JAC

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

Ekta H. Patel[1], Lynthia V. Paul[1], Ana I. Casanueva[1], Sheila Patrick[2] and Valerie R. Abratt[1]*

[1]*Department of Molecular and Cellular Biology, University of Cape Town, Rondebosch, South Africa;* [2]*Centre for Infection and Immunity, School of Medicine, Dentistry and Biomedical Sciences, Queen's University, Belfast BT9 7BL, UK*

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

*Corresponding author. Tel: +27-21-650-2183; Fax: +27-21-689-7573; E-mail: valerie.abratt@uct.ac.za

imidazole ring structure is thought to fragment producing two short-lived cytotoxic intermediates, acetamide and *N*-(2-hydroxyethyl)-oxamic acid.[5−7] Activation of the drug is likely to occur through interaction with a pyruvate oxidoreductase complex in an ATP-dependent reaction.[4,8] The pyruvate oxidoreductase complex is formed by a metabolic reaction between pyruvate and the oxidoreductase enzyme to facilitate pyruvate decarboxylation to form acetyl CoA. This reaction generates electrons that are accepted by ferredoxins or flavodoxins that have electron efficiency of -430 or -460 mV.[5] Studies on several *B. fragilis* species suggest that metronidazole activation occurs at a similar pyruvate oxidoreductase complex.[8−10] *Bacteroides* spp. can develop resistance to metronidazole via acquisition of nitroimidazole resistance genes (*nim*) that may be encoded on mobile genetic elements (reviewed by Patrick[11]). These encode a reductase that converts the nitro group of metronidazole into an amine, thus rendering it inactive.[3]

Overexpression of efflux pumps is known to play a role in the resistance of *Bacteroides* spp. to a range of antibiotics.[1] While this may also confer resistance to metronidazole, efflux pump inhibitors did not restore metronidazole susceptibility in a multiresistant *B. fragilis* that lacked *nim* genes,[12] suggesting that there are alternative mechanisms of resistance. In this study, we report the isolation and characterization of a *B. thetaiotaomicron* metronidazole-resistant mutant. We demonstrate for the first time a link between metronidazole resistance, the rhamnose catabolic pathway and the pyruvate oxidoreductase complex, and the overexpression of the regulator of the rhamnose catabolic pathway, RhaR.

Methods

Bacterial cultivation, strains and plasmids

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

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)[13] as previously described.[14] *Escherichia coli* strains were grown with aeration in Luria-Bertani (LB) broth[15] 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)[16] 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 an increase in optical density at 600 nm (OD_{600}) 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 timepoints by plating culture dilutions in triplicate on BHIS agar plates and incubation at 37°C for 72 h.

Tn4400′ transposon mutagenesis, Southern hybridization and identification of the transposon insertion site

The construction of a *B. thetaiotaomicron* VPI-5482 transposon mutant library was carried out using the transposon delivery vector, pYT646B, and the conjugation mutagenesis method of Tang and Malamy.[17] Selection of the transconjugant colonies was performed anaerobically by plating the conjugation mixture onto BHIS agar plates containing gentamicin and either tetracycline or erythromycin. Plasmid rescue was carried out according to Chen *et al.*[18] Putative metronidazole-resistant mutants were screened on BHIS agar plates containing metronidazole ranging in concentration from 0.5 to 8 mg/L. The mutant *B. thetaiotaomicron* Tn Met[R] was selected for further study. Southern blot analysis was performed according to standard protocols[15] using genomic DNA (20 μg) of both *B. thetaiotaomicron* VPI-5482 and the *B. thetaiotaomicron* Tn Met[R] mutant, as well as

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
B. thetaiotaomicron		
VPI-5482	clinical isolate: wild-type	ATCC 29148
Tn Met[R]	VPI-5482 containing the Tn4400′ transposon from pYT646B; (Tc[r])	this study
rhaR[(+)]	VPI-5482 containing *rhaR* gene on pLYLrhaR in cytoplasm; (Tc[r])	this study
E. coli		
DH5α	F^{−} *end A1 hsdR17*(r$_k^-$ m$_k^+$) *supE44 thi-l* mutant *recA1 gyrA96 relA1*Δ(*argF-lacZYA*)U169 Φ80d*lac*ZM15	15
S17-1	contains the transfer functions of RP4 integrated in the chromosome; *recA* Tp[r] Tra$_{RP4}$	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[r] (Tc[r]) Mob	22
pLYLrhaR	pLYL01 containing *rhaR* and 239 bp upstream of *rhaR*; Ap[r] (Tc[r]) Mob	14

Tc[r], tetracycline-resistant; Ap[r], ampicillin-resistant; Tp[r], 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.

Table 2. Oligonucleotides used for PCR

Designation	5′–3′ sequence	Use	Source or reference
L58	CAA TAA TGG ACC TCG TAA AAG	Tn junction PCR and plasmid rescue	17
TnOF	AAT CGT ACA TTC CAT GGC	Tn junction PCR	this study
TnRR	ACC GAA TTT GCG GTC TCC AT	Tn junction PCR	this study
Primer *c*	TAG CAA ACT TTATCC ATT CAG	Tn junction PCR	18
iKF	CCGTTATCTAAGCGGTGCC	DNA probe rhaK	14
iKR	CCGAAATTCTCTTCTTGCAGT	DNA probe rhaK	14
iIF	GCAATATTGATGAACTGCGTGCC	DNA probe rhaI	14
iIR	GATTTCTGCAATGAAATCTTCGC	DNA probe rhaI	14
iPF	GAAAATCGTCCGGCACTTGCC	DNA probe rhaP	14
iPR	TCATACCATCCGCTTCGAAGC	DNA probe rhaP	14
iAF	TATAGCCATCGGTAGCTTTTGCC	DNA probe rhaA	14
iAR	ATTACTAAAGGTTACGTTCAGCGCC	DNA probe rhaA	14
iOF	CTTATTTCGGTGCAGGATGCC	DNA probe fucO	14
iOR	CGAACATATCGCTCATAGCCC	DNA probe fucO	14
iRF	GCTGAAGGAGCATCC GTC	DNA probe rhaR	14
iRR	GTCTCATCGCCAGTTCCTCC	DNA probe rhaR	14
16SF	AGAGTTTGATCTTGGCTCAG	DNA probe 16S rRNA	14
16SR	ACGGTTACCTTGTTACGACTT	DNA probe 16S rRNA	14
cRF	GTGAGGAAGAGGATTCTCCGG	*rhaR* in pLYL01	14
cRR	ACAGATTTAAACGGAAAGCTTAAACTACATCG	*rhaR* in pLYL01	14

20 ng of the 13.1 kb transposon delivery vector, pYT646B, each digested to completion with restriction enzyme PvuII.[15] *B. thetaiotaomicron* total genomic DNA was prepared according to the method of Wehnert *et al.*[19] and *E. coli* plasmid DNA was prepared by the alkali-hydrolysis method of Ish-Horowicz and Burke.[20] The DNA probe used for hybridization was a 0.9 kb internal fragment of the pYT646B *tetQ* gene generated by digesting it with SacI and EcoRI. Hybridization and detection procedures were performed according to the manufacturers' 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.[18] The chromosomal DNA in the resulting ampicillin-resistant plasmid was sequenced using primer L58, derived from IS*4400*R in the transposon.[18] The primers listed in Table 2 were used to amplify the 5′ and 3′ junction ends of the transposon insertion in the *B. thetaiotaomicron* Tn met^R chromosomal DNA. The PCR fragments obtained were purified using the High Pure PCR Cleanup Kit (Roche) and sequenced as previously described.[14] *B. thetaiotaomicron* VPI-5482 sequence data were obtained from the NCBI website (GenBank accession number NC_004663). Analysis was done using the BLAST algorithm[21] and DNAMAN software V4.13.

RNA extraction and semi-quantitative dot blot hybridization

B. thetaiotaomicron wild-type and mutant cells for RNA extraction were grown anaerobically to late exponential phase in DMR or DMG and the total RNA was isolated.[14] RNA was treated with DNaseI and quantified using the NanoDrop® ND-100 spectrophotometer (NanoDrop Technologies, Inc.). For semi-quantitative RNA hybridization studies, equal amounts (1 μg) of the *B. thetaiotaomicron* RNA were spotted in equal volumes (2 μL) onto a nylon membrane (Roche). Hybridization and detection procedures were performed according to the manufacturer's instructions (Roche). Internal fragments of the genes of interest were used as DNA probes for

transcriptional hybridization studies. These were prepared by PCR amplification 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.*[14] 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 as 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 HindIII and SmaI sites of the shuttle vector pLYL01[22] and transformed into *E. coli* DH5α. The resultant plasmid, pLYLrhaR, was extracted, purified and retransformed into *E. coli* S17-1 according to standard methods.[15] Mobilization of pLYLrhaR from *E. coli* S17-1 (pLYLrhaR) into *B. thetaiotaomicron* VPI-5482 was performed by the conjugation procedure of Li *et al.*[22] 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 K, 4°C, 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,

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 Met[R], was isolated with an MIC of 8 mg/L metronidazole compared with the wild-type, which had an MIC of 0.8 mg/L.

Location of the transposon insertion

Southern hybridization confirmed the presence of a single transposon insertion within the chromosome of the *B. thetaiotaomicron* Tn Met[R] 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 OD_{600} 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 Met[R] 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 Met[R] 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
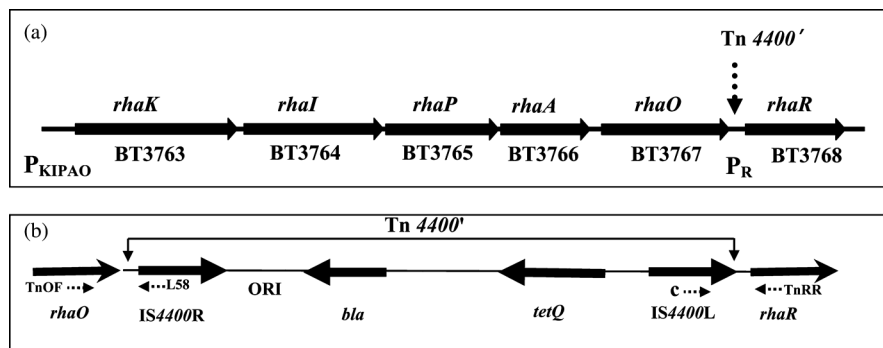

Figure 1. Genomic arrangement of the rhamnose gene cluster in *B. thetaiotaomicron* VPI-5482 (accession number NC 004663) and the *B. thetaiotaomicron* Tn Met[R] metronidazole-resistant mutant. (a) Position of the transposon insertion site. P_{KIAPO} and P_R indicate the two promoters that have been identified using sequencing and bioinformatic analysis.[14] The dashed arrow shows the position of the Tn*4400'* transposon insertion. (b) Schematic representation (not to scale) of the transposon insertion between *rhaO* and *rhaR*. IS*4400*L and IS*4400*R, 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.

```
GTATTTGCAACATCATAGAAATTGCATACCTTTGTTCCTCGGTTATATGTTTGCTCATCTGC

AACTTTTTTTTCTTTGGACGGACAATTAAAGCAAAGATAGCAAACTTTATCCATTCAGAGTG

AGAGAAAGGGGGACATTGTCTCTCTTTCCTCTCTGAAAAATAAATGTTTTTATTGCTTATTA

TCCGCACCCAAAAAGTTGCATTTATAAGTTGAACTCAAGAGTATAAGGTTTGTTTTTTATAA
                                                    -33
GCATCCGGAGGATCTGGACAGGTGCTCTGGATGTTTTTTTACTGTTTAGCGTGTGTTTTTTG
       -7        +1
AAAATAATTCCTACTTTTGTTCAATTCAATTGTATGAAAACACGCCTGTATGACTGAGGATA
```

Figure 2. Position of IS*4400*L in relation to the transcriptional start site of *rhaR*. Nucleotide sequence of IS*4400*L (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]

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 IS*4400*L 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 IS*4400*L sequence. This is in keeping with the findings of Bayley *et al.*[25] who first described the *B. fragilis* consensus promoter and reported its presence in the IS*4351* element, which is identical to IS*4400* used in this study. It is not unusual for IS elements to act as strong promoters and activate genes[26,27] and their role in naturally occurring antibiotic resistance is well documented. For example, Haggoud *et al.*[28] first identified the IS*1168* insertion sequence as being located upstream of the nitroimidazole resistance genes (*nim*), and Soki *et al.*[29] reported that nine *nimA* genes were activated by IS*1168* and three of the *nimC* genes were activated by IS*1170*. In *B. fragilis*, Rogers *et al.*[30] associated the IS*1224* insertion upstream of the *cepA* β-lactamase gene with up-regulation and Podglajen *et al.*[31] reported that IS*1186* activated the expression of the metallo-β-lactamase *cfiA* gene, causing resistance to carbapenems. They later identified the typical *Bacteroides* consensus promoter sequences within two IS elements, IS*1187* and IS*1188*, that were found upstream of *cfiA*.[32] Based on the analysis of the IS*4400*L in relation to RhaR, it is likely that these consensus promoter elements within the IS*4400* 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 Met[R]. 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*[(+)]. 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 $\log_{10}$ of the surviving fractions of the *B. thetaiotaomicron* Tn Met[R] mutant and *B. thetaiotaomicron rhaR*[(+)], 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 $\log_{10}$ surviving fraction of only −2.78 (±0.001). The *B. thetaiotaomicron* Tn Met[R] 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.

Pyruvate oxidoreductase and lactate dehydrogenase activity

The improved growth and a metronidazole resistance phenotype observed in both *B. thetaiotaomicron* Tn Met[R] and *B. thetaiotaomicron rhaR*[(+)] 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

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

Strain	Lactate dehydrogenase[a]	Pyruvate oxidoreductase[b]
B. thetaiotaomicron VPI-5482	3.24 ($\pm$3.27)	0.230 ($\pm$0.06)
B. thetaiotaomicron Tn Met^R	16.23 ($\pm$6.04)	0.035 ($\pm$0.05)
B. thetaiotaomicron $rhaR^{(+)}$	12.08 ($\pm$3.50)	0.022 ($\pm$0.02)

Data are the mean values of three experiments ($\pm$SD).
[a]Enzymatic activity expressed as μM NADH/mg of protein/min.
[b]Enzymatic activity expressed as μM methyl viologen/mg of protein/min.

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 Met^R 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.*,[9] 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 Met^R 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 Met^R 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 Met^R 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 Met^R 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.

Funding

Transparency declarations

None to declare.

References

1. Wexler HM. *Bacteroides*: the good, the bad, and the nitty-gritty. *Clin Microbiol Rev* 2007; **20**: 593–621.

2. Patrick S, Duerden BI. Gram-negative non-spore forming obligate anaerobes. In: Gillespie SH, Hawkey P, eds. *Principles and Practice of Clinical Bacteriology*. London: Wiley, 2006; 541–56.

3. Leiros HK, Kozielski-Stuhrmann S, Kapp U *et al*. Structural basis of 5-nitroimidazole antibiotic resistance: the crystal structure of NimA from *Deinococcus radiodurans*. *J Biol Chem* 2004; **279**: 55840–9.

4. Upcroft JA, Dunn LA, Wright JM *et al*. 5-Nitroimidazole drugs effective against metronidazole-resistant *Trichomonas vaginalis* and *Giardia duodenalis*. *Antimicrob Agents Chemother* 2006; **50**: 344–7.

5. Edwards DI. Nitroimidazole drugs—action and resistance mechanisms. II. Mechanisms of resistance. *J Antimicrob Chemother* 1993; **31**: 201–10.

6. Muller M. Mode of action of metronidazole on anaerobic bacteria and protozoa. *Surgery* 1983; **93**: 165–71.

7. Koch RL, Goldman P. The anaerobic metabolism of metronidazole forms N-(2-hydroxyethyl)-oxamic acid. *J Pharmacol Exp Ther* 1979; **208**: 406–10.

8. Diniz CG, Farias LM, Carvalho MA *et al*. Differential gene expression in a *Bacteroides fragilis* metronidazole-resistant mutant. *J Antimicrob Chemother* 2004; **54**: 100–8.

9. Narikawa S, Suzuki T, Yamamoto M *et al*. Lactate dehydrogenase activity as a cause of metronidazole resistance in *Bacteroides fragilis* NCTC 11295. *J Antimicrob Chemother* 1991; **28**: 47–53.

10. Britz ML, Wilkinson RG. Isolation and properties of metronidazole-resistant mutants of *Bacteroides fragilis*. *Antimicrob Agents Chemother* 1979; **16**: 19–27.

11. Patrick S. *Bacteroides*. In: Sussman M, ed. *Molecular Medical Microbiology*. London: Academic Press, 2002; 1921–48.

12. Pumbwe L, Wareham DW, Aduse-Opoku J *et al*. Genetic analysis of mechanisms of multidrug resistance in a clinical isolate of *Bacteroides fragilis*. *Clin Microbiol Infect* 2007; **13**: 183–9.

13. Holdeman LV, Moore WEC. *Anaerobe Laboratory Manual*. Blacksburg, VA: Virginia Polytechnic Institute and State University Anaerobe Laboratory, 1972.

14. Patel EH, Paul LV, Patrick S *et al*. Rhamnose catabolism in *Bacteroides thetaiotaomicron* is controlled by the positive transcriptional regulator RhaR. *Res Microbiol* 2008; **159**: 678–84.

15. Sambrook JT, Fritsch EF, Maniatis T. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1989.

16. Van Tassell RL, Wilkins TD. Isolation of auxotrophs of *Bacteroides fragilis*. *Can J Microbiol* 1978; **24**: 1619–21.

17. Tang YP, Malamy MH. Isolation of *Bacteroides fragilis* mutants with in vivo growth defects by using Tn*4400'*, a modified Tn*4400* transposition system, and a new screening method. *Infect Immun* 2000; **68**: 415–9.

18. Chen T, Dong H, Tang YP *et al*. Identification and cloning of genes from *Porphyromonas gingivalis* after mutagenesis with a modified Tn*4400* transposon from *Bacteroides fragilis*. *Infect Immun* 2000; **68**: 420–3.

19. Wehnert GU, Abratt VR, Woods DR. Molecular analysis of a gene from *Bacteroides fragilis* involved in metronidazole resistance in *Escherichia coli*. *Plasmid* 1992; **27**: 242–5.

20. Ish-Horowicz D, Burke JF. Rapid and efficient cosmid cloning. *Nucleic Acids Res* 1981; **9**: 2989–98.

21. Altschul SF, Madden TL, Schaffer AA *et al*. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 1997; **25**: 3389–402.

22. Li LY, Shoemaker NB, Salyers AA. Location and characteristics of the transfer region of a *Bacteroides* conjugative transposon and regulation of transfer genes. *J Bacteriol* 1995; **177**: 4992–9.

23. Abbe K, Takahashi S, Yamada T. Involvement of oxygen-sensitive pyruvate formate-lyase in mixed-acid fermentation by *Streptococcus mutans* under strictly anaerobic conditions. *J Bacteriol* 1982; **152**: 175–82.

24. Lindmark DG, Müller M. Hydrogenosome, a cytoplasmic organelle of the anaerobic flagellate *Tritrichomonas foetus*, and its role in pyruvate metabolism. *J Biol Chem* 1973; **248**: 7724–8.

25. Bayley DP, Rocha ER, Smith CJ. Analysis of *cepA* and other *Bacteroides fragilis* genes reveals a unique promoter structure. *FEMS Microbiol Lett* 2000; **193**: 149–54.

26. Mahillon J, Leonard C, Chandler M. IS elements as constituents of bacterial genomes. *Res Microbiol* 1999; **150**: 675–87.

27. Schneider D, Lenski RE. Dynamics of insertion sequence elements during experimental evolution of bacteria. *Res Microbiol* 2004; **155**: 319–27.

28. Haggoud A, Reysset G, Azeddoug H *et al*. Nucleotide sequence analysis of two 5-nitroimidazole resistance determinants from *Bacteroides* strains and of a new insertion sequence upstream of the two genes. *Antimicrob Agents Chemother* 1994; **38**: 1047–51.

29. Soki J, Gal M, Brazier JS *et al*. Molecular investigation of genetic elements contributing to metronidazole resistance in *Bacteroides* strains. *J Antimicrob Chemother* 2006; **57**: 212–20.

30. Rogers MB, Bennett TK, Payne CM *et al*. Insertional activation of *cepA* leads to high-level β-lactamase expression in *Bacteroides fragilis* clinical isolates. *J Bacteriol* 1994; **176**: 4376–84.

31. Podglajen I, Breuil J, Collatz E. Insertion of a novel DNA sequence, IS*1186*, upstream of the silent carbapenemase gene *cfiA*, promotes expression of carbapenem resistance in clinical isolates of *Bacteroides fragilis*. *Mol Microbiol* 1994; **12**: 105–14.

32. Podglajen I, Breuil J, Rohaut A *et al*. Multiple mobile promoter regions for the rare carbapenem resistance gene of *Bacteroides fragilis*. *J Bacteriol* 2001; **183**: 3531–5.

33. Xu J, Bjursell MK, Himrod J *et al*. A genomic view of the human-*Bacteroides thetaiotaomicron* symbiosis. *Science* 2003; **299**: 2074–6.

34. Gallegos MT, Schleif R, Bairoch A *et al*. AraC/XylS family of transcriptional regulators. *Microbiol Mol Biol Rev* 1997; **61**: 393–410.

35. Santos CL, Tavares F, Thioulouse J *et al*. A phylogenomic analysis of bacterial helix-turn-helix transcription factors. *FEMS Microbiol Rev* 2009; **33**: 411–29.

36. Casanueva AI, Paul L, Patrick S *et al*. An AraC/XylS family transcriptional regulator homologue from *Bacteroides fragilis* is associated with cell survival following DNA damage. *FEMS Microbiol Lett* 2008; **278**: 249–56.

37. Simon R, Priefer U, Puhler A. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. *Bio/Technology* 1983; **1**: 784–91.

38. Mahillon J, Chandler M. Insertion sequences. *Microbiol Mol Biol Rev* 1998; **62**: 725–74.