

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

Uyen T. Dang,^a Idalia Zamora,^a Kirk E. Hevener,^b Sudip Adhikari,^a Xiaoqian Wu,^c Julian G. Hurdle^{a,c,d}

Department of Biology, University of Texas at Arlington, Arlington, Texas, USA^a; Biomedical and Pharmaceutical Sciences, College of Pharmacy, Idaho State University, Meridian, Idaho, USA^b; Center for Infectious and Inflammatory Diseases, Institute of Biosciences and Technology, Texas A&M Health Science Center, Houston, Texas, USA^c; Department of Microbial and Molecular Pathogenesis, Texas A&M Health Science Center, College of Medicine, Bryan, Texas, USA^d

We characterized clinically occurring and novel mutations in the β subunit of RNA polymerase in *Clostridium difficile* (*CdRpoB*), conferring rifamycin (including rifaximin) resistance. The Arg₅₀₅Lys 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 *CdRpoB*. 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., Arg₅₀₅Lys) in *C. difficile* (*CdRpoB*) is the most common mutation among rifamycin-resistant clinical isolates (3, 5, 6). Other mutations in clinical isolates also occur at His₅₀₂, Ser₄₈₈, and Ser₅₅₀. 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 *CdRpoB* (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 \times the MIC. Mutations were identified in an \sim 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. 10⁴:10³ 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 \times 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 sen-

sitive 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 (OD₆₀₀) 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 \sim 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 *CdRpoB* 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 *CdRpoB* 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 *CdRpoB* homology model in the Schrödinger/Maestro alignment software (15). Next, the DNA subunit was transferred into the *CdRpoB* 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 μ g/ml, indicating that high-level rifaximin resistance is achievable in a single mutational step

Received 27 May 2016 Returned for modification 16 June 2016

Accepted 25 June 2016

Accepted manuscript posted online 5 July 2016

Citation Dang UT, Zamora I, Hevener KE, Adhikari S, Wu X, Hurdle JG. 2016. Rifamycin resistance in *Clostridium difficile* is generally associated with a low fitness burden. *Antimicrob Agents Chemother* 60:5604–5607. doi:10.1128/AAC.01137-16.

Address correspondence to Julian G. Hurdle, jhurdle@ibt.tamhsc.edu.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AAC.01137-16>.

Copyright © 2016, American Society for Microbiology. All Rights Reserved.

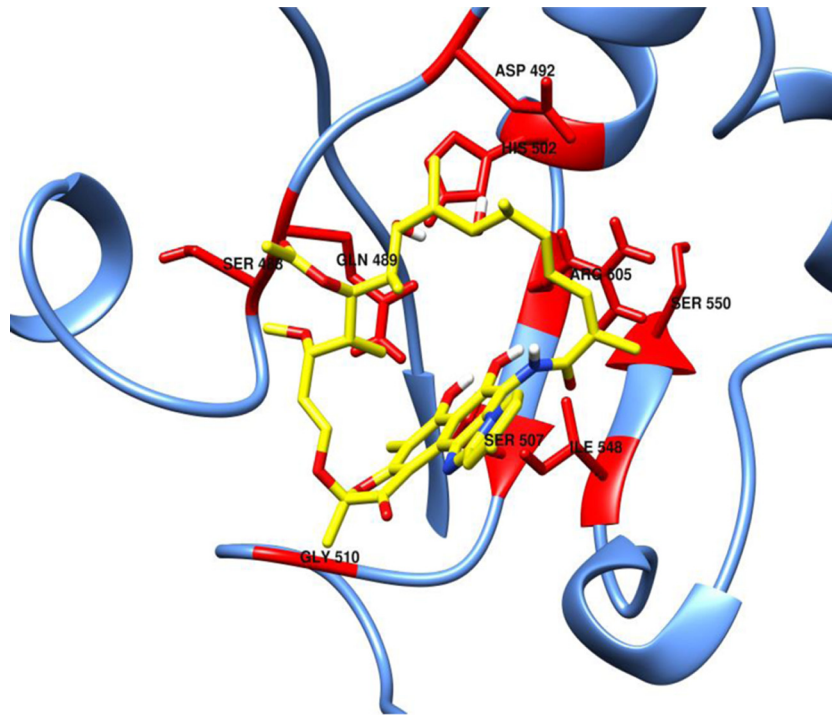


FIG 1 Model of *CdrPoB* with bound rifaximin. Mutational sites conferring rifaximin resistance are shown in red. Rifaximin is shown with yellow carbon atoms.

(Table 1). Most studies adopt a breakpoint of ≥ 32 $\mu\text{g/ml}$ to signify rifamycin resistance (5, 17). Several mutants possessed clinically occurring mutations, including His₅₀₂Asn, His₅₀₂Tyr, Arg₅₀₅Lys, Ser₄₈₈Tyr, Asp₄₉₂Tyr, Ser₅₅₀Phe, and Ser₅₅₀Tyr (3, 5, 6).

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

Strain	MIC ($\mu\text{g/ml}$)	Substitution	Fitness (<i>W</i>) ^a	Doubling time (min)
CD43 (parent)	0.125	None	1.00	97.3 \pm 4.8
CD43-D1	>1,024	Gln ₄₈₉ Leu	0.84 \pm 0.05	89.4 \pm 4.1
CD43-D2	>1,024	Asp ₄₉₂ Tyr	0.80 \pm 0.01	100.3 \pm 9.2
CD43-D3	>1,024	Asp ₄₉₂ Tyr	0.80 \pm 0.08	118.2 \pm 5.1
CD43-D4	>1,024	Asp ₄₉₂ Gly	1.2 \pm 0.12	92.3 \pm 6.0
CD43-A1	>1,024	His ₅₀₂ Tyr	1.20 \pm 0.13	ND ^b
CD43-A2	>1,024	His ₅₀₂ Asn	1.00 \pm 0.13	ND
CD43-D5	>1,024	Arg ₅₀₅ Lys	0.99 \pm 0.008	95.3 \pm 10.1
CD43-D6	>1,024	Arg ₅₀₅ Lys	ND	110.0 \pm 5.1
CD43-D7	>1,024	Gly ₅₁₀ Arg	0.85 \pm 0.12	90.3 \pm 5.5
CD43-D8	>1,024	Ser ₄₈₈ Tyr	0.85 \pm 0.02	ND
CD43-D9	>1,024	Ser ₅₅₀ Tyr	0.67 \pm 0.05	104.2 \pm 18.4
CD43-D10	>1,024	Ser ₅₅₀ Phe	1.26 \pm 0.13	ND
CD1769 (parent)	0.0625	None	1.00	97 \pm 9.5
CD1769-D1	>1,024	Asp ₄₉₂ Tyr	ND	98.9 \pm 9.2
CD1769-D2	>1,024	His ₅₀₂ Arg	0.91 \pm 0.3	102.1 \pm 10.7
CD1769-D3	>1,024	Arg ₅₀₅ Lys	1.02 \pm 0.15	102.0 \pm 4.4
CD1769-D4	>1,024	Ser ₅₀₇ Leu	0.57 \pm 0.07	267.5 \pm 37.1
CD1769-D5	>1,024	Leu ₅₈₄ Phe	1.24 \pm 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 Ser₅₀₇Leu, Gln₄₈₉Leu, Gly₅₁₀Arg, and Leu₅₈₄Phe.

With the exception of Ser₅₀₇Leu, Asp₄₉₂Tyr, and Ser₅₅₀Tyr, most mutations did not impose fitness costs on *C. difficile* (Table 1). *In vivo* studies indicated that the clinically occurring Arg₅₀₅Lys was as virulent as the wild type in terms of the time to mortality in

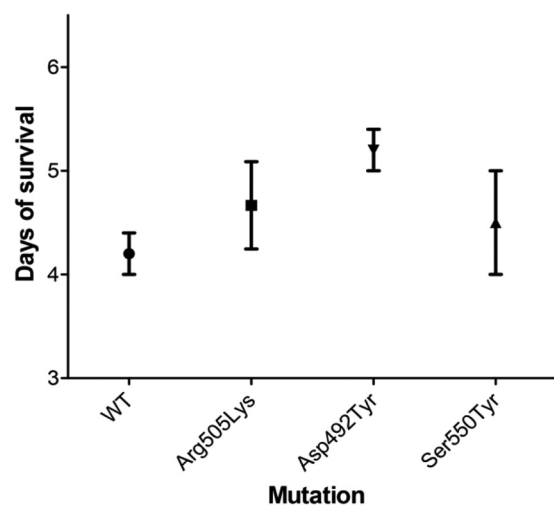


FIG 2 Comparison of virulence of rifaximin-resistant mutants in hamsters. WT, parent strain CD43; Arg₅₀₅Lys, mutant strain CD43-D5; Asp₄₉₂Tyr, mutant strain CD43-D3; Ser₅₅₀Tyr, mutant strain CD43-D9. No significant differences exist between the means as determined by one-way analysis of variance (ANOVA) ($P = 0.28$). The number of animals in each group was $n = 5$ for WT, $n = 6$ for Arg₅₀₅Lys, $n = 5$ for Asp₄₉₂Tyr, and $n = 4$ for Ser₅₅₀Tyr. CD43 mutants bearing His₅₀₂Asn and His₅₀₂Tyr strains were unavailable at the time of the experiments.

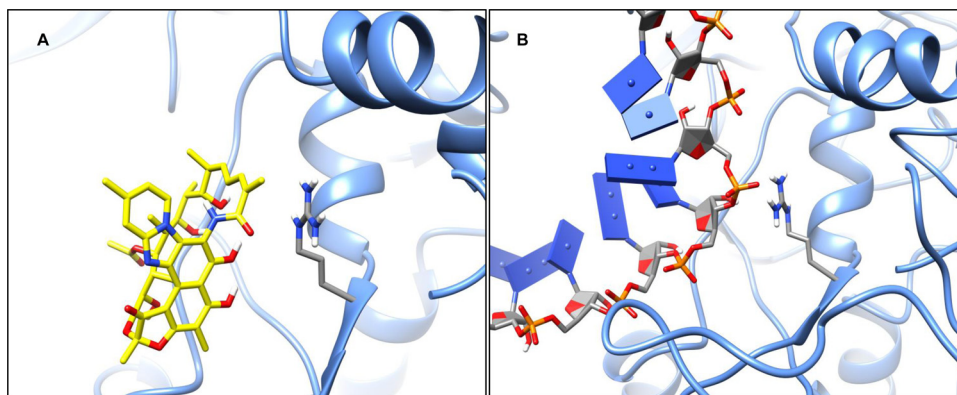


FIG 3 (A) *CdRpoB* model with rifaximin bound, highlighting the interaction with arginine-505. (B) *CdRpoB* model with DNA bound, highlighting the interaction with arginine-505.

the hamster model of *C. difficile* infection. Interestingly, the clinically occurring mutations Asp₄₉₂Tyr and Ser₅₅₀Tyr 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 *CdRpoB* 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 Arg₅₀₅Lys may correspond to minimal effects on DNA transcription. Similarly, histidine-502 mutations, including His₅₀₂Asn and His₅₀₂Tyr, 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 Arg₅₀₅Lys and the double substitution Ser₄₈₈Thr/Arg₅₀₅Lys or Arg₅₀₅Lys/Ile₅₄₈Met (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 His₅₀₂Tyr or His₅₀₂Tyr/Pro₄₉₆Ser 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 *CdRpoB* (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.

ACKNOWLEDGMENTS

Molecular graphics figures were generated with the University of California, San Francisco Chimera package. Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIGMS P41-GM103311).

J.G.H. acknowledges the receipt of funding from the National Center for Complementary and Integrative Health at the National Institutes of Health under grant 5R01AT006732. U.T.D. acknowledges the receipt of funding from the University of Texas System Louis Stokes Alliance for Minority Participation (LSAMP) and the American Society for Microbiology Undergraduate Research Fellowship (ASM-URF). K.E.H. acknowledges the ITHS Rising Stars Career Development Program at the University of Washington, which is supported by grants UL1TR000423, KL2TR000421, and TL1TR000422 from the NIH National Center for Advancing Translational Sciences through the Clinical and Translational Science Awards Program (CTSA).

FUNDING INFORMATION

This work, including the efforts of Kirk E. Hevener, was funded by HHS | NIH | National Center for Advancing Translational Sciences (NCATS) (UL1TR000423, KL2TR000421, and TL1TR000422). This work, including the efforts of Julian G. Hurdle, was funded by HHS | NIH | National Center for Complementary and Integrative Health (NCCIH) (5R01AT006732). This work, including the efforts of Uyen T. Dang, was funded by American Society for Microbiology (ASM).

REFERENCES

- Johnson S, Schriever C, Galang M, Kelly CP, Gerding DN. 2007. Interruption of recurrent *Clostridium difficile*-associated diarrhea epi-

- sodes by serial therapy with vancomycin and rifaximin. *Clin Infect Dis* 44:846–848. <http://dx.doi.org/10.1086/511870>.
2. Garey KW, Jiang ZD, Bellard A, Dupont HL. 2009. Rifaximin in treatment of recurrent *Clostridium difficile*-associated diarrhea: an uncontrolled pilot study. *J Clin Gastroenterol* 43:91–93. <http://dx.doi.org/10.1097/MCG.0b013e318144a4e97>.
 3. Curry SR, Marsh JW, Shutt KA, Muto CA, O'Leary MM, Saul MI, Pasculle AW, Harrison LH. 2009. High frequency of rifampin resistance identified in an epidemic *Clostridium difficile* clone from a large teaching hospital. *Clin Infect Dis* 48:425–429. <http://dx.doi.org/10.1086/596315>.
 4. Huang JS, Jiang ZD, Garey KW, Lasco T, Dupont HL. 2013. Use of rifamycin drugs and development of infection by rifamycin-resistant strains of *Clostridium difficile*. *Antimicrob Agents Chemother* 57:2690–2693. <http://dx.doi.org/10.1128/AAC.00548-13>.
 5. O'Connor JR, Galang MA, Sambol SP, Hecht DW, Vedantam G, Gerding DN, Johnson S. 2008. Rifampin and rifaximin resistance in clinical isolates of *Clostridium difficile*. *Antimicrob Agents Chemother* 52:2813–2817. <http://dx.doi.org/10.1128/AAC.00342-08>.
 6. Pecavar V, Blaschitz M, Hufnagl P, Zeininger J, Fiedler A, Allerberger F, Maass M, Indra A. 2012. High-resolution melting analysis of the single nucleotide polymorphism hot-spot region in the *rpoB* gene as an indicator of reduced susceptibility to rifaximin in *Clostridium difficile*. *J Med Microbiol* 61:780–785. <http://dx.doi.org/10.1099/jmm.0.041087-0>.
 7. Hurdle JG, O'Neill AJ, Ingham E, Fishwick C, Chopra I. 2004. Analysis of mupirocin resistance and fitness in *Staphylococcus aureus* by molecular genetic and structural modeling techniques. *Antimicrob Agents Chemother* 48:4366–4376. <http://dx.doi.org/10.1128/AAC.48.11.4366-4376.2004>.
 8. O'Neill AJ, Huovinen T, Fishwick CW, Chopra I. 2006. Molecular genetic and structural modeling studies of *Staphylococcus aureus* RNA polymerase and the fitness of rifampin resistance genotypes in relation to clinical prevalence. *Antimicrob Agents Chemother* 50:298–309. <http://dx.doi.org/10.1128/AAC.50.1.298-309.2006>.
 9. Wu X, Alam MZ, Feng L, Tsutsumi LS, Sun D, Hurdle JG. 2014. Prospects for flavonoid and related phytochemicals as nature-inspired treatments for *Clostridium difficile* infection. *J Appl Microbiol* 116:23–31. <http://dx.doi.org/10.1111/jam.12344>.
 10. Wu X, Hurdle JG. 2014. The *Clostridium difficile* proline racemase is not essential for early logarithmic growth and infection. *Can J Microbiol* 60:251–254. <http://dx.doi.org/10.1139/cjm-2013-0903>.
 11. Cherian PT, Wu X, Yang L, Scarborough JS, Singh AP, Alam ZA, Lee RE, Hurdle JG. 2015. Gastrointestinal localization of metronidazole by a lactobacilli-inspired tetramic acid motif improves treatment outcomes in the hamster model of *Clostridium difficile* infection. *J Antimicrob Chemother* 70:3061–3069. <http://dx.doi.org/10.1093/jac/dkv231>.
 12. Molodtsov V, Nawarathne IN, Scharf NT, Kirchhoff PD, Showalter HD, Garcia GA, Murakami KS. 2013. X-ray crystal structures of the *Escherichia coli* RNA polymerase in complex with benzoxazinorifamycins. *J Med Chem* 56:4758–4763. <http://dx.doi.org/10.1021/jm4004889>.
 13. Schrödinger. 2015. Biologics Suite 2015-3: Bioluminate, version 2.0. Schrödinger, LLC, New York, NY.
 14. Schrödinger. 2015. Schrödinger Release 2015-3: Prime, version 4.1. Schrödinger, LLC, New York, NY.
 15. Weixlbaumer A, Leon K, Landick R, Darst SA. 2013. Structural basis of transcriptional pausing in bacteria. *Cell* 152:431–441. <http://dx.doi.org/10.1016/j.cell.2012.12.020>.
 16. Marchese A, Salerno A, Pesce A, Debbia EA, Schito GC. 2000. *In vitro* activity of rifaximin, metronidazole and vancomycin against *Clostridium difficile* and the rate of selection of spontaneously resistant mutants against representative anaerobic and aerobic bacteria, including ammonia-producing species. *Chemotherapy* 46:253–266. <http://dx.doi.org/10.1159/000007297>.
 17. Carman RJ, Boone JH, Grover H, Wickham KN, Chen L. 2012. *In vivo* selection of rifamycin-resistant *Clostridium difficile* during rifaximin therapy. *Antimicrob Agents Chemother* 56:6019–6020. <http://dx.doi.org/10.1128/AAC.00974-12>.
 18. Hutton ML, Mackin KE, Chakravorty A, Lyras D. 2014. Small animal models for the study of *Clostridium difficile* disease pathogenesis. *FEMS Microbiol Lett* 352:140–149. <http://dx.doi.org/10.1111/1574-6968.12367>.
 19. Polivkova S, Krutova M, Petrlova K, Benes J, Nyc O. 2016. *Clostridium difficile* ribotype 176—a predictor for high mortality and risk of nosocomial spread? *Anaerobe* 40:35–40. <http://dx.doi.org/10.1016/j.anaerobe.2016.05.002>.