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Original Article

Initial expression levels of *nim*A are decisive for protection against metronidazole in *Bacteroides fragilis*

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ABSTRACT

Objectives: In the genus *Bacteroides*, the *nim* genes are resistance determinants for metronidazole, a nitroimidazole drug widely used against anaerobic pathogens. The Nim proteins are considered to act as nitroreductases. However, data from several studies suggest that the expression levels of Nim do not increase with increasing resistance which is conflicting with this notion. The impact of Nim protein levels on low-level metronidazole resistance, however, representing the early stage of induced resistance in the laboratory, has not been assessed as yet.
Methods: The *nimA* gene was cloned into two different plasmids and introduced into *B. fragilis* strain 638R. Expression levels of *nimA* mRNA were measured by RT-qPCR and compared to those in strain 638R harbouring plasmid pI417, the original clinical plasmid harbouring IS element IS*1168* with the *nimA* gene. Further, metronidazole susceptibility was assessed by Etest and the activity of pyruvate:ferredoxin oxidoreductase (PFOR) was measured in all strains after induction of high-level metronidazole resistance.
Results: The level of protection against metronidazole by *nimA* correleated with the level of expression of *nimA* mRNA. Further, the activity of PFOR in highly-resistant *B. fragilis* 638R was only preserved when expression levels of *nimA* were high.
Conclusions: Although the development of high-level metronidazole resistance in *B. fragilis* strains with a *nimA* gene is not caused by an increase of *nimA* expression as compared to the less resistant parent strains, *nimA* expression levels might be of decisive importance in the early stage of resistance development. This has potential implications for metronidazole resistance in clinical isolates.

1. Introduction

The 5-nitroimidazole drug metronidazole is a mainstay in the treatment of *Bacteroides* infections [1,2] and resistance rates have remained fairly low throughout the decades [3]. Still, in some parts of the world metronidazole resistance in *Bacteroides* spp., especially in *B. fragilis*, can be a problem [4,5]. Most of the metronidazole-resistant *Bacteroides* strains isolated to date carry an identified *nim* gene. The association of *nim* genes and metronidazole resistance was established about 30 years ago [6,7] and Nim proteins were believed to act as nitroreductases [8,9]. In principle, the reduction of the nitro group in metronidazole, i.e. either to a nitroimidazole anion (one electron transferred) or to a nitrosoimidazole (two electrons transferred), is a prerequisite for rendering the drug toxic. However, Nim proteins were hypothesized to transfer six electrons to the nitro group, resulting in the generation of a non-toxic aminoimidazole [8]. To date, however, direct evidence for the proposed nitroreductase activity of Nim proteins is still missing. Furthermore, it was shown that *nim* expression levels are not increased after the induction of high-level metronidazole resistance strains as compared to the far less resistant parent strains [10,11], thereby questioning a direct dose-response relationship between Nim proteins and metronidazole resistance. In contrast, the presence of *nimA* was shown to increase the activity of pyruvate:ferredoxin oxidoreductase (PFOR), a central metabolic enzyme in most anaerobes, including *B. fragilis* [12]. PFOR is iron-dependent because it harnesses iron-sulphur clusters for activity. The activity of PFOR was preserved even in a highly

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metronidazole-resistant strain harbouring the *nimA* gene whereas expression of this enzyme is shut down early in the development of resistance when resistance is induced in strains without a *nimA* gene [11]. Further, the presence of the *nimA* gene enabled unabated iron import via HmuY, a major factor in haemin import in *Bacteroides* [13], whereas *hmuY* expression was greatly suppressed in a highly resistant strain without the *nimA* gene [11]. These data suggest that the mode of action of NimA might be much more complex than suggested by the established model of Nim proteins acting as nitroreductases. On the other hand, certain observations imply that expression levels of *nim* could be of importance for the degree of protection against metronidazole. For example, the level of protection as conferred by *nimJ* was found to be far lower than that in a clinical isolate when the gene was expressed from a recombinant plasmid [14].

In order to obtain more information on the importance of *nimA* expression levels on the susceptibility to metronidazole, we cloned the *nimA* gene in to two different shuttle vectors (pFD288 and pFD340, respectively) and introduced them into strain *B. fragilis* 638R. Subsequently, the expression levels of *nimA* mRNA were measured by RT-qPCR and compared to those found in 638R harbouring the original clinical plasmid pIP417 which carries the IS*1168* element and the downstream *nimA* gene. The *nimA* expression levels were then compared to the levels of protection against metronidazole and PFOR activities as observed in these strains.

2. Materials and methods

2.1. Chemicals and growth media components

Wilkins-Chalgren anaerobe agar (WC) was purchased from Oxoid (Basingstoke, England) whereas Brain-heart-infusion broth (BHI) was purchased from Carl Roth (Karlsruhe, Germany). Supplementations for growth media, i.e. haemin, and vitamin K1 were all purchased from Sigma-Aldrich (St. Luis, USA). Anaerocult A pads for the generation of an anaerobic atmosphere in jars was purchased from Merck (Darmstadt, Germany). Metronidazole and antibiotics for selection media, i.e. spectinomycin, streptomycin, erythromycin, gentamycin, rifampicin, kanamycin, tetracycline were purchased from Sigma-Aldrich (St. Luis, USA). Paraquat dichloride hydrate, pyruvic acid, β-mercaptoethanol and coenzyme A for PFOR assays were also purchased from Sigma-Aldrich (St. Luis, USA). Etests were purchased from bioMérieux (Marcy-l'Étoile, France).

2.2. Bacterial strains and culture

Strain *B. fragilis* 638R and three transconjugant daughter strains were used in this study. The first transconjugant (638R pIP417 *nimA*) carries the *nimA* gene on clinical plasmid pIP417 together with the preceding IS element IS*1168* [6] and was already available to us prior to this study. The second transconjugant (638R pFD288 *nimA*) carries the *nimA* gene together with the preceding IS*1168* on plasmid pFD288 [15], and the third transconjugant (638R pFD340 *nimA*) carries the *nimA* gene only, cloned into the multiple cloning site (MCS) of pFD340 [16]. In pFD340 *nimA*, the *nimA* gene is under the transcriptional control of IS element IS*4351*. The sequence of IS*1168* and the downstream *nimA* gene can be accessed via GenBank number X71444. *B. fragilis* 638R and all daughter strains were routinely grown at 37 °C on WC agar plates. Batch cultures used for PFOR measurements were grown in supplemented BHI medium (5 μg ml^{-1} haemin and 1 μg ml^{-1} vitamin K1) in 14 mL tubes (Greiner Bio-One). Anaerobic growth inside anaerobic jars (Merck, Darmstadt, Germany) was achieved by applying Anaerocult A.

Escherichia coli NEB 10-beta (New England Biolabs Ipswich, MA, USA) which were used as plasmid donors and *E. coli* RK231 [17] which were used as helper cells in tri-parental mating experiments were cultivated in LB medium and LB on agar plates containing appropriate amounts of antibiotics. Streptomycin (150 μg ml^{-1}) and spectinomycin (150 μg ml^{-1}) select for the plasmid pFD288, ampicillin (100 μg ml^{-1}) for pFD340 in NEB 10-beta, and kanamycin (25 μg ml^{-1}) and tetracycline (2 μg ml^{-1}) to preserve mobilizing plasmids in RK231).

2.3. Cloning strategy and tri-parental mating

When cloning *nimA* into pFD288, the *nimA* gene was amplified together with the complete preceding IS*1168* sequence by PCR using appropriate primers. Sequences downstream of the *nimA* gene were not amplified. The primers introduced an *Eco*RI restriction site to the 5′ end and a *Pst*I site to the 3′ end of the amplicon. The amplicon was then inserted into the pFD288 vector using the *Eco*RI and *Pst*I restriction sites giving pFD288 *nimA*. When cloning *nimA* into pFD340, the *nimA* gene was amplified without the preceding sequence of IS*1168* save 20 bp upstream of the ATG codon containing the presumptive ribosomal binding site. The primers introduced a *Bam*HI restriction site to the 5′ end and a *Kpn*I site to the 3' end of the amplicon. The amplicon was then inserted into the MCS of the pFD340 vector using the *Bam*HI and *Kpn*I restriction sites giving pFD340 *nimA*. Primer sequences are given in Supplementary Table 1.

The conjugal transfer of plasmids from *E. coli* NEB 10-Beta to *B. fragilis* 638R was performed via tri-parental mating according to an established filter mating protocol [18] by using *E. coli* RK231 as the helper strain. Mating medium plates (BHI agar) were supplemented with cysteine (0.5 mg ml^{-1}) and haemin (5 mg ml^{-1}). Overnight batch cultures of each strain were diluted accordingly: 1:40 diultion of *B. fragilis* 638R and 1:100 for *E. coli* NEB 10-Beta. In pre-warmed broths diluted cultures were grown without antibiotics until OD_{600} was within the range of 0.3–1. The *E. coli* donor was placed on a nitrocellulose membrane circle together with helper strain RK231 and the recipient strain *B. fragilis* 638R in a ratio of 0.25/0.25/1. Mating plates were incubated aerobically at 37 °C overnight. Afterwards, cells were washed from filters with 1 ml of BHI–S broth and then plated on selective media BHI–S with rifampicin (20 μg ml^{-1}), gentamycin (100 μg ml^{-1}) and erythromycin (10 μg ml^{-1}). Rifampicin and gentamycin select against the *E. coli* donor and helper, whereas erythromycin selects for the presence of pFD288 or pFD340 in the recipient *B. fragilis* 638R. Plates were returned to anaerobic conditions and incubated anaerobically at 37 °C overnight for 24–48 h. Finally, transconjugants were purified on selective media and then screened for plasmid content.

2.4. Susceptibility testing

Susceptibility testing with Etests was performed as recently described [11].

2.5. Induction of metronidazole resistance in vitro

Metronidazole resistance was induced as previously described [11] by passaging 638R, 638R pIP417 *nimA*, 638R pFD288 *nimA*, and 638R pFD340 *nimA* on WC agar plates containing doubling metronidazole concentrations with every passage, i.e. 0.5, 1, 2, 4, 8, 16, 32 and 64 μg ml^{-1}.

2.6. RT-qPCR

RT-qPCR and subsequent quantitative analysis were exactly

performed as described earlier [11] using the *rpoD* and *gapdh* genes as internal standards. The primers used are given in Supplementary Table 1.

2.7. Measurement of plasmid yields from 638R

The densities of overnight cultures of 638R pIP417 *nimA*, 638R pFD288 *nimA*, and 638R pFD340 *nimA* were measured at $\lambda = 600$ nm in a UV/Vis spectrophotometer (Perkin-Elmer Lambda 25). Subsequently, the cells were processed for plasmid isolation using a GeneJET miniprep kit (Thermo). The concentrations of the isolated plasmids were measured at $\lambda = 260$ nm in a quartz cuvette and the amount of DNA was normalized to 10^9 cells of the respective cultures.

2.8. PFOR assay

PFOR activity in 638R and all daughter strains was measured as described before [11]. Briefly, assays were performed 1 ml reaction buffer containing 10 mM paraquat dichloride hydrate, 100 mM KH_2PO_4 pH 6.75, 250 mM β-mercaptoethanol, 2.5 mM sodium pyruvate, 0.1% Triton X-100, and 0.25 mM coenzyme A. The reaction buffer had been pre-incubated for 1h inside an anaerobic workstation (BugBox, Baker Ruskinn Technology Ltd) at 37 °C. Then, 10^5 cells in 50 μl 100 mM tris pH 7.5 were added, the cuvettes sealed with 2 layers of Parafilm™ and transferred to a UV/Vis spectrophotometer (Perkin-Elmer Lambda 25). PFOR activity was measured at $\lambda = 600$ nm over a period of 2 min at room temperature.

2.9. Statistical analysis

Statistical analysis was performed using GraphPad Prism 9 software. Details on the statistical tests applied are given in the respective figure legends.

3. Results

In order to assess if expression levels of the *nimA* gene do affect the degree of protection against metronidazole, the *nimA* gene was cloned into two different plasmids, pFD288 and pDF340. The *nimA* gene was amplified by PCR from clinical plasmid pIP417 which carries the IS*1168* and the downstream-positioned *nimA* gene. Strain 638R pIP417 *nimA*, previously designated as 638R *nimA* [11,19], had already been available to us prior to the start of this study. Shuttle vector pFD288 provides a multiple cloning site (MCS) but no plasmid-borne promoter driving the expression of the cloned gene [15]. Thus, in order to ensure transcription of *nimA*, the *nimA* gene was inserted into pFD288 together with the complete preceding IS*1168*. Shuttle vector pFD340, however, has its MCS positioned downstream of IS*4351* which provides a promoter [16]. This allowed cloning of the *nimA* gene into pFD340 without IS*1168* save 20 bp upstream of the start codon containing the translation initiation site. The resulting plasmids were propagated in *E. coli* NEB 10-Beta and introduced into *B. fragilis* 638R by tri-parental mating. The resulting strains were designated 638R pFD288 *nimA* and 638R pFD340 *nimA*, respectively.

We hypothesized that 638R pIP417 *nimA*, 638R pFD288 *nimA*, and 638R pFD340 *nimA* would display different expression levels of *nimA*, depending on the promoter and/or the number of plasmids per cell, and performed RT-qPCR on isolated RNA from all three strains. Indeed, the differences in *nimA* expression were profound, with the *nimA* mRNA level in 638 pIP417 *nimA* being approximately 7 times higher than in 638R pFD340 *nimA* (Fig. 1). In 638R pFD288 *nimA* the *nimA* mRNA level was intermediately high (Fig. 1). These differences in mRNA abundance were not due to significantly different numbers of plasmid copies per cell as determined by measuring the yields of each plasmid isolated from the harbouring strain 638R. In fact, pFD340 was present in numbers at least as high as pFD288 and pIP417, i.e. 110 ± 29 ng per 10^9 cells versus 75 ± 8 ng and 72 ± 21 ng per 10^9 cells, respectively. All three plasmids are approximately of the same size (between 8 and 9 kb).

Next, MICs were determined by Etests for 638R and the three daughter strains in order to test if the different *nimA* expression levels observed would lead to distinctly pronounced protection from metronidazole. This was indeed the case as protection from metronidazole was clearly strongest in 638R pIP417 *nimA*, whereas in 638R pFD340 *nimA* the MIC for metronidazole was barely higher than in the parental strain 638R (Table 1). In 638R pFD288 *nimA* the MIC for metronidazole was clearly elevated as compared to 638R but well below the MIC in 638R pIP417 *nimA* (Table 1).

Finally we measured PFOR activity in the parental strain 638R and the three *nimA*-harbouring daughter strains. In a recently published study, PFOR activity was found to be strongly elevated in 638R pIP417 *nimA* [11] as compared to 638R. Again, PFOR activity was about 2-fold higher in 638R pIP417 *nimA* than in 638R, whereas in 638R pFD288 *nimA* this effect was less pronounced (Fig. 2). In

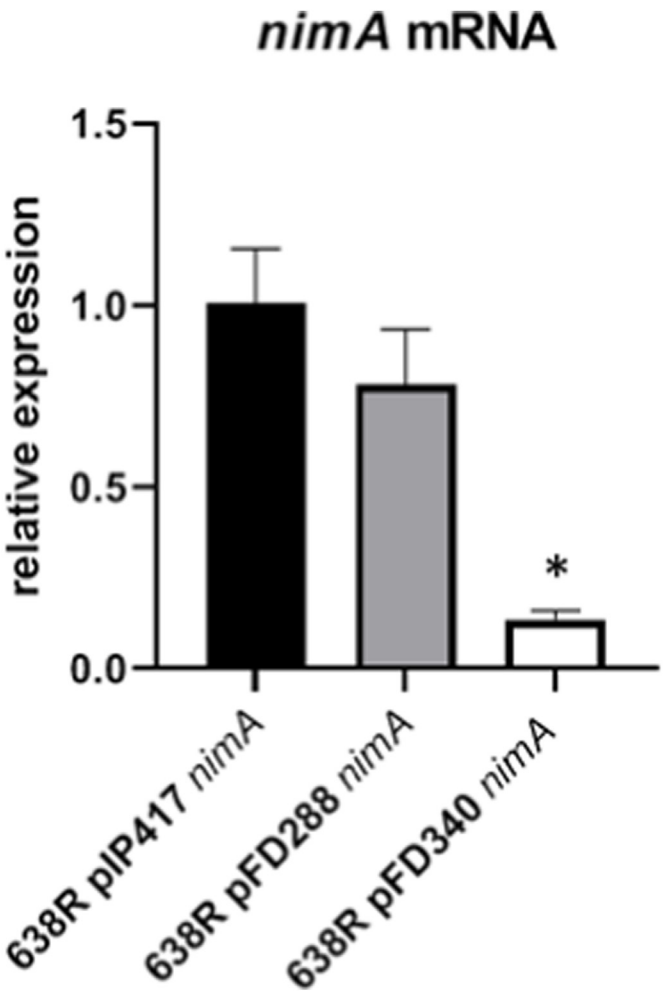

Fig. 1. A., relative abundance of *nimA* mRNA in 638R pIP417 nimA, 638R pFD288 *nimA*, and 638R pFD340 *nimA*. All strains were measured in three independent experiments in technical triplicates each. The asterisk indicates smaller than 638R pIP417 *nimA* with $p < 0.05$. Statistics: a Kruskal-Wallis test upon ranks with an ensuing Dunn's test for multiple analyses was performed. B., relative abundance of *nimA* mRNA in 638R pIP417 nimA after induction of high level resistance.

Table 1
Etests for metronidazole with *B. fragilis* 638R and its daughter strains.

Strain	638R (5 tests)	+ pIP417 *nimA* (3 tests)	+ pFD288 *nimA* (4 tests)	+ pFD340 *nimA* (6 tests)
MIC metronidazole **(μg ml^{-1})**	0.19–0.25	2–3	0.38–0.75	0.25–0.38

638R pFD340 *nimA* PFOR activity was approximately equal to the one in 638R. Thus, as observed before with the protection against metronidazole (Table 1), the expression level does affect the enhancing effect of *nimA* on PFOR activity. When high-level metronidazole was induced in 638R and the three daughter strains with a *nimA* gene, PFOR activity was only maintained in highly-resistant 638R pIP417 *nimA* (Fig. 2). This highly resistant strain showed a discernible but not statistically significant increase in *nimA* mRNA levels as compared to the 638R pIP417 *nimA* parent (Supplementary Fig. 1) and was not inhibited by Etest strips for metronidazole even at the highest concentration (256 μg ml^{-1}). In the other strains, PFOR activity had been lost upon induction of resistance (Fig. 2). This indicates that the *nimA*-specific route of resistance development as described previously [11] is also correlated with the expression level of *nimA*.

4. Discussion

The protective effect of *nim* genes in *Bacteroides* against metronidazole has been unequivocally and repeatedly demonstrated [6,13,20] but the mode of protection they confer has remained unresolved. Indirect evidence favoured the notion that Nim proteins could act as nitroreductases which deactivate metronidazole through reducing the nitro group of the drug to a comparably harmless amino group [8,9]. However, reduction of metronidazole by a Nim protein has not been demonstrated to date in an appropriate assay and the observation that Nim levels do not correspond with the level of resistance measured [10,11] are difficult to reconcile with a potential role of Nim proteins as nitroreductases. Indeed, the mode of action of Nim proteins could be much more complex and more indirect than anticipated. Recently it was shown that *nimA* elevates PFOR activity in *B. fragilis* 638R [11], probably by enhancing iron import. After induction of high-level metronidazole resistance, PFOR was still active in strain 638R pIP417 *nimA* but inactive in 638R [11]. This was accompanied by the preservation of *hmuY* expression in 638R pIP417 *nimA* with high-level induced resistance, but a sharp decrease of *hmuY* expression in highly resistant 638R. Since HmuY is a major factor in the import of haemin [13], the main source of iron in typical growth media for *B. fragilis*, this suggests that iron levels are much higher in resistant 638R pIP417 *nimA* than in resistant 638R.

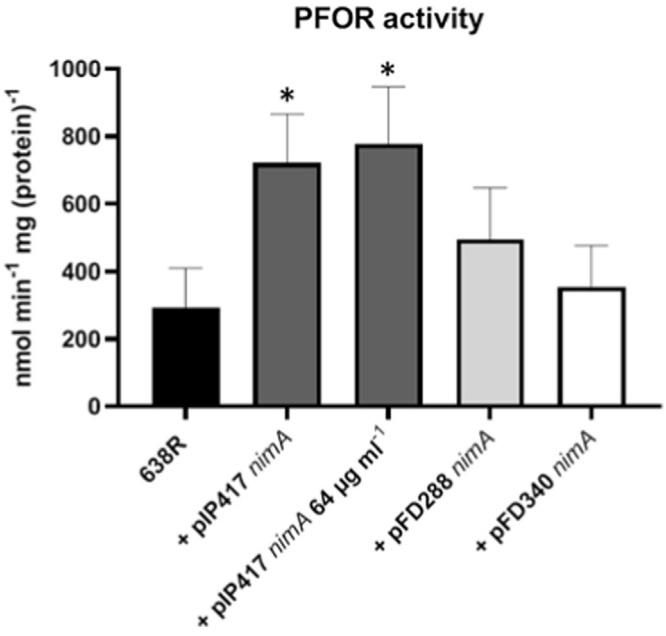

Fig. 2. PFOR activity in 638R, 638R pIP417 *nimA*, 638R pFD288 *nimA*, 638R pFD340 *nimA*, and metronidazole-resistant 638R pIP417 *nimA* cultivated in the presence of 64 μg ml^{-1} metronidazole (638R pIP417 *nimA* 64 μg ml^{-1}). Number of measurements: 638R, 8 times; 638R pIP417 nimA, 6 times; 638R pIP417 *nimA* 64 μg ml^{-1}, 4 times; 638R pFD288 *nimA*, 5 times; 638R pFD340 *nimA*, 5 times. Asterisks indicate higher than 638R with $p < 0.01$. Statistics: a Kruskal-Wallis test upon ranks with an ensuing Dunn's test for multiple analyses was performed.

The enhancing effect of *nimA* on PFOR activity could be reproduced in this study (Fig. 2), but it was clearly correlated with the expression level of *nimA* (Fig. 1). Indeed, the correlation of *nimA* expression levels and the degree of protection against metronidazole conferred (Table 1) or the degree of enhancement of PFOR activity, respectively, seems to follow a simple dose-response relationship. This contrasts with the observations in strains with induced metronidazole resistance in which *nim* expression levels are not further increased [10,11]. Thus, *nim* expression levels correlate with the degree of initial low-level protection against metronidazole but are not mediating higher-level protection. Furthermore, the expression level of *nimA* was shown to correlate with the path of resistance development because PFOR activity was retained after induction of high-level resistance in 638R pIP417 *nimA* only, i.e. the strain with the highest *nimA* expression level. The results of this study add another layer of complexity to the mode of action of *nim* genes but might provide a potential rationale for the observation that not all clinical isolates harbouring a *nim* gene display reduced susceptibility or resistance to metronidazole [21]. It is possible that the expression of *nim* genes in these strains remains beneath the threshold at which *nim*-mediated effects become manifest, quite comparably to strain 638R pFD340 *nimA* in this study.

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Declaration of competing interest

The authors declare that there is no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.anaerobe.2022.102630.

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