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Detection by PCR of the *nim* Genes Encoding 5-Nitroimidazole Resistance in *Bacteroides* spp.

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A PCR method was developed for detection of the *nim* genes encoding 5-nitroimidazole resistance in *Bacteroides* spp. Two PCR primers specific for *nim* genes were designed. They allowed amplification of a 458-bp fragment from all characterized plasmid- and chromosome-borne metronidazole resistance genes. The specificity of the method was tested with DNA from metronidazole-sensitive *Bacteroides* spp. strains and from other strains of unrelated species. Each DNA preparation was analyzed with and without an internal positive control to verify that the absence of PCR amplification product was not due to inhibition of the *Taq* polymerase inhibitors. By this technique, two newly discovered metronidazole-resistant clinical strains of *Bacteroides fragilis* were shown to harbor resistance genes undetectable by Southern blotting. In spite of the sequence divergence of the *nim* genes, the PCR method is thus suitable for epidemiological investigations. The amplification method also revealed that *nim*-related resistance genes were not present in either *Streptomyces* strain S6670, a natural producer of 2-nitroimidazole, or in *Enterococcus faecalis* strains, which have been suggested to possess metronidazole-inactivating enzyme.

Bacteroides species are major members of the normal colonic human microflora. Species most frequently isolated from the flora are *Bacteroides vulgatus*, *Bacteroides thetaiotaomicron*, *Bacteroides distasonis*, and, less frequently, *Bacteroides eggerthii* and *Bacteroides fragilis* (10). These gram-negative anaerobic bacteria are often involved in various infectious diseases (6), and the choice of suitable antibiotics for therapy is limited because *Bacteroides* spp. are frequently resistant to clinically relevant antimicrobial agents, including tetracycline, clindamycin, and β-lactam antibiotics (7). In contrast, the 5-nitroimidazole (5-NI) molecules are very potent anaerobicidal agents commonly used to treat or prevent *Bacteroides* infections. Metronidazole (MTR), ornidazole, and tinidazole are the compounds most often prescribed.

In spite of the wide use of these drugs during the last 30 years, few 5-NI-resistant strains have been isolated. A *B. fragilis* strain (NCTC11295) with a high level of resistance to MTR (MIC, 64 μg ml^{-1}) was isolated in 1978 from a patient with Crohn's disease following a long-term MTR regimen (11). Since then, other resistant strains have been found in clinical specimens (21). They are very rare, and the resistance is nontransferable, such that the potential for epidemiological dissemination is low. On the other hand, clinical *Bacteroides* spp. strains with reduced sensitivity to MTR (MICs of 5-NI lower than or near the standard breakpoint for MTR, i.e., 16 μg ml^{-1}) have also been isolated in France since 1986 (4). These *Bacteroides* strains present a new 5-Ni^r resistance phenotype characterized by higher levels of resistance to tinidazole or ornidazole than to MTR (4, 19), contrasting with the situation for strain NCTC11295. Two epidemiological studies conducted in French hospitals by Breuil et al. (1) and Patey et al. (17) show that the incidence of such resistant strains among *Bacteroides* isolates is increasing and is currently 3 to 5%. A subsequent molecular study (20) demonstrated that the moderate resistance phenotype of these clinical strains was in all cases associated with the presence of the 5-NI resistance gene (*nim*).

Four related *nim* genes have now been identified in *Bacteroides* species (8, 26). Three of them are located on low-copy-number mobilizable plasmids: pIP417 (7.7 kb, *nimA*) from *B. vulgatus* BV-17, pIP419 (10 kb, *nimC*) from *B. thetaiotaomicron* BT-13, and pIP421 (7.3 kb, *nimD*) from *B. fragilis* BF-F239. The fourth gene (*nimB*) was mapped to the chromosome of *B. fragilis* BF-8. In most other isolates (73%), the *nim* gene was also located on the chromosome and present in one or two copies (20). Most of both the plasmid-borne and chromosomally determined resistance markers are transferable by a conjugative process (2, 24). For example, the plasmid pIP417 has been transferred in vitro by conjugation into various sensitive strains and species of both *Bacteroides* and *Prevotella* (25). The transfer is presumed to have also occurred in vivo, as plasmids similar or identical to pIP417, first found in a *B. vulgatus* strain, have been detected in different clinical *B. fragilis* isolates (20). Thus, the mobilization of the *nim* genes could account for the spread of the 5-NI resistance among at least some gram-negative anaerobic bacteria. Monitoring for *nim* genes is therefore recommended for clinically important anaerobic pathogens.

Detection of 5-NI-resistant strains by conventional susceptibility testing methods requires conditions of strict anaerobiosis because of the usual inactivity of the drug in the presence of even low levels of oxygen (5, 13). This is particularly important for the moderately resistant strains, because of their low MICs. Even if the *nim* genes protect the bacteria more efficiently against tinidazole or ornidazole than against MTR (4, 24), MTR is still the most common 5-NI compound used in susceptibility testing and MIC determinations in clinical laboratories. The presence of the *nim* genes can be also detected by Southern blotting of DNA with specific [^{32}P]dATP-labeled probe, although the method presents some limitations. One is the necessity of using several probes as some *nim* genes are different enough not to cross-hybridize in high-stringency conditions (20). Southern blotting is also time-consuming, requires purified DNA and handling of radioactive products, and is thus difficult for nonspecialized laboratories. One consequence of these technical considerations is that the prevalence of the

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TABLE 1. *Bacteroides* spp. strains resistant to 5-NIs

Sp.	Strain	MIC of MTR[a]	5-Ni[r] determinant	Reference
B. fragilis	BF-8	34	Chromosome	20
B. vulgatus	BV-17	4	pIP417	20
B. thetaiotaomicron	BT-13	4	pIP419	20
B. fragilis	BF-F239	8	pIP421	20
B. fragilis	BF-152/94	32	Chromosome	This study
B. fragilis	BF-377/95	12	pIP417-like	This study
B. fragilis	BF-376/95	32	Unknown	This study

[a] MICs of MTR were determined on solid agar WC medium (Oxoid) by the E-test method and are expressed in micrograms per milliliter.

resistance *nim* genes among clinical isolates may be underestimated. We describe here an alternative technique, based on PCR (22), for screening anaerobic bacteria for *nim* genes.

MATERIALS AND METHODS

Bacterial strains. The *Bacteroides* spp. strains resistant to 5-NI used in this study are listed in Table 1. The other strains used to test the specificity of the PCR method are listed in Table 2.

Media and culture conditions. Anaerobic bacterial strains were cultured in an anaerobic chamber (Celster, Velizy, France) under 5% H_2–5% CO_2–90% N_2. Both Trypticase glucose yeast extract (TGY) medium (18) and Wilkins-Chalgren (WC) medium (Oxoid, Basingstoke, Hampshire, United Kingdom) were used for bacterial growth. The MIC of MTR was determined on solid WC agar medium, by the E-test method according to the supplier's instructions (AB BIODISK, Dalvägen, Sweden). Aerobic bacterial strains were cultured at 37°C in Luria broth (23) or brain heart infusion (Difco Laboratories, Detroit, Mich.). *Streptomyces* strains were cultured in aerobic conditions at 37°C in Pridham's starch medium as described by P. Jeannin: 10 g of starch liter^{-1}; 1 g of K_2HPO_4 liter^{-1}; 1 g of $MgSO_4$ liter^{-1}; 1 g of NaCl liter^{-1}; 1 g of $SO_4(NH_4)_2$ liter^{-1}; 2 g of $CaCO_3$ liter^{-1}.

DNA extraction from bacterial strains. Bacteria were harvested either by centrifugation of 2 ml of an overnight culture or by picking colonies from agar with a 1-μl inoculation loop. The target DNA was extracted as described by K. Wilson (27). The cells were resuspended in 600 μl of TE buffer (1×) (10 mM Tris hydrochloride [pH 7.4], 1 mM EDTA) containing proteinase K (to a final concentration of 100 μg ml^{-1}). The samples were incubated at 37°C for 1 h. To remove cell wall debris, polysaccharides, and other contaminating macromolecules, the NaCl concentration of the DNA solution was adjusted to 0.7 M and hexadecyltrimethylammonium bromide (CTAB), in 0.7 M NaCl, was added to a final concentration of 1%. The reaction mixture was incubated for 10 min at 65°C and then centrifuged at 13,000 rpm for 5 min. For DNA extraction, the supernatant was treated with 1 volume of chloroform-isoamyl alcohol (24:1) and then with 1 volume of phenol-chloroform-isoamyl alcohol (25:24:1). DNA was precipitated from the aqueous phase with 2 volumes of 100% ethanol for 20 min at −80°C. DNA was then pelleted by centrifugation (13,000 rpm for 15 min), washed with 70% ethanol, air dried, and resuspended in 100 μl of distilled water. A rapid DNA extraction procedure was also used. After centrifugation, the cells were resuspended in 100 μl of TE buffer (1×), pH 7.5. The bacterial suspension was incubated for 10 min at 95°C and then centrifuged at 13,000 rpm for 5 min. The supernatant containing the DNA was collected.

Oligonucleotide primers for PCR. Multiple alignment of the nucleotide sequences of the four *nim* genes (Fig. 1) was generated to select the following pair of primers: NIM-3 (5′-ATG TTC AGA GAA ATG CGG CGT AAG CG-3′) and NIM-5 (5′-GCT TCC TTG CCT GTC ATG TGC TC-3′). The corresponding sequences are at positions 1 to 26 and 436 to 458 for NIM-3 and NIM-5, respectively. Both were synthesized by Genset SA, Paris, France. The nucleotide sequences of the *nim* genes are available in the EMBL, GenBank, and DDBJ nucleotide sequence databases, under accession numbers X71444, X71443, X76948, and X76949 for *nimA*, *nimB*, *nimC*, and *nimD* genes, respectively. The pair of primers allows a 458-bp fragment amplification.

DNA amplification. Each PCR assay was performed in 100 μl of reaction mixture containing 10 μl of DNA sample, 25 μM tetramethylammonium chloride (Aldrich, Milwaukee, Wis.), 200 μM each deoxynucleoside triphosphate, 1 μM each primer, 10 μl of *Taq* polymerase buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mg of bovine serum albumin or gelatin ml^{-1}, pH 9), 1 U of *Taq* polymerase (Appligene Oncor), and distilled water. Target DNAs were amplified in a thermal cycler (model Crocodile III; Appligene Oncor). After an initial denaturation step at 94°C for 10 min, the reaction mixtures were subjected to 32 cycles of amplification consisting of denaturation at 94°C for 30 s, annealing at 62°C for 1 min, extension at 72°C for 1 min, and a final extension step at 72°C for 10 min.

To prevent cross-contamination with amplified DNA sequences, all reagents were aliquoted, autoclaved, and irradiated with a UV box (model UV crosslinker RPN 2500; Amersham Life Science). Microcentrifuge tubes, racks, gloves, and micropipettes were also irradiated. Sterile tips with aerosol barriers (ATGC, Inc.) were used, and all reagents were premixed. One set of micropipettes was reserved for handling amplified DNA. A negative control containing no template DNA was added to each set of amplification reaction mixtures.

Gel electrophoresis and Southern blot hybridization. After amplification, 20 μl of each PCR product was analyzed on a 1.3% (wt/vol) agarose gel by horizontal electrophoresis in TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM Na_2 EDTA, pH 8.25). After electrophoresis, the gel was stained with a solution containing 0.5 μg of ethidium bromide ml^{-1}, and DNA fragments were visualized under a UV light transilluminator (302 nm; UVP, Inc.). A 100-bp ladder (Gibco BRL) was used as a molecular weight marker: it consisted of 15 blunt-ended fragments between 100 and 1,500 bp in multiples of 100 bp and an additional fragment of 2,072 bp. Southern blot analysis was performed according to the method of Bryant and Tandeau de Marsac (3). The gel was denatured in 1.5 M NaCl–0.5 N NaOH and neutralized in 0.5 M Tris-HCl (pH 7.4)–1.5 M NaCl. The DNA fragments were transferred to a nylon membrane (Hybond-N$^+$; Amersham International) and covalently linked by heating. The nylon membranes were prehybridized at 65°C for 4 h in 10 ml of a solution containing 24× NET (0.36 M Tris-HCl [pH 7.5], 3.6 M NaCl, 24 mM EDTA) and 100× Denhardt solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 10% sodium dodecyl sulfate). Hybridization was performed at 60 or 50°C for 18 h in the prehybridization buffer containing the probe pFK726 radiolabeled with [^{32}P]dATP with the Boehringer Mannheim nick-translation kit (Boehringer-Mannheim France SA, Meylan, France). The Southern blots were

TABLE 2. *Bacteroides* spp. strains sensitive to 5-NIs and other strains used

Sp.	Strain	MIC[a]
Bacteroides sensitive strains[b]		
B. fragilis	BF-638R	0.19
	BF-2	0.125
	ATCC 9343	0.125
	IRL8	0.125
	BF-5/86	0.38
	BF-345	0.50
	BF-161/95	0.125
	BF-202/95	0.19
	BF-242/95	0.094
	BF-268/95	0.19
	BF-283/95	0.38
	BF-295/95	0.125
	BF-312/95	0.38
B. vulgatus	BV-1R	0.125
B. distasonis	BD-33/1	0.125
B. uniformis	BU-181/93	0.094
B. eggerthii	BE-560/95	0.125
B. thetaiotaomicron	BT-394/93	0.50
Other anaerobic strains[b]		
Prevotella bivia	PBi-17/92	0.50
Prevotella buccae	PBu-271/93	0.50
Fusobacterium nucleatum	FN-179/87	0.023
Fusobacterium mortiferum	ATCC 2557	0.023
Clostridium perfringens	CP-330/92	0.50
Clostridium botulinum	CB-468	0.006
Aerobic strains[c]		
Escherichia coli	HB101	
Staphylococcus aureus	NCTC 8325	
Helicobacter pylori	85P	
Acholeplasma laidlawii	JAI	
Lactobacillus sp.	10284	
Enterococcus faecalis	T22	
Enterococcus faecalis	Sc446	
Streptomyces sp.	S6670	

[a] MICs of MTR were determined on WC medium (Oxoid) by the E-test method and are expressed in micrograms per milliliter.

[b] These strains were from the Anaerobes Reference Center, Pasteur Institute, Paris, France.

[c] The first four strains were obtained from various laboratories at the Pasteur Institute, the *Lactobacillus* and *Enterococcus* strains were from E. Nagy, and the *Streptomyces* sp. strain was from P. Jeannin, Rhône-Poulenc-Rorer. All aerobic strains are resistant to up to 32 μg of MTR ml^{-1}, the E-test limit value.

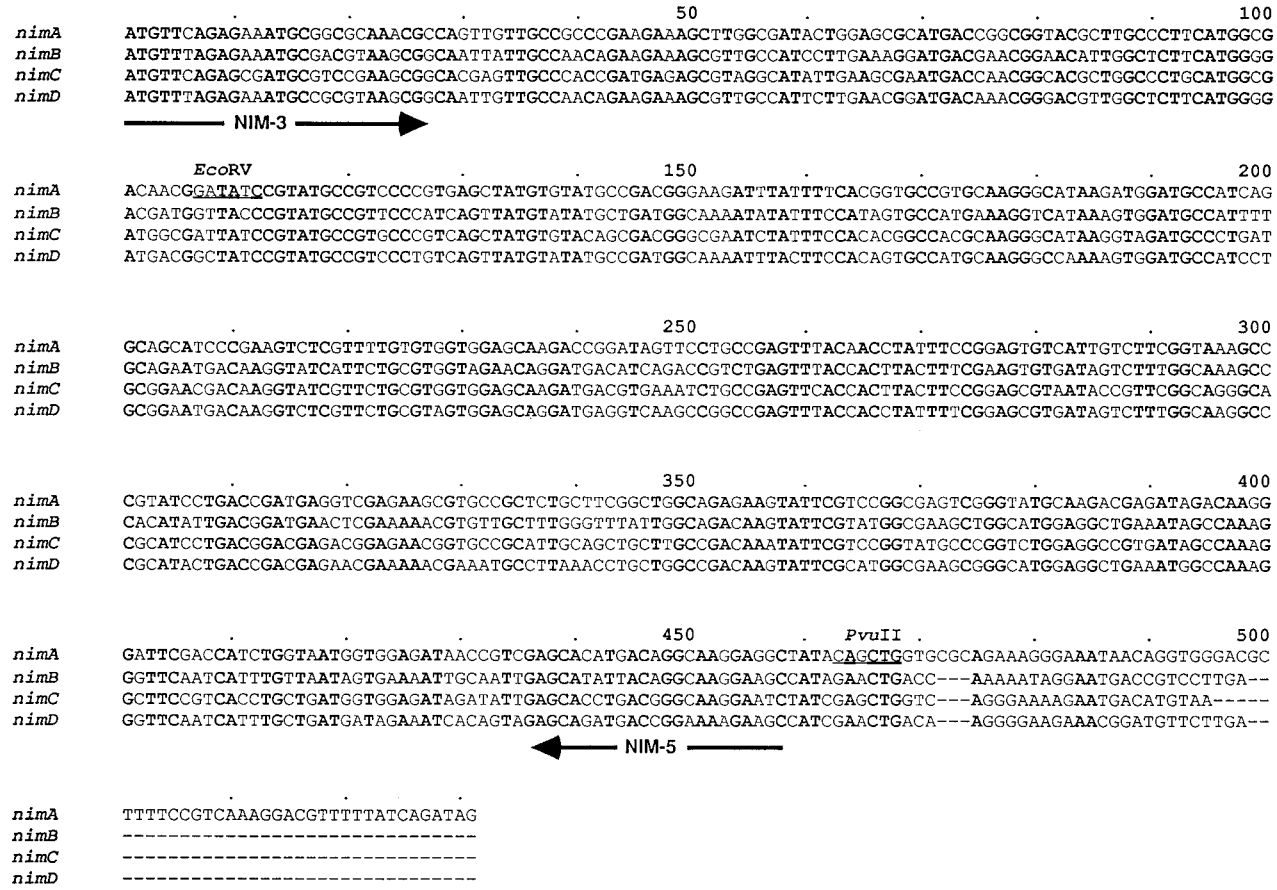

FIG. 1. Alignment of the nucleotide sequences of the four *nim* genes. The common nucleotides are indicated in boldface. The *Eco*RV and *Pvu*II restriction sites are underlined in the *nimA* sequence. Positions of the oligonucleotide primers NIM-3 and NIM-5 are indicated by arrows below the sequences.

then washed three times for 15 min in 6× NET and subjected to autoradiography for 1 or 2 h at −80°C with Hyperfilm-MP (Amersham International).

RESULTS

Evaluation of the PCR method for detection of *nim* genes. In preliminary studies, PCRs were performed with *Bacteroides* strains that carry the 5-Ni^r determinant either on a plasmid (BV-17, BT-13, BF-F239) or on the chromosome (BF-8). The sensitivity of the PCR method for detection of *nim* genes was evaluated by examining serial dilutions of total DNA extracted by the procedure described by K. Wilson (27). The PCR products generated with the primer pair NIM-3 and NIM-5 were analyzed on an ethidium bromide-stained electrophoresis gel (Fig. 2A). A distinct band with the expected size of 458 bp was detected in all strains carrying a *nim* gene, but with varying intensity. For BV-17 and BT-13, harboring the *nimA* and *nimC* genes, respectively, the highest template DNA dilution giving a visible fragment was 10^{-5} (lanes 13 and 20), whereas the detection limit was a dilution of 10^{-4} for BF-8 (*nimB*) (lane 6) and only 10^{-3} for BF-F239 (*nimD*) (lane 24).

To confirm that the product amplified by the PCR method was indeed a *nim* gene fragment, the amplified target sequences were transferred to a membrane by standard blotting and probed with [^{32}P]dATP-labeled plasmid pFK726. The recombinant plasmid pFK726 contains the internal *Eco*RV-*Pvu*II restriction fragment of the *nimA* gene in pUC19 (28). The restriction sites *Eco*RV and *Pvu*II are 106 and 468 nucleotides downstream from the ATG codon initiation of the *nimA* gene (Fig. 1). Hybridization was performed at 60°C, and membranes were exposed for 2 h (Fig. 2B). PCR products obtained from the *nimA* and *nimC* genes gave strong hybridization signals (lanes 9 to 13 and 16 to 20, respectively), and those of the *nimD* gene gave a weaker signal (lanes 22 to 24). In contrast, the probe hybridized very poorly with the PCR products from the *nimB* gene: no band was visible after 2 h of exposure (lanes 3 to 7), and only a faint band was visible after 16 h of exposure (data not shown). Similar results were obtained with hybridization assays performed at 50°C (data not shown).

Specificity of the PCR method. The specificity of the PCR method was first tested with MTR-sensitive strains of the *B. fragilis* group: 13 of *B. fragilis* and 1 each of *B. vulgatus*, *B. distasonis*, *B. uniformis*, *B. eggerthii*, and *B. thetaiotaomicron*. MICs of MTR were determined by the E-test method (Table 2). Each DNA sample was tested for amplification by the NIM-3–NIM-5 primer pair. The 458-bp fragment was not detected in any of the 18 strains tested by ethidium bromide-stained agarose gel electrophoresis (Fig. 3A). DNA samples from bacteria, phylogenetically closely or distantly related to the *B. fragilis* group, were also investigated with identical conditions. The strains included the anaerobes *Prevotella bivia*, *Prevotella buccae*, *Fusobacterium nucleatum*, *Fusobacterium mortiferum*, *Clostridium perfringens*, and *Clostridium botulinum* and the aerobes *Escherichia coli*, *Helicobacter pylori*, *Staphylococcus aureus*, *Acholeplasma laidlawii*, and *Lactobacillus* sp. None of these DNAs yielded an amplified fragment (Fig. 4A),

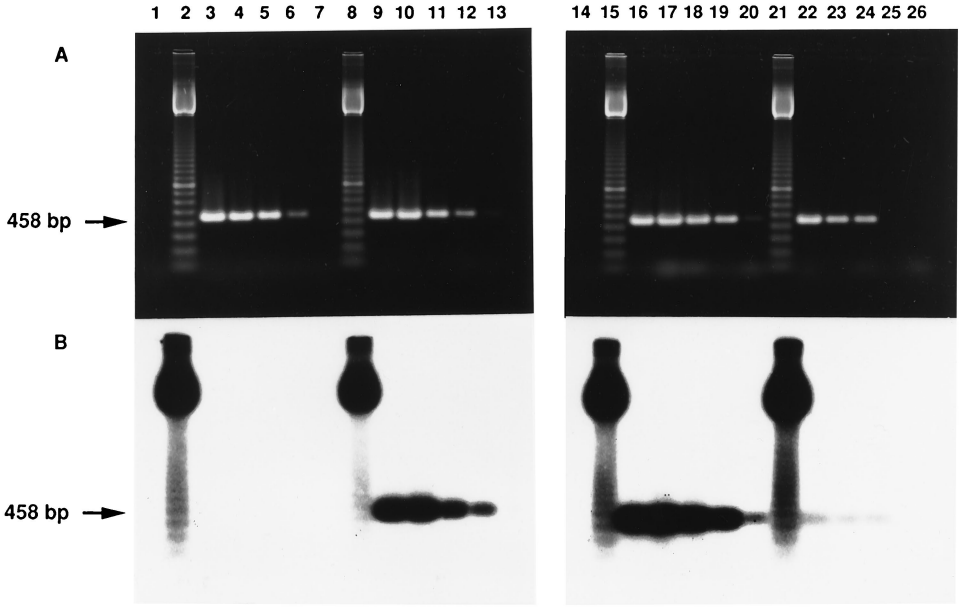

FIG. 2. Gel electrophoresis of PCR products from the four *nim* genes. (A) Ethidium bromide-stained gel of products amplified by the *nim* gene primers; (B) Southern blot hybridization with [^{32}P]dATP-labeled probe pFK726. Lanes 1 and 14, negative control (sterile distilled water); lanes 2, 8, 15, and 21, molecular size markers (fragments of 100 to 1,500 bp in multiples of 100 bp and an additional fragment of 2,072 bp); lanes 3 to 7, DNA from *B. fragilis* BF-8 serially diluted from 10^{-1} to 10^{-5}; lanes 9 to 13, DNA from *B. vulgatus* BV-17 serially diluted from 10^{-1} to 10^{-5}; lanes 16 to 20, DNA from *B. thetaiotaomicron* BT-13 serially diluted from 10^{-1} to 10^{-5}; lanes 22 to 26, DNA from *B. fragilis* BF-F239 serially diluted from 10^{-1} to 10^{-5}.

whereas DNA from *B. vulgatus* BV-17 used as a positive control did (lane 2).

To eliminate false-negative results due to *Taq* polymerase inhibitors, PCR experiments were conducted with test DNA alone (diluted 10^1-fold) and test DNA plus positive control DNA (diluted 10^2-fold) from BV-17 (Fig. 3B for sensitive *Bacteroides* spp. strains and Fig. 4B for the other strains). The 458-bp fragment was amplified from the added control DNA, but the intensity of the signal varied from strain to strain. This may be due to the presence of subinhibitory concentrations of *Taq* polymerase inhibitors or to nonspecific annealing of the primers to the DNA of the sensitive strain, thereby reducing PCR product yield. This was observed for strains *B. fragilis* BF-202/95 (Fig. 3, lane 12) and *Lactobacillus* sp. strain 10284 (Fig. 4, lane 14), the amplification product of which gave a very weak signal in standard loading conditions. When the PCR products were concentrated before loading, the 458-bp fragment was detected (data not shown).

Detection of *nim* genes in microorganisms carrying an undefined 5-NI resistance determinant. To validate the *nim* gene PCR method, DNAs from three recent 5-Ni^r clinical isolates of *B. fragilis* (BF-376/95, BF-377/95, and BF-152/94) were examined (Fig. 5A, lanes 11 to 13). The PCR test yielded the 458-bp amplified fragment for each of the three strains. In Southern blot analysis (Fig. 5B), the PCR product of strain BF-377/95 (lane 12) hybridized with the *nimA* gene probe, whereas no hybridization was observed for the two other strains (lanes 11 and 13). Subsequent genetic analysis of *B. fragilis* BF-377/95 has revealed that the amplified *nim* gene was located on a 7.7-kb plasmid, with the same restriction pattern as plasmid pIP417. The *nim* gene in BF-152/94 was located on the chromosome. The location of the *nim* gene from BF-376/95 has not yet been investigated.

PCR amplifications were also performed with DNAs extracted from two strains of *Enterococcus faecalis* which protect *B. fragilis* against the killing effect of MTR (15) and from

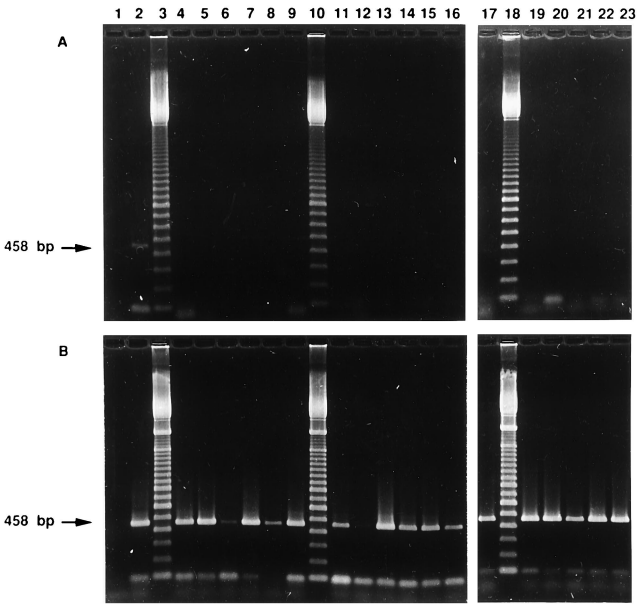

FIG. 3. Specificity of the PCR detection assay assessed with 5-NI-sensitive *Bacteroides* spp. strains. Shown is gel electrophoresis of PCR products from a 10^{-1} dilution of DNA samples, without (A) and with (B) addition of a 10^{-2} dilution of positive control DNA (*B. vulgatus* BV-17). Lane 1, negative control (sterile distilled water); lane 2, positive control; lanes 3, 10, and 18, molecular size markers (as in Fig. 2); lane 4, *B. fragilis* BF-638R; lane 5, *B. fragilis* BF-2; lane 6, *B. fragilis* ATCC 9343; lane 7, *B. fragilis* IRL8; lane 8, *B. fragilis* BF-5/86; lane 9, *B. fragilis* BF-345; lane 11, *B. fragilis* BF-161/95; lane 12, *B. fragilis* BF-202/95; lane 13, *B. fragilis* BF-242/95; lane 14, *B. fragilis* BF-268/95; lane 15, *B. fragilis* BF-283/95; lane 16, *B. fragilis* BF-295/95; lane 17, *B. fragilis* BF-312/95; lane 19, *B. vulgatus* BV-1R; lane 20, *B. distasonis* BD-33/1; lane 21, *B. uniformis* BU-181/93; lane 22, *B. eggerthii* BE-560/95; lane 23, *B. thetaiotaomicron* BT-394/93.

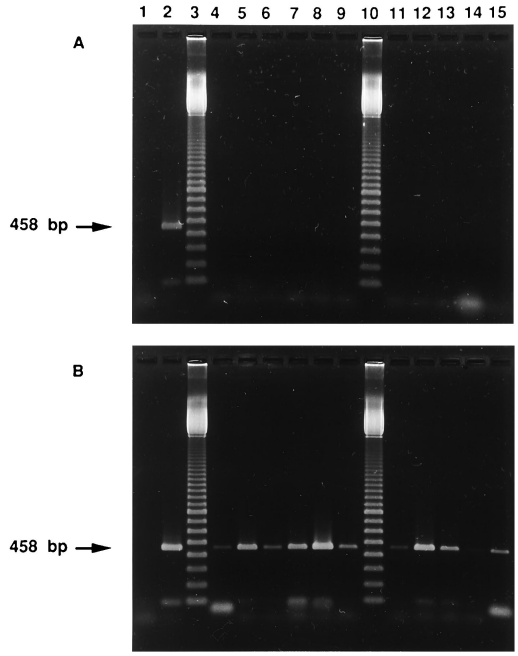

FIG. 4. Specificity of the PCR detection assay assessed with 5-NI-sensitive aerobic and anaerobic bacteria. Shown is gel electrophoresis of PCR products from a 10^{-1} dilution of DNA samples, without (A) and with (B) addition of a 10^{-2} dilution of positive control DNA (*B. vulgatus* BV-17). Lane 1, negative control (sterile distilled water); lane 2, positive control; lanes 3 and 10, molecular size markers (as in Fig. 2); lane 4, *P. bivia* PBi-17/92; lane 5, *P. buccae* PBu-271/93; lane 6, *F. mortiferum* ATCC 2557; lane 7, *F. nucleatum* FN-179/87; lane 8, *C. perfringens* CP-408/92; lane 9, *C. botulinum* CB-468; lane 11, *E. coli* HB101; lane 12, *S. aureus* NCTC8325; lane 13, *H. pylori* 85P; lane 14, *Lactobacillus* sp. strain 10284; lane 15, *A. laidlawii* JAI.

Streptomyces strain S6670, a 2-nitroimidazole producer (12), which may carry a nitroimidazole resistance gene (Fig. 5A, lanes 4 to 6). None of these DNA samples gave PCR products, except when DNA of *B. fragilis* BV-17 was added to the reaction mixture as a positive control (lanes 7 to 9). The same results were obtained by hybridization assays performed at 60°C with the *nimA* gene probe (Fig. 5B, lanes 4 to 9).

Validation of a routine *nim* gene amplification assay. The PCR-based method for *nim* gene detection was improved by using an easier and more rapid DNA extraction procedure, involving heating a bacterial suspension and recovering the DNA in the supernatant (see Materials and Methods). The PCR method was tested with DNAs extracted from the 5-NI-resistant strain BV-17 and from the 5-NI-sensitive strain BF-638R. The strains were cultivated in both liquid and solid WC medium, and in TGY, a medium more routinely used for isolation and growth of anaerobic bacteria. Amplification reactions were performed as described above, with the same set of primers (Fig. 6). In all growth media, the intensity of the signal was greater with DNAs extracted from 2 ml of an overnight culture (lanes 3, 4, 6, and 7) than with those from colonies scraped from plates (lanes 10, 11, 13, and 14). Moreover, better results were obtained when strains were grown in WC medium (lanes 3, 4, 10, and 11) than when they were grown in TGY medium (lanes 6, 7, 13, and 14). DNA extraction from bacteria cultivated in the rich TGY medium was less efficient, probably because of higher production of polysaccharides, which may also interfere with amplification reactions. Nevertheless, the PCR-based method was specific: no band was observed for the negative control strain BF-638R (lanes 5, 8, 12, and 15), whatever the medium or the DNA extraction procedure used.

DISCUSSION

A PCR-based method was developed for detection and screening of 5-NI resistance genes (*nim*) from *Bacteroides* spp. Moderate resistance to MTR, tinidazole, and ornidazole is conferred on clinical isolates by specific genes found either on the chromosome or on small mobilizable plasmids (19). A pair of primers (NIM-3 and NIM-5) were designed from the nucleotide sequences of the four identified *nim* genes for in vitro amplification of a 458-bp fragment. The fragment was obtained with all DNA samples tested from 5-Ni^r *Bacteroides* strains. The sensitivity of the method depended on the copy number of the *nim* genes. Plasmid-borne genes have higher copy numbers than chromosomal genes, and in view of the replication properties of the 5-Ni^r plasmids (9), we may assume that there are about 10 to 20 copies per cell. In contrast, there are never more than two *nim* gene copies on the chromosome (19). This could explain why the sensitivity of the PCR method is 10-fold higher for both BV-17 and BT-13 strains (plasmid *nim* genes) than for strain BF-8 (chromosomal *nim* gene). The specificity of the primers also affects sensitivity. The sequences of the target DNA have to be complementary to the primers used for PCR amplification. In this case, the choice of primer sequences was limited by the high degree of variability (55%) between the nucleotide sequences of the resistance genes (Fig. 1). The primers are less similar to the *nimD* gene sequence than to the *nimA* gene sequence, and this may be why the sensitivity is

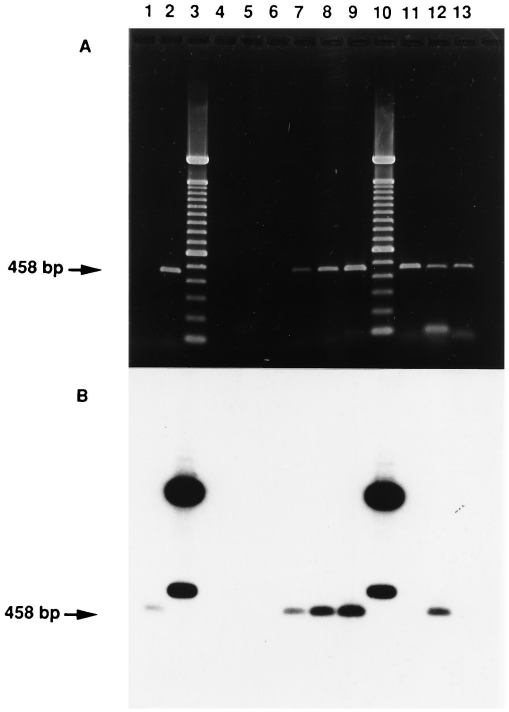

FIG. 5. Agarose gel electrophoresis (A) and Southern blot hybridization (B) of amplification products from microorganisms carrying an uncharacterized 5-NI resistance determinant. Lane 1, negative control (sterile distilled water); lane 2, positive control (*B. vulgatus* BV-17); lanes 3 and 10, molecular size markers (as in Fig. 2); lane 4, *E. faecalis* T22; lane 5, *E. faecalis* SC446; lane 6, *Streptomyces* sp. strain S6670; lanes 7 to 9, as in lanes 4 to 6, with addition of a 10^{-2} dilution of positive control DNA; lane 11, *B. fragilis* BF-152/94, lane 12, *B. fragilis* BF-377/95; lane 13, *B. fragilis* BF-376/95.

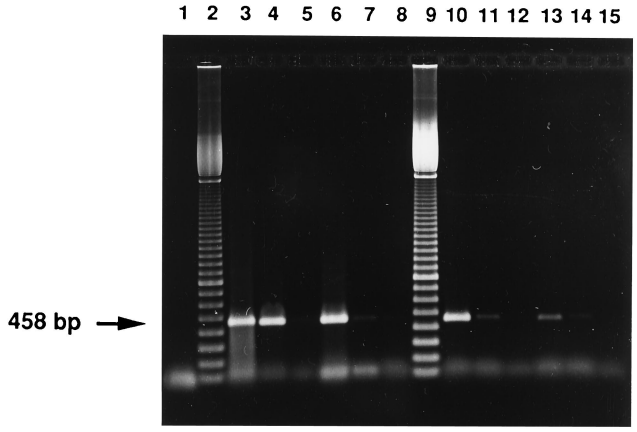

FIG. 6. Gel electrophoresis of PCR products amplified from DNA prepared by the rapid extraction procedure from the 5-Ni^r strain BV-17 and the 5-Ni^s strain BF-638R. Both strains were grown in WC (lanes 3 to 5) and TGY (lanes 6 to 8) liquid media and on solid agarose plates of WC (lanes 10 to 12) and TGY (lanes 13 to 15). Lane 1, negative control (sterile distilled water); lanes 2 and 9, molecular size markers (as in Fig. 2); lanes 3, 6, 10, and 13, a 10^{-1} dilution of DNA from BV-17; lanes 4, 7, 11, and 14, a 10^{-3} dilution of DNA from BV-17; lanes 5, 8, 12, and 15, a 10^{-1} dilution of DNA from BF-638R.

lower for BF-F239 than for BV-17 DNAs, although both genes are on a plasmid.

The technique is also very specific for the *nim* genes as no cross-reactivity was observed either with 5-NI-sensitive *Bacteroides* spp. or with the other microorganisms tested. For the 5-Ni^r *Bacteroides* spp. DNAs, the PCR products were detected by agarose gel electrophoresis and ethidium bromide staining, and the specificity was verified by hybridization with a *nimA* gene probe. The blotting detection was less sensitive than the gel-based technique: for example, amplified DNA from strain BF-8 was visible on the ethidium bromide-stained gel but not on the autoradiogram (Fig. 2B).

The presence of *nim* genes in recent 5-Ni^r isolates of *B. fragilis*, BF-377/95, BF-376/95, and BF-152/94, was detected by the PCR method. The resistance gene of strain BF-377/95 was shown to be similar to the *nimA* gene of *B. fragilis* BV-17 and located on a pIP417-like plasmid. The genes encoding 5-Ni^r in strains BF-376/95 and BF-152/94 gave the expected 458-bp fragment by PCR, but which in both cases did not hybridize with the *nimA* probe even under low-stringency conditions. Thus, the PCR method permits detection of novel 5-NI-encoding resistance genes that confirmed the great divergence at the nucleotide level of the *nim* genes. Sequencing of the PCR products of the newly detected genes will indicate the true diversity of the resistance genes among the *Bacteroides* species.

E. faecalis was first reported by Narikawa to inactivate in vitro MTR molecules (16). More recent studies have shown that *E. faecalis* strains protect *B. fragilis* group strains against the killing effect of MTR at concentrations up to four- to eightfold greater than the normal MIC (14). The protection is a characteristic specific to *E. faecalis* as strains of *Streptococcus* spp., *Staphylococcus aureus*, and *S. epidermidis* do not have the same effect (15). Sonicated cell extracts of *E. faecalis* inactivate MTR to the same extent as do living cells, suggesting enzymatic modification of MTR to an inactive product (14). The *nim* genes are of unknown ancestry; therefore, we looked for the presence of related genes in the *E. faecalis* genome. No PCR amplification product was obtained from DNAs extracted from two strains of *E. faecalis*, suggesting that the *nim* genes of *Bacteroides* spp. were of different origin (Fig. 5A). The *nim* genes might have been derived from an ancestral resistance gene in the genome of a natural producer organism. In fact, 5-NI molecules have never been found in living microorganisms, but a 2-nitroimidazole (azomycin) was identified in the *Streptomyces* sp. strain S6670 in Rhône-Poulenc's laboratories (12). The DNA of this strain was submitted to the *nim* gene PCR detection method, but no amplified product was obtained (Fig. 5A). The origin of the *nim* genes thus remains an open question.

The dissemination of 5-NI resistance is one of the most important antibiotic resistance issues for *Bacteroides* spp. It is important to monitor carefully the spread of the *nim* genes. The presence of moderate MTR resistance is sometimes difficult to detect by conventional susceptibility testing methods. Consequently, we developed an easier and more rapid PCR detection method useful for large epidemiological studies. The technique does not involve extensive purification of DNA and may be performed with bacterial cells cultivated in classical anaerobic growth media.

In conclusion, the PCR technique described in this study is sensitive and specific for *nim* genes, in spite of their nucleotide sequence diversity. It could be used to identify 5-NI-resistant *Bacteroides* strains both for research and for epidemiological investigations and avoids the ambiguity associated with classical MIC evaluations and the complexity of Southern blotting methods.

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