to Δ acrB or mutants)

Substrate	Abbr.	MW	Δ acrB	F615A	F617A	RA	RK	RD	RE	AA	F615A-RA	F617A-RA	AAA	Δ Loop
Spiramycin	SPR	841.1	16.0	1.0	1.0	1.0	1.0	1.0	2.0	1.0	1.0	1.0	1.0	2.0
Rifampicin	RIF	823.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Erythromycin	ERY	733.9	32.0	4.0	2.0	1.0	1.0	2.0	2.0	4.0	4.0	2.0	4.0	8.0
Novobiocin	NOV	612.6	16.0	2.0	2.0	1.0	1.0	2.0	2.0	4.0	2.0	2.0	4.0	8.0
Doxorubicin	DOX	543.5	64.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Fusidic acid	FSA	516.7	16.0	1.0	1.0	1.0	1.0	1.0	1.0	8.0	1.0	1.0	8.0	8.0
Minocycline	MIY	457.5	32.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	16.0
Tetracycline	TRC	444.4	4.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Rhodamin 6G	R6G	443.6	128.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	4.0	4.0	4.0	16.0
Clindamycin	CLD	425.0	32.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Moxifloxacin	MXF	401.4	32.0	4.0	4.0	2.0	2.0	2.0	2.0	4.0	4.0	4.0	4.0	4.0
Ofloxacin	OFL	361.4	8.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Lomefloxacin	LMF	351.4	16.0	2.0	2.0	4.0	4.0	4.0	4.0	2.0	4.0	4.0	4.0	4.0
Ciprofloxacin	CIP	331.3	4.0	1.0	1.0	1.0	1.0	2.0	2.0	1.0	1.0	1.0	1.0	1.0
Chloramphenicol	CLP	323.1	8.0	1.0	1.0	2.0	2.0	4.0	4.0	1.0	1.0	1.0	1.0	4.0
Benzalkonium	BZK	283.9	8.0	2.0	2.0	2.0	2.0	2.0	2.0	4.0	4.0	4.0	4.0	8.0
Acriflavine	ACR	259.7	4.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Nalidixic acid	NLD	232.2	4.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Carbonyl cyanide 3-chlorophenylhydrazone	CCP	179.6	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Phosphomycin	PPM	138.1	2.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

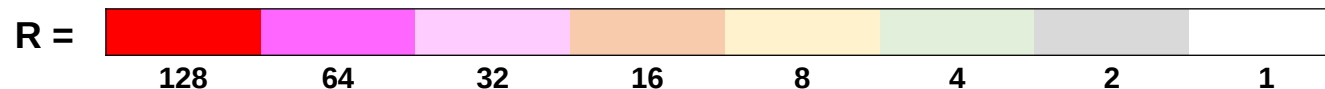


Table 2. Number of hydrogen bonds difference between the distal and proximal pockets.

Dist-Prox	AcrB	F615A	F617A	RA	RK	RD	RE	AA	F615A-RA	F617A-RA	AAA	Δ Loop
ACR	0	1	0	0	0	0	0	0	1	0	2	-1
BZK	0	0	0	0	0	0	0	0	0	0	0	0
CIP	-2	-2	-2	-3	-2	-1	-2	0	-3	-2	0	-3
CLD	-2	-1	-3	4	-1	-4	-1	-1	0	-1	-2	-1
CLP	-1	-2	2	-3	-2	-4	-2	1	-2	-4	-2	-1
DOX	-2	3	-4	0	-3	-2	-2	1	-3	-2	-1	-1
ERY	3	-1	2	5	2	5	2	-1	0	2	-1	-2
FSA	1	0	1	1	1	0	-1	0	-1	0	-1	0
LMF	0	0	2	0	0	0	0	3	0	2	0	-1
MIY	-3	3	-1	-4	-4	-3	-1	-2	2	-2	-2	2
MXF	-2	-5	-2	-1	-3	-2	-2	-4	-2	-1	-3	-2
NLD	-4	-3	-3	-4	-4	-4	-4	-2	-3	-4	-2	-2
NOV	1	1	3	-2	-3	0	2	3	-1	1	4	2
OFL	-1	-1	-2	-2	-2	-1	-1	-3	-1	-2	-1	-2
PPM	1	1	0	1	0	0	3	-1	2	1	2	4
R6G	-1	-1	-3	1	0	1	1	-1	-1	-1	-3	-1
SPR	-3	-5	-3	0	0	-1	-3	-2	-4	0	-3	-2
TRC	-1	0	0	0	0	-1	0	6	-1	0	6	-2
Distal # ^a	4	5	5	4	2	2	4	5	3	4	4	3
None # ^b	3	4	4	6	7	6	4	4	4	5	3	2
Proximal # ^c	11	9	9	8	9	10	10	9	11	9	11	13
Ratio ^d	2.8	1.8	1.8	2.0	4.5	5.0	2.5	1.8	3.7	2.3	2.8	4.3

^a Number of antibiotics with more hydrogen bonds in distal pocket. ^b Number of antibiotics with equal hydrogen bonds in distal and proximal pockets. ^c Number of antibiotics with more hydrogen bonds in proximal pocket. ^d Ratio of hydrogen bonds number in proximal to distal pockets.

Table 3. Number of atomic contact difference between the distal and proximal pockets.

Dist-Prox	AcrB	F615A	F617A	RA	RK	RD	RE	AA	F615A-RA	F617A-RA	AAA	ΔLoop
ACR	-6	-11	11	2	6	2	8	0	-11	-5	-3	3
BZK	0	11	17	5	3	-5	5	-4	-9	14	-2	-1
CIP	-5	-2	-8	-2	-4	-6	-5	-9	5	0	-7	-17
CLD	11	-12	0	14	-7	-3	-11	-12	-8	-4	-17	9
CLP	10	-6	-9	-1	-4	11	6	-4	-5	-2	-9	13
DOX	37	4	13	32	36	35	38	17	17	25	3	-22
ERY	-1	-1	14	15	24	-5	-1	-10	-4	9	-5	-12
FSA	29	3	33	23	24	22	26	-3	8	31	-4	3
LMF	5	-8	10	1	3	5	4	16	-8	2	0	1
MIY	9	8	8	8	6	15	7	3	7	10	1	-2
MXF	-2	-3	26	13	-3	-3	0	20	-5	22	21	14
NLD	11	-7	16	12	9	9	10	10	-6	15	-6	9
NOV	-13	-16	10	-14	32	25	6	-6	5	-6	-1	-15
OFL	1	-8	1	3	3	3	1	-10	-9	1	-12	14
PPM	15	12	10	16	14	8	3	20	12	16	15	8
R6G	15	-2	5	9	4	9	12	-5	8	1	-14	-8
SPR	-7	22	-2	-4	4	-18	-6	-16	-4	2	-9	1
TRC	33	14	16	26	30	-12	32	11	13	14	10	11
Distal #^a	11	7	14	14	14	11	13	7	8	13	5	11
None #^b	1	0	1	0	0	0	1	1	0	1	1	0
Proximal #^c	6	11	3	4	4	7	4	10	10	4	12	7
Ratio^d	0.5	1.6	0.2	0.3	0.3	0.6	0.3	1.4	1.3	0.3	2.4	0.6

^a Number of antibiotics with more atomic contacts in distal pocket. ^b Number of antibiotics with equal atomic contacts in distal and proximal pockets. ^c Number of antibiotics with more atomic contacts in proximal pocket. ^d Ratio of atomic contacts number in proximal to distal pockets.

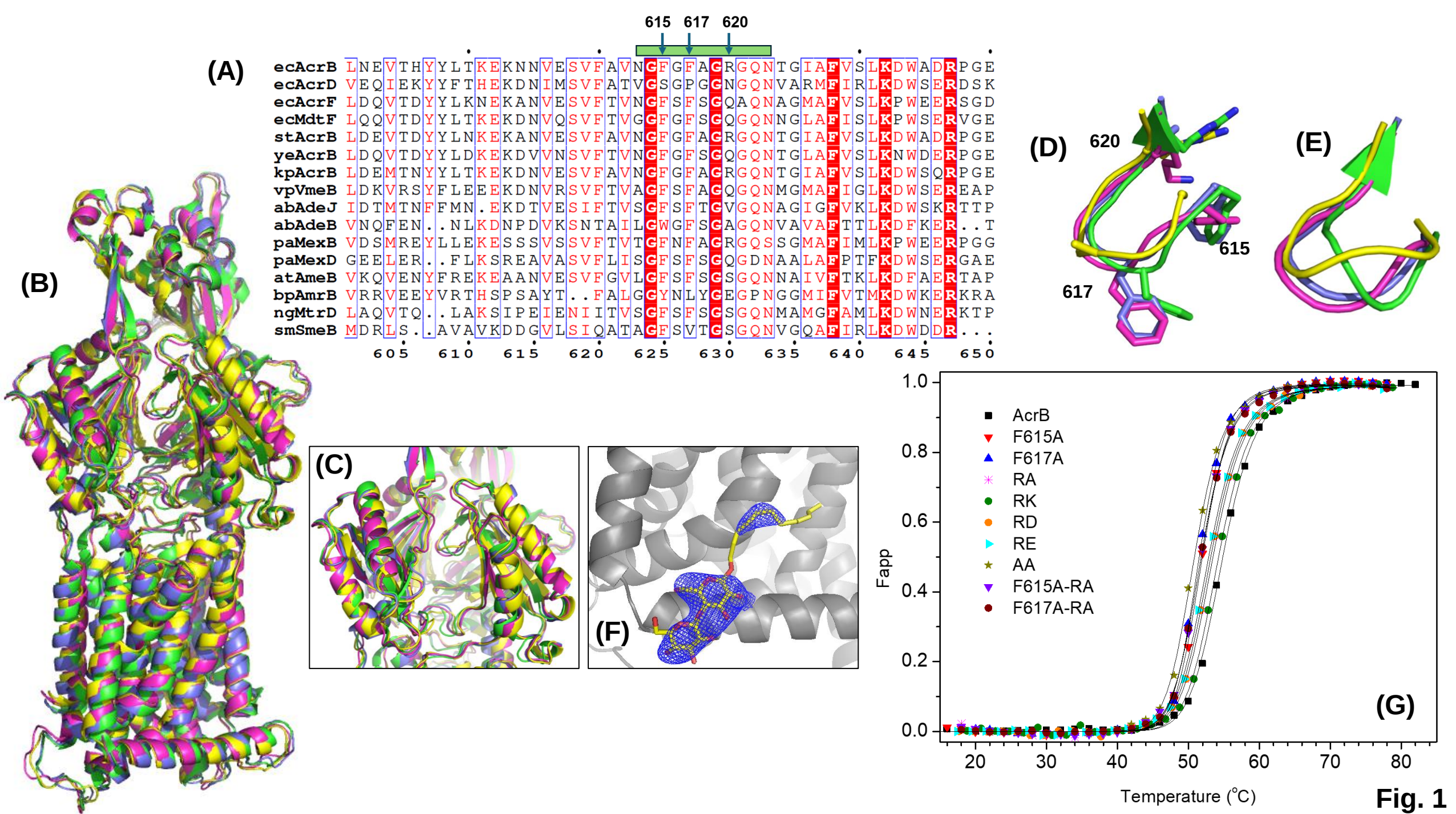
Table 4. (A) Number of hydrogen bonds found for the antibiotics within the tunnel-1 (T1) and -3 (T3) and their differences with those in distal pocket. (B) Number of atomic contact difference between the distal pocket and tunnel-1 (T1) or -3 (T3).

(A) HB	T1	T3	Dist-T1	Dist-T3	T1-T3	(B) Contacts	Dist-T1	Dist-T3
ACR	0	1	0	-1	-1	ACR	0	-7
BZK	0	0	0	0	0	BZK	0	-5
CIP	1	2	-1	-2	-1	CIP	-12	-11
CLD	4	2	-1	1	2	CLD	14	31
CLP	4	3	-3	-2	1	CLP	23	11
DOX	5	3	-3	-1	2	DOX	25	10
ERY	3	2	2	3	1	ERY	23	6
FSA	3	1	-1	1	2	FSA	30	35
LMF	3	0	-3	0	3	LMF	11	21
MIY	4	4	-2	-2	0	MIY	3	5
MXF	0	1	2	1	-1	MXF	28	8
NLD	0	2	0	-2	-2	NLD	11	7
NOV	3	4	-1	-2	-1	NOV	8	-15
OFL	0	2	0	-2	-2	OFL	21	5
PPM	3	3	1	1	0	PPM	19	17
R6G	2	1	-2	-1	1	R6G	-6	10
SPR	2	0	0	2	2	SPR	13	18
TRC	6	2	-5	-1	4	TRC	23	15
Distal / T1 # ^a			3	6	9	Distal # ^d	14	13
None # ^b			5	2	3	None # ^e	2	0
T1 & T3 / T3 # ^c			10	10	6	T1/T3 # ^f	2	4

^a Number of antibiotics with more hydrogen bonds in distal pocket or T1. ^b Number of antibiotics with equal hydrogen bonds in distal and proximal pockets or in T1 and T3. ^c Number of antibiotics with more hydrogen bonds in T1 and T3 or T3. ^d Number of antibiotics with more atomic contacts in distal pocket. ^e Number of antibiotics with equal atomic contacts in distal and T1 or T3. ^f Number of antibiotics with more atomic contacts in T1 or T3.

Table 5. Antibiotics similarities values. The colours reflect the similarity extent (Dark red for the highest and dark green for the lowest)

	SPR	ERY	NOV	DOX	FSA	MIY	TRC	R6G	CLD	MXF	LMF	OFL	CIP	CLP	BZK	ACR	NLD	PPM
SPR	1.000	0.209	0.132	0.140	0.185	0.136	0.138	0.095	0.194	0.128	0.091	0.118	0.092	0.082	0.149	0.013	0.070	0.098
ERY	0.209	1.000	0.131	0.200	0.205	0.135	0.137	0.120	0.200	0.127	0.101	0.132	0.119	0.092	0.169	0.015	0.079	0.113
NOV	0.132	0.131	1.000	0.203	0.141	0.167	0.169	0.167	0.145	0.237	0.255	0.250	0.259	0.185	0.216	0.173	0.220	0.130
DOX	0.140	0.200	0.203	1.000	0.188	0.241	0.224	0.286	0.179	0.259	0.231	0.250	0.260	0.255	0.239	0.273	0.217	0.119
FSA	0.185	0.205	0.141	0.188	1.000	0.207	0.190	0.129	0.208	0.179	0.170	0.145	0.151	0.118	0.222	0.019	0.125	0.125
MIY	0.136	0.135	0.167	0.241	0.207	1.000	0.857	0.179	0.154	0.216	0.208	0.229	0.239	0.205	0.275	0.190	0.190	0.139
TRC	0.138	0.137	0.169	0.224	0.190	0.857	1.000	0.182	0.180	0.220	0.213	0.208	0.191	0.238	0.316	0.167	0.195	0.143
R6G	0.095	0.120	0.167	0.286	0.129	0.179	0.182	1.000	0.111	0.292	0.261	0.255	0.267	0.178	0.186	0.389	0.220	0.079
CLD	0.194	0.200	0.145	0.179	0.208	0.154	0.180	0.111	1.000	0.191	0.156	0.178	0.186	0.175	0.250	0.023	0.128	0.207
MXF	0.128	0.127	0.237	0.259	0.179	0.216	0.220	0.292	0.191	1.000	0.688	0.774	0.767	0.256	0.306	0.353	0.484	0.121
LMF	0.091	0.101	0.255	0.231	0.170	0.208	0.213	0.261	0.156	0.688	1.000	0.821	0.885	0.250	0.265	0.400	0.556	0.100
OFL	0.118	0.132	0.250	0.250	0.145	0.229	0.208	0.255	0.178	0.774	0.821	1.000	0.852	0.278	0.294	0.387	0.536	0.133
CIP	0.092	0.119	0.259	0.260	0.151	0.239	0.191	0.267	0.186	0.767	0.885	0.852	1.000	0.257	0.273	0.414	0.577	0.103
CLP	0.082	0.092	0.185	0.255	0.118	0.205	0.238	0.178	0.175	0.256	0.250	0.278	0.257	1.000	0.357	0.233	0.233	0.120
BZK	0.149	0.169	0.216	0.239	0.222	0.275	0.316	0.186	0.250	0.306	0.265	0.294	0.273	0.357	1.000	0.250	0.250	0.130
ACR	0.013	0.015	0.173	0.273	0.019	0.190	0.167	0.389	0.023	0.353	0.400	0.387	0.414	0.233	0.250	1.000	0.417	0.042
NLD	0.070	0.079	0.220	0.217	0.125	0.190	0.195	0.220	0.128	0.484	0.556	0.536	0.577	0.233	0.250	0.417	1.000	0.087
PPM	0.098	0.113	0.130	0.119	0.125	0.139	0.143	0.079	0.207	0.121	0.100	0.133	0.103	0.120	0.130	0.042	0.087	1.000



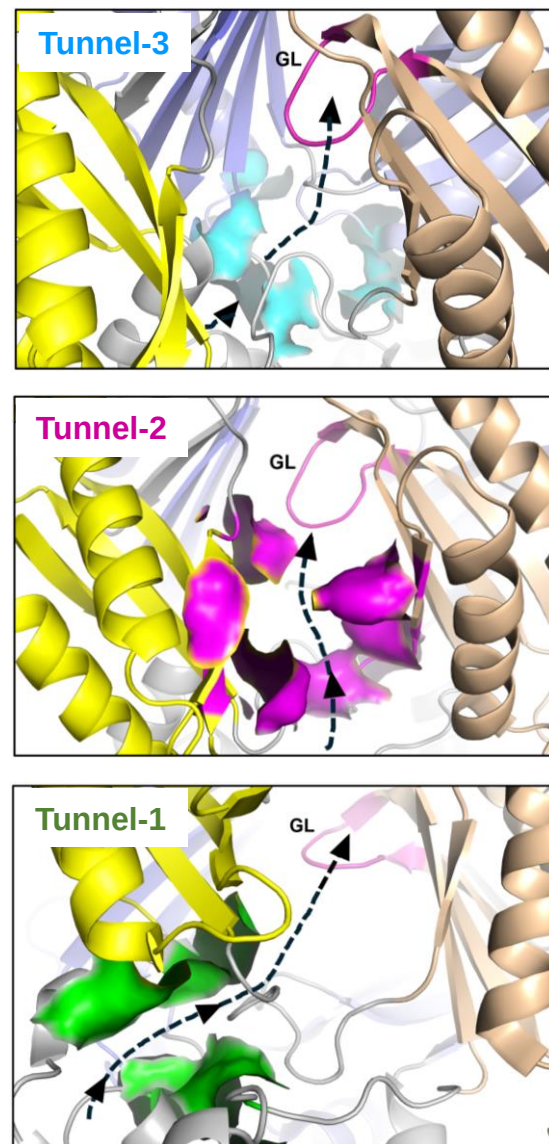
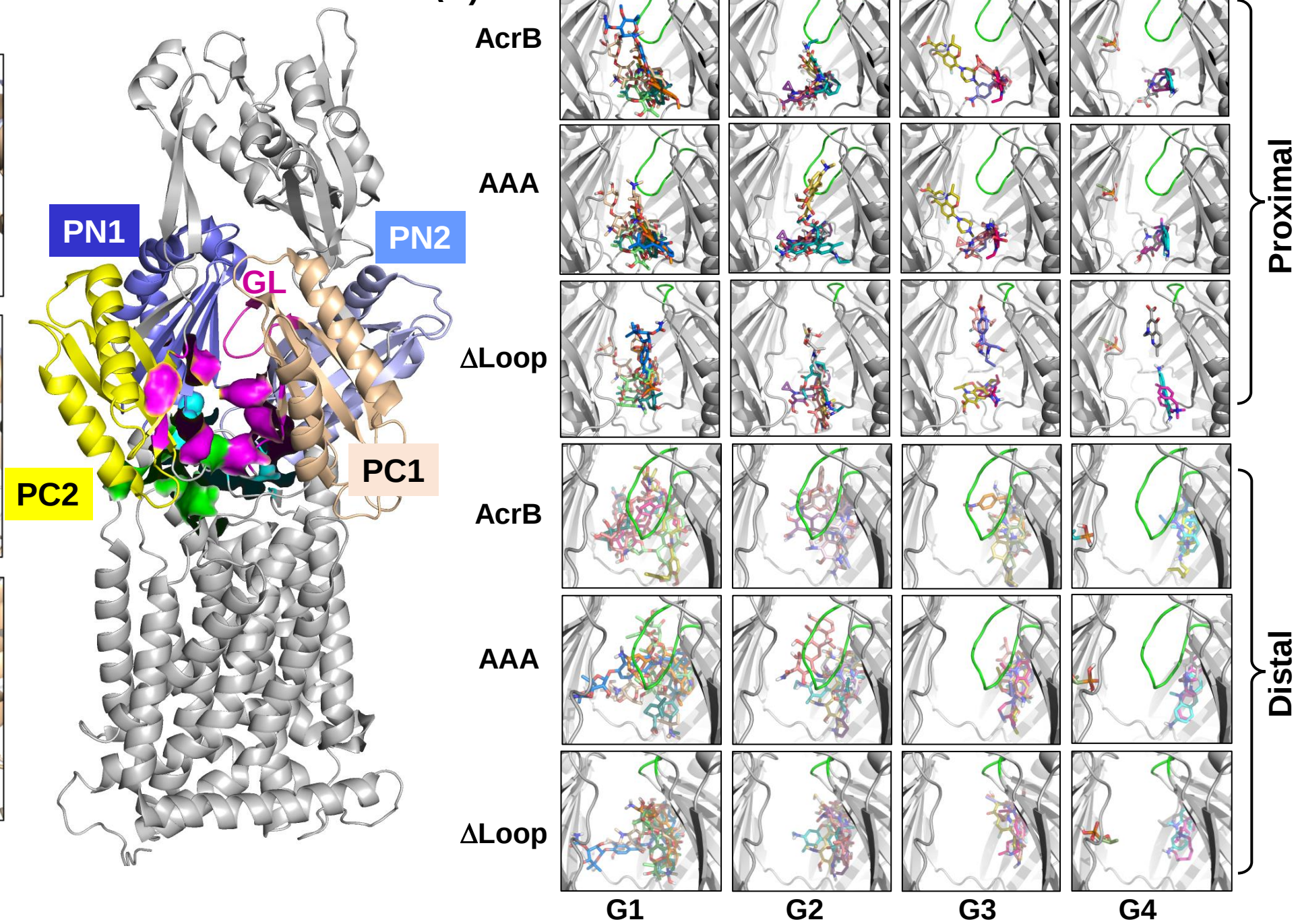
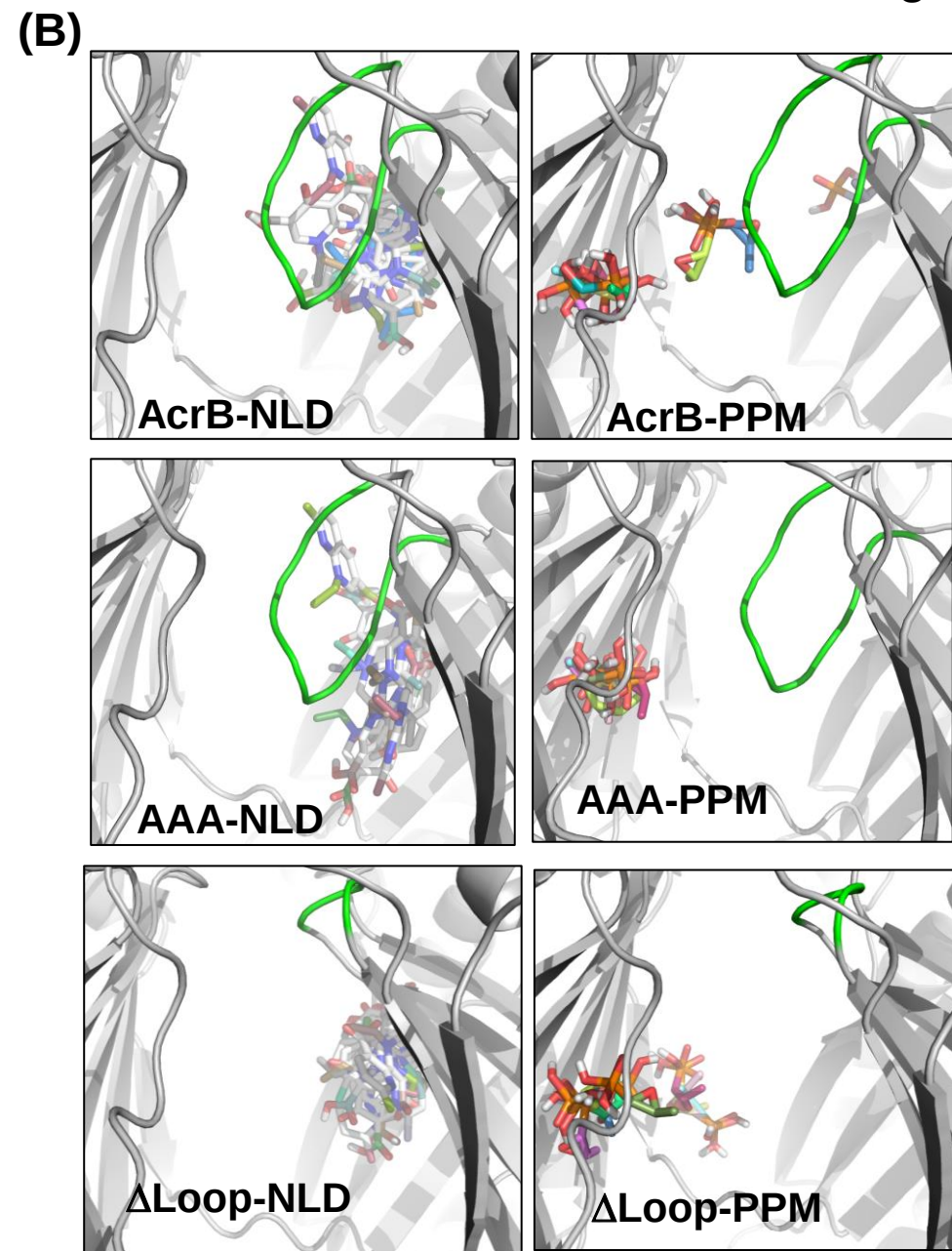
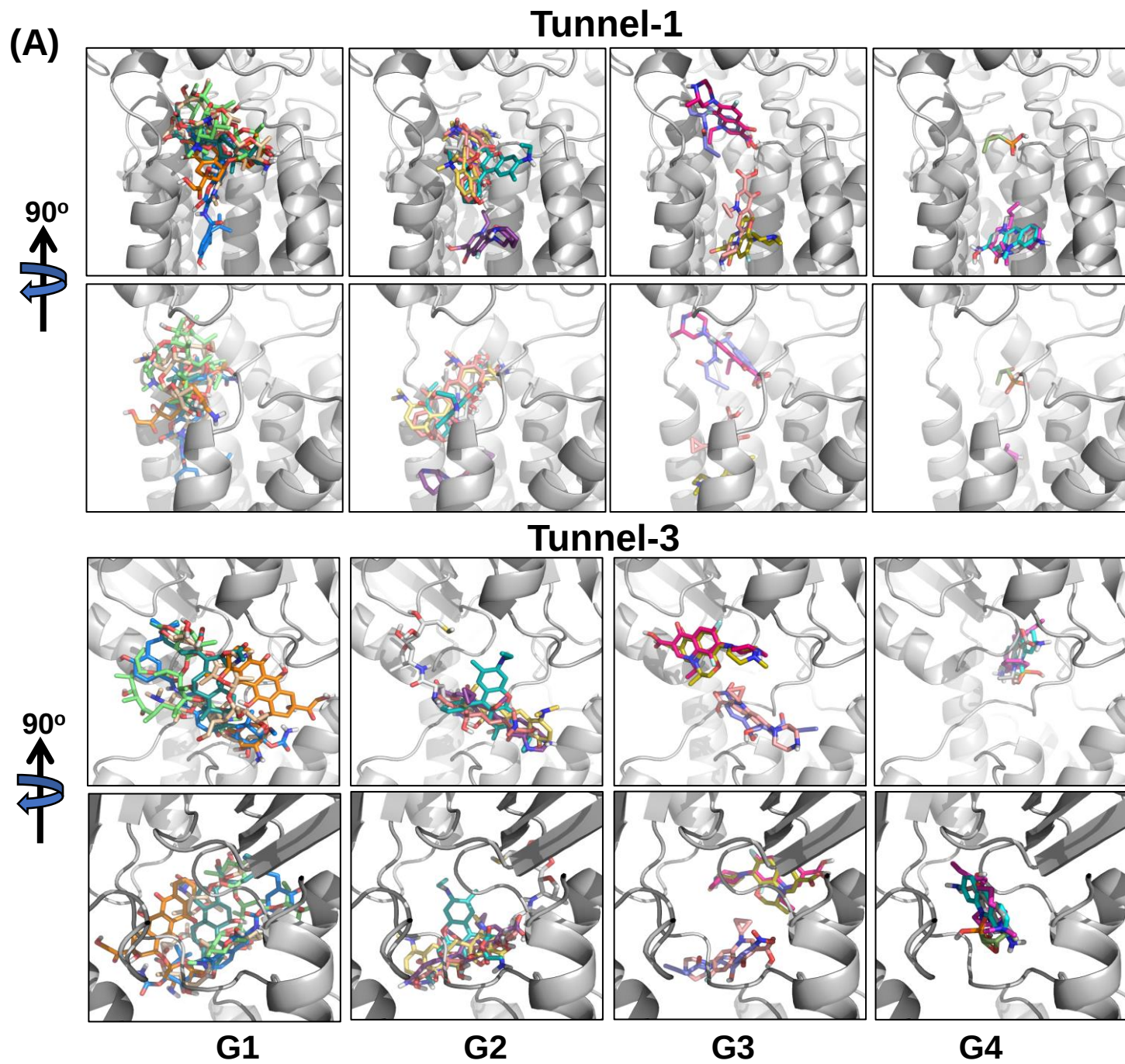
(A)**(B)****Fig. 2**

Fig. 3



33 parameters

5 parameters

F615A

AAA

Δ Loop

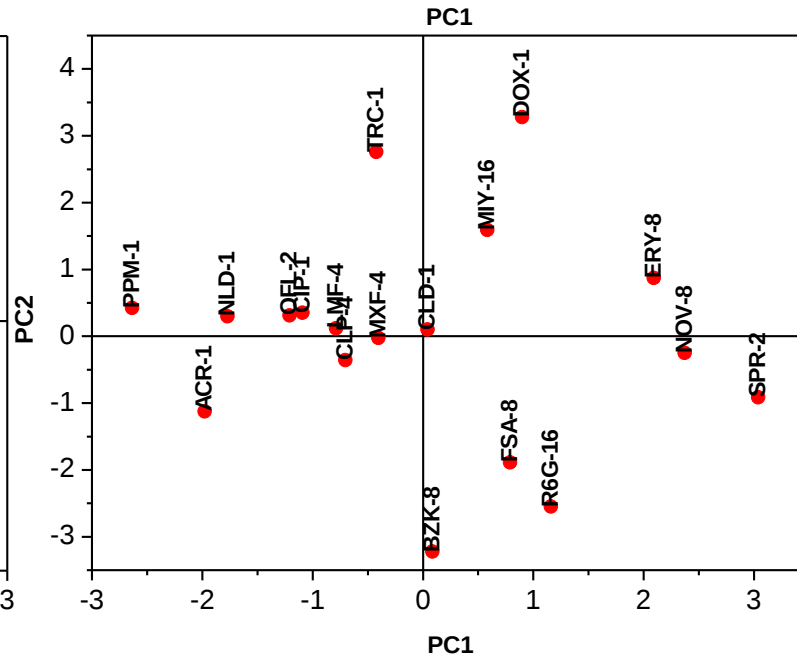
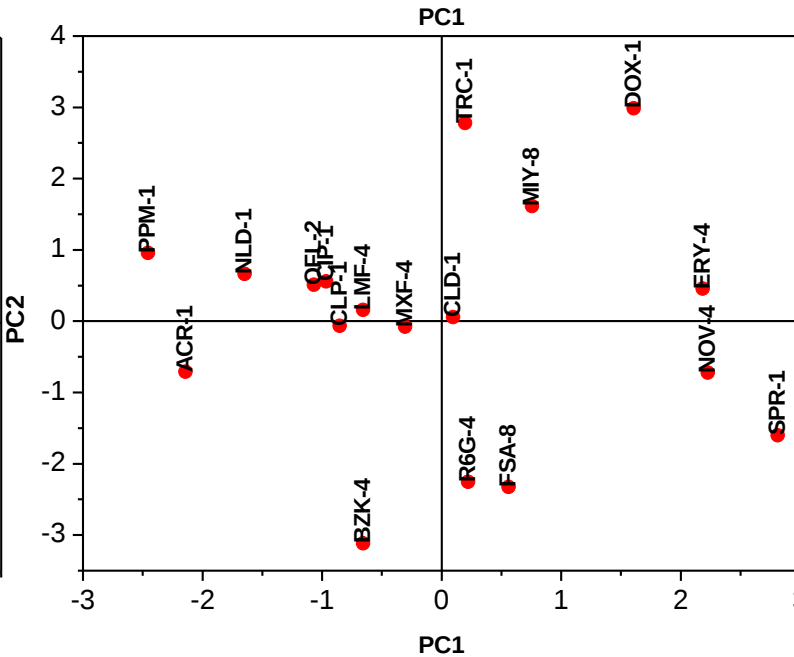
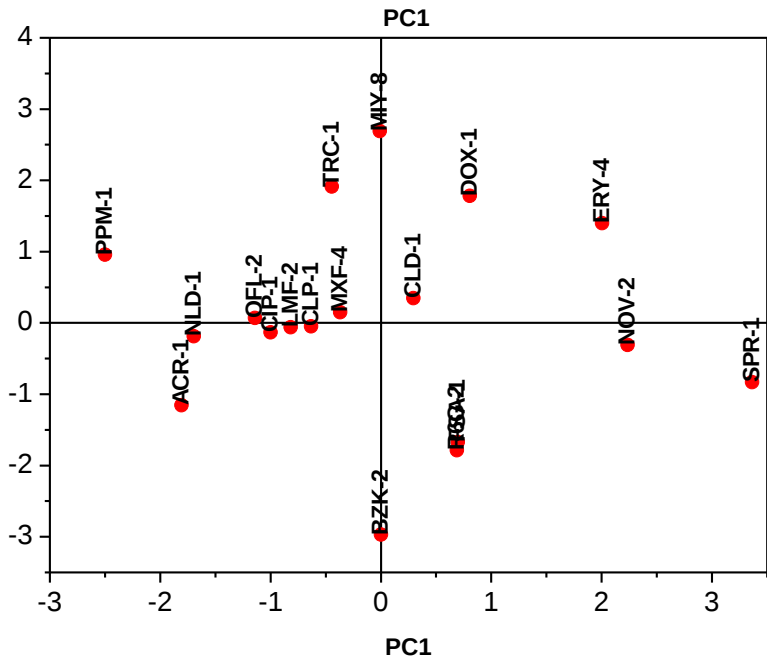
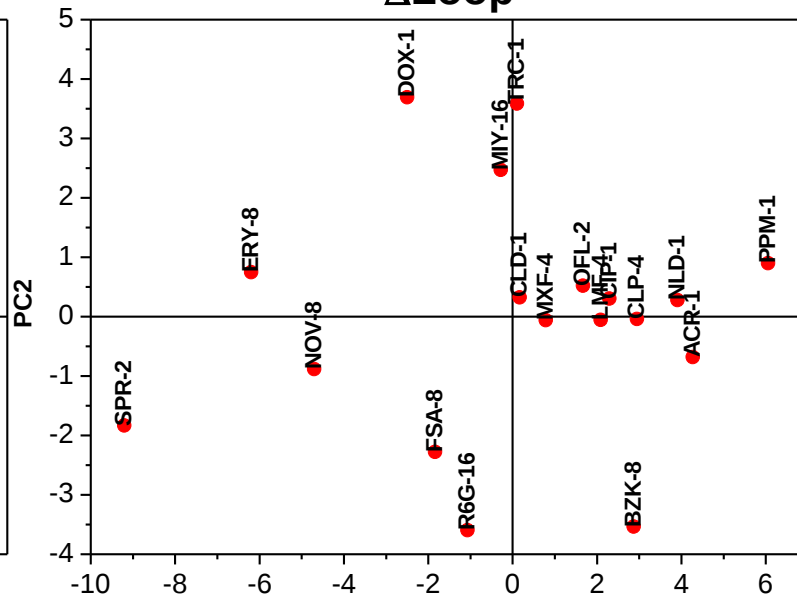
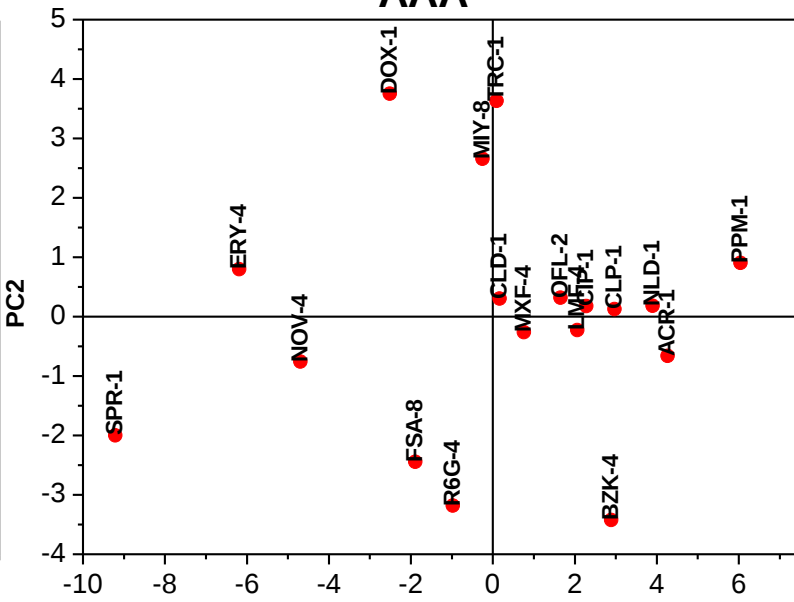
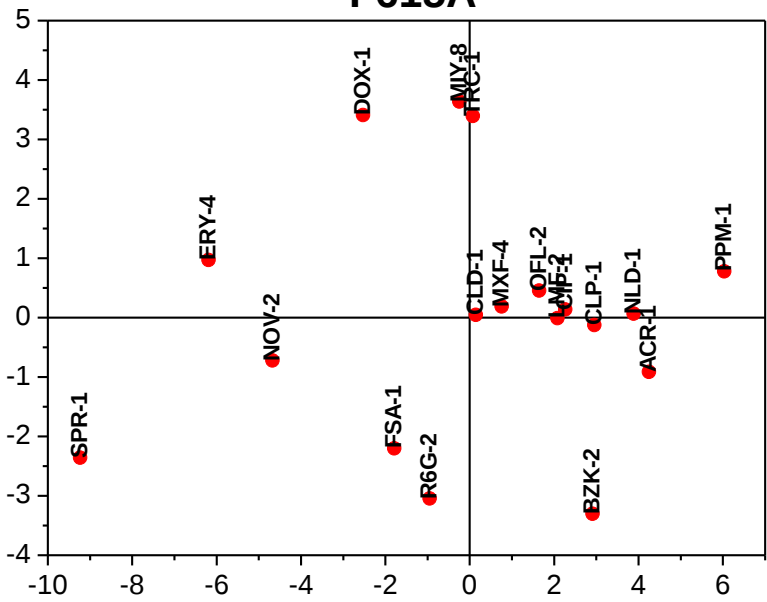


Fig. 4

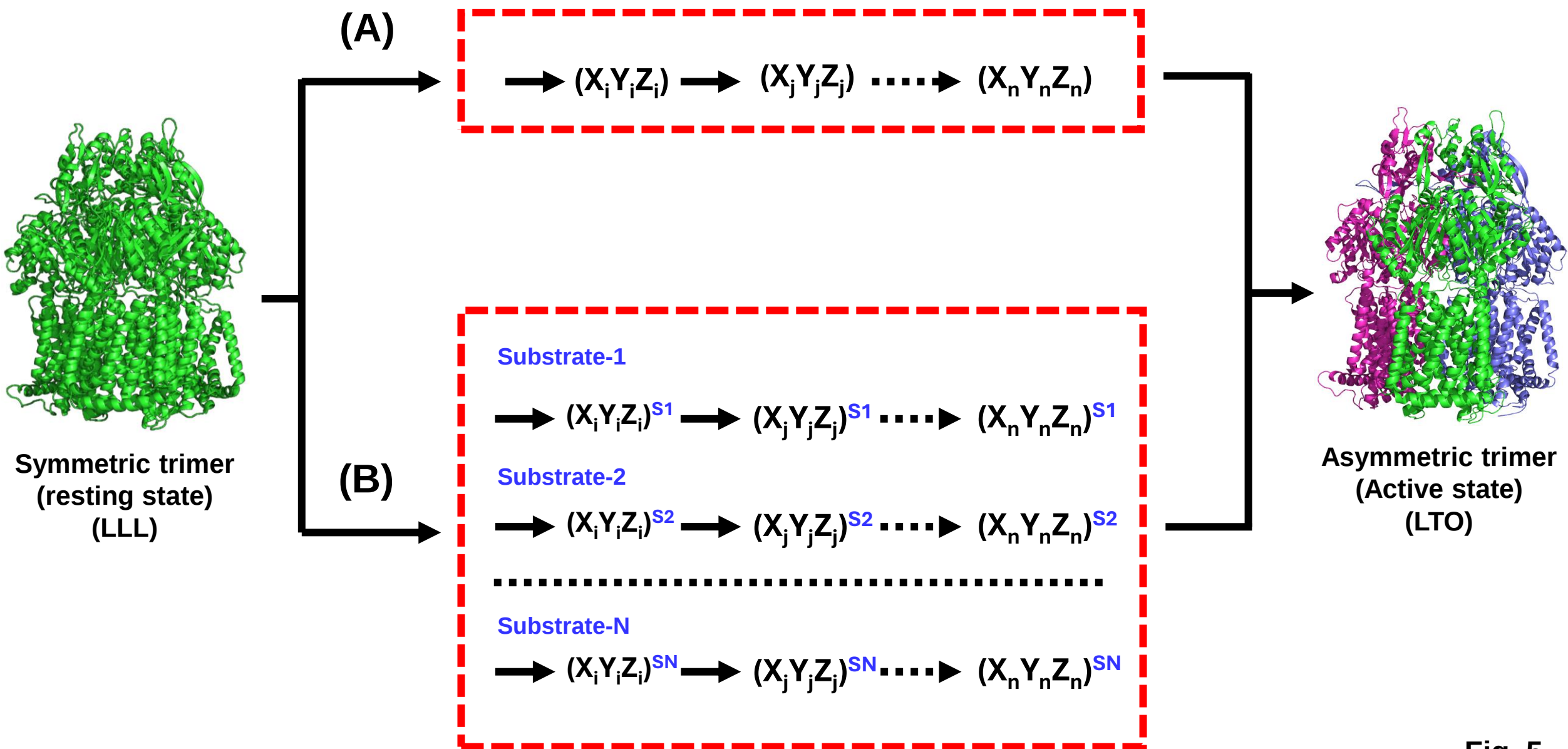


Fig. 5