

Rifaximin Resistance in *Escherichia coli* Associated with Inflammatory Bowel Disease Correlates with Prior Rifaximin Use, Mutations in *rpoB*, and Activity of Phe-Arg- β -Naphthylamide-Inhibitable Efflux Pumps

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***Escherichia coli* is implicated in the pathogenesis of inflammatory bowel disease (IBD). Rifaximin, a nonabsorbable derivative of rifampin effective against *E. coli*, improves symptoms in mild-to-moderate IBD. However, rifaximin resistance can develop in a single step *in vitro*. We examined the prevalence and mechanisms of rifaximin resistance in 62 strains of *E. coli* isolated from the ileal mucosa of 50 patients (19 with ileal Crohn's disease [L1+L3], 6 with colonic Crohn's disease [L2], 13 with ulcerative colitis [UC], 4 with symptomatic non-IBD diagnoses [NI], and 8 healthy [H]). Resistance (MIC > 1,024 mg/liter) was present in 12/48 IBD-associated ileal *E. coli* strains. Resistance correlated with prior rifaximin treatment ($P < 0.00000001$) but not with the presence of ileal inflammation ($P = 0.73$) or *E. coli* phylogroup. Mutations in a 1,057-bp region of *rpoB*, which encodes the bacterial target of rifaximin, were identified in 10/12 resistant strains versus 0/50 sensitive strains ($P < 0.000000001$) and consisted of seven amino acid substitutions. The efflux pump inhibitor Phe-Arg- β -naphthylamide (PA β N) lowered the MIC of 9/12 resistant strains 8- to 128-fold. Resistance was stable in the absence of rifaximin in 10/12 resistant strains after 30 passages. We conclude that IBD-associated ileal *E. coli* frequently manifest resistance to rifaximin that correlates with prior rifaximin use, amino acid substitutions in *rpoB*, and activity of PA β N-inhibitable efflux pumps, but not with the presence of ileal inflammation or *E. coli* phylogroup. These findings have significant implications for treatment trials targeting IBD-associated *E. coli*.**

Inflammatory bowel disease (IBD) is considered a consequence of uncontrolled intestinal inflammation in response to a combination of environmental, microbial, and immunological factors in a genetically susceptible individual, although its exact etiology remains unclear (1–3). The gut microbiota is increasingly implicated in the pathogenesis of both Crohn's disease (CD) and ulcerative colitis (UC) (4, 5). In particular, the detection of *Escherichia coli* antigens and DNA in granulomas and peri-ulcerative lesions in CD (6), and circulating antibodies against *E. coli* outer membrane porin C (OmpC) in patients with CD suggests the involvement of *E. coli* in the pathogenesis of CD (7). Studies have found selective enrichment of *E. coli* relative to *Firmicutes* in patients with Crohn's ileitis (ICD), termed dysbiosis, and a correlation between the severity of ileal inflammation and the density of *E. coli* colonization (8). Dysbiosis has also been implicated in the pathogenesis of UC, with an increase in *Escherichia* spp., *Rhodococcus* spp., and *Shigella* spp., relative to a decrease in anti-inflammatory *Lactobacillus* spp. and *Pediococcus* spp. in UC patients (9).

The potential role of gut microbes in IBD provides a rationale for antibiotic treatment, and drugs such as metronidazole, ciprofloxacin, and clofazimine have been used in the treatment of CD (10, 11), UC (12), and pouchitis (4). However, the success rate of antibiotic treatment varies dramatically with the different forms of IBD, and there is a lack of precise therapeutic guidelines (13). Moreover, IBD treatment involves long-term use of these antibiotics, which promotes emergence of resistance and leads to a poor patient tolerability profile due to adverse systemic side effects such as peripheral neuropathy, nausea, and diarrhea (13, 14). In light of

such concerns, the gut-selective antibiotic rifaximin has recently garnered much attention as a therapeutic option in IBD. Rifaximin is a semisynthetic derivative of rifampin with broad-spectrum *in vitro* activity against aerobic and anaerobic, Gram-positive and Gram-negative bacteria. It is virtually unabsorbed in the intestinal tract after oral administration (<0.4%) due to its pyridimidazole ring, and remains largely localized in the gastrointestinal tract, avoiding systemic circulation (15, 16). In the United States, it was originally approved for the treatment of uncomplicated Travelers' diarrhea (TD) and is currently used for treating disorders such as hepatic encephalopathy, small bowel bacterial overgrowth, irritable bowel syndrome, and IBD (15).

A growing number of studies have found rifaximin to be effective in the treatment of IBD. A multicenter, double-blind, placebo-controlled trial involving 83 patients with mild-to-moderate CD found that monotherapy with rifaximin at 800 mg for 12 weeks was superior to placebo in promoting clinical remission,

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only in patients with baseline concentrations of C-reactive protein above the upper reference limit (4, 17). A recent study involving 402 patients with moderately active CD found that administration of 800 mg of rifaximin-EIR (extended intestinal release) formulation twice daily for 12 weeks induced remission with few adverse events (18). Other studies have found rifaximin to be effective in the treatment of UC and antibiotic-dependent pouchitis (19–21). In light of such positive results, rifaximin is increasingly being prescribed for IBD treatment. However, the specific factors that determine positive and negative responses of a patient with IBD to rifaximin remain to be determined.

One factor that is likely to impact the ability of a patient to respond to rifaximin is the presence of antimicrobial resistance. Rifaximin, like other rifamycins, exerts its antimicrobial effects by binding to the β -subunit of bacterial DNA-dependent RNA polymerase and blocking the path of the elongating RNA transcript at the 5' end, thus inhibiting transcription (22). Point mutations in the *rpoB* gene, which encodes for the β subunit, have been implicated in resistance to rifaximin and its parent compound rifampin (23). Ninety-five percent of mutations associated with resistance to rifamycins map to four regions of *rpoB*: the N-terminal cluster (codons 143 to 148) and clusters I (codons 505 to 537), II (codons 563 to 575), and III (codons 684 to 690) (16, 22, 24, 25). Another potential mechanism implicated in resistance to rifaximin is activity of bacterial multidrug resistance (MDR) efflux pump systems that are capable of extruding chemically unrelated compounds, thus lowering the intracellular drug concentration (26). Phe-Arg- β -naphthylamide (PA β N) is a known inhibitor of MDR efflux pumps and has been shown to lower the MIC of rifaximin and rifampin in both resistant and sensitive *E. coli* strains *in vitro* (16, 27, 28).

We have previously reported that resistance to antimicrobials, including rifaximin, is common (71% in ICD patients) in *E. coli* associated with Crohn's ileitis (ICD) (1). In the present study, we investigated the molecular mechanisms of rifaximin resistance, and the association of resistance with *E. coli* phylogenetic group, prior rifaximin use, and the presence of ileal inflammation in *E. coli* isolated from the ileal mucosa of ICD, colonic CD (CCD), UC, symptomatic non-IBD (NI), and healthy (H) patients. The CCD, UC, and NI patients were included as a control group that did not present ileal inflammation but were likely to have received rifaximin treatment. The healthy patients were included as a control group that had neither ileal inflammation nor prior rifaximin treatment. Using this diverse group of patients, we found that resistance to rifaximin correlates with prior rifaximin use, amino acid substitutions in *rpoB*, and the activity of PA β N-inhibitable efflux pumps but not with the presence of ileal inflammation or *E. coli* phylogroup. Additionally, we found that resistance remains stable in the absence of rifaximin in 10/12 resistant strains over 30 passages. These findings have significant implications for treatment trials targeting IBD-associated *E. coli*.

MATERIALS AND METHODS

Patients and bacterial strains. We examined resistance to rifaximin in 62 *E. coli* strains isolated from the ileal mucosa of 50 patients. Patients with CD were categorized according to the Montreal Classification (29) as L1 (disease restricted to ileum), L2 (colonic involvement only), and L3 (ileal and colonic involvement). In the study group (patients showing ileal inflammation), 25 strains were previously obtained from 19 ICD (L1+L3) patients (6 with a history of rifaximin treatment) (1). In the control group

(patients without ileal inflammation), 9 strains were isolated from the ileum of 6 CCD (L2) patients (1 with history of rifaximin treatment), 14 strains were isolated from the ileum of 13 patients with UC (3 with history of rifaximin treatment), 4 strains were previously obtained from 4 patients with non-IBD (NI) related conditions (1 with history of rifaximin treatment) (1), and 10 strains were previously isolated from 8 healthy (H) patients (no known history of rifaximin use) (1). The 4 NI patients were investigated for signs of gastrointestinal disease with a final diagnosis not consistent with IBD (celiac disease [$n = 1$], *H. pylori* [$n = 1$], tubular adenoma [$n = 1$], lactose intolerance [$n = 1$]). The 10 H patients were undergoing surveillance endoscopy (with a history of polyps [$n = 3$], colorectal cancer [$n = 3$], and age-based surveillance endoscopy [$n = 4$]).

In all patients, the terminal ileum was intubated as part of the T1 standard of care. A single ileal biopsy was taken from each patient with standard single-use sterile endoscopic forceps into a sterile cryovial containing Luria-Bertani (LB) broth and glycerol (20%) on ice and stored at -80°C until processing. Ten *E. coli* colonies from each biopsy were screened by random amplified polymorphic DNA (RAPD)-PCR with informative primer 1283, and representative isolates from each individual that differed in overall genotype were selected for subsequent analyses (30). The major *E. coli* phylogenetic groups (A, B1, B2, and D) were determined by triplex PCR (1, 31). This study was approved by the Cornell University Committee on Human Subjects (protocol 05-05008). All patients gave signed informed consent to participate and to provide mucosal biopsies to the Tissue Bank (protocol 0603-859).

Determination of MIC of rifaximin. The MIC of rifaximin (Sigma-Aldrich, St. Louis, MO, USA) was determined by the agar dilution method using a breakpoint of 32 mg/liter between sensitive (≤ 32 mg/liter) and resistant (> 32 mg/liter) strains (32).

Mutations in *rpoB*. The presence of mutations in *rpoB* was determined by PCR amplification of a 1,057-bp long region, which includes clusters I, II, and III. The following primers and reaction conditions were used: 5'-AAG CTC ATC GAT ATC CGT AAC G-3' and 5'-GCA CGT CGC CAC GTT CAA CC-3'; 30 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, followed by elongation at 72°C for 10 min (16). The N-terminal cluster (codons 143 to 148) of *rpoB* was sequenced using the following primers: 5'-CTG CGC GTT AAA CTG CGT CTG GTG-3' and 5'-CAA CCG GGA CTT CGA TCA GTT TGA-3'. PCR was carried out in a MasterCycler Gradient (Eppendorf, Germany), and the products obtained were purified using a QIAquick PCR purification kit (Qiagen, Germany). PCR products were sequenced at the Cornell University Life Sciences Core Laboratories Center (Ithaca, NY, USA) and aligned with a reference sequence (*E. coli* K-12, substrain MG1655; GenBank accession no. NC_000913.2), using MegAlign (DNASTAR, Madison, WI, USA).

Determination of the role of bacterial efflux pumps. To determine the role of MDR efflux pumps in the development of resistance, the MIC of rifaximin in the presence or absence of the efflux pump inhibitor PA β N (Sigma-Aldrich, St. Louis, MO, USA) was measured using the agar dilution method (32). Cultures were grown in two sets of eight Mueller-Hinton agar plates each, one with rifaximin and the other with rifaximin plus PA β N (20 mg/liter). Rifaximin concentrations ranged from 8 to 1,024 mg/liter. A rifaximin stock solution of 50 mg/ml in methanol was used.

Stability of resistance to rifaximin. Resistant strains were streaked daily on LB agar plates in the absence of rifaximin selective pressure for 30 consecutive passages, representing 2,160 bacterial generations. Every 10 days, the MIC of rifaximin was determined for all passaged strains using the agar dilution method (32).

Statistical analysis. To identify differences between groups, the Fisher exact test (two-tailed) was used. $P < 0.05$ was considered statistically significant.

RESULTS

Resistance patterns. Resistance to rifaximin was present in 12/62 *E. coli* strains isolated from the ilea of 11/50 patients examined in

TABLE 1 Patterns of rifaximin resistance^a

Parameter ^b	Ileal inflammation present		Ileal inflammation absent						Total		
	CD (L1+L3)		CD (L2)		UC		NI			H	
Prior rifaximin treatment	Y	N	Y	N	Y	N	Y	N	Y	N	
No. of patients with resistant <i>E. coli</i> /total patients	5/6	0/13	2/2	0/4	4/4	0/9	0/1	0/3	0/0	0/8	11/50
No. of resistant <i>E. coli</i> strains/total no. of strains	6/7	0/18	2/2	0/7	4/4	0/10	0/1	0/3	0/0	0/10	12/62

^a Diagnoses: CD, Crohn's disease; L1+L3, ileal+ileocolonic; L2, colonic; UC, ulcerative colitis; NI, symptomatic non-IBD; H, healthy. Y, yes; N, no.

^b Resistance correlates with prior rifaximin treatment ($P < 0.00000001$) but not with the presence of ileal inflammation ($P = 0.73$).

the study (Table 1). All resistant strains displayed an MIC of $>1,024$ mg/liter, while sensitive strains displayed an MIC of 0.125 to 16 mg/liter. A total of 13/50 patients had received prior rifaximin treatment, 11 of whom showed presence of resistant strains; in the 2/13 patients who did not show presence of resistant strains, the treatment duration was <2 weeks, which may not be sufficient time to select for resistant strains. Overall, resistance showed a strong correlation with prior rifaximin treatment ($P < 0.00000001$), but not with the presence of ileal inflammation ($P = 0.73$) (Table 1). In patients without ileal inflammation (L2, UC, and NI), resistance correlated strongly with prior rifaximin treatment ($P < 0.000001$) as well. Among patients with rifaximin-resistant *E. coli*, treatment duration ranged from 13 days to 26 months. Rifaximin-resistant strain 60CM-1 was isolated 131 days after the last rifaximin dose, suggesting that resistance is persistent without continued selection pressure. The *E. coli* phylogenetic group was not associated with resistance (A, 4/15; B1, 4/17; B2, 2/17; D, 2/11).

Mutations in *rpoB*. Mutations in *rpoB* were identified in 10/12 resistant strains versus 0/50 sensitive strains ($P < 0.000000001$) (Table 2). In all but one strain, mutations were encoded by single amino acid substitutions at residues 513, 516, 518, 526, and 574 of the β -subunit of RNA polymerases; strain 58PP-1 was found to have two substitutions, at positions 513 and 574. Overall, seven

different amino acid substitutions were identified: Q513H (one strain), D516N (two strains), N518D (one strain), H526N (three strains), H526L (one strain), S574F (two strains), and S574Y (one strain) (Fig. 1). All polymorphisms were present in clusters I and II of *rpoB*; none were detected in the N-terminal cluster and cluster III.

Role of efflux pumps. The efflux pump inhibitor PA β N lowered the MIC of 75% (9/12) of resistant strains between 8- and 128-fold, rendering six strains sensitive with MIC of 8 to 32 mg/liter (Table 2). PA β N did not display any effect on the MIC of three resistant strains. In two of the nine strains affected by PA β N, no mutations in *rpoB* were detected.

Stability of resistant strains. After 30 passages in culture, 10/12 strains remained resistant (an MIC of $>1,024$ mg/liter) to rifaximin. The exceptions were strain 75TI-1, in which the MIC dropped from $>1,024$ to 128 mg/liter after 20 passages, and 58PP-1, in which the MIC dropped from $>1,024$ to 16 mg/liter after 10 passages. The basis of this change in MIC was investigated by sequencing the same 1,057-bp region of *rpoB*, which revealed that the Q513H and S574F substitutions in 58PP-1 had reverted back to wild type. In contrast, the N518D substitution was still present in 75TI-1, and the strain had maintained an active efflux mechanism with PA β N lowering the MIC of passaged 75TI-1 from 128 to <8 mg/liter, suggesting that a decrease in the activity

TABLE 2 Molecular mechanisms of rifaximin resistance^a

Diagnosis	Patient ID	<i>E. coli</i> strain ID	Rifx treatment		Strain type	MIC Rifx (mg/liter)	MIC Rifx + PA β N (mg/liter)	Mutations in <i>rpoB</i>	
			Duration (mo)	Time between last Rifx dose and ileal biopsy (days)				Codon	Amino acid substitution
CD (L2)	524	524-2	2.6	27	R	$>1,024$	128	526	H \rightarrow N
	14	14GK-1	24.5	0	R	$>1,024$	64	526	H \rightarrow N
CD (L1+L3)	75	75TI-1	5	6	R	$>1,024$	8	518	N \rightarrow D
	552	552-P4	5	0	R	$>1,024$	8	None	None
	552	552-2	5	0	R	$>1,024$	8	None	None
	24	24LW-1	16	0	R	$>1,024$	$>1,024$	516	D \rightarrow N
	32	32SY-1	26	0	R	$>1,024$	32	574	S \rightarrow F
	578	578-1	0.6	0	R	$>1,024$	128	516	D \rightarrow N
UC	40	40EM-1	0.4	0	R	$>1,024$	$>1,024$	526	H \rightarrow L
	48	48JD-1	Unknown	0	R	$>1,024$	32	526	H \rightarrow N
	58	58PP-1	7	0	R	$>1,024$	$>1,024$	513, 574	Q \rightarrow H, S \rightarrow F
	60	60CM-1	13	131	R	$>1,024$	8	574	S \rightarrow Y
CD (L1+L3)	541	541-1	NT	NT	S	32	8	None	None
	541	541-15	NT	NT	S	32	8	None	None
	41	41CB-1	NT	NT	S	16	8	None	None

^a Rifx, rifaximin; NT, no rifaximin treatment; R, resistant; S, sensitive; rifaximin resistant, MIC > 32 mg/liter; rifaximin sensitive, MIC ≤ 32 mg/liter.

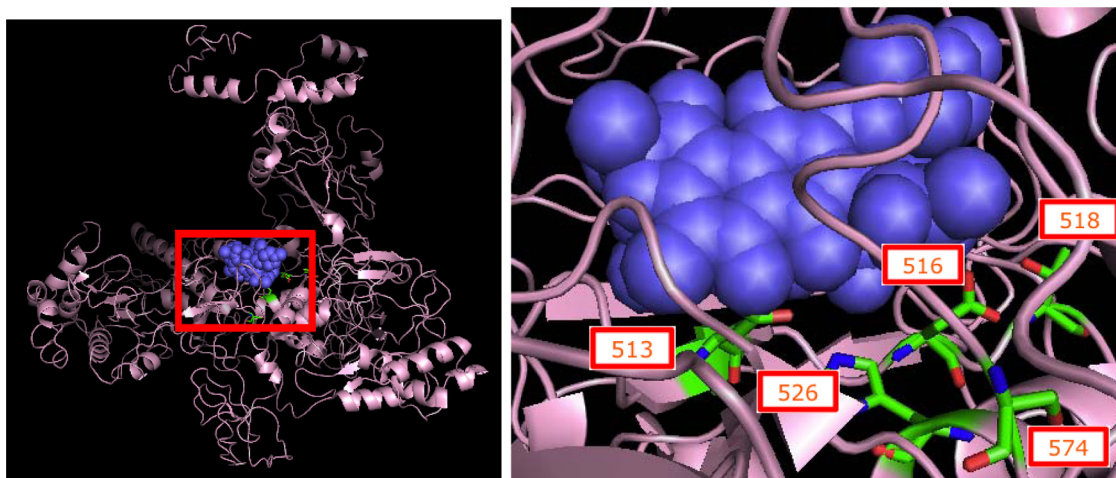


FIG 1 Mutated residues in the RNA polymerase β -subunit. (Left panel) *Thermus aquaticus* RNA polymerase β -subunit (pink diagram) with bound rifampin (purple spheres), the parent compound of rifaximin. (Right panel) A magnified view of bound rifampin and the *T. aquaticus* equivalents of the 5 amino acid codons (shown as RBG stick models) that were found to be mutated in our rifaximin resistant *E. coli* strains. All five codons occur close to the binding pocket of rifampin; 3/5 codons (513, 516, and 526) are known to interact directly with the antibiotic and affect its binding (22). Image of 116V (22) created using The PyMOL Molecular Graphics System (version 1.5.0.4; Schrödinger, LLC).

or expression level of PA β N-inhibitable efflux pumps was responsible for the decreased MIC of passaged 75TI-1. RAPD-PCR analysis of both passaged and nonpassaged parent strains with informative primer 1283 yielded identical banding patterns indicating the absence of substantial mutations and contamination during passage (30).

DISCUSSION

A growing number of studies have found rifaximin to be effective in the treatment of IBD, and it is increasingly prescribed for the treatment of CD and UC (4). However, the specific factors that determine the clinical response of a patient with IBD to rifaximin remain to be determined. One factor that can impact the ability of an individual to respond to rifaximin is the presence of antimicrobial resistance. The results of the present study indicate that *E. coli* isolated from the ileum of 11/38 (29%) patients with IBD (5/19 = 26% of L1+L3 patients, 2/6 = 33% of L2 patients, and 4/13 = 31% of UC patients) exhibit resistance to rifaximin with an MIC of >1,024 mg/liter. The presence of resistance correlates strongly with prior rifaximin treatment but not with the presence of ileal inflammation or with *E. coli* phylogroup. The observation of similar levels of resistance in the study group (with ileal inflammation: L1+L3) and the control group (without ileal inflammation: L2, UC, and NI) suggests that ileal inflammation does not play a role in selecting for rifaximin-resistant *E. coli*. Conversely, the strong correlation between resistance and prior rifaximin treatment in both the study group and the control group suggests that exposure to rifaximin selects for resistant *E. coli* independent of ileal inflammation.

Mutations in *rpoB* alone accounted for resistance in 3/12 resistant strains, activity of PA β N-inhibitable efflux pumps alone accounted for resistance in 2/12 strains, while a combined effect of both mechanisms accounted for resistance in the majority (7/12) of resistant strains. Thus, our findings suggest that both mutations in *rpoB* and the activity of PA β N-inhibitable efflux pumps, occurring together or independently, contribute to the development of rifaximin resistance. These results are consistent with a previous

study that examined *in vitro*-selected rifaximin-resistant *E. coli* mutants associated with traveler's diarrhea (16).

Mutations in *rpoB* have been widely reported in the development of resistance to rifamycins. We identified seven distinct polymorphisms occurring at five residues of the β -subunit of RNA polymerase in 10/12 resistant strains versus 0/50 sensitive strains. Four of these polymorphisms (Q513H, D516N, H526N, and H526L) occur at residues that are known to interact directly with rifampin, and substitutions at these have been shown to confer resistance to rifamycins (Fig. 1) (22, 33). Among these four polymorphisms, D516N (in *E. coli*) and H526L (in *Mycobacterium tuberculosis*) are known to be involved in the development of rifampin resistance but, to our knowledge, the specific Q513H and H526N changes have not previously been reported in the literature (22, 24). Of the three polymorphisms that do not directly interact with the drug, N518D has previously been identified in rifampin-resistant *E. coli*. Although residue 518 does not interact directly with the antibiotic, it is present in the drug's binding pocket and may indirectly affect binding to the β -subunit (22, 24). S574Y has previously been implicated in the development of rifaximin resistance *in vitro* in *E. coli*, even though this residue is not known to affect rifamycin binding directly or indirectly (16, 22, 34). S574F has been reported in the literature as a secondary mutation in a serially passaged rifampin-resistant strain carrying a primary mutation at another *rpoB* residue (34).

In addition to these well-characterized mutations in *rpoB*, our findings suggest that the activity of PA β N-inhibitable efflux pumps contributes to the development and maintenance of rifaximin resistance. PA β N is a broad-spectrum inhibitor of MDR efflux pumps in a variety of Gram-negative bacteria and is believed to exert its effects by inhibiting resistance nodulation-cell division (RND) type efflux pumps, although this proposition has been called into question by several studies (28, 35, 36). Our results replicate previous observations that PA β N lowers the MIC of rifampin and rifaximin *in vitro* in both resistant and sensitive *E. coli* strains (16, 27). PA β N lowered the MIC of 9/12 resistant strains 8

to 128 fold, rendering six strains sensitive to rifaximin (MIC, 8 to 32 mg/liter), while the other three strains exhibited low-level resistance (64 to 128 mg/liter); PA β N lowered the MIC of three sensitive strains 1- to 2-fold. In the two resistant strains with no mutations in *rpoB* (552-P4 and 552-2), PA β N lowered the MIC from >1,024 to 8 mg/liter, indicating that activity of PA β N-inhibitable efflux pumps can independently induce high-level rifaximin resistance. Intriguingly, both these strains were isolated from the same patient.

In 7/12 resistant strains both mechanisms of resistance were observed, raising questions about the extent to which each mechanism contributes to the development of resistance. Since PA β N lowers the MIC of sensitive strains as well, it appears that efflux pump mechanisms are constitutively active in all *E. coli* strains. It seems likely that resistant strains overexpress these efflux pumps, which may partially confer their resistant phenotype, together with *rpoB* polymorphisms that reduce the binding affinity of rifaximin for the β -subunit of RNA polymerase.

The finding that resistance remained stable in 10/12 strains after 30 consecutive passages in the absence of rifaximin selective pressure, and the observation that rifaximin resistant *E. coli* were isolated from a patient even after 131 days from the last rifaximin dose are in agreement with previous studies that have found rifaximin resistance to be generally stable (15, 23). However, a study evaluating the effect of rifaximin on commensal flora, including *E. coli* found that the majority of resistant strains regained sensitivity after discontinuing treatment (37). Of the two strains in our study where resistance did not remain stable, 75TI-1 showed a drop in MIC from >1,024 to 128 mg/liter after 20 passages even though the N518D mutation remained intact. However, since PA β N lowered the MIC of this strain from 128 to <8 mg/liter, it appears that activity of PA β N-inhibitable efflux pumps, rather than the N518D substitution, is the primary mechanism of resistance in this strain. In contrast, the drop in MIC of strain 58PP-1, from >1,024 to 16 mg/liter after 10 passages, was associated with reversion of the Q513H and S574F substitutions to wild type and not with the inhibitory effect of PA β N, suggesting that these mutations independently confer the high-level rifaximin resistance observed in this strain. To determine the functional effect of mutations in *rpoB* and distinguish it from the effect of PA β N-inhibitable efflux pumps, future experiments could examine the transcriptional activity of RNA polymerases isolated from resistant strains in the presence of various concentrations of rifaximin using a cell-free transcription assay.

Although the present study has focused on polymorphisms in *rpoB* and activity of PA β N-inhibitable efflux pumps, it is possible that there are other mechanisms contributing to resistance such as efflux pumps not inhibited by PA β N, structural modifications of rifaximin by bacteria, or a reduction in membrane permeability to the antibiotic. Studies on rifaximin resistance in *in vitro*-selected *E. coli* associated with Traveler's diarrhea (TD) have found no difference in the expression levels of outer membrane porin (Omp) proteins between resistant and sensitive strains (16). On the other hand, studies in *Bifidobacterium* have implicated reduction in membrane permeability due to specific changes in fatty acid composition, but not structural modifications of the antibiotic moiety by bacterial enzymatic activities in the development of rifaximin resistance (25, 38).

Although development of *in vivo* resistance to rifaximin during therapy has been reported in *Clostridium difficile* (39), studies in-

vestigating rifaximin resistance in TD-associated *E. coli* have generally found no clinically significant resistance (15, 40–42). However, one must note that therapy for TD is usually short-term, lasting 3 to 5 days, while therapy for IBD is long-term and may last for months or even years, creating a substrate for resistance development. Another important consideration in interpreting the results of this study is determining the clinical significance of the findings as rifaximin reaches concentrations of up to 8 mg/g of feces after 3 days of treatment with 800 mg daily (43). For most antibiotics, resistance breakpoints are set by CLSI guidelines based on the drug's plasma concentration. Since, rifaximin is largely unabsorbed and remains localized in the gut, there is no formal resistance breakpoint for it. Most studies, including ours, use a breakpoint of 32 mg/liter between sensitive (\leq 32 mg/liter) and resistant (>32 mg/liter) strains, although the manufacturers Alfa Wasserman recommend a resistance breakpoint of 64 mg/liter (15). Using either breakpoint, the strains described in this study are highly resistant with an MIC of >1,024 mg/liter and were isolated from patients in whom we would anticipate luminal rifaximin concentrations were in the range of 8 mg/g of feces and can therefore be considered clinically resistant.

The present study reveals valuable insights about the mechanisms and patterns of rifaximin resistance in a patient group with diverse disease phenotypes, although the broad applicability of our results is limited by the absence of information on temporal associations and dosing practices that may promote rifaximin resistance. In our study, resistance emerged in one patient after just 13 days of rifaximin treatment and was found to be stable in another patient even after 131 days from the last rifaximin dose. Since observations from two patients do not allow us to reach any broadly applicable conclusions, future studies should investigate rifaximin resistance in clinical settings where bacterial samples are obtained from IBD patients before, during, and after commencing antibiotic therapy in order to determine the temporal association between rifaximin therapy and resistance development.

Although the present study informs our knowledge of rifaximin resistance, it is clear that the mechanisms of action and clinical pharmacology of rifaximin in patients with IBD remain to be fully elucidated. Despite the high enteric concentrations rifaximin achieves, its bioavailability may be greatly limited by its hydrophobic nature and consequent insolubility in aqueous media (10, 11). This situation is further complicated by the increased solubility of rifaximin in the presence of bile acids, suggesting that it may have greater bioavailability and antimicrobial activity in the bile acid-rich small bowel than in the largely aqueous media of the colon (44). This may explain the absence of changes in the composition of the colonic microbiota, despite positive therapeutic responses in IBD patients (15, 45), and suggests differential responses to rifaximin may occur in patients with ileal versus colonic inflammation.

In conclusion, the present study clearly demonstrates that resistance to rifaximin in ileal IBD-associated *E. coli* correlates with prior rifaximin treatment, amino acid substitutions in *rpoB*, and activity of PA β N-inhibitable efflux pumps, but not with the presence of ileal inflammation and *E. coli* phylogroup. These findings have significant implications for treatment trials targeting IBD-associated *E. coli* and suggest that antimicrobial trials in IBD that are informed by knowledge of disease phenotype and antimicrobial susceptibility are more likely to demonstrate valid outcomes than those based on nonstratified empirical approaches.

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