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Three metronidazole-resistant *Prevotella bivia* strains harbour a mobile element, encoding a novel *nim* gene, *nimK*, and an efflux small MDR transporter

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Objectives: In this study we assess the antibiotic resistance genes in three metronidazole-resistant *Prevotella bivia* clinical isolates.

Methods: Strains were whole-genome sequenced. *De novo* assembly was performed and genes were annotated in RAST. Manual adjustments were made, when required, to the annotation and length of the genes.

Results: In all three strains a novel *nim* gene, *nimK*, was encountered located on a mobile genetic element (MGE). The *nimK* gene was associated with an IS*1380* family transposase. On the same MGE, genes encoding an efflux small MDR (SMR) transporter were present and were associated with a *crp/fnr* regulator.

Conclusions: This is the first description of the presence of a novel *nim* gene in metronidazole-resistant *P. bivia* clinical isolates. This gene is co-located with an efflux SMR transporter on an MGE, which has been named Tn*6456* (MG827401). The identification of these resistance genes on an MGE is worrisome, since this indicates the horizontal gene transfer of antibiotic and/or biocide resistance from one strain to the other.

Introduction

For decades, metronidazole has been the drug of choice when dealing with anaerobic infections. Nowadays, metronidazole-resistant anaerobic bacteria, mostly members of the Bacteroidaceae family, have been reported.[1] Several studies focusing on the mechanism of metronidazole resistance have been conducted. Metronidazole is a prodrug, and intracellular reduction by microbial nitroreductases results in the active molecule, a nitroso free radical. The activated form of this antibiotic is able to covalently bind to bacterial DNA, leading to cell death. This process takes place in an anaerobic environment. One of the resistance mechanisms used by bacterial cells is the production of an alternative reductase, encoded by the *nim* gene, converting metronidazole into a non-toxic molecule.[2] Several *nim* genes have been described, i.e. *nimA–H* and *nimJ* in *Bacteroides* species[3–5] and *nimI* in *Prevotella* species.[6] *nimA–D* are found to be associated with specific mobile genetic elements (MGEs), pIP417- and pIP419-like plasmids.[3]

Recently, the presence of a new *nim* gene, *nimH*, was revealed in two metronidazole-resistant *Bacteroides fragilis* strains.[7] *nimH* is the first *nim* gene associated with an IS*614B* transposase located on the chromosome. In this study, we describe another novel *nim* gene, assigned as *nimK*, which was encountered in three metronidazole-resistant *Prevotella bivia* strains. The gene was located on an MGE, inserted in the chromosome, and associated with an IS*1380* family transposase. Interestingly, on the same MGE, genes encoding an efflux small MDR (SMR) transporter were also identified.

Materials and methods

Bacterial strains

Three metronidazole-resistant *P. bivia* strains were found to harbour a novel *nim* gene. Strain UMCG-3721 was isolated from a gluteal infiltrate of a male patient. This *P. bivia* strain was cultured, and found to be resistant to amoxicillin, clindamycin and metronidazole, but susceptible to amoxicillin/clavulanic acid and meropenem. Unfortunately, data on the antimicrobial treatment are not available. Strain UMCG-93105 was isolated from an abdominal infection of a patient with small cell lung carcinoma, treated with chemotherapy. Perforation of the stomach was observed, resulting in peritonitis and septic shock. Antibiotic treatment was started with cefotaxime and metronidazole. The aerobic culture yielded *Escherichia coli*, *Streptococcus mitis* group and *Candida albicans*. The anaerobic culture revealed a *P. bivia* strain (UMCG-93105) resistant to amoxicillin, clindamycin and metronidazole, but susceptible to amoxicillin/clavulanic acid, piperacillin/tazobactam and meropenem. Based on these results, metronidazole

was switched to amoxicillin/clavulanic acid. Strain UMCG-8631 was obtained from a patient with perforating gastric trauma. After closure, a deep wound infection occurred, resulting in abdominal abscess formation. Pus from the abdominal abscess yielded *Klebsiella variicola*, *Enterococcus faecium*, *P. bivia* (UMCG-8631) and *Prevotella nigrescens*. The *P. bivia* strain was resistant to metronidazole and clindamycin, but susceptible to amoxicillin/clavulanic acid and meropenem. Drainage took place and the patient was treated with cefotaxime, metronidazole and teicoplanin. After obtaining the antibiotic susceptibility results, treatment was switched to piperacillin/tazobactam and vancomycin. The patient recovered from his trauma.

The *P. bivia* strains were subcultured on Brucella Blood Agar (Mediaproducts, Groningen, The Netherlands) and incubated for 48 h at 37°C in an anaerobic environment. After subsequent subculturing, the MIC of metronidazole was determined using Etest (bioMérieux, Marcy-l'Étoile, France). Breakpoints from EUCAST were applied. Identification at the species level was performed using MALDI-TOF MS as described previously.[8]

WGS, assembly and annotation

WGS was performed as described by Zhou *et al.*[9] *De novo* assembly was performed using CLC Genomics workbench version 7.0.4 (Qiagen, Hilden, Germany), using standard settings. Initial annotation of the genes was performed by uploading the draft genome in RAST (http://rast.nmpdr.org). Manual adjustments were made, when required, to the annotation and length of the genes.

nim and efflux gene description

The amino acid sequence of the NimK protein and other described Nim proteins were aligned, using the MUSCLE alignment, from the MEGA7 program.[10] A phylogenetic tree was calculated using the maximum likelihood method. The reliability of the branching was assessed by calculating 500 bootstraps.

The predicted structure of the NimK protein was assessed using the Phyre Protein Fold recognition server (http://www.sbg.bio.ic.ac.uk/phyre2)[11] and the predicted ligand binding site by using 3DLigandSite.[12] The Dali superimposed model was obtained by submitting the PDB file from the Phyre Protein Fold recognition server to the Dali server (ekhidna.biocenter.helsinki.fi).[13] Structures of the proteins, part of the encountered efflux system, were determined using the same server.

Results

WGS

Assembly of the reads of strain UMCG-3721 resulted in a 128-contig draft genome, representing 2604460 bp. The contig lengths varied from 511 to 161273 bp. The assembly of the reads of strain UMCG-93105 resulted in a 55-contig draft genome, contigs varying in length from 1092 to 272838 bp, representing 2395911 bp. The draft genome of strain UMCG-8631 consisted of 75 contigs, representing 2440195 bp. The length of the contigs varied from 1030 bp to 173810 bp.

nim genes

The MICs of metronidazole for strains UMCG-3721, UMCG-93105 and UMCG-8631 were 8, 6 and 12 mg/L, respectively. *nimA–J* were not encountered in the genomes using ResFinder 2.1 and a manual blast search using known *nim* gene sequences. However, based on a translated protein sequence of a gene in isolate UMCG-3721, a similarity match was found of 84% with a 5-nitroimidazole antibiotic resistance protein (WP_044266587).

Table 1. An overview of the proteins encoded by genes encountered in the MGE harbouring the *nimK* gene; the given percentage query and percentage identity apply for all three metronidazole-resistant *P. bivia* strains

Function	Percentage query[a]	Percentage identity[a]
DR left	NA	NA
Integrase (WP_044065638)	100	97.8
Mobilization protein (WP_005866998)	100	97.6
NimK	NA	NA
IS*1380* family transposase (WP_094446786)	100	99.5
QacE (OKZ20530)	100	82.1
Crp/Fnr regulator (WP_004326410)	97	62.6
DR right	NA	NA

NA, not applicable.
[a]The percentage was determined using blastp. If necessary, the length of the gene was adjusted.

An identical gene was identified in isolates UMCG-93105 and UMCG-8631. The predicted structure of NimK was split-barrel like (Figure S1, available as Supplementary data at *JAC* Online).The putative protein was 162 amino acids long and contained Pro49, His62 and Phe97, essential for the activity of the Nim protein (Figure S2).[14] The phylogenetic alignment is shown in Figure S3.

Mobile element and the efflux SMR transporter

The *nimK* gene was located on an MGE, harbouring genes for integrase and a mobilization protein. Downstream of the *nimK* gene, an IS element related to the IS*1380* family transposases (99.5% identity) was observed (Table 1). On the MGE a gene encoding a QacE family quaternary ammonium compound efflux SMR transporter was also encountered, with an amino acid identity of 82.1%. Downstream of the *qacE* gene, a gene related to the *crp/fnr* family transcriptional regulator was present (identity 62.6%). Between the *qacE* gene and the *crp/fnr* gene, repeat regions were encountered (Figure 1). Predicted protein structures and details on the encountered genes are shown in Figures S4 and S5 and Table S1. Direct IRs were encountered on both flanking sites of the MGE. The total length of the MGE was 7821 bp. All three sequenced strains harboured the same MGE, integrated at the same genomic position. The described MGE was assigned the Tn number Tn*6456*[15] and sequences were submitted to NCBI (accession number MG827401).

Discussion

In this manuscript, we report a previously undescribed *nim* gene (*nimK*) encountered in three metronidazole-resistant *P. bivia* strains. We did not find any matches in a BLAST search for this gene in publicly available isolates in PubMed. The *nimK* gene is located on an MGE, encoding its own genes for integration and

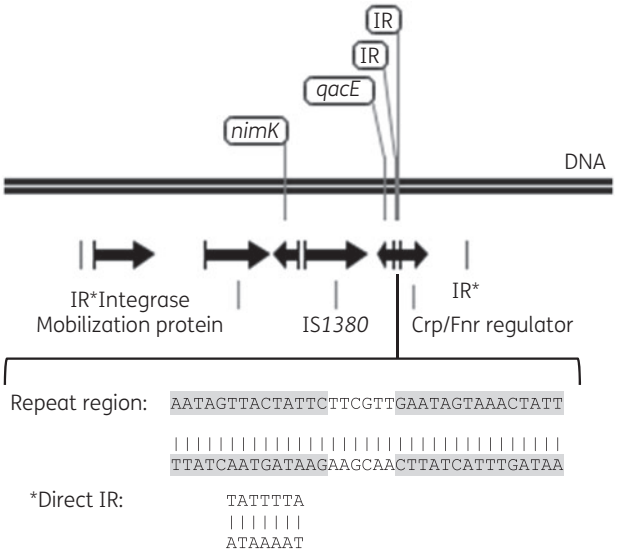

Figure 1. Schematic overview of the MGE harbouring the *nimK* and *qacE* genes.

excision and was associated with an IS*1380* family transposase, as is the case for *nimA–E*, *nimH* and *nimJ*.[3,7] The presence of *nimK* results in a faint band when the universal *nim* gene PCR was performed.

In addition to *nimK*, the MGE also harboured genes encoding a QacE efflux SMR transporter and a Crp/Fnr family transcriptional regulator. The mechanism of activation of transcription for the efflux SMR transporter in *Staphylococcus aureus* has been described by Grkovic *et al*.[16] A thorough search showed that there is no literature available on the prevalence of *qacE* genes and their function in Bacteroidaceae. The role of efflux SMR transporters in biocide resistance is described in a study by He *et al*.,[17] in which *Enterobacter cloacae* strains, harbouring the *sugE* gene, were shown to be resistant to several biocides.

In all three *P. bivia* strains, the *nimK* gene and the SMR efflux pump genes were located on the same MGE. Although strains harbouring a *nim* gene are not by definition phenotypically resistant to metronidazole, it is generally accepted that *nim* genes play a role in metronidazole resistance. To our knowledge, no currently available studies describe this phenomenon in *Prevotella* species. The presence of *nim* genes is mostly described in *Bacteroides* strains and rarely in *Prevotella* strains.[6] Steffens *et al*.[18] reported that overexpression of protein RecA, a DNA repair protein, in a *B. fragilis* strain caused metronidazole resistance. Furthermore, mutations in the ferrous transport fusion protein (FeoAB) resulted in metronidazole resistance in *B. fragilis*,[19] probably due to decreased cellular iron transport. Since the description of Tn*6456* is solely based on molecular data, it remains unclear whether the SMR efflux pump plays a role in the metronidazole resistance, and whether it plays a role in antibiotic/biocide resistance. However, these strains also harboured other resistance genes on their genome.

The detection of MGEs harbouring *nim* and efflux SMR transporter genes among anaerobic bacteria is worrisome. Horizontal gene transfer of these genes may cause a rapid emergence of resistance to the most commonly used antibiotics in anaerobic infections. Molecular surveillance of anaerobic resistance is certainly warranted.

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Transparency declarations

None to declare.

Supplementary data

Figures S1–S5 and Table S1 are available as Supplementary data at *JAC* Online.

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