Effects of Subinhibitory Concentrations of Antibiotics on Colonization Factor Expression by Moxifloxacin-Susceptible and Moxifloxacin-Resistant *Clostridium difficile* Strains[▽]

Cécile Denève,[1] Sylvie Bouttier,[1] Bruno Dupuy,[2] Frédéric Barbut,[3]
Anne Collignon,[1,4]* and Claire Janoir[1]

*EA 4043, Université Paris-Sud 11, Faculté de Pharmacie, 5 Rue Jean Baptiste Clément, 92296 Châtenay-Malabry, France[1];
Unité des Toxines et Pathogénie Bactérienne, Institut Pasteur, 25 Rue du Dr. Roux, 75724 Paris Cedex 15, France[2];
EA 2392, Université Pierre et Marie Curie, Paris 6, Hôpital St. Antoine, Assistance Publique-Hôpitaux de
Paris, Paris, France[3]; and Service de Microbiologie, Hôpital Jean Verdier,
Assistance Publique-Hôpitaux de Paris, Bondy, France[4]*

Recent outbreaks of *Clostridium difficile* infection have been related to the emergence of the NAP1/027 epidemic strain. This strain demonstrates increased virulence and resistance to the C-8-methoxyfluoroquinolones gatifloxacin and moxifloxacin. These antibiotics have been implicated as major *C. difficile* infection-inducing agents. We investigated by real-time reverse transcription-PCR the impact of subinhibitory concentrations of ampicillin, clindamycin, ofloxacin, and moxifloxacin on the expression of genes encoding three colonization factors, the protease Cwp84, the high-molecular-weight S-layer protein, and the fibronectin-binding protein Fbp68. We have previously shown in six non-NAP1/027 moxifloxacin-susceptible strains that the presence of ampicillin or clindamycin induced an upregulation of these genes, whereas the presence of fluoroquinolones did not. The objective of this study was to analyze the expression of these genes under the same conditions in four NAP1/027 strains, one moxifloxacin susceptible and three moxifloxacin resistant. Two in vitro-selected moxifloxacin-resistant mutants were also analyzed. Moxifloxacin resistance was associated with the Thr82→Ile substitution in GyrA in all but one of the moxifloxacin-resistant strains. The expression of *cwp84* and *slpA* was strongly increased after culture with ampicillin or clindamycin in NAP1/027 strains. Interestingly, after culture with fluoroquinolones, the expression of *cwp84* and *slpA* was only increased in four moxifloxacin-resistant strains, including the NAP1/027 strains and one of the in vitro-selected mutants. The overexpression of *cwp84* was correlated with increased production of the protease Cwp84. The historical NAP1/027 moxifloxacin-susceptible strain and its mutant appear to be differently regulated by fluoroquinolones. Overall, fluoroquinolones appear to favor the expression of some colonization factor-encoding genes in resistant *C. difficile* strains. The fluoroquinolone resistance of the NAP1/027 epidemic strains could be considered an ecological advantage. This could also increase their colonization fitness and promote the infection.

Antibiotic exposure is the most widely recognized risk factor for *Clostridium difficile* infection (CDI). It is assumed that antibiotics disturb the normal intestinal microbiota and its barrier effect, allowing subsequent *C. difficile* colonization and CDI. Broad-spectrum antibiotics, such as clindamycin, aminopenicillins, and cephalosporins, possess a high propensity to induce CDI (24, 50). The use of fluoroquinolones has recently been proposed as a significant risk factor for CDI due to emergent strains, such as the NAP1/027 epidemic strain, that are resistant to the C₈-methoxyfluoroquinolones gatifloxacin and moxifloxacin (5, 15, 35, 45, 55). This strain displays increased virulence, causing high morbidity and a high mortality rate. This results partly from the hyperproduction of toxins TcdA and TcdB that is caused by a frameshift mutation in the gene encoding negative regulator TcdC (37, 38, 39, 40, 46, 57).

It has been suggested that the increased virulence was probably insufficient to explain the wide spread of this strain (40). One explanation could come from its resistance to the C-8-methoxyfluoroquinolones, which have become the most frequently used antimicrobial class in North America in the last several years, as well as throughout Europe. In France, this epidemic strain emerged a short time after they began to be prescribed. The resistance to fluoroquinolones could then be seen as offering an ecological advantage for this strain. A role of antibiotic resistance in the emergence of epidemic strains has already been suggested for the highly clindamycin-resistant "J strain" in the United States and, more recently, for a fluoroquinolone-resistant, toxin-A-negative, toxin-B-positive clonal strain in Ireland (20, 33). In addition, the increased virulence of strain NAP1/027 and its sudden emergence could also be explained by its considerable ability to adapt to the host gut environment and its greater colonization capacity.

The colonization process, which is the first step of the pathogenesis, involves a variety of cell surface-associated proteins. Among these proteins, the Cwp66 adhesin and the Fbp68 fibronectin-binding protein have been shown to mediate attachment to Vero cells (28, 56). The high-molecular-weight S-layer protein, encoded by the 5′ end of the *slpA* gene, has been

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* Corresponding author. Mailing address: Université Paris-Sud 11, Faculté de Pharmacie, Département de Microbiologie, 5 Rue Jean Baptiste Clément, 92296 Châtenay-Malabry Cedex, France. Phone: 33 1 46 83 56 34. Fax: 33 1 46 83 55 37. E-mail: anne.collignon@u-psud.fr.
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TABLE 1. Bacterial strains used in this study

Strain	Properties[c]	Resistance to new FQ[a]	Amino acid substitution in[b]:	
			GyrA	GyrB
ATCC 43603	TcdA⁻ TcdB⁻; clinical isolate from a newborn carrier (Belgium)	−	None	None
ATCC 43603-M1	TcdA⁻ TcdB⁻; in vitro moxifloxacin-resistant mutant of ATCC 43603	+	Thr82→Ile (ACT→ATT)	Ser366→Ala (TCA→GCA)
CD196 (CIP10793)	TcdA⁺ TcdB⁺; historical NAP1/027, clinical isolate from CDI (France)	−	None	None
CD196-M1	TcdA⁺ TcdB⁺; in vitro moxifloxacin-resistant mutant from CD196	+	Thr82→Ile (ACT→ATT)	Gln434→Lys (CAA→AAA)
CD07-259	TcdA⁺ TcdB⁺; current NAP1/027, clinical isolate from CDI (Nord-Pas-de-Calais, France)	+	Thr82→Ile (ACT→ATT)	None
6296	TcdA⁺ TcdB⁺; current NAP1/027, REA-type BI-11, clinical isolate from CDI (United States)	+	None	None
6425	TcdA⁺ TcdB⁺; current NAP1/027, REA type BI-12, clinical isolate from CDI (United States)	+	Thr82→Ile (ACT→ATT)	None

[a] New FQ, new fluoroquinolone moxifloxacin; −, susceptible strain (MIC < 8 µg/ml); +, resistant strain (MIC ≥ 8 µg/ml).
[b] Compared to the GyrA and the GyrB sequences in strain 630.
[c] REA, restriction endonuclease analysis.

shown to bind to epithelial cell lines (8, 9, 10, 34). The Cwp84 cysteine protease possesses degrading activity on several components of the gut basal lamina and could contribute to the degradation of host tissue integrity and the dissemination of the infection (32). These proteins are able to induce an immune response in patients with CDI, arguing for their putative role in vivo in the colonization process (11, 18, 43, 44).

Antibiotics could also play a direct role in enhancing colonization. In a previous study, we showed that subinhibitory concentrations of ampicillin and clindamycin increased the expression of four colonization factor-encoding genes (*cwp66*, *slpA*, *fbp68*, and especially, *cwp84*) in six moxifloxacin-susceptible *C. difficile* strains. In contrast, ofloxacin and moxifloxacin had no effect on the expression of these genes in the fluoroquinolone-susceptible strains (14). In this study, we investigated the impact of the four antibiotics (ampicillin, clindamycin, ofloxacin, and moxifloxacin) on colonization factor expression by six moxifloxacin-susceptible or moxifloxacin-resistant strains, including four NAP1/027 clinical isolates and two in vitro-selected moxifloxacin-resistant mutants.

MATERIALS AND METHODS

***C. difficile* strains and growth conditions.** Four NAP1/027 *C. difficile* strains isolated in Europe (CD196 and CD07-259) and the United States (6296 and 6425) and two in vitro-selected moxifloxacin-resistant mutants were used in this study. Among the NAP1/027 isolates, strain CD196 was the historical moxifloxacin-susceptible, nonepidemic French strain (47). The two moxifloxacin-resistant mutants were selected in vitro from strain CD196 and strain ATCC 43603, a nontoxigenic clinical isolate from Belgium (Table 1). Bacteria were grown overnight at 37°C in an anaerobic cabinet (Jacomex), in peptone yeast glucose infusion broth (PYG; Difco Laboratories) containing or not containing subinhibitory concentrations of antibiotic. The antibiotics used in this study were ampicillin (Eurobio), clindamycin (Dalacine; Pfizer), ofloxacin (Sigma-Aldrich), and moxifloxacin (Izilox; Bayer).

MICs were determined by the broth dilution method with *Bacteroides thetaiotaomicron* ATCC 29741 as control and were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) breakpoints.

For RNA and protein extraction experiments, antibiotics were added to the growth medium to a final concentration of 0.5× MIC. All the cultures were incubated until the stationary phase was reached. Optical density measures and viable count determinations were assessed to exclude bias errors due to differential growth.

In vitro selection of moxifloxacin-resistant mutants. Two moxifloxacin-resistant mutants were selected from the moxifloxacin-susceptible strains CD196 (NAP1/027) and ATCC 43603 (non-NAP1/027). These two parental strains were chosen in order to evaluate the relative roles of the fluoroquinolone resistance and/or the genetic background of these strains in the increased gene expression. Bacteria were grown overnight in antibiotic-free medium and then spread on Columbia cysteine agar containing moxifloxacin at 1× and 2× MIC (2 µg/ml and 4 µg/ml moxifloxacin). After 3 days of incubation at 37°C in an anaerobic cabinet, colonies were subcultured onto Columbia cysteine agar plates containing 2× MIC of moxifloxacin. One colony of each strain was selected, and the MIC was confirmed. The mutants were designated ATCC 43603-M1 and CD196-M1. The stability of the mutants was assessed by repeated passages on PYG medium.

Molecular analysis of the *tcdC* gene and the quinolone resistance-determining region. Genomic DNA was extracted using either a GFX genomic DNA purification kit (GE Healthcare) or a QIAamp DNA kit (Qiagen) according to the manufacturer's instructions. Primers OBD81 and OBD82 were used to amplify a 720-bp fragment encompassing the entire *tcdC* gene as previously described (39). Primers gyrA1 (5′-AATGAGTGTTATAGCTGGAGG-3′) and gyrA1-R1 (5′-TCTTTTAACGACTCATCAAAG-3′) were used to amplify a 390-bp fragment of *gyrA* (nucleotide positions 71 to 460), and primers gyrB1 (5′-ATGTGATGAACTGGGGGTCTT-3′) and gyrB1-R1 (5′-TCAAAATCTTCTCCAATACCA-3′) were used to amplify a 390-bp fragment of *gyrB* (nucleotide positions 1059 to 1448). Purified amplicons were sequenced by using a BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions. Analysis was performed either with an ABI PRISM 310 or with a 3100 genetic analyzer (Perkin-Elmer Applied Biosystems).

Gene expression analysis. (i) RNA isolation and cDNA synthesis. Optical density measures and viable count determinations confirmed that all cultures reached stationary phase and that RNA extraction was performed on equivalent cell numbers. Total RNA was isolated from at least two independent cultures (biological replicate) by using Trizol reagent (Invitrogen) according to a previously described method (14). The RNA concentration and quality were determined using an Agilent Bioanalyzer 2100 (Roche) and RNA 6000 Nano kit reagents & supplies (Roche). For each RNA sample, absence of DNA contamination was controlled by performing a real-time PCR on RNA templates (with the same conditions as for the expression study) using primers specific for the *rrs* gene (encoding the 16S rRNA subunit). First-strand DNA synthesis was then performed on 5 µg of purified total RNA with random primers by the use of SuperScriptIII reverse transcriptase (Invitrogen) according to the manufacturer's instructions.

(ii) Real-time PCR. Primers for *cwp84*, *slpA*, *fbp68*, and *rrs* were designed from the genome sequences of *C. difficile* 630 and QCD-32g58 (Canadian NAP1/027) (Table 2). Real-time PCR was carried out on a LightCycler instrument using a LightCycler FastStart DNA MasterPLUS SYBR green I kit (Roche). The dilutions of cDNA used in the study were 1:5,000,000 for the *rrs* gene, 1:50 for *cwp84* and *fbp68*, and 1:5,000 for *slpA*. Five microliters of cDNA was added to 5 µl of

TABLE 2. Primers sequences used for real-time PCR

Gene	Forward primer sequence	Reverse primer sequence	Amplicon size (bp)
cwp84	TGGGCAACTGGTGGAAAATA	TAGTTGCACCTTGTGCCTCA	151
slpA	AATGATAAAGCATTTGTAGTTGGTG	TATTGGAGTAGCATCTCCATC	126
fbp68	AGTTCGTCAAGTTTTACCTGGTC	GGTCCTTCCAATTCCTCTAGGT	120
rrs	GGGAGACTTGAGTGCAGGAG	GTGCCTCAGCGTCAGTTACA	120

PCR mixture (2 μl master mix, 0.5 μM of each specific primer, and 2 μl RNase-free water). The following PCR profile was used for amplification: initial denaturation for 8 min at 95°C and amplification for 45 cycles of 5 s at 95°C, 5 s at 60°C, and 6 s at 72°C. An additional step from a start at 70 to 95°C (0.1°C/s) was performed to establish a melting curve and was used to verify the specificity of the real-time PCR for each primer pair.

(iii) Analysis of the results. The results were normalized to those for the _rrs_ gene and analyzed using the comparative critical threshold ($\Delta\Delta C_T$) method. For each RNA extract (two biological replicates under each condition), measurements were performed in triplicate for each gene tested. The comparison of the relative expression ratios of the two biological replicates for each strain allowed us to consider that genes were significantly upregulated if their relative expression level was found to be at least 3. Results were expressed as means ± standard deviations.

Analysis of Cwp84 production by immunoblotting. Glycine extracts containing S-layer proteins were prepared in triplicate from 2 ml of fresh, independent, overnight broth culture as previously described (9). The protein extract concentration was determined by the Bradford method, using Bio-Rad protein assay reagent with bovine serum albumin as a standard. Equivalent amounts of each protein extract (10 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a Hybond-P polyvinylidene difluoride membrane (Amersham, GE Healthcare) for immunoblotting. Immunoblotting with specific polyclonal anti-Cwp84 antibodies (1:4,000 dilution in blocking buffer) was performed as previously described (32). Quantitative differences in Cwp84 production were estimated by densitometric analysis with an imaging system (ImageJ), and results were expressed as means ± standard deviations.

Statistical analysis. The nonparametric Wilcoxon test was performed to evaluate the significance of differences in the expression levels of colonization factor-encoding genes between (i) in vitro-selected moxifloxacin-resistant mutants and their parental strains and (ii) moxifloxacin-susceptible and moxifloxacin-resistant strains. _P_ values of <0.05, with a two-tailed significance level, were considered significant.

RESULTS

Antimicrobial susceptibilities. Table 3 shows the MICs of ampicillin, clindamycin, ofloxacin, and moxifloxacin for the five clinical isolates and the two in vitro-selected moxifloxacin-resistant mutants studied. Ampicillin displayed MICs of ≥2 μg/ml for all the strains tested. Among the five clinical isolates, two strains were resistant to clindamycin (MIC = 128 μg/ml). The nontoxigenic strain ATCC 43603 and the historical NAP1/027 strain CD196 were susceptible to moxi-

TABLE 3. MICs of the antibiotics used in this study

Strain	MIC (μg/ml)[a]			
	Ampicillin	Clindamycin	Ofloxacin	Moxifloxacin
ATCC 43603	2	4	8	2
ATCC 43603-M1	ND	ND	256	64
CD196	8	128	8	2
CD196-M1	ND	ND	128	128
CD07-259	4	4	256	32
6296	2	1	32	8
6425	4	128	256	32

[a] ND, not determined.

floxacin (MIC = 2 μg/ml), and the three current NAP1/027 strains were resistant (MIC ≥ 8 μg/ml). All the strains were resistant to ofloxacin (MIC > 4 μg/ml), but the two moxifloxacin-susceptible strains showed a low level of resistance (MIC = 8 μg/ml).

Following selection for moxifloxacin resistance, the two mutant strains, ATCC 43603-M1 and CD196-M1, displayed high-level resistance to moxifloxacin (MICs of 64 and 128 μg/ml, respectively) and ofloxacin (MIC ≥ 128 μg/ml).

Detection of _gyrA_ and _gyrB_ mutations. The quinolone resistance-determining regions of _gyrA_ and _gyrB_ were sequenced from nucleotide codons 40 to 145 and 354 to 482, respectively. No mutation in _gyrA_ or _gyrB_ was found in the two moxifloxacin-susceptible strains, including the historical NAP1/027 strain. In contrast, in two of the three NAP1/027 moxifloxacin-resistant strains (6425 and CD07-259) and in the two in vitro-selected moxifloxacin-resistant mutants, a point mutation (C→T transition) in _gyrA_ was identified, leading to amino acid substitution Thr82→Ile (Table 1). An additional point mutation in _gyrB_ was found in each in vitro-selected moxifloxacin-resistant mutant, resulting in amino acid substitutions Ser366→Ala in mutant ATCC 43603-M1 and Gln434→Lys in mutant CD196-M1 (Table 1). No mutation was found in either _gyrA_ or _gyrB_ in the third NAP1/027 moxifloxacin-resistant clinical isolate (6296), which showed a low level of resistance to moxifloxacin (MIC = 8 μg/ml).

Analysis of partial deletion and frameshift mutation of the _tcdC_ gene. The _tcdC_ gene was sequenced from the four _C. difficile_ NAP1/027 strains and compared to the published _tcdC_ gene for strain 630. We showed that all NAP1/027 strains studied carry not only the 18-bp deletion in _tcdC_ at positions 330 to 347 but also the 1-bp deletion at position 117 relative to the ATG start codon. In addition, no single nucleotide polymorphism appears when the _tcdC_ genes of the four NAP1/027 strains are compared to each other.

Effects of subinhibitory concentrations of antibiotics on the expression of genes encoding colonization factors in _C. difficile_ NAP1/027 strains. The expression of _cwp84_, encoding a cysteine protease; _slpA_, encoding the precursor of the S-layer proteins; and _fbp68_, encoding a fibronectin-binding protein, was analyzed in the four _C. difficile_ NAP1/027 strains grown in the presence of subinhibitory concentrations of ampicillin, clindamycin, ofloxacin, or moxifloxacin (0.5× MIC). In the presence of the four antibiotics tested, a general upregulation of the _cwp84_ and _slpA_ genes in the four NAP1/027 strains was observed. However, the level of regulation varied between genes and strains (Fig. 1). The _fbp68_ gene was marginally influenced by the presence of these antibiotics (Fig. 1).

Exposure to subinhibitory concentrations of ampicillin or clindamycin led to the upregulation of _slpA_ and, especially,
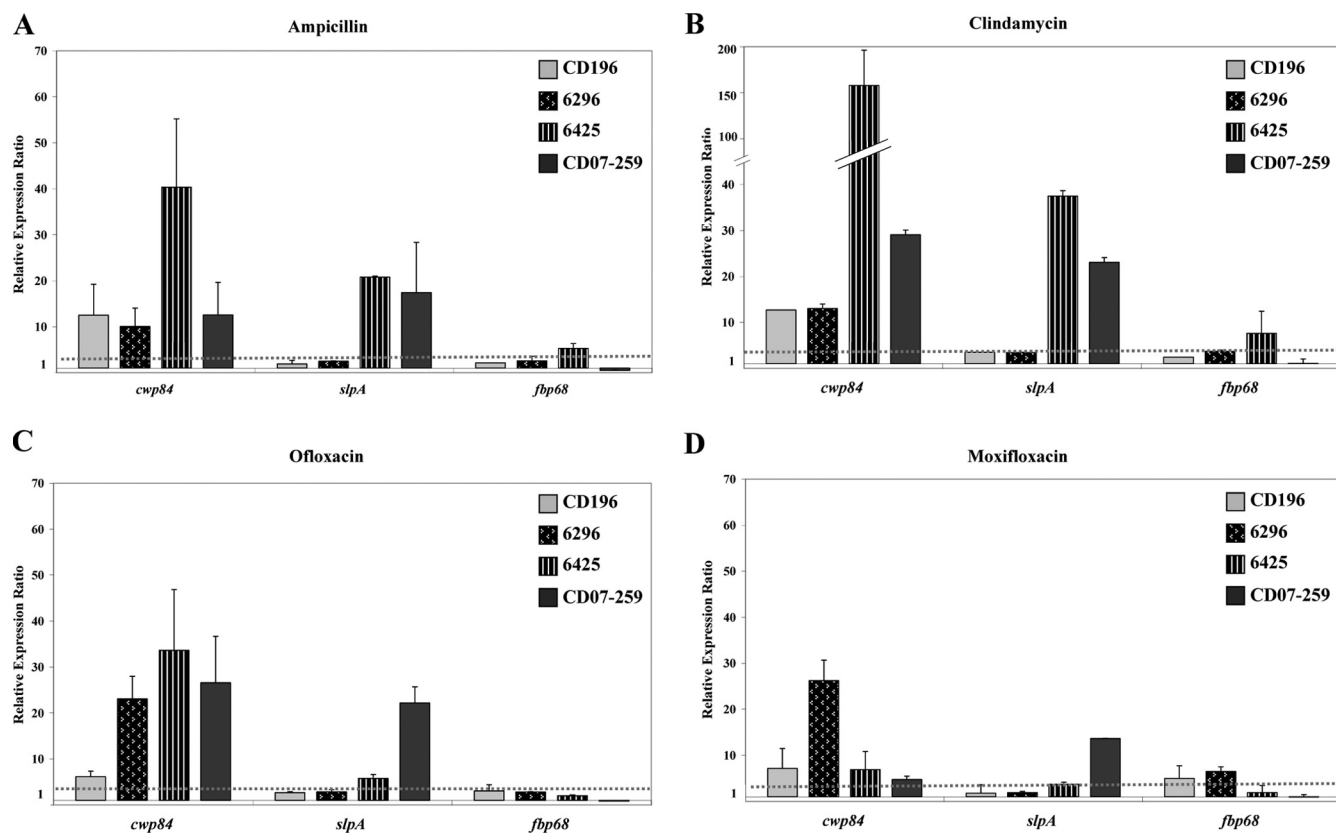
FIG. 1. Effects of subinhibitory concentrations of ampicillin (A), clindamycin (B), ofloxacin (C), and moxifloxacin (D) on the expression of *C. difficile* colonization factor-encoding genes in four NAP1/027 strains: one moxifloxacin susceptible (strain CD196) and three moxifloxacin resistant (strains 6296, 6425, and CD07-259). Bacteria were grown overnight in medium supplemented with 0.5× MIC of antibiotic. The data are the mean relative expression ratios (expression in bacteria grown with antibiotic versus expression in control grown in antibiotic-free medium) ± standard deviations. The dotted line represents the threshold value of upregulation (threefold).

cwp84. The levels of upregulation were variable and were not correlated with the levels of resistance to ampicillin and clindamycin in the strains tested (Fig. 1A and B).

In contrast, the impact of fluoroquinolones on the regulation of *slpA* and *cwp84* appears to differ in moxifloxacin-susceptible and moxifloxacin-resistant strains. Exposure to ofloxacin led to a strong increase in *cwp84* expression (23- to 34-fold) in the three moxifloxacin-resistant strains; exposure to moxifloxacin led to a strong, 26-fold increase in *cwp84* expression in one of the three moxifloxacin-resistant strains (6296) and a moderate increase in the two other resistant strains. Exposure to ofloxacin and moxifloxacin led to strong increases in the expression of *slpA* in strain CD07-259 (22- and 14-fold, respectively) and to weak increases in strain 6425 (6- and 4-fold, respectively). In contrast, in the historical NAP1/027 moxifloxacin-susceptible strain CD196, exposure to ofloxacin and moxifloxacin led to weak increases in the expression of *cwp84* (six- and sevenfold, respectively) and did not significantly modify the expression of *slpA* (Fig. 1C and D).

Effects of subinhibitory concentrations of antibiotics on the expression of genes encoding colonization factors in in vitro-selected moxifloxacin-resistant *C. difficile* mutants. In order to determine the differences in regulation observed between *C. difficile*-susceptible and -resistant strains exposed to fluoroquinolones, we analyzed the expression of *cwp84*, *slpA*, and

fbp68 in the presence of subinhibitory concentrations of ofloxacin or moxifloxacin (0.5× MIC) in in vitro-selected moxifloxacin-resistant mutants.

As previously observed, the expression of *fbp68* was not influenced by the presence of these antibiotics (Table 4).

In the NAP1/027 moxifloxacin-resistant mutant strain CD196-M1, exposure to ofloxacin and moxifloxacin led to nonsignificant upregulation in the expression of *cwp84* and *slpA* compared to their expression in its parental strain CD196 (Table 4).

In the nontoxigenic mutant strain ATCC 43603-M1, exposure to fluoroquinolones resulted in the general upregulation of *cwp84* and *slpA* expression (Table 4). The levels of overexpression of *cwp84* with ofloxacin and moxifloxacin were significantly higher ($P < 0.01$ and $P < 0.05$, respectively) than the level of expression in the moxifloxacin-susceptible parent; the expression of *slpA* was also upregulated, but not significantly so, compared to its expression in the parental strain ($P < 0.3$) (Table 4).

These results suggest that the acquisition of antibiotic resistance could modify, at least for those strains, the regulation of colonization factor genes in response to antibiotic stress.

Effects of subinhibitory concentrations of antibiotics on the production of Cwp84 protease. To correlate levels of gene upregulation and protein production, we analyzed the ef-

TABLE 4. Effects of subinhibitory concentrations of fluoroquinolones on the expression of genes encoding colonization factors of *C. difficile* in the two in vitro-selected moxifloxacin-resistant mutants and their parental strains

Gene	Antibiotic stress[a]	Mean relative expression ratio[b] ± SD					
		NAP1/027 strain			Non-NAP1/027 strain		
		CD196	CD196-M1	*P* value	ATCC 43603	ATCC 43603-M1	*P* value
cwp84	OFX	6.1 ± 1.3	11.7 ± 4.1	0.132[c]	3.2 ± 1.1	67.4 ± 34.0	0.009[c]
	MXF	7.1 ± 4.3	2.9 ± 0.2	0.485	3.1 ± 1.8	17.3 ± 5.4	0.024[c]
slpA	OFX	2.7 ± 0.2	4.3 ± 2.4	0.610	3.9 ± 0.1	32.8 ± 24.7	0.114
	MXF	1.8 ± 1.8	1.9 ± 0.8	0.575	2.9 ± 2.7	16.5 ± 11.8	0.262
fbp68	OFX	3.1 ± 1.3	2.3 ± 1.8	0.381	4.8 ± 1.7	2.1 ± 1.0	0.267
	MXF	5.0 ± 2.8	1.1 ± 0.9	0.048	2.1 ± 1.9	0.8 ± 0.2	0.800

[a] OFX, ofloxacin; MXF, moxifloxacin.
[b] Ratio of expression in bacteria grown with antibiotic versus expression in control grown without antibiotic. *P* values for the comparison of the upregulation of genes between the moxifloxacin-resistant mutant and its parental strain were derived using the Wilcoxon test.
[c] The difference between expression levels in the moxifloxacin-resistant mutant and its parental strain was significant.

fects of fluoroquinolones on the production of the Cwp84 protease. Immunoblotting was performed on glycine extracts of the four *C. difficile* NAP1/027 isolates, the ATCC 43603 parental strain, and the two in vitro-selected moxifloxacin-resistant mutants.

Densitometric analyses of immunoblots revealed that growth in the presence of subinhibitory concentrations of ofloxacin or moxifloxacin has a tendency to increase Cwp84 production in the three NAP1/027 moxifloxacin-resistant strains and the mutant strain ATCC 43603-M1 (Fig. 2). These results correlate with the increase of *cwp84* transcriptional expression previously observed.

The production of the protease was increased strongly in strain 6425 (approximately 130% in the presence of ofloxacin or moxifloxacin) and moderately in strains 6296, CD07-259, and ATCC 43603-M1. The production of the protease was also moderately increased in the historical NAP1/027 moxifloxacin-susceptible strain CD196, as well as in the mutant strain CD196-M1 (Fig. 2).

On the other hand, in the mutant strain ATCC 43603-M1, growth in the presence of ofloxacin or moxifloxacin, respectively, led to 25% and 20% increases in Cwp84 production, while the level of production was not significantly modified in its parental strain, ATCC 43603 (Fig. 2).
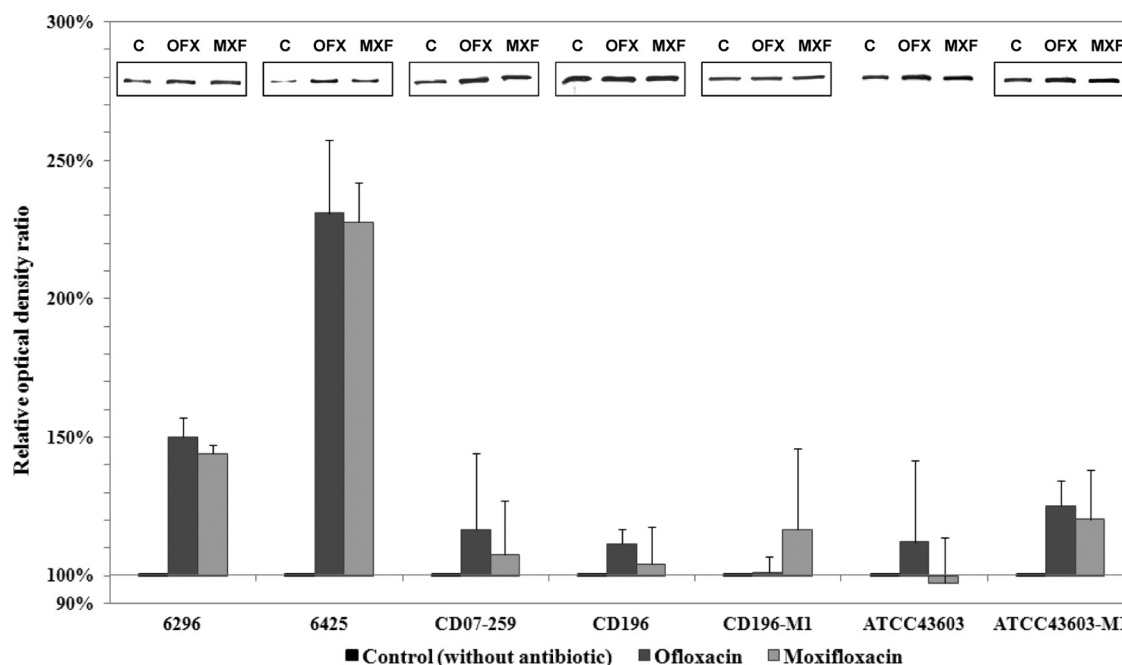

FIG. 2. Effect of subinhibitory concentrations of ofloxacin and moxifloxacin on the production of protease Cwp84 in three moxifloxacin-resistant NAP1/027 strains (6296, 6425, and CD07-259), a moxifloxacin-susceptible NAP1/027 strain (CD196), a non-NAP1/027 strain (ATCC 43603), and the two in vitro-selected moxifloxacin-resistant mutants (CD196-M1 and ATCC 43603-M1). Immunoblots and densitometry analyses of glycine extracts were performed. Anti-Cwp84 antibodies reacted with a unique band of 85-kDa protein. Histograms show mean relative optical density ratios (optical density of extract from bacteria grown with antibiotic versus optical density of extract from control grown in antibiotic-free medium) ± standard deviations; optical densities were determined by densitometry scanning. The inserts show examples of Cwp84 immunoblots for each strain grown in the absence (C, control without antibiotic) or presence of 0.5× MIC of ofloxacin (OFX) or moxifloxacin (MXF).

DISCUSSION

In this study, we investigated the influence of antibiotics on colonization factor expression in six moxifloxacin-susceptible or moxifloxacin-resistant *C. difficile* strains, including four NAP1/027 strains and two in vitro-selected moxifloxacin-resistant mutants.

No mutation was found in *gyrA* or *gyrB* in the two moxifloxacin-susceptible strains tested (Table 1). The high-level fluoroquinolone resistance of NAP1/027 strains CD07-259 and 6425 and of the in vitro-selected moxifloxacin-resistant mutants was associated with the usual amino acid substitution in GyrA (Thr82→Ile) (1, 2, 16, 19, 31, 51). An additional amino acid substitution in GyrB was found in each in vitro-selected mutant (20). In contrast, no mutation was found in *gyrA* or in *gyrB* in the NAP1/027 epidemic strain 6296. Since *C. difficile* lacks genes for topoisomerase IV, the moderate moxifloxacin resistance of this strain may then be explained by the reduction of the intracellular accumulation of the antibiotic, mediated by a drug efflux mechanism (1, 16). Nevertheless, several attempts to identify an efflux mechanism in *C. difficile* have failed (16, 20, 36).

There is increasing evidence that antibiotics at concentrations below the MIC possess biological activities that could influence the expression of virulence factors in many pathogenic gram-positive bacteria (13). In *Staphylococcus aureus*, the expression of the gene encoding the alpha-toxin Hla is induced to different degrees by exposure to subinhibitory concentrations of beta-lactams and fluoroquinolones and repressed by clindamycin (41). In contrast, clindamycin has been shown to stimulate the production of some exoproteins from staphylococci and *Streptococcus pyogenes* (29, 41, 49, 54).

Concerning *C. difficile*, the impact of subinhibitory concentrations of antibiotics on the production of TcdA and TcdB was subjected to numerous investigations, sometimes leading to disparate results (3, 21, 25, 30, 42, 48). However, some authors have suggested that the presence of antibiotics could facilitate toxin production (4, 23).

In contrast to these numerous studies of toxin expression, few studies have been performed on the impact of antibiotics on colonization factor expression. It has been previously shown that *C. difficile* grown in the presence of ampicillin produced greater levels of GroEL, a heat shock protein with adhesive properties (27). More recently, a study of the entire transcriptome of *C. difficile* strain 630 showed that antibiotic stress increased the transcription of genes encoding surface-associated proteins, including putative colonization factors (22).

We previously showed that exposure to ampicillin and clindamycin increased the expression of colonization factor-encoding genes by non-NAP1/027 moxifloxacin-susceptible *C. difficile* strains. This increase was correlated with increased adherence to Caco2-TC7 cells. In contrast, ofloxacin and moxifloxacin did not have such an effect (14). In this study, we showed that fluoroquinolones could have a different effect on *C. difficile* moxifloxacin-resistant strains. No significant difference in response to ampicillin and clindamycin stress was observed between moxifloxacin-resistant and moxifloxacin-susceptible strains. In contrast, the influence of ofloxacin on *cwp84* expression in moxifloxacin-resistant strains was strongly and significantly greater than in moxifloxacin-susceptible

strains, including the historical NAP1/027 strain CD196 (32.5-fold mean increase in moxifloxacin-resistant strains versus 3.4-fold mean increase in moxifloxacin-susceptible strains, $P < 0.01$). In addition, the influence of ofloxacin on *slpA* expression was also greater in moxifloxacin-resistant strains (13.6-fold mean increase versus 3-fold mean increase in moxifloxacin-susceptible strains), but this difference was not significant ($P = 0.052$). The influence of moxifloxacin on the expression of *cwp84* and *slpA* in moxifloxacin-resistant strains also appeared to be greater, but the changes in expression levels were not significantly different from those in moxifloxacin-susceptible strains.

In order to evaluate the respective roles of the acquisition of fluoroquinolone resistance and/or the genetic background of the strains in these differences in colonization factor gene regulation, we selected moxifloxacin-resistant mutants in vitro from one non-NAP1/027 strain (ATCC 43603) and the historical NAP1/027 moxifloxacin-susceptible strain (CD196). In the mutant strain ATCC 43603-M1, fluoroquinolones appear to upregulate colonization factor genes *cwp84* and *slpA*.

The regulation of genes appears to vary according to the gene. In our study, *cwp84* was the most upregulated gene in the presence of fluoroquinolones. The increased production of the protease Cwp84 observed in the glycine extracts did not correlate perfectly with the gene's overexpression. However, Cwp84 is not only associated with the cell wall but is also secreted (unpublished data). This could explain some of the discrepancies between gene upregulation and protein production.

In addition, an interstrain variability was observed despite the limited number of strains studied. This variability could concern the genetic environment of target genes and, also, regulatory genes. Although these NAP1/027 strains display the same *tcdC* organization and are clonally related, they appear to be differently regulated regarding their response to fluoroquinolone stress. This microheterogeneity and microevolution between hypervirulent NAP1/027 strains has already been suggested (52). We have shown here, for the NAP1/027 nonepidemic strain CD196, that the acquisition of fluoroquinolone resistance in the in vitro-selected mutant was insufficient to increase the expression of colonization factor genes in response to fluoroquinolone stress. The regulation of expression in this historical NAP1/027 strain appears to differ from that in the current NAP1/027 epidemic strains. This diversity of gene regulation between epidemic and nonepidemic strains has to be confirmed by the analysis of a larger panel of strains.

The molecular mechanisms of the regulatory effects of fluoroquinolones on colonization factor gene expression are not yet defined. Fluoroquinolones interact with DNA gyrase, leading to alterations in DNA supercoiling, which is known to affect gene transcription (17, 31). In addition, fluoroquinolones can induce the SOS response, which is recognized as a critical component of the response to environmental stress (12). Several studies have previously suggested that the stimulation or depression of bacterial gene expression by subinhibitory concentrations of many antibiotics, whatever their chemical structures and inhibitory actions, occurs at the transcriptional level (6, 26, 29).

In quinolone-resistant *S. aureus*, it was shown that the expression of the two fibronectin-binding proteins was increased

by subinhibitory concentrations of ciprofloxacin. This was correlated with an increased bacterial adhesion, and the authors have suggested that this may have contributed to the emergence of highly fluoroquinolone-resistant strains (6, 7).

In conclusion, our results suggest that antibiotics could lead to CDI not only by disruption of the barrier microbiota and its colonization resistance but also by inducing a bacterial stress response leading to enhanced expression of colonization factors, at least in the epidemic fluoroquinolone-resistant NAP1/027 strains studied. Antibiotics, then, may help *C. difficile* to colonize recently vacated niches. After oral administration, fluoroquinolones achieve high concentrations in human stools but the major part is bound to fecal material, leading to reduced amounts of active drug (53). These residual concentrations of fluoroquinolones may favor the adaptation of some *C. difficile* fluoroquinolone-resistant strains to the host environment. The genetic specificity and the colonization fitness of the *C. difficile* NAP1/027 epidemic strains combined with their antibiotic resistance may have contributed to their selection over other strains and their worldwide spread.

REFERENCES

1. **Ackermann, G., Y. J. Tang, R. Kueper, P. Heisig, A. C. Rodloff, J. Silva, Jr., and S. H. Cohen.** 2001. Resistance to moxifloxacin in toxigenic *Clostridium difficile* isolates is associated with mutations in *gyrA*. Antimicrob. Agents Chemother. **45:**2348–2353.
2. **Ackermann, G., Y. J. Tang-Feldman, R. Schaumann, J. P. Henderson, A. C. Rodloff, J. Silva, and S. H. Cohen.** 2003. Antecedent use of fluoroquinolones is associated with resistance to moxifloxacin in *Clostridium difficile*. Clin. Microbiol. Infect. **9:**526–530.
3. **Adams, D. A., M. M. Riggs, and C. J. Donskey.** 2007. Effect of fluoroquinolone treatment on growth of and toxin production by epidemic and nonepidemic *Clostridium difficile* strains in the cecal contents of mice. Antimicrob. Agents Chemother. **51:**2674–2678.
4. **Baines, S. D., J. Freeman, and M. H. Wilcox.** 2005. Effects of piperacillin/tazobactam on *Clostridium difficile* growth and toxin production in a human gut model. J. Antimicrob. Chemother. **55:**974–982.
5. **Biller, P., B. Shank, L. Lind, M. Brennan, L. Tkatch, G. Killgore, A. Thompson, and L. C. McDonald.** 2007. Moxifloxacin therapy as a risk factor for *Clostridium difficile*-associated disease during an outbreak: attempts to control a new epidemic strain. Infect. Control Hosp. Epidemiol. **28:**198–201.
6. **Bisognano, C., P. Vaudaux, P. Rohner, D. P. Lew, and D. C. Hooper.** 2000. Induction of fibronectin-binding proteins and increased adhesion of quinolone-resistant *Staphylococcus aureus* by subinhibitory levels of ciprofloxacin. Antimicrob. Agents Chemother. **44:**1428–1437.
7. **Bisognano, C., P. E. Vaudaux, D. P. Lew, E. Y. Ng, and D. C. Hooper.** 1997. Increased expression of fibronectin-binding proteins by fluoroquinolone-resistant *Staphylococcus aureus* exposed to subinhibitory levels of ciprofloxacin. Antimicrob. Agents Chemother. **41:**906–913.
8. **Calabi, E., F. Calabi, A. D. Phillips, and N. F. Fairweather.** 2002. Binding of *Clostridium difficile* surface layer proteins to gastrointestinal tissues. Infect. Immun. **70:**5770–5778.
9. **Calabi, E., S. Ward, B. Wren, T. Paxton, M. Panico, H. Morris, A. Dell, G. Dougan, and N. Fairweather.** 2001. Molecular characterization of the surface layer proteins from *Clostridium difficile*. Mol. Microbiol. **40:**1187–1199.
10. **Cerquetti, M., A. Molinari, A. Sebastianelli, M. Diociaiuti, R. Petruzzelli, C. Capo, and P. Mastrantonio.** 2000. Characterization of surface layer proteins from different *Clostridium difficile* clinical isolates. Microb. Pathog. **28:**363–372.
11. **Cerquetti, M., A. Pantosti, P. Stefanelli, and P. Mastrantonio.** 1992. Purification and characterization of an immunodominant 36 kDa antigen present on the cell surface of *Clostridium difficile*. Microb. Pathog. **13:**271–279.
12. **Cirz, R. T., M. B. Jones, N. A. Gingles, T. D. Minogue, B. Jarrahi, S. N. Peterson, and F. E. Romesberg.** 2007. Complete and SOS-mediated response of *Staphylococcus aureus* to the antibiotic ciprofloxacin. J. Bacteriol. **189:**531–539.
13. **Davies, J., G. B. Spiegelman, and G. Yim.** 2006. The world of subinhibitory antibiotic concentrations. Curr. Opin. Microbiol. **9:**445–453.
14. **Deneve, C., C. Delomenie, M. C. Barc, A. Collignon, and C. Janoir.** 2008. Antibiotics involved in *Clostridium difficile*-associated disease increase colonization factor gene expression. J. Med. Microbiol. **57:**732–738.
15. **Deshpande, A., C. Pant, A. Jain, T. G. Fraser, and D. D. Rolston.** 2008. Do fluoroquinolones predispose patients to *Clostridium difficile* associated disease? A review of the evidence. Curr. Med. Res. Opin. **24:**329–333.
16. **Dridi, L., J. Tankovic, B. Burghoffer, F. Barbut, and J. C. Petit.** 2002. *gyrA* and *gyrB* mutations are implicated in cross-resistance to ciprofloxacin and moxifloxacin in *Clostridium difficile*. Antimicrob. Agents Chemother. **46:**3418–3421.
17. **Drlica, K., and X. Zhao.** 1997. DNA gyrase, topoisomerase IV, and the 4-quinolones. Microbiol. Mol. Biol. Rev. **61:**377–392.
18. **Drudy, D., E. Calabi, L. Kyne, S. Sougioultzis, E. Kelly, N. Fairweather, and C. P. Kelly.** 2004. Human antibody response to surface layer proteins in *Clostridium difficile* infection. FEMS Immunol. Med. Microbiol. **41:**237–242.
19. **Drudy, D., L. Kyne, R. O'Mahony, and S. Fanning.** 2007. *gyrA* mutations in fluoroquinolone-resistant *Clostridium difficile* PCR-027. Emerg. Infect. Dis. **13:**504–505.
20. **Drudy, D., T. Quinn, R. O'Mahony, L. Kyne, P. O'Gaora, and S. Fanning.** 2006. High-level resistance to moxifloxacin and gatifloxacin associated with a novel mutation in *gyrB* in toxin-A-negative, toxin-B-positive *Clostridium difficile*. J. Antimicrob. Chemother. **58:**1264–1267.
21. **Drummond, L. J., D. G. Smith, and I. R. Poxton.** 2003. Effects of sub-MIC concentrations of antibiotics on growth of and toxin production by *Clostridium difficile*. J. Med. Microbiol. **52:**1033–1038.
22. **Emerson, J. E., R. A. Stabler, B. W. Wren, and N. F. Fairweather.** 2008. Microarray analysis of the transcriptional responses of *Clostridium difficile* to environmental and antibiotic stress. J. Med. Microbiol. **57:**757–764.
23. **Freeman, J., F. J. O'Neill, and M. H. Wilcox.** 2003. Effects of cefotaxime and desacetylcefotaxime upon *Clostridium difficile* proliferation and toxin production in a triple-stage chemostat model of the human gut. J. Antimicrob. Chemother. **52:**96–102.
24. **Freeman, J., and M. H. Wilcox.** 1999. Antibiotics and *Clostridium difficile*. Microbes Infect. **1:**377–384.
25. **Gerber, M., C. Walch, B. Loffler, K. Tischendorf, U. Reischl, and G. Ackermann.** 2008. Effect of sub-MIC concentrations of metronidazole, vancomycin, clindamycin and linezolid on toxin gene transcription and production in *Clostridium difficile*. J. Med. Microbiol. **57:**776–783.
26. **Goh, E. B., G. Yim, W. Tsui, J. McClure, M. G. Surette, and J. Davies.** 2002. Transcriptional modulation of bacterial gene expression by subinhibitory concentrations of antibiotics. Proc. Natl. Acad. Sci. USA **99:**17025–17030.
27. **Hennequin, C., A. Collignon, and T. Karjalainen.** 2001. Analysis of expression of GroEL (Hsp60) of *Clostridium difficile* in response to stress. Microb. Pathog. **31:**255–260.
28. **Hennequin, C., C. Janoir, M. C. Barc, A. Collignon, and T. Karjalainen.** 2003. Identification and characterization of a fibronectin-binding protein from *Clostridium difficile*. Microbiology **149:**2779–2787.
29. **Herbert, S., P. Barry, and R. P. Novick.** 2001. Subinhibitory clindamycin differentially inhibits transcription of exoprotein genes in *Staphylococcus aureus*. Infect. Immun. **69:**2996–3003.
30. **Honda, T., I. Hernadez, T. Katoh, and T. Miwatani.** 1983. Stimulation of enterotoxin production of *Clostridium difficile* by antibiotics. Lancet **1:**655.
31. **Hooper, D. C.** 1999. Mechanisms of fluoroquinolone resistance. Drug Resist. Updat. **2:**38–55.
32. **Janoir, C., S. Pechine, C. Grosdidier, and A. Collignon.** 2007. Cwp84, a surface-associated protein of *Clostridium difficile*, is a cysteine protease with degrading activity on extracellular matrix proteins. J. Bacteriol. **189:**7174–7180.
33. **Johnson, S., M. H. Samore, K. A. Farrow, G. E. Killgore, F. C. Tenover, D. Lyras, J. I. Rood, P. DeGirolami, A. L. Baltch, M. E. Rafferty, S. M. Pear, and D. N. Gerding.** 1999. Epidemics of diarrhea caused by a clindamycin-resistant strain of *Clostridium difficile* in four hospitals. N. Engl. J. Med. **341:**1645–1651.
34. **Karjalainen, T., A. J. Waligora-Dupriet, M. Cerquetti, P. Spigaglia, A. Maggioni, P. Mauri, and P. Mastrantonio.** 2001. Molecular and genomic analysis of genes encoding surface-anchored proteins from *Clostridium difficile*. Infect. Immun. **69:**3442–3446.
35. **Kuijper, E. J., B. Coignard, and P. Tull.** 2006. Emergence of *Clostridium difficile*-associated disease in North America and Europe. Clin. Microbiol. Infect. **12**(Suppl. 6):2–18.
36. **Lebel, S., S. Bouttier, and T. Lambert.** 2004. The *cme* gene of *Clostridium difficile* confers multidrug resistance in Enterococcus faecalis. FEMS Microbiol. Lett. **238:**93–100.
37. **Loo, V. G., L. Poirier, M. A. Miller, M. Oughton, M. D. Libman, S. Michaud, A. M. Bourgault, T. Nguyen, C. Frenette, M. Kelly, A. Vibien, P. Brassard, S. Fenn, K. Dewar, T. J. Hudson, R. Horn, P. Rene, Y. Monczak, and A. Dascal.** 2005. A predominantly clonal institutional outbreak of *Clostridium difficile*-associated diarrhea with high morbidity and mortality. N. Engl. J. Med. **353:**2442–2449.
38. **MacCannell, D. R., T. J. Louie, D. B. Gregson, M. Laverdiere, A. C. Labbe,**

F. Laing, and S. Henwick. 2006. Molecular analysis of *Clostridium difficile* PCR ribotype 027 isolates from eastern and western Canada. J. Clin. Microbiol. **44:**2147–2152.

39. **Matamouros, S., P. England, and B. Dupuy.** 2007. *Clostridium difficile* toxin expression is inhibited by the novel regulator TcdC. Mol. Microbiol. **64:** 1274–1288.

40. **McDonald, L. C., G. E. Killgore, A. Thompson, R. C. Owens, Jr., S. V. Kazakova, S. P. Sambol, S. Johnson, and D. N. Gerding.** 2005. An epidemic, toxin gene-variant strain of *Clostridium difficile*. N. Engl. J. Med. **353:**2433–2441.

41. **Ohlsen, K., W. Ziebuhr, K. P. Koller, W. Hell, T. A. Wichelhaus, and J. Hacker.** 1998. Effects of subinhibitory concentrations of antibiotics on alpha-toxin (*hla*) gene expression of methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* isolates. Antimicrob. Agents Chemother. **42:**2817–2823.

42. **Onderdonk, A. B., B. R. Lowe, and J. G. Bartlett.** 1979. Effect of environmental stress on *Clostridium difficile* toxin levels during continuous cultivation. Appl. Environ. Microbiol. **38:**637–641.

43. **Pechine, S., A. Gleizes, C. Janoir, R. Gorges-Kergot, M. C. Barc, M. Delmee, and A. Collignon.** 2005. Immunological properties of surface proteins of *Clostridium difficile*. J. Med. Microbiol. **54:**193–196.

44. **Pechine, S., C. Janoir, and A. Collignon.** 2005. Variability of *Clostridium difficile* surface proteins and specific serum antibody response in patients with *Clostridium difficile*-associated disease. J. Clin. Microbiol. **43:**5018–5025.

45. **Pepin, J., N. Saheb, M. A. Coulombe, M. E. Alary, M. P. Corriveau, S. Authier, M. Leblanc, G. Rivard, M. Bettez, V. Primeau, M. Nguyen, C. E. Jacob, and L. Lanthier.** 2005. Emergence of fluoroquinolones as the predominant risk factor for *Clostridium difficile*-associated diarrhea: a cohort study during an epidemic in Quebec. Clin. Infect. Dis. **41:**1254–1260.

46. **Pepin, J., L. Valiquette, and B. Cossette.** 2005. Mortality attributable to nosocomial *Clostridium difficile*-associated disease during an epidemic caused by a hypervirulent strain in Quebec. CMAJ **173:**1037–1042.

47. **Popoff, M. R., E. J. Rubin, D. M. Gill, and P. Boquet.** 1988. Actin-specific ADP-ribosyltransferase produced by a *Clostridium difficile* strain. Infect. Immun. **56:**2299–2306.

48. **Pultz, N. J., and C. J. Donskey.** 2005. Effect of antibiotic treatment on growth of and toxin production by *Clostridium difficile* in the cecal contents of mice. Antimicrob. Agents Chemother. **49:**3529–3532.

49. **Sawai, J., T. Hasegawa, T. Kamimura, A. Okamoto, D. Ohmori, N. Nosaka, K. Yamada, K. Torii, and M. Ohta.** 2007. Growth phase-dependent effect of clindamycin on production of exoproteins by *Streptococcus pyogenes*. Antimicrob. Agents Chemother. **51:**461–467.

50. **Spencer, R. C.** 1998. The role of antimicrobial agents in the aetiology of *Clostridium difficile*-associated disease. J. Antimicrob. Chemother. **41**(Suppl. C):21–27.

51. **Spigaglia, P., F. Barbanti, P. Mastrantonio, J. S. Brazier, F. Barbut, M. Delmee, E. Kuijper, and I. R. Poxton.** 2008. Fluoroquinolone resistance in *Clostridium difficile* isolates from a prospective study of *C. difficile* infections in Europe. J. Med. Microbiol. **57:**784–789.

52. **Stabler, R. A., D. N. Gerding, J. G. Songer, D. Drudy, J. S. Brazier, H. T. Trinh, A. A. Witney, J. Hinds, and B. W. Wren.** 2006. Comparative phylogenomics of *Clostridium difficile* reveals clade specificity and microevolution of hypervirulent strains. J. Bacteriol. **188:**7297–7305.

53. **Sullivan, A., C. Edlund, and C. E. Nord.** 2001. Effect of antimicrobial agents on the ecological balance of human microflora. Lancet Infect. Dis. **1:**101–114.

54. **Tanaka, M., T. Hasegawa, A. Okamoto, K. Torii, and M. Ohta.** 2005. Effect of antibiotics on group A *Streptococcus* exoprotein production analyzed by two-dimensional gel electrophoresis. Antimicrob. Agents Chemother. **49:**88–96.

55. **Walbrown, M. A., S. L. Aspinall, N. K. Bayliss, R. A. Stone, F. Cunningham, C. L. Squier, and C. B. Good.** 2008. Evaluation of *Clostridium difficile*-associated diarrhea with a drug formulary change in preferred fluoroquinolones. J. Manag. Care Pharm. **14:**34–40.

56. **Waligora, A. J., C. Hennequin, P. Mullany, P. Bourlioux, A. Collignon, and T. Karjalainen.** 2001. Characterization of a cell surface protein of *Clostridium difficile* with adhesive properties. Infect. Immun. **69:**2144–2153.

57. **Warny, M., J. Pepin, A. Fang, G. Killgore, A. Thompson, J. Brazier, E. Frost, and L. C. McDonald.** 2005. Toxin production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe. Lancet **366:**1079–1084.