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## Water-Mediated Network in the Resistance Mechanism of Fosfomycin

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No.	atom Name	atom Type	<b>RESP</b> Charge
1	01	OS	-0.39
2	02	02	-0.87
3	03	02	-0.87
4	04	ОН	-0.49
5	C1	СТ	-0.18
6	C2	СТ	0.14
7	C3	СТ	0.02
8	P1	Р	1.16
9	H1	H1	0.08
10	H2	H1	-0.01
11	H3	НС	0.01
12	H4	НС	0.01
13	H5	НС	0.01
14	Н6	НО	0.38

 Table S1. RESP charges of atoms of Fosfomycin

## Table S2. QM/MM Benchmark calculations on FomA

	Reactant	TS	Product
6-31g(d) Opt	0	17.31	-14.13
6-31g(d,p) SP	0	17.64	-14.48
6-31+g(d) SP	0	19.01	-11.83
6-311++g(d,p) SP	0	19.44	-12.22

	Lys18- ATP	dLys18- α-PO <sub>4</sub> (Å)	dLys18- β-,γ– bridging oxygen (Å)	<b>dHis58-</b> γ – PO <sub>4</sub> (Å)	<b>dHis58-</b> -Sub- PO <sub>4</sub> (Å)	Ser13- Water
Crystal FomA-FM (pdb: 3D41)	γ-PO <sub>4</sub>	4.40	5.35	3.45	3.88	$\beta$ -, $\gamma$ -bridging oxygen
Representative structure (MD Cluster)	α -,γ-ΡΟ4	3.82	3.76	3.28	2.74	Four-Water mediated H-bond with three water tethering ATP
Reactant (QM/MM)	α -,γ-ΡΟ4	2.92	3.84	3.28	3.71	Four-Water mediated H-bond with three water tethering ATP
TS (QM/MM)	α -,γ-ΡΟ4	2.87	3.78	3.56	2.70	Four-Water mediated H-bond with three water tethering ATP
Product (QM/MM)	α -,γ-ΡΟ4	2.86	3.58	3.15	2.95	Four-Water mediated H-bond with three water tethering ATP
Crystal FomA-FM (pdb: 3QUO)	γ <b>-</b> PO <sub>4</sub>	4.20	4.75	3.64	3.58	β-PO <sub>4</sub>

**Table S3.** Summary of key interactions in FomA from crystal, MD and QM/MM structures



**Figure S1.** ATP in the built reactant model (grey) and representative cluster structure of ATP-FM-Mg<sup>2+</sup>-FomA complex (cyan) shows significant conformational change. The built reactant model is shown in green ribbon representation, with the flexible glycine loop shown in red.

<mark>STW-FomA</mark> THA-IPK MEJ-IPK	K9 S13 K18 MGSSHHHHHHSSGLVPRGSHMTPDFLAIKVGGSLFSRKDEPGSLDDDAVTRFARNFARLA DPFTMMILKIGGSVITDKSAYRTARTYAIRSIVK GSHGGSMLTILKLGGSILSDKNVPYSIKWDNLERIAMEIKNAL : :*:***::: *. : : : : :
<mark>STW-FomA</mark> THA-IPK MEJ-IPK	H58 ETYRGRMVLISGGGAFGHGAIRDHDSTHAFS-LAGLTEATFEVKKRWAEK -VLSGIEDLVCVVHGGGSFGHIKAMEFGLPGPKNPRSSIGYSIVHRDMENLDLMV DYYKNQNKEIKLILVHGGGAFGHPVAKKYLKIEDGKKIFINMEKGFWEIQRAMRRFNNII . : :: ***:*** : : :*
<mark>STW-FomA</mark> THA-IPK MEJ-IPK	LRGIGVDAFPLQLAAMCTLRNGIPQLRSEVLRDVLDHGALPVLAGDALFDEHGKLWA IDAMIEMGMRPISVPISAL-RY-DGRFDYTPLIRYIDAGFVPVSYGDVYIKDEHSYGI IDTLQSYDIPAVSIQPSSFVVFGD-KLIFDTSAIKEMLKRNLVPVIHGDIVIDDKNGYRI : .: .: : ::: : : : : : : : : : : : : :
<mark>STW-FomA</mark> THA-IPK MEJ-IPK	D150 D171 FSSDRVPEVLLPMVEGRLRVVTLTDVDGIVTDGAGGDTILPEVDARS-PEQAYAALWGSS YSGDDIMADMAELLKPDV-AVFLTDVDGIYSKDPKR-NPDAVLLRDIDTNITFDRV ISGDDIVPYLANELKADL-ILYATDVDGVLIDNKPIKRIDKNNIYKIL-NYLSGSN *.*:::::::::::::::::::::::::::::::::::
<mark>STW-FomA</mark> THA-IPK MEJ-IPK	D208 T210K 216EWDATGAMHTKLDALVTCARR-GAECFIMRGDPGSDLE-FLTAPFSSWPAHVRSTRITTT QNDVTGGIGKKFESMVKMKSSVKNGVYLINGNHPERIGDIGKESFIGTVIR SIDVTGGMKYKIEMIRKNKCRGFVFNGNKANNIYKALLGEVEGTEIDFS . *.**.: *::::

**Figure S2.** Sequence alignment of AAK enzymes FomA and IPK using the Clustal Omega web server. Key residues in FomA and their corresponding residues in IPK are highlighted in red. Species names are abbreviated as follows: STW, *Streptomyces wedmorensis* (STW-FomA: BAA32493.1); THA, *Thermoplasma acidophilum* (THA-IPK: CAC11251.1); MEJ, *Methanocaldococcus jannaschii* (MEJ-IPK: AAB98024.1). The glycine-rich loop consisting residues 16-24 is underlined by red line.



**(B)** 

**Figure S3.** (A) RMSD values for Cα carbon of the backbone of ATP-FM-Mg<sup>2+</sup>-FomA complex (B) RMSD values for heavy atoms of ATP in the ATP-FM-Mg<sup>2+</sup>-FomA complex (plotted based on the last 50 ns trajectory).









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**Figure S4.** (A) Distance between the  $\gamma$ -phosphate of ATP and the phosphate group of fosfomycin from 3 replicas of 100-ns MD simulations of the ATP-FM-Mg<sup>2+</sup>-FomA complex (plotted based on the last 50 ns trajectory) (B). Distance between the  $\alpha$ -phosphate of ATP and Lys18from 3 replicas of 100-ns MD simulations of the ATP-FM-Mg<sup>2+</sup>-FomA complex (plotted based on the last 50 ns trajectory) (C). Distance between Lys18 and Asp208 from 3 replicas of 100-ns MD simulations of the ATP-FM-Mg<sup>2+</sup>-FomA complex (plotted based on the last 50 ns trajectory) (C). Distance between Lys18 and Asp208 from 3 replicas of 100-ns MD simulations of the ATP-FM-Mg<sup>2+</sup>-FomA complex (plotted based on the last 50 ns trajectory) (C).



**Figure S5.** The QM/MM optimized structure of the transition state shows that Gly12 on the  $\gamma$ -turn (orange) connecting to the flexible glycine rich loop (red) forms a H-bond with the  $\beta$ -,  $\gamma$ -bridging oxygen.



**Figure S6.** RMSF plots of the variants in comparison with the WT FomA. The glycine-rich loop comprising residues 16-24 is marked in pink and the loop connecting  $\alpha$ -helix 6 and  $\alpha$ -helix 7 is marked in red.







**Figure S7.** Representative structures of FomA mutants from MD simulations (a) H58L (b) S13A (c) D208A (d) D150A (e) S149A (f) T210A.