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:
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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:
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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:
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List of Abbreviations

AMR	Antimicrobial Resistance
BFT	*B. fragilis* toxin
BHIS	Supplemented Brain–Heart infusion broth
CNS	Central nervous system
CTns	Conjugative transposons
DMSO	Dimethyl sulfoxide
ERIC PCR	Enterobacterial Repetitive Intergenic Consensus PCR
EUCAST	The European Committee on Antimicrobial Susceptibility Testing
IS element	Insertion sequence element
LDH	Lactate dehydrogenase
MALDI-TOF MS	Matrix-Assisted Laser Desorption/Ionization Time-of-Flight mass Spectrometry
MBLs	Metallo-β-lactamases
MDR	Multidrug-Resistant
MGE	Mobile genetic element
MIC	Minimum Inhibitory Concentration
MTn	Mobilizable transposon
MTZ	Metronidazole
NCBI	National Center for Biotechnology Information
ORF	Open reading frame
PBP	Penicillin binding protein
PFOR	Pyruvate-ferrodoxin oxidoreductase
RACE PCR	Rapid amplification of cDNA ends
SCA	Supplemented Columbia Blood Agar
rTCA	Reductive or reverse TCA
TCA	Tricarboxylic cycle

1. INTRODUCTION

1.1 *The importance of Bacteroides species*

Human beings are colonized by a diverse collection of commensal microorganisms. The gut and gastrointestinal tract due to the availability of diverse nutrient sources derived from the host's diet make it a predilection habitat for a huge population of tens of trillions of these microorganisms that are normally acquired from the birth and neonatal period until the age of three years [1]. Concentrations of bacterial residents in the gut is roughly estimated at 10^{12}/ml, which is more compared to other body sites [2, 3]. These microbes are either harmless or beneficial to the host. They can strongly influence the host physiology in various aspects including the immune system function development, resistance to infection and the metabolism of nutrients [4]. 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 [5].

The nomenclature of the *Bacteroides* species has been discussed with some changes since the 1970s. First in the *B. fragilis* group there were subspecies which later attained the species level and the non-fragilis *Bacteroides* were reclassified mainly as *Prevotella* and *Porphyromonas* genera in the 1980s. And mostly by the development of high-throughput sequencing methods many more *Bacteroides* species were defined (>60), from which the *Parabacteroides* and *Phocaeicola* species were separated after the 2000s and 2010s, respectively. In my thesis by *Bacteroides* species I usually mean the more related and former *Bacteroides* spp. - the *Bacteroides*, *Parabacteroides* and *Phocaeicola* genera. As a part of human microbiota, anaerobic bacteria are primarily located on the mucosal surface and lumen of the gastrointestinal tract, oropharynx and female genital tract [6]. Despite being a beneficial provider for the healthy host and surrounding microbes residing close to them, some species of *Bacteroides* based on their location have a dual beneficial and pathogenic role in the host [3]. Under normal circumstances gut *Bacteroides* species and the host have a mutually beneficial relationship. *Bacteroides* species can provide numerous benefits to the host such as the

ability to utilize a wide variety of carbon sources. *B. fragilis* plays an important role in carbohydrate fermentation. Most importantly, they are the leading synthesizer of vitamin K in the human gut [5, 7, 8]. They can become opportunistic pathogens especially when they escape from their natural habitat as a consequence of surgery, trauma and injury or mucosal barrier disruption (especially in the case of cancer) to other normally sterile extra-intestinal body sites. Then they can be associated with a broad range of mono- and poly-microbial infections with other anaerobes and aerobic bacteria [6, 9]. Possible disease conditions and infection sites related to *Bacteroides* species are shown in **Figure 1**. *Bacteroides* species are the most frequent endogenous anaerobic opportunistic pathogens isolated from the gastrointestinal tract, but in rare cases they may be present in the oral cavity and upper respiratory tract, and female genitalia [5, 10]. Sometimes they cause serious infections such as intra-abdominal abscesses, obstetric–gynecological conditions, postoperative wounds, lung and brain abscesses, skin and soft tissue infections, appendicitis, diarrhoea and sepsis they are sometimes associated with oral infection and abscesses formation in the neck. *B. fragilis* and *B. vulgatus* are reported as the two main isolates identified from patients suffering from Crohn's disease [3, 5]. *Bacteroides* species are the most resistant in terms of the number of antibiotic resistance mechanisms and the antibiotic resistance levels attained among all the anaerobes. In many countries, recent studies have revealed regional variations in resistance to antimicrobial agents among *Bacteroides* species [11, 12].

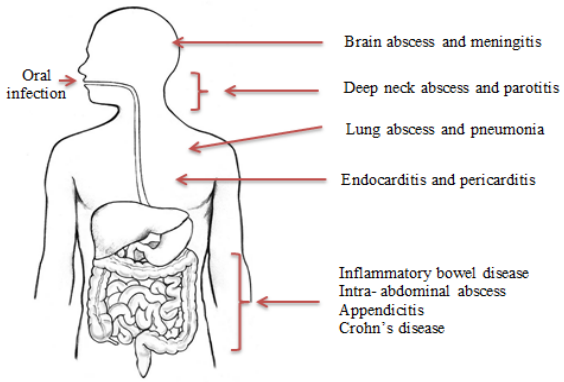

Figure 1. Disease and infection sites associated with *Bacteroides* species [3]

1.2 *Pathogenic characteristics and virulence factors of Bacteroides*

Normally *Bacteroides* species are commensals when residing in their natural habitat in the gut, but under favorable conditions they tend to become opportunistic pathogens especially when they translocate to another place in the body [13]. *Bacteroides* species translocate via the intestinal mucosa to other usually sterile tissues in the body other than the gut, ultimately inducing various disease conditions [14]. Numerous factors may be attributed to translocation from the gut to extra-intestinal body sites such as: 1) The disruption of the gut burrier; 2) A compromised immune system; 3) Extreme usage of antibiotics; 4) The elderly; 5) After surgery; 6) Circulatory problems and underlining diseases such as diabetes [3, 15, 16]. Mostly, during early penetration in the extra-intestinal organs aerobic bacteria dominate and damage the tissue. Later when the redox potential of oxygenated tissue decreases, anaerobic bacteria such as *Bacteroides* species start to thrive and this leads to the formation of an intra-abdominal abscess, inflammation and diarrhoea [14, 17]. *Bacteroides* species dissemination outside of the gastrointestinal tract lumen can consequently lead to some life-threatening disease like abscess formation in different body sites and bacteremia, occasionally present even in the central nervous system (CNS) [18]. Nevertheless, 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. In addition, they have other properties to avoid the host immune system, aerotolerance and mechanisms for antibiotic resistance [5]. The most important virulence factor is the enterotoxin of *B. fragilis* BFT that may cause diarrhoea in children. It has four isotypes called BFT-1, BFT-2, BFT-3, BFT-4 and the gene encoded enterotoxins are *bft1-4* located on a specific pathogenicity island. This enterotoxin which is also known as fragilysin is a about 20 kDa of non-lethal, heat-labile zinc-dependent metalloprotease capable of cleaving the E-cadherin protein of zonula adherence in the intestinal epithelial cells, leading to diarrhoea and the rearrangement of the actin cytoskeleton of the epithelial cells [19, 20].

1.3 *The antimicrobial resistance of Bacteroides*

Resistance to antimicrobial agents, which is a global issue in the clinical setting, remains a neglected concern in anaerobic bacteria mainly due to the great work needed for their isolation and identification [21]. 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, as shown in **Figure 2**. The resistance mechanisms of *Bacteroides* species to different antimicrobial agents are summarized in **Table 1**.

There is a relatively limited number of anti-anaerobic agents that are potentially effective for therapy and they can be prescribed in the case of anaerobic infections including those associated with the *Bacteroides* species such as β-lactams, β-lactam/β-lactamase inhibitor combinations, clindamycin, tigecycline, tetracycline, chloramphenicol, fluoroquinolones as well as the two most effective antimicrobial agents called carbapenems and metronidazole. Compared with most of the other anaerobic bacteria, resistance to antibiotics in *Bacteroides* strains is the most abundant according to some recent studies [6, 22-24].

Some studies have demonstrated that poor clinical outcomes are related to a failure in a direct appropriate anaerobic therapy [25]. Failure of a therapy against anaerobes may be due to several reasons including infection duration and severity, the patient's age, inaccuracies in the technique of susceptibility testing, the absence of surgical drainage, comorbidity, a lower pH at the site of the infection, impaired host immune defence, the deprivation of antimicrobial penetration at the site of the infection, the presence of an inactivation enzyme against antimicrobials and other antibiotic resistant factors [22]. Due to its natural location, *B. fragilis* in the human gut is normally exposed to various antimicrobial agents and some toxic compounds like bile [26, 27]. It has been found that the susceptibility levels in *B. fragilis* for most antimicrobial agents is continually in decline [28]. *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. The MDR of *B. fragilis* is

possibly linked to a failure of empiric therapy especially in those patients that have a history of an extensive exposure to antimicrobial agents [29-31]. Hence, there is a crucial need to monitor the pattern of resistance periodically for clinically important anaerobes. This will help the microbiologists and clinicians to further enhance their understanding of antimicrobial resistance in anaerobes and recommend better therapeutic approaches for improving the treatment outcomes by combatting resistant anaerobic bacteria [32].

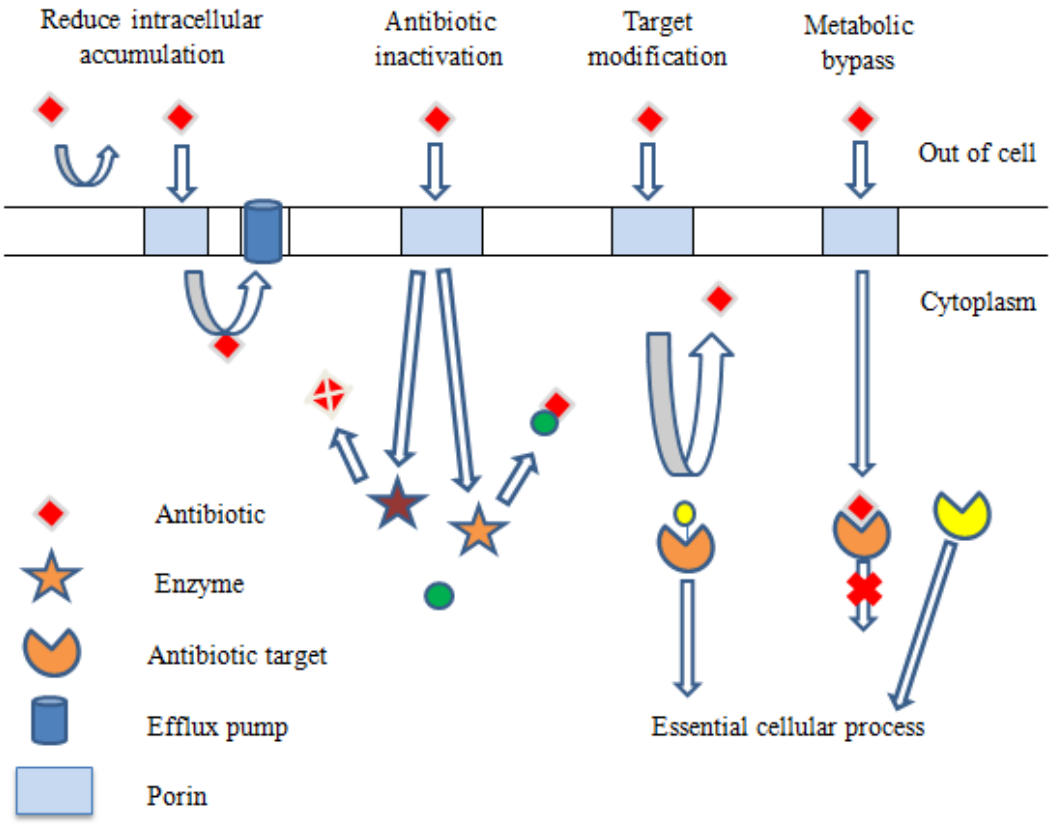

Figure 2. An overview of the antibiotic resistant mechanisms in bacteria

Table 1. Various antimicrobial resistance mechanisms of the *Bacteroides* species [6, 24, 33-36]

Antimicrobial agents	**Mechanisms of resistance**	**Genes or enzymes involved**
β-lactams	‣ Cephalosporinases	‣ *cepA*, *cfxA*
	‣ Metallo-β-lactamases	‣ *cfiA*, *crxA*
	‣ Modify affinity of target molecule	‣Penicillin binding proteins PBP1-2 alterations
	‣ Efflux pumps	‣ *bmeRABC5* (RND type)
	‣ Decreased uptake	‣ -
	‣ Loss of porin channels	‣ -
Clindamycin	‣ Methylation of the 23S rRNA	‣ *ermF*, *ermG*, *ermS*
	‣ Inactivation	‣ -
Fluoroquinolones	‣ DNA gyrase	‣ *gyrA*, *gyrB*
	‣ topoisomarase IV	‣ *parC*
	‣ Efflux pumps	‣ (MATE and RND type)
Metronidazole	‣ Reduction of the drug by nitroimidazole reductase	‣ *nimA-L*
	‣ Deficiency of iron uptake	‣ *feoAB*
	‣ Overexpression of DNA repair protein	‣ *RecA*
	‣ Increase in LDH activity	‣ -
	‣ Efflux pumps	‣ *bmeRABC5* (RND type)
Chloramphenicol	‣ Acetylation	‣ *cat*
	‣ Nitro-reduction	
Tetracycline	‣ Ribosomal protection	‣ *tetQ*
	‣ Efflux pumps	‣ *bmeRABC5* (RND type)
	‣ Enzymatic degradation (oxidative)	‣ *tetX*

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 [37]. For anaerobic bacteria several metronidazole resistance mechanisms have been described or suggested so far. The principle for these various mechanisms among organisms is the reduced uptake of the therapy and a modified reduction in efficiency [38, 39]. 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 [37]. The mode of action and the main metronidazole resistance mechanisms in *Bacteroides* are shown in **Figure 3**. A high level of metronidazole resistance (MIC 64 μg/mL) in *B. fragilis* (NCTC11295) was observed for the first time in 1978 from a patient who had a Crohn's disease with subsequent extensive metronidazole therapy [40]. Since then, other resistant strains from clinical specimens have been reported [41].

After the emergence of resistance to metronidazole, the prevalence of acquiring resistance among anaerobe remained overall moderately low, and several studies focused on this resistance issue in *Bacteroides*. These studies revealed that in most parts of the world metronidazole resistance is still at a lower level with (<3%) [12, 42-48], except for a reported rate of resistance in Spain (4.8%), South Africa (8.7%) and Pakistan (16-20%) [49-52]. In most Western countries it is less than 1% despite its long-term use since the 1960s. However, in different European countries it seems to have increased slightly. And in developing countries the metronidazole resistance rates in *B. fragilis* are sometimes very high-between 0.5 to 7.8% according to surveys from European countries, Canada, the USA, Asia, Africa and the Middle East [51, 53-56].

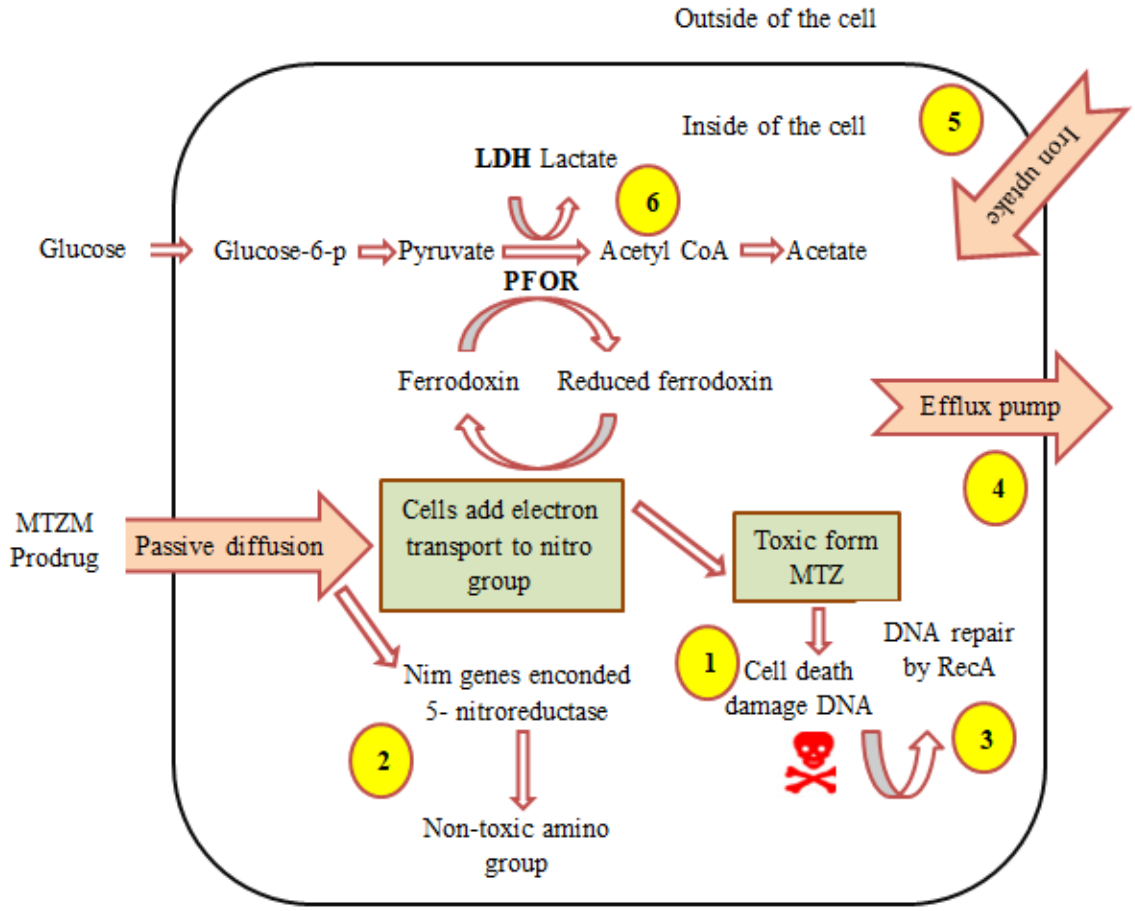

Figure 3. The metronidazole mode of action and main mechanisms of resistance in *Bacteroides* [35, 56]

1- The mode of action of Metronidazole: After MTZ transport to bacteria cytoplasm by passive diffusion, it is activated by electron transport from electron donors to the nitro group of MTZ prodrug molecule. This activated drug has cytotoxic effects and it damages bacterial genetic material such as DNA and it leads to cell death.

2- Nim-mediated resistance: The *nim* gene encoded nitro-reductase reduces the nitroimidazole drug of the 4 or 5-nitro group to the amino group and it produces a nontoxic inactive compound of 5-aminoimidazole.

3- An overexpression of the RecA protein: An increased DNA repair capacity gives rise to an increased resistance to metronidazole.

4- The efflux of the metronidazole drug: Through the overexpression of the efflux pump encoding genes such as *bmeRABC5* that confer metronidazole resistance.
5- A shortage of the ferrous iron transporter FeoAB: A lower level of iron is the reason for the deficiency of *feoAB*, which leads to the reduction of electron mediated metronidazole activation.
6- A metabolic change of the pathway linked to the reduction of pyruvate to lactate by lactate dehydrogenase which takes away pyruvate from pyruvate-ferredoxin/flavodoxin oxydoreductase and hence reduces metronidazole activation.

1.3.2 *The nim genes mediate metronidazole resistance*

Metronidazole resistance most of the time is coupled with the presence of *nim* genes, which was first described and reported as a transmissible or mobile resistance determinant of metronidazole [57, 58]. The *nim* genes (5-nitroimidasole resistance) encode nitroreductases that are thought to reduce metronidazole without producing harmful intermediates and they can be found most frequently among metronidazole-resistant *Bacteroides*, *Parabacteroides*, *Phocaeicola* and *Prevotella* strains [35]. Currently, we are aware of 12 *nim* gene types (*nimA-L*), they are 60-80% homologous and their expression is assumed to be activated by insertion sequence elements. These genes may locate on various amplicons like the chromosome or on small plasmids and each *nim* gene type may harbor a highly gene-specific IS element in their upstream regions [35, 36, 59]. The insertion sequence open reading frame (ORF) is in the reverse orientation of the *nim* genes ORF and they usually carry outward-oriented promoters that play an important role in the activation of *nim* genes transcription [59-61]. Furthermore, the expression of other resistance genes in *Bacteroides* species may be activated or regulated by insertion sequence elements such as those resistance genes linked with cefoxitin (*cepA*), macrolides (*erm*), cephalosporin (*cfxA*). These insertion sequence elements may be identical or similar with those described in carbapenem resistance *cfiA* gene positive strains [59-62]. In some cases the presence of the *nim* genes is linked to the presence of *cfiA* genes (the main effector of carbapenem resistant among *B. fragilis*), which helps induce multidrug-resistance [63, 64].

1.3.3 *β-lactam resistance mechanisms including carbapenems*

β-lactams are an important class of antibiotics most frequently prescribed in the hospital setting because of their efficacy, low toxicity and broad spectrum activity. This class of antibiotic includes penicillins, cephalosporins, monobactams and carbapenems [65]. β-lactam in combination with inhibitors of β-lactamase are also the drug of choice to combat infections related to the *Bacteroides* species [11, 12]. The action of all these agents involves inhibiting transpeptidases that are necessary for the final step of cell wall synthesis in bacteria [65, 66]. Among the *Bacteroides* species, the most common resistance against β-lactams occurs by various molecular mechanisms such as enzymatic inactivation like β-lactamases, reduced membrane permeability, the over expression of the multidrug efflux pump and the expression of alternative penicillin binding proteins (PBP_S) with a lower affinity to some β-lactams [67]. The resistance to β-lactam antibiotics is a worrisome problem in anaerobes mainly due to β-lactamase enzymes [65]. These enzymes are produced by bacteria to facilitate the hydrolysis of the β-lactam ring and render the antibiotic inactive or harm its effectiveness, making them unable to hydrolyze PBPs [65, 68].

The level of β-lactam antibiotic resistance varies among anaerobic bacteria. Nearly all *B. fragilis* strains display a resistance to penicillin G due to the production of the chromosomally located penicillinase enzyme. This rate decreases to 50% in the case of the *Prevotella* species and for *Porphyromonas* species it decreases to around 8-17% [24, 69]. At the molecular level currently two major classification schemes are used for β-lactamases: first there is the Ambler classification based on the molecular structure (the amino acid sequence) where β-lactamases may be divided into four distinct groups (A, B, C and D). Classes A, C and D have a serine residue in their enzymatic active site (serine β-lactamases), while class B is known for its zinc (Zn^{2+} ion) dependent metallo-β-lactamases (MBLs). The second is the Bush-Jacoby-Medeiros classification scheme of the enzymatic characteristics, which puts β-lactamases into three groups according to substrate hydrolysis similarities and the response to numerous inhibitors. Group 1 contains serine cephalosporinases enzymes; in group 2 there are mainly serine penicillinases; and in group 3 there are metallo-β-lactamases [65, 70, 71].

In *B. fragilis* the Class A β-lactamases (cephalosporinase encoded by *cepA* and *cfxA* genes) confer penicillin and group I and I cephalosporin and cephamycine resistance. These enzymes may be inhibited by a β-lactamases inhibitor such as clavulanic acid, sulbactam, or tazobactam. 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* [24, 67]. And in *B. xylanisolvens*, the carbapenemase enzyme encoded by the *crxA* gene was recently described [34]. Isolates harboring *cfiA* or *crxA* confer a high level of resistance to all β-lactams and any drug combination with β-lactamase inhibitors along with carbapenem which is usually regarded as an important therapy in the case of serious life-threatening infections [67].

It has been pointed out that in in *B. fragilis* isolates with a silent *cfiA* gene sometimes do not display any resistant phenotype. This is probably due to the expression of this gene at a lower level where it could not confer a resistance phenotype or have a decreased susceptibility to carbapenems. Susceptible strains to carbapenem may carry this silent *cfiA* gene. However, silent *cfiA* genes through a one-step mutation which involves an IS element insertion into the upstream region may be highly expressed and display resistance phenotype [24, 67, 72]. With *Bacteroides*, insertion sequence elements play a crucial role in driving the expression of the gene involved in carbapenem resistance. For the *cfiA* gene expression the existence of an IS element (e.g. I*S613*, IS*4351*, IS*614*B, IS*1169*, IS*1186* or IS*1187*) is necessary in the upstream region of this gene to act as a promoter [67, 73]. With the presence of *cepA* and *cfiA*, *B. fragilis* strains can be divided into distinct groups, namely division I (*cepA*-positive, *cfiA*-negative) and division II (*cepA*-negative, *cfiA*-positive) [74, 75].

1.4 *Mobile genetic elements in Bacteroides*

Like other bacteria, to exchange the genetic material information the *Bacteroides* species have a variety of mechanisms available to do this. The genome of the *Bacteroides* species is incredibly flexible and it has the capacity to activate and repress phenotypic functions like AMR [5, 9]. The presence of mobile genetic elements (MGEs) is not only important to molecular biologists, but it is important for the clinical microbiologists and infectious disease specialists too, since these elements 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 [5]. As summarized in **Table 2**, the *Bacteroides* species carry various conjugative and mobilizable elements.

1.4.1 *Insertion sequence elements*

IS elements are small segments of double stranded integrative DNA with (< 2.5 kb) that encode a transposase enzyme which is implicated in the transposition activity and adaptability of prokaryote genomes [67, 76]. They are very common in all bacterial phyla and in their genome a wide range of IS elements copy numbers can occur [76, 77]. Within bacterial chromosomes IS elements can move and are involved in the modulation of nearby gene expression with the help of outward-oriented promoters [67]. Additionally, they may take part in gene dissemination by transferring genetic material from the bacterial chromosomes to other mobile element vectors such as plasmids and phages. The IS elements are bordered by inverted repeat (IR) sequences, and during the integration IS elements usually induce target site duplications of a small number of nucleotides [67]. 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* [9, 59, 78].

1.4.2 *Plasmids*

Plasmids are very common among *Bacteroides* and there are two different types of plasmids harbored by these species: cryptic and antibiotic resistance plasmids. Cryptic plasmids can be found in approximately 20-50% of the strains with a small molecular size and despite their widespread dissemination, cryptic plasmids do not encode any antibiotic resistance genes or induce the production of protein associated with virulence. They have the capacity to replicate and be mobilized by horizontal gene transfer [5, 79]. These cryptic plasmids can be characterized into three major classes according to the size of the plasmid: class I (2.7 kb), class II (A, 4.2 kb; B, 5.0 kb; C, 7.9 kb), and class III (5.6 kb) [79, 80].

In the *Bacteroides* species, two groups of resistance plasmids have been discovered so far, namely conjugative and mobilizable plasmids, which have the ability to replicate as independent elements in the host cell. Several plasmids have a transfer origin *oriT* sequence and a *trans* acting mobilization gene which lets them transfer by conjugation e.g. *B. fragilis* pBF4 (41 kb), *B. ovatus* pBI136 (80.6 kb) [79]. There are also multiple mobilizable plasmids that do not have the ability to perform auto-transfer, but via mobilization they may be transferred by other chromosomal elements like *B. fragilis* pBFTM10 (15 kb) [5]. Different genes conferring resistance to antimicrobial agent such as clindamycin, chloramphenicol, metronidazole, carbapenem have been found on plasmids in the *Bacteroides* species. For example, genes conferring metronidazole resistance like *nimA-F* have been identified on a transferable plasmid in clinical isolates worldwide [81]. The carbapenem resistance gene *cfiA* has also been found on a 6.4 kb plasmid in clinical strains [82].

1.4.3 *Conjugative transposons*

Transposons are divided into conjugative and mobilizable transposons and they are incapable of self-replication unaided, in contrast to plasmids. Most of these transposons are located on the bacterial chromosome and copied along with the genomic DNA. Conjugative transposons have the ability to excise from and integrate into chromosomal DNA using a specific mechanism [79]. More than 80% of *Bacteroides* species isolates

carry at least one conjugative transposon often designated as CTns. In *Bacteroides* clinical isolates, conjugative transposons have been mostly responsible not only for disseminating genes, but also for transferring genes that confer resistance to tetracycline and erythromycin antibiotics [83]. The size of conjugative transposons, which is highly distributed among *Bacteroides* strains ranges from 52 kb to 150 kb, and the best characterized CTn is the *B. thetaiotaomicron* CTnDOT (65 kb) [79, 83]. Many transposons in *Bacteroides* harbor the *tetQ* gene, so they are often called tetracycline resistance factors and they may confer resistance to tetracycline and by tetracycline exposure most of the conjugative transposons can be induced to be transferred [79]. Moreover, there are many CTns that harbor the regulatory system *rteABC* gene cluster, which plays a role in the regulation of conjugal transfer [84, 85]. Lots of *B. frsgilis* CTns also harbor the genes *ermB* (CTnBST), *ermF* (CTnDOT), and *ermG* (CTNGERM1), which confer resistance to erythromycin [86].

1.4.4 *Mobilizable transposons*

Mobilizable transposons (MTns) compared to conjugative transposons are smaller with a size of 5 kb to 15 kb. They are located on the chromosome, but harbor genes encoded for the product for the excision, integration and mobilization of these genetic elements [79]. Similar to mobilizable plasmids, MTns have no ability to perform an auto-transfer among species. They can transfer material to the recipient only in the presence of other elements like conjugative transposons and the TcR helper elements [79]. Some of the most common and well-characterized MTns among *Bacteroides* include *B. fragilis* Tn4399 (9.6 kb) [87], *B. vulgatus* Tn4555 (12.5 kb) [88], *B. fragilis* Tn5520 (4.69 kb) [89], *B. fragilis* cLV25 (15.3 kb) [90], *B. uniformis* NBU1 (10.3 kb), and *B. uniformis* NBU2 (11.1 kb) [91].

Table 2. Summary of mobile genetic elements found in the *Bacteroides* species [79]

Conjugative Elements	**Mobilizable Elements**
Self-transmissible	Not self-transmissible
Encode DNA-processing function	Encode DNA-processing function
Encode conjugation apparatus (CA) or mating channel for completely autonomous transfer	Do not encode CA. Transfer is dependent on CA formed by co-resident conjugative element(s)
Almost always harbor antibiotic resistance genes	May harbor antibiotic resistance genes
Conjugative Plasmids	**Mobilizable Plasmids**
Have *oriT* and *trans*-acting mobilization gene(s)	Have *oriT* and *trans*-acting mobilization gene(s)
Can replicate independently	Can replicate independently
May integrate into recipient chromosome	May integrate into recipient chromosome
E.g. pBF4, pBI136	E.g. pBFTM10
Conjugative Transposons	**Mobilizable Transposons**
Located on a chromosome	Located on a chromosome
Do not replicate extra-chromosomally	Do not replicate extra-chromosomally
52 kb -150 kb	5 kb -15 kb
Also referred to as “Tet elements” since most carry the tetracycline resistance gene tetQ	-
E.g. CTnDOT, BFT-37, CTnERL, Tcr Emr 7853, Tcr Emr DOT, CTnGERM1, CTnBST, CTn9343, CTn86	E.g. Tn4399, Tn4555, Tn5520, cLV25, NBU1, NBU2

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 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. The clinical isolates were collected in four Hungarian microbiological centers between 2014 and 2016 [92], and six clinical isolates obtained in the USA were also included (these strains were provided by the collection of Dr. David Hecht and initially obtained from antibiotic susceptibility studies in the USA in which *cfiA*-positive strains were reported [93]). The species composition is shown in **Table 3**. Fecal strains were collected in Germany, Belgium, Slovenia, Hungary, and Turkey [94, 95]. The *B. fragilis* strain carrying the pPE2-1 plasmid was described previously [96]. 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 matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS; Biotyper®, Bruker Daltonics, Bremen, Germany) [97]. Antibiotic susceptibility testing was performed to obtain minimum inhibitory concentrations as previously described [92, 94, 95] 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).

Table 3. The species composition of *Bacteroides, Parabacteroides, and Phocaeicola* in clinical and normal microbiota isolates

Species	**Clinical isolates (n=406)**	**Normal microbiota (n=184)**
B. fragilis	236	25
B. thetaiotaomicron	79	24
Ph. vulgatus/dorei	26	36
B. ovatus/xylanisolvens	24	47
B. uniformis	12	6
B. stercoris	1	4
B. cellulosilyticus	1	4
B. caccae	4	9
B. intestinalis	1	3
B. salyersiae	2	0
B. nordii	2	2
B. clarus	0	1
B. eggerthii	0	1
B. faecis	0	1
B. finegoldii	0	4
P. distasonis	17	12
P. goldsteinii	1	0
P. johnsonii	0	3
P. merdae	0	1
Ph. coprocola	0	1

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, as listed in **Table 4**. For PCR, chromosomal DNA was obtained from each isolate by suspending bacterial colonies in 100 µL of DNase/RNase-free H_2O to give a 0.5 McFarland density. The suspended colonies were then incubated in a heating block at 99.5°C for 12 min, centrifuged at 14 000 rpm for 2 min, and then stored at −20°C until further use. 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 (Thermo Fisher Scientific) using the conditions given in **Table 4**.

To confirm some species, we conducted 16S rRNA sequencing as the "gold standard" using the pairs of primers and cycling conditions listed in **Table 4**. To detect the genetic element of *crxA*, we used the Emerald PCR master mix (Takara Bio Inc., Shiga, Japan) as recommended by the supplier. For nucleotide sequencing, the PCR mixture volume was increased (50 µL), and the products were purified using a PCR cleanup kit (HighPure, Roche, Basel, Switzerland). Nucleotide sequencing was performed at a core facility via capillary sequencing (Eurofins, Luxembourg City, Luxembourg). Sequence homology analysis was conducted by comparing the sequences obtained with those recorded in the GenBank database (National Center for Biotechnology Information, US National Institutes of Health, https://www.ncbi.nlm.nih.gov/) or uploaded for species identification to the EzBioCloud webpage (https://www.ezbiocloud.net/) [98].

Table 4. PCR primer sequences and reaction conditions used in this study

Gene/PCR	Primer/Sequence (5'… 3')	Type of PCR and reaction condition	Ref.
cblA	cblA1: TGAAAACCGGATTGACAGTC	RT, 95 °C 15s, 55 °C 30s,	This study
	cblA2: GAAACAATGCTGTCCAGACT	72 °C 30s; 35x	
crxA	crxA-F: ACCGTTGGCAAAAGTAAGTT	RT, 95 °C 15s, 55 °C 30s,	[34]
	crxA-R: TTCACACGCGCAATTGTATT	72 °C 1m; 35x	
*bla*HGD1	HGD-11: CGAATACATACAGCCCTTTG	RT, 95 °C 15s, 55 °C 30s,	This study
	HGD-12: TTCGGTAGCAGTTATGGAAA	72 °C 30s; 35x	
*bla*OXA347	oxa347-1: GCGGTAAGACTGGATTAAGT	RT, 95 °C 15s, 55 °C 30s,	This study
	oxa347-2: TCAACATTCAATCCGTCTGT	72 °C 30s; 35x	

pbbA	pbbA1: TGAAAACGACATGCATGAGA	RT, 95 °C 15s, 58 °C 20s,	[96]
	pbbA2: GCACCAGTGTTTTTCAATGG	72 °C 30s; 35x	
16S rRNA	E8F: AGAGTTTGATCCTGGCTCAG	EP, 94 °C 15s, 56 °C 20s,	[94]
	E533R: TIACCGIIICTICTGGCAC	72 °C 1m; 35x	
ERIC PCR	ERIC1: ATGTAAGCTCCTGGGGATTCAC	EP, 94 °C 15s, 50 °C 1m,	[99]
	ERIC2: AAGTAAGTGACTGGGGTGAGCG	72 °C 3m; 35x	
crxA	crxAEi1: AGTGAAGCATCAATATGGGA	EP, 98 °C 1m, 55 °C 1m,	This study
	crxAEi2: TCCTCGTTCTAATGATTTTCA	72 °C 6m; 35x	
crxA	crxAEo1: GAGTGGAAACGAGTGTCTAT	EP, 98 °C 1m, 55 °C 1m,	This study
	crxAEo2: CTGCTACAACTGTTTGTGAT	72 °C 6m; 35x	

Note: RT= Real-time PCR, EP= End-point PCR, ERIC= Enterobacterial repetitive intergenic consensus PCR

3.1.3 *β-glucosidase assay*

MALDI-TOF MS usually gives ambiguous identification results for *Ph. vulgatus*/*Ph. dorei*. Therefore, we used the 4-nitrophenyl-β-D-glucopyranoside hydrolysis assay to differentiate between *Ph. vulgatus* and *Ph. dorei* [100, 101]. We found the method with the best setting after conducting some optimization experiments. Namely, the substrate was dissolved in H_2O or DMSO and incubated at room temperature for 4 or 24 h. The final reaction setup included 100 µL of 50 mg/mL substrate (in water) and 100 µL of a 2 McFarland density cell suspension in water, and the reaction was stopped after 4 h by adding 5 µL of 1 M Na_2CO_3 (pH 11). Water served as the blank (background absorbance), and *B. fragilis* 638R and *Ph. vulgatus* ATCC 29327 served as the positive and negative controls, respectively. The absorbance of the produced 4-nitrophenol was measured at 405 nm in a microplate reader (PR 3100 TSC model, Bio-Rad, Hercules, CA, USA), and the values obtained were corrected by the absorbance mean of the blank samples.

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. ERIC-PCR was performed as previously described [99]. The IR Biotyper method included the following steps. Seven times a loopful (7 × 10 µL) of bacterial culture was collected and suspended in 55 µL of 70% ethanol in a 1.5-mL e-tube containing sterile metal rods. To get a uniform suspension, a multi-vortexer was used. After vortexing for 1.5 min and waiting for 5 min, 60 µL of sterile water was added. The 15 µL of the bacterial suspension was spotted onto the IRBT silicon plate together with 12 µL of two spots each of the IR test standard 1 and IR test standard 2 suspension, which were contained in the IR Biotyper Kit (Bruker Daltonics) for quality control, and dried at 37°C until a dry film formed over 20–30 min. Three technical replicates were used for each sample. Next, the dried silicon plate was inserted into the IRBT spectrometer (Bruker Daltonics) to acquire the spectra at default analysis settings using OPUS 7.5 software (Bruker Daltonics). The spectra that met the default quality criteria of absorption (0.4 arbitrary units [AU] < D value < 2 AU), signal/noise (<150 × 10^{-6} AU), signal/water (<300 × 10^{-6} AU), and fringes (<100 × 10^{-6} AU) were determined as "quality pass" in the IRBT® analysis. The *B. xylanisolvens* isolates that failed to pass the default quality criteria were re-examined. The spectra obtained with "quality pass" were analyzed to build the dendrogram. The software contains a feature that automatically proposes a cutoff that defines up to which distance spectra are considered to be in the same cluster. MALDI-TOF MS typing was performed by taking the spectra generated by the Sirius instrument (Bruker Daltonics) [97] and the built-in dendrogram-making feature of MBT Compass Explorer software (Bruker Daltonics).

3.1.5 *Bioinformatics*

ERIC PCR fragment similarity patterns were analyzed using GelJ software [102], 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 [99, 103] from our collection of glycerol stocks stored at -80^{o}C. After their inoculation onto supplemented Columbia blood agar plates (300 mg/l L-cystein, 5% defibrinated sheep blood, 2.5% laked sheep blood and 1 µg/ml vitamine K1) 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) from BHIS (Brain Heart Infusion, BHI, broth supplemented with 0.25% Yeast extract, 5 µg /ml hemin and 1 µg /ml vitamine K1) cultures [99] 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 listed in **Table 5** using the $\Delta\Delta C_T$ method. For the RT-qPCR experiments, we used the StepOne RT-PCR instrument (Life Technologies) with the following cycling parameters given in **Table 5** and a melting curve record from72 °C to 95 °C using the Verso 1-Step SYBRGreen mastermix (Life Technologies).

Table 5. PCR primer sequences and reaction conditions used in this study

Gene	Primer/Sequence (5'… 3')	Reaction condition	Ref.
nimB	nimB-RT1: CTTCATGGGGACGATGGTTA nimB-RT2: ATCCGTCAATATGTGGGCTT	55°C 20m, 95 °C 15m, 95°C 15s, 55 °C 30s, 72°C 30s, 35x; 72°C 75s	This study
nimE	nimE-RT1: TCGTAAACGACGGTTGTTGC nimE-RT2: TGCATAATAGCGTCCACCTT	55°C 20m, 95 °C 15m, 95°C 15s, 55 °C 30s, 72°C 30s, 35x; 72°C 75s	This study
Gap	gapBF1: AGCCATTGTAGCAGCTTTTT gapBF3: GAAGACGGGATGATGTTTC	55°C 20m, 95 °C 15m, 95°C 15s, 55 °C 30s, 72°C 30s, 35x; 72°C 75s	This study

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

The promoter of the IS*1186* element was identified earlier by Podglajen et al. [104] and we did this for the IS*Bf6* element by a 5'-RACE kit (Roche), as described earlier [34] 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 listed in **Table 9** and **Table S1** with known genetic backgrounds were stored in 20% glycerol stocks at −80 °C and cultivated on a supplemented Columbia blood agar medium (SCA, Columbia base, supplemented with 2.5% defibrinated sheep blood, 0.5% laked sheep blood, 0.3 mg/mL L-cysteine, 1 µg/mL vitamin K_1) or in supplemented brain–heart infusion broth (BHIS, brain–heart infusion broth supplemented with 2.5% yeast extract, 10 µg/mL hemin and 1 µg/mL vitamin K_1) under anaerobic conditions (85% N_2, 10% H_2, 5% CO_2, the Concept 400 anaerobic cabinet (Ruskinn, Bridgend, UK)) at 37 °C. The strains included both *nim*-positive and *nim*-negative *B. fragilis* strains whose *cfiA* gene statuses are known (see **Table 9)**. In parallel with metronidazole the MIC determinations, we used the same SCA plates for inoculations of 5 mL of BHIS for RNA isolation and cell suspensions to determine MICs. To test the effect of C_4-dicarboxylic acids on metronidazole resistance, we used a semi-defined M9-based agar medium (48 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.5 mM NaCl, 1.6 mM NH_4Cl, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1% casein peptone Type I (Neogene), 0.625% yeast extract, 10 mM glucose or 15 mM C_4-dicarboxylic acid (oxaloacetate/D(-) malate/fumarate/succinate), 10 µg/mL hemin, 1 µg/mL vitamin K_1) to perform MIC measurements.

3.3.2 *Metronidazole MIC measurements*

Metronidazole MICs were measured using a gradient method (Etest, bioMérieux, Hungary Ltd. Budapest, Hungary). First, McFarland density suspensions were made in a phosphate-buffered saline solution (137 mM NaCl, 2.7 mM KCl, 100 mM Na_2HPO_4 and 1.8 mM KH_2PO_4, pH 7.4), with which we inoculated the surface of SCA plates by cotton swabs, and applied the Etest strips, and after anaerobic cultivation at 37 °C for 48 h, we read the plates.

3.3.3 *RT-qPCR*

We extracted total RNA from 5 mL BHIS cultures for RT-qPCR experiments using the HighPure RNA Isolation Kit (Roche). The quantity and quality of RNA were assessed using the Qubit 4 fluorometer and the Qubit RNA BR and RNA Integrity kits (Thermo Fisher Scientific). Of the 32 candidate genes identified in previous proteomic studies [105], we chose 18 and designed primer pairs using the Primer3 Plus software (www.primer3plus.com). During the primer design, we took into account the possibility that the *cfiA*-positive and *cfiA*-negative strains might differ in their respective sequences. Therefore, the consensus nucleotide sequences of the selected genes were obtained from the complete genomic sequences of the *cfiA*-negative and *cfiA*-positive strains *B. fragilis* 638R (GenBank Acc. No. NC_016776) and *B. fragilis* 3130 (GenBank Acc. No. LJVI01), respectively, to design primer pairs. We used the *gap, rrn*, and *rpoD* genes as endogenous controls. The nucleotide sequences of the primers used by us are shown in **Table S1**. 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 (QuantStudio 3, Thermo Fisher Scientific) 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.

3.3.4 *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*

In this study, we screened clinical and normal microbiota isolates of *Bacteroides* species (n = 590) for less frequently investigated β-lactamase genes (see **Table 6)**. All the *B. uniformis* strains included in both collections harbored *cblA*. Similarly, in all *Ph. vulgatus*/*Ph. dorei* strains, we detected *bla*HGD1. Because MALDI-TOF MS cannot discriminate between *Ph. dorei* and *Ph. vulgatus* strains, we took the advantage of differences in their ability to produce β-glucosidase, demonstrating that all the strains of both species carry this β-lactamase gene **Figure S1**. This species identification was confirmed by 16S rRNA sequencing for five *Ph. vulgatus* and six *Ph. dorei* isolates. 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 for *bla*OXA347. *pbbA* was only detected in three clinical isolates (two *B. thetaiotaomicron* and one *B. ovatus*). 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 (see **Table 7)**.

Table 6. The number of isolates and prevalence of rare resistance genes distribution in *Bacteroides* species

Isolates	**Prevalence of resistance genes**				
	*bla*HGD1	*bla*OXA347	*cblA*	*crxA*	*pbbA*
Normal microbiota (184)	16+20 (*Ph. vulg/dorei*)	0	6 (*B. uni*)	6 (*B. xyl*)	0
Clinical (406)	16+10 (*Ph. vulg*/*dorei*)	6	12 (*B. uni*)	3 (*B. xyl*) [34]	3

Note: *B. uni= Bacteroides uniformis*, *B. xyl= Bacteroides xylanisolvens*, *Ph. vulg/dorei= Phocaeicola vulgatus or dorei*

At the same time, the carbapenem resistance determinant gene *crxA*, which is specific for *B. xylanisolvens* strains, was detected in three clinical [34] and six normal microbiota strains. We previously found by applying bioinformatics methods that *crxA* resides on a small genomic island [34]. Therefore, we studied whether the eight *B. xylanisolvens* strains carried this gene on the same island. However, PCR primers specific for this island (inside of the element, crxAEi1 and crxAEi2; see **Table 4**, **Figure 4, Figure S2**) 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 (crxAEo1 and crxAEo2; see **Table 4**, **Figure 4, Figure S2**) designed to recognize the borders of the *crxA* element did not amplify the gene. Because of these heterogeneities, we performed genetic relatedness experiments (typing, see **Figure S4**) for these 8 *crxA*-positive *B. xylanisolvens* strains and 13 additional *crxA*-negative *B. xylanisolvens* strains, and we found that the *crxA*-positive strains were located on different branches.

Table 7. The MIC values of E-test results for *B. fragilis* 638R with and without a transformed *pbbA* gene

Strains	**Antibiotics and MIC value**						
	AMP	XL	AMC	PTC	PZP	FOX	IMP
***B. fragilis* 638R**	2	0.25	0.064	0.064	0.25	2	0.032
***B. fragilis* 638R/pPE2-1**	>256	0.5	0.5	256	4	2	0.064

Note: AMP= Ampicillin, XL= Amoxicillin/clavulanate (bioMeriux fix ratio), AMC= Amoxicillin/clavulanate (Liofilchem fixed inhibitor concentration), PTC= Piperacillin, PZP= Piperacillin/tazobactam, FOX= Cefoxitin, IMP= Imipenem

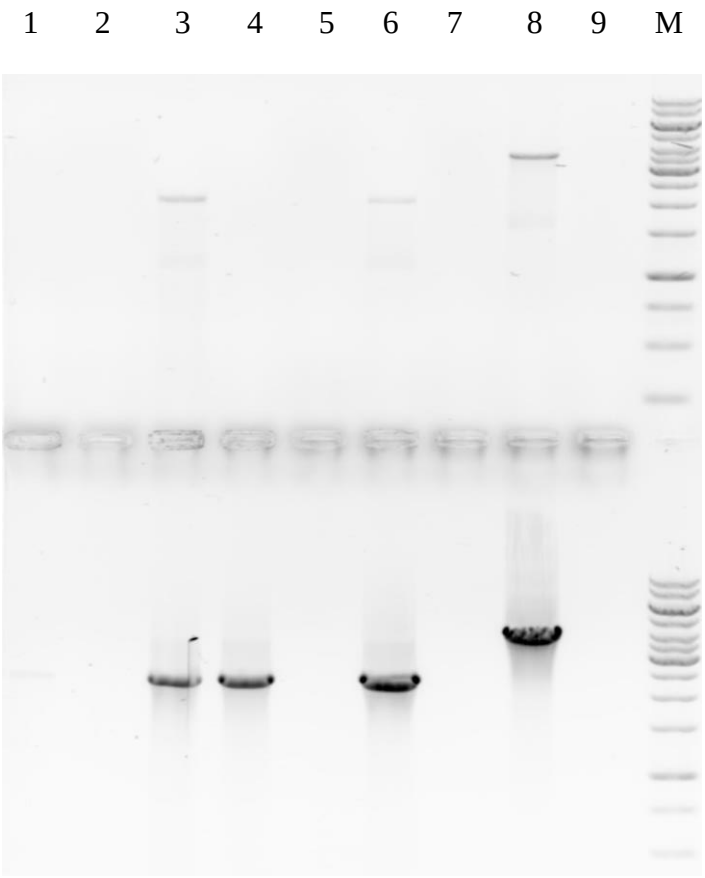

Figure 4. Gel picture of a *crxA* element PCR; lanes 1-8 represent *B. xylanisolvens* strains (D85, SZ9, P8, Hu1, P6A1, 1A, P4A1, 14880); lane 9 is the negative control and M 1kb ladder

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 (see **Table 8)**. This means that identical *nim* gene-IS element combinations have the same expression levels as the resistance genes. However, the metronidazole resistance levels did not correlate with the expressions of the *nim* genes, and the associations were not significant; so they appear to be independent (see **Table 8)**. **Figure 5** tells us that 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 (see **Figure S5)**.

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 same *nim* gene-IS element configurations of the strains were epidemiologically and genetically unrelated, as we found in previous studies [99, 103]. The IS*1186* and IS*Bf6* elements had recognizable *Bacteroides*-specific promoter sequences (the consensus, TtTg _33 TnnTAnnTTTGY _7, was determined earlier [106]) that could drive the expression of the *nimB* and *nimE* genes, respectively.

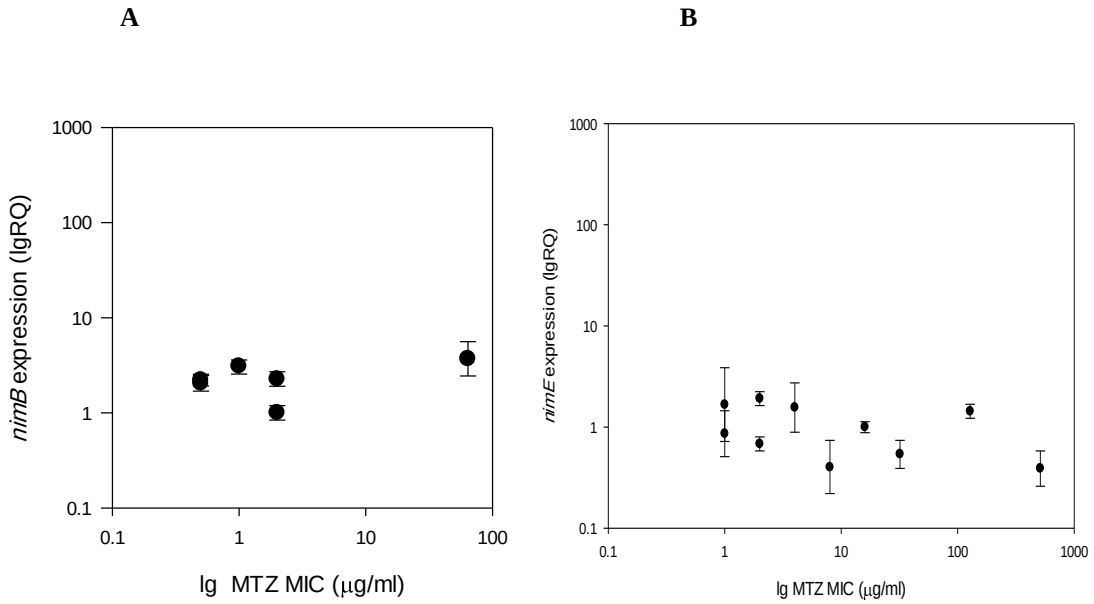

Figure 5. A representation of the uniform expression of *nim* genes and the apparent independence of the metronidazole MICs

Table 8. Data on the metronidazole resistance levels and *nim* gene expressions of our test strains

***B. fragilis* strain and *nim* gene**	**Statistics**	**MTZ MIC (µg/ml)**	**Relative expression (RQ[a])**		
			mean	**RQ (min)**	**RQ (max)**
nimB					
BF8		2	1.00	0.84	1.19
KSB-R		64	3.69	2.44	5.60
1672		1	3.90	2.56	3.60
O:21		0.5	2.21	1.92	2.55
768a		2	2.26	1.90	2.70
13038		0.5	2.04	1.69	2.47
	range[b]	0.5-64	2.69		
	$\log_2$[c]		7 1.88		
	correlation MIC-RQ[d]	r=-0.506 (p=0.126)			
nimE					
Q3		1	1.66	0.72	3.86
Q4		1	0.86	0.51	1.45
Q5		32	0.54	0.39	0.74
Q6		>256	0.39	0.26	0.58
Q7		4	1.56	0.89	2.73
Q8		2	0.68	0.58	0.80
Q9		16	1.00	0.88	1.13
Q10		8	0.40	0.22	0.74
Q11		128	1.43	1.22	1.68
Q12		2	1.91	1.63	2.24
	range[b]	1- >256	1.52		
	$\log_2$[c]		>8 0.604		
	correlation MIC-RQ[d]	r=0.441 (p=0.356)			

[a] RQ stands for relative expression values got from RT-qPCR experiments where an RQ of 1.00 is that for the control sample and we used the $\Delta\Delta C_T$ method to calculate the RQs (the data values were obtained from three technical replicates).
[b] Ranges of the metronidazole MICs and the gene expression means.
[c] Log_2 values of the ranges.
[d] Parameters of the correlations (correlation coefficients and their significance) between the metronidazole MICs and the *nim* gene expressions.

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

4.3.1 *Connection between the metronidazole MICs and nim gene expression*

Table 9 shows the results of the metronidazole MICs and the expression levels of the *nim* genes of the eight *nim*-positive strains. Similar to the results of our previous studies [105, 107], 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 [59, 108, 109], and they are associated with metronidazole resistance in field studies [110, 111]. 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 [107].

Table 9. The strains used, their properties and results of the RT-qPCR experiment

B. fragilis	**MTZ[a] MIC (µg/ml)**	***nim* (IS)**	***nim* expression (Rq[b])**	***cfiA***	**Ref**
GBR13	>256	E (IS*Bf6*)	0.352	+	[81]
388/2	>256	E (IS*Bf6*)	1.778	+	[93]
Q5	256	E (IS*Bf6*)	1.411	+	[103]
20584	256	E (IS*Bf6*)	1	+	This study
Q6	256	E (IS*Bf6*)	0.187	-	[103]
DOR18i3	256	D (IS*1169*)	0.403	+	This study
18807i2	(0.5-)>256[c]	-	n.a.	-	This study
Q11	64	E (IS*Bf6*)	0.856	+	[103]
WI1	32	-	n.a.	+	[112]
KSB-R	32	B (IS*1186*)	0.109	+	[113]
SY46	0.25	-	n.a.	-	[92]
SZ69	0.25	-	n.a.	+	[92]
638R	0.125	-	n.a.	-	[114]
SZ26	0.125	-	n.a.	+	[92]
SE61	0.064	-	n.a.	-	[92]

Note: [a] MTZ stands for metronidazole. [b] Rq is a relative quantity determined by the $\Delta\Delta C_T$ method. [c] Heterogeneous resistance phenotype

4.3.2 *An examination of the roles of 18 genes in metronidazole resistance*

To investigate this, we measured the expression levels of 18 genes selected according to the results of previous research or from our recent investigations [105] using RT-qPCR to study 15 *B. fragilis* strains. The cross-correlations between gene expressions and the correlation between gene expression and metronidazole MICs for all 15 *B. fragilis* strains are shown in **Table 11**. The cross-correlations between certain genes 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—$r = 0.593$, $p = 0.0192$, **Table 11**) are located on the same operon [115]. 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 (see **Table S2** and **Table S3**). 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 (*cf.* **Table S2** and **Table S3**). 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 (see **Table 12**) 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 (see **Table 12**). The genes listed above differ from the list in **Table 12** because the list above includes and excludes *ldh* and *nanH*, respectively. We are currently unable to explain this finding, although the inclusion of *nanH* suggests there is a link between metronidazole resistance/*nim* positivity and virulence.

4.3.3 *An examination of addition of the C_4-dicarboxylic acids on metronidazole resistance*

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. The results are shown in **Table 10**. 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.

Table 10. The effect of C_4-dicarboxylic acid supplementation on metronidazole MICs for selected *nim*-positive and -negative *B. fragilis* strains

Strain	***nim***	**Media (MICs in µg/ml)**					
		SCA	**M9-G[a]**	**M9-O**	**M9-M**	**M9-F**	**M9-S**
638R	-	0.125	0.25	0.064	0.032	0.032	0.032
638R/*nimA*	*nimA*	4	4	4	4	2	4
KSB-R	*nimB*	16	>256[b]	16	256	16	>256
19811	-	32	>256	64	>256	16	>256
18807i2	-	(0.25-)>256[c]	0.5	0.25	0.125	0.125	0.125
Q5	*nimE*	>256	>256	>256	>256	256	>256

[a] M9 media supplemented with glucose (G), oxaloacetic acid (O), malate (M), fumarate (F) and succinate (S). [b] Significantly increased metronidazole resistance values are highlighted in yellow. [c] Heterogeneous resistance phenotype.

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. This latter finding supports our assumption that is noted below (e.g., that the metronidazole resistance is highly dependent on reducing cofactor(s)).

Table 11. Cross-correlation values between the examined gene expressions and the metronidazole MICs for 15 *B. fragilis* strains [a]

	S3	**acr5**	***acr15***	***crpF***	***frdC***	***feoAB***	***fldA***	***fprA***	***frdA***	***galK***	***gatMZ***	***Ldh***	***mdh***	***nanH***	***porMZ***	***pgk***	***relA***	**MIC [b]**
L20	0.486	−0.318	0.243	−0.346	0.725	0.0393	−0.479	0.421	0.789	0.154	0.149	−0.0964	0.621	−0.393	0.257	0.704	0.679	−0.423
	0.0639	0.24	0.374	0.199	0.00178	0.883	0.0685	0.113	$2x10^{-7}$	0.575	0.584	0.724	0.0129	0.142	0.346	0.00302	0.00504	0.113
S3		0.214	0.429	−0.296	0.511	−0.175	0.025	0.275	0.443	0.579	0.31	0.0286	0.421	−0.321	0.414	0.643	0.614	−0.25
		0.433	0.107	0.275	0.0498	0.523	0.923	0.312	0.0946	0.0231	0.252	0.913	0.113	0.235	0.12	0.00934	0.0143	0.359
acr5			0.461	0.136	−0.343	−0.113	0.393	0.05	−0.132	0.486	−0.125	0.211	−0.111	0.25	0.443	−0.0536	−0.168	0.227
			0.0808	0.62	0.204	0.676	0.142	0.852	0.629	0.0639	0.648	0.441	0.686	0.359	0.0946	0.842	0,54	0.41
acr15				0.4	−0.0179	−0.0536	0.0821	0.307	0.546	0.629	−0.133	0.664	0.0107	0.343	0.639	0.125	0.104	0.132
				0.134	0.944	0.842	0.763	0.257	0.0339	0.0116	0.629	0.00654	0.964	0.204	0.00988	0.648	0.705	0.629
crpF					−0.3	0.211	0.0929	−0.214	0.0429	0.075	−0.262	0.646	−0.25	0.468	0.439	−0.368	−0.25	0.491
					0.269	0.441	0.734	0.433	0.873	0.783	0.339	0.00882	0.359	0.0757	0.0975	0.171	0.359	0.0597
frdC						0.179	−0.336	0.486	0.593	−0.193	0.528	−0.311	0.7	−0.614	0.25	0.75	0.814	−0.669
						0.514	0.214	0.0639	0.0192	0.481	0.0413	0.252	0.00326	0.0143	0.359	0.000786	$2x10^{-7}$	0.00614
feoAB							0.259	0.45	−0.132	−0.37	−0.0403	0.247	0.182	−0.316	0.39	−0.218	0.261	−0.0118
							0.339	0.0889	0.629	0.167	0.883	0.367	0.506	0.24	0.146	0.426	0.339	0.964
fldA								−0.05	−0.536	0.118	−0.0685	0.468	−0.207	0.286	0.143	−0.386	−0.0429	0.274
								0.852	0.0382	0.667	0.802	0.0757	0.449	0.293	0.602	0.15	0.873	0.312
fprA									0.218	−0.179	0.27	−0.0536	0.575	−0.454	0.386	0.461	0.471	−0.426
									0.426	0.514	0.319	0.842	0.0241	0.0861	0.15	0.0808	0.0732	0.11
frdA										0.279	0.157	0.143	0.393	−0.1	0.357	0.55	0.489	−0.361
										0.306	0.566	0.602	0.142	0.714	0.185	0.0325	0.0618	0,18
galK											−0.475	0.482	−0.25	0.382	0.339	0.0643	−0.075	0.457
											0.0708	0.0662	0.359	0.154	0.209	0.812	0.783	0.0834
gatMZ												−0.5	0.596	−0.58	−0.00403	0.463	0.483	−0.683
												0.0556	0.0183	0.0231	0.985	0.0782	0.0662	0.00471
ldh													−0.3	0.564	0.421	−0.346	−0.0893	0.517
													0.269	0.0275	0.113	0.199	0.743	0.0463
mdh														−0.689	0.364	0.836	0.825	−0.528
														0.00409	0.176	$2x10^{-7}$	$2x10^{-7}$	0.0413
nanH															−0.0214	−0.521	−0.543	0.446
															0.934	0.0446	0.0353	0.0917
porMZ																0.254	0,45	0.0798
																0.353	0.0889	0.773
pgk																	0.786	−0.586
																	$2x10^{-7}$	0.0211
relA																		−0.629
																		0.0116

[a] Odd and even rows with correlation coefficients and significance values, respectively. The color-coding denotes the following: yellow—$0.5 < r < 0.7$, $0.05 > p > 0.01$, orange: $r > 0.7$, $p < 0.01$; abbreviations in Table S1. [b] Metronidazole MIC.

Table 12. Significance values of the one-way variance analyses of the gene expressions and the metronidazole MICs depending on the genetic background in 15 *B. fragilis* strains

	L20	**S3**	***acr5***	***acr15***	***crpF***	***frdC***	***feoAB***	***fldA***	***fprA***	***frdA***	***galK***	***gat***	***ldh***	***mdh***	***nanH***	***por***	***pgk***	***relA***	**MIC**
nim	n.s.[a]	n.s.	n.s.	n.s.	n.s.	0.006	n.s.	n.s.	n.s.	n.s.	n.s.	0.029	n.s.	0.001	0.04	n.s.	0.001	0.001	0.001
cfiA	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

[a] n.s.-not significant

5. DISCUSSION

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

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 from molecular Classes A2 and D (see **Figure S3**), 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 (ERIC, IR, MALDI-TOF MS; **Figure S4**), 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. Whole-genome analyses clarifying these assumptions are ongoing in our laboratory. *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 [93]. This gene has been rarely detected (Denmark [116] and Hong Kong [117]), but carbapenem resistance in cases where this gene was detected could not be confirmed. This is why further studies are needed.

Although β-lactam antibiotics have a broad spectrum and they are prescribed most frequently in hospital settings, these agents are difficult to use because of the production of β-lactamases, which represent the most common cause of β-lactam resistance in anaerobes [65]. 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 [118]. Most previous studies extensively focused on the detection and prevalence of *cfiA* in *B. fragilis* strains [93, 119, 120].

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.

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

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 [121, 122]. 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 [64], 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* [105, 123, 124]. Next, we plan to extend the range of genes whose expression could be

studied in other RT-qPCR experiments that would be based on our current RNASeq and mass spectrometry measurements to determine their association with Nim action and metronidazole resistance. These experiments 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.

5.3 *A proteomics-based RT-qPCR and functional analysis of the expressions of 18 genes in Bacteroides fragilis strains with different 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 (see above). 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* (described in [124], *acr5* (*bmeB*, described in [125] and *por* (described in [126]). This may be applied to genes not examined here (*recA*, *sod* and *rhaA*), as their role was demonstrated in metronidazole resistance earlier [127-129]. 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 (mentioned above). In addition, the roles of enzymes involved in the central metabolism in *B. fragilis* should also be considered. The central metabolism varies greatly among bacteria [130], 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(see **Figure S6**) [131]. Previously, we found that hemin depletion causes metronidazole susceptibility in both *nim*-negative and *nim*-positive strains of *B. fragilis* [121]. 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.

The Nim enzymes are nitro-reductases that can transfer either six [63] or two electrons to the nitro group of metronidazole, yielding either an amino or a nitroso imidazole, respectively [108]. Recently, in vivo and in vitro experiments demonstrated that a *nim* group enzyme encoded by *Clostridioides difficile* strains is a nitro-reductase [132]. In this latter study, it was also confirmed that metronidazole resistance in *C. difficile* is dependent on hemin [133] in experiments involving the direct addition of metronidazole to assay its modification using recombinant NimB and by a transcriptomic analysis. Moreover, genetic (transposon mutagenesis) and biochemical (aromatic nitro-reduction to amine) tests have proven that the *nimB* gene of some *C. difficile* strains is responsible for their metronidazole resistance. However, in the in vitro experiments, the metronidazole concentration used, 5 mM, was much higher than that to which the bacteria are usually exposed (the 4 μg/mL breakpoint concentration corresponds to 23.4 μM—roughly a 160-fold difference). 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. We also argue that the C_4-dicarboxylic acid uptake rates probably do not affect these processes because one transport protein, the anaerobic C_4-dicarboxylic carrier protein, is responsible for their uptake with similar efficiencies in *Escherichia coli* [134]. The ortholog of this carrier protein is present in the genomes of *B. fragilis* strains (in an unpublished analysis). The observed increase in *ldh* gene expression is consistent with previous findings, revealing the importance of reducing cofactors in metronidazole resistance in anaerobic bacteria [126]. 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. Perhaps withdrawing hydrogen/reducing cofactors from metronidazole activation are involved in this process. 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. Earlier modeling studies just focused on laboratory strains of *B. fragilis*. For instance, based on the roles of a limited set of proteins analyzed by two-dimensional protein electrophoresis and northern blotting, Diniz et al. proposed that *ldh* and *por* participate in metronidazole activation at certain low levels [126, 135]. However, their model was not confirmed by Paunkov et al. [122]. Also, de Freitas et al. analyzed the transcriptome-wide effect of metronidazole on a large number of proteins, and they confirmed that, along with some other proteins, the concentration of activating ferredoxin is important in alleviating metronidazole stress [136]. 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 [105]. 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. The activated metronidazole radical acts by reducing *nim* and redox cofactor proteins and thiol compounds of the proteome [137]. 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. Hence the process of developing resistance to metronidazole is also complex (see **Figure 6**).

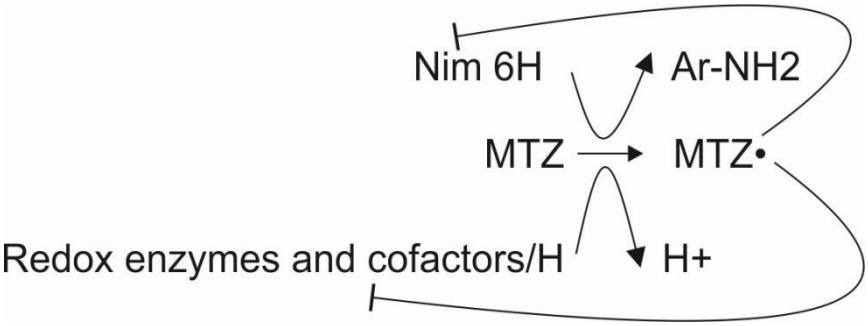

Figure 6. Interactions of the participants in metronidazole resistance. Lines with the ⊣ symbol mean inhibition.

Earlier work suggested that ferredoxin is responsible for reducing metronidazole [126]; however, we were unable to find a role for PFOR (negative association), as we observed increased PFOR and PFOR activities in laboratory metronidazole-resistant *B. fragilis* strains [122]. 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. The starting point for this study was a proteomic analysis of metronidazole resistant laboratory strains [105], but only some of them proved to be effective in metronidazole resistance at the population level; therefore, 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. SUMMARY

Resistance to antimicrobial agents, which is a global problem in a clinical setting, remains a neglected concern in anaerobic bacteria mainly due to the enormous work required for their isolation and identification. *Bacteroides* species belonging to the order Bacteroidales are strict anaerobic Gram-negative bacilli and major constituents of the gastrointestinal tract. They are also the most frequently isolated endogenous anaerobic opportunistic pathogens, sometimes causing serious infections. *Bacteroides* are generally regarded as the most resistant anaerobic bacteria, with many resistance mechanisms and resistance rates. The most useful antibiotics against them are carbapenems, 5-nitroimidazoles, β-lactam/β-lactamase combinations and tigecycline.

- In this study, we screened the prevalence of rare β-lactamase genes in *Bacteroides* species strains from clinical specimens and normal microbiota and we examined the genetic properties of the strains carrying these genes. *bla*HGD1, *bla*OXA347, *cblA*, *crxA*, and *pbbA* were detected via real-time polymerase chain reaction in collections of *Bacteroides* strains taken from clinical (n = 406) and fecal (n = 184) samples. To examine the genetic background of the samples, end-point PCR, FT-IR, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry were used. All the *B. uniformis* isolates were positive for *cblA* in both collections. Although *crxA* was *B. xylanisolvens*-specific and associated with carbapenem resistance, it was only found in six fecal and three clinical *B. xylanisolvens* strains. Moreover, the *crxA*-positive strains were not clonal among *B. xylanisolvens* (unlike *cfiA* in *B. fragilis*), implicating a rate of mobility or emergence by independent evolutionary events. The *Phocaeicola* (*B.*) *vulgatus*/*Ph. dorei*-specific gene *bla*HGD1 was detected among all *Ph. vulgatus*/*Ph. dorei* isolates from fecal (n = 36) and clinical (n = 26) samples. No *bla*OXA347-carrying isolate was found in European collections, but all the US samples (n = 6) were positive. For three clinical isolates belonging to *B. thetaiotaomicron* (n = 2) and *B. ovatus* (n = 1), *pbbA* was detected on mobile genetic elements, and *pbbA*-positive strains displayed non-susceptibility to piperacillin or piperacillin/tazobactam phenotypically. Based on these observations, β-lactamases produced by rare β-

lactamase genes in *Bacteroides* species strains should not be neglected because they may encode important resistance phenotypes.

- In this study we examined whether the same *nim* gene-insertion sequence (IS) element combinations give rise to the same expression levels as they harbor shared IS element-borne promoters. From our quantitative analysis, we found that the expressions of the *nimB* and *nimE* genes with their cognate IS elements were similar, but the metronidazole resistance of these strains were more diverse.

- Previously, we reported that metronidazole MICs do not depend on the expression levels of *nim* genes in *B. fragilis* strains and we compared the proteomes of metronidazole-resistant laboratory *B. fragilis* strains with those of their susceptible parent strains. Here, we used RT-qPCR to correlate the expression levels of 18 candidate genes in a panel of selected, *nim*-gene positive and *nim*-gene negative *B. fragilis* clinical strains with their metronidazole MICs. Metronidazole MICs were correlated with the expression of certain tested genes. Specifically, lactate dehydrogenase expression correlated positively, whereas cytochrome fumarate reductase/succinate dehydrogenase, malate dehydrogenase, phosphoglycerate kinase redox and *gat* (GCN5-like acetyltransferase), and *relA* (stringent response) regulatory gene expressions correlated negatively with metronidazole MICs. This result provides evidence for the involvement of carbohydrate catabolic enzymes in metronidazole resistance in *B. fragilis*. And it was supported by direct substrate utilization tests. However, the exact roles of these genes/proteins should be determined in deletion–complementation tests. Moreover, the exact redox cofactors participating in metronidazole activation need to be clarified.

8. 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.

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DEDICATION

I would like to dedicate this PhD thesis to my family members:

My beloved parents: Father and Mother

My Brothers and Sisters

My Nieces and Nephews

APPENDICES

Supplementary Materials

Table S1. RT-qPCR target genes and primer sequences

	Gene id. Bf 638R	MS[a] FC	Ref.	Description/Name	Cellular pathway[b]	Primer designation	sequence F(1)[d]	sequence R(2)[d]
1	BF638R_0963	8,30	[124]	FprA family A-type flavoprotein (*fprA*)	1	fprA1/2	TAACGACCGTAACANGCACC	GATCAGTGCCACCATTTCAT
2	BF638R_1663	10,55	[124]	galactokinase (*galK*)	1	galK1/2	TGGCAGANGCAAAAGCTGAT	YTCGTAAAGTTTGCTCATGC
3	BF638R_1728	-2,95	[124]	exo-alpha-sialidase (*nanH*)	2	nanH1/2	TGTGTGCTATACCGGTAGTT	GTCTGGCTCTCTTTAGCATC
4	BF638R_3176	8,40	[124]	BmeB15 (*acr15*)	2	act15-1/2	AGTATCGATCACCTTCGGAA	TACTTGCTGTCGTTCAGTTC
5	BF638R_3999	9,10	[124]	BmeB5 (*acr5*)	2	acr51/2	CCTATCGACTTGTATCCGGA	ATTCAGCGTATTCTCCAACG
6	BF638R_2281	4,00	[124]	RelA/SpoT family protein (*relA*)	5	relA_1/2	CGTGACCGTTATATTGCCAA	ACTGGCATTTCTGTTTCTTCA
7	BF638R_1701	22,80	[124]	50S ribosomal protein L20	4	L20_31/2	CGTGACCGTAGAAACAAGAA	AAGTCAGCCAAAACCTTACG
8	BF638R_4035	15,10	[124]	30S ribosomal protein S3	4	S3-1/2	GTGGCCAGGAAGTTGATAAG	ACAATCACAGCATCCAGTTC
9	BF638R_3828	-	This study	malate dehydrogenase (*mdh*)	1	mdh-1/2	GACTCGTGAAGAACTGATCG	AGTCATTGTATCCATCGGGT
10	BF638R_1473	-	[92]	lactate dehydrogenase (*ldh*)	1	ldh-1/2	TCGGACCGTATCATNGACGA	TATTCTCCAGAGTCGTTGCT
11	BF638R_3133	-	This study	phospho-glycerate kinase (*pgk*)	1	pgk-1/2	TTCGAAAACTTCACTCACGG	TACCCGGAAGAACTTTTCCT
12	BF638R_1421	-	[113]	*feoAB*	3	feoAB-1/2	GCTTCCGTAAACGNATTGTG	GTCGNCGCAGAGAGATTTCA
13	BF638R_2696	4,1	[124]	flavodoxin (*fldA*)	1	fldA-1/2	ATTTTACGGTTCCACAACGG	TCACTAACTCGTCGTTCAGT
14	BF638R_4500	-	This study	fumarate reductase (*frdA*)	1	frdA-1/2	ATCTGGGTTCCGAAGAAGAT	GCGTTCCAGATAGAAGTCAC
15	BF638R_4499	11,50	[124]	succinate dehydrogenase/fumarate reductase cytochrome b subunit (*frdC*)	1	frdC-1/2	TGGGCTAAAATGCAGTTACC	CAGAAACCATGAGTCAGGTG
16	BF638R_2263	2,40	[124]	Crp/Fnr family transcriptional regulator (*crpF*)	5	crpF-1/2	CGTGATTTTGACGGAATCG	TAATCGATCATTTCTCCCCG
17	BF638R_1544	-3,20	[124][c]	GNAT family N-acetyltransferase (*gat*)	5	gatMZ-1/2[c]	TTCCCTATCGTAGCATACCC	TCTGGTGTCCACTCTGTATC
18	BF638R_3194	43,12	This study[c]	pyruvate-flavodoxin/ferredoxin oxidoreductase (*por*)	1	porMZ-1/2[c]	ATGACATTCCGTATCCAGGT	GTTGTTTGCTCTTCACGTTG
	Endogeneous controls							
19	BF638R_0945		This study	glyceraldehyde-phosphate dehydrogenase (*gap*)	-	gap1/3BF	AGCCATTGTAGCAGCTTTTT	GAAGACGGGATGATGTTTC
20	6 16S rRNA		This study	16S rRNA (*rrn*)	-	rrnBF-1/2	TCCTGTTTGATACCCACACT	GCTCAACCGTAAAATTGCAG
21	BF638R_2767		This study	*rpoD*	-	rpoD_BF1/2	CCAATCTTCGTTTCGTCGTA	TTCATCAAACTTCTCGGCAG

[a] FC means fold change in the proteomic study. [b] The numbers designate the following pathways - 1: glycolysis, carbohydrate metabolism, redox processes, 2: cell-wall, efflux, virulence, 3: heme/Fe2+ metabolism, 4: protein synthesis, 5: nucleic acids, repair, stress, gene regulation. [c] These items were selected from the different paralog genes of these proteins in the B. fragilis genomes based on differential expressions, the pyruvate-ferredoxin oxidoreductase gene here was selected on its differential expression in RNASeq experiments (our unpublished results). [d] Forward (1) and reverse (2) orientations.

Table S2. Cross-correlation values of gene expressions metronidazole resistance for eight *nim*-positive *B. fragilis* strains[a]

	S3	*acr5*	*acr15*	*crpF*	*frdC*	*feoAB*	*fldA*	*fprA*	*frdA*	*galK*	*gatMZ*	*ldh*	*mdh*	*nanH*	*porMZ*	*pgk*	*relA*	MIC[b]
L20	0,476	0,429	0,762	-0,167	0,31	-0,333	-0,214	0,286	0,881	0,762	-0,247	0,143	0,119	0,429	0,381	0,5	0,357	-0,334
	0,207	0,26	0,0212	0,662	0,423	0,387	0,578	0,46	2E-07	0,0212	0,537	0,705	0,749	0,26	0,321	0,182	0,353	0,387
S3		0,548	0,333	-0,333	0,548	0,143	0,357	0,619	0,0952	0,738	0,577	-0,119	0,429	0,0238	0,667	0,595	0,81	-0,124
		0,139	0,387	0,387	0,139	0,705	0,353	0,086	0,794	0,0287	0,12	0,749	0,26	0,931	0,0588	0,102	0,0096	0,749
acr5			0,286	-0,0476	0,119	-0,333	-0,262	0,476	0,143	0,452	0,412	-0,429	0,857	-0,19	0,714	0,81	0,262	0,272
			0,46	0,885	0,749	0,387	0,498	0,207	0,705	0,233	0,29	0,26	0,00178	0,619	0,0374	0,0096	0,498	0,498
acr15				0,0238	0,143	-0,429	0,0238	0,5	0,81	0,5	-0,247	0,476	0,19	0,786	0,262	0,357	0,119	-0,136
				0,931	0,705	0,26	0,931	0,182	0,0096	0,182	0,537	0,207	0,619	0,0149	0,498	0,353	0,749	0,705
crpF					0	-0,619	-0,119	-0,452	0,0476	0,0952	-0,577	0,333	0,214	0,381	0,0952	0,286	0	0,568
					0,977	0,086	0,749	0,233	0,885	0,794	0,12	0,387	0,578	0,321	0,794	0,46	0,977	0,12
frdC						0,0238	-0,0476	0,429	0,262	0,476	0,0825	-0,167	-0,167	0,0476	0,167	0,452	0,405	-0,507
						0,931	0,885	0,26	0,498	0,207	0,839	0,662	0,662	0,885	0,662	0,233	0,29	0,182
feoAB							0,619	0,0714	-0,452	-0,357	0,577	0,0476	-0,357	-0,548	-0,5	-0,619	0,0476	-0,148
							0,086	0,839	0,233	0,353	0,12	0,885	0,353	0,139	0,182	0,086	0,885	0,705
fldA								0,0952	-0,333	0,0238	0,412	0,595	0,0238	0,0952	-0,119	-0,333	0,429	0,309
								0,794	0,387	0,931	0,29	0,102	0,931	0,794	0,749	0,387	0,26	0,423
fprA									0,167	0,167	0,577	-0,19	0,286	0,0952	0,286	0,357	0,0714	-0,321
									0,662	0,662	0,12	0,619	0,46	0,794	0,46	0,353	0,839	0,423
frdA										0,5	-0,577	0,333	-0,143	0,619	0,0476	0,286	0	-0,383
										0,182	0,12	0,387	0,705	0,086	0,885	0,46	0,977	0,321
galK											-0,0825	0,0714	0,333	0,381	0,738	0,738	0,833	-0,0865
											0,839	0,839	0,387	0,321	0,0287	0,0287	0,00526	0,794
gatMZ												-0,412	0,412	-0,577	0,247	0,0825	0,247	0,0856
												0,29	0,29	0,12	0,537	0,839	0,537	0,794
ldh													-0,19	0,667	-0,333	-0,333	0,0714	0,272
													0,619	0,0588	0,387	0,387	0,839	0,498
mdh														-0,0714	0,738	0,69	0,31	0,667[c]
														0,839	0,0287	0,0474	0,423	0,0588
nanH															0,143	0,143	0,119	-0,0618
															0,705	0,705	0,749	0,839
porMZ																0,857	0,667	0,185
																0,00178	0,0588	0,619
pgk																	0,5	0,124
																	0,182	0,749
relA																		0,0741
																		0,839

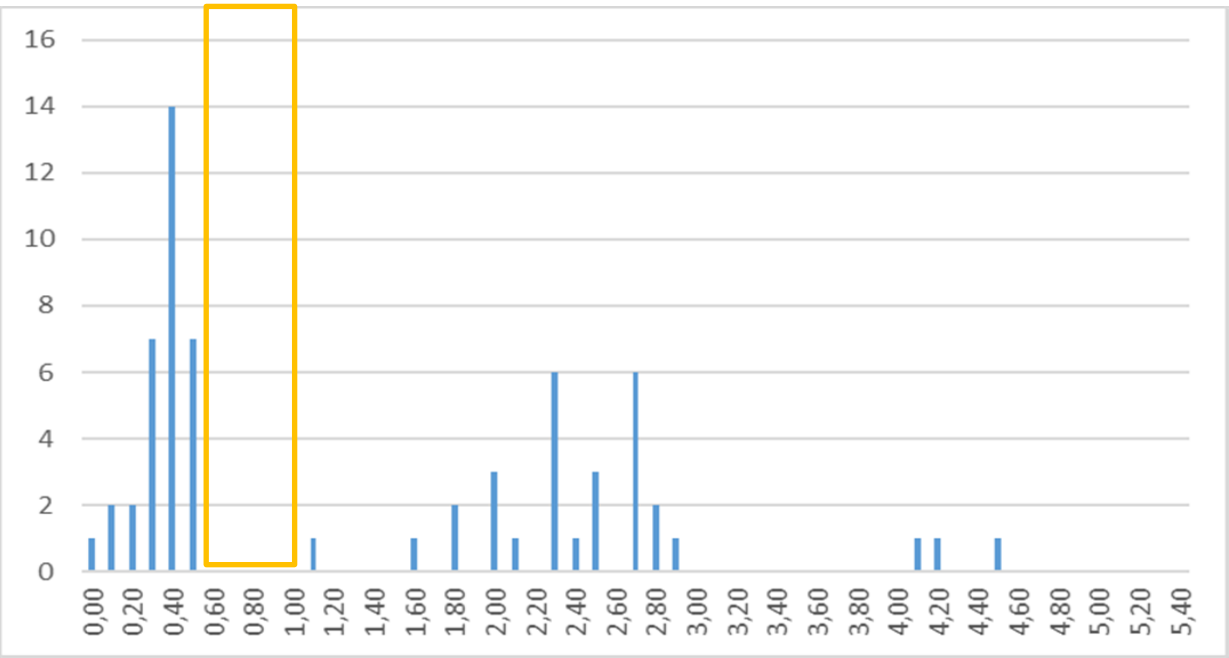
[a] The colour-coding denotes the following: yellow – 0.5 < r < 0.7, 0.05 > p >0.001, orange: r > 0.7, p<0.001, , abbreviations in Table S1. [b] Metronidazole MIC. [c] Borderline significance

Table S3. Cross-correlation values of gene expressions metronidazole resistance for seven *nim*-negative *B. fragilis* strains[a]

	S3	*acr5*	*acr15*	*crpF*	*frdC*	*feoAB*	*fldA*	*fprA*	*frdA*	*galK*	*gatMZ*	*ldh*	*mdh*	*nanH*	*por*	*pgk*	*relA*	MIC[b]
L20	0,643	-0,786	-0,0357	-0,536	0,821	-0,198	-0,786	0,143	0,679	0,464	-0,018	-0,464	0,643	-0,821	0,0357	0,964	0,536	0,128
	0,0956	0,0251	0,905	0,181	0,0145	0,602	0,0251	0,72	0,0735	0,255	0,905	0,255	0,0956	0,0145	0,905	2E-07	0,181	0,72
S3		-0,107	0,607	-0,25	0,5	-0,577	-0,429	-0,214	0,679	0,857	0,0541	-0,0357	0,25	-0,321	0	0,75	0,5	0,0551
		0,781	0,121	0,545	0,217	0,15	0,297	0,602	0,0735	0,00609	0,843	0,905	0,545	0,438	0,968	0,0384	0,217	0,843
acr5			0,464	0,536	-0,75	-0,018	0,821	-0,179	-0,286	0,0714	-0,234	0,714	-0,679	0,893	0,179	-0,714	-0,214	0,147
			0,255	0,181	0,0384	0,905	0,0145	0,66	0,491	0,843	0,545	0,0545	0,0735	2E-07	0,66	0,0545	0,602	0,72
acr15				0,429	0,143	-0,18	-0,0357	-0,0357	0,5	0,321	0,252	0,571	-0,0357	0,0714	0,321	0,0714	0,464	-0,257
				0,297	0,72	0,66	0,905	0,905	0,217	0,438	0,545	0,15	0,905	0,843	0,438	0,843	0,255	0,545
crpF					-0,143	0,631	0,536	0,0357	0,214	-0,357	0,27	0,857	0	0,464	0,643	-0,607	0,25	0,0551
					0,72	0,0956	0,181	0,905	0,602	0,388	0,491	0,00609	0,968	0,255	0,0956	0,121	0,545	0,843
frdC						0	-0,786	0	0,75	0,143	0,432	-0,321	0,643	-0,857	0,107	0,75	0,5	-0,128
						0,968	0,0251	0,968	0,0384	0,72	0,297	0,438	0,0956	0,00609	0,781	0,0384	0,217	0,72
feoAB							0,252	0,595	0,18	-0,667	-0,0909	0,523	0,324	0,0541	0,775	-0,396	0,36	0,232
							0,545	0,121	0,66	0,0735	0,781	0,181	0,438	0,843	0,0251	0,341	0,388	0,602
fldA								-0,214	-0,393	-0,0714	-0,378	0,607	-0,607	0,964	0,214	-0,821	-0,357	0,496
								0,602	0,341	0,843	0,341	0,121	0,121	2E-07	0,602	0,0145	0,388	0,217
fprA									0,179	-0,393	-0,36	0,25	0,429	-0,286	0,607	0,0714	0,571	-0,0367
									0,66	0,341	0,388	0,545	0,297	0,491	0,121	0,843	0,15	0,905
frdA										0,393	0,0901	0,286	0,607	-0,464	0,607	0,607	0,857	0,239
										0,341	0,781	0,491	0,121	0,255	0,121	0,121	0,00609	0,545
galK											-0,252	-0,0714	0	0,0357	-0,143	0,571	0,214	0,385
											0,545	0,843	0,968	0,905	0,72	0,15	0,602	0,341
gatMZ												-0,144	0,306	-0,306	-0,27	0,0541	-0,018	-0,722[c]
												0,72	0,438	0,438	0,491	0,843	0,905	0,0545
ldh													-0,0714	0,571	0,786	-0,5	0,429	0,257
													0,843	0,15	0,0251	0,217	0,297	0,545
mdh														-0,643	0,357	0,607	0,75	-0,11
														0,0956	0,388	0,121	0,0384	0,781
nanH															0,0714	-0,786	-0,393	0,349
															0,843	0,0251	0,341	0,388
por																-0,107	0,75	0,404
																0,781	0,0384	0,341
pgk																	0,5	-0,0184
																	0,217	0,905
relA																		0,11
																		0,781

[a] The colour-coding denotes the following: yellow – 0.5 < r < 0.7, 0.05 > p >0.001, orange: r > 0.7, p<0.001, abbreviations in Table S1. [b] Metronidazole MIC. [c] Borderline significance

Fig. S1. Separation of β-glucosidase assay: negative and positive samples



Note: The optical density with <0.60 is treated as a negative and ≥1.0 as a positive β-glucosidase; and the cut-off range is bracketed with a yellow rectangle.

Fig. S2. The *crxA* element examined

crxAEo1∨	DUT93_21650	PLP-dependent aminotransferase family protein
crxAEi1∨	DUT93_21645	hypothetical protein
	DUT93_21640	hypothetical protein
	DUT93_21635	Fic family protein
	DUT93_21630	MATE family efflux transporter
	DUT93_21625	alpha/beta hydrolase
	crxA	subclass B1 metallo-beta-lactamase
	DUT93_21615	IS1380 family transposase
	DUT93_21610	cupin domain-containing protein
crxAEi2∧	DUT93_21605	hypothetical protein
crxAEo2∧	DUT93_21600	GNAT family N-acetyltransferase

Fig. S3. Phylogeny of the main Classes A and D β-lactamases of the *Bacteroides* species

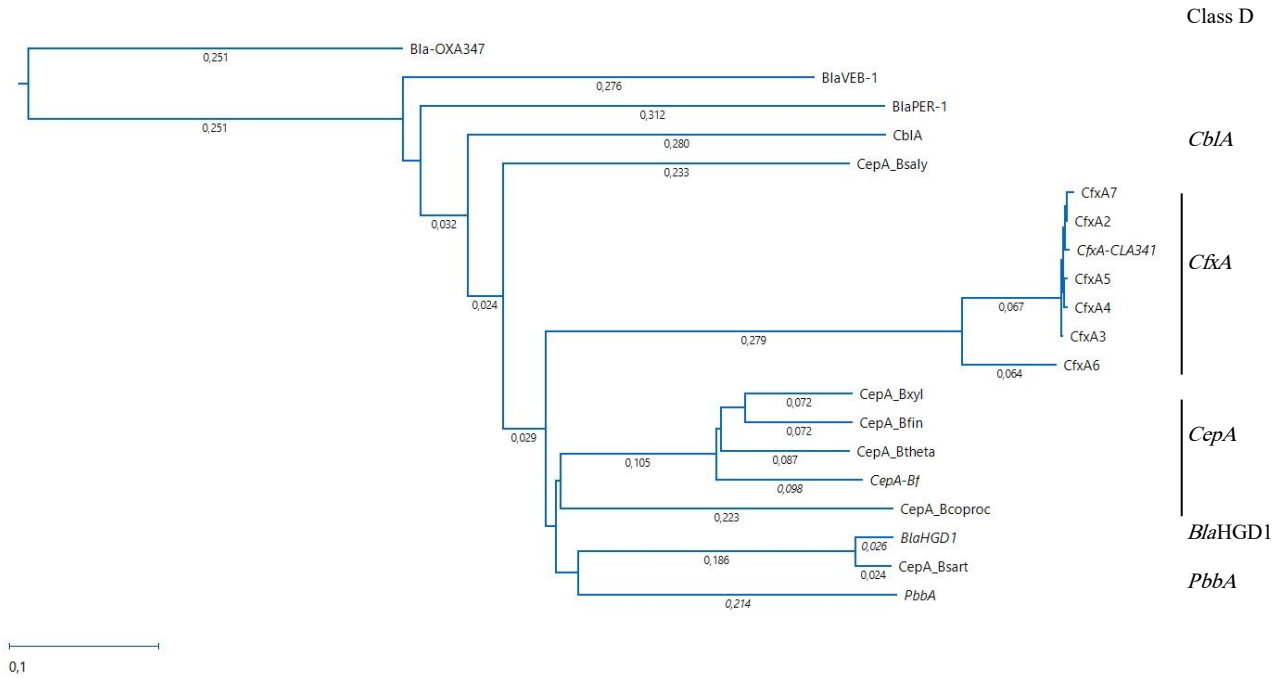
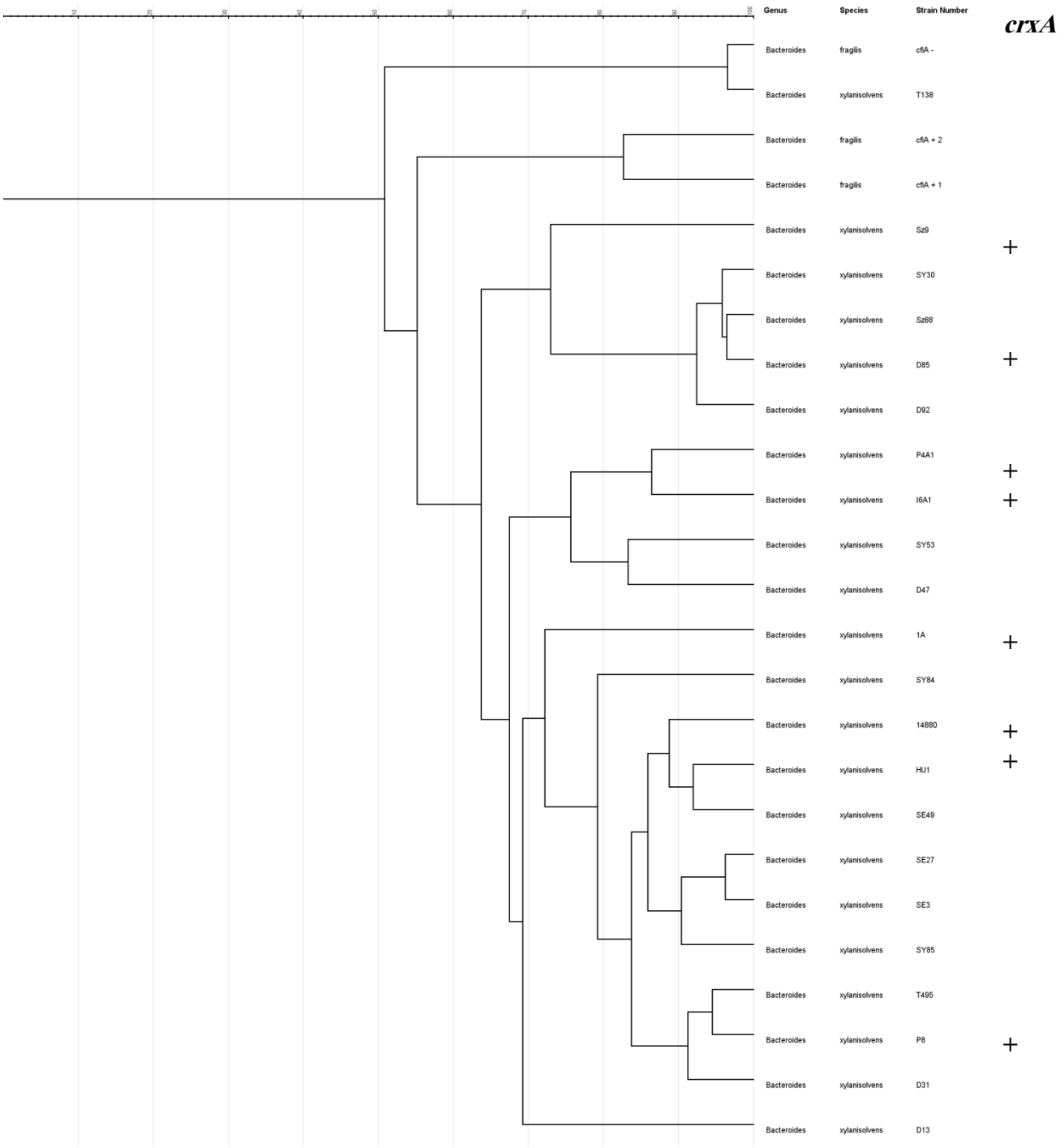
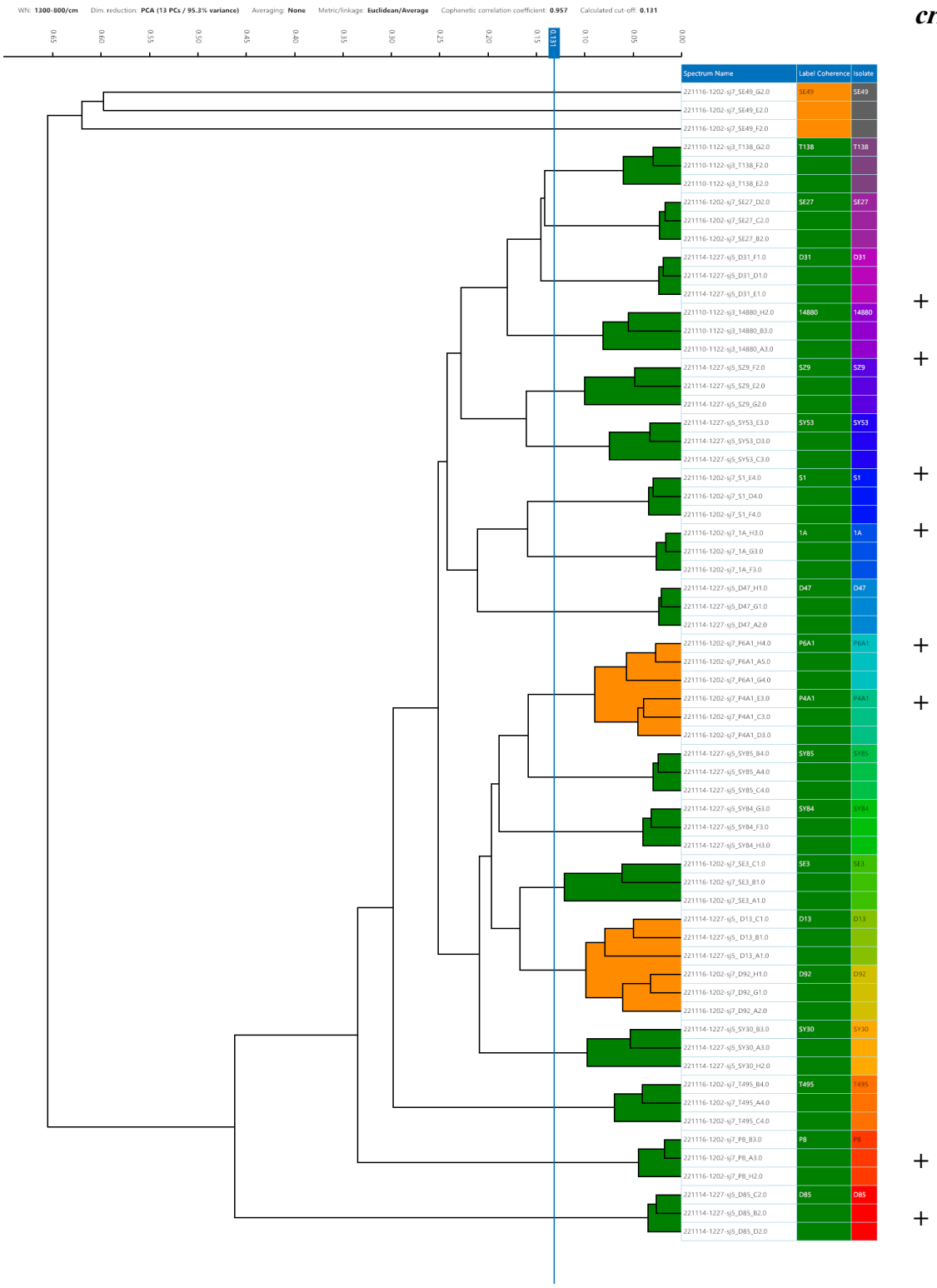


Fig. S4. Typing of the selected *B. xylanisolvens* strains

A ERIC PCR typing



B Infrared spectra typing



C MALDI-TOF MS typing

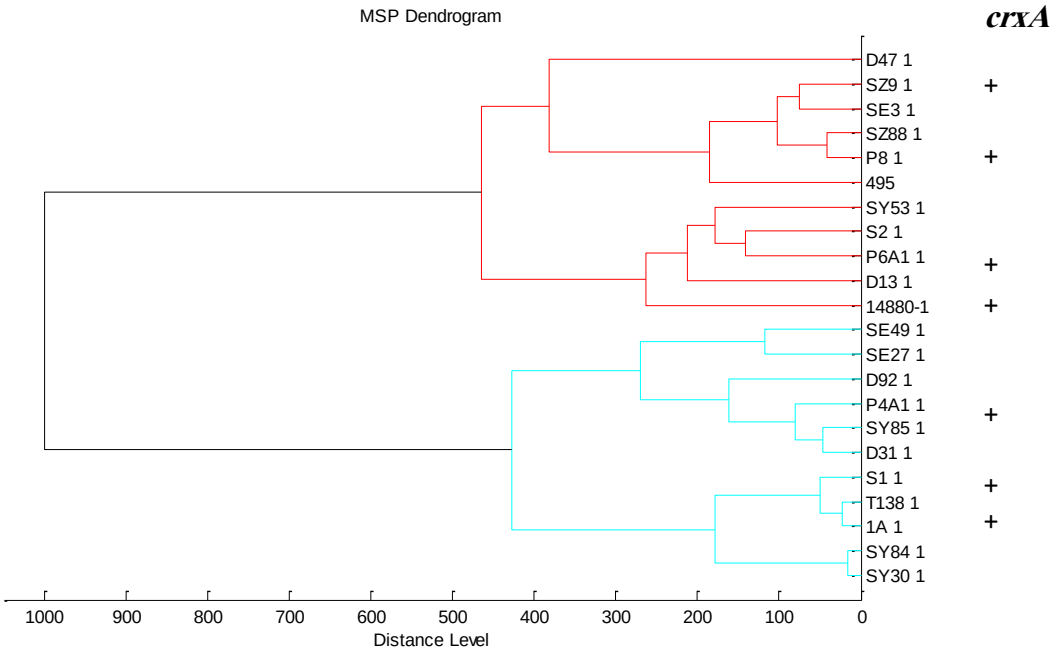


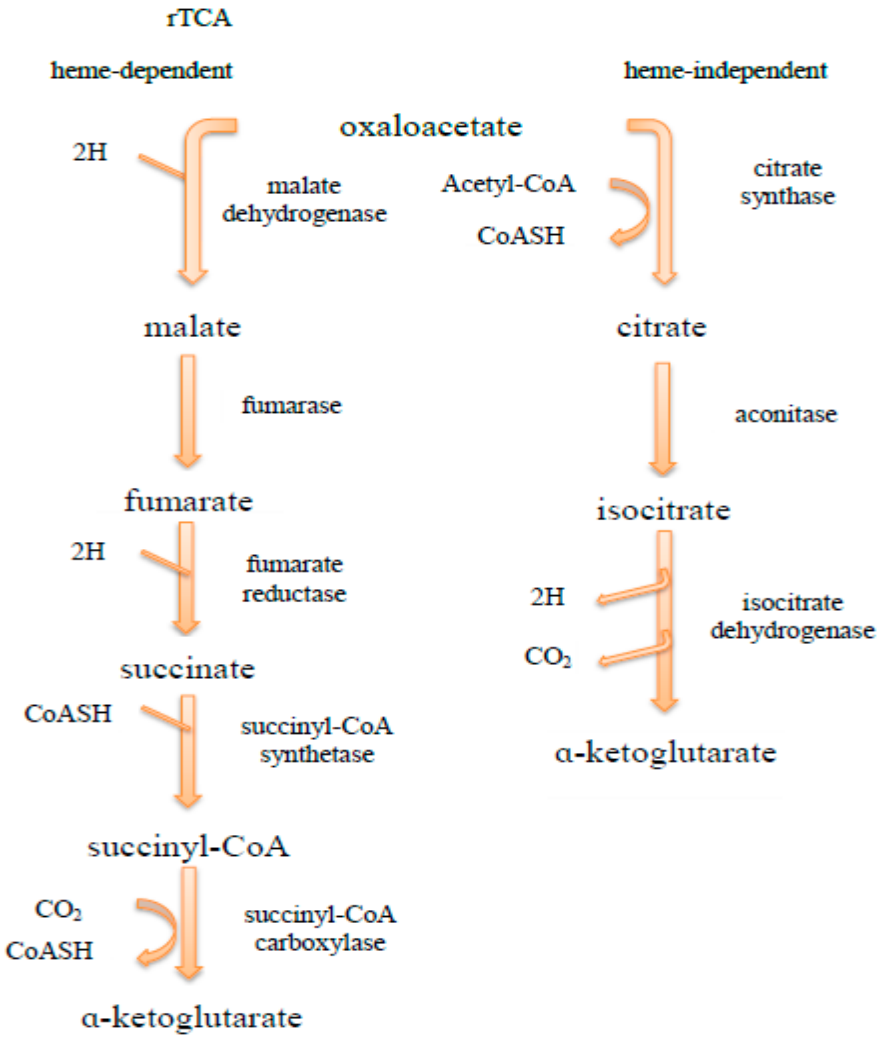
Fig. S5. Determination of outward-oriented promoters by a 5'RACE experiment in IS*Bf6* for the *ninE* genes

CCCATTGACAACCGGTCTTCAACTTGTACAAGATGGCGTTTACCACCTCTATGAGACAACTTTGAGTG
1 >
GCATAACCACGTTTTGCCTTGGAAAGATGAGGCAATATTTCATTTTTTATCGTATCTTTGTCGAGCAT
2
AGTGTACATTGGACTTTCACTATATATGAGTTTGGTCACCATAATATAGGGAACAATTCAATTTGTAC
>
GCTGTGCTTTAACATTTTGATACGATTTTAAGAGAATGAAATAAGTTTAAATCACTTC]ATTGTTTAA

TACTAAAGAAACAGTAAT**ATG**

The > symbol denotes the transcription initiation sites we obtained. The proposed promoter sequences have been underlined and numbered at the leftmost nucleotides. Here,] denotes the IS*Bf6* border. The ATG in bold is the start codon of the *nimE* gene

Fig. S6. The tricarboxylic acid pathways of *B. fragilis*



OTHER PUBLICATIONS

- **PRESENTATIONS AND ABSTRACTS RELATED TO THE TOPIC OF THE THESIS**

➢ ***Verbal presentations***

I. **Bakhtiyar Mahmood**, Zain Baaity, David Leitsch, Katalin Burian, Elisabeth Nagy, Jozsef Soki. Investigation of the same *nim* gene-insertion sequence configurations on the expression of the *nim* genes and metronidazole resistance of *Bacteroides fragilis* strains. *The 6th Central European Conference/Forum for Microbiology, Kecskemet Hungary 13th October -15th October 2021. Acta Microbiologica et Immunologica Hungarica 2021; 68 (Suppl. 1).*

II. **Bakhtiyar Mahmood**, Ana Paunkov, Malgorzata Kupc, Elisabeth Nagy, David Leitsch, József Sóki. Detection of the expression of 18 genes expected to participate in metronidazole resistance by RT-qPCR of *Bacteroides fragilis* strains with or without *nim* genes and various metronidazole MICs. *The 19th International Congress of the Hungarian Society for Microbiology, Budapest Hungary 5th July – 7th July 2023.*

➢ ***Poster and abstract presentations***

I. **Bakhtiyar Mahmood**, David Leitsch, Zain Baaity, Elisabeth Nagy, Jozsef Soki. Effects of the same *nim* gene-insertion sequence configurations on the expression of the *nim* genes and metronidazole resistance of *Bacteroides fragilis* strains. *The 16th Biennial International Congress of the Anaerobe Society of the Americas; Seattle, Washington, USA 28th July- 31st July 2022.*

II. **Bakhtiyar Mahmood**, Zain Baaity, Elisabeth Nagy, Jozsef Soki. Investigations on the clonality of the novel *crxA* metallo-β-lacatamase gene-carrying *Bacteroides xylanisolvens* strains. *The 16th Biennial International Congress of the Anaerobe Society of the Americas; Seattle, Washington, USA 28th July- 31st July 2022.*

III. **Bakhtiyar Mahmood**, Elisabeth Nagy, Jozsef Soki. Detection and analysis of the genetic background of rare β-lactamase genes of *Bacteroides* strains. *The General Meeting of Hungarian Microbiological Society 2022 and 15th Fermentation Colloquium, Kecskemet Hungary 12th October -14th October 2022.*

IV. **Bakhtiyar Mahmood**, Elisabeth Nagy, Jozsef Soki. Detection and analysis of the genetic background of rare β-lactamase genes of *Bacteroides fragilis* group strains. *33rd ECCMID, Copenhagen Denmark 15th April – 18th April 2023*.

V. **Bakhtiyar Mahmood**, Ana Paunkov, Malgorzata Kupc, Elisabeth Nagy, David Leitsch, József Sóki. RT-qPCR detection of the expression of 18 selected genes of *Bacteroides fragilis* strains with or without *nim* genes and various metronidazole MICs. *Anaerobe 2023 Conference, Cardiff UK 12th July –14th July 2023.*

- **PRESENTATIONS AND ABSTRACTS NOT RELATED TO THE TOPIC OF THE THESIS**

I. **Bakhtiyar Mahmood**, Zain Baaity, Katalin Burian, Elisabeth Nagy, Jozsef Soki. Phenotypic and molecular characterization of a multidrug-resistant *Bacteroides vulgatus* isolate that also has imipenem resistance. *31st ECCMID, Vienna Austria* (Online) *9th July – 12th July2021*.

II. **Bakhtiyar Mahmood**, Zain Baaity, Katalin Burian, Elisabeth Nagy, Jozsef Soki. Phenotypic and molecular characterization of an imipenem resistant *Phocaeicola vulgatus* isolate. *4th MedPECS2021 P109. Pecs Hungary* (Online) *15th May 2021*.

III. **Bakhtiyar Mahmood**, Katalin Burian, Elisabeth Nagy, Jozsef Soki. Examination of the antibiotic resistance mechanisms of a multidrug-resistant *Phocaeicola* (*Bacteroides*) *vulgatus* isolate and the role of a novel β-lacatamase gene on imipenem resistance in *Bacteroides* isolates. *The 6th Central European Conference/Forum for Microbiology, Kecskemet Hungary 13th October -15th October 2021. Acta Microbiologica et Immunologica Hungarica 2021; 68 (Suppl. 1).*

IV. Zain Baaity, **Bakhtiyar Mahmood**, Katalin Burian, Elisabeth Nagy, Jozsef Soki. Characterization of heteroresistance to imipenem in *Bacteroides fragilis* strains. *31st ECCMID, Vienna Austria* (Online) *9th July – 12th July2021*.

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Novel and rare β-lactamase genes of *Bacteroides fragilis* group species: Detection of the genes and characterization of their genetic backgrounds

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ABSTRACT

Objectives: This study screened the prevalence of rare β-lactamase genes in *Bacteroides fragilis* group strains from clinical specimens and normal microbiota and examined the genetic properties of the strains carrying these genes.
Methods: *bla*HGD1, *bla*OXA347, *cblA*, *crxA*, and *pbbA* were detected by real-time polymerase chain reaction in collections of *Bacteroides* strains from clinical (n = 406) and fecal (n = 184) samples. To examine the genetic backgrounds of the samples, end-point PCR, FT-IR, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry were used.
Results: All *B. uniformis* isolates were positive for *cblA* in both collections. Although *crxA* was *B. xylanisolvens*-specific and associated with carbapenem resistance, it was only found in six fecal and three clinical *B. xylanisolvens* strains. Moreover, the *crxA*-positive strains were not clonal among *B. xylanisolvens* (contrary to *cfiA* in *B. fragilis*), implicating a rate of mobility or emergence by independent evolutionary events. The *Phocaeicola* (*B.*) *vulgatus/P. dorei*-specific gene *bla*HGD1 was detected among all *P. vulgatus/P. dorei* isolates from fecal (n = 36) and clinical (n = 26) samples. No *bla*OXA347-carrying isolate was found from European collections, but all US samples (n = 6) were positive. For three clinical isolates belonging to *B. thetaiotaomicron* (n = 2) and *B. ovatus* (n = 1), *pbbA* was detected on mobile genetic elements, and *pbbA*-positive strains displayed non-susceptibility to piperacillin or piperacillin/tazobactam phenotypically.
Conclusions: Based on these observations, β-lactamases produced by rare β-lactamase genes in *B. fragilis* group strains should not be overlooked because they could encode important resistance phenotypes.

1. Introduction

Bacteroides fragilis group (*Bacteroides*, *Parabacteroides*, and *Phocaeicola*) species belonging to the order Bacteroidales are strict anaerobic gram-negative bacilli and major constituents of the gastrointestinal tract. They are also the most frequently isolated endogenous anaerobic opportunistic pathogens, sometimes causing serious infections such as intra-abdominal abscesses, obstetric–gynecological conditions, post-operative wounds, lung and brain abscesses, skin and soft tissue infections, bacteremia, and diarrhea [1]. Among all anaerobes, *B. fragilis* group species are the most resistant species regarding the number of antibiotic resistance mechanisms and the antibiotic resistance levels attained. In many countries, recent studies revealed regional variations in resistance to antimicrobial agents among *B. fragilis* group species [2,3]. In developed countries, timely antibiotic susceptibility testing is performed to facilitate empiric therapy [4,5]. However, data on antimicrobial resistance in anaerobic pathogens in developing countries are rarely reported because of laborious processes required for susceptibility testing, and most data in the literature do not obey the guidelines from EUCAST and CLSI [6]. The most useful antibiotics for severe *B. fragilis* group infections are wide-spectrum β-lactams (β-lactam/β-lactamase inhibitor combinations or carbapenems), metronidazole, and tigecycline. Various molecular mechanisms are involved among *B. fragilis* species for β-lactams, e.g., β-lactamase production and reduced affinity of some β-lactams for penicillin-binding proteins or efflux pumps [7]. In *B. fragilis* group species, β-lactamase production is the most frequent cause of penicillin and cephalosporin resistance, and *cepA* or *cepA*-related gene products are responsible for this resistance. *cfxA* is another frequently identified β-lactamase gene that is responsible for high resistance to cefoxitin and other β-lactams, and it is carried on the mobilizable transposon MTn*4555* in *Bacteroides* species [8].

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List of abbreviations

DMSO	Dimethyl sulfoxide
EUCAST	The European Committee on Antimicrobial Susceptibility Testing
PCR	Polymerase chain reaction

Isolates of *B. fragilis* group species can be resistant to carbapenems [4,9,10]. This is usually mediated by the chromosomally located genes *cfiA* (also known as *ccrA*) and *crxA*, which encode class B metallo-β-lactamases in *B. fragilis* and *B. xylanisolvens*, respectively. These zinc-dependent metallo-β-lactamase gene products can confer strong resistance to almost all β-lactams including cephamycins and carbapenems as well as β-lactam/β-lactamase inhibitor combinations [11–13]. Based on the presence of *cepA* and *cfiA*, *B. fragilis* strains can be divided into distinct groups: division I (*cepA*-positive, *cfiA*-negative) and division II (*cepA*-negative, *cfiA*-positive) [12]. However, the presence of *cfiA* usually does not confer carbapenem resistance, but the upstream presence of an insertion sequence element harboring a promoter with high activity is necessary [14].

Information on the distribution of rare or newly described β-lactam resistance genes in *B. fragilis* group species is limited, which prompted us to assess the prevalence of these genes. Thus, we aimed to detect five infrequent β-lactam resistance genes, namely *bla*HGD1, *bla*OXA347, *cblA*, *crxA*, and *pbbA*, in *B. fragilis* group strains isolated from normal microbiota and clinical specimens. Moreover, the lack of knowledge on the phenotypes and genetic background of some of these resistance genes also led us to investigate these genes via various techniques.

2. Materials and methods

2.1. Bacterial isolates and cultivation

In total, 590 *B. fragilis* group strains including clinical (n = 406) and normal microbiota (n = 184) isolates were previously collected. The clinical isolates were collected in four Hungarian microbiological centers between 2014 and 2016 [15], and six clinical isolates obtained in the USA were also included (these strains were provided by the collection of Dr. David Hecht and initially obtained from antibiotic susceptibility studies in the USA in which *cfiA*-positive strains were reported [16]). Species composition is shown in Table S1. Fecal strains were collected in Germany, Belgium, Slovenia, Hungary, and Turkey [17,18]. *B. fragilis* strain carrying the pPE2-1 plasmid was described previously [19]. All isolates were revived from −70 °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 matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS; Biotyper®, Bruker Daltonics, Bremen, Germany) [20]. Antibiotic susceptibility testing was performed to obtain minimum inhibitory concentrations as previously described [15,17,18] by agar dilution, new MIC measurement for amoxicillin/clavulanate, piperacillin/tazobactam we used gradient method using Etest (bioMérieux fixed ratio) or MIC test strips (Liofilchem fixed inhibitor concentration).

2.2. Polymerase chain reaction and nucleotide sequencing

All 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, as listed in Table 1. For PCR, chromosomal DNA was obtained from each isolate by suspending bacterial colonies in 100 μL of DNase/RNase-free H_2O to give a 0.5 McFarland density. The suspended colonies were then incubated in a heating block at 99.5 °C for 12 min, centrifuged at 14 000 rpm for 2 min, and then stored at −20 °C until further use. 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 (Thermo Fisher Scientific) using the conditions presented in Table 1. To confirm some species, we conducted 16S rRNA sequencing as the "gold standard" using the pairs of primers and cycling conditions listed in Table 1. To detect the genetic element of *crxA*, we used Emerald PCR master mix (Takara Bio Inc., Shiga, Japan) as recommended by the supplier. For nucleotide sequencing, the PCR mixture volume was increased (50 μL), and the products were purified using a PCR cleanup kit (HighPure, Roche, Basel, Switzerland). Nucleotide sequencing was performed at a core facility via capillary sequencing (Eurofins, Luxembourg City, Luxembourg). Sequence homology analysis was conducted by comparing the obtained sequences with those recorded in the GenBank database (National Center for Biotechnology Information, US National Institutes of Health, https://www.ncbi.nlm.nih.gov/) or uploaded for species identification to the EzBioCloud webpage (https://www.ezbiocloud.net/) [21].

Table 1
PCR primer sequences and reaction conditions used in this study.

Gene/PCR	Primer/Sequence (5' … 3')	Type of PCR and reaction condition	Ref.
cblA	cblA1: TGAAAACCGGATTGACAGTC cblA2: GAAACAATGCTGTCCAGACT	RT, 95 °C 15s, 55 °C 30s, 72 °C 30s; 35x	This study
crxA	crxA-F: ACCGTTGGCAAAAGTAAGTT crxA-R: TTCACACGCGCAATTGTATT	RT, 95 °C 15s, 55 °C 30s, 72 °C 1m; 35x	[13]
***bla*HGD1**	HGD-11: CGAATACATACAGCCCTTTG HGD-12: TTCGGTAGCAGTTATGGAAA	RT, 95 °C 15s, 55 °C 30s, 72 °C 30s; 35x	This study
***bla*OXA347**	oxa347-1: GCGGTAAGACTGGATTAAGT oxa347-2: TCAACATTCAATCCGTCTGT	RT, 95 °C 15s, 55 °C 30s, 72 °C 30s; 35x	This study
pbbA	pbbA1: TGAAAACGACATGCATGAGA pbbA2: GCACCAGTGTTTTTCAATGG	RT, 95 °C 15s, 58 °C 20s, 72 °C 30s; 35x	[19]
16S rRNA	E8F: AGAGTTTGATCCTGGCTCAG E533R: TIACCGIIICTICTGGCAC	EP, 94 °C 15s, 56 °C 20s, 72 °C 1m; 35x	[17]
ERIC PCR	ERIC1: ATGTAAGCTCCTGGGGATTCAC ERIC2: AAGTAAGTGACTGGGGTGAGCG	EP, 94 °C 15s, 50 °C 1m, 72 °C 3m; 35x	[22]
crxA	crxAEi1: AGTGAAGCATCAATATGGGA crxAEi2: TCCTCGTTCTAATGATTTTCA	EP, 98 °C 1m, 55 °C 1m, 72 °C 6m; 35x	This study
crxA	crxAEo1: GAGTGGAAACGAGTGTCTAT crxAEo2: CTGCTACAACTGTTTGTGAT	EP, 98 °C 1m, 55 °C 1m, 72 °C 6m; 35x	This study

Note: RT = Real-time PCR, EP = End-point PCR, ERIC = Enterobacterial repetitive intergenic consensus PCR.

2.3. β-glucosidase assay

MALDI-TOF MS usually gives ambiguous identification results for *P. vulgatus/P. dorei*. Therefore, we used the 4-nitrophenyl-β-D-glucopyranoside hydrolysis assay to differentiate between *P. vulgatus* and *P. dorei* [23,24]. Our method was set after some optimization experiments. Namely, the substrate was dissolved in H_2O or DMSO and incubated at room temperature for 4 or 24 h. The final reaction setup included 100 μL of 50 mg/mL substrate (in water) and 100 μL of a 2 McFarland density cell suspension in water, and the reaction was stopped after 4 h by adding 5 μL of 1 M Na_2CO_3 (pH 11). Water served as the blank (background absorbance), and *B. fragilis* 638R and *P. vulgatus* ATCC 29327 served as the positive and negative controls, respectively. The absorbance of the produced 4-nitrophenol was measured at 405 nm in a microplate reader (PR 3100 TSC model, Bio-Rad, Hercules, CA, USA), and the obtained values were corrected by the absorbance mean of the blank samples.

2.4. 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. ERIC-PCR was performed as previously described [22]. The IR Biotyper method included the following steps. Seven times a loopful (7 × 10 μL) of bacterial culture was collected and suspended in 55 μL of 70% ethanol in a 1.5-mL e-tube containing sterile metal rods. To obtain a uniform suspension, a multi-vortexer was used. After vortexing for 1.5 min and waiting for 5 min, 60 μL of sterile water were added. The, 15 μL of the bacterial suspension were spotted onto the IRBT silicon plate together with 12 μL of two spots each of the IR test standard 1 and IR test standard 2 suspension, which were contained in the IR Biotyper Kit (Bruker Daltonics) for quality control, and dried at 37 °C until a dry film formed over 20–30 min. Three technical replicates were used for each sample. Finally, the dried silicon plate was inserted into the IRBT spectrometer (Bruker Daltonics) to acquire the spectra at default analysis settings using OPUS 7.5 software (Bruker Daltonics). The spectra that met the default quality criteria of absorption (0.4 arbitrary units [AU] < D value < 2 AU), signal/noise (<150 × 10^{-6} AU), signal/water (<300 × 10^{-6} AU), and fringes (<100 × 10^{-6} AU) were determined as "quality pass" in the IRBT® analysis. The *B. xylanisolvens* isolates that failed to pass the default quality criteria were re-examined. The spectra obtained with "quality pass" were analyzed to build the dendrogram. The software contains a feature that automatically proposes a cutoff that defines up to which distance spectra are considered to be in the same cluster. MALDI-TOF MS typing was performed by taking the spectra generated by the Sirius instrument (Bruker Daltonics) [20] and the built-in dendrogram-making feature of MBT Compass Explorer software (Bruker Daltonics).

2.5. Bioinformatics

ERIC PCR fragment similarity patterns were analyzed using GelJ software [25], and the *Bacteroides* β-lactamase sequence similarity dendrogram was drawn using the Lasergene 17 suite (DNAStar, Madison WI, USA).

3. Results and discussion

In this study, we screened clinical and normal microbiota isolates of *B. fragilis* group species (n = 590) for less frequently investigated β-lactamase genes (Table 2). All *B. uniformis* strains included in both collections harbored *cblA*. Similarly, in all *P. vulgatus/P. dorei* strains, we detected *bla*HGD1. Because MALDI-TOF MS cannot discriminate between *P. dorei* and *P. vulgatus* strains, we took the advantage of differences in their ability to produce β-glucosidase, revealing (Fig. S1) that all strains of both species carry this β-lactamase gene. This species identification was confirmed by 16S rRNA sequencing for five *P. vulgatus* and six *P. dorei* isolates. 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 for *bla*OXA347. *pbbA* was only detected in three clinical isolates (two *B. thetaiotaomicron* and one *B. ovatus*). This latter gene exerts resistance to piperacillin and decreased piperacillin/tazobactam susceptibility, but it does not significantly affect amoxicillin/clavulanate resistance (Table 3). Meanwhile, the carbapenem resistance determinant gene *crxA*, which is specific for *B. xylanisolvens* strains, was detected in three clinical [13] and six normal microbiota strains. We previously determined by bioinformatics that *crxA* resides on a small genomic island [13]. Therefore, we studied whether the eight *B. xylanisolvens* strains carried this gene on the same island. However, PCR primers specific for this island (inside of the element, crxAEi1 and crxAEi2; Table 1, Fig. S2, Fig. 1) detected its presence in only five strains including the prototype strain *B. xylanisolvens* 14880. Additionally, two of strains might carry this gene at different genomic locations because other primers (crxAEo1 and crxAEo2; Table 1, Fig. S2, Fig. 1) designed to recognize the borders of the *crxA* element did not amplify the gene. Because of these heterogeneities, we performed genetic relatedness experiments (typing, Fig. S4) for these 8 *crxA*-positive *B. xylanisolvens* strains and 13 additional *crxA*-negative *B. xylanisolvens* strains, revealing that the *crxA*--positive strains were located on different branches.

In this study, we analyzed the prevalence of "rare" β-lactamase genes of *B. fragilis* group strains from normal microbiota and cases of infection from several countries, and as necessary, other traits of the strains, especially their genetic backgrounds, were also examined. Most current knowledge is about *cepA*, *cfxA*, and *cfiA*; therefore, this exhaustive study on 590 strains provides important new data. The included genes had extremely different amino acid sequences from molecular Classes A2 and D (Fig. S3), and they represent 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 *P. vulgatus* and *P. 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

Table 2
The number of isolates and prevalence of rare resistance genes distribution in *B. fragilis* group species.

Isolates	Prevalence of resistance genes				
	*bla*HGD1	*bla*OXA347	*cblA*	*crxA*	*pbbA*
Normal microbiota (184)	16 + 20 (*P. vulg/dorei*)	0	6 (*B. uni*)	6 (*B. xyl*)	0
Clinical (406)	16 + 10 (*P. vulg/dorei*)	6	12 (*B. uni*)	3 (*B. xyl*) [13]	3

Note: *B. uni* = *Bacteroides uniformis*, *B. xyl* = *Bacteroides xylanisolvens*, *P. vulg/dorei* = *Phocaeicola vulgatus or dorei*.

Table 3
The MIC value of E-test result for *B. fragilis* 638R with and without transformed *pbbA* gene.

Strains	Antibiotics and MIC value						
	AMP	XL	AMC	PTC	PZP	FOX	IMP
B. fragilis 638R	2	0.25	0.064	0.064	0.25	2	0.032
B. fragilis 638R/pPE2-1	>256	0.5	0.5	256	4	2	0.064

Note: AMP= Ampicillin, XL = Amoxicillin/clavulanate (bioMérieux fix ratio), AMC= Amoxicillin/clavulanate (Liofilchem fixed inhibitor concentration), PTC= Piperacillin (bioMérieux fix ratio), PZP= Piperacillin/tazobactam (Liofilchem fixed inhibitor concentration), FOX= Cefoxitin, IMP= Imipenem.

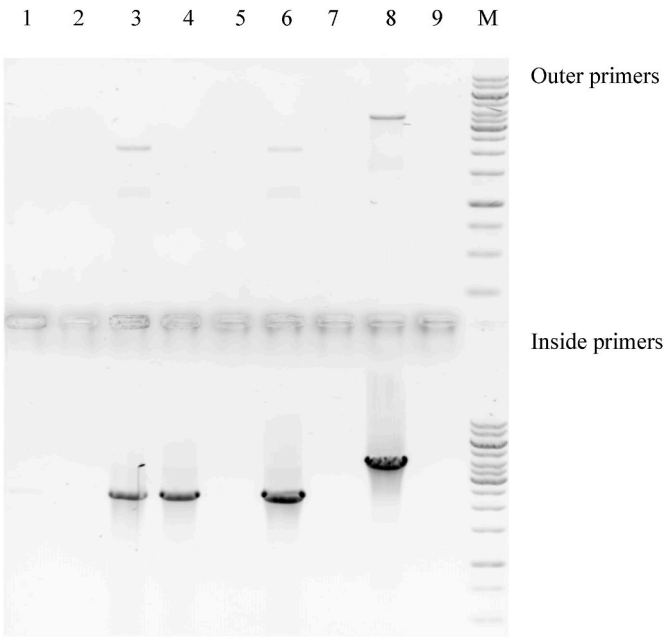

Fig. 1. Gel picture of *crxA* element PCR; lane 1–8 represent *B. xylanisolvens* strains (D85, SZ9, P8, Hu1, P6A1, 1A, P4A1, 14880); lane 9 negative control and M 1 kb ladder.

schemes (ERIC, IR, MALDI-TOF MS; Fig. S4), as the topologies of the detected dendrogram and the positions of the *crxA*-positive strains differed. Based on the observed dendrogram topologies, we suspect limitations in the use of IR Biotyper or MALDI-TOF MS in this case. The picture could be diversified via *crxA* element detection because the already 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. Whole-genome analyses clarifying these assumptions are ongoing in our laboratory. *pbbA* was found in some of the species of our collections, but it was rarely detected. The gene conferred strong piperacillin resistance, potentially accounting some of the cases of moderate β-lactam/β-lactamase resistance among *B. fragilis* group strains. *bla*OXA347 was found in six *B. fragilis* group strains with imipenem resistance that were obtained in the USA during *Bacteroides* antibiotic susceptibility studies in the 1990s [16]. This gene has been rarely detected (Denmark [26] and Hong Kong [27]), but carbapenem resistance in cases in which this gene was detected could not be confirmed; thus, further studies are needed.

Although β-lactam antibiotics have a broad spectrum and they are prescribed most frequently in hospital settings, these agents are difficult to use because of the production of β-lactamases, which represent the most common cause of β-lactam resistance in anaerobes [28]. 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 [29]. Most previous studies extensively focused on the detection and prevalence of *cfiA* in *B. fragilis* strains [16,30,31].

The limitation of this study is that exact carriage rates for some of the genes cannot be established appropriately since we have relatively low number of strains for the normal microbiota for some of the European countries. Additionally, for the Hungarian clinical strains we also 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 at other places. Thus, our data are just approximating the real cases.

4. Conclusions

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

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Ethical approval

Not required.

CRediT authorship contribution statement

Bakhtiyar Mahmood: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Károly Péter Sárvári:** Methodology, Writing – review & editing. **Laszló Orosz:** Software, Writing – review & editing. **Elisabeth Nagy:** Writing – review & editing. **József Sóki:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare they have no conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.anaerobe.2024.102832.

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The effects of identical *nim* gene-insertion sequence combinations on the expression of the *nim* genes and metronidazole resistance in *Bacteroides fragilis* strains

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ABSTRACT

In this study we examined whether the same *nim* gene-insertion sequence (IS) element combinations give rise to the same expression levels as they harbor shared IS element-borne promoters. From our quantitative analysis, we found that the expressions of the *nimB* and *nimE* genes with their cognate IS elements were similar, but the metronidazole resistance of these strains were more diverse.

Metronidazole is a highly efficient antibiotic for the treatment of anaerobic infections with less than 1% resistance rates in Western countries despite its long-term usage since the 1960s. However, this varies in different European regions and it seems also to increase slightly. Additionally, in developing countries the metronidazole resistance rates are sometimes very high. Resistance may occur via various mechanisms (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 *Bacteroides* spp [1]. Now we know 12 *nim* gene types (*nimA-L*), which are 60–80% homologous and IS elements upregulate their expression [2,3]. Each *nim* gene type can harbor a highly gene-specific IS element in their upstream regions [2]. With their mobility, the metronidazole resistance phenotype caused by *nim* genes can also be experimentally transferred [2], as they are associated by metronidazole resistance [4,5] and it was suggested that they are 5-nitroimidazole reductases [6]. However, there are also doubts about their specific resistance determinant nature. This is because not all *nim*-positive *Bacteroides* strains are resistant [2], their expression does not correlate with the resistance levels [7] and 5-nitroimidazole resistance is not stable. The *nim*-positive strains can lose their resistance upon storage [8], and higher resistance can be induced by metronidazole, which sometimes can revert to the original levels [9]. These findings also led us to ask whether the IS element are true regulators of the 5-nitroimidazole resistance caused by *nim* genes. This is why we decided to study the correspondence of expressions of two well-characterized *nim* gene type-IS element associations (*nimB*-IS*1186* and *nimE*-IS*Bf6*) [10,11] and to see how it is related to metronidazole resistance.

We chose 6 *nimB*-IS*1186* and 10 *nimE*-IS*Bf6* construct-carrying *Bacteroides fragilis* strains [10,11] from our collection of glycerol stocks stored at −70 °C. After their inoculation onto supplemented Columbia blood agar plates (300 mg/l L-cystein, 5% defibrinated sheep blood, 2.5% laked sheep blood and 1 μg/ml vitamine K_1) and anaerobic cultivation, we measured their actual metronidazole resistance levels by gradient tests (Etest, bioMerieux). We prepared total RNA (HighPure RNA Isolation Kit, Roche) from BHIS (Brain Heart

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Infusion, BHI, broth supplemented with 0.25% Yeast extract, 5 μg/ml hemin and 1 μg/ml vitamine K_1) cultures [10] and examined the expression levels of the *nim* genes in RT-qPCR experiments with specific primers for the *nim* genes (*nimB*: nimB-RT1 CTTCATGGGGACGATGGTTA and nimB-RT2 ATCCGTCAATATGTGGGCTT, and *nimE*: nimE-RT1 TCGTAAACGACGGTTGTTGC and nimE-RT2 TGCATAATAGCGTCCACCTT) and for the glyceraldehyde 3-phosphate dehydrogenase gene (*gap*: gapBF1 AGCCATTGTAGCAGCTTTTT and gapBF3 GAAGACGGGATGATGTTTC) as an endogenous control using the $\Delta\Delta C_T$ method. For the RT-qPCR experiments we used the StepOne RT-PCR instrument (Life Technologies) with the following cycling parameters (55 °C 20 min, 95 °C 15 min; 95 °C 15 s, 55 °C 30 s, 72 °C 30 s, 35x; 72 °C 75 s and a melting curve record from 72 °C to 95 °C) using the Verso 1-Step SYBRGreen mastermix (Life Technologies). The promoter of the IS*1186* element was identified earlier by Podglajen et al. [12] and we did this for the IS*Bf6* element by 5′-RACE PCR (rapid amplification of cDNA ends, Roche), as described earlier [13] but with the following primers (SP1: CATTTTTTCAAGAATTGCCAC, SP2: CTCTCTTCTTGTGGCAAC and SP3: GTTTACGACGCATTTCTCT). The correlation calculations were carried out using the Spearman rank method with the Sigmaplot 12 software package (Sigmaplot, Germany).

The variations in *nim* gene expressions were much less, and practically constant on a $\log_2$ scale compared to variations in metronidazole resistance levels (Table 1). It demonstrates that identical *nim* gene-IS element combinations have the same expression levels of the resistance genes. However, the metronidazole resistance levels did not correlate with the expressions of the *nim* genes, and the associations were not significant; so they appear independent (Table 1). Fig. 1 shows the constant expression of the *nimB* and *nimE* genes, which are independent of the metronidazole MICs. The *nimE* gene of *B. fragilis* Q8 had two *ISBf6* element-born promoters (34 and 114 bp upstream of the right end of ISBf6), as revealed by 5′-RACE (Fig. S1).

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 from the analyzed *nim* gene IS element pairs. We attribute the small variations of the *nim* gene's expressions to other strain-specific differences, e.g. levels of reducing agents or enzymes, some low-level nitroresuctase or efflux activites, etc. The same *nim* gene-IS element configurations of the strains were epidemiologically and genetically unrelated, as we found in previous studies [10,11]. The IS*1186* and IS*Bf6* elements had recognizable *Bacteroides*-specific promoter sequences (the consensus, TtTg −33 TnnTAnnTTTGY −7, was determined earlier [14]) that could drive the expression of the *nimB* and *nimE* genes, respectively.

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 competition. 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 [15,16]. 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 cofactor that reduces metronidazole to an active compound [17], but it remains to be confirmed. 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 metronidazole resistance of *Clostridioides difficile* and *B. fragilis* [18–20]. Next, we plan to extend the range of genes whose expression could be studied by more RT-qPCR experiments which would be based on our current RNASeq and mass spectrometry measurements to

Table 1
Data on the metronidazole resistance levels and *nim* gene expressions of our test strains.

B. fragilis strain and *nim* gene	Statistics	MTZ MIC (μg/ml)	Relative expression (RQ[a])		
			mean	RQ (min)	RQ (max)
nimB					
BF8		2	1.00	0.84	1.19
KSB-R		64	3.69	2.44	5.60
1672		1	3.90	2.56	3.60
O:21		0.5	2.21	1.92	2.55
768a		2	2.26	1.90	2.70
13038		0.5	2.04	1.69	2.47
	range[b]	0.5−64	2.69		
	$\log_2$[c]		7 1.88		
	correlation MIC-RQ[d]	r = −0.506 (p = 0.126)			
nimE					
Q3		1	1.66	0.72	3.86
Q4		1	0.86	0.51	1.45
Q5		32	0.54	0.39	0.74
Q6		>256	0.39	0.26	0.58
Q7		4	1.56	0.89	2.73
Q8		2	0.68	0.58	0.80
Q9		16	1.00	0.88	1.13
Q10		8	0.40	0.22	0.74
Q11		128	1.43	1.22	1.68
Q12		2	1.91	1.63	2.24
	range[b]	1->256	1.52		
	$\log_2$[c]		>8 0.604		
	correlation MIC-RQ[d]	r = 0.441 (p = 0.356)			

[a] RQ stands for relative expression values from RT-qPCR experiments where the RQ of 1.00 is that for the control sample and we used the $\Delta\Delta C_T$ method for the calculations of RQs (the data were from three technical replicates).
[b] Ranges of the metronidazole MICs and the gene expression means.
[c] Log_2 values of the ranges.
[d] Parameters of the correlations (correlation coefficients and their significance) between the metronidazole MICs and the *nim* gene expressions.

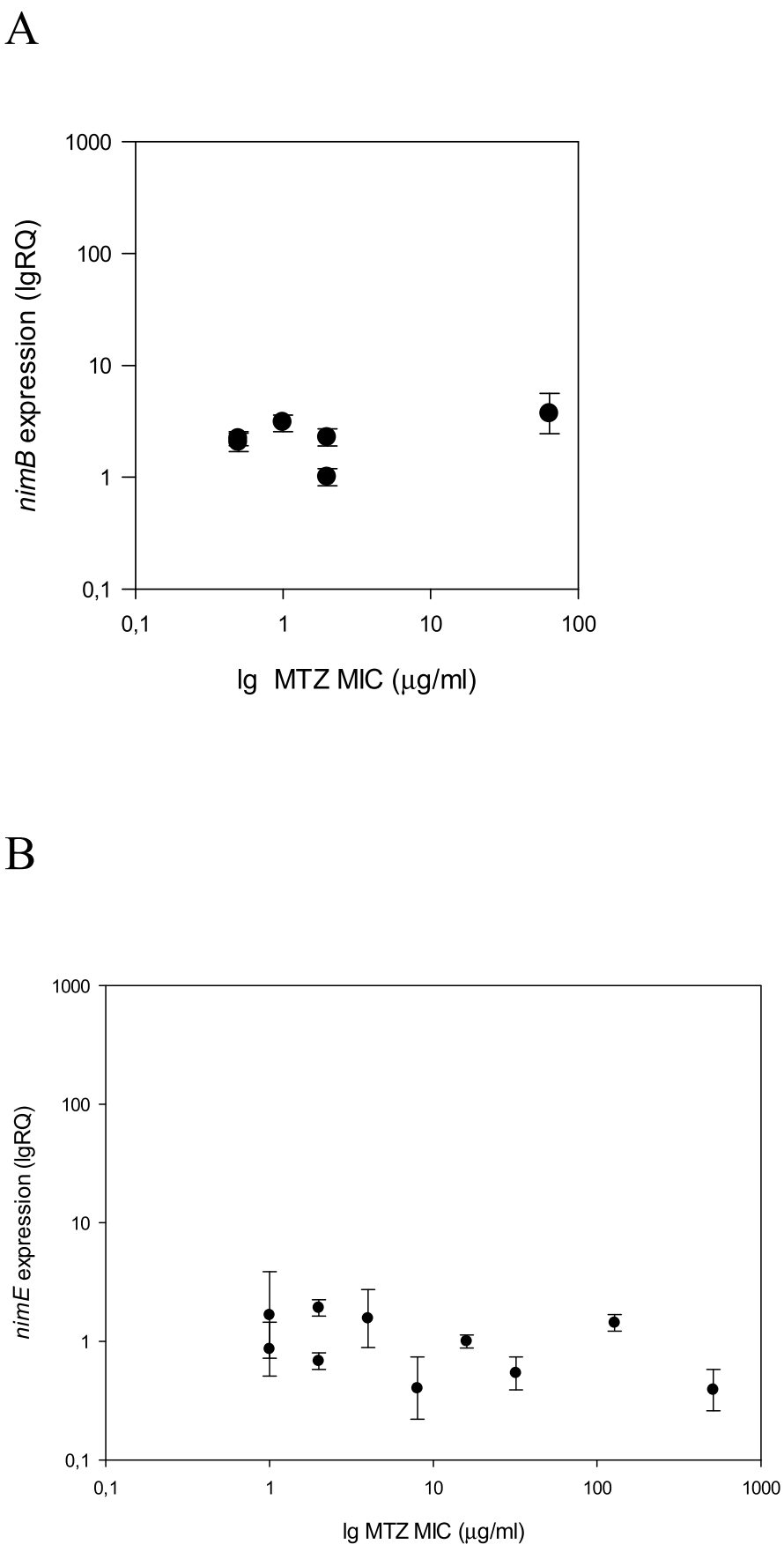

Fig. 1. A representation of the uniform expression of *nim* genes and the apparent independence of the metronidazole MICs.

determine their association with Nim action and metronidazole resistance. These experiments 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.

In summary, from our results we revealed that (i) the *nimB* and *nimE* genes are uniformly regulated by their IS elements in different strains; (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* spp. These results and our assumptions should help clarify the role of *nim* gene-IS element combinations and the mechanism of metronidazole resistance among anaerobic pathogens.

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Declaration of competing interest

The authors declare they have no conflict of interest.

Data availability

All the obtained and used data are contained in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.anaerobe.2023.102739.

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PUBLICATION III.

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Article

Proteomics-Based RT-qPCR and Functional Analysis of 18 Genes in Metronidazole Resistance of *Bacteroides fragilis*

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Abstract: Previously, we reported that metronidazole MICs are not dependent on the expression levels of *nim* genes in *B. fragilis* strains and we compared the proteomes of metronidazole-resistant laboratory *B. fragilis* strains to those of their susceptible parent strains. Here, we used RT-qPCR to correlate the expression levels of 18 candidate genes in a panel of selected, clinical *nim* gene-positive and -negative *B. fragilis* strains to their metronidazole MICs. Metronidazole MICs were correlated with the expression of certain tested genes. Specifically, lactate dehydrogenase expression correlated positively, whereas cytochrome fumarate reductase/succinate dehydrogenase, malate dehydrogenase, phosphoglycerate kinase redox and *gat* (GCN5-like acetyltransferase), and *relA* (stringent response) regulatory gene expressions correlated negatively with metronidazole MICs. This result provides evidence for the involvement of carbohydrate catabolic enzymes in metronidazole resistance in *B. fragilis*. This result was supported by direct substrate utilization tests. However, the exact roles of these genes/proteins should be determined in deletion–complementation tests. Moreover, the exact redox cofactor(s) participating in metronidazole activation need to be identified.

Keywords: *Bacteroides*; metronidazole; *nim*; resistance mechanism; RT-qPCR

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1. Introduction

Species within the former *Bacteroides fragilis* group (BFG) (which are now classified within the *Bacteroides*, *Parabacteroides*, and *Phocaeicola* genera) are the most frequently isolated opportunistic anaerobic pathogens, which are also important members of the mammalian intestinal microbiota. Of these, *B. fragilis* is the most pathogenic and accounts for 50% of clinical isolates; however, it is only a minor population among intestinal strains [1]. They are highly resistant to most antimicrobial agents through the use of several antibiotic resistance mechanisms. Metronidazole is a prominent choice to treat infections of *B. fragilis* because metronidazole is an anti-anaerobic drug that usually elicits low levels of resistance among obligately anaerobic pathogens [2]. However, metronidazole resistance levels among *Bacteroides* have increased somewhat and increased greatly in developed and developing countries, respectively [3,4]. The best known and most investigated metronidazole resistance mechanism of BFG strains is mediated by the *nim* genes, a few of which were originally discovered in the 1980s and 1990s [5]. There are now 12 known homologs of *nim* (i.e., *nimA-L*) that share 50–80% amino acid homologies, and they are all proposed to act as nitro-reductases [6,7]. They have been localized to both the plasmid and chromosome, and they all require an upstream copy of a *Bacteroides*-specific insertion sequence (IS) element with promoter sequences to function in metronidazole resistance [5]. Besides

nim genes, the other proposed metronidazole resistance mechanisms among BFG strains include increased lactate dehydrogenase, decreased pyruvate-ferredoxin oxidoreductase (PFOR), efflux, reduced iron uptake, and increased DNA repair [5]. However, these latter diverse mechanisms do not operate in all *nim*-negative-resistant strains and are sometimes found only in laboratory strains after the induction of metronidazole resistance. The *nim*-mediated mechanism, which is most prevalent among *Bacteroides*, still has some open questions. However, it is known that the metronidazole MICs of *nim*-positive strains are sometimes low and unstable, and the expression level of the *nim* genes correlates poorly with metronidazole MICs, which tend to be flexible. To explain this low correlation, the action of auxiliary factors has been proposed [8].

B. fragilis strains can be classified into two divergent divisions based on genetic differences (e.g., differences in alleles or genetic elements, most importantly the carbapenem resistance gene, *cfiA*) that can be mapped to specific loci in the genome of this important species [9], and, by our observations, it is also related to the gene expressions of the *B. fragilis* strains.

The aim of this study was to search for factors, in addition to *nim* genes, that affect the metronidazole resistance of *B. fragilis*. We have previously revealed that hemin- and iron-uptake mechanisms are involved in metronidazole resistance, and *nim*-negative and *nim*-positive *B. fragilis* strains behave differently in these regards [10]. Previously, we analyzed the proteomics of *nimA*-positive and -negative *B. fragilis* laboratory strains [11]. In addition, here, we analyzed the expression of 18 genes previously identified as resistance candidates in a proteomic study in a collection of *nim*-positive and -negative clinical *B. fragilis* strains, and we correlated the expression levels of these genes to measurements of metronidazole MICs to identify possible auxiliary factor(s) involved in metronidazole resistance of *B. fragilis*. Finally, we studied the effects of C_4-dicarboxylic acid supplementation on metronidazole MICs to better understand the hemin dependence of metronidazole resistance.

2. Results and Discussion

2.1. Connection between the Metronidazole MICs and nim Gene Expression

Table 1 shows the results of the metronidazole MICs and the expression levels of the *nim* genes of the eight *nim*-positive strains. Similarly to the results of our previous studies [8,11], metronidazole MICs and the expression levels of the *nim* genes were independent of each other ($r = 0.185$, $p = 0.619$, $r^2 = 0.0342$).

Table 1. Strains used, their properties and RT-qPCR experiment results.

B. fragilis	MTZ [a] MIC (μg/mL)	*nim* (IS)	*nim* experession (Rq [b])	*cfiA*	Ref.
GBR13	>256	E (IS*Bf6*)	0.352	+	[12]
388/2	>256	E (IS*Bf6*)	1.778	+	[13]
Q5	256	E (IS*Bf6*)	1.411	+	[14]
20584	256	E (IS*Bf6*)	1	+	This study
Q6	256	E (IS*Bf6*)	0.187	-	[14]
DOR18i3	256	D (IS*1169*)	0.403	+	This study
18807i2	(0.5−) > 256 [c]	-	n.a.	-	This study
Q11	64	E (IS*Bf6*)	0.856	+	[14]
WI1	32	-	n.a.	+	[15]
KSB-R	32	B (IS*1186*)	0.109	+	[16]
SY46	0.25	-	n.a.	-	[17]
SZ69	0.25	-	n.a.	+	[17]
638R	0.125	-	n.a.	-	[18]
SZ26	0.125	-	n.a.	+	[17]
SE61	0.064	-	n.a.	-	[17]

[a] MTZ stands for metronidazole. [b] Rq is relative quantity determined by the $\Delta\Delta C_T$ method. [c] Heterogeneous resistance phenotype.

However, *nim* genes are known resistance factors because they transfer the resistance phenotype in conjugation experiments [19–21], and they are associated with metronidazole resistance in field studies [4,22]. Therefore, 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 [8].

2.2. Examination of the Roles of 18 Genes in Metronidazole Resistance

To investigate this possibility, we measured the expression levels of 18 genes selected according to the results of previous research or from our recent investigations [11] using RT-qPCR to study 15 *B. fragilis* strains. The cross-correlations between gene expressions and the correlation between gene expression and metronidazole MICs for all 15 *B. fragilis* strains are shown in Table 2. The cross-correlations between certain genes 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—$r = 0.593$, $p = 0.0192$, Table 2) are located on the same operon [23]. 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 (Tables S2 and S3), 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 (*cf.* Tables S2 and 3). 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 (Table 3) demonstrated that *frdC*, *gat*, *mdh*, *nanH* (sialidase), *pgk*, and *relA* gene expression depended on the presence of the *nim* gene; however, the *cfiA* gene status did not affect the expression of the studied genes (Table 3). The genes listed above differ from the list in Table 3 because the list above includes and excludes *ldh* and *nanH*, respectively. We are currently unable to explain this finding, although the inclusion of *nanH* indicates a link between metronidazole resistance/*nim* positivity and virulence.

Although we found no significant association of the examined genes among the *nim*-negative and *nim*-positive strains separately, the combined data signalized some good associations in the case of the whole strain set (see above). Therefore, we conclude that no particular enzyme is exclusively correlated with metronidazole resistance in both the *nim*-negative and -positive strains. However, some of these genes have previously been found to cause metronidazole resistance, e.g., *feoAB* (described in [24]), *acr5* (*bmeB*, described in [25]) and *por* (described in [26]). This may be applied to genes not examined here (*recA*, *sod* and *rhaA*), as their role has been demonstrated in metronidazole resistance earlier [27–29]. So, on the population level, these genes do not exert a general role; they are important only in individual cases/strains. However, it should be noted that in both *nim*-positive and *nim*-negative strains, the possible exceptions of *mdh* and *gat* could be significant, respectively (mentioned above).

Table 2. Cross-correlation values between the examined gene expressions and the metronidazole MICs for 15 *B. fragilis* strains [a].

	S3	**acr5**	***acr15***	***crpF***	***frdC***	***feoAB***	***fldA***	***fprA***	***frdA***	***galK***	***gatMZ***	***ldh***	***mdh***	***nanH***	***porMZ***	***pgk***	***relA***	**MIC [b]**
L20	0.486	−0.318	0.243	−0.346	0.725	0.0393	−0.479	0.421	0.789	0.154	0.149	−0.0964	0.621	−0.393	0.257	0.704	0.679	−0.423
	0.0639	0.24	0.374	0.199	0.00178	0.883	0.0685	0.113	2×10^{-7}	0.575	0.584	0.724	0.0129	0.142	0.346	0.00302	0.00504	0.113
S3		0.214	0.429	−0.296	0.511	−0.175	0.025	0.275	0.443	0.579	0.31	0.0286	0.421	−0.321	0.414	0.643	0.614	−0.25
		0.433	0.107	0.275	0.0498	0.523	0.923	0.312	0.0946	0.0231	0.252	0.913	0.113	0.235	0.12	0.00934	0.0143	0.359
acr5			0.461	0.136	−0.343	−0.113	0.393	0.05	−0.132	0.486	−0.125	0.211	−0.111	0.25	0.443	−0.0536	−0.168	0.227
			0.0808	0.62	0.204	0.676	0.142	0.852	0.629	0.0639	0.648	0.441	0.686	0.359	0.0946	0.842	0,54	0.41
acr15				0.4	−0.0179	−0.0536	0.0821	0.307	0.546	0.629	−0.133	0.664	0.0107	0.343	0.639	0.125	0.104	0.132
				0.134	0.944	0.842	0.763	0.257	0.0339	0.0116	0.629	0.00654	0.964	0.204	0.00988	0.648	0.705	0.629
crpF					−0.3	0.211	0.0929	−0.214	0.0429	0.075	−0.262	0.646	−0.25	0.468	0.439	−0.368	−0.25	0.491
					0.269	0.441	0.734	0.433	0.873	0.783	0.339	0.00882	0.359	0.0757	0.0975	0.171	0.359	0.0597
frdC						0.179	−0.336	0.486	0.593	−0.193	0.528	−0.311	0.7	−0.614	0.25	0.75	0.814	−0.669
						0.514	0.214	0.0639	0.0192	0.481	0.0413	0.252	0.00326	0.0143	0.359	0.000786	2×10^{-7}	0.00614
feoAB							0.259	0.45	−0.132	−0.37	−0.0403	0.247	0.182	−0.316	0.39	−0.218	0.261	−0.0118
							0.339	0.0889	0.629	0.167	0.883	0.367	0.506	0.24	0.146	0.426	0.339	0.964
fldA								−0.05	−0.536	0.118	−0.0685	0.468	−0.207	0.286	0.143	−0.386	−0.0429	0.274
								0.852	0.0382	0.667	0.802	0.0757	0.449	0.293	0.602	0.15	0.873	0.312
fprA									0.218	−0.179	0.27	−0.0536	0.575	−0.454	0.386	0.461	0.471	−0.426
									0.426	0.514	0.319	0.842	0.0241	0.0861	0.15	0.0808	0.0732	0.11
frdA										0.279	0.157	0.143	0.393	−0.1	0.357	0.55	0.489	−0.361
										0.306	0.566	0.602	0.142	0.714	0.185	0.0325	0.0618	0,18
galK											−0.475	0.482	−0.25	0.382	0.339	0.0643	−0.075	0.457
											0.0708	0.0662	0.359	0.154	0.209	0.812	0.783	0.0834
gatMZ												−0.5	0.596	−0.58	−0.00403	0.463	0.483	−0.683
												0.0556	0.0183	0.0231	0.985	0.0782	0.0662	0.00471
ldh													−0.3	0.564	0.421	−0.346	−0.0893	0.517
													0.269	0.0275	0.113	0.199	0.743	0.0463
mdh														−0.689	0.364	0.836	0.825	−0.528
														0.00409	0.176	2×10^{-7}	2×10^{-7}	0.0413
nanH															−0.0214	−0.521	−0.543	0.446
															0.934	0.0446	0.0353	0.0917
porMZ																0.254	0,45	0.0798
																0.353	0.0889	0.773
pgk																	0.786	−0.586
																	2×10^{-7}	0.0211
relA																		−0.629
																		0.0116

[a] Odd and even rows with correlation coefficients and significance values, respectively; the color-coding means the following: yellow—0.5 < r < 0.7, 0.05 > *p* >0.01, orange: r > 0.7, *p* < 0.01; abbreviations in Table S1. [b] Metronidazole MIC.

Table 3. Significances of the one-way variance analyses of the gene expressions and the metronidazole MICs depending on the genetic background in 15 *B. fragilis* strains.

	L20	**S3**	***acr5***	***acr15***	***crpF***	***frdC***	***feoAB***	***fldA***	***fprA***	***frdA***	***galK***	***gat***	***ldh***	***mdh***	***nanH***	***por***	***pgk***	***relA***	**MIC**
nim	n.s.[a]	n.s.	n.s.	n.s.	n.s.	0.006	n.s.	n.s.	n.s.	n.s.	n.s.	0.029	n.s.	0.001	0.04	n.s.	0.001	0.001	0.001
cfiA	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

[a] n.s.—non-significant.

In addition, the roles of enzymes involved in the central metabolism in *B. fragilis* should also be considered. The central metabolism varies greatly among bacteria [30], 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 (Figure S1) [31]. Previously, we found that hemin depletion causes metronidazole susceptibility in both *nim*-negative and *nim*-positive strains of *B. fragilis* [10]. Thus, heme may be a rate-limiting factor in the metronidazole resistance of *B. fragilis*, as proposed above. Our results show 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 can decrease the cellular concentrations of reducing cofactor, which diminishes metronidazole activation, thus inducing resistance.

The Nim enzymes are nitro-reductases that can transfer either six [6] or two electrons to the nitro group of metronidazole, yielding either an amino or a nitroso imidazole, respectively [20]. Recently, in vivo and in vitro experiments demonstrated that a *nim* group enzyme encoded by *Clostridioides difficile* strains is a nitro-reductase [7]. In this latter study, it was also confirmed that metronidazole resistance in *C. difficile* is dependent on hemin [32] through experiments involving the direct addition of metronidazole to assay its modification by recombinant NimB and by transcriptomic analysis. Moreover, genetic (transposon mutagenesis) and biochemical (aromatic nitro-reduction to amine) tests have proven that the *nimB* gene of some *C. difficile* strains is responsible for their metronidazole resistance. However, in the in vitro experiments, the metronidazole concentration used, 5 mM, was much higher than that to which the bacteria are usually exposed (the 4 μg/mL breakpoint concentration corresponds to 23.4 μM—a ca. 160-fold difference). 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.

2.3. Examination of Addition of C_4-dicarboxylic Acids on Metronidazole Resistance

We were also interested in how the addition of intermediates of the rTCA pathway affects 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. The results are shown in Table S4, with some representative plates shown in Figure S2. 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. This latter finding supports our assumption that is noted above (e.g., that the metronidazole resistance is highly dependent on reducing cofactor(s)). Moreover, 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. 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. We

also argue that the C_4-dicarboxylic acid uptake rates probably do not affect these processes because one transport protein, the anaerobic C_4-dicarboxylic carrier protein, is responsible for their uptake with similar efficiencies in *Escherichia coli* [33]. The ortholog of this carrier protein is present in the genomes of *B. fragilis* strains (our unpublished analysis). The observed increase in *ldh* gene expression is consistent with previous findings, showing the importance of reducing cofactors in metronidazole resistance in anaerobic bacteria [26]. 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. It is possible that withdrawing hydrogen/reducing cofactors from metronidazole activation may be involved in this process. 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. Earlier modeling studies have focused only on laboratory strains of *B. fragilis*. For example, based on the roles of a limited set of proteins analyzed by two-dimensional protein electrophoresis and northern blotting, Diniz et al. proposed that *ldh* and *por* participate in metronidazole activation at certain low levels [26,34]. However, their model was not confirmed by Paunkov et al. [35]. Also, de Freitas et al. analyzed the transcriptome-wide effect of metronidazole on a large number of proteins, and they confirmed that, along with some other proteins, the concentration of activating ferredoxin is important in alleviating metronidazole stress [36]. Based on the results of proteomic studies, Paunkov et al. developed models of how *nim* and other proteins act in *nim*-dependent and -independent metronidazole-resistant *B. fragilis* strains [11].

2.4. Proposal for the Interactions of Redox and Other Proteins in Metronidazole Resistance

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 highlight the importance of reducing cofactors that are needed for both metronidazole activation and inactivation. The activated metronidazole radical acts by reducing *nim* and redox cofactor proteins and thiol compounds of the proteome [37]. Thus, the metronidazole resistance mechanism of *B. fragilis* is complex and nonlinear. This complexity can explain why metronidazole MICs and *nim* gene expression do not always correlate, especially long after the isolation of strains from clinical specimens. Thus, the process of developing resistance to metronidazole is also complex (Figure 1).

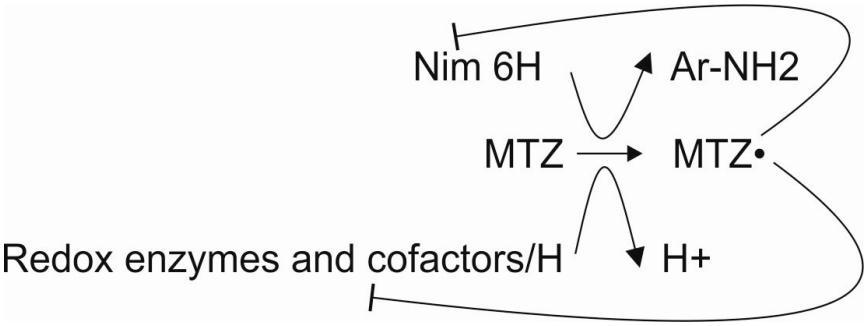

Figure 1. Interactions of the participants in metronidazole resistance. Lines with the ⊣ symbol mean inhibition.

Earlier work suggested that ferredoxin is responsible for reducing metronidazole [26]; however, we were unable to find a role for PFOR (negative association), as we observed increased PFOR and PFOR activities in laboratory metronidazole-resistant *B. fragilis* strains [35]. Thus, more work is needed to determine significant associations

between gene expression and metronidazole MICs in "field" strains *nim*-negative and -positive *B. fragilis*. In particular, more strains need to be analyzed to prove the roles of those genes. In addition, the roles of the genes that had positive or negative correlations with metronidazole resistance should be confirmed by deletion–complementation analysis. In particular, *frdC* is a good candidate for these experiments because it also contains heme. Identifying with more certainty which redox cofactor activates metronidazole also remains a future task. The starting point for this study was a proteomic analysis of metronidazole resistant laboratory strains [11], but only some of them proved to be effective in metronidazole resistance on a population level; therefore, we believe that the genes that had a role in our study (*ldh*, *frdC*, *mdh*, *pgk*, *gat* and *relA*) are significant/valid contributors in this important kind of antibiotic resistance mechanism.

3. Materials and Methods

3.1. Bacterial Strains and Cultivation

Sixteen *B. fragilis* test strains (Tables 1 and S1) with known genetic backgrounds were stored in 20% glycerol stocks at −80 °C and cultivated on supplemented Columbia blood agar medium (SCA, Columbia base, supplemented with 2.5% defibrinated sheep blood, 0.5% laked sheep blood, 0.3 mg/mL L-cysteine, 1 μg/mL vitamin K_1) or in supplemented brain–heart infusion broth (BHIS, brain–heart infusion broth supplemented with 2.5% yeast extract, 10 μg/mL hemin and 1 μg/mL vitamin K_1) under anaerobic conditions (85% N_2, 10% H_2, 5% CO_2, Concept 400 anaerobic cabinet (Ruskinn, Bridgend, UK)) at 37 °C. The strains include both *nim*-positive and -negative *B. fragilis* strains whose *cfiA* gene statuses are known (Table 1). Parallelly with metronidazole MIC determinations, we used the same SCA plates for inoculations of 5 mL of BHIS for RNA isolation and cell suspensions to determine MICs. To test the effect of C_4-dicarboxylic acids on metronidazole resistance, we used a semi-defined M9-based agar medium (48 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.5 mM NaCl, 1.6 mM NH_4Cl, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1% casein peptone Type I (Neogene), 0.625% yeast extract, 10 mM glucose or 15 mM C_4-dicarboxylic acid (oxaloacetate/D(-) malate/fumarate/succinate), 10 μg/mL hemin, 1 μg/mL vitamin K_1) to perform MIC measurements.

3.2. Metronidazole MIC Measurements

Metronidazole MICs were measured using a gradient method (Etest, bioMérieux, Hungary Ltd., Budapest, Hungary). First, McFarland density suspensions were made in a phosphate-buffered saline solution (137 mM NaCl, 2.7 mM KCl, 100 mM Na_2HPO_4 and 1.8 mM KH_2PO_4, pH 7.4), with which we inoculated the surface of SCA plates by cotton swabs, and applied the Etest strips, and after anaerobic cultivation at 37 °C for 48 h, we read the plates.

3.3. RT-qPCR

We extracted total RNA from 5 mL BHIS cultures for RT-qPCR experiments using the HighPure RNA Isolation Kit (Roche). The quantity and quality of RNA were assessed using the Qubit 4 fluorometer and the Qubit RNA BR and RNA Integrity kits (Thermo Fisher Scientific, Waltham, MA, USA). Of 32 candidate genes identified in previous proteomic studies [11], we chose 18 and designed primer pairs using the Primer3 Plus software (www.primer3plus.com). During primer design, we took into account the possibility that the *cfiA*-positive and -negative strains may differ in their respective sequences. Therefore, the consensus nucleotide sequences of the selected genes were obtained from the complete genomic sequences of the *cfiA*-negative and -positive strains *B. fragilis* 638R (GenBank Acc. No. NC_016776) and *B. fragilis* 3130 (GenBank Acc. No. LJVI01), respectively, to design primer pairs. We used the *gap*, *rrn*, and *rpoD* genes as endogenous controls. The nucleotide sequences of the primers used are shown in Table S1. 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 (QuantStudio 3, Thermo Fisher Scientific) 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.

3.4. 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, Erkrath, Germany).

4. Conclusions

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 independently of the presence of *nim* genes. This finding emphasizes 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. Thus, the list of examined genes should also be increased.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antibiotics13030207/s1 Table S1. RT-qPCR target genes and primer sequences. Table S2. Cross-correlation values of gene expressions metronidazole resistance for eight *nim*-positive *B. fragilis* strains. Table S3. Cross-correlation values of gene expressions metronidazole resistance for seven *nim*-negative *B. fragilis* strains. Figure S1. The tricarboxylic acid pathways of *B. fragilis*. Figure S2. Examples of the Etest results on modified M9 medium. Table S4. Effects of C_4 dicarboxylic acid supplementation on metronidazole MICs of selected *nim*-positive and negative *B. fragilis* strains.

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