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## Metronidazole Resistance in *Prevotella* spp. and Description of a New *nim* Gene in *Prevotella baroniae*<sup>∇</sup>

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**Nonduplicate clinical isolates of *Prevotella* spp. recovered from patients hospitalized between 2003 and 2006 in two French tertiary-care teaching hospitals were investigated for their susceptibility to metronidazole and the presence of *nim* genes. Of the 188 strains tested, 3 isolates displayed reduced susceptibility to metronidazole after 48 h of incubation, while 27 additional isolates exhibited heterogeneous resistance after prolonged incubation; all 30 of the isolates were *nim* negative. Among the remaining 158 isolates, 7 *nim*-positive isolates were detected. All of these strains were identified as *Prevotella baroniae* by 16S rRNA gene sequence analysis and contained a new *nim* gene, named *nimI*, as determined by DNA sequence analysis. Chromosomal localization of this single-copy gene was demonstrated in all clinical isolates as well as in type strain *P. baroniae* DSM 16972 by using Southern hybridization. No known associated insertion sequence elements were detected upstream of the *nimI* gene in any of the *nim*-positive strains by PCR mapping. After prolonged exposure to metronidazole, stable resistant subpopulations could be selected in *nimI*-positive *Prevotella* isolates ( $n = 6$ ) as well as in *nim*-negative *Prevotella* isolates ( $n = 6$ ), irrespective of their initial susceptibility to this antibiotic. This study is the first description of a new nitroimidazole resistance gene in *P. baroniae* which seems to be silent and which might be intrinsic in this species. Moreover, our findings highlight the fact that high-level resistance to metronidazole may be easily induced in both *nim*-positive and *nim*-negative *Prevotella* sp. strains.**

The genus *Prevotella* includes strictly anaerobic, gram-negative, moderately saccharolytic, bile-sensitive rods formerly belonging to the genus *Bacteroides* (33). These bacteria, which are part of the human oral, intestinal, and urogenital floras, may be involved in various infections, including infections of the head and neck, respiratory tract, central nervous system, and abdominal and urogenital tracts, as well as bacteremia (19). Metronidazole is commonly used for the treatment of infections caused by anaerobic organisms. For a long time, it has been considered that acquired resistance to this antibiotic is rare among anaerobes, despite its extensive use. However, recent studies have shown that this resistance is no longer uncommon among these organisms (3, 16, 18, 22, 23, 26, 36). Reduced susceptibility to 5-nitroimidazole drugs is generally associated with the presence of a nitroimidazole reductase encoded by a *nim* gene. This enzyme converts 4- or 5-nitroimidazole to 4- or 5-aminoimidazole, thus avoiding the formation of the toxic nitroso radicals that are essential for antimicrobial activity (4). Currently, seven *nim* genes, named *nimA* to *nimG*, which are either plasmid or chromosomally encoded, have been described (30), while a new *nim* gene, *nimH* (GenBank accession number FJ969397), has been described in *Bacteroides fragilis*.

*Prevotella* spp. have only rarely been investigated for the presence of *nim* genes in studies concerning metronidazole

susceptibility (20, 23, 27). This led us to investigate a large panel of clinical *Prevotella* strains that belong to different species and that were isolated from patients hospitalized in two French tertiary-care teaching hospitals for the presence of *nim* genes and for the type of *nim* genes that they carry.

### MATERIALS AND METHODS

**Bacterial isolates, identification, and culture.** One hundred eighty-eight nonduplicate *Prevotella* isolates recovered from patients hospitalized between 2003 and 2006 in two French tertiary-care teaching hospitals (university hospital center of Nancy,  $n = 157$ ; university hospital center of Poitiers,  $n = 31$ ) were investigated. These strains were isolated from patients with various clinically significant infections (head and neck,  $n = 49$ ; skin and soft tissues,  $n = 36$ ; intra-abdominal,  $n = 30$ ; pleuropulmonary,  $n = 25$ ; urogenital,  $n = 20$ ; osteoarticular,  $n = 13$ ; bacteremia,  $n = 11$ ; other,  $n = 4$ ). Isolates were identified by phenotypic methods as well as by 16S rRNA gene sequence analysis, if necessary (*Prevotella bivia*,  $n = 41$ ; *Prevotella buccae*,  $n = 35$ ; *Prevotella denticola*,  $n = 28$ ; *Prevotella melaninogenica*,  $n = 17$ ; *Prevotella disiens*,  $n = 10$ ; *Prevotella nanceiensis*,  $n = 10$ ; *Prevotella oris*,  $n = 9$ ; *Prevotella baroniae*,  $n = 7$ ; *Prevotella nigrescens*,  $n = 5$ ; *Prevotella oralis*,  $n = 5$ ; *Prevotella* spp.,  $n = 5$ ; *Prevotella loescheii*,  $n = 3$ ; *Prevotella salivae*,  $n = 3$ ; *Prevotella veroralis*,  $n = 3$ ; *Prevotella bergensis*,  $n = 2$ ; *Prevotella intermedia*,  $n = 2$ ; *Prevotella corporis*,  $n = 1$ ; *Prevotella heparinolytica*,  $n = 1$ ; *Prevotella multififormis*,  $n = 1$ ) (1, 17, 19). The strains were stored in brucella broth containing 15% (wt/vol) glycerol at  $-80^{\circ}\text{C}$  prior to the assays. For all experiments, the strains were grown at  $37^{\circ}\text{C}$  on brucella agar supplemented with 5% sheep blood, hemin, and vitamin K<sub>1</sub> (BBA) under anaerobic conditions.

**Metronidazole susceptibility testing.** The MICs of metronidazole were determined on BBA by the agar dilution method, according to CLSI standards (document M11-A7) (5), and by the Etest method (AB Biodisk, Solna, Sweden), according to the manufacturer's instructions. Readings were performed after incubation for 48 h at  $35^{\circ}\text{C}$  under anaerobic conditions. The MIC results were interpreted in accordance with the guidelines of the European Committee on Antimicrobial Susceptibility Testing (susceptibility, MIC  $\leq 4$   $\mu\text{g/ml}$ ; resistance, MIC  $\geq 8$   $\mu\text{g/ml}$ ) (11). *Bacteroides fragilis* ATCC 25285<sup>T</sup> and *Bacteroides thetaiotaomicron* ATCC 29741 were included as controls. As it has previously been shown that metronidazole resistance may be detected only after prolonged in-

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cubation (9, 14, 25), a second reading of all tests was performed after an additional 72 h. All assays were performed in duplicate.

**Detection of *nim* genes and IS elements.** DNA was extracted by using a QIAamp DNA minikit (Qiagen Inc., Hilden, Germany). The strains were investigated for the presence of *nim* genes by PCR with universal primers NIM-3 and NIM-5, as described previously (25, 38). For the insertion sequence (IS) element-specific PCR amplifications, previously published primers were used (15, 35, 37, 38). By using the forward primer specific to the amplified IS element and the reverse NIM-5 primer, further PCR experiments were performed to determine whether the IS was upstream of the *nim* gene, as described by S6ki et al. (34, 35). The following positive control strains containing *nim* genes and IS elements were used: *B. fragilis* 638R(pIP417) (*nimA*, IS1168), *B. fragilis* BF8 (*nimB*, IS1168), *B. thetaiotaomicron* BT13(pIP419) (*nimC*, IS1170), *B. fragilis* 638R(pIP421) (*nimD*, IS1169), *B. fragilis* BF388(pBF388c) (*nimE*, ISBf6), and *B. fragilis* BF6712 (IS612). *B. fragilis* ATCC 25285<sup>T</sup> was included as a *nim*-negative control. The IS element and *nim* gene PCR products were sequenced by using an ABI Prism BigDye Terminator sequencing kit on an ABI Prism 3100 automated sequencer (Applied Biosystems, Les Ulis, France). The nucleotide sequences were analyzed by using SeqScape software (version 2.5; Applied Biosystems). The sequences were compared to those deposited in the GenBank database by using the BLAST program (2). The sequences of the Nim proteins were determined by using the TRANSLATE program of the ExpASY proteomics server (www.expasy.org). The protein sequences were analyzed and aligned by using the BioEdit sequence alignment editor program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). A phylogenetic dendrogram was generated by the neighbor-joining method with MEGA software, version 2.1 (21).

**Localization of *nim* genes.** Plasmid extraction and Southern hybridization of *nim*-positive strains were used to determine whether the *nim* genes were present in either plasmid or chromosomal DNA, as described previously (14). Briefly, plasmid DNA was extracted with a plasmid miniprep kit (Qiagen). Uncut plasmid DNA and EcoRI-digested chromosomal DNA were run on electrophoresis gels and then transferred by capillarity to nylon membranes (Hybond-N+; Amersham Biosciences). *Nim*-specific digoxigenin-labeled probes were obtained by PCR, as described above, with primer pair NIM-3 and NIM-5 with a deoxynucleoside triphosphate mixture containing 0.1 mM digoxigenin-dUTP (Roche Diagnostics). The hybridization of the probes was detected with a CSPD chemiluminescence system (Roche Diagnostics). *B. fragilis* 638R(pIP417) and *B. fragilis* BF8 were used as positive and negative controls for plasmid and chromosomal localization, respectively.

**Selection and stability of metronidazole resistance.** Eleven clinical isolates (five *nim*-positive strains and six *nim*-negative strains), as well as *Prevotella baroniae* DSM 16972<sup>T</sup> and *B. fragilis* 25285<sup>T</sup>, were used to test for the induction of metronidazole resistance. For this purpose, bacterial suspensions were inoculated on BBA plates containing metronidazole at increasing concentrations, as previously described by Schaumann et al. (32). The susceptibility of colonies growing in the presence of the highest concentration was determined by the agar dilution method. The stability of the resistance was evaluated by determining the MIC of metronidazole after three consecutive subcultures on drug-free BBA. As described by Pel6ez et al. (29), we considered that the resistance was stable when the MIC of metronidazole for *Prevotella* spp. was maintained (within  $\pm 1$  dilution) after the passages. The experiments were performed in duplicate.

**Nucleotide sequence accession numbers.** The 16S rRNA and *nim* gene sequences of the *P. baroniae* isolates have been deposited in the GenBank database under accession numbers FJ940875 to FJ940882 and FJ940883 to FJ940890, respectively.

## RESULTS

Of the 188 clinical isolates tested, 3 were found to be resistant to metronidazole either by the agar dilution method (isolates LBN 464 and LBN 467) or by the Etest method (isolate LBN 298) after 48 h of incubation (Table 1). For 30 isolates, slowly growing colonies were visualized inside the inhibition zone of the Etest strip after prolonged incubation. Colonies from within the inhibition zone were subcultured without antibiotic and were retested by the agar dilution method to determine whether the MIC had changed from its original value. These subpopulations showed MICs ranging from 8 to 32  $\mu\text{g/ml}$ .

PCR amplification with universal primers targeting the *nim*

TABLE 1. MICs of metronidazole for *nimI*-positive strains and *nim*-negative strains exhibiting reduced susceptibility to metronidazole

Strain <sup>a</sup>	Presence of <i>nim</i> gene	MIC determined by agar dilution ( $\mu\text{g/ml}$ )	
		Original	SGC <sup>b</sup>
<i>P. baroniae</i>			
DSM 16972 <sup>T</sup>	+	2	NA <sup>c</sup>
LBN 427	+	4	NA
LBN 430	+	4	NA
LBN 432	+	2	NA
LBN 466	+	2	NA
LBN 475	+	4	NA
LBP 9	+	2	NA
LBP 19	+	1	NA
<i>P. bivia</i>			
LBN 330	–	2	16
LBN 331	–	2	16
LBN 332	–	4	32
LBN 333	–	2	32
LBN 334	–	2	16
LBN 336	–	2	32
LBN 339	–	2	16
LBN 343	–	1	16
LBN 346	–	1	16
LBN 350	–	2	16
LBN 361	–	2	32
LBN 365	–	4	8
LBN 371	–	4	32
LBN 374	–	2	8
LBN 445	–	0.5	16
LBN 464	–	8	16
LBN 467	–	16	32
LBN 478	–	4	8
LBP 4	–	2	32
LBP 38	–	2	8
LBP 51	–	2	32
LBP 52	–	4	16
LBP 65	–	4	32
LBP 69	–	2	8
LBP 78	–	4	8
<i>P. nanceiensis</i>			
LBN 293b	–	2	32
LBN 297	–	2	16
LBN 298 <sup>d</sup>	–	4	32
LBN 410	–	1	32
LBP 66	–	2	8

<sup>a</sup> LBN, Laboratoire de Bact6riologie de Nancy; LBP, Laboratoire de Bact6riologie de Poitiers.

<sup>b</sup> SGC, slowly growing colonies within the inhibition zone of the Etest strip.

<sup>c</sup> NA, not applicable (absence of slowly growing colonies).

<sup>d</sup> Strain with reduced susceptibility to metronidazole (MIC, 16  $\mu\text{g/ml}$ ), as determined at 48 h by the Etest method.

genes yielded products of about 460 bp for seven clinical isolates. All of these strains were genotypically identified as *P. baroniae*. The same *nim* gene was amplified from *P. baroniae* DSM 16972<sup>T</sup>. All *P. baroniae* strains tested were considered susceptible to metronidazole by the agar dilution method and the Etest method (Table 1). The distance matrix constructed by using the Similarity table program of the PHYLIP package (13) showed that the *P. baroniae* strains shared from 97.1 to 100% of their *nim* gene nucleotide positions. The *nim* gene detected in the *P. baroniae* type strain (GenBank accession no.

TABLE 2. Induction and stability of resistance to metronidazole in 11 clinical *Prevotella* isolates, *P. baroniae* DSM 16972<sup>T</sup>, and *B. fragilis* ATCC 25285<sup>T</sup>

Strain ( <i>nimI</i> gene <sup>a</sup> )	MIC (μg/ml)		
	Original	Postinduction	Poststability
<i>P. baroniae</i> DSM 16972 <sup>T</sup> (+)	2	16	16
<i>P. baroniae</i> LBN 427 (+)	4	128	128
<i>P. baroniae</i> LBN 430 (+)	4	128	128
<i>P. baroniae</i> LBN 432 (+)	2	64	64
<i>P. baroniae</i> LBN 475 (+)	4	256	256
<i>P. baroniae</i> LBP19 (+)	1	128	128
<i>P. bivia</i> LBN 332 (-)	4	>256	>256
<i>P. bivia</i> LBN 371 (-)	4	>256	>256
<i>P. bivia</i> LBN 467 (-)	16	>256	>256
<i>P. buccae</i> LBN 465 (-)	0.5	128	64
<i>P. nanceiensis</i> LBN 293b (-)	2	256	256
<i>P. nanceiensis</i> LBN 410 (-)	1	256	256
<i>B. fragilis</i> ATCC 25285 <sup>T</sup> (-)	0.5	256	128

<sup>a</sup> +, positive; -, negative.

FJ940883) showed the highest degree of identity with *nimH* (63.8%), followed by *nimF* (63.2%), *nimD* (62.8%), *nimG* (62.5%), *nimC* (62.1%), *nimE* (61.7%), *nimA* (60.1%), and *nimB* (59.7%). This potentially new *nim* gene was named *nimI*. The predicted amino acid sequence alignment of NimI with homologs from other anaerobic bacteria showed that NimI formed a homogeneous group distinct from the other Nim types, with which NimI exhibited 55.9 to 64.4% identity. Insertion elements that have been associated with *nim* genes (IS1168, IS1169, IS1170, ISBf6, and IS612) (35, 37) were not found in any of the *nimI*-positive strains except the type strain, in which IS1168 was detected. However, PCR mapping and sequencing showed that this sequence was not localized upstream of the *nimI* gene.

No plasmid was found in any of the *P. baroniae* isolates tested or in *B. fragilis* BF8, whereas a *nimA*-carrying plasmid of about 7 to 8 kb was detected in *B. fragilis* 638R(pIP417), as expected (15). To determine whether the *nimI* gene was present in chromosomal DNA, hybridizations were performed with *nimA*, *nimB*, and *nimI* gene probes on chromosomal blots with EcoRI-digested DNA. No hybridization with any of the strains tested with the *nimA* gene probe was found. A positive hybridization was observed for *B. fragilis* BF8 with the *nimB* gene probe and for all *P. baroniae* strains with the *nimI* gene probe. In all cases, only one copy of the *nimI* gene was observed.

Experiments performed to detect whether metronidazole-resistant subpopulations could be induced or selected from *nim*-positive ( $n = 6$ ) or *nim*-negative ( $n = 6$ ) *Prevotella* strains showed that all isolates exhibited significantly enhanced MICs (8× to 256× the original MIC) after several passages on plates containing increasing concentrations of metronidazole (Table 2). The induced resistance was stable in all strains after subculture in the absence of metronidazole and also after storage and freezing. For the *nimI*-positive strains, no differences in the *nim* gene sequences were found in strains that converted from metronidazole susceptibility to metronidazole resistance.

## DISCUSSION

In the few studies examining the distribution of *nim* genes in *Prevotella* spp. (20, 23, 27), *nim*-positive *Prevotella* strains have rarely been found. Lubbe et al. (23) detected one *nimA*-positive strain (*Prevotella bivia*) among seven strains screened, while Katsandri et al. (20) recovered one *nimC*-positive strain (*Prevotella oralis*) and two *nimE*-positive isolates (*P. oralis*, *Prevotella buccalis*) from among 57 isolates tested. In our study, no *nim* gene was amplified from any of the 30 isolates exhibiting reduced susceptibility to metronidazole after 48 h of incubation and/or isolates that presented slowly growing resistant subpopulations. This suggests that other potential resistance mechanisms may exist for these isolates, such as decreased pyruvate:ferredoxin oxidoreductase activity, overexpression of efflux pumps, or alterations of the rhamnose catabolism pathway (7, 10, 28, 29, 31). However, a potentially novel *nim* gene, *nimI*, was present and silent in 7 of the 158 remaining clinical isolates, for which neither increased initial MICs nor slowly growing resistant populations were observed. For *Bacteroides* spp., it has been reported that strains harboring silent *nim* genes may become resistant to metronidazole after prolonged exposure to this antibiotic (14, 22) and that this phenomenon may be a consequence of the activation of the *nim* gene as a result of point mutations, the insertion of an IS element promoter immediately upstream of the gene, or the formation of a new promoter following insertion (14, 37). In the present study, metronidazole resistance could be selected after prolonged in vitro exposure to this drug in all *nim*-positive *Prevotella* strains tested. The possibility that mutation within the *nimI* gene might have led to the conversion from silent to constitutive expression was ruled out by *nim* gene sequencing in the pre- and postinduction states. However, none of the known IS elements that have been associated with *nim* genes were detected upstream of *nimI*. These findings are in accordance with those of previous studies that have reported that *nim*-positive strains may be metronidazole resistant in the absence of known activating IS elements (14, 20, 22). This suggests that, as for other *nim* genes, the expression of *nimI* may be modulated by mechanisms other than those related to the presence of IS elements, although it cannot be ruled out that new IS elements that are not recognized by the primers used may be involved.

In *Bacteroides* spp., it has been shown that exposure to metronidazole can select for resistant subpopulations exhibiting either a nonstable phenotype that reverted to susceptibility during growth in the absence of the antibiotic or a constitutive phenotype that remained resistant after removal of the drug (14, 22, 32). In the present study, metronidazole resistance could be selected and remained stable in both the *nim*-positive and the *nim*-negative *Prevotella* strains tested, as well as in *B. fragilis* ATCC 25285<sup>T</sup>. Other authors have also shown that metronidazole-resistant mutants may be selected from *nim*-negative *Bacteroides* sp. isolates, including the *B. fragilis* type strain (7, 32). In contrast, Gal and Brazier (14) as well as Löfmark et al. (22) did not observe any selection of resistant mutants when *nim*-negative *Bacteroides* isolates were tested. Possible reasons for the discrepancies between the findings of those studies can be attributed, at least partially, to the use of different protocols for the selection of metronidazole-resistant

subpopulations. It has been reported that the selection of microbial subpopulations that are resistant to the therapy administered may lead to the failure of treatment for infections caused by different bacterial species, including *Prevotella* spp. (12, 24, 25, 29). Furthermore, Diniz et al. have also shown that the in vivo selection of resistant *Bacteroides* isolates by low doses of metronidazole may lead to enhanced pathogenicity (6). In the present study, the review of the charts of patients with *P. baroniae* infections revealed that treatment failure was not observed in the only patient who was treated with metronidazole. However, no conclusion can be drawn from this observation since that patient also received another active antibiotic. The failure of metronidazole treatment was observed in two patients infected with *nim*-negative *Prevotella* isolates, isolates LBN 293b (*Prevotella nanceiensis*) and LBN 465 (*P. buccae*), both of which were initially considered susceptible to metronidazole. High-level metronidazole resistance could be induced in both strains, while slowly growing subpopulations within the inhibition zone of the Etest strip were observed only for strain LBN 293b. That strain was isolated from blood cultures (25), while strain LBN 465 was recovered from pancreatic necrotic tissue in a patient with acute pancreatitis. For that patient, who remained febrile during treatment with metronidazole and who was operated on again, a metronidazole-resistant *P. buccae* isolate (MIC, 32 µg/ml) was obtained from a surgical drainage sample. That strain exhibited the same pulsed-field gel electrophoresis pattern as strain LBN 465 after DNA digestion was performed by using XbaI (data not shown). The patient became afebrile after treatment with metronidazole was changed to treatment with imipenem. Thus, the in vivo selection of a resistant subpopulation, which was responsible for treatment failure, was demonstrated and corroborated the previous observation that the initial isolate became resistant after prolonged in vitro exposure to metronidazole.

It is noteworthy that *nimI* was found in all *P. baroniae* isolates tested, including the type strain originating from the United Kingdom (8) and clinical isolates obtained in two geographically distant sites in France. *NimI* was also recently detected in two other *P. baroniae* clinical strains isolated in our laboratory, whereas this gene was not detected in 33 type strains belonging to other *Prevotella* species. Moreover, a phylogenetic tree based on *NimI* amino acid sequences showed that *NimI* formed a new homogeneous group distant from the other *Nim* types involved in metronidazole resistance in anaerobic bacteria (data not shown). These results, associated with the fact that *nimI* had a chromosomal localization, suggest that this gene might be intrinsic in *P. baroniae*. However, further studies with a larger number of strains are needed to confirm this hypothesis. It is interesting to note that 16S rRNA gene sequencing was necessary to unambiguously identify all *P. baroniae* strains, suggesting that the clinical implication of this species might be underestimated, since molecular identification is not widely used in routine clinical laboratories.

In conclusion, this study led to the description of a new *nim* gene which seems to be intrinsic to the species *P. baroniae*. This finding underscores the importance of a correct identification at the species level, at least for isolates responsible for severe infections. Moreover, we showed for the first time that subpopulations exhibiting high-level resistance to metronidazole can be selected from both silent *nimI*-positive and *nim*-nega-

tive *Prevotella* sp. isolates after prolonged exposure to this antibiotic. These findings not only confirm that *nim* genes are not the only factors involved in the decreased susceptibility of *Prevotella* spp. to metronidazole but also emphasize the importance of careful susceptibility testing of anaerobes and the usefulness of 16S rRNA gene-based identification methods, especially in cases of severe infections or treatment failures.

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