Supplemental Material for

Rifamycin Resistance in *Clostridium difficile* is Generally Associated with a Low Fitness Burden

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COMPUTATIONAL METHODS

***Cd*RpoB Homology Model.** A homology model of the *C. difficile* RNA polymerase subunit B (RpoB) was generated using the Schrödinger molecular modeling package (BioLuminate with Advanced Homology Modeling), the known *C. difficile* RpoB sequence (strain 630; accession no. CAJ66881.1), and the x-ray crystal structures of *E. coli* RNA polymerase in complex with rifampin (PDB ID 4KMU) (1, 2). ClustalW, interfaced from Schrödinger, was used to generate the sequence alignments between the RpoB subunit of the proteins (45% identity, 60% positives, 15% gaps) (3). The knowledge-based model building method was used and rifampicin ligand from 4KMU was included in the model building. During model building, side chain rotamers were retained for conserved residues and optimized by minimization for residues not derived from the template. The structure of rifaximin was substituted for rifampin and minimized in place following the completion of the homology model.

***Cd*RpoB Mutations and Rifaximin Binding Energy Calculations.** The relative binding affinities (reported in kcal/mol) for rifaximin with the known resistance mutations in the *Cd*RpoB model were calculated using the Prime MM-GBSA software included in the Schrödinger molecular modeling suite.(4) Prior to simulations the separated ligand (rifaximin) and protein were prepared using the Schrödinger protein preparation tool and as described above. The VSGB 2.0 solvation model and OPLS3 force field (both included in the Schrödinger software package, were used for the simulations. Protein flexible residues were permitted as defined by a 6 Å radius around the ligand (rifaximin) binding site. Full minimization sampling method was enabled. Relative binding affinities were calculated by comparing the MMGBSA

dG bind values for the native complex with those calculated for the resistance mutation complexes (**Table S1**, below).

*Cd*RpoB/DNA structure preparation. The DNA & C-chain RpoB from *Thermus thermophilus* RNA polymerase x-ray crystal structure (4GZY) were aligned with the *Cd*RpoB homology model using the Schrödinger/Maestro alignment software (5). Following this, the DNA subunit was transferred into the *Cd*RpoB model and the complex was refined by restrained minimization using the OPLS3 force field with water solvation potential (extended cutoff, force field charges) with the Schrödinger software to a convergence of heavy atom RMSD 0.6 Å.

SUPPORTING RESULTS

Binding Energy Calculation Results

Modeling of binding energies for rifaximin to the homology model of *Cd*RpoB is tabulated below. Modeling of *Cd*RpoB containing double mutations as reported by Curry et al.(6) were performed. The R505K changes significantly impacts binding of rifaximin, but appears to lack an *in vitro* or *in vivo* fitness cost. It is unclear why second site mutations are required in mutants containing an R505K change or if R505K may act as a compensatory to other mutations.

Table S1. Changes in binding energy of rifaximin to *Cd*RpoB for clinically relevant mutations/allelic sites.

Rifaximin- MM/GBSA Calculations		
Mutation	**dG bind**	**ddG Native**
WT	-105.578	0
S488Y	-68.762	36.816
D492Y	-85.357	20.221
H502N	-86.056	19.522
H502Y	-74.597	30.981
R505K	-52.112	53.466
S550F	-72.325	33.253
S550Y	-73.508	32.07
R505K/S488T	-54.677	50.901
R505K/I548K	-55.061	50.517
R505K/I548M	-67.615	37.963

MM/GBSA = Molecular mechanics energies combined with generalized Born and surface area continuum solvation. dG=delta G; ddG= delta deltaG= difference in Gibbs energy for ligand binding to WT and mutant.

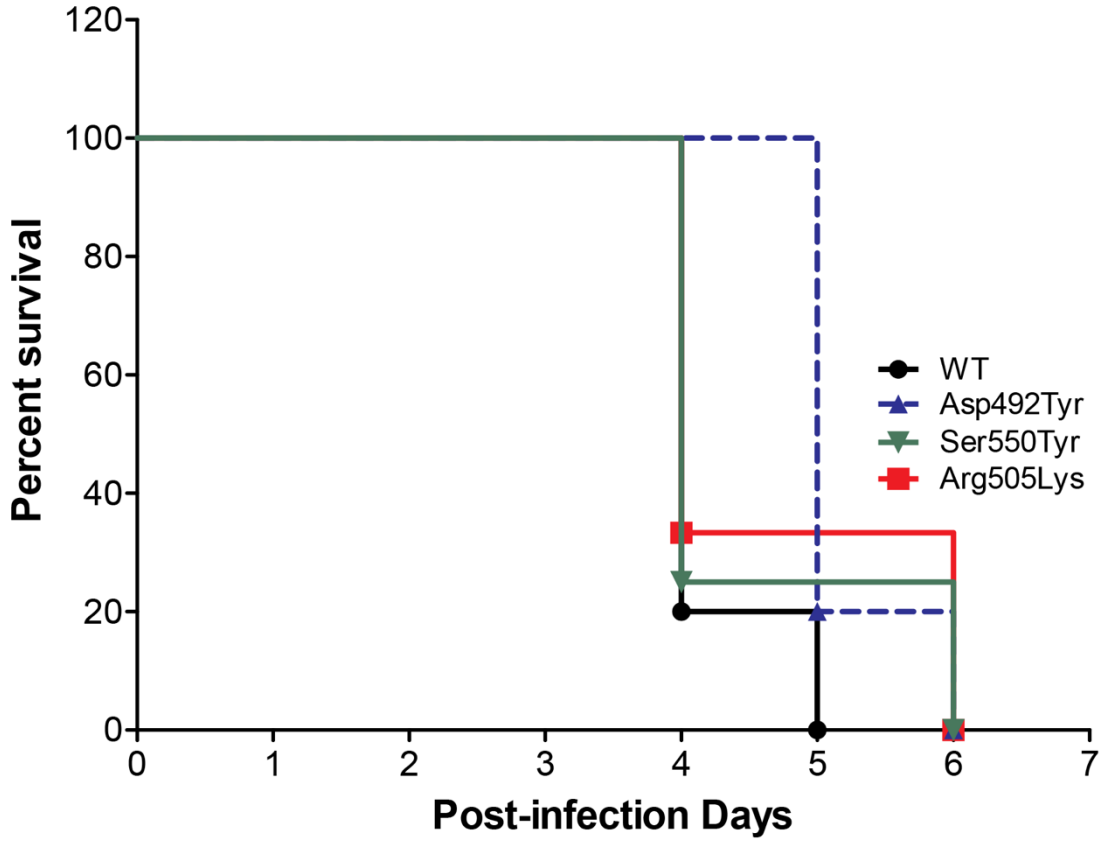

Figure S1. Kaplan-Meier survival analysis of hamsters infected with wild type or rifaximin-resistant mutants containing a point mutation. WT = parent strain CD43; $Arg_{505}Lys$ = mutant strain CD43-D5; $Asp_{492}Tyr$ = mutant strain CD43-D3; and $Ser_{550}Tyr$ = mutant strain CD43-D9. No significant differences exist between animal survival, as determined by Log-rank (Mantel-Cox) Test (P=0.7366). The number of animals in each group were: n=5 for WT; n=6 for $Arg_{505}Lys$; n=5 for $Asp_{492}Tyr$; and n=4 for $Ser_{550}Tyr$.

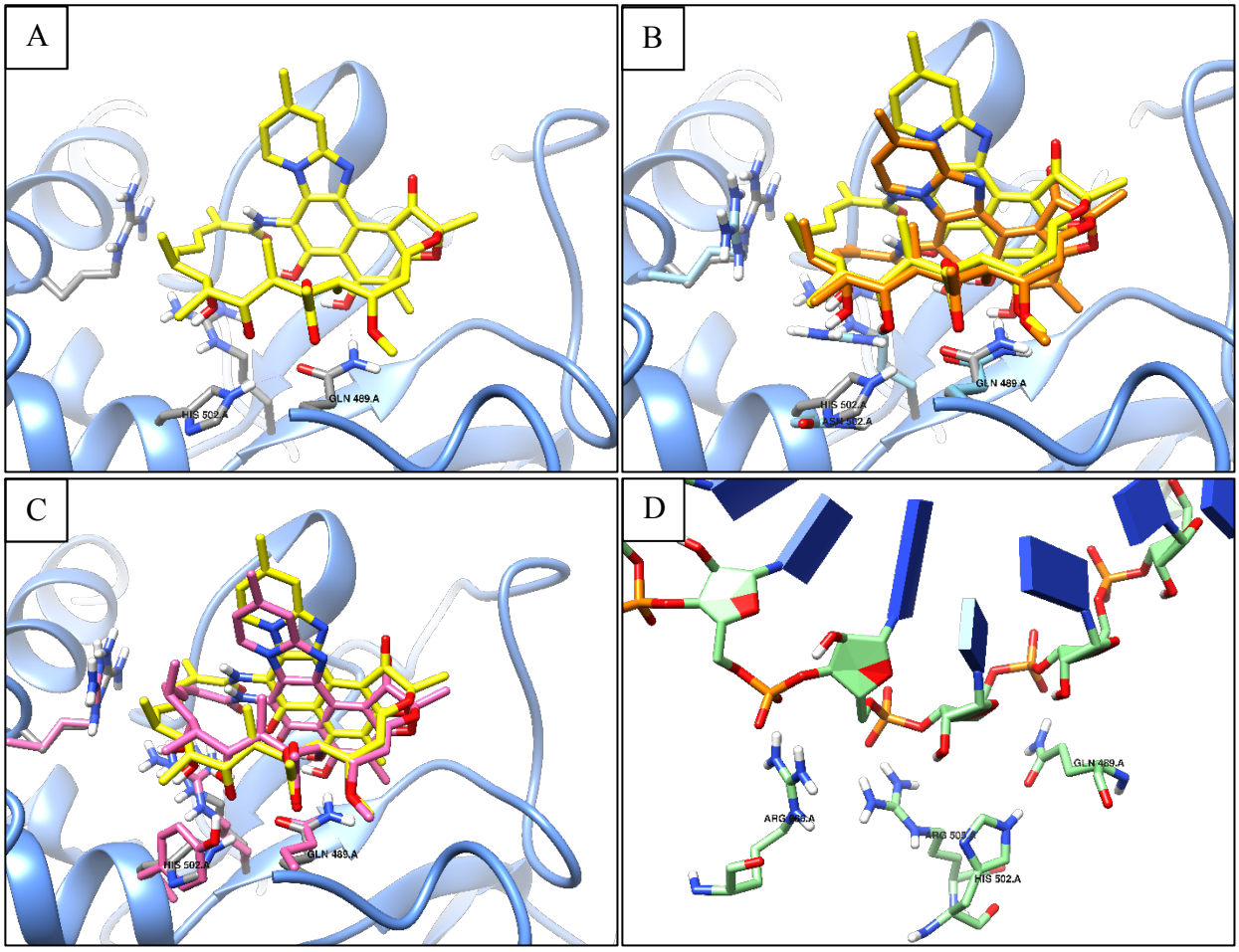

Figure S2. H502 Mutation Interactions. **A**. Native *Cd*RpoB with bound rifaximin shows hydrogen bonding network between H502, Q489, and phenolic –OH of rifaximin. **B**. H502N mutation results in disruption of H-bond network in active site and movement of R505 leading to minor positional changes in binding conformation of RFX (orange carbons). **C**. H502Y mutation results in disruption of active site H-bond network and minor positional changes in binding conformation of rifaximin (pink carbons). The Y502 hydroxyl group is predicted to engage the rifaximin 10-position hydroxyl group in a hydrogen bond interaction. **D**. Model of DNA bound to RpoB active site shows that H502 does not directly engage DNA backbone, whereas R666, R505, and Q489 engage DNA backbone in electrostatic interactions.

SUPPORTING REFERENCES

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