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Haemin deprivation renders *Bacteroides fragilis* hypersusceptible to metronidazole and cancels high-level metronidazole resistance

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Background: Infections with *Bacteroides fragilis* are routinely treated with metronidazole, a 5-nitroimidazole antibiotic that is active against most anaerobic microorganisms. Metronidazole has remained a reliable treatment option, but resistance does occur, including in *B. fragilis*.

Objectives: In this study we tested whether haemin, a growth supplement for *B. fragilis in vivo* and *in vitro*, had an influence on the susceptibility of resistant *B. fragilis* strains to metronidazole. We further tested whether haemin-deprived *B. fragilis* would be more susceptible to oxygen and oxidative stress. Metronidazole has been described to cause oxidative stress, which we argued would be exacerbated in haemin-deprived *B. fragilis* because the bacteria harness haemin, and the iron released from it, in antioxidant enzymes such as catalase and superoxide dismutase.

Methods: Haemin was omitted from growth media and the effect on metronidazole susceptibility was monitored in susceptible and resistant *B. fragilis* strains. Further, haemin-deprived *B. fragilis* were tested for resistance to aeration and hydrogen peroxide and the capacity for the removal of oxygen.

Results: Omission of haemin from the growth medium rendered metronidazole-resistant *B. fragilis* strains, including an MDR isolate from the UK, highly susceptible to metronidazole. Haemin deprivation further rendered *B. fragilis* highly susceptible to oxygen, which was further exacerbated in resistant strains. *B. fragilis* was incapable of scavenging oxygen when haemin was omitted.

Conclusions: We propose that haemin deprivation overrules resistance mechanisms by rendering *B. fragilis* hypersusceptible to metronidazole due to a compromised antioxidant defence. Monitoring of haemin concentrations is imperative when conducting metronidazole susceptibility testing in *B. fragilis*.

Introduction

Bacteroides fragilis is a very common commensal in the human gut.[1] It can, however, also cause severe infections, either in the intestinal tract or in ectopic sites, mainly through formation of abscesses.[2] Presently, carbapenems and metronidazole are the most reliable options for the treatment of *B. fragilis* infections.[3] Despite being relatively rare, treatment failures with metronidazole due to resistance do occur.[4] In many cases, resistant *B. fragilis* strains harbour a *nim* gene,[5] but not always.[6] Nim proteins are believed to act as nitroreductases, which reduce the nitro group in metronidazole to a non-reactive amino group through a transfer of six electrons.[7]

Previously, haemin was found to have a marked protective effect against metronidazole and hydrogen peroxide in the closely related oral pathogen *Porphyromonas gingivalis*.[8] Haemin is also standardly added to growth media for *B. fragilis* and has a strong impact on the metabolism[9] and the activity of catalase.[10]

In this study we determined susceptibility to metronidazole in a highly metronidazole-resistant clinical isolate, either with or without supplementation of haem in the growth medium. Likewise, the effect of haemin on metronidazole susceptibility was determined in a laboratory strain with a *nim* gene and compared to its *nim*-free parent.

Materials and methods

Strains used in the study

The MDR strain *B. fragilis* R19811, with a reported metronidazole MIC of 256 mg/L, was originally isolated in the UK from a 48-year-old woman[6]

suffering from sepsis, and does not harbour any known *nim* gene.[11] Strain *B. fragilis* 638R has been used as recipient for the *nimA* gene located on plasmid pI417.[12] The *nimA* gene is positioned behind insertion element IS*1168*.[13] The transconjugant of 638R harbouring pI417 is referred to as 638R *nimA* throughout the manuscript. Strain *B. fragilis* 388/1 was isolated in Kuwait and was reported to display intermediate metronidazole resistance (MIC of 16 mg/L);[14] it has a *nimE* gene.[15]

Growth media

For routine subculture, *B. fragilis* strains were grown on Wilkins–Chalgren (WC) anaerobe agar (Oxoid). Susceptibility assays were performed on brain heart infusion (BHI) plates (Carl Roth), either with or without 1 mg/L vitamin K (Carl Roth) and 5 mg/L haemin (Sigma–Aldrich). All cultures were maintained in anaerobic jars using Anaerocult A (Merck) for generating an anaerobic atmosphere (0% O_2 and 18% CO_2). Etests were purchased from bioMérieux.

Susceptibility testing

Susceptibility tests were performed on BHI agar plates with Etests (three times each). Plates were transferred to anaerobic jars and incubated for 48 h at 37°C. Etest images were obtained using Bio-Rad GelDoc XR.

Oxygen-scavenging measurements

Oxygen-scavenging measurements were performed as described previously[16] but with a larger number of cells (2×10^9).

Experiments on oxygen and hydrogen peroxide exposure

Cells were grown in BHI medium with or without vitamin K and haemin until an OD_{600} of 0.5 was reached, and serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) were prepared. Five microlitres of each dilution was spotted onto BHI agar plates. Before anaerobic incubation, cells were exposed to air inside an aerobic incubator at 37°C for 0, 48, 72 and 96 h. After air exposure, cells were incubated for 48 h at 37°C inside anaerobic jars.

Microbial susceptibility to hydrogen peroxide was assayed by applying a sterile blank disc (Oxoid) saturated with 10 μL of 500 mM hydrogen peroxide on the agar plates, followed by incubation for 48 h at 37°C. A hydrogen peroxide concentration of 500 mM had been found to be optimal in preliminary experiments. Disc assay images were obtained using Bio-Rad GelDoc XR.

Catalase assay

Overnight cultures were harvested (centrifugation for 10 min at 3000 × **g**) and washed with 0.05 M phosphate buffer (pH 7.0). For lysis, an inverted-cup sonicator set on 10 cycles of pulse-on time 30 sec every 30 sec at 4°C was used. Cell debris was removed (12 000 × **g**, 20 min, 4°C). Bovine liver catalase was used as standard. Catalase activity was measured in 0.05 M phosphate buffer, with 5 mM hydrogen peroxide in a UV transparent cuvette (λ = 240 nm) at room temperature. Ten micrograms of protein from the cell extract were used per reaction.

Results

Omission of haemin from growth medium abolishes metronidazole resistance

Based on observations with *P. gingivalis* that haemin protected bacteria from metronidazole,[8] it was speculated that haemin would also affect the susceptibility of *B. fragilis* to metronidazole. Etests were initially performed with the MDR strain *B. fragilis* R19811[6] (henceforth referred to as R19811), which is highly resistant to metronidazole but lacks a *nim* gene,[11] and with the strain *B. fragilis* 638R (henceforth referred to as 638R). A transconjugant of 638R, which harboured plasmid pI417 carrying a *nimA* gene (henceforth referred to as 638R *nimA*), was also available. High-level resistance in R19811 (Table 1) and reduced susceptibility to metronidazole in 638R *nimA* was observed when Etests for metronidazole were performed with haemin and vitamin K1. After omission of vitamin K1 alone, no effect was observed (data not shown). Thus, for convenience, supplementation of vitamin K1 alongside haemin was dropped in the subsequent experiments. Next, the same strains were tested without supplementation of haemin. Before performing the Etests, cells had previously been subcultured without haemin at least three times. For R19811, Etests on plates without haemin gave metronidazole MICs that were 80- to 250-fold lower than with haemin (Table 1). In 638R *nimA*, a similar effect was observed (Table 1). In order to test whether this effect was specific for metronidazole, the same strains were also assayed for imipenem, tetracycline, clindamycin and erythromycin (Table 1). Overall, the MICs of these antibiotics were only slightly affected, or even unaffected, without haemin.

Table 1. Metronidazole, tetracycline, erythromycin, clindamycin and imipenem Etest MICs, either with or without haemin supplementation (performed three times each)

		MIC (mg/L)				
Strains	Haemin	Metronidazole	Tetracycline	Erythromycin	Clindamycin	Imipenem
R19811	+	48/48/16	32/32/32	2/2/3	<0.016/<0.016/<0.016	>32/>32/>32
	−	0.19/0.19/0.38	32/32/32	0.38/0.38/0.38	<0.016/<0.016/<0.016	>32/>32/>32
638R *nimA*	+	1/3/1.5	0.094/0.094/0.094	0.38/0.38/0.38	0.047/0.047/0.047	0.047/0.047/0.094
	−	0.032/0.032/0.047	0.064/0.064/0.064	0.032/0.047/0.047	0.064/0.125/0.125	0.016/0.016/0.016
638R	+	0.25/0.25/0.25	0.064/0.064/0.064	0.25/0.38/0.38	0.25/0.25/0.25	0.064/0.064/0.064
	−	0.064/0.016/0.047	0.094/0.047/0.094	0.125/0.125/0.125	0.064/0.064/0.064	0.047/0.047/0.047
388/1	+	256/256/256	32/32/32	2/2/1	<0.016/<0.016/<0.016	0.25/0.25/0.25
	−	0.125/0.19/0.5	32/32/32	0.19/0.19/0.19	<0.016/<0.016/<0.016	0.25/0.25/0.25

+, growth medium supplemented with haemin; −, growth medium not supplemented with haemin.

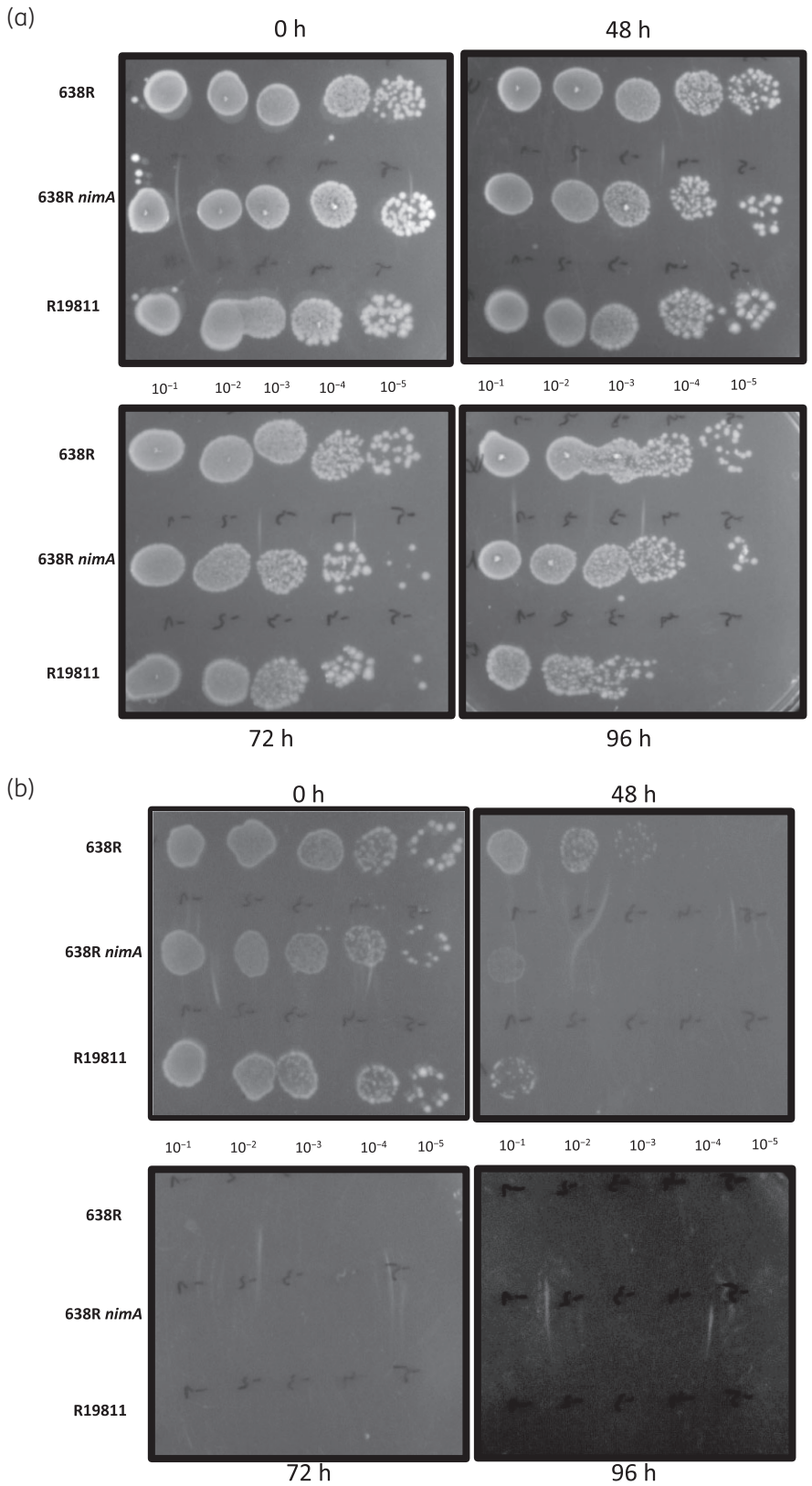

Figure 1. (a) Cell survival assays with haemin supplementation following oxygen exposure for the time periods indicated. Dilutions are indicated in the middle between upper and lower panels. (b) Cell survival assays without haemin supplementation with oxygen exposure for the time periods indicated. Dilutions are indicated in the middle between upper and lower panels.

In addition to R19811 and 638R *nimA*, a highly metronidazole-resistant Kuwaiti isolate, *B. fragilis* 388/1[14] with an identified *nimE* gene,[15] was also tested. Again, a similar effect was observed. With haemin supplementation, growth of *B. fragilis* 388/1 was not inhibited by metronidazole at all, indicating resistance with an MIC of >256 mg/L. Without haemin supplementation, growth was inhibited at 0.25 mg/L metronidazole (Table 1).

Impact of haemin deprivation on the antioxidant defence

It was hypothesized that haemin deprivation would negatively affect the antioxidant defence in *B. fragilis*. This, in turn, might render cells more susceptible to metronidazole because it is known that metronidazole targets the cellular redox system.[7] The negative effect of haemin deprivation on catalase function was confirmed with catalase activity decreasing to only 5%–15% of original levels (R19811: 5 versus 80 U/mg/min; 638R *nimA*: 2 versus 26 U/mg/min; 638R: 6 versus 45 U/mg/min; and 388/1: 11 versus 58 U/mg/min). As expected, haemin deprivation diminished the ability of the tested strains to survive in the presence of oxygen (Figure 1). R19811, 638R *nimA* (Figure 1) and 388/1 were clearly more susceptible to oxygen than WT 638R, with the numbers of surviving cells after 48 h being smaller by about two orders of magnitude.

We hypothesized that the increased oxygen susceptibility of haemin-deprived *B. fragilis* could be due to an impairment of oxygen removal mechanisms. Thus, removal of oxygen from growth medium by R19811, 638R *nimA* and 638R was measured with an oxygen sensor. After growth with haemin, very pronounced oxygen-scavenging rates could be observed, with 50% to 65% of the oxygen in the test medium being removed after 60 min (Figure S1, available as Supplementary data at *JAC* Online). After growth without haemin, however, oxygen scavenging was almost completely inhibited (Figure S1).

As catalase depends on haemin for activity, we hypothesized that haemin deprivation would render *B. fragilis* more susceptible to hydrogen peroxide. Thus, all strains were tested for hydrogen peroxide susceptibility in disc diffusion assays, either with or without haemin. Surprisingly, haemin deprivation increased susceptibility to hydrogen peroxide minimally, if at all (data not shown).

Discussion

In this study we present evidence that omission of haemin from the growth medium can specifically nullify high-level metronidazole resistance in *B. fragilis*. Our results suggest that haemin deprivation renders *B. fragilis* more susceptible to metronidazole, thereby possibly overruling resistance mechanisms in place. Metronidazole induces oxidative damage in the anaerobic cell,[7] which might be further exacerbated in haemin-deprived *B. fragilis*. In many body sites where it causes abscesses, *B. fragilis* cannot avoid oxygen. Exposure to oxygen, however, elicits the formation of reactive oxygen species such as superoxide or hydrogen peroxide. Since one of the most important enzymes involved in hydrogen peroxide removal, i.e. catalase, depends on haemin it was expected that haemin-deprived *B. fragilis* would be more susceptible to hydrogen peroxide but this was not the case. Possibly, however, metronidazole deactivates other enzymes involved in hydrogen peroxide removal such as thioredoxin peroxidase, AhpCF or rubrerythrins.[17] It would be important to find an explanation for the enhanced susceptibility to oxygen in haemin-deprived metronidazole-resistant *B. fragilis* (Figure 1). Possibly, this weakness could be exploited for countermeasures against metronidazole-resistant *B. fragilis* in the future.

Finally, it is important to note that enhanced susceptibility to metronidazole through haemin deprivation is not restricted to *B. fragilis* as similar observations were made very recently in *Clostridioides difficile*.[18,19] It is likely that haemin concentrations in the medium have a very strong effect on metronidazole susceptibility testing of anaerobes in general, which should be taken into account by the bodies responsible for antibiotic resistance monitoring.

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Transparency declarations

The authors declare that there are no competing financial interests.

Supplementary data

Figure S1 is available as Supplementary data at *JAC* Online.

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