Overexpression of the rhamnose catabolism regulatory protein, RhaR: a novel mechanism for metronidazole resistance in Bacteroides thetaiotaomicron

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Objectives: The aim of the investigation was to use in vitro transposon mutagenesis to generate metronidazole resistance in the obligately anaerobic pathogenic bacterium Bacteroides thetaiotaomicron, and to identify the genes involved to enable investigation of potential mechanisms for the generation of metronidazole resistance.

Methods: The genes affected by the transposon insertion were identified by plasmid rescue and sequencing. Expression levels of the relevant genes were determined by semi-quantitative RNA hybridization and catabolic activity by lactate dehydrogenase/pyruvate oxidoreductase assays.

Results: A metronidazole-resistant mutant was isolated and the transposon insertion site was identified in an intergenic region between the rhaO and rhaR genes of the gene cluster involved in the uptake and catabolism of rhamnose. Metronidazole resistance was observed during growth in defined medium containing either rhamnose or glucose. The metronidazole-resistant mutant showed improved growth in the presence of rhamnose as compared with the wild-type parent. There was increased transcription of all genes of the rhamnose gene cluster in the presence of rhamnose and glucose, likely due to the transposon providing an additional promoter for the rhaR gene, encoding the positive transcriptional regulator of the rhamnose operon. The B. thetaiotaomicron metronidazole resistance phenotype was recreated by overexpressing the rhaR gene in the B. thetaiotaomicron wild-type parent. Both the metronidazole-resistant transposon mutant and RhaR overexpression strains displayed a phenotype of higher lactate dehydrogenase and lower pyruvate oxidoreductase activity in comparison with the parent strain during growth in rhamnose.

Conclusions: These data indicate that overexpression of the rhaR gene generates metronidazole resistance in B. thetaiotaomicron

Keywords: antibiotic resistance, anaerobes, rhamnose regulation

Introduction

Bacteroides fragilis and Bacteroides thetaiotaomicron are members of the normal resident gastrointestinal microbiota, and are important opportunistic pathogens that are capable of causing life-threatening infections such as peritonitis, serious gynaecological sepsis, bacteraemia, and soft tissue and brain abscesses.1,2 Metronidazole [1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole] is commonly used in the treatment of these anaerobic microbial infections. In addition, it is used for routine prophylaxis during gastrointestinal tract and gynaecological surgery. It is administered as an inert prodrug that is activated intracellularly under anaerobic conditions by the reduction of its nitro group. This generates an active toxic derivative, which causes single- and double-strand DNA breaks.3 In certain anaerobic protozoa, activation of metronidazole involves a low intracellular redox potential process that results in the reduction of the 5-nitro group forming the transient toxic radicals.4 The

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Plasmids

Strains

Tcr, tetracycline-resistant; Apr, ampicillin-resistant; Tpr, trimethoprim-resistant; Mob, mobilization region on plasmid.

| Strain or plasmid | Relevant characteristics | Source or reference |
|-------------------|--------------------------|---------------------|
| **Strains** B. thetaiotaomicron | | |
| VPI-5482 | clinical isolate: wild-type | ATCC 29148 |
| Tn MetR | VPI-5482 containing the Tn4400' transposon from pYT646B; (Tc') | this study |
| E. coli rhaR<sup>(+)</sup> | VPI-5482 containing rhaR gene on pLYLrhaR in cytoplasm; (Tc') | this study |
| DH5α | F<sup>−</sup> end A1 hsdR17<sup>(tk− m<sub>K</sub>)</sup> supE44 thi-l mutant recA1 gyrA96 relA1Δ(argF-lacZYA)U169 φ80dlacZM15 | 15 |
| S17-1 | contains the transfer functions of RP4 integrated in the chromosome; recA Tp' T<sub>fr</sub>R<sub>4</sub> | 37 |
| **Plasmids** | | |
| pYT646B | delivery vector carrying Tn4400' transposon | 17 |
| pLYL01 | 2.6 kb SstI fragment containing the tetQ gene from pNFD13-2 cloned into the AatII site of pFD160R; Ap' (Tc') Mob | 22 |
| pLYLrhaR | pLYL01 containing rhaR and 239 bp upstream of rhaR; Ap' (Tc') Mob | 14 |

Te', tetracycline-resistant; Ap', ampicillin-resistant; Tp', trimethoprim-resistant; Mob, mobilization region on plasmid.

Parentheses around the antibiotic resistance phenotype indicate that it is expressed only in E. coli. If there are no parentheses, then the antibiotic resistance phenotype is expressed in B. thetaiotaomicron only.

B. thetaiotaomicron cultures were grown anaerobically in brain heart infusion broth supplemented with haemin, menadione and cysteine (BHIS) or BHIS agar (1.5% w/v) as previously described. Escherichia coli strains were grown with aeration in Luria-Bertani (LB) broth or on LB agar plates (1.5% w/v) at 37°C. The antibiotics gentamicin (200 mg/L), ampicillin (100 mg/L), tetracycline (2 mg/L) and erythromycin (10 mg/L) were added to BHIS or LB whenever necessary. E. coli strains carrying plasmids were grown in LB medium supplemented with ampicillin (100 mg/L). Carbohydrate utilization studies were performed by growing B. thetaiotaomicron in defined medium (DM) supplemented with 0.1% of either glucose (DMG) or rhamnose (DMR) (Sigma-Aldrich) as the sole carbon source. Growth of the organisms was measured as increase in optical density at 600 nm (OD<sub>600</sub>) over time. Metronidazole survival studies were performed by growing B. thetaiotaomicron cultures to mid-exponential phase in DMR or DMG broth followed by exposure to 15 mg/L metronidazole. Viable counts were determined anaerobically at various time-points by plating culture dilutions in triplicate on BHIS agar plates and incubation at 37°C for 72 h.

**Methods**

**Bacterial cultivation, strains and plasmids**

The bacterial strains and plasmids used in this study are listed in Table 1.

| Table 1. Bacterial strains and plasmids |
|---------------------------------------|
| Strain or plasmid | Relevant characteristics | Source or reference |
|-------------------|--------------------------|---------------------|
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| DH5α | F<sup>−</sup> end A1 hsdR17<sup>(tk− m<sub>K</sub>)</sup> supE44 thi-l mutant recA1 gyrA96 relA1Δ(argF-lacZYA)U169 φ80d lacZ M15 | 15 |
| S17-1 | contains the transfer functions of RP4 integrated in the chromosome; recA Tp' T<sub>fr</sub>R<sub>4</sub> | 37 |
| **Plasmids** | | |
| pYT646B | delivery vector carrying Tn4400' transposon | 17 |
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| pLYLrhaR | pLYL01 containing rhaR and 239 bp upstream of rhaR; Ap' (Tc') Mob | 14 |
were used as DNA probes for hybridization and detection procedures were performed according to the manufacturer’s instructions (Roche). Hybridization and detection procedures were performed according to the manufacturer’s instructions. For identification of the transposon insertion site, genomic DNA from the mutant was digested with HindIII and the fragment adjacent to the transposon was isolated by plasmid rescue. The chromosomal DNA in the resulting ampicillin-resistant plasmid was sequenced using primer L58, derived from IS4400R in the transposon. The primers listed in Table 2 were used to amplify the 5’–3’ junction ends of the transposon insertion in the B. thetaiotaomicron Tn metR gene. Hybridization and detection procedures were performed according to the manufacturer’s instructions. For identification of the transposon insertion site, genomic DNA from the mutant was digested with HindIII and the fragment adjacent to the transposon was isolated by plasmid rescue.

The full-length rhaR gene, including 239 bp upstream of the gene, was PCR amplified using gene-specific primers (Table 2). The 16S rRNA gene of B. thetaiotaomicron VPI-5482 was used as an internal control (Table 2). The PCR, DNA hybridization, detection procedures and analysis were carried out as previously described by Patel et al. Signal intensities were measured using a densitometer and analysed with GeneSnap 6.05 software (Synoptics Ltd). Results from duplicate experiments were expressed as the ratio of the gene-specific transcription signal of mutant cells compared with wild-type cells (induction ratio).

**Construction of B. thetaiotaomicron rhaR overexpresser strain**

The full-length rhaR gene, including 239 bp upstream of the gene, was PCR amplified using primers cRF and cRR (Table 2). The PCR product was digested with HindIII and XmnI, cloned into the shuttle vector pLYL01 and transformed into E. coli DH5α. The resultant plasmid, pLYLrhaR, was extracted, purified and retransformed into E. coli S17-1 according to standard methods. Mobilization of pLYLrhaR from E. coli S17-1 (pLYLrhaR) into B. thetaiotaomicron VPI-5482 was performed by the conjugation procedure of Li et al. The resulting transconjugant, B. thetaiotaomicron rhaR(+) was grown on BHIS agar with tetracycline (2 mg/L).

**Preparation of cell-free extracts (CFEs) and enzyme assays**

B. thetaiotaomicron VPI-5482 cultures for CFEs were grown anaerobically to late log phase in DMR broth. Cells were harvested by centrifugation (8,000 rpm, 10 min), pellets were washed three times under anaerobic conditions in 10 mM Tris–HCl, pH 8.0 (for the lactate dehydrogenase assay) or 10 mM phosphate buffer with 250 μM β-mercaptoethanol, pH 7.0 (for the pyruvate oxidoreductase assay), and resuspended in 5 mL of the same buffers, saturated with nitrogen.
respectively. Cells were disrupted by sonication using an MSE sonicator at an amplitude of 18–24 μm for periods of 30 s/mL of cell suspension in tubes chilled at 0°C. Cell debris was removed by centrifugation (8 K, 4°C, 20 min). The supernatant was collected (CFE) and protein concentrations were determined using Bio-Rad reagents according to the manufacturer’s instructions (Bio-Rad Laboratories). Lactate dehydrogenase activity was measured anaerobically at 37°C by the rate of decrease in absorbance of NADH at a wavelength of 340 nm using a Beckman DU®530 Lifescience UV/Vis Spectrophotometer according to the method of Abbe et al.23 Enzyme activity was recorded as μM NADH/mg of protein/min at 340 nm. Pyruvate oxidoreductase activity was measured spectrophotometrically at an absorbance of 600 nm using a Beckman DU®530 Lifescience UV/Vis Spectrophotometer, with methyl viologen as the electron acceptor at 37°C under anaerobic conditions using the method of Lindmark and Müller.24 The enzyme activity was recorded as μM methyl viologen/mg of protein/min at 600 nm. All the experiments were performed in triplicate.

Results and discussion
Isolation of the metronidazole-resistant B. thetaiotaomicron mutant
Transposon mutagenesis was used to create metronidazole-resistant B. thetaiotaomicron mutants.17 Tetracycline-resistant transconjugants were detected at frequencies of 2×10⁻⁶ to 4×10⁻⁵ per E. coli donor cell. An average of 40% of tetracycline-resistant transconjugants were co-integrates, compared with the 4% co-integrate formation in B. fragilis reported by Tang and Malamy.17 The B. thetaiotaomicron transposon mutant bank (~2000 isolates) was screened for metronidazole resistance on BHIS agar using metronidazole concentrations varying from 0.5 to 8 mg/L. A mutant, B. thetaiotaomicron Tn MetR metronidazole-resistant mutant. (a) Position of the transposon insertion site. P KIPAOr and P R indicate the two promoters that have been identified with transcriptional activity. Location of the transposon insertion
Southern hybridization confirmed the presence of a single transposon insertion within the chromosome of the B. thetaiotaomicron Tn MetR mutant (data not shown). Plasmid rescue and junction PCR of the chromosomal DNA were performed in order to determine the precise location of the transposon within the mutant. The transposon insertion was found to be 141 bp upstream of rhaR, the gene encoding the positive transcriptional regulator of rhamnose catabolism in B. thetaiotaomicron (Figure 1a and b). We have demonstrated previously that this region contains the rhaR gene promoter sequence.14

Growth studies under inducing conditions
Growth studies were performed in order to investigate the effect of L-rhamnose or d-glucose on the growth of the metronidazole-resistant mutant. Results indicated that the mutant strain grown in DMR attained a higher cell density after 10 h when compared with the parent strain, with OD₆₀₀ values of 1.00±0.02 and 0.65±0.03, respectively. There was no significant difference in the cell density of the wild-type and mutant when cells were grown in DMG. This observation suggested that the transposon was improving the capacity of the cells to utilize rhamnose, possibly through increasing the levels of RhaR, the positive transcriptional regulator. The transposon did not, however, appear to affect the catabolism of glucose in the mutant cells.

Effect of the transposon on transcription
The levels of transcription of the genes of the rhamnose cluster were examined to determine if this was related to the enhanced growth of the MetR mutant when compared with the wild-type. Semi-quantitative transcriptional dot blot hybridization studies were performed using RNA extracted from cells grown in DMR or DMG. Results indicated that the transposon insertion in the intergenic region upstream of rhaR caused increased transcription of all the rhamnose genes in B. thetaiotaomicron Tn MetR as compared with the wild-type strain. In the presence of rhamnose, the genes rhaKIPAOr all showed an increase in transcription, expressed as induction ratios of mutant versus wild-type, of 3.4, 3.8, 3.4, 4.4, 3.4 and 1.7, respectively. During growth in glucose, an increase in transcription of these genes was also seen, with induction ratios of 11.1, 19.5, 14.4, 18.8, 15.5 and 1.5, respectively. The internal control 16S rRNA gene was transcribed at the same level in both wild-type and mutant strains.

![Figure 1](https://academic.oup.com/jac/article-abstract/64/2/267/716825)

**Figure 1.** Genomic arrangement of the rhamnose gene cluster in B. thetaiotaomicron VPI-5482 (accession number NC 004663) and the B. thetaiotaomicron Tn MetR metronidazole-resistant mutant. (a) Position of the transposon insertion site. P KIPAOr and P R indicate the two promoters that have been identified using sequencing and bioinformatic analysis.14 The dashed arrow shows the position of the Tn4400′ transposon insertion. (b) Schematic representation (not to scale) of the transposon insertion between rhaO and rhaR: IS4400R and IS4400L, insertion sequence elements; arrows indicate positions of primers used to confirm the insertion site (Table 2); tetQ, gene expressing tetracycline resistance in Bacteroides species; bla, gene expressing β-lactamase (ampicillin resistance) in E. coli; rhaO and rhaR, chromosomal genes of B. thetaiotaomicron VPI-5482.
under both growth conditions. The results show that even a relatively low level of increase in transcription of the positive regulator, RhaR, can bring about higher levels of transcription of all the genes of the rhamnose operon, even in the absence of rhamnose. In the presence of rhamnose, increased transcription resulted in improved utilization of the substrate and hence better growth as compared with the wild-type parent. During growth in glucose, the increased transcription of the rhamnose catabolic genes brought no additional nutrient benefit to the mutant cells and the same growth response as the wild-type was observed.

Based on the location of the transposon and the orientation of IS4400L relative to the transcriptional direction of rhaR (Figure 1b), it is possible that the IS element could be acting as an additional promoter leading to increased transcription. Figure 2 illustrates the DNA sequence obtained from sequence analysis of the transposon insertion site relative to the rhaR promoter region and coding sequence. Three possible typical Bacteroides putative −7 hexamers (consensus TAnnTTTG) were identified within the IS4400L sequence. This is in keeping with the findings of Bayley et al. who first described the B. fragilis consensus promoter and reported its presence in the IS4351 element, which is identical to IS4400 used in this study. It is not unusual for IS elements to act as strong promoters and activate genes and their role in naturally occurring antibiotic resistance is well documented. For example, Haggoud et al. first identified the IS1168 insertion sequence as being located upstream of the nitromidazole resistance genes (nim), and Soki et al. reported that nine nimA genes were activated by IS1168 and three of the nimC genes were activated by IS1170. In B. fragilis, Rogers et al. associated the IS1224 insertion upstream of the cepA β-lactamase gene with up-regulation and Podgajen et al. reported that IS1186 activated the expression of the metallo-β-lactamase cfaA gene, causing resistance to carbapenems. They later identified the particular Bacteroides consensus promoter sequences within two IS elements, IS1187 and IS1188, that were found upstream of cfaA. Based on the analysis of the IS4400L in relation to RhaR, it is likely that these consensus promoter elements within the IS4400L element are increasing the activation of the rhaR gene, as observed in the transcriptional studies.

Metronidazole resistance

The transposon insertion in the intergenic region between rhaO and rhaR was shown to increase the transcription of the rhamnose genes in B. thetaiotaomicron Tn MetR. It was, therefore, possible that higher levels of RhaR were causing the metronidazole resistance phenotype. In order to test this hypothesis, RhaR, the positive transcriptional regulator of the rhamnose operon,14 with its putative promoter region, was overexpressed in B. thetaiotaomicron VPI-5482 through introducing the rhaR gene into the parent strain on a multicopy plasmid to create B. thetaiotaomicron rhaR(+)L. Overexpression of the rhamnose genes in this strain was tested at the transcriptional level in defined medium supplemented with rhamnose, and the results indicated that overexpression of the RhaR protein resulted in increased transcription of these genes (data not shown). In order to test the metronidazole resistance phenotype, cells grown in defined medium supplemented with rhamnose or glucose were exposed to 15 mg/L metronidazole for 40 min. Results showed that the log10 of the surviving fractions of the B. thetaiotaomicron Tn MetR mutant and B. thetaiotaomicron rhaR(+)L, grown in DMR, were −1.65 (+0.007) and −1.88 (+0.010), respectively. These were more resistant than the parent strain, which had a log10 surviving fraction of only −2.78 (+0.001). The B. thetaiotaomicron Tn MetR mutant cells grown in DMG also showed metronidazole resistance relative to the parental strain, with the log surviving fractions of the mutant and wild-type being −1.75 (+0.013) and −2.62 (+0.001), respectively. This result supports the previously observed metronidazole resistance during isolation of the mutant on BHIS agar, a glucose-containing medium. These data represent the mean values of three experiments, with the standard deviation for the experiments shown in brackets. These results confirm the relationship between increased levels of RhaR and the observed metronidazole resistance, and demonstrate that the phenotype is not directly dependent on the catabolism of rhamnose, but suggest an additional as yet unknown regulatory role for the RhaR protein in relation to metronidazole resistance.

Figure 2. Position of IS4400L in relation to the transcriptional start site of rhaR. Nucleotide sequence of IS4400L (italics) upstream of the B. thetaiotaomicron rhaR promoter region and nucleotide sequence; −7 and −33 promoter recognition sequences, the +1 transcriptional start site (bold underlined) and the ATG start codon of rhaR (bold, box) are shown. The putative −7 hexamers within the IS element are shown in bold italics and are underlined.38

Pyruvate oxidoreductase and lactate dehydrogenase activity

The improved growth and a metronidazole resistance phenotype observed in both B. thetaiotaomicron Tn MetR and B. thetaiotaomicron rhaR(+)L during growth in the presence rhamnose could be due to increased expression of RhaR affecting the enzymes responsible for metronidazole activation. Given the proposed role of pyruvate oxidoreductase in metronidazole activation in anaerobes, and the role of lactate dehydrogenase...
in catabolism, biochemical assays were undertaken to determine the activity of the lactate dehydrogenase and pyruvate oxidoreductase in the metronidazole-resistant strains during growth in DMR (Table 3). The results showed that the lactate dehydrogenase activity of B. thetaiotaomicron Tn MetR and B. thetaiotaomicron rhaR(+) during growth in DMR, was 4–5 times higher than B. thetaiotaomicron VPI-5482, whereas pyruvate oxidoreductase activity in these resistant strains was 7–10 times lower in comparison with the parent strain (Table 3). These results suggested that the lower pyruvate oxidoreductase activity observed in the resistant strains could be contributing to the observed metronidazole resistance phenotype through a lack of metronidazole activation.

The mechanism of metronidazole resistance in bacteria is not well understood. However, in vitro studies have shown that resistant strains displayed higher levels of lactate or lactate dehydrogenase and, on occasion, no detectable levels of pyruvate oxidoreductase, a key enzyme that is thought to activate metronidazole.5 There have been no previous reports on lactate dehydrogenase or pyruvate oxidoreductase activity in any B. thetaiotaomicron metronidazole-resistant strains. Narikawa et al.6 however, reported high levels of lactate dehydrogenase in a B. fragilis metronidazole-resistant strain. In a more recent study, Diniz et al.8 studied a B. fragilis metronidazole-resistant mutant strain, generated by exposure to a sub-lethal concentration of metronidazole, and reported that lactate dehydrogenase activity was up-regulated, but flavodoxin was down-regulated, and there was no detectable pyruvate oxidoreductase activity in the resistant strain. They could not, however, recreate the level of metronidazole resistance observed in the spontaneous mutant by targeted genetic knock-out of either the pyruvate-ferredoxin oxidoreductase gene (porA) or flavodoxin genes. It is, therefore, possible that during growth in rhamnose metronidazole resistance in B. thetaiotaomicron Tn MetR and B. thetaiotaomicron rhaR(+) could be attributed to the lack of metronidazole activation via an altered electron flux driven by the up-regulation of rhamnose catabolism. It is possible that any mutations that give rise to the up-regulation of catabolic pathways associated with a concomitant decrease in pyruvate oxidoreductase activity generate metronidazole resistance within the Bacteroides spp.

The metronidazole resistance phenotype was, however, also seen in the B. thetaiotaomicron Tn MetR mutant cells grown in glucose, where the products of rhamnose catabolism would not have been a factor. While the results in the presence of rhamnose suggested that resistance was potentially due to reduced activation of metronidazole, the fact that the resistance phenotype was also observed in the presence of glucose makes this unlikely. It is, therefore, probable that the RhaR protein encoded by the rhaR gene may also possess additional, as yet unidentified, regulatory functions outside of the rhamnose operon that could affect the cellular responses to metronidazole. Xu et al.33 analysed the B. thetaiotaomicron genome sequence and observed that transcriptional regulatory genes, many of them of unknown function, are particularly well represented. We have previously reported that the RhaR protein shows conserved consensus domains typical of transcriptional regulators from the AraC helix-turn-helix (AraC/XylS) family of one-component sensor-regulator proteins.14 Members of the AraC family have been characterized as being involved in regulating three types of systems, namely pathogenesis, carbon metabolism and certain stress responses, and in the context of regulating stress responses, the proteins exert their regulatory role only when they are overproduced.34,35 We have previously shown that the reg gene product of B. fragilis, also a member of the AraC family, although lacking the putative cupin superfamily domain, is involved in cell survival following exposure to several damaging agents, including metronidazole.36 An equivalent gene, BT 1847, is present in the B. thetaiotaomicron genome. It remains to be determined if overexpression of the RhaR protein of B. thetaiotaomicron Tn MetR is functioning in a similar way to improve cell survival in the presence of metronidazole damage by inducing transcription of other, as yet unidentified, genes. Future work will involve proteomic comparison of the B. thetaiotaomicron wild-type and B. thetaiotaomicron Tn MetR mutant strains to identify the proteins that are up-regulated in response to the overexpression of the RhaR protein.

This is the first study to report a link between a carbohydrate catabolism regulatory protein and metronidazole resistance and presents a potentially novel mechanism for the generation of metronidazole resistance in Bacteroides spp.

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**Table 3.** Enzymatic activity of lactate dehydrogenase and pyruvate oxidoreductase during growth in rhamnose

| Strain                  | Lactate dehydrogenase | Pyruvate oxidoreductase |
|-------------------------|-----------------------|-------------------------|
| B. thetaiotaomicron VPI-5482 | 3.24 (±3.27)          | 0.230 (±0.06)           |
| B. thetaiotaomicron Tn MetR | 16.23 (±6.04)         | 0.035 (±0.05)           |
| B. thetaiotaomicron rhaR(+) | 12.08 (±3.50)         | 0.022 (±0.02)           |

Data are the mean values of three experiments (±SD).

aEnzymatic activity expressed as μM NADH/mg of protein/min.

bEnzymatic activity expressed as μM methyl viologen/mg of protein/min.
Metronidazole resistance in *Bacteroides thetaiotaomicron*

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**Transparency declarations**

None to declare.

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