

# Two Multidrug-Resistant Clinical Isolates of *Bacteroides fragilis* Carry a Novel Metronidazole Resistance *nim* Gene (*nimJ*)

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**Two multidrug-resistant *Bacteroides fragilis* clinical isolates contain and express a novel *nim* gene, *nimJ*, that is not recognized by the “universal” *nim* primers and can confer increased resistance to metronidazole when introduced into a susceptible strain on a multicopy plasmid. HMW615, an appendiceal isolate, contains at least two copies of *nimJ* on its genome, while HMW616, an isolate from a patient with sepsis, contains one genomic copy of *nimJ*. *B. fragilis* NimJ is phylogenetically closer to *Prevotella baroniae* NimI and *Clostridium botulinum* NimA than to the other known *Bacteroides* Nim proteins. The predicted protein structure of NimJ, based on fold recognition analysis, is consistent with the crystal structures derived for known Nim proteins, and specific amino acid residues important for substrate binding in the active site are conserved. This study demonstrates that the “universal” *nim* primers will not detect all *nim* genes with the ability to confer metronidazole resistance, but *nimJ* alone cannot account for the very high metronidazole MICs of these resistant clinical isolates.**

*Bacteroides* spp. are Gram-negative anaerobic bacteria that are usually present as a commensal in the human and animal gut microbiomes. A recent metagenomic analysis (1–3) reported that *Bacteroides* species account for 27.8% (mean) of the gut bacteria with a broad standard deviation (16.6%) and range (0.1 to 64.9%) (2). The metagenomic studies (3) also support the earlier reports that the species *Bacteroides fragilis* is a relatively small proportion of the gut *Bacteroides* (4–6), although identification to the species level, data collection, and analyses have become much more complex. When *B. fragilis* moves out of its niche in the gut, it becomes an opportunistic pathogen and can cause serious infection. *B. fragilis* has been implicated in almost every type of infection and is the anaerobe most frequently isolated from patients with intra-abdominal sepsis, necrotizing skin, perforated and gangrenous appendicitis, and soft tissue infections (1, 7).

*B. fragilis* is inherently resistant to a wide variety of drugs (1). *B. fragilis* strains that are resistant to previously effective agents are being isolated with increasing frequency and are challenging current therapeutic regimens. These strains are often associated with adverse outcomes, including increased morbidity and mortality. To date, metronidazole is among the few drugs still considered reliable for the treatment of *B. fragilis* infections and is the most commonly prescribed drug worldwide for this purpose (8). In the last several years, however, metronidazole-resistant strains have been isolated more frequently and have often been associated with adverse outcome, including death (9) or amputation (10). Metronidazole was first introduced against protozoal infections in the middle of the 20th century (11). As administered, it is an inactive prodrug, and activation requires the partial reduction of the nitro group (12, 13) in metronidazole to the toxic nitroso radical intermediate that then binds to DNA, causing single- and double-stranded DNA breakage (14). Most pathogens do not have this activation mechanism and are thus intrinsically resistant. In sensitive organisms, “active” metronidazole resistance is often attributed to the nitroimidazole resistance gene (*nim*). While the exact mechanism is not known, the generally accepted premise is that *nim* codes for a nitroimidazole reductase that reduces the nitro group of 4- or 5-nitroimidazole to an amino group to make the

inactive compound 5-aminoimidazole, thus avoiding the formation of toxic nitroso radicals that are essential for antimicrobial activity (15). The *nim* homologs are found in both Gram-positive and -negative genera of aerobic and anaerobic bacteria and archaea, suggesting that the *nim* gene family is ancient and widespread. The *nim* genes can be found on the chromosome, or more significantly, on mobilizable plasmids that pose a significant threat to the continuing utility of 5-nitroimidazole drugs.

Molecular detection of *nim* genes in *Bacteroides* isolates was described in 1996 using specific primers that were assumed to detect conserved sequences in all of the *nim* gene types (16). Identification of the *nim* type was accomplished by restriction analysis of the amplicon produced by these primers with Hsp92II, resulting in unique banding patterns for *nimA* to *-F* (17). To date, eight *nim* genes (*nimA* to *-H*) have been described in *B. fragilis* (18, 19), and an additional *nimI* gene was described in *Prevotella* (a related anaerobic Gram-negative genus) (19). To our knowledge, most clinical studies determine if a strain is *nim* positive or *nim* negative based on the outcome of a strain-specific PCR using these primers.

However, it has become increasingly clear that the mere presence of the *nim* gene is not the sole determining factor for the metronidazole resistance observed in an isolate. Increasing numbers of *nim*-negative metronidazole-resistant strains have been found (18, 20). Furthermore, metronidazole-resistant strains can be easily induced from *nim*-negative *B. fragilis* (20). Thus, although *nim*-based resistance is generally considered the most important mechanism of resistance to metronidazole in *Bacteroides* species, it is already acknowledged that other mechanisms are involved, such as increased efflux gene transcription levels, altera-

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Use in this study	Source or reference
<b>Strains</b>		
<i>B. fragilis</i>		
ATCC 25285		ATCC type strain
638R		Clinical isolate (51)
HMW610		Clinical isolate (10)
HMW615		Clinical isolate (23)
HMW616		Clinical isolate (9)
638R/pMCL140		This study
638R/pMCL140:610nimE	Transcription and activity of <i>nim</i> gene in <i>B. fragilis</i>	This study
638R/pMCL140:615nimJ	Transcription and activity of <i>nim</i> gene in <i>B. fragilis</i>	This study
638R/pMCL140:616nimJ	Transcription and activity of <i>nim</i> gene in <i>B. fragilis</i>	This study
<i>E. coli</i>		
DH5 $\alpha$		Invitrogen
Stellar		Invitrogen
Stellar/pMCL140:610nimE	Used to introduce pMCL140:: <i>nim</i> into <i>B. fragilis</i>	This study
Stellar/pMCL140:615nimJ	Used to introduce pMCL140:: <i>nim</i> into <i>B. fragilis</i>	This study
Stellar/pMCL140:616nimJ	Used to introduce pMCL140:: <i>nim</i> into <i>B. fragilis</i>	This study
DH5 $\alpha$ /pSportI		This study
DH5 $\alpha$ /pSportI:610nimE		This study
DH5 $\alpha$ /pSportI:615nimJ		This study
DH5 $\alpha$ /pSportI:616nimJ		This study
HB101/pRK231	<i>E. coli</i> helper with mobilizing plasmid	
<b>Plasmids</b>		
pMCL140	Plasmid for overexpression in <i>B. fragilis</i>	L. Comstock (35)
pMCL140:610nimE		This study
pMCL140:615nimJ		This study
pMCL140:616nimJ		This study
pSportI	Plasmid for overexpression in <i>E. coli</i>	

tions in the DNA repair system, metabolic changes, and lack of activation of the metronidazole molecule (21–24).

In previous studies, we investigated two *nim*-negative, metronidazole-resistant (and multidrug-resistant) clinical isolates of *B. fragilis* (10). HMW615 (aka WAL 272) was a clinical isolate from a pediatric patient with appendicitis and was originally obtained from the R. M. Alden Research Laboratory, Los Angeles, CA (23). Detailed examination of *B. fragilis* HMW615 indicated that no single mechanism of those evaluated could account for the extremely high levels of clinical resistance observed and that either this was caused by multiple simultaneous resistance mechanisms, or some as yet unknown factor was involved. A second isolate, *B. fragilis* HMW616 (aka W1), was originally isolated from a patient in the United Kingdom who eventually died of sepsis (25). This isolate was resistant to metronidazole,  $\beta$ -lactams,  $\beta$ -lactam- $\beta$ -lactamase inhibitor combinations, carbapenems, macrolides, and tetracyclines. Although microbiological cure was apparently achieved with linezolid, the patient ultimately died. No *nim* genes (9) were detected using “universal” *nim* primers and restriction fragment length polymorphism (RFLP) profiling (18). *B. fragilis* HMW610 was isolated from an American soldier who was injured in a blast accident while deployed in Afghanistan; after being thrown into a sewage-infested river he developed leg abscesses with multidrug-resistant *B. fragilis* (10); the leg ultimately was amputated, but the patient survived. Two plasmids were isolated from *B. fragilis* HMW610: (i) an 8.3-kb plasmid shown to carry a *nimE* gene with confirmed similarity to the sequenced fragment of

pBF388c (an 8.3-kb plasmid present in a metronidazole-resistant *B. fragilis* strain isolated in Kuwait) (26) and (ii) a 5.5-kb plasmid identical to pHAG1 (isolated from HMW616 [previously W1]) (9) and the class III plasmid pBFB35, which is widespread throughout *Bacteroides* strains (27). This report describes a new *nim* gene in *B. fragilis*, *nimJ*, that is not recognized by universal *nim* primers. *nimJ* can confer increased resistance to metronidazole in *B. fragilis* 638R (a metronidazole-susceptible, *nim*-negative laboratory isolate) when introduced on a multicopy plasmid. The data indicate, however, that the presence of the *nimJ* gene is not solely responsible for the high metronidazole MICs seen in the two clinical isolates and that there are other unknown factors contributing to increased metronidazole resistance in these isolates.

## MATERIALS AND METHODS

**Strains and culture conditions.** All strains used are listed in Table 1. All strains were grown at 37°C as described previously (28) using brain heart infusion medium supplemented with 15  $\mu$ g/ml hemin (BHIS) for *Bacteroides* isolates (Anaerobe Systems, Morgan Hill, CA) and Luria-Bertani (LB) agar or broth (Sigma) for *Escherichia coli*. *Bacteroides* isolates were incubated anaerobically (5% carbon dioxide, 5% hydrogen, 90% nitrogen), and *E. coli* was incubated either aerobically or anaerobically. *E. coli* DH5 $\alpha$  was used as the host to determine whether the pSPORT vector containing the *nim* homolog could confer metronidazole resistance in anaerobically grown *E. coli*. Ampicillin (50  $\mu$ g/ml), erythromycin (10  $\mu$ g/ml), rifampin (10  $\mu$ g/ml), gentamicin (10  $\mu$ g/ml), and kanamycin (40  $\mu$ g/ml) were used for selection as indicated.

TABLE 2 Primers used in this study

Primer	Sequence
Cloning primers for expression in <i>B. fragilis</i>	
610nimE-pMCL140-F	GAACGTTGGATCCCCGGGTAATACTAAAGATCAGTAATATGTTTCAGAGAAATGC
610nimE-pMCL140-R	TCGAGCTCGGTACCCGGGTAATCATCTAAATCGTTTTTCAAGAGGTTTTCTC
615nimJ-pMCL140-F	GAACGTTGGATCCCCGGGAGTGTTCGCGAATTAAGGCTATGAATG
615nimJ-pMCL140-R	TCGAGCTCGGTACCCGGGTTGTCATATACGTACCTCTATTACTTTTTGATGC
616nimJ-pMCL140-F	GAACGTTGGATCCCCGGGTAAGGAGTATTAGGATTGCTATGAGTGAATTTAG
616nimJ-pMCL140-R	TCGAGCTCGGTACCCGGGTTGTCATATACGTACCTCTATTACTTTTTGATGC
Cloning primers for expression in <i>E. coli</i>	
610nimE-pSport-F	CGGTCCGGAATCCCGGGTAATACTAAAGATCAGTAATATG
610nimE-pSport-R	TGAGCTCGT CGACCCGGG TACTCATCT AAATCGTTTTTTC
615nimJ-pSport-F	CGGTCCGGAATCCCGGGAGTGTTCGCGAATTAAGGC
615nimJ-pSport-R	TGAGCTCGTCCGACCCGGGTTGTCATATACGTACCTCTAT
616nimJ-pSport-F	CGGTCCGGAATCCCGGGTAAGGAGTATTAGGATTGCTA
616nimJ-pSport-R	TGAGCTCGTCCGACCCGGGTTGTCATATACGTACCTCTAT
qRT-PCR primers	
610nimE-qRT-F	TATCGTTTTGCGTTGTGGAA
610nimE-qRT-R	CATCAGACAAACCTGGCTCA
615-616nimJ-qRT-F	TGACAAGGCTTCGTTCTGTG
615-616nimJ-qRT-R	GTCGAAACGAATCATCAGCA

**Molecular methods.** DNA preparation, restriction digestions, gel electrophoresis, and analysis were done as previously described (28).

**Genome sequencing.** *B. fragilis* strains HMW610, HMW615, and HMW616 were submitted to the Broad Institute and sequenced as part of the Human Microbiome Project, *Bacteroides* Group Sequencing Project, Broad Institute of Harvard and MIT (<http://www.broadinstitute.org/>). The Broad Institute sequencing project utilized the 454 whole-genome shotgun methodology and Newbler (454 Life Sciences) assembly. This sequencing project was supported by the National Institute of Allergy and Infectious Disease/National Institutes of Health-funded Genome Sequencing Center for Infectious Diseases at the Broad Institute. The clinical isolates HMW610, -615, and -616 have been given the Broad Institute designations HMPREF1203, HMPREF1204, and HMPREF1205, respectively. Fasta files of the genome sequences and associated annotations were downloaded from the Broad Institute.

**Genomic analysis.** The RAST Annotation Server (29) was used for comparative genome analysis. This server assigns protein-encoding genes (PEGs) to subsystems, a subsystem being a set of functional roles that an annotator has decided should be thought of as related. For RAST analysis, all submitted sequences were downloaded from either the Broad Institute (for the clinical isolates) or NCBI (for the reference strains) and submitted to the RAST server. Genome comparisons were done using the sequence comparison feature of the SEED server (30). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 (31). Amino acid sequences were aligned using ClustalW, and the phylogenetic tree was generated by the maximum likelihood method with bootstrap replications set to 500.

**Prediction of NimJ protein structure.** Predicted protein sequences were submitted to the Phyre Protein Fold recognition server (32), and the predicted models were viewed with Jmol (an open-source Java viewer for chemical structures in three dimensions [3D]). The predicted Protein Data Bank (PDB) structure generated by Phyre was submitted to the Dali server ([http://ekhidna.biocenter.helsinki.fi/dali\\_server/start](http://ekhidna.biocenter.helsinki.fi/dali_server/start)); this program compares a query structure supplied by the user against the database of known structures (PDB) carrying out automatic comparisons of protein structures determined by X-ray crystallography or nuclear magnetic resonance (NMR) and returns the list of structural neighbors (33). The predicted PDB structure for NimJ was also threaded directly on the known crystal structure of NimA from *Deinococcus radiodurans* (DrNimA) soaked with metro-

nidazole, pyruvate, and acetate (1W3R.pdb) (34) using the Dali Lite server ([http://ekhidna.biocenter.helsinki.fi/dali\\_lite/start](http://ekhidna.biocenter.helsinki.fi/dali_lite/start)) by the sum-of-pairs method. The similarity of compared intramolecular distances was measured by Dali Z scores with structures having significant similarities being assigned a Z score above 2 (resulting from similar folds).

**Transcription levels of *nimJ* in HMW610, -615, and -616.** Transcription levels of *nimJ* were determined using quantitative reverse transcription-PCR (qRT-PCR). Total RNA for qRT-PCR was prepared from early to mid-log growth strains (optical density at 600 nm [OD<sub>600</sub>] of 0.6 to 0.8) using the RNeasy minikit with RNAprotect bacterial reagent (Qiagen, Chatsworth, CA). Contaminating DNA was removed with the RNA-free DNase kit (Qiagen) using the manufacturer's recommended protocol. qRT-PCR studies were done with the Power SYBR green RT-PCR mix and the StepOnePlus instrument (Applied Biosystems, Chatsworth, CA). The primers used for qRT-PCR analysis are listed in Table 2. The comparative threshold cycle ( $\Delta\Delta C_T$ ) method was used to determine the relative transcription levels using 16S RNA as the endogenous control. The analysis program is part of the StepOne software.

**Cloning the *nim* gene in an expression vector and plasmid mobilization to *B. fragilis* 638R.** The expression vector pMCL140, noted for its robust transcription levels of inserted genes (35), was a kind gift from Laurie Comstock (Channing Laboratory, Harvard Medical School). SmaI (New England BioLabs, Ipswich, MA) was used to linearize pMCL140. *nim* genes were PCR amplified using *nimJ*- or *nimE*-specific cloning primers (Table 2) and Phusion DNA polymerase (New England BioLabs, Ipswich, MA) with template DNA from appropriate *B. fragilis* strains. Cloning was done using Phusion DNA polymerase (New England BioLabs) and the In-Fusion HD kit (Clontech, Mountain View, CA). After the In-Fusion HD reaction, the mix was used to transform *E. coli* Stellar cells, and the transformant mixture was plated on ampicillin-containing LB (50  $\mu$ g/ml). Ampicillin-resistant colonies were selected, and the presence of the cloned *nim* gene was confirmed by PCR analysis and subsequent sequencing of the plasmid insert. The *nim*-containing vectors were then introduced to *B. fragilis* 638R by a triparental mating technique using *E. coli* DH5 $\alpha$ /pMCL140::*nimJ* (HMW615 or HMW616, respectively) and *E. coli* DH5 $\alpha$ /pMCL140::*nimE* (HMW610) as donors, *B. fragilis* 638R as the recipient host, and *E. coli* HB101/pRK231 as the mobilizer (36, 37). Transconjugants were selected on BHIS plates containing erythromycin, rifampin, and gentamicin (10  $\mu$ g/ml of each). The *nimJ* gene was also

TABLE 3 *nim* genes in *B. fragilis* HMW610, -615, and -616

Strain ( <i>nim</i> homologs)	Supercontig no.	Position		Broad Institute locus		Recognized by universal primers <sup>c</sup>
		Start	Stop	tag <sup>a</sup>	RAST ID <sup>b</sup>	
HMW615	1.1	238	735	HMPREF1204_00002	figl1073387.3.peg.0.9	No
HMW615	1.2	1550932	1551429	HMPREF1204_02912	figl1073387.3.peg.1957	No
HMW615	1.5	215	712	HMPREF1204_04081	figl1073387.3.peg.4096	No
HMW616	1.2	1736505	1736008	HMPREF1205_01450	figl6666666.20506.peg.2695	No
HMW610	1.5	1033	458	HMPREF1203_04663	figl6666666.20571.peg.4745	Yes

<sup>a</sup> HMPREF1204\_00002, HMPREF1204\_02912, and HMPREF1204\_04081 are annotated in the Broad annotation as *Bacteroides fragilis* HMW615 hypothetical protein (498 nucleotides [nt]). HMPREF1205\_01450 is annotated in the Broad annotation as *Bacteroides fragilis* HMW616 hypothetical protein (498 nt). HMPREF1203\_04663 is annotated in the Broad annotation as *Bacteroides fragilis* HMW610 hypothetical protein (513 nt). All of these genes are annotated by the RAST server as coding for pyridoxamine 5'-phosphate oxidase-related, FMN binding.

<sup>b</sup> fig, fellowship for interpretation of genomes; peg, protein-encoding gene.

<sup>c</sup> NIM-3 (5'-ATG TTC AGA GAA ATG CCG CGT AAG CG-3') and NIM-5 (5'-GCT TCC TTG CCT GTC ATG TGC TC-3').

cloned into pSport (Invitrogen, NY) using the In-Fusion HD cloning kit as described above. pSPORT-*nimJ* was mobilized into *E. coli* DH5 $\alpha$  for the MIC evaluation.

**Screening for *nimJ* in laboratory constructs and in other clinical isolates.** Colony PCR analysis was done using OneTaq DNA polymerase (New England Biolabs) following the manufacturer's recommendation. The primers used for detection or cloning of the particular *nim* gene were the qRT-PCR or cloning primers listed in Table 2, respectively. The parameters of the PCR for the presence or absence of *nim* were 30 s at 94°C, 30 s at 55°C, and 30 s at 68°C for 31 cycles with a final cycle of 11 min at 68°C. PCR-generated amplicons were sequenced by Laragen (Culver City, CA).

**MICs.** MICs were determined using the Etest methodology (bioMérieux SA Marcy l'Etoile, France) which is comparable to the NCCLS agar dilution method for testing anaerobic bacteria (38, 39). The cells were adjusted to 1 McFarland standard (OD<sub>600</sub> of 0.257) in phosphate saline buffer (pH 7.4). The cells were spread on *Brucella* blood agar plates (Anaerobe Systems, Morgan Hill, CA) using sterile cotton swabs. The Etest strip was placed in the center, and the plates were incubated anaerobically for 16 to 24 h.

## RESULTS

**Genome sequencing of multidrug-resistant clinical isolates.** Metronidazole-resistant *B. fragilis* strains HMW615 and -616 were found to be *nim* negative based on PCR using the previously published "universal" *nim* primers (16). In order to gain more information about the nature of the metronidazole resistance seen, these strains were submitted to the Broad Center for genome sequencing (*Bacteroides* Group Sequencing Project, Broad Institute of Harvard and MIT). At the same time, we submitted *B. fragilis* HMW610, also a virulent and metronidazole (and multi-drug)-resistant clinical isolate that was previously designated *nim* positive using the universal *nim* primers (16); the strain carried the *nimE* gene on an 8.3-kb plasmid (10). Genome sequencing confirmed the absence of *nimA* to *-H* genes in HMW615 and HMW616. Since the Broad Center annotated a large part of the *B. fragilis* genome(s) as hypothetical proteins, we decided to reannotate using the RAST annotation server (<http://rast.nmpdr.org>), which assigns genes to subsystem-based protein families. While studying the RAST genome annotations, we noted that HMW615 peg9 was annotated as a gene coding for a pyridoxamine 5'-phosphate oxidase-related protein. (This is the family that includes *nim* genes.) The Annotation Overview tool then detected three apparent homologs of this gene (named *nimJ*) within the HMW615 genome (Table 3). Using BLAST analysis with this gene within the RAST program, we found a nearly identical gene in the HMW616

with 99% nucleotide (495/498) and 98% amino acid (163/165) identical residues. The previously described *nimE* gene detected in HMW610 (10) has 56% nucleotide and 57% amino acid similarity with *nimJ*. The presence of the *nimJ* sequences in *B. fragilis* HMW615 and HMW616 was further confirmed by PCR followed by sequencing.

**Alignments of the predicted NimJ protein sequences to those of other *Bacteroides* Nim proteins and phylogenetic analysis of NimJ.** Phylogenetic analysis of the predicted NimJ protein sequence indicated that it is more closely related to Nim proteins from *Prevotella baroniae* and *Clostridium botulinum* than to other Nim proteins described in *Bacteroides* (40, 41). Based on these results, these sequences were included for the subsequent phylogenetic analysis and tree building (Fig. 1). HMW610 Nim has an identical amino acid sequence to the published sequence for *B. fragilis* NimE, whereas NimJ from HMW615 and HMW616 is closest to *P. baroniae* NimI and similar to *C. botulinum* NimA.

**The predicted protein structure of NimJ is consistent with that of known Nim proteins.** The predicted protein sequence of NimJ was submitted to the Phyre Protein Fold recognition server (32), and the predicted models were viewed with Jmol (an open-source Java viewer for chemical structures in 3D). Molecules that belong to the PNPOx-like superfamily and catalyze flavin mononucleotide (FMN)-mediated redox reactions, including Nim reductases and other flavin-nucleotide-binding

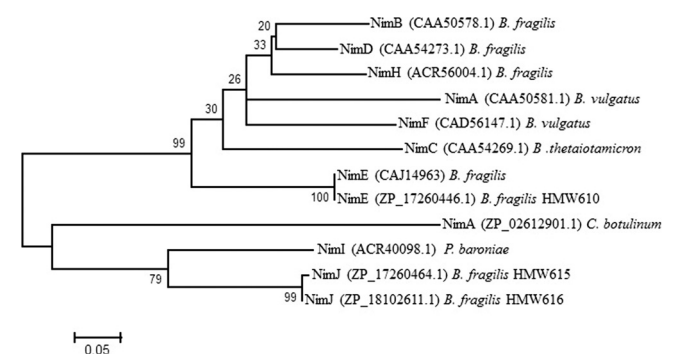
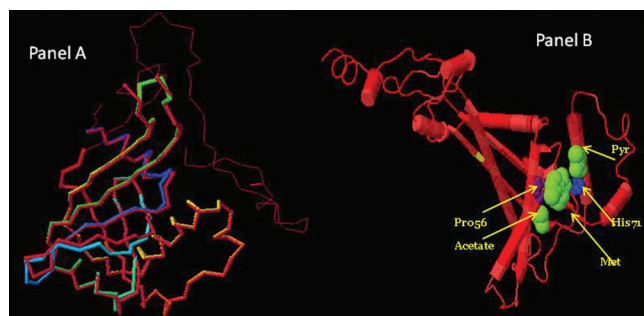


FIG 1 Phylogenetic analysis of *B. fragilis* NimJ. Phylogenetic analysis was performed with the MEGA 5 program using a MUSCLE alignment. The phylogenetic tree was reconstructed using the maximum likelihood method, and reliability for internal branching was assessed using the bootstrapping method (500 bootstrap replicates).



**FIG 2** (A) Dali Lite superimposed models of NimJ and closest PDB match. Phyre-predicted PDB structure of *B. fragilis* of NimJ superimposed on the closest match in the PDB database. The highest match was 2furA2 (Z score, 23.3). The NimJ backbone is rainbow colored from the N terminal to the C terminal. (B) Dali pairwise superimposed models: 1W3R.pdb (DR NimA soaked with metronidazole, pyruvate, and acetate) and PDB file of HMW615 NimJ (generated from Phyre server). The yellow band is tyrosine 111. The positions of metronidazole, pyruvate, and acetate molecules are indicated. DR NimA is in the cartoon mode, colored by sequence conservation (identical sequence is in red) with HMW615 NimJ.

(FMN) proteins, exist as dimers, have a characteristic fold, and possess beta-barrel structural elements (42, 43). Recent crystal studies identified specific, conserved residues (Pro45, His59, and Tyr94) that are part of the active site of Nim proteins (42, 43). Phyre analysis of the predicted protein sequence for NimJ predicted a similar fold structure for NimJ, including the predicted beta barrel. The conserved residues that are found in the active site are also present in the predicted protein sequence of NimJ in the predicted active site.

The predicted PDB structure file generated by Phyre was submitted to the Dali server, which compared the query structure against the database of known structures (PDB) (33). Matches were found with multiple PDB structures of NIM-like proteins with the highest match (Z score, 23.3) to 2furA/2, a putative FMN-binding protein (TA1372) from *Thermoplasma acidophilum*. The predicted superimposition of the two structures is shown in Fig. 2A.

The crystal structure of the Nim protein has been shown to be slightly altered when crystalized with its natural ligands, metronidazole, pyruvate, and acetate (43). Therefore, the Dali Lite server was also used to thread the predicted PDB file for NimJ onto the known crystal structure of DR NimA soaked with metronidazole, pyruvate, and acetate (1W3R.pdb) (34). The Dali Z score was 12.8. (structures that have significant similarities have a Z score above 2 and usually have similar folds). The predicted superimposition of the two structures is shown in Fig. 2B.

**Prevalence of *nimJ* in clinical isolates.** *B. fragilis* clinical isolates from the United States ( $n = 15$ ) and from Groote Schuur Hospital, South Africa ( $n = 23$ ) (44), were examined using the *nimJ* qRT-PCR primers, but the gene was not found in any of these clinical isolates. A search of the Broad database of unfinished *Bacteroides* genome sequences in the Human Microbiome Project with the *nimJ* sequence also did not find any other strains with the *nimJ* gene.

**Overexpression of *nimE* and *nimJ* confers higher MICs of metronidazole for *B. fragilis* 638R.** The *nim* alleles (610*nimE*, 615*nimJ*, and 616*nimJ*) were cloned and expressed in *B. fragilis* 638R using pMCL140, and the overexpression was confirmed us-

**TABLE 4** Difference in transcription levels of *nim* genes between clinical isolates and *B. fragilis* 638R carrying pMCL140::*nim*

Sample	Target	RQ range <sup>a</sup>	Avg fold change (RQ avg) <sup>b</sup>
638R/pMCL140::610 <i>nimE</i> HMW610	610 <i>nimE</i> 610 <i>nimE</i>	0.7–1 2.37–3.46	3.41
638R/pMCL140::615 <i>nimJ</i> HMW615	615-616 <i>nimJ</i> 615-616 <i>nimJ</i>	0.22–1 2.47–2.73	4.26
638R/pMCL140::616 <i>nimJ</i> HMW616	615-616 <i>nimJ</i> 615-616 <i>nimJ</i>	0.76–1 1.27–1.83	1.76

<sup>a</sup> The comparative  $C_T$  ( $\Delta\Delta C_T$ ) method was used to determine the relative quantitation (RQ) using 16S RNA as the endogenous control in the samples and in the reference sample. (*B. fragilis* 638R/pMCL:*nim* was chosen as the reference sample for each set.) Two biological and two technical replicates were used for each determination.

<sup>b</sup> The RQ values of the two biologic repeats were averaged and then normalized to 1 to determine the fold difference between the clinical isolate and the corresponding *B. fragilis* 638 isolate containing its *nimJ* gene on a multicopy plasmid.

ing qRT-PCR (for detection of transcription levels). In related studies, the levels of transcription of a gene insert in pMCL140 introduced into *B. fragilis* 638R were found to be ~40-fold above the baseline (data not shown). In these studies, the expression of *nim* from the chromosomes of HMW610 (*nimE*), HMW615 (*nimJ*), and HMW616 (*nimJ*) was compared to those of the corresponding alleles carried on the pMCL140 vector introduced into *B. fragilis* 638R. Transcription levels were ~2- to 4-fold higher in the clinical isolate than the corresponding levels from the strain with a plasmid carrying the gene (Table 4).

**Determination of MICs.** All four biological replicates of each allele conferred ~4- to 6-fold increases in the MICs of metronidazole when introduced into *B. fragilis* 638R compared to 4 biological replicates of *B. fragilis* 638R harboring the vector pMCL140 (Table 5). There was no difference in the metronidazole MIC for *E. coli* DH5 $\alpha$  carrying pSport/*nimJ* compared to the MIC for *E. coli* DH5 $\alpha$  carrying pSportI (data not shown).

## DISCUSSION

These studies were a continuation of the previous analyses of three virulent, multidrug-resistant and metronidazole-resistant clinical isolates of *Bacteroides fragilis* (HMW610, HMW615, and HMW616). Metronidazole is currently the most commonly prescribed antibiotic worldwide for infections involving *Bacteroides*. Since the time that *nim* genes were first described in *Bacteroides*,

**Table 5** MICs of metronidazole for *B. fragilis* clinical isolates and laboratory constructs

Strain	MIC or MIC range ( $\mu\text{g/ml}$ )	Fold change <sup>a</sup>
ATCC 25285	0.19–0.25	
638R/pMCL140	0.03	
638R/pMCL140:610 <i>nimE</i>	0.17	~4
638R/pMCL140:615 <i>nimJ</i>	0.19	~4
638R/pMCL140:616 <i>nimJ</i>	0.14	~4
HMW610	8–16	
HMW615	6–8	
HMW616	8–24	

<sup>a</sup> Fold change between strain carrying empty pMCL140 plasmid and strain carrying plasmid with *nim* insert.

the presence of this gene (particularly with a related insertion sequence) has been considered the premier mechanism for metronidazole resistance. Moreover, the presence of *nim* genes on plasmids suggests the potential for horizontal gene transfer (45), especially in the gastrointestinal tract, which is a very favorable environment for gene transfer and the likely source for *B. fragilis* isolates. Although *nim*-based resistance is an important mechanism of resistance to metronidazole, it has already been acknowledged that other mechanisms must be involved. *B. fragilis* HMW615 and *B. fragilis* HMW616, both *nim*-negative isolates when tested with the “universal” primers, had previously been analyzed. They showed increased efflux gene transcription levels, and their high metronidazole MICs were somewhat lowered by addition of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; an inhibitor of the energy-driven resistance-nodulation-division [RND] family of *Bacteroides* multidrug efflux [*bme*] pumps) (9, 23). However, since metronidazole efflux systems alone did not cause the high levels of clinical resistance observed, we concluded that they were clearly not the only contributing factor and other, as yet unknown, factors were involved. The current genome sequence analysis of *B. fragilis* HMW615 and *B. fragilis* HMW616 strains was therefore undertaken to identify genomic differences that might account for the increased resistance to metronidazole. The clinical isolate *B. fragilis* HMW610 [*nimE* positive] was submitted at the same time for sequencing and subsequent characterization.

The three isolates came from different parts of the world and were all isolated as pathogenic strains; all strains were multidrug resistant and metronidazole resistant. We added the RAST annotation to that provided by Broad and found that many proteins annotated only as “hypothetical proteins” in Broad could be assigned to a specific family in RAST; this was the case with the multiple alleles of the *nim*-like gene (now called *nimJ*) observed in HMW615 as well as the close homolog in HMW616. The universal *nim* primers did not amplify the *nimJ* sequences. Interestingly, although the gene sequences of the “universal *nim*” primers and *nimJ* are different, the predicted amino acid sequence at the site of primer binding in NimJ is identical to that of the “classical” Nim proteins (FREMRRK). Differences in codon usage in *nimJ* for several of the amino acids, therefore, result in the amino acid sequences at these sites being identical.

The predicted protein sequence of NimJ is highly homologous to that of the classical Nim proteins, and the predicted fold structure analysis identified the Nim crystal structure as the best match. The FMN domain and conserved residues (Pro45, His59, and Tyr94) that are considered to be important for the reductase reaction are all conserved. This initial observation strongly suggested that *nimJ* could code for a 5-nitroimidazole reductase, as is suggested for the other well-studied Nim proteins, and we therefore proceeded with further study of the functional characteristics and phenotype conferred by this gene.

Previous studies have shown that the plasmid-carried *nimA* (pIP417 from *B. vulgatus*) and *nimC* (pIP417 from *B. thetaiotaomicron*) genes transferred metronidazole resistance to metronidazole-sensitive *B. fragilis* strain 638R (46). It was also shown that a 1- to 2-kb fragment (containing *nimA* or *nimC*, respectively) of the native plasmids cloned into pBI191 (46) and introduced into *B. fragilis* 638R transferred levels of metronidazole resistance comparable to that of the clinical isolate. In these cases, the cloned fragment included known *Bacteroides* insertion sequence (IS) el-

ements located a few base pairs upstream of the *nim* gene; these IS elements were shown to promote expression of the *nim* genes (47, 48). Chromosomal fragments containing the *nimB* gene from plasmid-free *nim*-positive isolate *B. fragilis* 8 could be cloned into plasmids and transfer metronidazole resistance to *B. fragilis* 638R (49); the transferred resistance determinant was located on a 1.6-kb fragment. In the present study, the *nimJ* gene (cloned together with 20 flanking base pairs into the strong promoter expression vector pMCL140) was introduced to the metronidazole-sensitive recipient *B. fragilis* 638R and was able to increase metronidazole resistance ~4- to 6-fold. No obvious IS elements upstream of the *nim* genes were detected. To our knowledge, this is the first time that the *nim* gene was cloned on an expression plasmid without accompanying IS elements and shown to confer increased MICs of metronidazole for the host strain. It is possible that inclusion of the sequence upstream of *nimJ* could result in a stronger transcription signal, and this is currently being evaluated.

Both Broad analysis and RAST analysis of HMW615 identified three *nimJ* homologs (HMPREF1204\_00002 (supercontig 1.1), HMPREF1204\_02912 (supercontig 1.2), and HMPREF1204\_04081 (supercontig 1.5)). It could not definitively be proved that all three homologs were unique genes on the HMW615 genomes because the HMW615 supercontigs have not been assembled into a finished genome, and there may be overlap between them. HMPREF1204\_00002 and HMPREF1204\_04081 could be distinguished from each other based on differences in a region 9 kb downstream of their respective *nimJ* genes. Supercontig 1.2 containing HMPREF1204\_2912 ended before this region, so this strategy could not be used to distinguish between it and the others. Upstream sequence was not available for HMPREF1204\_00002 and HMPREF1204\_04081 to enable differences or similarities between the homologs to be identified.

The presence of adjacent insertion elements has been associated with *nim* genes in *Bacteroides* (45). A gene coding for an IS4 family transposase that shares 99 to 100% similarity with other IS4 family transposases was found upstream of HMPREF1204\_2912 (in the opposite orientation). In pWAL610, there is also a transposase of the IS4/5 class adjacent to *nimE*, in the opposite orientation; however, this transposase has little similarity at the nucleotide level to the transposase upstream of HMPREF1204\_02912. The closest homolog to *nimJ*, *nimI* of *Prevotella baroniae*, had no known insertion sequence elements detected upstream of *nimI* (19). Genes coding for NimI were found in all of the clinical isolates tested. It is noteworthy that *nimI* was found in all *P. baroniae* isolates tested, but not in 33 type strains belonging to other *Prevotella* species. The authors commented that NimI formed a new homogeneous group distant from the other Nim types involved in metronidazole resistance in anaerobic bacteria confirmed by the phylogenetic analysis reported in this work. Since the species *P. baroniae* has not yet been sequenced, no further conclusions can be reached about the genomic context of *nimI* in relation to possible similarities to *nimJ*.

In summary, we have identified a new *nim* gene, *nimJ*, that increases resistance to metronidazole when introduced into *B. fragilis* 638R. However, overexpression of the *nimJ* gene in *B. fragilis* 638R does not confer the same degree of resistance found on the original clinical isolate, suggesting that the isolates carry additional unknown resistance factors. The data are consistent with the published literature concluding that screening for *nim* is not advisable for diagnosis of or screening for metronidazole resis-

tance clinical isolates (50). The work presented here has clearly demonstrated that at least one *nim* gene, *nimJ*, will not be recognized by the universal primers, and this may also be the case with other unknown *nim* alleles. In addition, it is clear that the mere presence and transcription of a *nim* gene are not solely responsible for the high MICs seen in the metronidazole-resistant clinical isolates. These studies clearly suggest that MIC determination is more reliable screening method for metronidazole resistance.

The discovery of the contribution of the *nimJ* to metronidazole resistance is significant in the context of clinical drug resistance surveillance studies and should be taken into account when predicting clinical outcomes. These studies confirm that metronidazole resistance in clinical isolates of *B. fragilis* is multifactorial. Hopefully, the current generation of genomic and transcriptomic data combined with functional analysis will lead to a better understanding of this problem and may suggest therapeutic directions.

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