Proteins released during high toxin production in *Clostridium difficile*

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The mechanism by which toxins A and B are released by *Clostridium difficile* is unknown and information about the other extracellular proteins of this bacterium is limited. The authors identified exported proteins from *C. difficile* strain VPI 10463 during conditions promoting high toxin production. Toxins A and B were released in a 1:1 ratio and the proportion of toxin in the extracellular fraction reached 50% during the stationary phase as compared to a proportion of <1% for typical cytoplasmic proteins, showing that toxin export was not due to bacterial lysis. A 47 kDa protein, released with similar kinetics to the toxins, was processed and showed weak similarity to the channel-forming protein TolC. Another protein released during high toxin production was unprocessed and showed similarity to XkdK encoded by the prophage PBSX in *Bacillus subtilis*, a protein supposedly exported via phage-specific holins. The two most abundant extracellular *C. difficile* proteins, found during both high and low toxin production, were processed and identified as shed S-layer proteins. As shown by N-terminal sequencing and PCR-based methods, there was a considerable sequence variation of the S-layer gene *slpA* in different serogroup reference strains. To conclude, *C. difficile* uses the classical Sec-dependent and probably also holin-like pathways to secrete a comparatively small repertoire of proteins.

Keywords: extracellular proteins, outer membrane efflux proteins, PBSX prophage, S-layer

INTRODUCTION

Clostridium difficile-associated diarrhoea (CDAD), occasionally complicated by pseudomembranous colitis (PMC), has become a major hospital problem usually caused by antibiotics that perturb the large-bowel microflora, facilitating colonization and overgrowth by *C. difficile* (Lyerly & Wilkins, 1995). The virulence factors of *C. difficile* comprise two closely related toxins, A and B, with predicted molecular masses of 308 and 270 kDa, respectively (Dove *et al.*, 1990; von Eichel-Streiber *et al.*, 1992). The toxins are endocytosed by the intestinal epithelial cells and glycosylate small GTP-binding proteins, e.g. Rho, Rac and Cdc42, resulting in disruption of the actin cytoskeleton and finally, cell death (von Eichel-Streiber *et al.*, 1996).

The *tcdA* (*toxA*) and the *tcdB* (*toxB*) genes are part of a 19.6 kb pathogenicity locus (PaLoc, toxigenic element), which comprises five ORFs (Braun *et al.*, 1996; von Eichel-Streiber *et al.*, 1992; Hammond & Johnson,

1995). Several mRNAs are transcribed from the toxigenic element, including a 17.5 kb polycistronic transcript (Hammond *et al.*, 1997; Hundsberger *et al.*, 1997). One of the genes, *tcdD* (*txeR*), encodes a 22 kDa protein necessary for transcription of the toxin genes (Moncrief *et al.*, 1997) and was recently shown to act as an alternative sigma factor (Mani & Dupuy, 2001).

The toxin yield or activity may differ up to 10⁶-fold between toxin-positive strains (Lyerly & Wilkins, 1986). The toxins are expressed mainly during the late exponential growth phase and the stationary phase (Kamiya *et al.*, 1992; Ketley *et al.*, 1986), and limiting nutrient levels (e.g. glucose, amino acids, biotin) lead to up-regulation of toxin expression (Dupuy & Sonenshein, 1998; Haslam *et al.*, 1986; Karlsson *et al.*, 1999, 2000; Yamakawa *et al.*, 1994, 1996). Toxin export occurs by an unknown mechanism during the stationary phase (Ketley *et al.*, 1984), and may be affected by the oxidation–reduction potential of the medium, heat shock, and certain antibiotics (Onderdonk *et al.*, 1979).

Fig. 1. Release kinetics of toxins and other proteins by *C. difficile* strain VPI 10463. (a) Growth curve (○, OD₆₀₀; △, total count) and proportion of extracellular toxins A and B measured by enzyme immunoassay (□). (b) Extracellular proteins analysed by SDS-PAGE and silver staining. A volume corresponding to 0.5 µg intracellular protein was applied in each lane. Arrows point to proteins of interest. (c) Identification of toxins A and B in cell extract (CE) and supernatant (CS) fractions from PY cultures by immunoprecipitation. Approximately 100 µl culture extract was precipitated and 50% of the precipitated proteins were applied in each lane. (d) Relative intensity of toxins A (●) and B (◇) and two other proteins (□, 47 kDa; △, 205 kDa) measured using Totallab software (Amersham Biosciences).

The toxin export mechanism remains obscure, but due to their large size and lack of secretion signal peptides,

one hypothesis is that the toxins are externalized by bacterial lysis. The aim of this study was to investigate the toxin export kinetics and to identify other extracellular proteins and putative virulence factors in the culture supernatant of *C. difficile*. Bacterial lysis could not explain release of toxins in the high-toxin-producing strain *C. difficile* VPI 10463. Five other extracellular proteins were identified and characterized at the protein and the genomic levels.

METHODS

Strains, growth media and toxin measurements. The *C. difficile* strains were CCUG 19126 (VPI 10463), CCUG 37766–37787 (serotype reference strains) from the Culture Collection, University of Göteborg, Sweden, and strain 630. Bacteria were grown in peptone-yeast with (PYG) or without (PY) glucose. Medium was prepared as described previously (Karlsson *et al.*, 1999). Toxin yield was measured using the Ridascreen *C. difficile* Toxin A/B enzyme immunoassay kit (r-Biopharm).

Sample preparation and membrane fractionation. Culture samples were centrifuged at 16000 *g* for 3 min; the resulting supernatants were removed and stored at –20 °C for later analysis. The cell pellet was dissolved in 1 ml sterile water and sonicated on ice for 3 × 30 s at 100 W (Labsonic 1510, B. Braun). The cell extracts were centrifuged at 5000 *g* for 5 min, giving a low-speed pellet (LSP). The supernatant was removed and further centrifuged at 50000 *g* for 20 min, resulting in a high-speed pellet (HSP) and a soluble fraction. The LSP and the HSP were resuspended in PBS and all samples were stored at –20 °C. Protein was measured using a kit (Bio-Rad) and a BSA standard curve according to the manufacturer's instructions. Proteins in culture supernatants were precipitated in 10% trichloroacetic acid.

SDS-PAGE and immunoprecipitation. Proteins were analysed by SDS-PAGE (ExcelGel 8–18%, Amersham Biosciences) on a Multiphor II horizontal slab gel apparatus and stained with silver. The gels were digitized by scanning (Scanjet 3c/T, Hewlett Packard) and analysed using the Totallab software (Amersham Biosciences). Immunoprecipitation was performed in microtitre wells coated with antibodies against toxin A (PCG-4, r-Biopharm) or toxin B (2CV, r-Biopharm). Antibody (10 µg ml^{–1} in 40 mM Na₂CO₃, 60 mM NaHCO₃, pH 9.6) was coated in microtitre wells by incubation for 1 h at 37 °C. The wells were washed four times with PBS containing 0.05% (v/v) Tween 20, pH 7.4. The wells were loaded with cell extract, culture supernatant medium or PBS (negative control), incubated for 1.5 h at 25 °C, and washed four times with PBS. Fifty microlitres of SDS sample buffer was added to each well and the samples were heated to 95 °C for 5 min prior to analysis by SDS-PAGE.

Two-dimensional (2-D) PAGE. For 2-D PAGE, 40 µl aliquots of each protein sample (see above) were mixed with 160 µl buffer [9.9 M urea, 4% (v/v) Igepal CA630, 2.2% (v/v) Pharmalytes 3–10, 100 mM DTT, 2% (w/v) CHAPS]. Proteins were focused at 20 °C on 180 mm IPG Drystrip pH 4–7 (Amersham Biosciences). The second dimension was run on 12% SDS-PAGE and proteins were stained with silver. PDQuest (Bio-Rad) were used to quantify proteins. The chemicals were obtained from Sigma except for Pharmalytes (Amersham Biosciences). Proteins were transferred to PVDF membranes,

Fig. 2. *C. difficile* proteins analysed by 2-D PAGE. Samples from 24 h PY and PYG cultures (OD_{600} 1.3–1.5) are shown in the panels. The toxins were not visualized because 2-D PAGE resolves large proteins poorly. (a–d) Samples from strain VPI 10463: (a) extracellular proteins from PY; (b) extracellular proteins from PYG; (c) high-speed pellet from PY (containing large protein complexes and proteins associated with the cell membrane and cell wall); (d) soluble (cytoplasmic) fraction from PY. (e, f) Samples from strain 630: (e) extracellular proteins from PY; (f) high-speed pellet from PY. The horizontal axes represent pH of the isoelectric focusing gradient; the vertical axes represent molecular masses in kDa, based on migration of a molecular mass standard in the SDS electrophoresis gel.

stained with Coomassie brilliant blue, and spots of interest were excised and N-terminally sequenced.

PCR amplification and restriction cleavage of *slpA*. The primers used in the detection of the surface layer gene *slpA* were 5'-TATAATGTTGGGAGGAATTTAAGA-3' and 5'-CAAATCCAAATTCACATTTGTAC-3'. The PCR was performed using Expand Long Template PCR System from Boehringer Mannheim (as specified by the manufacturer). After an initial denaturation at 92 °C for 2 min, the PCR cycle conditions were 92 °C for 10 s, 40 °C for 30 s, and 68 °C for 2 min (30 cycles). The PCR products were cleaved with the restriction enzymes *RsaI* (Boehringer Mannheim) and *SauIII*A (Amersham Pharmacia Biotech). PCR and digestion products were separated on 0.8% agarose gels and visualized by ethidium bromide.

RESULTS

Kinetics of toxin release by strain VPI 10463

Toxin production by *C. difficile* is high in complex media (PY) but lowered by >20-fold in media containing excess glucose (PYG) (see Introduction). In PY, about 50% of the total toxin had become externalized after 40 h (Fig. 1a). Few spores (<0.1%) were found and only a small fraction of the population (<1%) were broken or lysed. SDS-PAGE of PY cultures revealed two proteins, with mobilities of 290 kDa and 270 kDa (Fig. 1b), not observed in PYG (not shown). The amount of these proteins increased as *C. difficile* entered into stationary phase (Fig. 1b), and immunoprecipitation verified their identity as toxins A and B (Fig. 1c). The toxins co-immunoprecipitated using the toxin B antibody, and the identities of the bands, were further

confirmed by Western blotting (not shown). By analysing the intensities of the toxin A and B bands on silver-stained gels, the ratio of total toxin A:toxin B was estimated to be 3:1 whereas the extracellular ratio was approximately 1:1 (Fig. 1d). These ratios were constant for at least 24 h in stationary phase, further arguing against bacterial lysis as the route of release of toxins A and B. A 47 kDa protein was released with similar kinetics as the toxins (Fig. 1d) whereas most other extracellular proteins remained at constant levels (Fig. 1d, 205 kDa protein).

Identification of extracellular proteins in strain VPI 10463 by 2-D PAGE

The extracellular protein pattern of strain VPI 10463 was much less complex than those of the membrane and intracellular fractions (Fig. 2; compare a and b with c and d) and also less complex than the extracellular protein pattern of strain 630 (compare Fig. 2e with 2a). Since strain 630 sporulates efficiently, presumably giving rise to partial lysis, and produces lower amounts of toxin than strain VPI 10463 during these conditions, we focused on the differences in extracellular protein between PY and PYG cultures of strain VPI 10463. The toxins, being $\approx$ 300 kDa in molecular mass, were not visualized since 2-D PAGE resolves large (150 kDa) proteins poorly. Four other extracellular proteins more abundant in PY than in PYG cultures (Fig. 2, spots 3–6) were identified.

As measured by the PDQuest 2-D software, the amount of spot 3 (a 47 kDa protein) increased by >10-fold

Table 1. Extracellular proteins of *C. difficile* grown in PY and PYG medium identified by N-terminal sequence analysis and database searches

Spot*	Mol. mass (kDa)	Growth medium	N-terminal sequence	Match in strain 630 database†	Highest homology‡
1	50	PY	AAKASIADENSPVKLTLSKSDXKKDL	None	–
2	36	PY	DDTKVETGDQGYTVV	Partial (ORF 1)	CwlB/LytC
3	47	PY	SEKEILTARLAV	Complete, internal	TolC (weak)
4	40	PY	AIGLPSINISSK	Complete, N-terminal	XkdK
5	39	PY	MXDIKLDXFXKX	Complete, N-terminal	FixB
6	30	PY	MKILVXVKQVXX	Complete, N-terminal	FixA
7	41	PYG	AETTQVKKETIT	Complete, internal (ORF 2)	CwlB/LytC
8	38	PYG	AETTQVKKETIT	Complete, internal (ORF 2)	CwlB/LytC
9	24	PYG	TSLKIADEVGLD	Complete, internal (ORF 1)	CwlB/LytC
10	48	PY	ANDTIASQDTPAKVV	Complete, internal (ORF 1)	CwlB/LytC
11	35	PY	ATTGTQGYTVVKND	Complete, internal (ORF 1)	CwlB/LytC

* Spots 1–9 were from *C. difficile* strain VPI 10643; spots 10 and 11 were from *C. difficile* strain 630. See Fig. 2.

† Searches were made in the *C. difficile* strain 630 sequence database by the BLAST algorithm at http://www.sanger.ac.uk/Projects/C_difficile/blast_server.shtml. ORF 1 and ORF 2 refer to Fig. 3(c). The N-terminal sequences of spot 2 showed a partial match with ORF 1 of strain 630 (cf. the underlined amino acids of spot 11 from strain 630). Internal means that the protein sequence started downstream of the predicted start codon of the ORF, i.e. the protein was processed at its N-terminus.

‡ *C. difficile* contigs were exported to ORF finder at <http://www.ncbi.nlm.nih.gov/gorf/>, and the entire ORF was subjected to further pBLAST searches against the redundant database. CwlB/LytC and XkdK are from *B. subtilis*, TolC, FixA and FixB from *E. coli*.

Fig. 3. Genes encoding extracellular proteins and their organization on the *C. difficile* chromosome. The panels show (a) the 49 kDa TolC-like ORF, (b) the 40 kDa XkdK-like ORF and (c) the 72 kDa S-layer ORF (*slpA*). ORFs 1 and 2 refer to those in Table 1. The genes encoding the extracellular proteins are shown in grey and adjacent genes in white. Similarities to other known proteins are indicated (see Results and Discussion for further information).

between OD₆₀₀ 0.5 and OD₆₀₀ 1.3 in PY (not shown) and was >10-fold less abundant in PYG supernatants (compare Fig. 2a with 2b). Its N-terminal sequence matched with an ORF encoding a 49 kDa protein in the *C. difficile* database and the C-terminal part showed weak similarity to outer-membrane efflux proteins of Gram-negative bacteria (Table 1, Fig. 3a, Fig. 4a).

The protein corresponding to spot 4 (a 40 kDa protein) showed homology to XkdK (Table 1, Fig. 4b) encoded by the prophage PBSX in *Bacillus subtilis*. The *C. difficile* *xkdK*-like gene was located in a chromosomal segment of strain 630 that comprised several ORFs homologous to those from PBSX (Fig. 3b; XkdK, XkdM, XkdP shown). The *B. subtilis* PBSX prophage is induced by the SOS response to e.g. the DNA-damaging agent mitomycin C (Okamoto *et al.*, 1968; Seaman *et al.*, 1964). However, neither the *C. difficile* XkdK homologue nor the toxins showed altered levels after addition of mitomycin C to PY or PYG cultures, a

treatment that evidently caused induction of SOS since the cells started to form long filaments (not shown).

The PY-specific extracellular proteins 5 and 6 (39 and 30 kDa, respectively, Table 1) matched with two ORFs encoding proteins of 38 and 22 kDa, respectively, showing high similarity to FixB and FixA in *Escherichia coli*. The amounts of these were estimated to be <1% of those in the intracellular fraction (compare Fig. 2a with 2d, spots 5 and 6) and their presence in the extracellular fraction may thus represent a small fraction of the bacterial population being lysed. Importantly, <1% lysed cells cannot explain the efficient release of toxins from strain VPI 10463. The amounts of extracellular FixA and FixB were higher in strain 630 (Fig. 2e), confirming that this strain was more prone to lyse.

Identification of the *C. difficile* surface layer proteins

The two most abundant extracellular proteins in strain VPI 10463 were present in both PY and PYG culture supernatants (Fig. 2a and 2b, spot 1 [50 kDa] and 2 [36 kDa]) and these were highly abundant in the high-speed pellet fraction, which contained mainly membrane vesicles, cell wall fragments and large protein complexes (compare Fig. 2c with Fig. 2a). In strain 630, two abundant proteins with similar molecular masses (48 and 35 kDa) and different pI values than those of strain VPI 10463 were found in the extracellular (Fig. 2e, spots 10 and 11) and particularly in the pellet fraction (Fig. 2f).

The N-terminal sequence of the 50 kDa protein (spot 1) from strain VPI 10463 did not show homology to any protein in the *C. difficile* strain 630 genome database whereas the N-terminus of the 36 kDa protein (spot 2) showed partial similarity to the 72 kDa *C. difficile* S-layer protein SlpA (Fig. 3c, Table 1). Spots 7 and 8 found in PYG cultures (Fig. 2b) matched with ORF 2 from the same genomic fragment and spot 9 matched with SlpA (Table 1). The N-terminal sequences of spots 10 and 11 from strain 630 both matched with the SlpA ORF (Table 1). At least six other proteins less abundant than spots 1 and 2 were observed in both PY and PYG culture supernatants of strain VPI 10463 but were not further characterized. These results imply an extracellular or surface-located proteolytic activity mainly involved in processing of the *C. difficile* S-layer protein.

The *C. difficile* surface layer gene shows inter-strain diversity

As reported by Calabi *et al.* (2001) and Karjalainen *et al.* (2001) we found that the gene segment containing the *C. difficile* S-layer gene *slpA* comprised *secA* and several additional genes with high similarity to *slpA*. The amino acid sequences of SlpA and the SlpA-like ORFs were characterized by a constant part showing significant homology to N-acetylmuramoyl-L-alanine amidase (CwlB/LytC) and amidase enhancer protein (LytB) from *B. subtilis* (Table 1, Fig. 3c). The N-terminus of all the N-acetylmuramoyl-L-alanine amidase-like ORFs contained a typical Sec-dependent signal peptide, and the

Fig. 4. Similarity of the *C. difficile* 47 kDa and 40 kDa ORFs to those of other known proteins. (a) Multiple alignment of the C-terminus of the 47 kDa protein from *C. difficile* and the outer-membrane efflux proteins TliF, ZapD and TolC from *Pseudomonas fluorescens*, *Proteus mirabilis* and *E. coli*, respectively. The consensus sequence of outer-membrane efflux proteins (pfam02321) is also included. (b) Alignment of part of the 40 kDa protein and XdkK from *C. difficile* and *B. subtilis*, respectively. The sequences were aligned using CLUSTALW at <http://www2.ebi.ac.uk/clustalw/> and visualized by BOXSHADE at http://www.ch.embnet.org/software/BOX_form.html using default settings. Identical and similar amino acids are marked with black and grey, respectively.

predicted cleavage site in SlpA from strain 630 was identical to that found in the extracellular form. However, no typical secondary protease processing site was found that would allow the further cleavage of the expected 72 kDa SlpA protein to give the finally sized S-layer proteins of apparent molecular masses 48 and 35 kDa in strain 630.

To test whether other *C. difficile* strains contained the *slpA* gene, a PCR with primers directed to the region upstream of *slpA* and to the 5' part of *secA* was applied to 18 serogroup type strains. All strains except those belonging to serogroups A, A5 and S4 yielded one major PCR product of approximately 2900 bp, although that of serogroup H was slightly larger (Fig. 5a). Thus the upstream region of *slpA* and *secA* and the size of the intervening DNA appeared to be conserved in most strains of *C. difficile*. Digestion of the PCR fragments using the restriction enzymes *Sau*III and *Rsa*I revealed that each type strain yielded a distinct and unique banding pattern (Fig. 5b, c). The pattern of strain 630 and VPI 10463 was identical to that of the serogroup C and G type strains, respectively. In summary, these results showed that the *C. difficile* S-layer genes contain both constant and variable domains and the latter may be useful for grouping and typing of various *C. difficile* isolates.

DISCUSSION

It has been reported that export of toxins A and B by *C. difficile* correlates with a decrease in the number of vegetative cells and a concomitant appearance of spores

(Kamiya *et al.*, 1992; Ketley *et al.*, 1984), suggesting that the toxins are released by lysis during the sporulation process. Another study showed no correlation between release of toxin A and sporogenesis (Ketley *et al.*, 1986), and this was confirmed here in the high-toxin-producing and low-sporulating strain VPI 10463. The small amounts of typical cytoplasmic proteins such as FixA and FixB in the culture supernatant of strain VPI 10463 probably reflected a small fraction of lysed cells (<1%), but this cannot explain the efficient toxin release (50%). As the toxins lack typical Sec-dependent signal peptides, this result indicates that the toxins are exported via another mechanism. Strain 630 had a more complex extracellular protein pattern than strain VPI 10463, most likely reflecting more lysis due to increased sporulation efficiency. Lysis associated with sporulation to release toxin is probably not important for disease development, as we have found that strain VPI 10463 caused severe diarrhoea in gnotobiotic mice (unpublished).

As compared to *B. subtilis* (Antelmann *et al.*, 2001) the repertoire of extracellular proteins in *C. difficile* was small (about 15 vs 200 proteins), possibly reflecting the different ecological niches of the organisms. The 47 kDa extracellular *C. difficile* protein found during high toxin production (PY cultures), was identified as an ORF with weak similarity to outer-membrane efflux proteins (OEPs) of Gram-negative bacteria, e.g. TolC (Fig. 4a). This ORF contained a putative signal peptide for export via the Sec-dependent pathway (Economou, 1999; Izard & Kendall, 1994). The extracellular form was truncated downstream of the predicted signal peptide cleavage site, suggesting that its release was a

Fig. 5. Restriction enzyme cleavage patterns of PCR products from the *slpA-secA* gene segment in *C. difficile*. (a) Electrophoretic analysis of PCR products from 10 serotype reference strains, VPI 10463 and 630 (results from serotypes A3, A4, A5, A8, A10, S1, S3 and S4 not shown). *HindIII*-digested λ DNA was used as size marker (M). (b) Electrophoretic analysis of *SauIII*A digests of the PCR products. (c) As in (b) but *RsaI* digests.

result of further proteolytic digestion. The two adjacent ORFs encoded proteins similar to cation/multidrug resistance efflux pumps of the AcrB/D/F family and periplasmic membrane fusion proteins of the HlyD family (Fig. 4a). These proteins, including TolC, are thought to form a continuous channel that traverses both the inner and the outer membrane in Gram-negative bacteria, and enable direct transport of molecules to the extracellular compartment via proton-motive force (Paulsen *et al.*, 1996; Zgurskaya & Nikaido, 2000). A similar architecture is found also in the type I secretion system which transports toxins in some Gram-negative bacteria, but the multidrug efflux pump protein is replaced by an ATPase. The three genes found in *C. difficile* may thus constitute a novel type of transporter system in Gram-positive bacteria that traverses both the cell membrane and the cell wall. The PY-specific XkdK-like protein neither contained a typical signal peptide, nor was processed at its N-terminus. Its gene was found in a segment on the *C. difficile* chromosome containing several genes similar to those encoded by the phage-like element PBSX in *B. subtilis* (Krogh *et al.*, 1996). *B. subtilis* also releases PBSX

proteins including XkdK into the culture supernatant during growth in LB medium, most likely via phage-specific holins (Antelmann *et al.*, 2001). Interestingly, the *tcdE* gene located between the genes for toxin A and B in the pathogenicity locus of *C. difficile* encodes a putative holin (Tan *et al.*, 2000), suggesting that the toxins and the XkdK homologue may be secreted via similar mechanisms.

The S-layer of *C. difficile* is composed of two proteins with apparent molecular masses of 32–36 and 45–47 kDa and has been characterized previously at the genomic, protein and structural levels (Calabi *et al.*, 2001; Cerquetti *et al.*, 2000; Karjalainen *et al.*, 2001; Mauri *et al.*, 1999; Takeoka *et al.*, 1991; Waligora *et al.*, 2001). The two highly abundant extracellular and membrane fraction proteins in strain 630 were identical to the S-layer protein SlpA-630/C253 (Karjalainen *et al.*, 2001). The differences in size and particularly pI between the S-layer proteins of strains 630 and VPI 10463 as shown by 2D-PAGE indicate significant sequence variation of the S-layer protein among different strains. A significant variation in pI of these proteins was also observed for other serogroup type strains (unpublished). The restriction cleavage pattern of PCR products from the *slpA-secA* region of various serogroup reference strains verified this sequence variability and also that strains 630 and VPI 10463 showed patterns identical to reference strains representing those of serogroup C and G, respectively. Using PCR ribotyping (Stubbs *et al.*, 1999), we found strain 630 to be of PCR ribotype 12, whose reference strain again belongs to serogroup C. Strain 630 has however been assigned to serogroup X (cf. http://www.sanger.ac.uk/Projects/C_difficile/), a result that is not in agreement with these data. This indicates that serotyping may give ambiguous results (or that strain 630 has been assigned to the wrong serogroup), or that the strain has switched its *slpA* gene. Strain VPI 10463 clustered by PCR ribotyping close to a group belonging to serogroup G (unpublished). The PCR ribotype of VPI 10463 is not determined according to the nomenclature of Stubbs *et al.* (1999) but the N-terminal sequences of its S-layer proteins were identical to those of a strain belonging to PCR ribotype 1 (Calabi *et al.*, 2001), whose reference strain belongs to serogroup G. These data show that S-layer genomic typing methods should be developed for improved molecular typing of *C. difficile* strains.

The C-terminal part of SlpA showed significant homology to *N*-acetylmuramoyl-L-alanine amidase (CwlB/LytC) and amidase enhancer protein (LytB) from *B. subtilis* (Lazarevic *et al.*, 1992), which confirmed recent findings (Calabi *et al.*, 2001; Karjalainen *et al.*, 2001). This motif may thus have cell wall peptidoglycan binding properties. Karjalainen *et al.* (2001) suggested that the N-terminal part of *C. difficile* SlpA has homology to the SLH domain present in many S-layer proteins (cf. Lupas *et al.*, 1994), but like Calabi *et al.* (2001) we did not find such similarity at the sequence level. It is possible, however, that this part of the C.

difficile S-layer protein shows weak similarities to that of the SLH domain.

Considering the highly competitive situation of closely related organisms in their natural habitats, it is obvious that the S-layer has to contribute to diversification rather than to conservation. With respect to this, the importance of S-layer variation during different stress conditions such as those imposed by the immune system of a host in response to an S-layered pathogen or drastic changes in the growth and environmental conditions for non-pathogens is conceivable (Dworkin & Blaser, 1997; Luckevich & Beveridge, 1989; Sa'ra *et al.*, 1996). A protein named Cwp66 encoded in the same chromosomal segment as SlpA was shown to have putative adhesive properties to eukaryotic cells (Waligora *et al.*, 2001). Moreover, it was shown by RT-PCR that most ORFs in the S-layer gene segment were simultaneously transcribed (Calabi *et al.*, 2001), although the protein yield (as found here) may differ significantly between the ORFs. Whether the different S-layer ORFs in this chromosomal segment have similar or different regulatory control is yet to be verified. An interesting question is whether these genes are important for disease development and whether changes of organization or expression of the S-layer genes occur *in vivo*.

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