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Antibiotics involved in *Clostridium difficile*-associated disease increase colonization factor gene expression

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Clostridium difficile is the most common cause of antibiotic-associated diarrhoea. Antibiotics are presumed to disturb the normal intestinal microbiota, leading to depletion of the barrier effect and colonization by pathogenic bacteria. This first step of infection includes adherence to epithelial cells. We investigated the impact of various environmental conditions *in vitro* on the expression of genes encoding known, or putative, colonization factors: three adhesins, P47 (one of the two S-layer proteins), Cwp66 and Fbp68, and a protease, Cwp84. The conditions studied included hyperosmolarity, iron depletion and exposure to several antibiotics (ampicillin, clindamycin, ofloxacin, moxifloxacin and kanamycin). The analysis was performed on three toxigenic and three non-toxigenic *C. difficile* isolates using real-time PCR. To complete this work, the impact of ampicillin and clindamycin on the adherence of *C. difficile* to Caco-2/TC7 cells was analysed. Overall, for the six strains of *C. difficile* studied, exposure to subinhibitory concentrations (1/2 MIC) of clindamycin and ampicillin led to the increased expression of genes encoding colonization factors. This was correlated with the increased adherence of *C. difficile* to cultured cells under the same conditions. The levels of gene regulation observed among the six strains studied were highly variable, *cwp84* being the most upregulated. In contrast, the expression of these genes was weakly, or not significantly, modified in the presence of ofloxacin, moxifloxacin or kanamycin. These results suggest that, in addition to the disruption of the normal intestinal microbiota and its barrier effect, the high propensity of antibiotics such as ampicillin and clindamycin to induce *C. difficile* infection could also be explained by their direct role in enhancing colonization by *C. difficile*.

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INTRODUCTION

Pseudomembranous colitis and diarrhoea have long been recognized as important adverse effects of antimicrobial chemotherapy, even before *Clostridium difficile* was recognized as the aetiological agent (Johnson & Gerding, 1998). This Gram-positive anaerobic bacillus is now considered an important nosocomial enteropathogen. It is associated with considerable morbidity and attributable mortality, especially since the emergence and dissemination of the *C. difficile* NAP1, PCR ribotype 027 epidemic strain, which demonstrates increased virulence and fluoroquinolone resistance (McDonald *et al.*, 2005; Pepin *et al.*, 2005b; Tachon *et al.*, 2006; Warny *et al.*, 2005). The major virulence factors are the two toxins A and B, which cause severe tissue damage and influence the immune response, leading to the clinical manifestations of moderate to severe diarrhoea and the sometimes fatal condition pseudomembranous colitis (Cloud & Kelly, 2007). However, the first step of pathogenesis is the colonization process, including adherence to gut epithelial cells. This step involves an array of adhesion factors, some of which have recently been identified. The cell surface-associated proteins Cwp66, Fbp68, GroEL and the S-layer protein P47, encoded by the 5′-part of the *slpA* gene, have been shown to mediate attachment to epithelial cell lines (Calabi *et al.*, 2002; Cerquetti *et al.*, 2000; Hennequin *et al.*, 2001b, 2003; Karjalainen *et al.*, 2001; Waligora *et al.*, 2001). The flagellar cap protein FliD is involved in the attachment to mucus, the first barrier encountered during colonization (Tasteyre *et al.*, 2001a, b). In addition, *C. difficile* displays some surface proteolytic activity (Poilane *et al.*, 1998; Seddon & Borriello, 1992). We recently characterized the Cwp84 cysteine protease, which possesses degrading activity on several extracellular matrix proteins and which could contribute to degradation of host tissue integrity (Janoir

Abbreviations: CDAD, *Clostridium difficile*-associated disease; C_t, threshold cycle; $\Delta\Delta C_t$, comparative critical threshold.

et al., 2007). Most of these surface-associated proteins are able to induce an immune response in patients with *C. difficile*-associated disease (CDAD), which confirms that these proteins are expressed during the course of the infection and could be involved *in vivo* in the colonization process (Cerquetti *et al.*, 1992; Drudy *et al.*, 2004; Pechine *et al.*, 2005a, b).

Antibiotic exposure has been highlighted as the most important risk factor for *C. difficile* infection, especially exposure to broad-spectrum antibiotics, such as clindamycin, aminopenicillins and cephalosporins (Freeman & Wilcox, 1999; Spencer, 1998). Until recently, fluoroquinolones were seldom associated with *C. difficile* infection, but, since the emergence of the resistant NAP1/027 strain, the use of the new fluoroquinolones, such as moxifloxacin or gatifloxacin, is now considered an important risk factor for CDAD (Bartlett, 2006; Pepin *et al.*, 2005a). Antibiotics are presumed to disturb the normal colonic microbiota, impairing the barrier effect and allowing subsequent colonization and infection by *C. difficile*. However, we could hypothesize that antibiotics may also play a role in enhancing the expression of virulence or colonization factors (Starr & Impallomeni, 1997). Several studies have examined the impact of antibiotics on toxin production, but their conclusions were divergent (Adams *et al.*, 2007; Baines *et al.*, 2005; Barc *et al.*, 1992; Drummond *et al.*, 2003; Nakamura *et al.*, 1982; Onderdonk *et al.*, 1979; Pultz & Donskey, 2005). Our own studies of adhesion have previously shown that the adherence of *C. difficile* to cultured cell lines could be regulated by environmental conditions (Hennequin *et al.*, 2001a; Waligora *et al.*, 1999).

This study was performed to evaluate the impact of various environmental conditions, including antibiotics, on both the expression of genes encoding colonization factors of *C. difficile* and the adherence of *C. difficile* to cultured cells.

METHODS

Bacterial strains and growth conditions. A total of six European clinical isolates of *C. difficile*, toxigenic and non-toxigenic, belonging to a limited number of serogroups, were used in this study (Table 1). Bacteria were grown overnight at 37 °C in an anaerobic cabinet (Jacomex) in tryptone yeast glucose infusion broth (Difco Laboratories) either containing or not containing additional components diluted from stock solutions to appropriate final concentrations. To evaluate the impact of high osmolarity and iron depletion, 200 mM NaCl or 50 μM of the iron chelator deferoxamine (Sigma-Aldrich) was added to the growth media. Antibiotics chosen for the study were: ampicillin (Eurobio) and clindamycin (Dalacine; Pfizer), two antimicrobial agents that have been associated with CDAD for a long time; ofloxacin (Sigma-Aldrich); moxifloxacin (Izilox; Bayer), a new fluoroquinolone that has been recently associated with CDAD; and kanamycin (Sigma-Aldrich), which has never been associated with CDAD because of its inactivity against anaerobic bacteria. MICs of all antibiotics for the six strains were determined by the broth dilution method; for the assay, antibiotics were added in the growth medium to a final concentration of half the MIC. For kanamycin, we chose to work with a concentration of 8 μg ml^{-1}, which approximates the mean concentration used for the other antibiotics. All cultures were incubated overnight (approx. 16 h) until the stationary phase was reached (OD_{620} at least 1.3), to exclude bias errors due to a differential growth.

RNA isolation and cDNA synthesis. Total RNA was isolated from 5 ml fresh overnight broth culture using Trizol reagent (Invitrogen) according to a previously described method (Savariau-Lacomme *et al.*, 2003). Briefly, after bacterial lysis in buffer containing 50 mM Tris (pH 8.0), 25 % sucrose and 10 mg lysozyme ml^{-1}, RNA was extracted with Trizol and then precipitated in absolute ethanol and stored until use at −80 °C. Before cDNA synthesis, RNA was washed with 70 % ethanol and dissolved in RNase-free water. RNA concentration and quality were determined using the Bioanalyser Agilent 2100 (Roche) and RNA 6000 nano reagent and supplies kit (Roche). Checks for DNA contamination were (a) a classical PCR on RNA templates and (b) real-time PCR, under the same conditions used for the expression study, using specific primers for the *rrs* gene. When needed, DNase–RNase free treatment (DNase I; Invitrogen) was performed on RNA extracts and the absence of DNA contamination was confirmed by the same methods. First-strand DNA synthesis was then performed on 5 μg purified total RNA with random primers using SuperScriptIII reverse transcriptase (Invitrogen), according to the manufacturer's instructions.

Real-time PCR. Primers for *cwp84*, *cwp66*, *slpA*, *fbp68*, *rrs*, *gluD* and *gyrA* were designed from known gene sequences by using the Primer3 primer design software (Table 2). Real-time PCR was carried out on a LightCycler instrument (Roche Diagnostics) using the LightCycler Fast Start DNA Master^{PLUS} SYBR Green I kit (Roche). The cDNA dilutions used in this study were 1/50, 1/500 or 1/5000 for the genes encoding colonization factors and 1/5 000 000 for the *rrs* gene; they were chosen in order to obtain a threshold cycle (C_t) value between 20 and 30. Five microlitres of the appropriate dilution was added to 5 μl PCR mixture (2 μl master mix, 0.5 μl of each specific primer at

Table 1. Bacterial strains used in this study

Strain	Serogroup	Toxin production	Origin*	Source
ATCC 43593	B	$TcdA^-$ $TcdB^-$	New-born carrier	Belgium
CO 109	B	$TcdA^-$ $TcdB^-$	ND	France
ATCC 43596	C	$TcdA^+$ $TcdB^+$	PMC	Belgium
630	C	$TcdA^+$ $TcdB^+$	PMC	UK
ATCC 43603	X	$TcdA^-$ $TcdB^-$	New-born carrier	Belgium
79-685	S3	$TcdA^+$ $TcdB^+$	PMC	France

*ND, Not determined; PMC, pseudomembranous colitis.

Table 2. Primer sequences used for real-time PCR

Target gene	Function of encoding protein	5′ primer	3′ primer	Amplicon size (bp)
cwp84	Cell wall protein – protease	TGGGCAACTGGTGGAAAATA	TAGTTGCACCTTGTGCCTCA	151
cwp66	Cell wall protein – adhesin	TTGGTGGCTTAGGTAATGAAGA	CATCATTTGCATCTGCCTTTT	123
slpA	Surface layer protein (C-ter. part) – adhesin	AATGATAAAGCATTTGTAGTTGGTG	TATTGGAGTAGCATCTCCATC	126
fbp68	Fibronectin binding protein	AGTTCGTCAAGTTTTACCTGGTC	GGTCCTTCCAATTCCTCTAGGT	120
rrs	16S RNA ribosomal subunit	GGGAGACTTGAGTGCAGGAG	GGGAGACTTGAGTGCAGGAG	120
gluD	Glutamate dehydrogenase	ATGCAGTAGGGCCAACAAAA	ATGCAGTAGGGCCAACAAAA	135
gyrA	DNA gyrase subunit A	CTCGTATTGTTGGGGACGTT	ATCCC ATCAACAGAACCAA	146

10 μM and 2 μl RNase-free water). Thermal cycling conditions were as follows: initial denaturation at 95 °C for 8 min, followed by 45 cycles of denaturation at 95 °C for 5 s, hybridization at 60 °C for 5 s and extension at 72 °C for 6 s. Fluorescence measurements were recorded during each extension step. An additional step starting from 70 to 95 °C (0.1 °C s^{-1}) was performed to establish a melting curve and was used to verify the specificity of the real-time PCR reaction for each primer pair.

Analysis of the results. For each measurement, a C_t value was determined and the results were analysed using the comparative critical threshold ($\Delta\Delta C_t$) method. In order to choose a housekeeping gene for normalization of the results, the expression stabilities of three genes, *rrs* (encoding the 16S rRNA subunit), *gluD* (encoding glutamate dehydrogenase) and *gyrA* (encoding DNA gyrase subunit A), were evaluated for the toxigenic 79-685 and the non-toxigenic ATCC 43603 strains, under the following conditions: high sodium concentration, iron depletion and a subinhibitory concentration of ampicillin. The 16S rRNA gene, which was consistently more stably expressed (variation of C_t <5 %) than *gluD* and *gyrA* (variation of C_t between 10 and 25 %), was then chosen for normalization during this study, as in other studies (Eleaume & Jabbouri, 2004; Tasara & Stephan, 2007). For each condition (200 mM NaCl, 50 μM deferoxamine or subinhibitory concentrations of antibiotics), measurements of cDNA (synthesized from RNA extracted from at least two independent cultures) were carried out in duplicate or triplicate for each gene. Comparison of relative expression ratios obtained from different analyses of the same sample (corresponding to one RNA extraction) allowed us to calculate technical reproducibility values and data with non-satisfactory values were discarded. Comparison of relative expression ratios obtained from the analysis of different samples (corresponding to different RNA extractions) for each strain allowed us to calculate the biological replicate and to define that a $\Delta\Delta C_t$ between 1 and 2.5 could be considered as a physiological change. We then considered that genes were significantly down- or upregulated if their relative expression level was found to be at least 2.5. Results were expressed as means and standard deviations.

Adherence assays. The enterocyte-like Caco-2/TC7 cell line was used between passages 20 and 25. Cells were routinely grown in Dulbecco's modified Eagle's minimum essential medium (DMEM, 4.5 g glucose l^{-1}; Life Technologies) supplemented with 15 % fetal calf serum (Boehringer) and 1 % non-essential amino acids (Life Technologies). The Caco-2/TC-7 monolayers were prepared in 24-well TPP tissue culture plates (ATGC, Paris, France) and used at late post-confluence (15 days after seeding). Prior to adherence assays, cells were washed twice with PBS and 0.5 ml DMEM was added to each well. Bacteria were grown as previously described, with or without subinhibitory concentrations of antibiotics, washed twice with PBS and 0.5×10^9 c.f.u. were added to each well. Bacteria and cells were incubated together for 1 h at 37 °C under anaerobic conditions and the plates were then washed three times with PBS in order to discard non-adherent bacteria. For each plate, 12 wells were used to count cell-adherent bacteria after fixation and staining (Gram stain) and 12 wells were used for enumeration of cell-adherent bacteria after culture. Briefly, cells were lysed with 0.5 ml cold water per well during 1 h at 4 °C and appropriate dilutions were spread on Columbia cysteine agar plates supplemented with 5 % horse blood (bioMérieux). Bacterial colonies were counted after 24–48 h of incubation and results were expressed as c.f.u. of cell-adherent bacteria ml^{-1}. Assays were carried out in triplicate in two separate experiments.

RESULTS AND DISCUSSION

MICs

Results of MICs of the antibiotics used in this study for the six strains are shown in Table 3. All the strains were resistant to ampicillin (MIC 2 and 4 μg ml^{-1}), three strains were susceptible to clindamycin (MIC <8 μg ml^{-1}) and three strains were resistant (MIC ⩾8 μg ml^{-1}). All the strains were resistant to ofloxacin (MIC >4 μg ml^{-1}) and susceptible to moxifloxacin (MIC <8 μg ml^{-1}).

Amplification of genes encoding colonization factors

Because of high interstrain variability, the amplification of the *cwp66* gene could only be performed for three strains, 79-685, ATCC 43603 and CO109. For the same reason,

Table 3. MICs of the antibiotics used in this study

Strain	MIC (μg ml^{-1})			
	Ampicillin	Clindamycin	Ofloxacin	Moxifloxacin
ATCC 43593	2	4	16	2
CO 109	2	4	16	2
ATCC 43596	4	32	16	2
630	4	16	8	2
ATCC 43603	2	4	8	2
79-685	2	8	16	4

amplification of the *slpA* gene could not be performed for the ATCC 43593 strain.

Effect of high osmolarity and iron depletion on *C. difficile* gene expression

We chose to analyse the impact of high osmolarity and iron depletion, two environmental conditions that deviate from the physiological norm (Bermudez *et al.*, 1997; Conte *et al.*, 1994). This analysis was performed on the toxigenic 79-685 strain and the non-toxigenic ATCC 43603 strain for three genes, *cwp84*, *cwp66* and *slpA*. Exposure to iron depletion did not significantly modify the expression of genes, which was consistent with a previous study on the expression of *groEL* (Hennequin *et al.*, 2001a). However, iron depletion has been shown to promote the expression of adherence factors in other bacteria, such as *Staphylococcus aureus* (Clarke *et al.*, 2004; Morrissey *et al.*, 2002), and to increase *C. difficile* adherence to Vero cells (Waligora *et al.*, 1999). This could suggest that other, unknown adherence factors may be involved in the adherence of *C. difficile*. In contrast, exposure to high osmolarity led to high overexpression of the genes studied, with the exception of *slpA* (Fig. 1): the expression of *cwp84* was increased 44- and 27-fold for the toxigenic and the non-toxigenic strains, respectively; the expression of *cwp66* was also increased by high osmolarity, but to a lesser extent (24- and 10-fold, respectively); and the expression of *slpA* was moderately increased (relative expression ratios of two to four). These results were correlated with the three- to fourfold increase in *C. difficile* cell adherence after exposure to high sodium concentrations (Waligora *et al.*, 1999) and the high increase in *groEL* expression (Hennequin *et al.*, 2001a). In addition, high sodium concentration has already been shown to increase the expression of colonization factors in *Salmonella typhi* (Leclerc *et al.*, 1998).

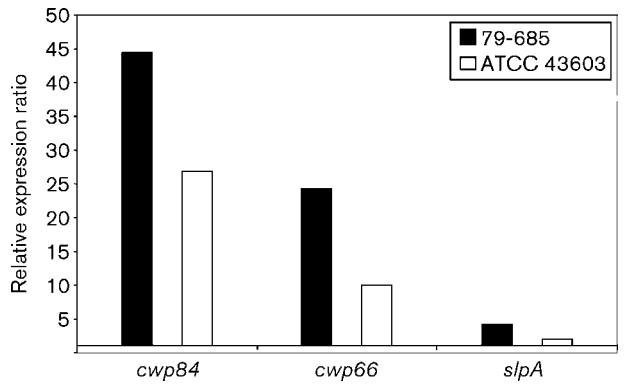

Fig. 1. Real-time RT-PCR analysis of relative expression of three genes encoding colonization factors of two strains of *C. difficile* grown under high sodium concentration.

Effect of subinhibitory concentrations of antibiotics on *C. difficile* gene expression

For all the strains studied, the expression of colonization factors was influenced by exposure to subinhibitory concentrations of antibiotics in the growth medium, albeit to different extents. Overall, the range of expression was variable among the genes studied. The gene *cwp84*, encoding a cysteine protease (Janoir *et al.*, 2007), was the most upregulated, with relative expression ratios ranging from 1 to 41. In contrast, the genes encoding the three adhesins Cwp66, the S-layer protein P47 and Fbp68 showed a moderate overexpression, with relative expression ratios ranging from 1 to 11. Whichever strain was tested, the two antibiotics representing old and new fluoroquinolones only marginally modified the expression of the four genes studied; their relative expression ratios were between one and six (Fig. 2a, b). In contrast, the influence of ampicillin and clindamycin on the expression of these genes was clear (Fig. 2c, d). Exposure to ampicillin led to a 2- to 27-fold increase in the expression of *cwp84* and a 2- to 10-fold increase in the expression of *cwp66*, *slpA* and *fbp68*. In the presence of subinhibitory concentrations of clindamycin, the increase in the expression of *cwp84* was clearly the most important, with relative expression ratios ranging from 2 to 41; the expression of *fbp68* was moderately increased (relative expression ratios between 1 and 11) and that of *cwp66* and *slpA* was moderate or weak (less than sevenfold). The presence of kanamycin led to relative expression ratios $\leqslant 5$ for the two strains studied: relative expression ratios ranged from 1.3 to 2.3 for the toxigenic 79-685 strain and from 3.2 to 5 for the non-toxigenic strain ATCC 43603 (data not shown). To conclude, even though there was a tendency towards upregulation of these genes observed for all the strains studied, the levels of regulation were highly variable and were not correlated with the resistance level of the strains.

Effect of subinhibitory concentrations of antibiotics on *C. difficile* adherence

Since Cwp66, Fbp68 and the S-layer protein P47 are involved in the adherence of *C. difficile* (Calabi *et al.*, 2002; Cerquetti *et al.*, 2002; Hennequin *et al.*, 2003; Waligora *et al.*, 2001), we studied the impact of exposure to ampicillin and clindamycin on *C. difficile* adherence to Caco-2/TC7 cells. We investigated the adherence of ATCC 43603, one of the strains displaying the greatest upregulation of these genes, grown in presence of half the MIC of ampicillin and clindamycin. Counts of Gram-stained cell-adherent bacteria showed that exposure to ampicillin and clindamycin increased the adherence of *C. difficile* to Caco-2/TC7 cells. This was supported by the results from cultures of cell-adherent bacteria: the adherence was increased 2.2-fold by ampicillin and 1.8-fold by clindamycin (data not shown), which was consistent with the increased expression of genes encoding adhesins previously observed in this study.

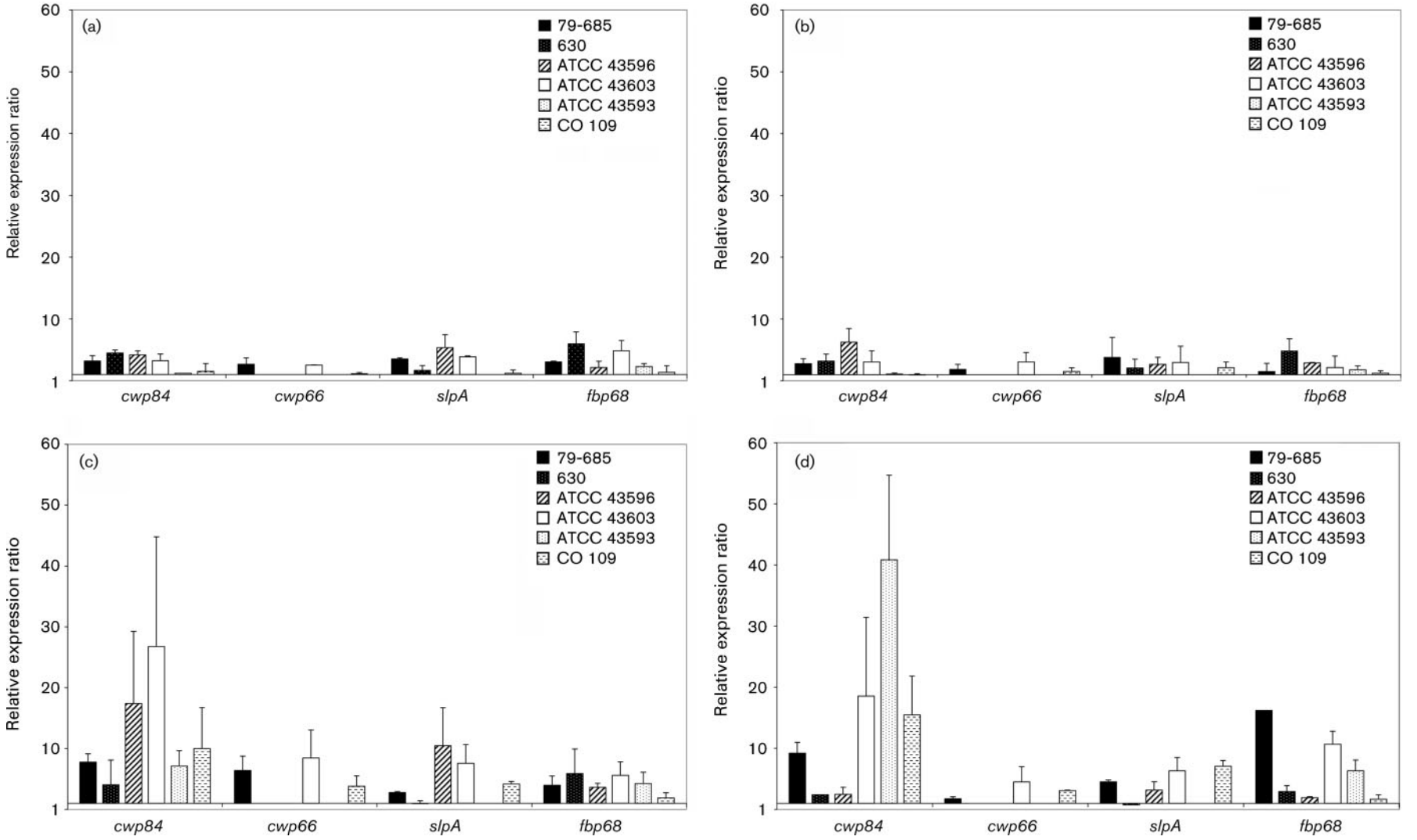

Fig. 2. Real-time RT-PCR analysis of relative expression of four genes encoding colonization factors of six strains of *C. difficile* grown under subinhibitory concentrations of (a) ofloxacin, (b) moxifloxacin, (c) ampicillin and (d) clindamycin, compared with controls grown without any additional component. The data are the means of at least two independent experiments ± standard deviations.

Broad-spectrum antimicrobial agents do not always lead to *C. difficile* proliferation and infection. Among the four antibiotics we have studied, both clindamycin and moxifloxacin have a broad-spectrum activity, including against anaerobes (Behra-Miellet *et al.*, 2002), but only clindamycin possesses a high propensity to induce CDAD due to 'classical' non-epidemic strains of *C. difficile*. In contrast, ampicillin and ofloxacin have a weak activity against anaerobic bacteria (Sullivan *et al.*, 2001), but ampicillin is recognized as a risk factor for CDAD, whereas ofloxacin is not. This could suggest that factors others than reduced colonization resistance could favour colonization by *C. difficile*. One factor is undoubtedly the susceptibility of *C. difficile* strains; however, another factor could be positive regulation of virulence factors by antibiotics. Although several studies on the effect of antibiotics on toxin production have been performed (Drummond *et al.*, 2003; Nakamura *et al.*, 1982; Pultz & Donskey, 2005), no consistent effect emerged. Interstrain variability may account for these divergent results. It has been shown, however, that exposure to ceftriaxone led to increased production of toxins, compared with exposure to piperacillin/tazobactam, which is less frequently involved in CDAD (Baines *et al.*, 2005; Freeman *et al.*, 2003).

In our study, we investigated the impact of exposure to various antibiotics on the expression of genes encoding colonization factors of non-NAP1/027 strains. We have shown that exposure to ampicillin and clindamycin increased the expression of some colonization factors of *C. difficile*, namely three adhesins, Cwp66, the S-layer protein P47 and Fbp68, and a cysteine protease, Cwp84. Moderate overexpression of *slpA* and *fbp68* correlated with moderate increases in adherence to Caco-2/TC7 cells. The Cwp84 protease is able to degrade some of the components of the gut basal lamina (Janoir *et al.*, 2007), therefore the observed overexpression of *cwp84* may facilitate dissemination of the infection.

In contrast, the two fluoroquinolones studied did not significantly modify the expression of the colonization factors of the six fluoroquinolone-susceptible strains studied. Consequently, some antibiotics, such as ampicillin and clindamycin, could lead to CDAD, not only by disrupting the barrier microbiota, but also by facilitating gut colonization by enhancing the expression of genes encoding colonization factors. We can hypothesize that antibiotics can induce stress conditions which interact on regulatory genes, in particular genes involved in two-component regulatory systems, which have been described in the *C. difficile* genome (Sebaihia *et al.*, 2006). Our results indicate that other stress conditions, such as hyperosmolarity, can also enhance the gene expression of colonization factors.

These results on the impact of antibiotics on colonization by *C. difficile* were obtained from a limited number of strains and therefore need to be extended to a larger panel of a variety of strains, including hypervirulent epidemic NAP1/027 strains, in order to confirm the relevance of our findings to other clinical situations.

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