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

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

The nonabsorbed rifamycin antibiotic rifaximin has been considered an adjunctive therapy to reduce the recurrence of *Clostridium difficile* infection 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 clinically occurring and novel rifamycin resistance mutations in terms of their impacts on the growth and competitive fitness of *C. difficile* and by *in silico* structural modeling of the *Cd*RpoB (Fig. 1).

The rifamycin-susceptible *C. difficile* strains were CD43 and CD1679 (both epidemic ribotype 027) and were kindly provided by 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 that prevented growth on BHITY agar (9). Spontaneous mutants were recovered by plating aliquots of overnight cultures onto selective agars containing rifaximin at 4× the MIC. Mutations were identified in an ~200-bp 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 coinoculated 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× the rifaximin MIC and on nonselective BHITY agar (7, 8). W was calculated from $\ln[N_R(24)/N_R(0)]/\ln[N_S(24)/N_S(0)]$, 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 at 600 nm (OD_{600}) over 48 h at 37°C in a BioTek 2 microplate reader (10). Effects on virulence were assessed in the hamster model of *C. difficile* infection as described previously (11) using a spore inoculum of ~200 spores. Animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Texas at Arlington and adhered to the USDA Animal Welfare Act (9 CFR, parts 1 to 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 no. 4KMU) in the Schrödinger molecular modeling suite (12, 13). Changes in the relative binding affinities for rifaximin in the 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 the *Thermus thermophilus* X-ray crystal structure (PDB no. 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 root mean square deviation (RMSD) of 0.6 Å. Further method details may be found in the supplemental material.

In both strains, rifaximin resistance arose at a frequency of 10^{-8}, which is consistent with prior reports (16); rifaximin MICs against all mutants were >1,024 μg/ml, indicating that high-level rifaximin resistance is achievable in a single mutational step

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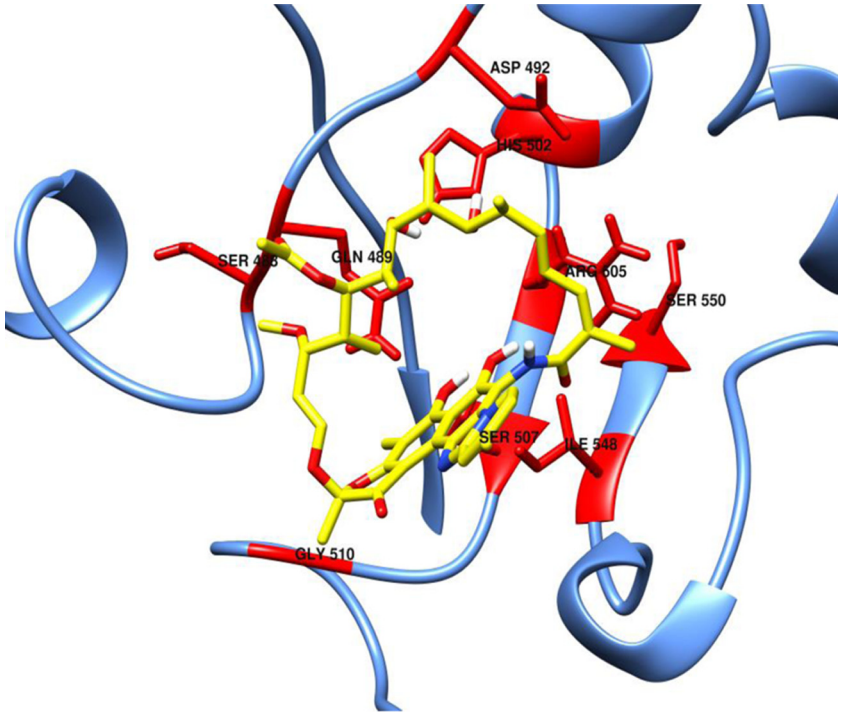

FIG 1 Model of *Cd*RpoB with bound rifaximin. Mutational sites conferring rifaximin resistance are shown in red. Rifaximin is shown with yellow carbon atoms.

(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).

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	>1,024	$Gln_{489}Leu$	0.84 ± 0.05	89.4 ± 4.1
CD43-D2	>1,024	$Asp_{492}Tyr$	0.80 ± 0.01	100.3 ± 9.2
CD43-D3	>1,024	$Asp_{492}Tyr$	0.80 ± 0.08	118.2 ± 5.1
CD43-D4	>1,024	$Asp_{492}Gly$	1.2 ± 0.12	92.3 ± 6.0
CD43-A1	>1,024	$His_{502}Tyr$	1.20 ± 0.13	ND[b]
CD43-A2	>1,024	$His_{502}Asn$	1.00 ± 0.13	ND
CD43-D5	>1,024	$Arg_{505}Lys$	0.99 ± 0.008	95.3 ± 10.1
CD43-D6	>1,024	$Arg_{505}Lys$	ND	110.0 ± 5.1
CD43-D7	>1,024	$Gly_{510}Arg$	0.85 ± 0.12	90.3 ± 5.5
CD43-D8	>1,024	$Ser_{488}Tyr$	0.85 ± 0.02	ND
CD43-D9	>1,024	$Ser_{550}Tyr$	0.67 ± 0.05	104.2 ± 18.4
CD43-D10	>1,024	$Ser_{550}Phe$	1.26 ± 0.13	ND
CD1769 (parent)	0.0625	None	1.00	97 ± 9.5
CD1769-D1	>1,024	$Asp_{492}Tyr$	ND	98.9 ± 9.2
CD1769-D2	>1,024	$His_{502}Arg$	0.91 ± 0.3	102.1 ± 10.7
CD1769-D3	>1,024	$Arg_{505}Lys$	1.02 ± 0.15	102.0 ± 4.4
CD1769-D4	>1,024	$Ser_{507}Leu$	0.57 ± 0.07	267.5 ± 37.1
CD1769-D5	>1,024	$Leu_{584}Phe$	1.24 ± 0.07	ND

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

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 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

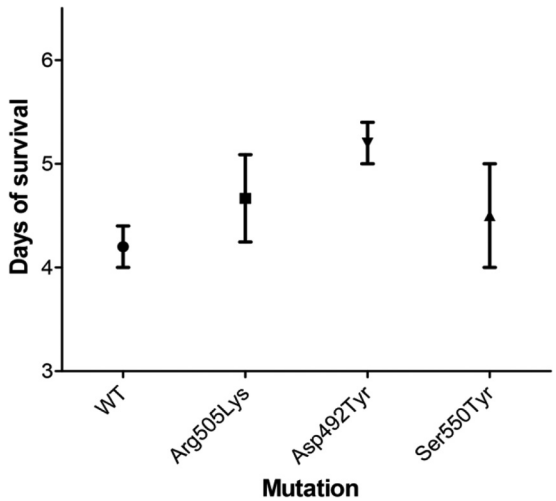

FIG 2 Comparison of virulence of rifaximin-resistant mutants in hamsters. WT, parent strain CD43; $Arg_{505}Lys$, mutant strain CD43-D5; $Asp_{492}Tyr$, mutant strain CD43-D3; $Ser_{550}Tyr$, mutant strain CD43-D9. No significant differences exist between the means as determined by one-way analysis of variance (ANOVA) ($P = 0.28$). The number of animals in each group was $n = 5$ 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.

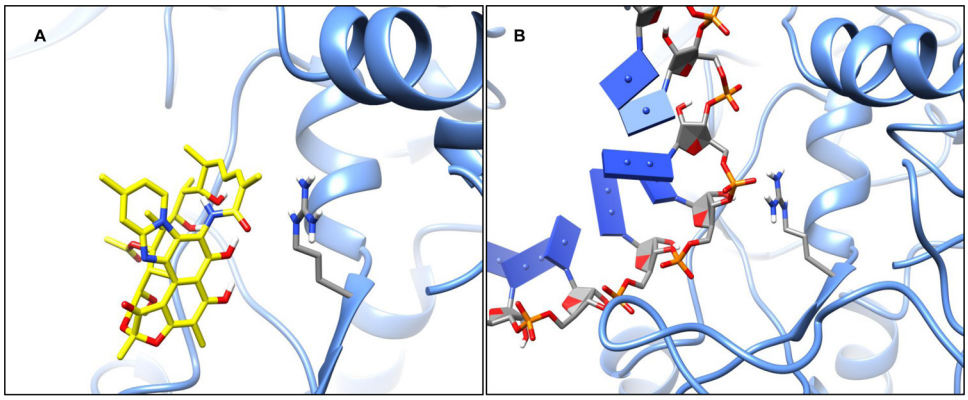

FIG 3 (A) *Cd*RpoB model with rifaximin bound, highlighting the interaction with arginine-505. (B) *Cd*RpoB model with DNA bound, highlighting the interaction with arginine-505.

the hamster model of *C. difficile* infection. Interestingly, the clinically occurring mutations $Asp_{492}Tyr$ and $Ser_{550}Tyr$ that imposed moderate (20%) and significant (33%) *in vitro* fitness costs did not appear to affect *in vivo* virulence (Fig. 2; see also Fig. S1 in the supplemental material). This may also imply that the hamster model of *C. difficile* infection 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 rifaximin (Fig. 3A). Therefore, a change to lysine-505 results in the 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 (see Fig. S2A in the supplemental material). This leads to a conformational change in the rifaximin binding site and an energetic cost between 20 and 30 kcal/mol (see Fig. S2B and C). Based on 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* (see Fig. S2D). The effects of other mutations on rifaximin binding are shown in Table S1 in the supplemental material.

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 *C. difficile* infection. The isolates recovered contained the change $Arg_{505}Lys$ and the double substitution $Ser_{488}Thr/Arg_{505}Lys$ or $Arg_{505}Lys/Ile_{548}Met$ (see Fig. 1 for amino acid sites relative to rifaximin). Interestingly, from the initial study period from 2001 to 2002 to the second study period in 2005, Curry et al. (3) observed a 10% decrease in the proportion of rifamycin-resistant *C. difficile* isolates, which they suggested was 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, which resulted 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* may be maintained in clinical settings even without selection pressure.

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