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Downregulation of an NfsA-like nitroreductase causes metronidazole resistance in *Gardnerella vaginalis*

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ABSTRACT

Objectives: The facultatively anaerobic or microaerophilic bacteria of the genus *Gardnerella* are the most common species associated with bacterial vaginosis (BV). The treatment of BV is greatly complicated by the high rates of resistance to metronidazole and clindamycin among *Gardnerella* strains. We wanted to identify factors contributing to metronidazole resistance in *G. vaginalis* and performed high-throughput mass spectrometry on a selection of susceptible and resistant strains.
Methods: We wanted to identify factors contributing to metronidazole resistance in *G. vaginalis* and performed high-throughput mass spectrometry on a selection of susceptible and resistant strains.
Results: We identified the downregulation of a novel nitroreductase, GvNR1, as the only relevant change in protein expression in a metronidazole-resistant derivative of the susceptible type of strain ATCC 14018 (emended *Gardnerella* genospecies 1; i.e. *G. vaginalis* sensu stricto). When tested in in vitro enzyme assays, GvNR1 displayed very marked nitroreductase activity with several nitro drugs, including metronidazole and other 5-nitroimidazoles. GvNR1 also rendered *Escherichia coli* BL21-AI much more susceptible to metronidazole when expressed in this strain. In metronidazole-resistant *G. vaginalis* clinical isolates (>8 µg/mL according to Clinical and Laboratory Standards Institute), GvNR1 was found to be downregulated as well. In resistant clinical strains 33 other proteins were found to be differentially expressed. Of note, these include thioredoxin reductase and vaginolysin. The lowered expression levels of GvNR1 in resistant strains were not caused by mutations in the GvNR1 gene or in sequences directly flanking the gene but by downregulation of mRNA expression. This might explain why *G. vaginalis* becomes resistant to metronidazole so rapidly.
Conclusions: We propose GvNR1 as a novel metronidazole resistance determinant in *G. vaginalis*.

1. Introduction

The Gram-variable, facultative anaerobic/microaerophilic bacteria of the genus *Gardnerella* (family Bifidobacteriaceae) are associated with bacterial vaginosis (BV) [1], the most prevalent vaginal infection that affects 23 % to 29 % of all women of reproductive age worldwide [2]. Although *Gardnerella* species are not the only presumptive aetiological agents of BV, they are certainly the most common [1], especially *G. vaginalis* (also termed *Gardnerella* genome species 1).

Treatment of BV is mainly based on the administration of clindamycin or 5-nitroimidazoles such as metronidazole [3]. Although treatment with either antimicrobial is initially often successful, recurrence of symptoms after some time is very common (50 % to 80 % of all cases), and cases refractory to treatment are not rare [4]. This is often attributable to antimicrobial resistance in the bacteria causing BV, either pre-existent or emerging after treatment. In *Gardnerella*, resistance to 5-nitroimidazoles is particularly common [5], especially after repeated treatments [6,7] when resistance rates up to 80 % can be observed [7]; additionally resistance to clindamycin develops quickly after treatment (i.e. to resistance rates higher than 50 %) [4]. There is, therefore, a need for alternative, novel treatments such as *Gardnerella*-specific endolysins [8,9] or vaginal microbiome transplantations [10].

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Throughout the last 60 years plus, metronidazole has remained an overall highly reliable drug against microaerophiles and anaerobes [11], notable exceptions being *Helicobacter pylori* and *Gardnerella* spp. From the biochemical point of view, the latter is an exceptional case because it constitutes the only microaerophilic/anaerobic pathogen treated with metronidazole or other 5-nitroimidazoles that does not express the enzyme pyruvate:ferredoxin oxidoreductase (PFOR). This enzyme was originally believed to be essential for the reduction of the nitro group in metronidazole, a step required to convert the inactive pro-drug to its active form [12]. Despite the absence of PFOR, however, metronidazole is active against *Gardnerella*, although many strains seem to be intrinsically resistant and resistance can develop exceptionally fast [7].

We wanted to elucidate the molecular mechanism behind metronidazole resistance in *G. vaginalis* and applied high-throughput proteomics to compare the protein expression profiles of the metronidazole-susceptible type strain ATCC 14018 and a derivative metronidazole-resistant strain generated in vitro in a previous study [9]. In addition, we compared the protein expression profile of ATCC 14018 with the profile of one metronidazole-susceptible clinical isolate (UGent 09.01) and the profiles of two resistant clinical isolates (UGent 09.07 and BV50.1) to identify factors which may further contribute to metronidazole resistance in vivo [8,9].

2. Materials and methods

2.1. Bacterial strains and culture conditions

Gardnerella strains, including the type strain *G. vaginalis* ATCC 14018 and clinical isolates *G. vaginalis* UGent 09.07, *G. vaginalis* UGent 09.01, and *G. vaginalis* BV50.1, had been obtained from the Laboratory of Bacteriology, University of Ghent, Belgium for prior studies [8,9]. All strains belong to *Gardnerella* genome species 1 (i.e. *G. vaginalis* sensu stricto). The MIC of metronidazole for the strains tested were as follows: ATCC 14018: 8 µg/mL; UGent 09.01: 8 µg/mL; UGent 09.07: 64 µg/mL; BV50.1: 32 µg/mL. According to the European Committee on Antimicrobial Susceptibility Testing, all strains had MICs of metronidazole higher than the defined breakpoint concentration of 4 µg/mL. However, according to the less stringent definition by the Clinical and Laboratory Standards Institute, ATCC 14018 and UGent 09.01 qualify as only borderline-resistant or even metronidazole-susceptible, respectively (breakpoint concentration: >8 µg/mL). Further, a highly metronidazole-resistant derivative of ATCC 14018, grown with sublethal concentrations of metronidazole for nine and 25 subcultures, respectively, had been generated in a previous study [9]. These strains are able to grow in the presence of ≥ 512 µg/mL metronidazole. All *G. vaginalis* strains were cultured on chocolate agar plates (Becton Dickinson) under anaerobic conditions using anaerobic chambers equipped with anaerobic atmosphere generation bags (O_2: <0.1 %, CO_2: 7 %-15 %) provided by AnaeroGen (Thermo Scientific). Plates were incubated for 24 to 48 hours.

Escherichia coli (*E. coli*) 10-beta and BL21-AI cells (New England Biolabs) were used for plasmid amplification and protein expression, respectively. These strains were grown on Luria-Bertani (LB) agar plates or in LB broth (Carl Roth) supplemented with ampicillin (100 µg/mL). Cultures were incubated aerobically at 37 °C.

Liquid cultures of all *G. vaginalis* strains were grown in New York City broth III, containing 10 mM HEPES (Sigma Aldrich), 15 g/L proteose peptone (Sigma Aldrich), 3.8 g/L yeast extract (Thermo Fisher Scientific), 86 mM sodium chloride (Carl Roth), 28 mM α-D-glucose (Sigma Aldrich), and 10 % (v/v) horse serum (Thermo Fisher Scientific).

2.2. Expression of recombinant hexahistidine-tagged nitroreductase in *E. coli*

Genomic DNA from *G. vaginalis* was used as a template to amplify the nitroreductase 1 gene (GvNTR1). The forward primer included an NdeI restriction site, whereas the reverse primer included an XhoI restriction site and a hexahistidine tag for protein purification. Primer sequences are given in Supplementary Table S1. PCR products were ligated into pET17b vectors and propagated in *E. coli* 10-beta cells. After plasmid isolation, BL21-AI cells were transformed with the constructs and selected on LB agar plates containing 100 µg/mL ampicillin.

Recombinant expression was performed by overnight incubation at 20 °C with 0.05 % L-arabinose. Cells were harvested and disrupted using a mortar, and proteins were purified using Ni-NTA spin columns (Qiagen). The recombinant nitroreductase is referred to as recGvNR1.

2.3. Susceptibility testing in *E. coli* BL21-AI

Antimicrobial susceptibility was assessed by collecting *E. coli* BL21-AI colonies grown on LB agar using a sterile swab. The colonies were then suspended in phosphate buffered saline and evenly spread onto fresh LB plates. Etest for metronidazole (bioMérieux) strips were applied after having allowed the plates to air-dry for 15 minutes. The plates were then placed in anaerobic jars and incubated at 37 °C for 24 hours. MICs were determined by observing the point where the ellipse of growth inhibition intersected with the Etest strip.

2.4. Nitroreductase activity assay and cytochrome c assay

Nitroreductase activity was assessed by measuring nicotinamide adenine dinucleotide phosphate (NADPH) oxidation (λ = 340 nm; $\Delta\varepsilon 340$ = 6.2 mM^{-1} cm^{-1}). Reaction mixtures contained 100 mM KH_2PO_4 (pH 6.75), 0.2 mM NADPH, and 5 µg recGvNR. Metronidazole, tinidazole, ornidazole, ronidazole, azomycin, and nitrofurantoin were all purchased from Sigma and used at a concentration of 100 µM.

The cytochrome c assay was performed as described in a prior study [13]. Reaction mixtures contained 100 mM KH_2PO_4 (pH 6.75), 0.2 mM NADPH, 50 µM cytochrome c, 100 µM test drugs, and either 1 µg recGvNR for azomycin or 5 µg recGvNR for 5-nitroimidazole drugs. Readouts were made at λ = 550 nm ($\Delta\varepsilon 550$ = 20 mM^{-1} cm^{-1}).

2.5. Protein isolation for proteomics analysis

G. vaginalis was cultured overnight in 20 mL New York City III broth and 4 × 10^9 cells were harvested by centrifugation at 3000 × *g* for 10 minutes at 4 °C. Pellets were washed with 1 mL water, centrifuged again, and resuspended in 120 µL denaturing protein sample buffer (7 M urea, 2 M thiourea, 1 % DTT, 1 % Biolyte ampholytes (Bio-Rad Laboratories), 4 % CHAPS). Cells were sonicated at 4 °C for 10 cycles (30-second pulses on and off) and centrifuged at 20,000 × *g* for 20 minutes at 4 °C. Supernatants were collected, and protein concentrations were determined using the Bradford assay.

2.6. Protein digest and clean-up

For protein digestion, filter-aided sample preparation 3 kDa ultrafiltration units (Pall Corporation) were used as described earlier [14]. In brief, filters were prepared with 8 M urea in 50 mM Tris, and subsequently 30 µg of protein was added. Next, proteins

were reduced with 20 mM DTT for 30 minutes at 37 °C and alkylated with 60 mM IAA for 30 minutes at 25 °C in darkness on the filter. After the samples were washed twice with 100 µL 50 mM Tris, Trypsin/LysC (Promega) was added to the samples at a final enzyme concentration of 1 : 25 (enzyme to protein w/w) for overnight digestion at 37 °C. Peptides were eluted from the filter using 3 × 50 µL 50 mM Tris, and the collected eluate was acidified with concentrated TFA to a pH < 2. Pierce C18 spin columns (Thermo Fisher Scientific) were then used for desalting and clean-up. Columns were activated using 2 × 200 µL 50 % ACN and equilibrated using 2 × 200 µL 5 % ACN, 0.5 % TFA, centrifuging at 1500 × *g* between every step. Samples were then eluted using 2 × 20 µL 70 % ACN and 0.1 % TFA. Finally, samples were evaporated to dryness using a vacuum centrifuge at 45 °C, and resolubilized in 300 µL 0.1 % TFA for liquid chromatography-tandem mass spectrometry analysis.

2.7. Mass spectrometry and data analysis

Samples were analysed using a nano-high-performance liquid chromatography (HPLC) Ultimate 3000 RSLC system (Dionex) coupled to a high-resolution Q-Exactive HF Orbitrap mass spectrometer (Thermo Fisher Scientific). The liquid chromatography system was equipped with a 5 mm Acclaim PepMap µ-precolumn (300 µm inner diameter, 5 µm particle size, 100 Å pore size) for sample pre-concentration and desalting. All solvents, solvent gradients and tandem mass spectrometry parameters were identical to those recently published [13]. A 25 cm Acclaim PepMap C18 column (75 µm inner diameter, 2 µm particle size, 100 Å pore size) was used for peptide separation. Samples were injected into the nanoHPLC in technical duplicates. Database search was performed using the Proteome Discoverer Software 3.1.1.93 (Thermo Fisher Scientific) using the Sequest HT search engine. The digestion enzyme was trypsin with a maximum of two missed cleavages allowed. Carbamidomethylation was set as a fixed modification. Oxidation (M), deamidation (NQ), acetylation (Protein N-term), and Met-loss + acetyl (Protein N-Term (M)), Met-loss (Protein N-term (M)), and Gln -> pyro-Glu (Q) were set as variable modifications. The precursor mass tolerance was set to 10 ppm, and the fragment mass tolerance to 0.02 Da. Spectra were searched in the NCBI *Gardnerella vaginalis* database (tx585528, strain ATCC14018, 4874 sequences, www.ncbi.nlm.nih.gov, downloaded on 2025.03.21). The common Repository of Adventitious Proteins (cRAP) database was used to automatically filter out common contaminants (www.thegpm.org/crap/). Further search parameters were set as previously published, and intensity-based label-free quantification, as well as statistical evaluation of data were also performed as recently described [14].

2.8. Reverse transcription-quantitative PCR

Reverse transcription-quantitative PCR (RT-qPCR) was carried out as previously described with slight modifications [15]. Total RNA was extracted from approximately 1 × 10^9 mid-log phase cells (OD_{6nn} = 0.4–0.6) using the GeneJet RNA Purification Kit (Thermo Fisher Scientific). The RNA was then diluted with nuclease-free water to a final concentration of 10 ng/µL and stored at −20 °C until use. Reactions were run on a CFX Connect Real-Time System (Bio-Rad) using the Luna Universal One-Step RT-qPCR Kit. Reactions (20 µL each) were prepared in low-profile, non-skirted 96-well plates sealed with optically clear adhesive sheets (Thermo Scientific). Each reaction contained 10 µL of 2× Luna Universal One-Step reaction mix, 1 µL of 20× Luna WarmStart RT enzyme mix, 0.8 µL of each primer (10 µM), 30 ng of RNA, and nuclease-free water. The RT-qPCR thermal cycling included: reverse transcription at 55 °C for 10 minutes, initial denaturation at 95 °C for 1 minute, followed by 45 cycles of denaturation at 95 °C for 1 second and extension at 60 °C for 30 seconds (with fluorescence measurement). A melt curve was generated at the end of the run, increasing the temperature from 60 °C to 95 °C in 0.5 °C increments every 5 seconds. All mRNA levels were quantified across three independent biological replicates, each performed in duplicate. The 16S rRNA gene served as the internal control for normalization. Expression levels of nitroreductase mRNA were analysed using the comparative Ct method (2^−ΔΔCt) as described by Livak and Schmittgen [16]. Primer sequences are given in Supplementary Table S1.

2.9. PCR amplification of nitroreductase 1 genes and sequencing

The nitroreductase 1 genes were amplified by PCR from the genomes of the strains studied using the following primers: forward – 5' ACAGAGCTGCAACAAAATAA 3'; reverse – 5' GCGCAAGGATTTACTTTTAAGTAC 3'. PCR was performed using HotStarTaq DNA Polymerase (Qiagen) using the following program: (1) 95 °C, 15 minutes; (2) 95 °C, 30 seconds; (3) 50 °C, 30 seconds; (4) 68 °C, 75 seconds; [steps 2 to 4 cycled 35 ×]; and (5) 68 °C, 10 minutes. PCR fragments were cleaned using PCR purification columns (Qiagen) and submitted to Eurofins Genomics for Sanger sequencing. DNA sequences were then aligned using the BLAST Global Align Tool at the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

3. Results

3.1. Metronidazole resistance in G. vaginalis type strain ATCC 14018 is caused by the downregulation of expression of an NfsA-like nitroreductase

To identify proteins differentially expressed in metronidazole-resistant ATCC 14018, cultures of the metronidazole-susceptible parent ATCC 14018 (14018^s) and of a derivative metronidazole-resistant strain of ATCC 14018, passaged with sublethal concentrations of metronidazole for nine subcultures ($14018^{r\ 9\times}$) and for 25 subcultures ($14018^{r\ 25\times}$) [9], were used for high-throughput mass spectrometry (i.e. nano-HPLC-tandem mass spectrometry with subsequent data analysis). There were no consistent and statistically significant changes (all data available in Supplementary Table 2) between the resistant strains and 14018^s with the exception of an InlB B-repeat-containing protein of unknown function (WP_009994333) and of an NADPH-dependent oxidoreductase (GenBank entry TCH81729). A BLAST search revealed that the latter is a predicted nitroreductase with an NfsA-like domain.

To confirm the predicted function of TCH81729 as a nitroreductase, we cloned the respective gene into the protein expression vector pET17b for recombinant expression in *E. coli* BL21-AI. The expressed protein was used in a nitroreductase assay with a selection of nitroaromatic drugs, including metronidazole and other 5-nitroimidazoles, the 2-nitroimidazole azomycin, and the nitrofuran nitrofurantoin. Indeed, TCH81729 proved to be a potent nitroreductase, displaying high reduction rates even with 5-nitroimidazoles, which have a very low single electron reduction potential (Table 1). Overall, reduction rates increased with increasing single electron reduction potential (E^1_7), with the exception of the nitrofuran nitrofurantoin, which was more slowly reduced than its comparably high potential would have suggested. After confirmation of the enzyme's function, we named the protein *G. vaginalis* nitroreductase 1 (GvNR1). We noticed that the enzyme did not display any background activity (i.e. oxidation of NADPH) in the absence of nitro compounds, indicating that the enzyme does not react with oxygen. This is compatible with GvNR1's homology to *E. coli* NfsA, which is an oxygen-insensitive nitroreductase. To further confirm this assumption, we performed a cytochrome c-coupled nitroreductase assay. Oxygen-sensitive nitroreductases form superoxide,

Table 1
Activity of recombinant GvNR1 with selected nitroimidazoles and the nitrofuran nitrofurantoin (100 µM each).

Nitro drug	E^1_7 [mV]	Vmax [nmol min^{-1} mg^{-1}]	Turnover rate [s^{-1}]
Metronidazole	−486	727 ± 112	0.3
Tinidazole	−464	4,554 ± 382	2.3
Ornidazole	−467	3,834 ± 779	2
Ronidazole	−466	3,777 ± 157	1.9
Azomycin	−418	14,889 ± 914	7.5
Nitrofurantoin	−255	8,840 ± 745	4.3

All measurements were performed at least three times.

Table 2
Etest with BL21-AI either bearing pET recGvNR1 or empty pET 17b as control.

Strain	MIC metronidazole [µg mL^{-1}]
BL21-AI pET recGvNR1 (no L-arabinose)	24
BL21-AI pET recGvNR1 (0.2 mM L-arabinose)	3
BL21-AI pET 17b (no L-arabinose)	24
BL21-AI pET 17b (0.2 mM L-arabinose)	24

The break point concentration of metronidazole according to the European Committee on Antimicrobial Susceptibility Testing is 4 µg/mL (https://www.eucast.org/clinical_breakpoints).

either directly by transferring an electron from NADPH to molecular oxygen, or indirectly by reducing the nitro group of a nitro compound with a single electron first, followed by the subsequent transfer of this electron to molecular oxygen. Superoxide then reduces cytochrome c in the assay buffer. When performing the assay, no reduction of cytochrome c could be observed with GvNR1, regardless of which nitroaromatic compound was used (data not shown), indicating that GvNR1 is an oxygen-insensitive nitroreductase.

Finally, we wanted to test the importance of GvNR1 for metronidazole reduction. Unfortunately, we could not perform any gene complementation assays in *G. vaginalis* because no suitable plasmids or other genetic tools were available at the time of our study. Instead, we measured the metronidazole susceptibility of *E. coli* BL21-AI harbouring pET GvNR1 in the presence and absence of inducer (arabinose) by Etest. Indeed, when expression of GvNR1 was induced upon the addition of arabinose, BL21-AI cells became much more susceptible to metronidazole (Table 2). In BL21-AI carrying the pET17b plasmid without the GvNr1 gene, addition of arabinose had no effect.

3.2. The expression of GvNR1 is also downregulated in metronidazole-resistant G. vaginalis clinical isolates alongside other changes in protein expression

As a next step we analysed three clinical *Gardnerella* genospecies 1 isolates, of which two are metronidazole-resistant (UGent 09.07 and BV50.1) and one is metronidazole-susceptible (Ugent 09.01), by mass spectrometry, and compared the protein expression profiles with that of strain 14018^s. For this analysis, the latter was re-analysed to include all four strains in one single proteome analysis. We searched for proteins which were consistently up- or downregulated in the resistant strains as compared with the susceptible strains. Only proteins which were up- or downregulated at least twofold in the resistant strains were taken into account (all data available in Supplementary Table 3). GvNR1 was found to be downregulated in the resistant clinical isolates, albeit to a lesser degree than in $14018^{r\ 9\times}$ and $14018^{r\ 25\times}$ (Table 3). However, this is mirrored by the clearly lower level of resistance of the former [9]. Expression of thioredoxin-disulfide reductase, more commonly designated as thioredoxin reductase, was also downregulated in the resistant isolates. The downregulated proteins included transporters, metabolic enzymes, cell wall synthesizing enzymes, ribosomal factors, and transcription factors/repressors. In total, 30 proteins were downregulated in the resistant strains, and four were upregulated. Interestingly, the *G. vaginalis* pathogenicity factor vaginolysin was found upregulated by at least a factor of 2 in the resistant strains, as was pullulanase. The InlB B-repeat-containing protein of unknown function (WP_009994333) found in the previous analysis was not detected in either of the strains in the second analysis.

3.3. The expression of the GvNR1 gene is downregulated at the mRNA level

We sequenced the GvNR1 gene and its flanking sequences, presumably including the promoter, in all strains and searched them for mutations (Supplementary Figure 1). The sequence was completely unaltered in $14018^{r\ 9\times}$ and $14018^{r\ 25\times}$ as compared with the susceptible parent, indicating that the loss of GvNR1 expression was not due to a frameshift mutation or deletion of the gene. In the clinical strains alterations in the GvNR1 gene as compared with 14018^s were noted. No nonsense mutations, however, were identified. The amino acid sequence of GvNR1 in resistant UGent 09.07 differs from that in 14018^s by only three amino acid residues. These changes, however, are also present in the GvNR1 gene of susceptible UGent 09.01, ruling out that they are causative for resistance. In the resistant strain BV50.1, six mismatches in the amino acid sequence as compared with 14018^s were identified (Supplementary Figure 1). A BLAST search, however, revealed that the sequence in BV50.1 is identical to numerous others in the database, including that of ATCC 49145, which is as equally susceptible to metronidazole as ATCC 14018 is [17]. In the downstream sequences, numerous differences were detected between the strains (Supplementary Figure 1), however, again in susceptible and resistant strains alike.

With the sequencing analysis showing that resistance was not caused by mutations in the GvNR1 gene, we next measured the mRNA levels of GvNR1 in all strains by RT-qPCR. Indeed, mRNA levels of GvNR1 were found to be below the detection limit in $14018^{r\ 9\times}$ and $14018^{r\ 25\times}$ and severalfold lower in UGent 09.07 and BV50.1 than in 14018^s and UGent 09.01 (Table 4).

4. Discussion

In this study we identified a potent nitroreductase in *Gardnerella* genospecies 1, GvNR1, which was not expressed in a highly metronidazole-resistant derivative strain of ATCC 14018. Interestingly, its activity was several orders of magnitude higher than observed with other nitroreductases (e.g. RdxA from *H. pylori*) [18]. GvNR1 is clearly an oxygen-insensitive nitroreductase and transfers at least two electrons to nitro groups, resulting in the formation of nitroso compounds (e.g. 5-nitrosoimidazoles). Nitroso compounds are highly reactive and rapidly bind to sulfhydryl groups, either free or of cysteines in proteins [19]. Accordingly, expression of GvNR1 in *E. coli* BL21-AI greatly increased metronidazole susceptibility. Due to a lack of appropriate genetic tools available, we could not demonstrate the effect of GvNR1 overexpression directly in *G. vaginalis*. However, based on the data presented, we conclude that the loss of GvNR1 expression can render *G. vaginalis* resistant to metronidazole. This resembles the situation in *H. pylori* where the loss of the *rdxA* gene results in metronidazole resistance [reviewed in 11]. In the *G. vaginalis* strains studied herein, however, the sequence of the nitroreductase gene remains unaltered. Instead, its expression is actively downregulated at the transcriptional level (Table 4). This might explain why metronidazole resistance can develop so rapidly in *G. vaginalis*.

Table 3
List of proteins either downregulated (red) or upregulated (green) in resistant UGent 09.07 and BV50.1 as compared to susceptible ATCC 14018 and UGent 09.01.

No.	Description	GenBank	ATCC 14018 vs. UGent 09.01	ATCC 14018 vs. UGent 09.07	ATCC 14018 vs. BV50.1	UGent 09.01 vs. UGent 09.07	UGent 09.01 vs. BV50.1
1	NADPH-dependent oxidoreductase (= GvNR1)	TCH81729	**−2.8**	**3.3**	**4.9**	**9.4**	**13.8**
2	Thioredoxin-disulfide reductase	TCH82796	1.5	**4.3**	**5.4**	**2.9**	**3.7**
3	Aldo/keto reductase	TCH81262	−1.1	**2.4**	**4.5**	**2.7**	**5.1**
4	Glutamate 5-kinase	TCH81251	−1.1	**3.8**	**3.1**	**4.2**	**3.4**
5	S-adenosyl-L-homocysteine hydrolase	BAQ32890	1.0	**2.1**	**2.1**	**2.2**	**2.2**
6	3-dehydroquinate dehydratase	BAQ33542	−1.1	**2.3**	**4.1**	**2.5**	**4.4**
7	Transcriptional repressor NrdR	TCH82599	1.3	**3**	**2.9**	**2.4**	**2.3**
8	Transcriptional regulator MraZ	TCH82597	−1.1	**2**	**2.5**	**2.3**	**2.9**
9	Holliday junction DNA helicase RuvA	BAQ33338	−1.3	**6.8**	**4.8**	**5.2**	**3.3**
10	Excinuclease ABC subunit UvrA	TCH82168	−1.0	**2.1**	**3.6**	**2.2**	**3.8**
11	EF-Ts	BAQ33291	−1.1	**2**	**2.2**	**2.3**	**2.5**
12	50S ribosomal protein L23	TCH82026	1.1	**2.2**	**2.2**	**2**	**2**
13	16S rRNA (cytosine(1402)-N(4))-methyltransferase RsmH	TCH82596	1.8	**6.1**	**4.1**	**3.3**	2.3
14	tRNA (adenosine(37)-N6)-threonylcarbamoyltransferase TsaD	TCH81875	**2.6**	**8**	**7.7**	**3.1**	**3**
15	HflX GTPase family protein	BAQ33028	1.0	**5.3**	3.0	**5.2**	3.0
16	Cold-shock protein	WP_004112652	−1.3	**2.5**	**4.4**	**3.3**	**5.7**
17	ABC transporter ATP-binding protein (heavy metal transport)	WP_004574971	−1.2	**6.9**	**5.9**	**8.3**	**7.1**
18	ABC transporter ATP-binding protein (LolD-like)	TCH82096	−1.3	**3.2**	**8.1**	**4**	**10.2**
19	ABC transporter ATP-binding protein (LolD-like)	TCH81628	1.6	**16.8**	**11.5**	**10.6**	**7.3**
20	Phosphate ABC transporter substrate-binding protein PstS	TCH81274	2.0	**11.3**	**13.1**	**5.7**	**6.7**
21	HAD-superfamily subfamily IIA hydrolase	BAQ33090	1.0	**2.5**	**6.5**	**2.6**	**6.8**
22	HAD family phosphatase	TCH81820	−1.7	**2.2**	**3.3**	**3.7**	**5.6**
23	HAD family phosphatase	TCH82480	−1.4	**2.1**	**4.3**	**3**	**6.2**
24	Inosine-uridine nucleoside N-ribohydrolase	BAQ33380	−1.4	2.1	**4**	**2.9**	**5.5**
25	NUDIX domain-containing protein	TCH82793	−1.2	**2.1**	**2**	**2.5**	**2.4**
26	Conserved hypothetical protein (phosphatase PhoE)	BAQ33551	−1.4	**3.3**	**2.1**	**4.4**	**2.8**
27	UDP-N-acetylmuramate–L-alanine ligase	TCH82587	**2.8**	**5.7**	**8.2**	**2.1**	**3**
28	Laccase domain-containing protein	TCH81263	1.1	**3.5**	**3.1**	**3**	**2.7**
29	Mbeg1-like protein (triacylglycerol lipase)	WP_004138632	1.3	**2.9**	**3.1**	**2.2**	**1.3**
30	Hypothetical protein GAVG_1032	BAQ33684	−1.4	∞	**4.1**	∞	**5.8**
31	Pullulanase (type I)	TCH81974	**−2.2**	**−4.6**	**−4.8**	**−2.2**	**−2.2**
32	Vaginolysin	TCH82751	**3.2**	**−2.1**	**−7.5**	**−6.5**	**−23.7**
33	Putative fucose transport protein	BAQ33908	**−4.5**	**−22.5**	**−11**	**−5**	**−2.4**
34	Hypothetical protein GAVG_0972	BAQ33624	−1.3	**−5.6**	**−3.6**	**−4.4**	**−2.8**

Changes larger than factor 2, which are statistically significant ($P \leq 0.05$), are given in bold.

Table 4
mRNA levels of GvNR1 in all strains were normalised against the GvNR1 mRNA level in ATCC 14018^{s}.

Strain	mRNA level as compared to ATCC 14018^{s}
ATCC 14018^{s}	100 %
ATCC 14018$^{r\ 9\times}$	–
ATCC 14018$^{r\ 25\times}$	–
UGent 09.01	32 %–159 %
UGent 09.07	3 %–20 %
BV50.1	19 %–25 %

The range as determined in three independent experiments is given.

The physiological function of GvNR1 is unclear because no compensatory changes could be observed in the protein expression profiles of 14018$^{r\ 9\times}$ and 14018$^{r\ 25\times}$, which could have given indication of physiological changes brought about by the downregulation of GvNR1. In fact, expression differences between 14018^{s} and 14018$^{r\ 9\times}$ or 14018$^{r\ 25\times}$, respectively, were minimal. In this context it is necessary to emphasize that the metabolism of *Gardnerella*, and that of other Bifidobacteriaceae for that matter, differs greatly from most other anaerobic bacteria and also anaerobic protozoa. *Gardnerella* does not have a PFOR, which has been proposed to be the decisive factor for metronidazole reduction in the past [12]. Indeed, when metronidazole resistance is induced in vitro, PFOR expression is almost always strongly downregulated [11]. This is often accompanied by reduced iron uptake [11,15,20], leading to numerous changes in protein expression [20,21]. None of this was observed in 14018$^{r\ 9\times}$ or 14018$^{r\ 25\times}$, underscoring that the mere loss of GvNR1 might be sufficient to cause metronidazole resistance.

In the metronidazole-resistant clinical strains UGent 09.07 and BV50.1, more differences than only downregulation of GvNR1 could be observed. In total, 34 proteins were differentially expressed as compared with metronidazole-sensitive 14018^{s} and UGent 09.01, with clearly more proteins downregulated (30) than upregulated (4; Table 2). Among the downregulated enzymes was also thioredoxin reductase, which had been shown to have marked nitroreductase activity in anaerobic parasites [13,22,23]. Overall, the differences might be indicative of decelerated growth, with effectors of cell wall synthesis (protein 25 in Table 2) and lipoprotein transport (proteins 16 and 17 in Table 2) being downregulated. Also factors of the protein expression machinery were downregulated (proteins 11 to 14 in Table 2). Conversely, vaginolysin was upregulated in UGent 09.07 and BV50.1. Vaginolysin may enhance nutrient uptake by lysing erythrocytes [24]. This indicates that metronidazole resistance in clinical *G. vaginalis* could be accompanied by an elevated need for nutrients, constituting a possible disadvantage for these strains. The role of GvNR1 in this, however, is unclear at present, and the sample size of four strains is too small to make reliable predictions. Importantly, a homolog of GvNR1 exists in all *Gardnerella* species, including those which are intrinsically resistant to metronidazole such as genospecies 7b (*Gardnerella swidsinksii*) belonging to clade 4 [25,26]. It will be informative to study the function and expression of GvNR1 in these *Gardnerella* species.

When studying a larger number of isolates, it is possible that other resistance determinants will emerge. In this context, it is interesting to note that intrinsically resistant *Gardnerella* species have an *aruH* gene, whereas susceptible *Gardnerella* species do not [26]. A possible role for the transaminase AruH in metronidazole resistance, and whether its selective presence in intrinsically metronidazole-resistant *Gardnerella* species is causative for resistance or only correlated with it, remains to be established.

5. Conclusions

In this study we identified nitroreductase 1 (GvNR1) as a metronidazole-resistant determinant in *G. vaginalis*. The enzyme displayed very high nitroreductase activity in vitro, and its expression was lower in metronidazole-resistant *G. vaginalis* strains. To the best of our knowledge, GvNR1 is the first metronidazole-resistant determinant to be described in *G. vaginalis*. This could prove relevant in the clinic because metronidazole resistance rates in *Gardnerella* are generally very high, complicating treatment of BV considerably.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2025.107681.

References

[1] Shvartsman E, Hill JE, Sandstrom P, MacDonald KS. *Gardnerella* revisited: species heterogeneity, virulence factors, mucosal immune responses, and contributions to bacterial vaginosis. Infect Immun 2023;91:e0039022. doi:10.1128/iai.00390-22.
[2] Peebles K, Velloza J, Balkus JE, McClelland RS, Barnabas RV. High globalb and costs of bacterial vaginosis: a systematic review and meta-analysis. Sex Transm Dis 2019;46:304–11. doi:10.1097/OLQ.0000000000000972.
[3] Workowski KA, Bachmann LH, Chan PA, Johnston CM, Muzny CA, Park I, et al. Sexually transmitted infections treatment guidelines, 2021. MMWR Recomm Rep 2021;70:1–187. doi:10.15585/mmwr.rr7004a1.
[4] Muzny CA, Sobel JD. The role of antimicrobial resistance in refractory and recurrent bacterial vaginosis and current recommendations for treatment. Antibiotics (Basel) 2022;11:500. doi:10.3390/antibiotics11040500.
[5] Nagaraja P. Antibiotic resistance of *Gardnerella vaginalis* in recurrent bacterial vaginosis. Indian J Med Microbiol 2008;26:155–7. doi:10.4103/0255-0857.40531.
[6] Bannatyne RM, Smith AM. Recurrent bacterial vaginosis and metronidazole resistance in *Gardnerella vaginalis*. Sex Transm Infect 1998;74:455–6.
[7] Schwebs T, Kieninger AK, Podpera Tisakova L, Oberbauer V, Berdaguer R, Mtshali A, et al. Evaluation of metronidazole resistance of vaginal swab isolates from South African women treated for bacterial vaginosis. Antibiotics (Basel) 2024;13(12):1217. doi:10.3390/antibiotics13121217.
[8] Landlinger C, Tisakova L, Oberbauer V, Schwebs T, Muhammad A, Latka A, et al. Engineered phage endolysin eliminates *Gardnerella* biofilm without damaging beneficial bacteria in bacterial vaginosis *ex vivo*. Pathogens 2021;10:54. doi:10.3390/pathogens10010054.
[9] Landlinger C, Oberbauer V, Podpera Tisakova L, Schwebs T, Berdaguer R, Van Simaey L, et al. Preclinical data on the Gardnerella-specific endolysin PM-477 indicate its potential to improve the treatment of bacterial vaginosis through enhanced biofilm removal and avoidance of resistance. Antimicrob Agents Chemother 2022;66:e0231921. doi:10.1128/aac.02319-21.
[10] Lev-Sagie A, Goldman-Wohl D, Cohen Y, Dori-Bachash M, Leshem A, Mor U, et al. Vaginal microbiome transplantation in women with intractable bacterial vaginosis. Nat Med 2019;25:1500–4. doi:10.1038/s41591-019-0600-6.
[11] Leitsch D. A review on metronidazole: an old warhorse in antimicrobial chemotherapy. Parasitology 2019;146:1167–78. doi:10.1017/S0031182017002025.
[12] Narikawa S. Distribution of metronidazole susceptibility factors in obligate anaerobes. J Antimicrob Chemother 1986;18:565–74. doi:10.1093/jac/18.5.565.
[13] Leitsch D, Kolarich D, Binder M, Stadlmann J, Altmann F, Duchêne M. *Trichomonas vaginalis*: metronidazole and other nitroimidazole drugs are reduced by the flavin enzyme thioredoxin reductase and disrupt the cellular redox system. implications for nitroimidazole toxicity and resistance. Mol Microbiol 2009;72:518–36. doi:10.1111/j.1365-2958.2009.06675.x.
[14] Mayr AL, Hummel K, Leitsch D, Razzazi-Fazeli E. A comparison of bottom-up proteomic sample preparation methods for the human parasite *Trichomonas vaginalis*. ACS Omega 2024;9:9782–91. doi:10.1021/acsomega.3c10040.

[15] Paunkov A, Sóki J, Leitsch D. Modulation of iron import and metronidazole resistance in *Bacteroides fragilis* harboring a *nimA* gene. Front Microbiol 2022;13:898453. doi:10.3389/fmicb.2022.898453.
[16] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 2001;25:402–8. doi:10.1006/meth.2001.1262.
[17] Knupp de Souza DM, Diniz CG, Filho DS, Andrade de Oliveira LM, Coelho DM, Talha LS, et al. Antimicrobial susceptibility and vaginolysin in *Gardnerella vaginalis* from healthy and bacterial vaginosis diagnosed women. J Infect Dev Ctries 2016;10:913–19. doi:10.3855/jidc.7161.
[18] Olekhnovich IN, Goodwin A, Hoffman PS. Characterization of the NAD(P)H oxidase and metronidazole reductase activities of the RdxA nitroreductase of *Helicobacter pylori*. FEBS J 2009;276:3354–64. doi:10.1111/j.1742-4658.2009.07060.x.
[19] Ehlhardt WJ, Beaulieu BB Jr, Goldman P. Nitrosoimidazoles: highly bactericidal analogues of 5-nitroimidazole drugs. J Med Chem 1988;31:323–9. doi:10.1021/jm00397a009.
[20] Paunkov A, Hummel K, Strasser D, Sóki J, Leitsch D. Proteomic analysis of metronidazole resistance in the human facultative pathogen *Bacteroides fragilis*. Front Microbiol 2023;14:1158086. doi:10.3389/fmicb.2023.1158086.
[21] Mayr AL, Paunkov A, Hummel K, Razzazi-Fazeli E, Leitsch D. Comparative proteomic analysis of metronidazole-sensitive and resistant *Trichomonas vaginalis* suggests a novel mode of metronidazole action and resistance. Int J Parasitol Drugs Drug Resist 2024;26:100566. doi:10.1016/j.ijpddr.2024.100566.
[22] Leitsch D, Kolarich D, Wilson IB, Altmann F, Duchêne M. Nitroimidazole action in *Entamoeba histolytica*: a central role for thioredoxin reductase. PLoS Biol 2007;5:e211. doi:10.1371/journal.pbio.0050211.
[23] Leitsch D, Burgess AG, Dunn LA, Krauer KG, Tan K, Duchêne M, et al. Pyruvate:ferredoxin oxidoreductase and thioredoxin reductase are involved in 5-nitroimidazole activation while flavin metabolism is linked to 5-nitroimidazole resistance in *Giardia lamblia*. J Antimicrob Chemother 2011;66:1756–65. doi:10.1093/jac/dkr192.
[24] Gelber SE, Aguilar JL, Lewis KL, Ratner AJ. Functional and phylogenetic characterization of vaginolysin, the human-specific cytolysin from *Gardnerella vaginalis*. J Bacteriol 2008;190:3896–903. doi:10.1128/JB.01965-07.
[25] Schuyler JA, Mordechai E, Adelson ME, Sobel JD, Gygax SE, Hilbert DW. Identification of intrinsically metronidazole-resistant clades of *Gardnerella vaginalis*. J Bacteriol 2008;190:3896–903. doi:10.1128/JB.01965-07.
[26] Innamorati KA, Earl JP, Barrera SC, Ehrlich RL, Aiyeku J, Gordon A, et al. Metronidazole response profiles of *Gardnerella* species are congruent with phylogenetic and comparative genomic analyses. Genome Med 2025;17:28. doi:10.1186/s13073-025-01446-4.