Investigation of the molecular mechanisms of metronidazole and β-lactam antibiotic resistance of *Bacteroides* species

Ph.D. Thesis

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PUBLICATIONS

- **LIST OF PUBLICATIONS RELATED TO THE TOPIC OF THE THESIS**

I. **Mahmood B**, Sárvári KP, Orosz L, Nagy E, Sóki J. Novel and rare β-lactamase genes of *Bacteroides fragilis* group species: Detection of the genes and characterization of their genetic backgrounds. Anaerobe. 2024 Feb;86:102832: https://doi.org/10.1016/j.anaerobe.2024.102832
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II. **Mahmood B**, Juhász H, Leitsch D, Sóki J. The effects of identical *nim* gene-insertion sequence combinations on the expression of the *nim* genes and metronidazole resistance in *Bacteroides fragilis* strains. Anaerobe. 2023 Jun;81:102739: https://doi.org/10.1016/j.anaerobe.2023.102739
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III. **Mahmood B**, Paunkov A, Kupc M, Burian K, Nagy E, Leitsch D, Sóki J. Proteomics-Based RT-qPCR and Functional Analysis of 18 Genes in Metronidazole Resistance of *Bacteroides fragilis.* Antibiotics. 2024 Feb;13:13030207: https://doi.org/10.3390/antibiotics13030207
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1. INTRODUCTION

1.1*The importance of Bacteroides species*

Human beings are colonized by a diverse collection of commensal microorganisms. Anaerobic bacteria are regarded as major constituents of this population of which *Bacteroides* species belonging to the order Bacteroidales are strict anaerobic Gram-negative non-spore forming rods, but bile-resistant comprising approximately 25% of the residents of the intestinal microbiota. They can become opportunistic pathogens. Sometimes they cause serious infections such as intra-abdominal, lung and brain abscesses, appendicitis, diarrhoea and sepsis.

1.2 *Pathogenic characteristics and virulence factors of Bacteroides*

Besides the conspicuous pathogenic characteristic of *B. fragilis* most other *Bacteroides* species have their direct and indirect virulence factors that include the production of polysaccharide capsules, fimbriae, adhesions along with enzymes for tissue destruction such as enterotoxin, fibrinogenases, neuraminidase and haemolysins. These virulence properties allow them to adhere, invade and destroy the tissues in the body.

1.3*The antimicrobial resistance of Bacteroides*

The antimicrobial resistance mechanisms of bacteria can generally be grouped into four categories like enzymatic drug inactivation either extra or intracellularly, the efflux pump, drug target modification and a metabolic bypass that is also valid for anaerobes. It has been found that the susceptibility levels in *B. fragilis* for most antimicrobial agents is continually in decline. *B. fragilis* strains displaying multidrug resistant phenotypes emerged with a resistance to different classes of antimicrobial agents and the MDR *B. fragilis* isolates have often been identified during antibiotic treatment.

1.3.1 *Metronidazole resistance mechanisms and prevalence*

Nowadays clear evidence demonstrates that resistance to metronidazole is a complex process and it is most likely to be multifactorial and probably even now include unknown mechanisms. Resistance to metronidazole may occur via nitro-reductases, reduced reduction effectiveness, DNA repair or efflux, but the best characterized one is mobile and it is mediated by *nim* genes originally described in the 1980s and 1990s, mainly for the *Bacteroides*

species. Studies revealed that in most parts of the world metronidazole resistance in *Bacteroides* is still at a lower level with (<3%), except for a reported rate of resistance in Spain (4.8%), South Africa (8.7%) and Pakistan (16-20%). In most Western countries it is less than 1% despite its long-term use since the 1960s.

1.3.2 *β-lactam resistance mechanisms including carbapenems*

The resistance to β-lactam antibiotics is a worrisome problem in anaerobes mainly due to β-lactamase enzymes. These enzymes are produced by bacteria to facilitate the hydrolysis of the β-lactam ring and render the antibiotic inactive. The most important emerging problem is the appearance of Ambler's class B metallo β-lactamases (carbapenemase) enzyme encoded by *cfiA/ccrA* genes in *B. fragilis*. And in *B. xylanisolvens*, the carbapenemase enzyme encoded by the *crxA* gene was recently described.

1.4 *Mobile genetic elements in Bacteroides*

Bacteroides species have a variety of mechanisms available to exchange the genetic material. The presence of mobile genetic elements (MGEs) play a vital role in the dissemination of antibiotic resistance genes. In the *Bacteroides* species MGEs include plasmids, transposons and conjugative transposons, and all of these are implicated in the transference of antimicrobial resistance genes except bacteriophages. Insertion sequence elements are small segments of double stranded integrative DNA with (< 2.5 kb). Within bacterial chromosomes IS elements can move and are involved in the modulation of nearby gene expression with the help of outward-oriented promoters. The existence of the IS elements in the upstream region of antimicrobial resistance genes such as *nim*, *cfiA*, *cfxA*, *cepA*, and *erm* has a close connection with the activation of these genes and high levels of AMR in *Bacteroides.*

2. AIMS AND OBJECTIVES

The general objective of this study was to investigate the existing gaps in the resistance mechanisms of the metronidazole and β-lactam antibiotics in the *Bacteroides* species and to characterize their phenotypic and genetic background at the molecular level in order to better understand how antimicrobial resistance in this microorganism takes place.

The specific aims of the study were as follows:

- To screen for rare β-lactamase genes in *Bacteroides* strains isolated from clinical specimens and normal microbiota to determine the prevalence of these genes which were described earlier, and examine the genetic properties of the strains carrying these genes.

- To examine whether the identical *nim* gene-insertion sequence (IS) element combinations give rise to the same expression levels because they harbor shared IS element-born promoters in metronidazole resistance.

- To study the expression level of 18 selected genes from various cellular pathways by RT-qPCR and their correlation with metronidazole MICs in a panel of selected *nim* gene-positive and *nim* gene-negative *B. fragilis* clinical strains.

3. MATERIALS AND METHODS

3.1 *An Investigation of novel and rare β-lactamase genes in Bacteroides strains*

3.1.1 *Bacterial isolates and cultivation*

In total, 590 *Bacteroides* species strains including clinical (n = 406) and normal microbiota (n = 184) isolates were collected earlier. All the isolates were revived from −80°C and subcultured on Columbia blood agar supplemented with 5% defibrinated sheep blood, 2.5% laked sheep blood, 300 mg/L L-cysteine, and 1 mg/L vitamin K_1. The inoculated plates were incubated anaerobically (85% N_2, 10% H_2, and 5% CO_2) in an anaerobic cabinet (Concept 400, Ruskinn, UK) and/or in a jar with gas pack at 37°C for 48 h. Identification was performed using MALDI. Antibiotic susceptibility testing was performed to obtain minimum inhibitory concentrations as previously described by agar dilution. For new MIC measurements for amoxicillin/clavulanate, and piperacillin/tazobactam, we applied the gradient method using Etest (bioMérieux fixed ratio) or MIC test strips (Liofilchem at a fixed inhibitor concentration).

3.1.2 *Polymerase chain reaction and nucleotide sequencing*

All the isolates were tested for the presence of the resistance genes *bla*HGD1, *bla*OXA347, *cblA*, *crxA*, and *pbbA* using a pair of specific primers for each gene. For end-point PCR, each 20-µL reaction mixture contained 10 µL of master mix (DreamTaq, Thermo Fisher Scientific, Waltham, MA, USA), 0.4 µL of each primer, 2 µL of DNA template, and 7.2 µL of DNase/RNase-free H_2O. For real-time (RT)-PCR, each 10-µL reaction mixture contained 5 µL of master mix (QuantiNova, Qiagen, Hilden, Germany), 1 µL of ROX, 0.2 µL of each primer (35 pmol/µL), 2.6 µL of DNase/RNase-free H_2O, and 1 µL of DNA template. Reactions were run on a StepOne RT-PCR instrument. To confirm some species, we conducted 16S rRNA sequencing. To detect the genetic element of *crxA*, we used the Emerald PCR master mix as recommended by the supplier. Sequence homology analysis was conducted with recorded sequences in https://www.ncbi.nlm.nih.gov/) or uploaded for species identification to the EzBioCloud webpage (https://www.ezbiocloud.net/).

3.1.3 *β-glucosidase assay*

We used the 4-nitrophenyl-β-D-glucopyranoside hydrolysis assay to differentiate between *Ph. vulgatus* and *Ph. dorei.* For positive and negative controls we used *B. fragilis* 638R and *Ph. vulgatus* ATCC 29327, respectively. The absorbance of the produced 4-nitrophenol was measured at 405 nm in a microplate reader.

3.1.4 *Molecular typing of B. xylanisolvens strains*

In this study, three molecular typing methods were applied: enterobacterial repetitive intergenic consensus (ERIC)-PCR, IR Biotyper®, and MALDI-TOF MS.

3.1.5 *Bioinformatics*

ERIC PCR fragment similarity patterns were analyzed using GelJ software, and the *Bacteroides* β-lactamase sequence similarity dendrogram was drawn using the Lasergene 17 suite (DNAStar, Madison WI, USA).

3.2*An investigation of the expression levels of identical nim gene-insertion sequence combinations in Bacteroides fragilis strains*

3.2.1 *Bacterial strain cultivation and the E-test*

We chose 6 *nimB*-IS*1186* and 10 *nimE*-IS*Bf6* construct-carrying *Bacteroides fragilis* strains from our collection of glycerol stocks stored at -80^{o}C. After their inoculation onto supplemented Columbia blood agar plates and anaerobic cultivation, we measured their actual metronidazole resistance levels Minimum Inhibitory Concentration (MIC) value by gradient tests (Etest, bioMérieux) and the result was interpreted according to criteria of the EUCAST guidelines.

3.2.2 *RNA preparation and RT-qPCR*

Total RNA was prepared using the HighPure RNA Isolation Kit (Roche) and then we examined the expression levels of the *nim* genes in RT-qPCR experiments with specific primers for the *nim* genes and the glyceraldehyde 3-phosphate dehydrogenase gene as an endogenous control using the $\Delta\Delta C_T$ method. For the RT-qPCR experiments, we used the StepOne RT-PCR instrument.

3.2.3 *RACE PCR (rapid amplification of cDNA ends)*

The promoter of the IS*1186* element was identified earlier by Podglajen et al. and we did this for the IS*Bf6* element by a 5'-RACE kit (Roche), as described earlier but with the following primers (SP1: CATTTTTTCAAGAATTGCCAC, SP2: CTCTCTTCTTGTGGCAAC and SP3: GTTTACGACGCATTTCTCT).

3.2.4 *Statistical analysis*

The correlation calculations were carried out using the Spearman rank method with the Sigmaplot 12 software package (Sigmaplot, Germany) and the significance level was set to 0.05 (i.e. $p<0.05$).

3.3 *A proteomics-based RT-qPCR and functional analysis of the expressions of 18 genes in Bacteroides fragilis strains with different metronidazole resistance*

3.3.1 *Bacterial strains and cultivation*

Sixteen *B. fragilis* test strains with known genetic backgrounds were stored in 20% glycerol stocks at −80 °C and cultivated on a supplemented Columbia blood agar medium and incubated under anaerobic conditions at 37 °C. The strains included both *nim*-positive and *nim*-negative *B. fragilis* strains. To test the effect of C_4-dicarboxylic acids on metronidazole resistance, we used a semi-defined M9-based agar medium to perform MIC measurements. To measure Metronidazole MICs we used a gradient method (Etest, bioMérieux, Hungary Ltd. Budapest, Hungary). After anaerobic cultivation at 37 °C for 48 h, we read the plates.

3.3.2 *RT-qPCR*

Of the 32 candidate genes identified in previous proteomic studies, we chose 18 and designed primer pairs using the Primer3 Plus software (www.primer3plus.com). We used the *gap*, *rrn*, and *rpoD* genes as endogenous controls. The 10 μL RT-qPCR reactions contained 5.6 μL kit components (Verso 1-step SYBR RT-PCR mastermix, Thermo Fisher Scientific), 0.2 μL each primer (35 μM), 3 μL H_2O, and 1 μL total RNA. The reactions were incubated in an RT-PCR instrument in 100 μL 96-well plates using the following conditions: 35 cycles consisting of 55 °C 20 min, 95 °C 15 min; 95 °C 15 s, 55 °C 30 s, 72 °C 30 s. The melting curves were recorded using 3 technical replicates. We detected the expression of the *nim* genes in 8 *nim*-positive *B. fragilis* strains by amplifying *nim* PCR products using the same conditions as those described above, except the 35 PCR cycles consisted of two steps (55 °C 20 min, 95 °C 15 min; 95 °C 15 s, 60 °C 1 min; melting curve) because three *nim* gene types were included.

3.3.3 *Data analysis*

We used the amplification threshold values (C_T) from RT-qPCR experiments to calculate the ratios of gene expression by the $\Delta\Delta C_T$ method. The calculations were performed by the Relative Quantitation App on the Thermo Fisher Scientific webpage (www.thermofisher.com). One-way variance (ANOVA), Spearman's rank, and cross-correlation values were calculated using SigmaPlot 12 software (Sigmaplot, Germany).

4. RESULTS

4.1 *An investigation of novel and rare β-lactamase genes in Bacteroides strains*

All the *B. uniformis* strains included in both collections harbored *cblA*. Similarly, in all *Ph. vulgatus*/*Ph. dorei* strains, we detected *bla*HGD1. β-glucosidase, demonstrating that all the *Ph. dorei* and *Ph. vulgatus* strains carry this β-lactamase gene. This species identification was confirmed by 16S rRNA sequencing. Unexpectedly, we did not identify a strain that carried *bla*OXA347 among the recent European strains; however, all (n = 6) isolates previously obtained from US collections were positive. *pbbA* was only detected in three clinical isolates. This latter gene is resistant to piperacillin and it has a decreased piperacillin/tazobactam susceptibility, but it does not have a significant effect on amoxicillin/clavulanate. Carbapenem resistance gene *crxA*, which is specific for *B. xylanisolvens* strains, was detected in three clinical and six normal microbiota strains. We previously found that *crxA* resides on a small genomic island. Therefore, we studied whether the eight *B. xylanisolvens* strains carried this gene on the same island. However, PCR primers specific for this island detected its presence in only five strains including the prototype strain *B. xylanisolvens* 14880. Also, two of the strains might carry this gene at different genomic locations because other primers did not amplify the gene. Genetic relatedness experiments revealed that *crxA*-positive *B. xylanisolvens* strains were located on different branches.

4.2 *An investigation of the expression levels of identical nim gene-insertion sequence combinations in Bacteroides fragilis strains*

The variations in *nim* gene expressions were far fewer, and practically constant on a $\log_2$ scale compared to variations in metronidazole resistance levels. This means that identical *nim* gene-IS element combinations have the same expression levels as the resistance genes. There is no correlation of the metronidazole MICs with the constant expression of the *nimB* and *nimE* genes. The *nimE* gene of *B. fragilis* Q8 had two IS*Bf6* element-born promoters (34 and 114 bp upstream of the right end of IS*Bf6*), as found by 5′-RACE.

In these experiments we confirmed that the same IS element background produces similar gene expression levels of the *nim* genes, but we also observed the non-dependence of the resistance for the *nim* gene IS element pairs we analyzed. We attribute the small variations of the *nim* gene expressions to other strain-specific differences, e.g. the levels of reducing agents or enzymes, some low level nitroreductase or efflux activities, etc. The IS*1186* and IS*Bf6* elements had recognizable *Bacteroides*-specific promoter sequences (the consensus, TtTg _33 TnnTAnnTTTGY _7, was determined earlier) that could drive the expression of the *nimB* and *nimE* genes, respectively.

4.3 *A proteomics-based RT-qPCR and functional analysis of the expressions of 18 genes in Bacteroides fragilis strains with different metronidazole resistance*

The results show that metronidazole MICs and the expression levels of the *nim* genes were independent of each other ($r = 0.185$, $p = 0.619$, $r^2 = 0.0342$). However, *nim* genes have known resistance factors because they transfer the resistance phenotype in conjugation experiments, and they are associated with metronidazole resistance in field studies. Hence there is a need to account for this lack of correlation even with the same *nim* gene and IS element pairs. Previously, we proposed the existence of rate-limiting factors that influence the metronidazole resistance of *B. fragilis* strains.

To investigate this, we measured the expression levels of 18 genes selected according to the results of previous research or from our recent investigations using RT-qPCR to study 15 *B. fragilis* strains. The cross-correlations between certain gene expressions were very strong ($r > 0.7$, $p < 0.01$), indicating their common regulation, although not all genes (except for *frdA* and *frdC*, whose expressions correlated well—$r = 0.593$, $p = 0.0192$) are located on the same operon. Moreover, we detected highly significant correlations between the expression of some genes and metronidazole MICs. In particular, lactate dehydrogenase (*ldh*) expression correlated positively, whereas cytochrome b fumarate reductase/succinate dehydrogenase (*frdC*), malate dehydrogenase (*mdh*), phosphoglycerate kinase (*pgk*) catabolic and *gat* (GCN5-related acetyltransferase toxin), and *relA* (stringent response regulator) regulatory gene expressions correlated negatively with metronidazole MICs. Within the *nim*-positive and *nim*-negative strains, we detected cross-correlations between gene expressions;

however, we found no significant association between metronidazole MICs and gene expressions, except for *mdh* and *gat*, which tended to correlate with metronidazole MICs in the *nim*-positive and *nim*-negative groups, respectively. In addition, the gene cross-correlations of the full set did not overlap with those in the *nim*-positive and *nim*-negative groups of strains. The lack of statistical confirmation may be due to the low number of strains in each group (eight *nim*-positive and seven *nim*-negative strains). However, one-way variance analysis demonstrated that *frdC*, *gat*, *mdh*, *nanH* (sialidase), *pgk*, and *relA* gene expression depended on the presence of the *nim* gene; but the *cfiA* gene status did not affect the expression of the genes examined.

We also wanted to know how the addition of intermediates of the rTCA pathway affected metronidazole MICs. We expected that higher oxaloacetate or fumarate concentrations would decrease the redox intermediate concentration (e.g., NADH), thus decreasing metronidazole activation and MICs. In these experiments, we used modified M9 minimal medium supplemented with Tryptone, hemin, vitamin K1, and glucose or C_4-dicarboxylic acid. Out of six *nim*-negative or *nim*-positive strains, four showed no significant difference in metronidazole MICs compared to those obtained on supplemented Columbia agars. However, the MICs of one *nim*-positive and one *nim*-negative strains increased in response to glucose, malate, and succinate addition, whereas no changes were observed in response to oxaloacetate or fumarate addition.

5. DISCUSSION

- In this study, we analyzed the prevalence of "rare" β-lactamase genes in *Bacteroides* species strains from normal microbiota and cases of infection from several countries, and where necessary, other traits of the strains, especially their genetic backgrounds, were also examined. Most of our current knowledge is about *cepA*, *cfxA*, and *cfiA*; therefore, this exhaustive study on 590 strains provides important new data. The genes included in our study had very different amino acid sequences and they have various biochemical activities. *cblA* was found in a large set of *B. uniformis* strains (n = 18), making it 100% specific for this species. *bla*HGD1 was specific for two closely related *Phocaeicola* species, namely *Ph. vulgatus* and *Ph. dorei*. *crxA* was found in both intestinal and infectious *B. xylanisolvens* strains. We demonstrated that the *crxA*-positive strains were not clonal

using different typing schemes, as the topologies of the detected dendrogram and the positions of the *crxA*-positive strains differed. Based on the observed dendrogram topologies, we suspect that there are limitations in the use of the IR Biotyper or MALDI-TOF MS in this case. The picture could be extended with *crxA* element detection because the previously described case for the prototype strain (*B. xylanisolvens* 14880) was not found for all positive strains. We anticipate that the described *crxA* element can be inserted at different genomic locations or there are other *crxA* element(s) different from the prototype. *pbbA* was found in some of the species of our collections, but it was rarely detected. The gene conferred strong piperacillin resistance, potentially accounting for some of the cases of moderate β-lactam/β-lactamase resistance among *Bacteroides* spp. strains. *bla*OXA347 was found in six *Bacteroides* strains with imipenem resistance that were obtained in the USA during *Bacteroides* antibiotic susceptibility studies in the 1990s. This gene has been rarely detected in (Denmark and Hong Kong), but carbapenem resistance in cases where this gene was detected could not be confirmed. This is why further studies are needed. Resistance to antimicrobial agents is less extensively studied in anaerobic bacteria than in aerobic bacteria. However, anaerobes comprise the majority of human gut commensal bacteria, and they can serve as reservoirs of antimicrobial resistance genes for the related organisms.

The limitation of this study is that the exact carriage rates for some of the genes cannot be established appropriately since we had a relatively low number of strains for the normal microbiota in some of the European countries. And for the Hungarian clinical strains we found low rates of *crxA* and *pbbA* genes that may vary in other circumstances. *bla*OXA347 could only be detected in strains isolated earlier in the USA so the situation may have changed since then and in other places. Therefore our data results may only roughly represent the true picture.

- The detected ‘independence’ of the metronidazole resistance levels in the expression of the *nim* genes does not mean that they are not true resistance factors, but we expect that other processes restrict the action of *nim* genes. The nature of these factors may be epistatic, rate-limiting or competitive. As a limiting factor we recently found that when the medium is hemin-deprived, it reverts to the susceptibilities of the strains with *nim*-dependent and *nim*-independent resistance mechanisms. Hence, a lack of hemin (as a cofactor of redox enzymes) may limit the level of redox enzyme produced

reduced co-factors whose nature should be investigated. At present ferredoxin is thought to be the co-factor that reduces metronidazole to an active compound, but this needs to be checked. Iron, as chelated in hemin, might also be necessary in such processes. An earlier and two more recent articles also reported that iron is an important co-factor in the metronidazole resistance of *Clostridioides difficile* and *B. fragilis*. RT-qPCR experiments with extend the range of genes and will provide an insight into the roles of other factors that are required for Nim proteins to act, initiate full clearance, and tell us how these genetically well-characterized genes participate in metronidazole resistance.

- Although we found no significant association of the genes in question among the *nim*-negative and *nim*-positive strains separately, the combined data indicated several good associations in the case of the whole strain set. Therefore, we conclude that no particular enzyme is exclusively correlated with metronidazole resistance in both the *nim*-negative and *nim*-positive strains. However, some of these genes have previously been found to cause metronidazole resistance, e.g., *feoAB*, *acr5 bmeB*, and *por*. This may be applied to genes not examined here (*recA*, *sod* and *rhaA*), as their role was demonstrated in metronidazole resistance earlier. So, at the population level, these genes do not exert a general role; they are important only in individual cases/strains. However, it should be remarked that in both *nim*-positive and *nim*-negative strains, the possible exceptions of *mdh* and *gat* might be significant, respectively.

The central metabolism varies greatly among bacteria e.g., the central metabolism of *Bacteroides* differs greatly from that of γ-Proteobacteria, and the latter comprises glycolysis and parts of the tricarboxylic cycle (TCA). However, instead of a complete TCA cycle, *Bacteroides* have a reductive or reverse TCA (rTCA) branch that is heme dependent, as well as a branch that is heme independent. Previously, we found that hemin depletion causes metronidazole susceptibility in both *nim*-negative and *nim*-positive strains of *B. fragilis*. Thus, heme may be a rate-limiting factor in the metronidazole resistance of *B. fragilis*, as proposed above. Our results indicate that the expression of genes from the glycolytic and rTCA pathways (*pgk*, *frdC*, and *mdh*) correlate negatively with the metronidazole MICs, whereas that of *ldh* correlates positively. These latter changes may decrease the cellular concentrations of the reducing cofactor, which diminishes metronidazole activation, thus inducing resistance. It is possible that the hemin dependence

of metronidazole resistance is due to the hemin dependence of the NimB protein; however, this does not explain the hemin dependence of *nim*-negative strains.

In addition, our findings are consistent with the previous observation on the flexibility of metronidazole MICs and the idea of a rate-limiting step(s) involved in *nim* action in metronidazole resistance. Here we propose the following mechanism: the addition of malate and succinate forces the cells to reduce the levels of these compounds at the expense of the pool of reducing cofactors, thus leading to decreased metronidazole activation. The observed increase in *ldh* gene expression is consistent with previous findings, revealing the importance of reducing cofactors in metronidazole resistance in anaerobic bacteria. This means that the pyruvate level is the main mediator in this latter process. However, we did not observe a differential expression of *por* during our experiment. The involvement of *frdC* (a cytochrome b enzyme) in metronidazole resistance is noteworthy because it can explain, at least partly, the heme dependence of the metronidazole resistance of *B. fragilis.* Additionally, the negative correlation of the regulatory genes (*relA* and *gat*) suggests that a high metabolic state is required for metronidazole to act on cells because these genes have a role in decreasing cellular metabolism. This study is the first to examine the role of multiple proteins/genes on metronidazole resistance in clinical *B. fragilis* strains.

Based on the results of proteomic studies, Paunkov et al. developed models of how *nim* and other proteins act in *nim*-dependent and *nim*-independent metronidazole-resistant *B. fragilis* strains. Here, we propose that a limited number of genes/proteins are correlated with metronidazole resistance in *B. fragilis* at the population level. In this study, we highlighted the importance of reducing cofactors that are needed for both metronidazole activation and inactivation. Thus, the metronidazole resistance mechanism of *B. fragilis* is complex and nonlinear. This complexity could explain why metronidazole MICs and *nim* gene expression do not always correlate, especially long after the isolation of strains from clinical specimens. So more work is needed to determine significant associations between gene expression and metronidazole MICs in "field" strains of *nim*-negative and *nim*-positive *B. fragilis*. In particular, more strains need to be analyzed to determine the roles of these genes. And the roles of the genes that had positive or negative correlations with metronidazole resistance should be confirmed with a

deletion–complementation analysis. In this case, *frdC* is a good candidate for these experiments because it also contains heme. Identifying with more certainty which redox cofactor activates metronidazole is also a future task. We think that the genes that had a role in our study (*ldh*, *frdC*, *mdh*, *pgk*, *gat* and *relA*) are significant/valid contributors in this key antibiotic resistance mechanism.

6. CONCLUSIONS

Based on our current observations, β-lactamases encoded rare β-lactamase genes should not be overlooked because they may confirm important resistance phenotypes. They represent very different proteins, and they may be species-specific. *crxA* is genetically polyclonal among *B. xylanisolvens* strains. Moreover, a recently described mobilizable gene, called *pbbA* is carried on some plasmids among *Bacteroides* species strains, and it could increase resistance to piperacillin and piperacillin/tazobactam.

In summary, from our results we found that (i) the *nimB* and *nimE* genes are uniformly regulated by their IS elements in different strains; and (ii) the metronidazole resistance of our test strains did not correlate with the expression levels of these *nim* genes. Therefore, we expect that auxiliary factors play a part in the *nim*-mediated metronidazole resistance of *Bacteroides* species. These results and our main assumptions should help clarify the role of *nim* gene-IS element combinations and the mechanism of metronidazole resistance among anaerobic pathogens.

In this study, we assessed the connection between metronidazole MICs and the expression of 18 genes in a wide selection of *B. fragilis* clinical strains. The expression of metabolic genes *ldh*, *frdC*, *mdh*, and *pgk* correlated with metronidazole resistance, independent of the presence of *nim* genes. This finding means that redox intermediates may be crucial in both metronidazole activation and enzymatic inactivation. However, the exact identities of the enzymes and intermediates involved in both processes need to be confirmed experimentally. Roles for some regulatory proteins (*gat*, *relA*) were also found and not all (genes)/proteins could be examined here as they were differentially expressed at the protein level. This is why the list of examined genes should also be increased.

7. NOVEL FINDINGS

- It was confirmed on a large number of normal microbiota and clinical strains that the *cblA* gene is *B. uniformis*/specific and all strains carry it.
- *bla*HGD1 behaves similarly – it is *Ph. vulgatus*/*dorei*-specific.
- The *pbbA* gene is implicated in the resistance to piperacillin or piperacillin/tazobactam.
- *crxA*-positive *B. xylanisolvens* isolates were found not to be genetically related by different typing methods.
- *nim* genes are usually under transcriptional control by gene-specific insertion sequence elements; and here we determined the transcriptional start sites of the promoters of the *nimE* gene-specific IS*Bf6* element.
- However the IS elements with specific promoters permit a constant expression, which is not reflected in metronidazole resistance (*nimB*-IS*1186* and *nimE*-IS*Bf6*).
- Over all, it was demonstrated that the *nim* gene expression is similar in strains with different genetic backgrounds.
- Other pathways should be sought to better understand the resistance determinant function of *nim*-genes.
- This study is the first to examine the role of multiple proteins/genes on metronidazole resistance in clinical *B. fragilis* strains.
- In the case of 15 *B. fragilis* strains the roles of *ldh, frdA, mdh, pgk, gat* and *relA* genes was demonstrated in metronidazole resistance, and this establishes the dependence of metronidazole susceptibility and resistance on reducing cofactors and cellular metabolic activity.
- The rTCA intermediates may affect metronidazole resistance and account for its hemin-dependence.