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Multidisciplinary Analysis of a Nontoxigenic *Clostridium difficile* Strain with Stable Resistance to Metronidazole

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Stable resistance to metronidazole in a nontoxigenic *Clostridium difficile* strain was investigated at both the genomic and proteomic levels. Alterations in the metabolic pathway involving the pyruvate-ferredoxin oxidoreductase were found, suggesting that reduction of metronidazole, required for its activity, may be less efficient in this strain. Proteomic studies also showed a cellular response to oxidative stress.

Clostridium difficile infection (CDI), the main cause of nosocomial infectious diarrhea, has increased in recent years (1). Metronidazole, a prodrug activated after the reduction of its nitro group (2), is recommended for the treatment of initial and moderate CDI cases.

A direct association between *in vitro* susceptibility to metronidazole and treatment efficacy is not always observed in CDI (3).

C. difficile strains showing resistance to metronidazole are rare, and laboratory manipulation of these strains frequently results in a MIC decrease toward the susceptibility range (4, 5). Therefore, the investigation of resistance mechanisms in *C. difficile* has proved difficult.

In other anaerobes, metronidazole resistance has been associated with drug inactivation by *nim* genes, altered activity of nitroreductases such as pyruvate-ferredoxin oxidoreductase (Pfo), a proteomic response to oxidative stress (2), and mutations of the ferric uptake regulator (*fur*) and oxygen-independent coproporphyrinogen III oxidase (*hemN*), as recently reported in a North American pulsed-field gel electrophoresis type 1 (NAP1) *C. difficile* strain (5).

In a previous study, we investigated metronidazole susceptibility in different *C. difficile* PCR ribotypes (6). In that study, the average MIC of metronidazole for PCR ribotype 010 strains was higher than that for widespread epidemic PCR ribotype strains, i.e., 7-fold compared to PCR ribotype 027 and 9-fold compared to PCR ribotype 001 or 078.

In the present study, *C. difficile* 7032989, a nontoxigenic strain with stable resistance to metronidazole, was analyzed by genome sequencing and quantitative proteomic analysis in comparison with *C. difficile* 7032985, a strain showing reduced susceptibility to metronidazole. Both strains were isolated in Spain and were typed as PCR ribotype 010 at the Cardiff Reference Centre (6). *C. difficile* 7032994, a metronidazole-susceptible strain also belonging to PCR ribotype 010, and *C. difficile* 630, a toxigenic reference strain (7), were used as controls in this study.

All *C. difficile* strains were stored at −80°C. Susceptibility assays were performed immediately after thawing and also after serial passages in medium without antibiotics by the agar dilution method (8). Antibiotic resistance was defined as a MIC of ≥32 μg/ml, according to the Clinical and Laboratory Standards Institute (7). The MICs were 0.25, 2, 4, and 32 μg/ml for *C. difficile* 630, 7032994, 7032985, and 7032989, respectively (6). PCR ribotype 010 strains were sequenced by Next-Generation Sequencing, 7032985 and 7032994 were sequenced via Illumina HiSeq, and 7032989 was sequenced via Roche 454. Contigs were assembled by using Velvet software (kmer 55 and 99; options, -short -fastq -unused_reads -read_trkg) (9) and reorganized on the basis of *C. difficile* reference strain 630 (7). Contigs that did not match the reference were placed at the end to obtain each genome scaffold. The genomes of strains 7032989, 7032985, and 7032994 are available in the MicroScope database with the links https://www.genoscope.cns.fr/agc/microscope/mage/viewer.php?S_id=2122, https://www.genoscope.cns.fr/agc/microscope/mage/viewer.php?S_id=2341, and https://www.genoscope.cns.fr/agc/microscope/mage/viewer.php?S_id=3220, respectively. Mauve Software (10) was used to compare the whole-genome sequences of PCR ribotype 010 strains 7032994, 7032985, and 7032989 with the available genome sequences of several *C. difficile* PCR ribotype strains (GenBank database accession numbers CAMY00000000, CAMJ00000000, AM180355.1, CAMS00000000, CAMH00000000, FN665652.1, CAMP00000000, FN545816.1, CAMM00000000, CAMC00000000, CAMB00000000, CAMR00000000, CAMZ00000000, CAMD00000000, CANC00000000, and CANB00000000 for PCR ribotypes 001, 005, 012, 014, 015, 017, 020, 027, 075, 078, 079, 095, 106, 126, 156, and 165, respectively).

The phylogenetic analysis of *C. difficile* strains 7032994, 7032985, and 7032989 in comparison with strains belonging to other PCR ribotypes showed that the three PCR ribotype 010 strains were closely related, with distances between the strains ranging from 0.02 to 0.06 (Fig. 1).

DNA sequencing did not show the presence of the pathogenicity locus (PaLoc) in any of the PCR ribotype 010 strains investi-

Received 21 January 2014 **Returned for modification** 10 March 2014
Accepted 29 May 2014
Published ahead of print 9 June 2014
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doi:10.1128/AAC.02350-14

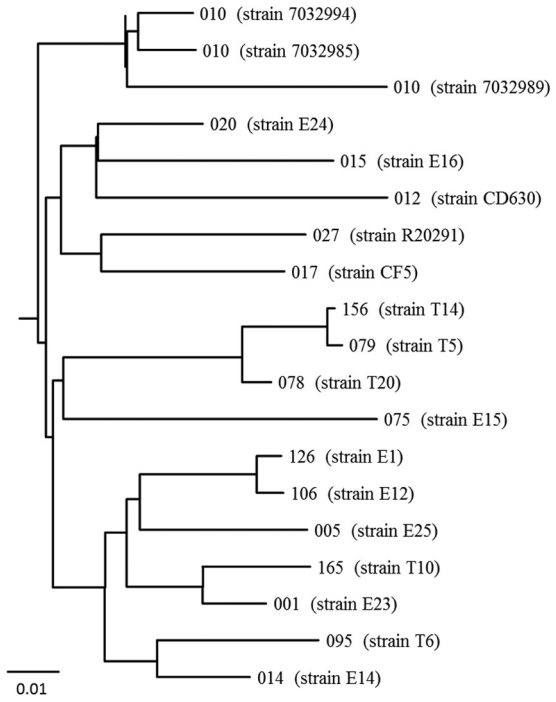

FIG 1 Phylogenetic tree showing relationships between *C. difficile* strains 7032994, 7032985, and 7032989, belonging to PCR ribotype 010, and other *C. difficile* PCR ribotypes. PCR ribotype 012 is represented by *C. difficile* reference strain 630. The GenBank database accession numbers of PCR ribotypes 001, 005, 012, 014, 015, 017, 020, 027, 075, 078, 079, 095, 106, 126, 156, and 165 are CAMY00000000, CAMJ00000000, AM180355.1, CAMS00000000, CAMH00000000, FN665652.1, CAMP00000000, FN545816.1, CAMM00000000, CAMC00000000, CAMB00000000, CAMR00000000, CAMZ00000000, CAMD00000000, CANC00000000, and CANB00000000, respectively.

gated. Additionally, the genomic analysis did not show the presence of *nim* genes or deletions or mutations in the *pfo*, *hemN*, or *fur* gene in any of the strains (data not shown). Thus, quantitative proteomic experiments were undertaken.

Strains were grown in brucella agar (Oxoid) plates containing vitamin K_1 (0.5 mg/liter), hemin (5 mg/liter), and 5% defibrinated sheep red blood cells. Proteins were extracted from cultures grown until mid-log phase (optical density at 600 nm of 0.5) in brain heart infusion broth (BHI; Oxoid) (11) without antibiotic or in BHI containing metronidazole at 0.1 μg/ml for *C. difficile* 630, 0.4 μg/ml for 7032994, 1 μg/ml for 7032985, and 10 μg/ml for 7032989. Extraction assays were performed in triplicate. Following centrifugation, pellets were washed with Tris-EDTA (pH 7.5) containing a protease inhibitor cocktail (Roche) and maintained at −80°C. Pellets were resuspended in Tris-EDTA, sonicated on ice, and cleared by centrifugation.

Following denaturation and reduction by 0.1% RapiGest (Waters) and 5 mM dithiothreitol, respectively, 50 μg of each extract was trypsin digested (Promega) overnight at 37°C. The digestion reaction was stopped with 0.1% formic acid, and peptide mixtures were desalted with Oasis cartridges (Waters). Label-free analysis was performed with a nanoACQUITY UPLC column coupled to a Synapt G2 mass spectrometer (Waters) by the Hi3 method (12). Data were processed with the ProteinLynx Global Server 2.5.2 software (Waters) for protein identification and quantification. Each extract was digested twice, and each digestion product was analyzed in triplicate by data-independent liquid chromatography-mass spectrometry. Significant protein level differences were established at >1.5-fold (13).

Although the amounts of 95% of the proteins were similar, some alterations were observed in both 7032989 and 7032985 before and after exposure to metronidazole (Table 1), suggesting a common cellular response. In particular, the DNA repair protein RecA increased following antibiotic pressure in both 7032989 and 7032985 but not in the control strains and the polyribonucleotide nucleotidyltransferase (PNP) protein was detected in both 7032989 and 7032985 only in the absence of antibiotic. PNP is associated with RNA processing and mRNA quality control (14). Alterations in transcription due to a decrease in this protein's activity could interfere with the response of strains 7032989 and 7032985 to metronidazole stress.

In this study, the aminoacyl-tRNA proteins cysteinyl-tRNA synthetase (CysS) and seryl-tRNA synthetase (SerS) were not detected in strains 7032989 and 7032985 in the presence of antibiotic. On the contrary, in susceptible strain 7032994, CysS was observed under both conditions, together with tyrosyl-tRNA synthetase and threonyl-tRNA synthetase (data not shown). In other bacteria, a decrease in the concentration of aminoacyl-tRNAs has been associated with slower cell growth (15), a characteristic also

TABLE 1 Protein quantification in *C. difficile* strains 630, 7032994, 7032985, and 7032989 before and after metronidazole exposure

	Normalized value[b]							
	630		7032994		7032985		7032989	
Protein[a]	Before[c] MZ	After[d] MZ	Before MZ	After MZ	Before MZ	After MZ	Before MZ	After MZ
Pfo	7.4	8.34	7.89	8.03	5.65	9.65	4.40	4.92
Pnp	ND[e]	ND	0.9	0.86	0.92	ND	0.89	ND
Bcd	0.27	ND	0.23	0.21	0.52	0.47	0.52	0.27
CysS	ND	ND	0.35	0.28	0.56	ND	0.56	ND
SerS	ND	ND	ND	ND	0.32	ND	0.43	ND
FtnA	0.13	0.13	0.15	0.16	0.19	0.22	0.22	ND
RecA	ND	0.14	ND	0.4	0.15	0.58	0.13	0.46

[a] Pfo, pyruvate ferredoxin oxidoreductase; Pnp, polyribonucleotide nucleotidyltransferase; Bcd, butyryl coenzyme A dehydrogenase; CysS, cysteinyl-tRNA synthetase; SerS, seryl-tRNA synthetase 1; FtnA, ferritin; RecA, protein recombinase A.
[b] Nanograms of protein/nanograms on column.
[c] Before metronidazole (MZ) exposure.
[d] After metronidazole (MZ) exposure.
[e] ND, not detected.

reported for *C. difficile* colonies showing higher metronidazole MICs (4, 5). Impaired activity of aminoacyl-tRNAs in strains 7032989 and 7032985 could result in posttranslational variations of proteins relevant for metronidazole activation.

The protein ferritin was not detected in metronidazole-resistant strain 7032989 under antibiotic pressure, suggesting restriction of the storage of iron, an element relevant to the oxidation-reduction balance of bacterial cells (16). Under the same experimental conditions, a significant butyryl coenzyme A dehydrogenase (Bcd) concentration decrease was also observed. It has been proposed that a cytoplasmic complex formed by Bcd and electron transport flavoproteins could catalyze the reduction of ferredoxin in other *Clostridium* species. This process could result in a decreased amount of reduced ferredoxin in the metronidazole-resistant strain (17).

Interestingly, the Pfo concentration was stable in resistant strain 7032989 before and after metronidazole exposure (4.4 and 4.9 ng, respectively), whereas it increased significantly from 5.6 to 9.6 ng in strain 7032985 following antibiotic pressure. In *C. difficile* 630 and 7032994, the concentration of Pfo was significantly higher than in the metronidazole-resistant strain, both in the presence and in the absence of metronidazole. Gene expression of the locus including the *pfo* gene (18 genes in total) was investigated by real-time quantitative reverse transcription (qRT)-PCR in both 7032989 and 7032985. cDNA synthesis and qRT-PCR were performed as previously described (18). The qRT-PCR analysis did not show significant differences in the expression of the *pfo* gene or of surrounding genes in either strain before and after antibiotic exposure (data not shown), suggesting that the variations observed in *C. difficile* 7032985 could be posttranslational.

In other anaerobes, metronidazole activation has been related to higher enzymatic activity of electron carriers mediating the pyruvate-oxidizing pathway, such as Pfo (trichomonads), pyruvate dehydrogenase (*C. perfringens*), or flavodoxins and hydrogenases (*C. acetobutylicum*) (2). A recent proteomic study of a NAP1 *C. difficile* strain reported a reduction in iron uptake under antibiotic pressure, possibly because of mutations in the genes *hemN* and *fur*, which could result in a defect in the electron transport required to metronidazole activation (19). In this study, we did not observe variations in those genes and the production of relative products. Instead, we found that the Pfo concentration in resistant strain 7032989 remained stable at a low concentration, with or without antibiotic, so it could be hypothesized that the electron transfer required for metronidazole activation is less efficient in this strain than in the other strains investigated. These results also suggest that *C. difficile* strains resistant to metronidazole can show peculiar alterations in their enzymes or metabolic pathways.

The average MIC of metronidazole for the PCR ribotype 010 strains we have analyzed so far was higher than that for epidemic PCR ribotypes (6). According to the European Committee on Antimicrobial Susceptibility Testing epidemiological cutoff (MIC, >2 μg/ml) (20), the majority of those strains can be classified as having reduced susceptibility to metronidazole. PCR ribotype 010 strains are mostly nontoxigenic, but the possibility that they acquire the PaLoc *in vivo* cannot be excluded. Actually, it was recently shown that the PaLoc was successfully transferred to nontoxigenic isolates *in vitro* by genetic recombination (21).

In conclusion, our results indicate that a metabolic pathway associated with *pfo* expression is altered in stably metronidazole-resistant strain 7032989, opening new perspectives in the complete characterization of this intriguing and complex resistance mechanism in *C. difficile*.

Nucleotide sequence accession numbers. The sequences for strains 7032989, 7032985, and 7032994 can be found under EMBL accession numbers PRJEB6600, PRJEB6601, and PRJEB6602, respectively.

ACKNOWLEDGMENTS

We thank Federico Giannoni and François Wasels for technical advice and support.

The research leading to these results received funding from the European Community's Seventh Framework Programme FP7/2007-2013 under grant agreement 2378942.

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