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

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ABSTRACT

We characterized clinically occurring and novel mutations in the β subunit of RNA polymerase (*Cd*RpoB), conferring rifamycin (including rifaximin) resistance in *Clostridium difficile*. The $Arg_{505}Lys$ substitution did not impose an *in vitro* fitness cost, which could be one one reason for its dominance among rifamycin-resistant clinical isolates. These observations were supported through structural modeling of the *Cd*RpoB. In general, most mutations lacked *in vitro* fitness costs, suggesting that rifamycin resistance may in some cases persist in the clinic.

TEXT

The non-absorbed rifamycin antibiotic rifaximin has been considered as an adjunctive therapy to reduce the recurrence of *Clostridium difficile* infection (CDI) following vancomycin treatment (1, 2). Rifaximin, which is approved for the treatment of traveler's diarrhea inhibits DNA transcription by selectively binding to the β subunit of RNA polymerase (RpoB). Substitutions in the rifamycin resistance-determining region (RRDR) of RpoB confer resistance to rifamycins, including rifaximin, in clinical isolates of *C. difficile* (3, 4). An arginine to lysine substitution at position 505 (i.e. $Arg_{505}Lys$) in *C. difficile* (*Cd*RpoB) is the most common mutation among rifamycin-resistant clinical isolates (3, 5, 6). Other mutations in clinical isolates also occur at His_{502}, Ser_{488} and Ser_{550}. However, it is unknown whether fitness costs influence the spectra of rifamycin resistance alleles among *C. difficile* isolates.

Fitness cost is a leading factor that affects the clinical prevalence of specific resistance alleles (7, 8). In the present study, we characterized both clinically occurring and novel rifamycin resistance mutations in terms of their impact on the growth and competitive fitness of *C. difficile* and by *in silico* structural modeling of the *Cd*RpoB (**Figure 1**).

The rifamycin-susceptible *C. difficile* strains were CD43 and CD1679 (both epidemic ribotype 027), from Dr. Scott Curry at the University of Pittsburgh. They were cultivated in Brain Heart Infusion Tryptone Yeast (BHITY) broth or agar at 37°C in a Whitley A35 anaerobic workstation (Don Whitley Scientific). The MIC of rifaximin was defined as the lowest concentration of drug preventing growth on BHITY agar (9). Spontaneous mutants were recovered by plating aliquots of overnight cultures onto selective agars containing rifaximin at 4 × MIC. Mutations were identified in a ~200bp PCR amplicon containing the RRDR (3). The competitive fitness (*W*) of rifaximin-resistant mutants was determined by pairwise competition

between the wild type parents and their respective derivative mutants (7, 8). Briefly, aliquots from overnight cultures of wild type and mutant bacteria were co-inoculated in BHITY broth at a 10:1 ratio (*ca.* 10^4:10^3 CFU/mL) and grown for 24 h. The numbers of mutant and wild type bacteria at the start and at the end of the experiments were determined by plating onto selective agar containing 4 × rifaximin MIC and on nonselective BHITY agar (7, 8). *W* was calculated from the $\ln[N_R(24)/N_R(0)]$ /$\ln[N_S(24)/N_S(0)]$, where $N_R(t)$ and $N_S(t)$ indicate the numbers of resistant and sensitive bacteria, respectively, at time *t* (0 or 24 h) (8). Doubling times in BHITY broth were calculated in Graphpad Prism 5 from automated optical density readings (OD600nm) over 48 h at 37°C in a Biotek 2 microplate reader (10). Effects on virulence were assessed in the hamster model of CDI as described (11) using a spore inocula of ~200 spores. Animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Texas at Arlington and in adherence to the USDA Animal Welfare Act (9 CFR, Parts 1–3). Using the RpoB sequence of *C. difficile* CD630, a homology model of *Cd*RpoB was generated from the x-ray crystal structure of *Escherichia coli* RNA polymerase in complex with rifampin (PDB 4KMU) in the Schrödinger molecular modeling suite (12, 13). Changes in the relative binding affinities for rifaximin in mutated *Cd*RpoB model were calculated using the Prime MM-GBSA software in the Schrödinger molecular modeling suite (14). To assess the impact on RpoB DNA interaction, the DNA and C-chain RpoB from *Thermus thermophilus* x-ray crystal structure (PDB 4GZY) were aligned with the *Cd*RpoB homology model in the Schrödinger/Maestro alignment software (15). Next, the DNA subunit was transferred into the *Cd*RpoB model and refined by restrained minimization to a convergence of heavy atom RMSD 0.6 Å. Further method details are found in the supplementary.

In both strains, rifaximin resistance arose at a frequency of 10^{-8}, consistent with prior reports (16); the rifaximin MICs against all mutants were >1024 µg/mL, indicating the high-level rifaximin resistance is achievable in a single mutational step (**Table 1**). Most studies adopt a breakpoint of ≥32 µg/mL to signify rifamycin resistance (5, 17). Several mutants possessed clinically occurring mutations including $His_{502}Asn$, $His_{502}Tyr$, $Arg_{505}Lys$, $Ser_{488}Tyr$, $Asp_{492}Tyr$, $Ser_{550}Phe$ and $Ser_{550}Tyr$ (3, 5, 6). We also identified previously unreported changes, including $Ser_{507}Leu$, $Gln_{489}Leu$, $Gly_{510}Arg$ and $Leu_{584}Phe$.

With the exception of $Ser_{507}Leu$, $Asp_{492}Tyr$ and $Ser_{550}Tyr$, most mutations did not impose a fitness costs on *C. difficile* (**Table 1**). *In vivo* studies indicated that the clinically occurring $Arg_{505}Lys$ was as virulent as the wild type, in terms of the time to mortality in the hamster model of CDI. Interestingly, the clinically occurring mutations $Asp_{492}Tyr$ or $Ser_{550}Tyr$ that imposed moderate (20%) and significant (33%) *in vitro* fitness costs, did not appear to affect *in vivo* virulence (**Figure 2; Figure S1**). This may also imply that the hamster model of CDI may be inadequate to assess subtle differences in fitness costs, due to its remarkable susceptibility to *C. difficile* (18).

Modeling of the *Cd*RpoB with bound rifaximin suggests that Arginine-505 engages in an energetically favorable, Pi-stacking interaction with the polyene moiety (16Z, 18E) in the central scaffold of both rifaximin (**Fig 3A**). Therefore, a change to Lysine-505 results in loss of the Pi-stacking interaction, leading to rifamycin resistance. According to our computational predictions, a *ca.* 40 kcal/mol relative energetic cost to rifaximin binding occurs with Lysine-505. From the DNA bound model, Arginine-505 interacts with the phosphate backbone via a charge-charge interaction (**Fig 3B**). Due to the cationic nature of Lysine-505, the charge-charge interaction with bound DNA is maintained. We suggest that the low fitness costs of $Arg_{505}Lys$

may correspond to minimal effects on DNA transcription. Similarly, Histidine-502 mutations, including $His_{502}Asn$ and $His_{502}Tyr$, are predicted to disrupt an active site hydrogen-bond network involving Glutamine-489 and a phenolic group on rifaximin (**Figure S2A**). This leads to a conformational change in the rifaximin binding site and an energetic cost between 20 and 30 kcal/mole (**Figure S2B & S2C**). Based upon our DNA bound model, the Histidine-502 residue does not directly engage DNA when bound, which may explain the low fitness cost in *C. difficile* (**Figure S2D**). The effects of other mutations on rifaximin binding are shown in the Supplementary Table S1.

The apparent lack of fitness costs for clinically occurring resistance alleles suggests that rifamycin-resistant mutants may in some cases persist in patients and clinical settings. Indeed, Curry et al.(3) reported the isolation of rifamycin-resistant *C. difficile* in patients who previously received a rifamycin antibiotic in the preceding 6 months prior to the onset of CDI. The isolates recovered contained the change $Arg_{505}Lys$; double substitutions $Ser_{488}Thr/Arg_{505}Lys$ or $Arg_{505}Lys/Ile_{548}Met$ (see **Figure 1** for amino acids sites relative to rifaximin**)**. Interestingly, from the initial study period of 2001-2002 to the second period in 2005, Curry et al.(3) observed a 10% decrease in the proportion of rifamycin-resistant *C. difficile* isolates, which was suggested to be due to a decrease in rifamycin exposure and increased infection control measures. Carman et al.(17) also reported the rise of rifaximin resistance during therapy, resulting from two strains carrying either $His_{502}Tyr$ or $His_{502}Tyr/Pro_{496}Ser$ substitutions. About 45 days after therapy, the two rifaximin-resistant isolates were still present, at the time of recurrence. From our studies we predict that the mutations in the Curry et al. (3) and Carman et al. (17) studies either lacked or were associated with low fitness costs. However, it is unclear why some rifamycin-resistant clinical isolates contain double resistance mutations in *Cd*RpoB (3, 5, 19) and if any of these

resistance changes may also be compensatory. Nonetheless, our study suggests that in some cases high-level rifamycin resistance in *C. difficile* could be maintained in clinical settings, even without selection pressure.

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TABLES AND FIGURES

Table 1. Impact of rifaximin resistance alleles on the fitness and growth of *C. difficile*.

Strain	MIC (μg/mL)	Substitution	Fitness (*W*)[a]	Doubling time (min)
CD43 (Parent)	0.125	none	1.00	97.3 ± 4.8
CD43-D1	>1024	$Gln_{489}Leu$	0.84 ± 0.05	89.4 ± 4.1
CD43-D2	>1024	$Asp_{492}Tyr$	0.80 ± 0.01	100.3 ± 9.2
CD43-D3	>1024	$Asp_{492}Tyr$	0.80 ± 0.08	118.2 ± 5.1
CD43-D4	>1024	$Asp_{492}Gly$	1.2 ± 0.12	92.3 ± 6.0
CD43-A1	>1024	$His_{502}Tyr$	1.20 ± 0.13	*ND*
CD43-A2	>1024	$His_{502}Asn$	1.00 ± 0.13	*ND*
CD43-D5	>1024	$Arg_{505}Lys$	0.99 ± 0.008	95.3 ± 10.1
CD43-D6	>1024	$Arg_{505}Lys$	*ND*	110.0 ± 5.1
CD43-D7	>1024	$Gly_{510}Arg$	0.85 ± 0.12	90.3 ± 5.5
CD43-D8	>1024	$Ser_{488}Tyr$	0.85 ± 0.02	*ND*
CD43-D9	>1024	$Ser_{550}Tyr$	0.67 ± 0.05	104.2 ± 18.4
CD43-D10	>1024	$Ser_{550}Phe$	1.26 ± 0.13	*ND*
CD1769 (Parent)	0.0625	none	1.00	97 ± 9.5
CD1769-D1	>1024	$Asp_{492}Tyr$	*ND*	98.9 ± 9.2
CD1769-D2	>1024	$His_{502}Arg$	0.91 ± 0.3	102.1 ± 10.7
CD1769-D3	>1024	$Arg_{505}Lys$	1.02 ± 0.15	102.0 ± 4.4
CD1769-D4	>1024	$Ser_{507}Leu$	0.57 ± 0.07	267.5 ± 37.1
CD1769-D5	>1024	$Leu_{584}Phe$	1.24 ± 0.07	*ND*

[a]By convention the fitness of wild type is designated as 1. MICs were done using two independent cultures in duplicates. A minimum of three independent replicates were performed to calculate *W* and doubling times. *ND*-Not determined.

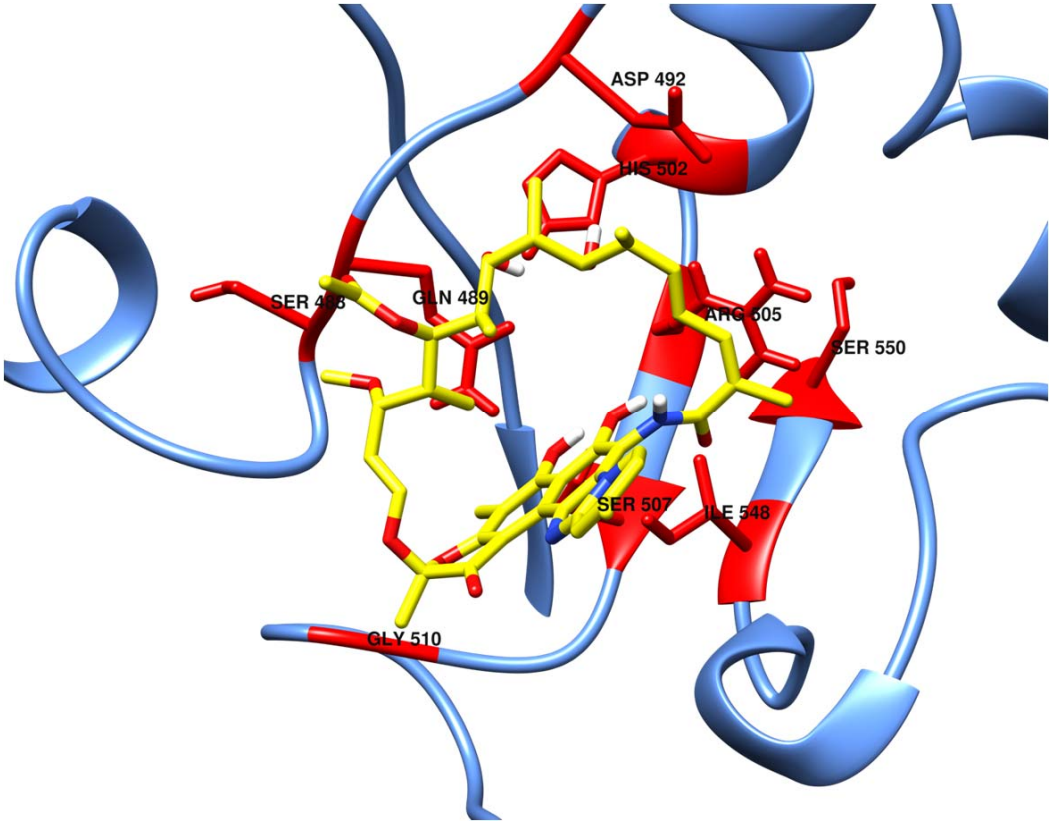


Figure 1. Model of *Cd*RpoB with bound rifaximin. Mutational sites conferring rifaximin resistance are shown in red. Rifaximin is shown with yellow carbon atoms.

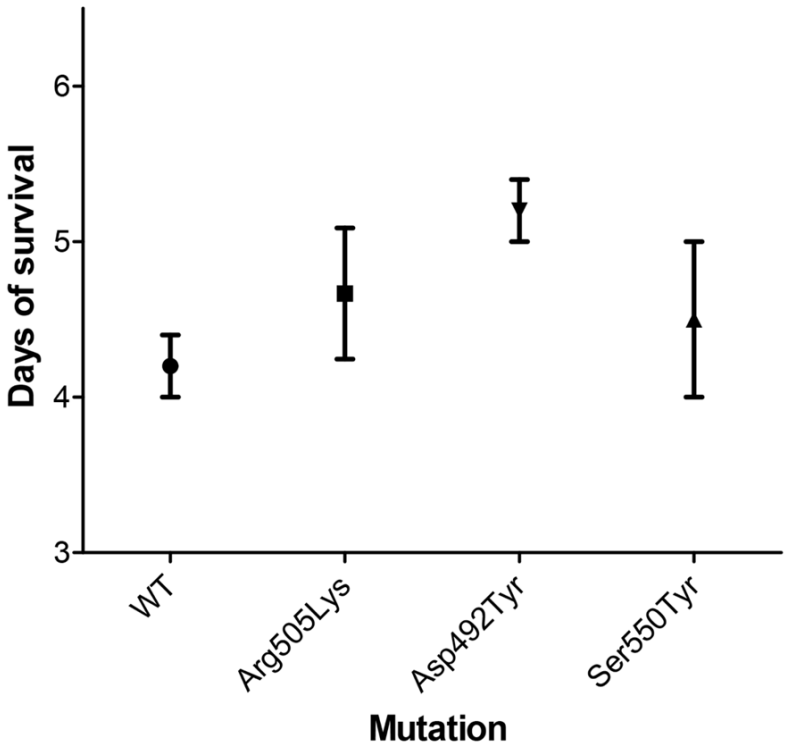
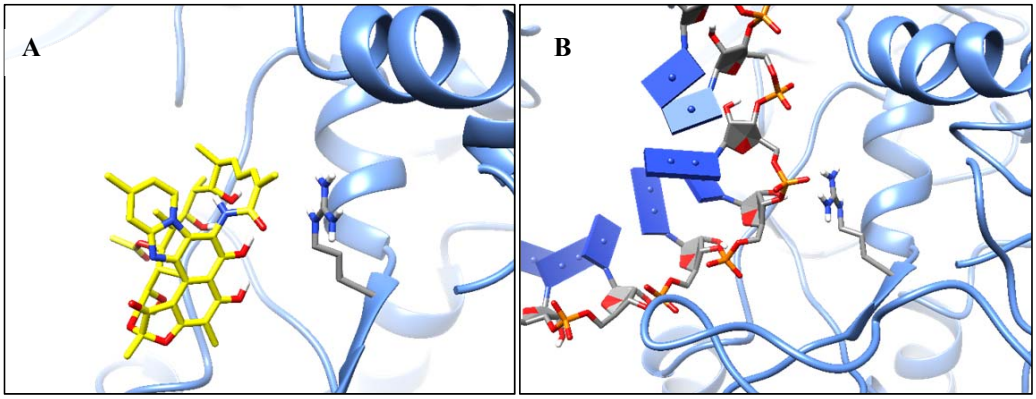


Figure 2. Comparison of virulence of rifaximin-resistant mutants in hamsters. WT = parent strain CD43; $Arg_{505}Lys$ = mutant strain CD43-D5; $Asp_{492}Tyr$ = mutant strain CD43-D3; and $Ser_{550}Tyr$ = mutant strain CD43-D9. No significant differences exist between the means, as determined by one-way ANOVA (P=0.28). 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 $Ser_{550}Tyr$. CD43 mutants bearing $His_{502}Asn$ and $His_{502}Tyr$ strains were unavailable at the time of the experiments.



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167 **Figure 3. A.** *Cd*RpoB Model with rifaximin bound, highlighting interaction with Arginine-505.

168 **B.** *Cd*RpoB Model with DNA bound highlighting interaction with Arginine-505.

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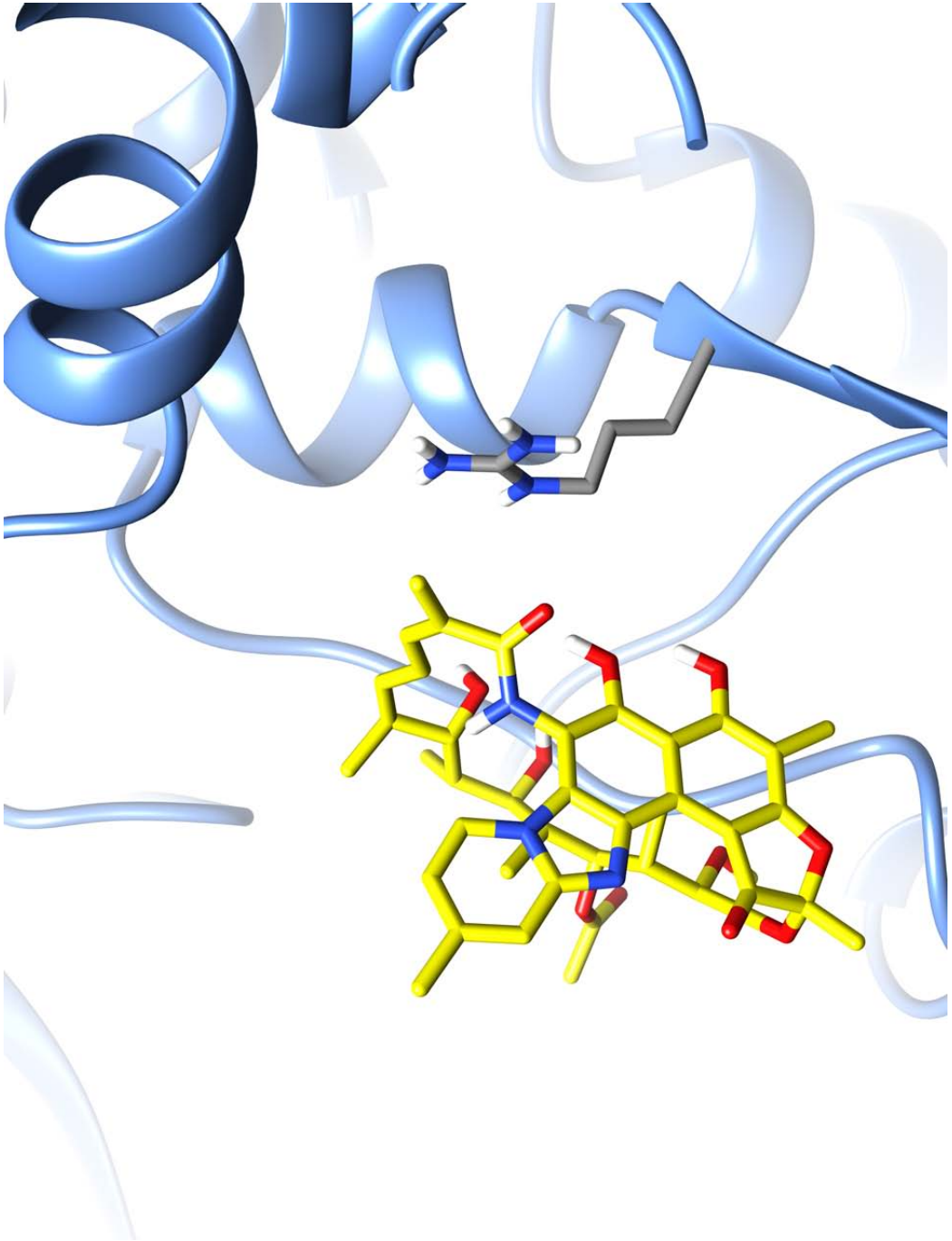
Figure 1

Figure 2



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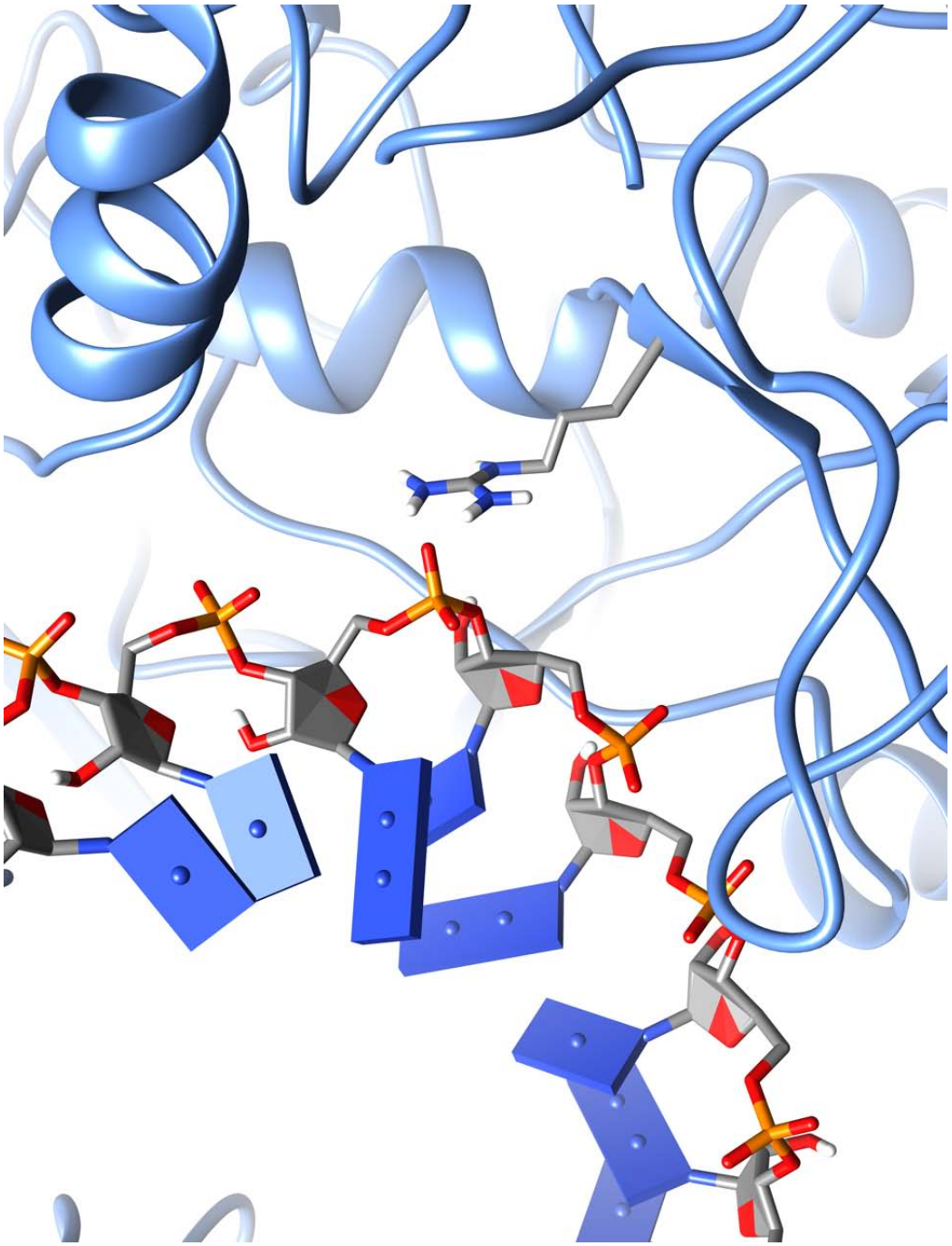

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