In vitro selection, via serial passage, of Clostridium difficile mutants with reduced susceptibility to fidaxomicin or vancomycin

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Objectives: Current treatments for Clostridium difficile infection include vancomycin, metronidazole and fidaxomicin. LFF571 is an experimental agent undergoing evaluation in humans for the treatment of moderate C. difficile infection. Reduced susceptibility of C. difficile to fidaxomicin or LFF571 in vitro can be mediated by single point mutations in genes encoding the targets, whereas the mechanism(s) mediating reduced susceptibility to vancomycin in vitro remains elusive. To further characterize mechanisms reducing susceptibility of C. difficile to vancomycin, fidaxomicin or LFF571 in vitro, selections via serial passage at low cell density were performed, followed by whole-genome sequencing.

Methods: C. difficile strain ATCC 43255 and three clinical isolates were subjected to 10 passages on medium containing a range of concentrations of fidaxomicin, LFF571 or vancomycin. Genomic DNA from isolates with reduced susceptibility was sequenced using Illumina Whole Genome Sequencing.

Results: Clones exhibiting decreased susceptibility to fidaxomicin harboured mutations in rpoB and CD22120 (marR homologue). Clones exhibiting decreased susceptibility to vancomycin harboured mutations in rpoC and also in CD2725, CD3659 and sdaB, which encode a putative N-acetylglucosamine transferase, exonuclease and L-serine deaminase, respectively. All mutations resulted in non-synonymous substitutions. No clones with reduced susceptibility to LFF571 were selected in this study.

Conclusions: Reduced susceptibility to fidaxomicin and vancomycin was associated with mutations mediating target modifications (RNA polymerase and cell wall, respectively), as well as with mutations that may contribute to reduced susceptibility via other mechanisms. The MIC of LFF571 was unaffected for those mutants with reduced susceptibility to fidaxomicin or vancomycin.

Keywords: LFF571, MarR, RNA polymerase, MurG

Introduction

Clostridium difficile is a Gram-positive, anaerobic, spore-forming bacterium that is the major cause of nosocomial antibiotic-associated diarrhea and pseudomembranous colitis. Treatments for C. difficile infection (CDI) include metronidazole, vancomycin and fidaxomicin.1 LFF571 is an investigational agent under evaluation for efficacy and safety in the treatment of moderate CDI. Single-step mutants of C. difficile with reduced susceptibility to LFF571 were selected in vitro at frequencies of ≤1.2 × 10^{-9} and harboured single amino acid substitutions in the target, elongation factor Tu.2 Single-step mutants of C. difficile with reduced susceptibility to fidaxomicin and vancomycin were selected in vitro at frequencies of ≤7.2 × 10^{-7},3,4 and ≤1.1 × 10^{-9},3 respectively. Changes in RNA polymerase are associated with reduced susceptibility to fidaxomicin,5 while the mechanism(s) mediating reduced susceptibility of C. difficile clinical isolates to vancomycin are uncharacterized. Here, we attempted to select C. difficile mutants at low cell density by serial passaging on vancomycin, fidaxomicin or LFF571. Mutations conferring reduced susceptibility were identified by whole-genome sequencing.

Materials and methods

Organisms

C. difficile NB95009 is CLSI MIC quality control strain ATCC 70057. NB95013 is strain ATCC 43255. NB95026 (untyped), NB95031 (REA type AA) and NB95047 (REA type J) are clinical isolates from Dr Donald Low at Mount Sinai Hospital, Toronto, Canada.6
Antibiotics

LFF571 and fidaxomicin were obtained from Novartis, Clindamycin, tetracycline, rifampicin and ampicillin were from Sigma-Aldrich (St Louis, MO, USA). Vancomycin and moxifloxacin were from US Pharmacopeia (Rockville, MD, USA). Erythromycin was from Abbott Laboratories (Chicago, IL, USA). Moxifloxacin was obtained as Avelox® from Merck (Whitehouse Station, NJ, USA). Linezolid was purchased as Zyvox® from Pharmacia and Upjohn (New York, NY, USA).

Serial passage of C. difficile

Serial passage experiments were conducted in six-well tissue culture-treated plates containing Brucella agar (Remel, Lenexa, KS, USA), 5% sheep blood (BD Biosciences, San Jose, CA, USA), 0.5 mg/L vitamin K and 5.0 mg/L haemin (Remel/Thermo Scientific, Lenexa, KS, USA). Test agents were serially diluted 2-fold from 9.6 g/L in 100% DMSO, and dilutions spanning the agar MICs were transferred to assay plates containing molten agar. Two microlitres of a C. difficile suspension (0.5 McFarland standard) was used to inoculate 10^8 cfu per well. Following incubation at 37°C for 48 h in an anaerobic chamber (Coy Lab Products, Grass Lake, MI, USA), the lowest concentration of compound on which no visible growth was observed was recorded as the MIC. Culture growing at one dilution below the MIC was used to inoculate the subsequent passage, and this was repeated for a total of 10 passages. The compound concentration range of each new passage was based on the MIC from the previous passage. A sample of the inoculum for each passage was sub-streaked without drug and frozen at −80°C. When reduced susceptibility was observed, single, isolated clones were obtained from the frozen mixed populations.

Antimicrobial susceptibility testing

Determination of MICs was performed according to CLSI guidelines. MICs of LFF571 and comparator antibiotics for C. difficile were within CLSI quality control ranges.

Sequence analysis of C. difficile strains

Genomic DNA was isolated from C. difficile using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA) and whole-genome fragment libraries were prepared using the Nextera paired-end sample preparation kit (Illumina, San Diego, CA, USA). Sequencing was performed using DNA Illumina Whole Genome Sequencing technology and analysed using the Illumina Pipeline version 1.3 software. Reads of fidaxomicin-passaged NB95013 and NB95026 DNA were aligned to the reference C. difficile strain 630 genome sequence using SOAP software version 1.11-1.0.4;16,17 read alignment was confirmed with Velvet version 1.1.05.18 Reads of vancomycin-passaged NB95013 and NB95026 were aligned to C. difficile 630 using BWA version 0.5.9. Single-nucleotide polymorphisms (SNPs) were called with SAMtools version 1.18.11,12 Whole-genome sequences were aligned to C. difficile 630 because complete reconstruction of each parental sequence was not performed. For comparison of mutant and parental strains, alignments of the appropriate regions from the whole-genome sequences were created with Vector NTI version 11.1. The N-terminal methionine residues were excluded from the amino acid sequence alignments and numbering.

Results

Serial passaging of C. difficile on vancomycin, fidaxomicin or LFF571

C. difficile were serially passaged 10 times on vancomycin, fidaxomicin or LFF571 to select for mutants with reduced susceptibility. When passaged on vancomycin, NB95013 progeny displayed an 8-fold decrease in susceptibility following the 7th passage and NB95026 progeny displayed a 16-fold decrease in susceptibility following the 5th passage. The susceptibility phenotypes then remained unchanged through the 10th passages. When passaged on fidaxomicin, a 16-fold decrease in susceptibility was observed following the 7th passage of strain NB95013, and a 64-fold decrease was observed following the 10th passage of strain NB95026. There were <2-fold changes in vancomycin and fidaxomicin susceptibility for strain NB95031 and a 4-fold shift in fidaxomicin susceptibility following 10 passages of NB95047. No mutants with reduced susceptibility to LFF571 were selected using this passaging procedure.

Antimicrobial susceptibilities of selected C. difficile

Individual C. difficile clones were isolated from the cultures demonstrating reduced susceptibility following 10 serial passages. MICs for the isolated clones increased 4- to 16-fold for fidaxomicin and 8-fold for vancomycin (Table 1), consistent with the phenotypes of the source populations. We further tested susceptibility to LFF571 and 10 antibiotics in clinical use. The MICs of the non-selecting antibiotics increased ≤4-fold against the clones selected on fidaxomicin or vancomycin compared with the parental strains (Table 1).

Sequencing of C. difficile strains with reduced susceptibility

To locate mutations associated with reduced susceptibility to vancomycin, the genomes of NB95013-JAL0863 and NB95026-JAL0865 were sequenced (Table 2). NB95013-JAL0863 harboured a set of three mutations, resulting in a P108L substitution in UDP-N-acetylgalactosamine-N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylgalactosamine transferase encoded by marG/CD2725, a stop codon after amino acid 326 in an RNA single-stranded DNA exonuclease encoded by CD3659, and a single amino acid deletion in a stretch of alanines (292–295) in L-serine dehydrogenase (encoded by sdaB). NB95026-JAL0865 had a single mutation encoding a D244Y substitution in the RNA polymerase subunit β’ (encoded by rpoC).

A similar approach was used to identify mutations associated with reduced susceptibility to fidaxomicin. The genomes of NB95013-JAL0859 contained a deletion in CD22120, resulting in a frameshift after amino acid 117 of a homologue of the MarK family of transcriptional regulators. Clone NB95026-JAL0861 had a mutation in rpoB (RNA polymerase subunit β), encoding a Q1073R substitution.

Discussion

We identified mutations conferring decreased susceptibility to fidaxomicin or vancomycin in C. difficile selected by serial passaging at low cell density. Mutations in rpoB or CD22120 were associated with decreased susceptibility to fidaxomicin. Previous reports have shown mutations in rpoB or rpoC are associated with reduced susceptibility in vitro10 and an rpoC mutant was isolated from a CDI patient who received fidaxomicin during a Phase III clinical trial. Lysine and histidine substitutions at the Q1073 position of RpoB have been associated with reduced susceptibility to fidaxomicin. In contrast, identification of mutations
Mutants of *C. difficile* with reduced susceptibility to fidaxomicin or vancomycin

Table 1. MICs of antibiotics for selected *C. difficile* clones

<table>
<thead>
<tr>
<th>Strain</th>
<th>Selecting agent</th>
<th>LFF571</th>
<th>FDX</th>
<th>VAN</th>
<th>MET</th>
<th>CLI</th>
<th>RIF</th>
<th>ERY</th>
<th>AMP</th>
<th>MXF</th>
<th>TET</th>
<th>LZD</th>
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<tbody>
<tr>
<td>NB95013</td>
<td>none</td>
<td>1</td>
<td>0.25</td>
<td>2</td>
<td>1</td>
<td>0.004</td>
<td>2</td>
<td>0.05</td>
<td>1</td>
<td>0.25</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>NB95013·JAL0859</td>
<td>FDX</td>
<td>0.5</td>
<td>1</td>
<td>4</td>
<td>0.5</td>
<td>0.004</td>
<td>2</td>
<td>0.05</td>
<td>2</td>
<td>0.25</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>NB95013·JAL0863</td>
<td>VAN</td>
<td>1</td>
<td>0.12</td>
<td>16</td>
<td>1</td>
<td>0.004</td>
<td>4</td>
<td>0.05</td>
<td>1</td>
<td>0.25</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>NB95026</td>
<td>none</td>
<td>0.5</td>
<td>0.25</td>
<td>2</td>
<td>0.5</td>
<td>0.002</td>
<td>2</td>
<td>0.05</td>
<td>32</td>
<td>0.25</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>NB95026·JAL0861</td>
<td>FDX</td>
<td>0.5</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>0.002</td>
<td>2</td>
<td>1</td>
<td>32</td>
<td>0.5</td>
<td>4</td>
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<tr>
<td>NB95026·JAL0865</td>
<td>VAN</td>
<td>0.5</td>
<td>0.12</td>
<td>16</td>
<td>1</td>
<td>0.002</td>
<td>4</td>
<td>1</td>
<td>32</td>
<td>0.25</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

AMP, ampicillin; CLI, clindamycin; ERY, erythromycin; FDX, fidaxomicin; LZD, linezolid; MET, metronidazole; MXF, moxifloxacin; RIF, rifampicin; TET, tetracycline; VAN, vancomycin.

Table 2. Genomic changes in *C. difficile* selected for reduced susceptibility to vancomycin or fidaxomicin

<table>
<thead>
<tr>
<th>Strain</th>
<th>Selecting agent</th>
<th>Gene name</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
</tr>
</thead>
<tbody>
<tr>
<td>NB95013·JAL0859</td>
<td>FDX</td>
<td>marR/CD22120</td>
<td>ΔT349</td>
<td>frameshift after amino acid 117</td>
</tr>
<tr>
<td>NB95013·JAL0863</td>
<td>VAN</td>
<td>murG/CD2725</td>
<td>C326T</td>
<td>P108L</td>
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<tr>
<td></td>
<td></td>
<td>CD3659</td>
<td>G982T</td>
<td>E327Stop</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sdaB/CD3222</td>
<td>ΔAGC (879–881)</td>
<td>ΔA295</td>
</tr>
<tr>
<td>NB95026·JAL0861</td>
<td>FDX</td>
<td>rpoB/CD0066</td>
<td>A3221G</td>
<td>Q1073R</td>
</tr>
<tr>
<td>NB95026·JAL0865</td>
<td>VAN</td>
<td>rpoC/CD0067</td>
<td>G733T</td>
<td>D244Y</td>
</tr>
</tbody>
</table>

FDX, fidaxomicin; VAN, vancomycin.

In CD22120 (encodes a homologue to the multidrug resistance-associated transcriptional regulator MarR) represents, to our knowledge, the first report of a mechanism outside of RNA polymerase (RNAP) that may alter susceptibility to fidaxomicin. Although MarR is associated with multidrug resistance, we did not observe reduced susceptibility to antibiotics other than fidaxomicin in this study. The role of this regulator in fidaxomicin resistance requires further study.

Mechanisms reducing susceptibility of *C. difficile* to vancomycin have not been previously reported. Here, we identified four mutations in two clones of *C. difficile*; three mutations were found together in a derivative of strain NB95013 and one mutation was identified in a derivative of NB95026. Among the substitutions in NB95013·JAL0863 was a conserved proline to leucine change (P108L) in MurG, which follows the second of three ‘G loops’ involved in binding the charged phosphate groups of the substrate UDP-GlcNAc. MurG converts lipid I to lipid II during the membrane-bound stage of peptidoglycan biosynthesis. Alterations in this pathway may affect vancomycin activity since the drug inhibits cell wall formation by binding to the D-Ala-D-Ala portion of lipid II. More study is required to understand how P108L affects MurG function and vancomycin susceptibility. The strain NB95026·JAL0865 mutation was found in rpoC, which has been previously associated with susceptibility to RNAP inhibitors such as rifampicin and fidaxomicin. rpoC mutations are also associated with reduced susceptibility to antibacterials that do not inhibit RNAP, including daptomycin and the antimicrobial surfactant zwittermicin A. rpoC mutations may lead to global gene expression changes that affect multiple pathways. However, to our knowledge, the D244Y substitution in β’ reported here has not previously been associated with decreased antibacterial susceptibility. In the linear sequence of β’ from *Escherichia coli*, the analogous D256 is relatively close to two residues at which substitutions resulting in reduced fidaxomicin susceptibility occur (L249 and S263). In the three-dimensional structure of RNAP, however, D256 is not part of the fidaxomicin resistance determinant as previously defined. Specifically, D256 is located at the tip of a loop in which the base of the loop has been shown to be part of the fidaxomicin resistance determinant, but the tip has not (R. Landick, University of Wisconsin-Madison and R. Ebright, Rutgers University, personal communication).

Overall, our results identify genotypic changes in clones of *C. difficile* selected, via serial passage, for reduced susceptibility to fidaxomicin or vancomycin in vitro. Intriguingly, we were unable to select for reduced susceptibility to LFF571 using the same approach. In a previous study, we selected single-step mutants with reduced susceptibility to LFF571 at a frequency of ≤10^{-8} with two of the strains used in the current study. The difference is that serial passage exposes cells to subinhibitory concentrations of an antibiotic for a prolonged period of time, whereas the single-step procedure uses super-inhibitory concentrations for only one passage. Serial passage therefore potentially allows multiple mutations to accumulate over generations. Our results suggest that it is unlikely that an accumulation of mutations selected at higher frequencies than the gain-of-function tuf mutation would confer a reduction in susceptibility of *C. difficile* exposed repeatedly to LFF571. None of the mutations identified here reduced susceptibility to other antibiotics tested, including LFF571. Since no in vitro
susceptibility test interpretive criteria have been established for vancomycin or fidaxomicin for the treatment of CDI, the clinical significance of these changes, if any, remains to be determined.

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