Available online at www.sciencedirect.com
SCIENCE DIRECT®
Research in Microbiology 155 (2004) 843–846
Research in Microbiology
www.elsevier.com/locate/resmic

Plasmid-related β-lactamase production in *Bacteroides fragilis* strains

Viviane Nakano, Gabriel Padilla, Marilis do Valle Marques, Mario Julio Avila-Campos *

Department of Microbiology, Institute of Biomedical Sciences, University of São Paulo, Av. Prof. Lineu Prestes 1374, São Paulo, 05508-900 SP, Brazil

Received 16 January 2004; accepted 22 June 2004

Available online 10 August 2004

Abstract

Twenty *Bacteroides fragilis* group species isolated from children with and without diarrhea were analyzed. Antibiotic susceptibility was performed using an agar dilution method; *β*-lactamase production was determined using a nitrocefin method, and plasmids were extracted using a commercial Miniprep System. MIC values ranged from 16 to 256 μg/ml for penicillin, 4–128 μg/ml for amoxicillin/clavulanic acid, ⩽0.25–256 μg/ml for clindamycin, and 16–256 μg/ml for penicillin. β-Lactamase was detected in all isolates. Only five isolates harbored plasmids varying from 7.8 to 1.8 kb. Loss of 6.4- and 3.8-kb plasmids in *B. fragilis* C68c was related to antibiotic resistance. Low molecular weight plasmids of 2.8–1.8 kb were stable. PCR amplification of *cfiA* and *cepA* genes was observed using total DNA, and the *cfiA* gene was also amplified from the 6.4-kb plasmid.

Keywords: *Bacteroides fragilis* group; Antimicrobial susceptibility; β-Lactamase; Plasmid

1. Introduction

Studies have reported that plasmids in *Bacteroides* spp. are grouped into three classes: class I, 2.8-kb plasmid, class II, 4.2-, 5.0- and 7.9-kb, and class III, 5.5-kb [2]. In addition, Sóki et al. [9] described three other different classes: class I, 1.8 MDa, class II, 2.6 MDa, and class III, 3.7 MDa, with the latter plasmid most frequently observed in this microbial group, and present in 66% of plasmid-positive isolates. *Bacteroides vulgatus* isolated from intra-abdominal infection and presenting a 2.6-MDa plasmid has also been observed [5]. It has been demonstrated that the wide distribution and high carriage rate of these plasmids could be caused by mobilization, and that members of these plasmid classes are found in geographically distant regions [10].

The goals of this study were to determine the plasmid profile and its association with antimicrobial resistance in species of the *Bacteroides fragilis* group.

2. Materials and methods

Ten bacteria of the *B. fragilis* group isolated from children with diarrhea (*B. fragilis* P1-2, *B. vulgatus* P15f, *B. distasonis* P36b, *B. fragilis* P36d, *B. ovatus* P23e, *B. vulgatus* P23j, *B. fragilis* P35a, *B. distasonis* P40a, *B. uniformis* P65h, and *B. fragilis* P70i) and ten from children without diarrhea (*B. distasonis* C19c, *B. vulgatus* C43a, *B. vulgatus* C56b, *B. distasonis* C58g, *B. distasonis* C59e, *B. fragilis* C68c, *B. fragilis* C68e, *B. fragilis* C68h, *B. fragilis* C71h, and *B. fragilis* C74d) were analyzed. Bacteria were isolated and identified at the Anaerobe Laboratory, Department of Microbiology, University of São Paulo. All strains were stored in 10% skim milk at −80 °C.

Antimicrobial susceptibility was performed using an agar dilution method in Wilkins–Chalgren agar [11]. The antibiotics were the following: ampicillin and clindamycin (Luper Ind. Farm. Ltd., SP, Brazil); amoxicillin/clavulanic acid (Smithkline Beecham Ltd., SP, Brazil); penicillin G (Prodoti Lab. Farm. Ltd., SP, Brazil). All tests were performed in duplicate. The breakpoints used for antibiotics were: ampicillin, 4 μg/ml; clindamycin, 8 μg/ml; amoxi-

* Corresponding author.
E-mail address: mariojac@usp.br (M.J. Avila-Campos).

0923-2508/$ – see front matter
doi:10.1016/j.resmic.2004.06.011

cillin/clavulanic acid, 8 μg/ml; and penicillin G, 8 μg/ml [4]. Reference strain *B. fragilis* ATCC 25285 was used as control.

The antibiotic concentrations ranged from 0.25 to 512 μg/ml. Media without antimicrobials were used as control. Bacterial inocula were standardized to $c = 1.5 \times 10^8$ CFU/ml. Media were inoculated using a Steer's replicator delivering a final inoculum of $c = 1.5 \times 10^5$ CFU/spot. Plates were incubated in anaerobiosis (90% N_2/10% CO_2) at 37 °C for 48 h. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of each antimicrobial agent able to inhibit macroscopic bacterial growth.

Strains resistant to penicillin G were tested to verify β-lactamase production using a nitrocefin method (Oxoid Ltd., São Paulo, SP, Brazil). β-Lactamase-positive *B. fragilis* ATCC 43858 was used as control.

Plasmid DNA from all tested strains was extracted by using a Concert™ Rapid Plasmid Miniprep System (GibcoBRL). DNA was analyzed by electrophoresis on 0.8% agarose gel. A plasmid-positive *E. coli* J53 pACYC 184 strain was used as control. Plasmid-positive *B. fragilis* species were cured by exposition to different ethidium bromide (EtBr) concentrations (from 0.25 to 64 μg/ml) in BHI broth [7]. Plasmid DNA from 10 colonies randomly selected was extracted and analyzed by agarose gel electrophoresis in order to verify the plasmid loss.

Moreover, the presence of *cfiA* and *cepA* genes related to β-lactamase production was evaluated by PCR from purified total DNA and plasmids 6.4- and 3.8-kb isolated from strain C68c. Primers for *cepA*, 5′-TTT CTG CTA TGT CCT GCC C-3′ and 5′-ATC TTT CAC GAA GAC GGC-3′, to provide a specific 780-bp band, and primers for *cfiA*, 5′-ATG GTA CCT TCC AAC GGG-3′ and 5′-CAC GAT ATT GTC GGT CGC-3′; to provide a specific 353-bp band, were designed from published sequences [3]. The reaction mixtures were prepared in a total volume of 25 μl containing 15 ng of DNA, 1.0 U of *Taq* polymerase (Invitrogen Ltd., SP, Brazil), 200 μM deoxynucleoside triphosphate, 1X PCR buffer, 1.5 mM $MgCl_2$, and 0.4 μM of each primer. The PCR conditions were as follows: denaturing at 94 °C for 30 s; annealing at 55 °C for 1 min (*cepA*) or 56 °C for 1 min (*cfiA*) and elongation at 72 °C for 5 min. The cycles were repeated 35 times for *cfiA* and *cepA*. Water was used as a negative control. All PCR amplification products were detected on 1.5% agarose gel stained with ethidium bromide (0.5 μg/ml).

3. Results and discussion

From the 20 analyzed species of the *B. fragilis* group, 15 strains did not harbor plasmids and showed MIC values ranging from 16 to 256 μg/ml for penicillin; 4–128 μg/ml for amoxicillin/clavulanic acid; ⩽0.25–256 μg/ml for clindamycin; 16–256 μg/ml for penicillin (Table 1). These results showing resistance to antibiotics and the absence of plasmids suggest that these resistances are chromosomally mediated, as proposed by Pestana et al. [6].

Table 1
Antimicrobial susceptibility, β-lactamase production and plasmid profile in *B. fragilis* group species isolated from 10 children with (P) and 10 without (C) diarrhea

Species	Amp 4[a]	A/Ac 8[a]	Cli 8[a]	Pe 8[a]	β-Lactamase production	Plasmid band
					Nitrocefin method	
B. fragilis P1-2	256	8	256	256	+	−
B. vulgatus P15f	256	4	256	256	+	−
B. distasonis P36b	256	16	256	256	+	−
B. fragilis P36d	256	16	⩽0.25	256	+	−
B. ovatus P23e	256	32	256	256	+	−
B. vulgatus P23j	16	4	256	8	+	+
B. fragilis P35a	16	64	⩽0.25	256	+	+
B. distasonis P40a	256	16	⩽0.25	256	+	−
B. uniformis P65h	64	2	⩽0.25	16	+	−
B. fragilis P70i	16	2	1	16	+	−
B. distasonis C19c	64	16	8	256	+	−
B. vulgatus C43a	256	32	256	256	+	−
B. vulgatus C56b	256	64	2	256	+	−
B. distasonis C58g	256	128	4	256	+	−
B. distasonis C59e	256	32	4	256	+	−
B. fragilis C68c	256	64	256	256	+	+
B. fragilis C68e	16	1	256	16	+	+
B. fragilis C68h	16	4	1	16	+	+
B. fragilis C71h	256	16	⩽0.25	256	+	−
B. fragilis C74d	16	4	⩽0.25	16	+	−

P: bacteria isolated from diarrhea; C: bacteria isolated from normal stool; Amp, ampicillin; A/Ac, amoxicillin/clavulanic acid; Cli, clindamycin; Pe, penicillin; β-lactamase production and plasmid band: +, presence; −, absence.
[a] Breakpoint used for antibiotics, expressed in μg/ml.

On the other hand, from the 20 tested *B. fragilis* group species, all were β-lactamase-positive using a nitrocefin method. Moreover, five of the 20 isolates harbored plasmids (Table 1) and from these isolates two different plasmid profiles were observed. Low molecular weight plasmids were found in two species isolated from children with diarrhea, *B. vulgatus* P23j (6.4, 3.8, 2.8, and 1.8 kb) and *B. fragilis* P35a (7.8, 5.0, and 2.7 kb); and the other three isolates from a child without diarrhea, *B. fragilis* C68c, *B. fragilis* C68e and *B. fragilis* C68h harboring 6.4-, 3.8-, 2.8-, and 1.8-kb plasmids. *B. vulgatus* P23j isolated from a diarrhea stool showed a similar plasmid profile as *B. fragilis* C68c, *B. fragilis* C68e and *B. fragilis* C68h, isolated from normal stool. Interestingly, the latter three strains showed different MIC values, ranging from 1 to 256 μg/ml, in terms of resistance to antibiotics (Table 1). These differences in resistance to antibiotics suggest that these species may not be considered as derivate clones from a wild strain.

The *B. fragilis* C68c isolate lost two of the plasmids (6.4 and 3.8 kb) after treatment with EtBr, and its antibiotic resistance was decreased: for ampicillin, from 256 to 8 μg/ml; for amoxicillin/clavulanic acid, from 64 to 0.5 μg/ml; for clindamycin, from 256 to $\leqslant$0.25 μg/ml; and for penicillin G, from 256 to 8 μg/ml. On the other hand, when derived clones from the *B. fragilis* C68c strain were again evaluated, the biochemical and physiological characteristics were not altered (data not shown). It is important to note that 2.8- and 1.8-kb low molecular weight plasmids, considered as very stable elements, were observed after EtBr treatment.

The presence of genes conferring resistance to β-lactam antibiotics was assessed by PCR amplification using specific primers for *cfiA* and *cepA* genes. The *cfiA* gene was detected on the total DNA and 6.4-kb plasmid, but not on the other three plasmids, and the *cepA* gene was only amplified from total DNA (Fig. 1). These results suggest a strong correlation between the antibiotic resistance pattern and the presence of the 6.4-kb plasmid. Moreover, these results indicate that a *cepA* homolog may be present in the chromosome or in one of the small plasmids, and that there is a *cfiA* gene in the 6.4-kb plasmid. The loss of this gene is probably the cause of reduced resistance to β-lactams when the plasmids were cured, although there was residual β-lactamase production, as observed by the nitrocefin method (data not shown). Pestana et al. [6] observed that, from five tested *B. fragilis* strains, the presence of plasmids was observed in only one strain, responsible for penicillin and clindamycin resistance.

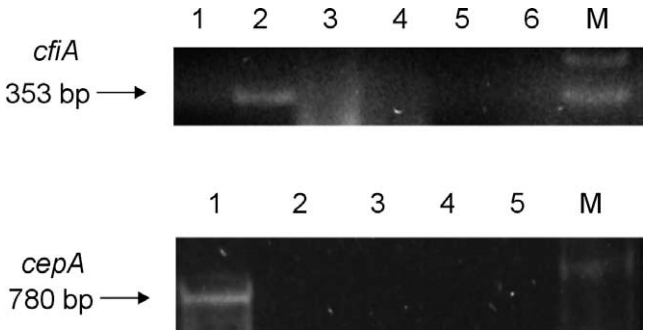

Fig. 1. Agarose gel electrophoresis of *cfiA* and *cepA* genes amplified by PCR, from *B. fragilis* C68c and plasmids. *cfiA* gene: lane 1, total DNA; lane 2, 6.4-kb plasmid; lane 3, 3.8-kb plasmid; lane 4, 2.8-kb plasmid; lane 5, 1.8-kb plasmid; lane 6, negative control; lane 7, 1-kb plus DNA ladder. *cepA* gene: lane 1, total DNA; lane 2, 6.4-kb plasmid; lane 3, 3.8-kb plasmid; lane 4, 2.8-kb plasmid; lane 5, 1.8-kb plasmid; lane 6, 1-kb plus DNA ladder.

The presence of low molecular weight plasmids is frequently observed in *Bacteroides* spp. Surveys have reported that 20–50% of clinical and fecal isolates possess plasmids ranging in size from 2.7 to >80 kb [1]. On the other hand, both clinical and intestinal *Bacteroides* isolates have also been observed, displaying plasmids ranging from 2.0 to 5.0 MDa [9,12].

In this study, plasmids with similar low molecular weight were observed and their presence was correlated with antibiotic resistance. However, the occurrence of small cryptic plasmids with the same molecular size in different *Bacteroides* strains from different parts of the world may be due to their horizontal mobilization by several different conjugative elements, which may promote their spread through the indigenous microbiota [8].

The wide distribution of plasmids may signal a potential problem if they acquire antibiotic resistance or pathogenicity determinants, which may render treatment of bacteroides infections more difficult.

Acknowledgements

The authors thank Ms. Zulmira Alves de Souza for her technical support. This study was supported by a grant from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) Proc. no. 02/02680-4.

References

[1] H.A. Beul, C.H. von Eichel-Streiber, M. Schreiner, F.P. Schwindling, D. Weinblum, E.J. Zöllner, M. Dierich, Characterization of cryptic plasmids in clinical isolates of *Bacteroides fragilis*, J. Med. Microbiol. 20 (1985) 39–48.

[2] D.R. Callihan, F.E. Young, V.L. Clark, Identification of three homology classes of small, cryptic plasmids in intestinal *Bacteroides* species, Plasmid 9 (1983) 17–30.

[3] M. Gutacker, C. Valsangiacomo, J.C. Piffaretti, Identification of two genetic groups in *Bacteroides fragilis* by multilocus enzyme electrophoresis: Distribution of antibiotic resistance (*cfiA*, *cepA*) and enterotoxin (*bft*) encoding genes, Microbiology 146 (2000) 1241–1254.

[4] National Committee for Clinical Laboratory Standards, Methods for Dilution Antimicrobial Susceptibility Testing of Anaerobic Bacteria—Fourth Edition: Approved Standard M11-A4, NCCLS, Villanova, PA, USA, 1997.

[5] E. Nagy, É. Dányi, J. Földes, Plasmid analysis of clinical isolates of *Bacteroides fragilis* group strains, Acta Microbiol. Hung. 37 (1990) 367–373.

[6] A.C.N.R. Pestana, C.G. Diniz, L.M. Farias, M.A.R. Carvalho, Plasmid-related resistance to clindamycin and penicillin G in a *Bacteroides vulgatus* strain, Anaerobe 5 (1999) 447–449.

[7] V. Salisbury, R.W. Hedges, N. Datta, Two modes of curing transmissible bacterial plasmid, J. Gen. Microbiol. 70 (1972) 443–452.
[8] J.C. Smith, A.C. Parker, A gene product related to Tral is required for the mobilization of *Bacteroides* mobilizable transposons and plasmids, Mol. Microbiol. 20 (1996) 741–750.
[9] J. Sóki, I. Szöke, E. Nagy, Characterization of 5.5-kb cryptic plasmid present in different isolates of *Bacteroides* spp. originating from Hungary, J. Med. Microbiol. 48 (1999) 25–31.
[10] J. Sóki, I. Kerekes, I. Marczinovits, E. Nagy, Molecular relatedness of the prevalence cryptic plasmids of *Bacteroides* species isolates in Hungary, Anaerobe 6 (2000) 179–185.
[11] V.L. Sutter, A.L. Barry, T.D. Wilkins, R.L. Zabransky, Collaborative evaluation of a proposed dilution method of susceptibility testing of anaerobic bacteria, Antimicrob. Agents Chemother. 16 (1979) 495–502.
[12] B.L. Wallace, J.E. Bradley, M. Rogolsky, Plasmid analyses in clinical isolates of *Bacteroides fragilis* and other *Bacteroides* species, J. Clin. Microbiol. 14 (1981) 383–388.