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Molecular analysis of the carbapenem and metronidazole resistance mechanisms of *Bacteroides* strains reported in a Europe-wide antibiotic resistance survey

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ABSTRACT

Here we examine the carbapenem and metronidazole resistance mechanisms of 640 *Bacteroides* strains reported in the 2008–2009 European antibiotic susceptibility survey. Of the 22 strains with elevated imipenem minimum inhibitory concentrations (≥4 μg/mL), 10 were *cfiA*-positive and out of these 5 carried activating insertion sequence (IS) elements in the upstream regions of the *cfiA* genes. However, resistant strains with *cfiA* genes but with no activating IS elements were found ($n = 2$) as well as a resistant strain with no *cfiA* gene. In the former the resistance phenotypes by Etest were heterogeneous, whilst in the latter no carbapenemase production was seen; both mechanisms have been rarely observed, examined and characterised. Interestingly, few ($n = 3$) *nim*-positive strains were found, including one metronidazole-resistant strain harbouring *nimE* activated by IS*Bf6*, and two susceptible strains harbouring chromosomally located *nim* genes.

1. Introduction

Bacteroides spp. represent one of the most significant groups of anaerobic bacteria. They are important constituents of the intestinal microbiota, from where they can cause severe anaerobic infections ranging from those of the soft tissue and upper respiratory tract to sepsis and various abscesses [1]. *Bacteroides* spp. can harbour the highest number of antibiotic resistance mechanisms and have the highest antibiotic resistance prevalences among all pathogenic anaerobes [2]. Because of their special and usually long culture requirements, temporary records of antibiotic resistance rates is considered a good and recommended practice worldwide. Such monitoring was performed mostly in the USA [3] and Europe [4], the latter under the organisation of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) Study Group for Antimicrobial Resistance in Anaerobic Bacteria (ESGARAB), whose name was changed to the ESCMID Study Group on Anaerobic Infections to cover a broader interest. The general trend is almost 100% resistance to penicillins, cephalosporins and tetracycline, a rising moderate resistance prevalence to cefoxitin, clindamycin and moxifloxacin, and very low prevalences for carbapenems, β-lactam/β-lactamase combinations, metronidazole and tigecycline [3,4]. Following antibiotic resistance monitoring for *Bacteroides* in 2000, molecular analyses were carried out to determine the metronidazole and carbapenem resistance mechanisms [5,6]. These investigations demonstrated the roles of the *nim* and *cfiA* genes and their activating insertion sequence (IS) elements in metronidazole and carbapenem resistance mechanisms, respectively.

Carbapenem-resistant *Bacteroides* isolates usually belong to the *Bacteroides fragilis* group, with the *cfiA* resistance gene being chromosomal and the majority of *cfiA*-positive strains being susceptible phenotypically because of the lack of upregulating IS elements [1]. The best-characterised metronidazole resistance mechanism among *Bacteroides* strains is due to the *nim* genes (*nimA–F*) that may occur in all *Bacteroides* species, and they are either located on well-characterised plasmids or on the chromosome. The majority of *nim*-positive *Bacteroides* isolates studied harbour a *nim* gene and a corresponding IS element pair [6]. It is of interest that the *cfiA*-positive *B. fragilis* isolates form a subgroup within this species. The *cfiA*-negative and *cfiA*-positive strains are therefore often classified as Division I and II, respectively, and can be distinguished by differences in DNA–DNA homology rates and by molecular typing methods such as randomly amplified polymorphic DNA polymerase chain reaction (RAPD-PCR), ribotyping, multilocus enzyme electrophoresis, sequence typing and

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Table 1
Primers and PCR conditions originally designed and applied in this study.

PCR	Primers	Sequence 5′→3′	PCR conditions
cfiA	cfiA-RT1 cfiA-RT2	AATCGAAGGATGGGGTATGG CGGTCGGTGAATCGGTGAAT	95 °C for 5 min; 35 cycles of 95 °C for 15 s, 59 °C for 1 min, 72 °C for 30 s; melting 72–95 °C
nim[a]	nim3 nim5	ATGTTCAGAGAAATGCGGCGTAAGCG GCTTCCTTGCCTGTCATGTGCTC	95 °C for 10 min; 35 cycles of 95 °C for 30 s, 62 °C for 1 min, 72 °C for 1 min; melting 72–95 °C

[a] The method of Trinh and Reysset was adapted to real-time PCR [13].

matrix-assisted laser-desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry [7–12]. The Ambler class A cephalosporinase gene, *cepA*, and the enterotoxin *bft* genes were reported to occur exclusively in Division I strains [10].

This study investigated the prevalences of the *cfiA* and *nim* genes, the imipenem and metronidazole resistance mechanisms in the majority of *Bacteroides* strains reported in the 2008 European *Bacteroides* antibiotic resistance survey.

2. Materials and methods

2.1. Bacterial strains and cultivation

A total of 640 isolates belonging to the *Bacteroides* and *Parabacteroides* genera (486 *B. fragilis*, 54 *Bacteroides thetaiotaomicron*, 36 *Bacteroides ovatus*, 33 *Bacteroides vulgatus*, 8 *Bacteroides uniformis*, 7 *Parabacteroides distasonis*, 4 *Parabacteroides merdae*, 3 *Bacteroides eggerthii*, 3 *Bacteroides massiliensis*, 3 *Bacteroides nordii*, 2 *Bacteroides caccae* and 1 *Bacteroides stercoris*) were analysed from the collection sent to the central laboratory (Institute of Clinical Microbiology, University of Szeged, Szeged, Hungary) for the 2008–2009 European *Bacteroides* antibiotic susceptibility survey (participating countries: Belgium, Croatia, Czech Republic, Finland, France, Germany, Greece, Hungary, Italy, Spain, Sweden, The Netherlands and Turkey). Isolate identification was carried out by routine clinical methods. Strains were stored at −70 °C in CryoBank vials (Mast Diagnostica, Rheinfeld, Germany) and were cultivated at 37 °C anaerobically on Columbia agar supplemented with 5% (v/v) sheep blood, 5 g/L haemin and 1 g/L vitamin K_1, or in BHIS broth [brain–heart infusion broth supplemented with 0.5% (w/v) yeast extract, 5 g/L haemin and 1 g/L vitamin K_1] in an anaerobic cabinet (Concept 400; Ruskinn Technology Ltd., Bridgend, UK) under a gas composition of 85% N_2, 10% H_2 and 5% CO_2 for 48 h. Antibiotic resistance results were obtained from the susceptibility measurements done previously by the agar dilution method [4] or by Etest (bioMérieux, Marcy-l'Étoile, France) as recommended by the supplier. The following control strains were used: *B. fragilis* TAL3636 (*cfiA*); *B. fragilis* 638R (pIP417) (*nimA*); *B. fragilis* BF-8 (*nimB*); *B. fragilis* 638R (pIP419) (*nimC*); *B. fragilis* 638R (pIP421) (*nimD*); and *B. fragilis* 388 (*nimE*).

2.2. Real-time PCR detection of the cfiA and nim genes

Bacterial template DNA samples for the real-time PCR analysis were prepared by incubating 100 μL of 0.5 McFarland turbidity suspensions at 100 °C for 10 min, which were stored at −30 °C until use. Real-time PCR experiments were carried out in an MXPro3000 instrument (Stratagene, Santa Clara, CA) with the following reaction setup: 1× MasterMix [iQ™ (Bio-Rad Hungary, Budapest, Hungary) with 1× EvaGreen® (Biotium Inc., Hayward, CA) for *nim*; or Brilliant III (Stratagene/Agilent, Santa Clara, CA) for *cfiA* and *bft*], 0.7 μM of each primer and 2 μL of template DNA preparation in 10 μL final volumes in 96-well PCR reaction plates. The nucleotide sequences of the newly used primers and the cycling conditions chosen during this study are shown in Table 1. Positive reactions were identified by the starting amplification cycle, melting curves showing the correct melting temperatures, and in rare cases where it was required to compare the size of the products with those of the positive controls in 1.2% agarose gel electrophoresis.

Table 2
Analysis of the imipenem resistance mechanism of strains with elevated imipenem minimum inhibitory concentrations (MICs) (≥4 μg/mL).

Strain	Imipenem MIC (μg/mL)	*cfiA*	Upstream region	Mechanism
Bacteroides fragilis SW42	4	–	–	Other[a]
B. fragilis SW46	4	–	–	Other
B. fragilis SW83	4	–	–	Other
B. fragilis TR38	4	–	–	Other
B. fragilis HU25	4	–	–	Other
B. fragilis FI63	4	–	–	Other
Bacteroides eggerthii GR67	4	–	–	Other
Bacteroides thetaiotaomicron BEM28	4	–	–	Other
Parabacteroides merdae GR70	4	–	–	Other
B. fragilis DE14	4	+	280 bp[b]	Silent with increased MIC
B. fragilis HU51	4	+	280 bp[b]	Silent with increased MIC
B. fragilis IT15	4	+	IS*4351*	IS-activated
Bacteroides stercoris HU59	8	–	–	Other
B. thetaiotaomicron BEA22	8	–	–	Other
B. fragilis HU92	8	+	280 bp[b]	Silent with increased MIC
B. fragilis TR27	16	+	IS*1187*	IS-activated
B. fragilis TR31	16	+	IS*1187*	IS-activated
B. fragilis HU61	32	+	280 bp[b]	Heteroresistant
B. fragilis NLH3	>32	+	IS*Bf11*	IS-activated
B. fragilis FR41	>32	+	280 bp[b]	Heteroresistant
B. fragilis FI87	>32	+	IS*614B*	IS-activated
B. fragilis FI37	>32	–	–	Other

[a] The effects are not caused by *cfiA*.
[b] The 280-bp PCR fragment displays no insertion upstream of *cfiA*.