The LMW surface-layer proteins of *Clostridium difficile* PCR ribotypes 027 and 001 share common immunogenic properties

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The aim of this study was to investigate the S-layer proteins (SLPs) of the hypervirulent *Clostridium difficile* PCR ribotype 027 and compare them with those of PCR ribotype 001 and other PCR ribotypes involved in *C. difficile* infection and outbreaks, by molecular analysis and immunological assays. It has been demonstrated previously that PCR ribotype 027 SlpA is conserved in *C. difficile* strains belonging to this PCR ribotype and that it is a new variant, showing 88 % identity with SlpA of PCR ribotype 001. As the low-molecular-weight (LMW) SLPs of *C. difficile* are immunodominant antigens, attention was focused on this region of the genome. Sequencing of strains of different PCR ribotypes (001, 012, 014, 017, 027 and 078) showed that SlpA was conserved among strains belonging to the same PCR ribotype. Comparison of the LMW SLP region among these strains identified ten regions with sequence identity between PCR ribotypes 027 and 001, and low conservation with the other PCR ribotypes. In particular, two of these regions corresponded to areas predicted to be surface exposed. Three specific peptides, including those of the two surface-exposed regions, were recognized by human sera against PCR ribotypes 027 and 001 and by a rabbit polyclonal serum against the SLPs of PCR ribotype 027. In contrast, these peptides were not recognized by a polyclonal serum against the SLPs of PCR ribotype 012 used as a control. These results confirm the antigenic role of the LMW SLP and suggest that it may have a role in evasion of the host immune response.

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INTRODUCTION

Although two large proteins, toxin A (TcdA) and toxin B (TcdB), are recognized as the principal virulence factors of *Clostridium difficile*, a number of additional factors have been suggested to play a role in infection, including flagella, fimbriae, degradative enzymes and cell-surface proteins (Hennequin *et al.*, 2001; Sára & Sleytr, 2000; Tasteyre *et al.*, 2001; Waligora *et al.*, 2001).

The surface (S) layer is a predominantly surface-associated crystalline or paracrystalline array, composed of identical protein or glycoprotein subunits, found in many prokaryotes (Sára & Sleytr, 2000). Previous studies have shown that *C. difficile* expresses on its surface two superimposed and structurally different S-layer lattices: the inner is arranged in a hexagonal symmetry, whereas the outer has a square symmetry (Cerquetti *et al.*, 2000). Biochemical

characterization of isolated S-layer proteins (SLPs) from different *C. difficile* strains has demonstrated that each layer is composed of a distinct protein subunit that displays a slight variation in molecular mass among strains: the high-molecular-weight (HMW) SLP (~40 kDa) and the low-molecular-weight (LMW) SLP (~35 kDa) (Calabi *et al.*, 2001; Cerquetti *et al.*, 2000). These proteins have been demonstrated not to be glycosylated in strains representing different PCR ribotypes (Qazi *et al.*, 2009). Both SLPs are encoded by a single gene, *slpA*, and are produced from the post-translational cleavage of a common precursor: the HMW SLP is derived from the C-terminal portion of the precursor, whereas the LMW SLP is derived from the N-terminal portion (Calabi *et al.*, 2001). Sequence variability is particularly evident in the LMW SLP but is also present in the HMW SLP, giving rise to a range of protein sizes (Calabi & Fairweather, 2002; Eidhin *et al.*, 2006). Studies on S-layer adhesive properties have demonstrated that chemical removal of the SLPs or treatment with anti-SLP antibodies can abolish adherence of *C.*

Abbreviations: CDI, *Clostridium difficile* infection; HMW, high molecular weight; LMW, low molecular weight; SLP, surface-layer protein.

difficile to human HeLa or mouse 929 cells (Takumi *et al.*, 1991). The specific binding of purified *C. difficile* SLPs to both gastrointestinal tissues and several extracellular matrix proteins has also been demonstrated (Calabi *et al.*, 2002). The HMW SLP appears to be immunologically conserved among strains (Cerquetti *et al.*, 2000; Takeoka *et al.*, 1991), whereas the LMW SLP is less conserved. The LMW SLP is an immunodominant antigen and, in fact, is recognized the most consistently by specific antibodies from sera of patients infected by *C. difficile* (Pantosti *et al.*, 1989).

In the present study, we investigated the SLPs of the hypervirulent *C. difficile* PCR ribotype 027 and compared them with those of PCR ribotype 001 and other PCR ribotypes involved in *C. difficile* infection (CDI) and outbreaks, by molecular analysis and immunological assays.

METHODS

C. difficile strains and typing. Fifteen *C. difficile* strains belonging to different PCR ribotypes were used in this study. The characteristics of these strains are shown in Table 1. Strains were cultured under anaerobic conditions on *Brucella* agar plates containing vitamin K₁ (0.5 mg l⁻¹), haemin (5 mg l⁻¹) and 5% defibrinated sheep red blood cells and in brain–heart infusion (BHI) broth (Oxoid) for SLP extraction.

Typing of *C. difficile* strains was performed by PCR ribotyping using the method of Bidet *et al.* (1999). The genomic DNA fingerprinting patterns produced by PCR ribotyping were compared and analysed using GelCompar II version 6.0 (Applied Maths). DNA fingerprinting patterns produced by PCR ribotyping were compared with those of a collection of the predominant PCR ribotypes circulating in Europe. This collection of strains was kindly supplied by Dr Ed Kuijper (on

behalf of the European Centre for Disease Prevention and Control, Stockholm, Sweden).

SDS-PAGE. SLPs were obtained by low-pH glycine extraction (Calabi *et al.*, 2001). Briefly, a 24 h culture in BHI broth was centrifuged at 4500 g for 15 min and washed twice with PBS. The pellet was treated with 0.2 M glycine (pH 2.2). After 15 min incubation at room temperature, the mixture was centrifuged and the supernatant was neutralized with 2 M Tris and separated by SDS-PAGE, using a 12% gel, following the Laemmli system (Sambrook *et al.*, 1989). After electrophoresis, the gel was rinsed in H₂O and stained with Coomassie Brilliant Blue to visualize the proteins.

Sequence analysis. A set of primers was used to amplify the *slpA* gene of *C. difficile* strains (Table 2). PCR conditions were: 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 46–48 °C (depending on the melting temperature of the primers) for 1 min and 72 °C for 1 min, with a final extension at 72 °C for 5 min.

Nucleotide sequence analysis was performed using Lasergene version 8.0 software (DNASTAR), the National Center for Biotechnology Information BLAST server (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and the European Bioinformatics Institute CLUSTAL W server (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>).

Visualization of the LMW SLP structure (LMW SLP_{1–262}) for identification of surface-exposed regions was performed using the Swiss-PDB Viewer for the published crystal structure (PDB no. 3CVZ) (Fagan *et al.*, 2009).

Human and rabbit sera. Human sera were obtained from two patients at the Brussels St Luc University Hospital, Brussels, Belgium, who were infected with *C. difficile* strains belonging to PCR ribotypes 027 and 001, respectively. The PCR ribotype 027 serum was collected in 2009 from a patient chronically positive for *C. difficile* PCR ribotype 027 since 2008. The PCR ribotype 001 serum was collected in 2010 from a patient who had one CDI episode during his hospitalization for pancreatitis.

As it was not possible to obtain sera from patients infected by *C. difficile* strains other than PCR ribotypes 027 and 001, polyclonal antibodies against the SLPs of the PCR ribotype 012 *C. difficile* C253 clinical isolate (Cerquetti *et al.*, 2000) were prepared in adult rabbits by Primm Srl, Milan, Italy, using as antigen both the HMW and LMW protein bands excised from an SDS-PAGE gel. Each animal received four doses of antigen: the first immunization with 200 µg in complete Freund's adjuvant and the other three at days 21, 28 and 35 with 200, 100 and 100 µg in incomplete Freund's adjuvant, respectively. The same procedure was used to prepare polyclonal antibodies against the SLPs of PCR ribotype 027 *C. difficile* R20291.

Rabbit pre-immune serum and a commercial human serum (type AB; Euroclone) were used as negative controls.

Peptide synthesis and immunodetection. For dot blot analysis, 11 synthetic peptides from the SLP variable region were synthesized by Invitrogen (Table 3). Chemical activation of nitrocellulose membranes was performed following the procedure described by Måsson *et al.* (1993). Briefly, membranes were treated with 2.5% 1,6-diaminohexane solution (pH 11.9) for 30 min, washed for 24 h with H₂O and activated with 1% glutaraldehyde, 0.5 M NaHCO₃/Na₂CO₃ (pH 10) for 30 min. The membranes were then washed for 3 h with H₂O and 10 µg each peptide was spotted on the activated nitrocellulose membrane.

Immunodetection was performed using sera from patients with severe CDI or sera from rabbits immunized with SLPs. Human sera were used at a 1:1000 dilution in 0.9% NaCl/10 mM Tris/HCl buffer containing 3% BSA and incubated for 2 h at room temperature. Detection was performed using a 1:2000 dilution of anti-human IgG

Table 1. *C. difficile* strains used in this study

<i>C. difficile</i> strain	PCR ribotype	Source or reference*
001-3	001	ECDC collection
630	012	Wüst <i>et al.</i> (1982)
C252	012	ISS collection
TR1	012	ISS collection
C253	012	Cerquetti <i>et al.</i> (2000)
ATCC 43600	014	ECDC collection
FII3	014	ESGCD collection
IT0855	014	ISS collection
ATCC 43598	017	ECDC collection
CIH4	017	ESGCD collection
DI4	017	ESGCD collection
R20291	027	ECDC collection
CD5	078	ISS collection
IT0844	078	ISS collection
IT1008	078	ISS collection

*ECDC, European Centre for Disease Prevention and Control; ISS, Istituto Superiore di Sanità; ESGCD, ESCMID Study Group on *Clostridium difficile* (collection of 2005).

Table 2. Primers used in the study for *slpA* amplification

PCR ribotype	GenBank accession no. of reference sequence (<i>slpA</i> gene position, nt)	Primer pairs	Primer positions in reference sequence (nt)
001	DQ060625 (107–2357)	slpR1/slp001r2 slp001f5/slp001r1 slp001f4/slp15 slp001f3/slp15–16f slp001f2/slpR3–16f slp001f1/slpR4	38–57/500–481 311–330/1003–981 801–820/1285–1265 1152–1171/1821–1803 1581–1600/2203–2184 2052–2070/2557–2536
012	AM180355 (3252559–3254718)	slp2/slp1 slp4/slp3 slp5 /slp6 slp7/slp8 slp9/slp10	3254109–3254132/3254642–3254619 3253659–3253683/3254232–3254209 3253303–3253327/3253803–3253780 3252853–3252576/3253432–3253409 3252503–3252526/3252980–3252955
014	DQ060638 (28–2226)*	slp014f1/slp014r2 slp014f2/slp014r3 slp014f3/slp014r4 slp014f5/slp014r5	1–20/710–692 610–630/1291–1271 1181–1201/1850–1832 1793–1810/2321–2301
017	DQ060640 (108–2252)	slpR1/slp017r6 slp017f4/slp017r5 slp017f3/slp017r3 slp017f1/slp017r2 slpR3–16r/slp017r1	38–57/640–622 432–451/1070–1051 940–960/1639–1631 1531–1549/2010–1991 1771–1792/2448–2427
027	FN538970 (3081644–3083920)	slpR3/slpR3–16r slpR3–16f/slp16 slp15–16f/slp15–16r slp15/slpR1	187698–187681/3082218–3082200 3081818–3081837/3082756–3082741 3082200–3082218/3082986–3082967 3082736–3082756/3083990–3083971
078	DQ060643 (108–2288)	slpR1/slp078r1 slp078f2/slp078r4 slp18r1/slp18f1 slp078f5/slp078r6 slp078f7/slpR3	38–57/510–491 431–450/1409–1391 1101–1121/1903–1884 1701–1719/2440–2421 2241–2260/2802–2785

*SlpA of the *C. difficile* strains of PCR ribotype 014 sequenced in this study had an amino acid change at position 211 in comparison with this sequence.

Table 3. Synthetic peptides used in the study

Peptide	Sequence	Position in SlpA amino acid sequence*
1	KVETGDQG	30–37
2	QSKYKKAVEQLQKG	42–55
3	LDGSITEIK	57–65
4	FFEGTLASTIKVG	67–79
5	DASKLLFTQVDNKLND	86–101
6	TTSKLVALK	122–130
7	VFDTAFTDSTETA	174–186
8	DGKTEKIVKLGDSD	216–229
9	PVITKLRI	263–270
10	NKTDLNTLYRVLNGDEADTN	298–317
11	LGDGDYVDF	102–110

*NCBI protein no. CBE06198 (*C. difficile* strain R20291).

(whole molecule) alkaline phosphatase conjugate (Sigma-Aldrich Biotechnology) and SigmaFast BCIP/NBT (Sigma-Aldrich Biotechnology) according to the manufacturer's instructions. Rabbit sera were used at 1:50 000 or 1:10 000 dilutions and detection was performed using a 1:2000 dilution of anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate (Sigma-Aldrich Biotechnology).

RESULTS AND DISCUSSION

In the present study, SlpA of *C. difficile* PCR ribotype 027 was analysed and compared with that of five other PCR ribotypes (001, 012, 014, 017 and 078) that are frequently involved in sporadic CDI and outbreaks (Bauer *et al.*, 2011). We demonstrated previously that SlpA is conserved in *C. difficile* strains belonging to PCR ribotype 027 and that it is a new variant, showing 88% identity to SlpA of PCR ribotype 001 (Spigaglia *et al.*, 2011).

We focused our attention on the LMW SLP of *C. difficile* as it has been demonstrated to be an immunodominant antigen (Pantosti *et al.*, 1989).

Except for PCR ribotypes 001 and 027, only one or two *slpA* sequences were found in GenBank for the other PCR ribotypes considered in this study. In particular, three *slpA* sequences were found for PCR ribotype 001 (GenBank accession nos DQ060625, DQ060626 and AJ300676), two for PCR ribotype 012 (GenBank accession nos AM180355 and AJ291709), one for PCR ribotype 014 (GenBank accession no. DQ060638), two for PCR ribotype 017 (GenBank accession nos DQ060640 and AJ300677) and one for PCR ribotype 078 (GenBank accession no. DQ060643). Eight PCR ribotype 027 epidemic strains were sequenced previously in our laboratory, confirming that the *slpA* sequence is conserved in this PCR ribotype (Spigaglia *et al.*, 2011). Conservation of the SlpA sequence among strains belonging to the other PCR ribotypes was verified by sequencing the *slpA* gene of other human *C. difficile* clinical isolates (Table 1) in order to obtain at least four sequences for each PCR ribotype. The results indicated that all strains belonging to the same PCR

ribotype had an identical SlpA sequence, and the sequence of each PCR ribotype, except that of PCR ribotype 014, was identical to those found previously by other authors in *C. difficile* strains of the same type. All PCR ribotype 014 *C. difficile* isolates analysed, including the reference strain from the ECDC collection, showed a sequence that differed from the reference SlpA we used for PCR ribotype 014 (GenBank accession no. DQ060638), obtained by Eidhin *et al.* (2006), by one amino acid substitution (Leu₂₁₁→Ser), whereas it was identical to that found in the *C. difficile* strain R13699, typed as PCR ribotype 066 in the same study. As strain R13699 was reported as non-toxicogenic, although it is known that PCR ribotype 066 strains are toxigenic and belong to toxinotype V or VI, as reported by Rupnik *et al.* (2001), it is likely that a mistake occurred in typing.

SlpA glycine extracts of the reference *C. difficile* strains representing the different PCR ribotypes were examined by

Fig. 1. Multiple alignments of the predicted amino acid sequences of the SlpA variable region from strains of PCR ribotypes 001, 012, 014, 017, 027 and 078. Numbers in parentheses show the nucleotide numbers taking missing nucleotides into account. The alignment was performed using SlpA variants representative of each PCR ribotype. One SlpA variant was identified in each PCR ribotype, except for PCR ribotype 014, for which two variants, SlpA-14a and SlpA-14b, were found. These variants differ by the amino acid substitution Leu₂₁₁→Ser. Blocks of conserved residues at a given position are highlighted in cyan, whilst consensus residues derived from the occurrence of >50% of a single residue at a given position are highlighted in green. Residues conserved in all sequences are indicated by red text with yellow shading. Peptides showing sequence identity between PCR ribotypes 001 and 027 and low conservation with the other PCR ribotypes are underlined and numbered from 1 to 10. Peptide 11 is a sequence with 100% identity among all PCR ribotypes. The two peptides of PCR ribotypes 001 and 027 with sequence identity that are predicted to be surface exposed are underlined in red. Diamonds indicate residues with high solvent accessibility.

Fig. 2. Mapping of residues that are highly conserved and surface exposed in PCR ribotypes 001 and 027 onto the crystal structure of the LMW SLP₁₋₂₆₂. Conserved residues are shown in a surface representation superimposed onto a ribbon diagram of the structure. Residues predicted to have high surface accessibility are shown in yellow. Several of the most conserved residues have been labelled.

SDS-PAGE. All strains showed two distinct SLPs, and the same pattern of proteins was observed for PCR ribotypes 027 and 001 (data not shown).

Multiple alignments of the predicted amino acid sequences of the LMW SLPs of *C. difficile* strains of PCR ribotypes 001, 012, 014, 017, 027 and 078 are shown in Fig. 1. The SlpA sequence variant found in the *C. difficile* strains belonging to a certain PCR ribotype is denoted with the number of the PCR ribotype (SlpA-1 for PCR ribotype 001, etc.). The two SlpA variants identified in *C. difficile* strains belonging to PCR ribotypes 014 were denominated SlpA-14a (strain R12885, DQ060638) and SlpA-14b (strains ATCC 43600, FII3 and IT0855). Ten regions with sequence identity between PCR ribotypes 027 and 001 but with low conservation with the other PCR ribotypes were identified. In particular, two of these regions corresponded to areas predicted to be surface exposed on the basis of the solvent accessibility data given for the single amino acids in the published crystal structure of LMW SLP₁₋₂₆₂ (Fagan *et al.*, 2009) (Fig. 2).

Synthetic peptides (numbered 1–10) were designed based on the ten regions with sequence identity between PCR ribotypes 027 and 001 (Table 3 and Fig. 1), whereas peptide 11 was designed based on a region conserved in all PCR ribotypes. Peptides 4, 5, 7 and 8 were recognized by human sera against PCR ribotype 027 or 001 (Fig. 3a). The same peptides, except for peptide 5, were also recognized by the

rabbit polyclonal serum against the SLPs of PCR ribotype 027 (Fig. 3b). In contrast, peptides 2, 3, 5, 6 and 9 were recognized by the polyclonal serum against the SLPs of PCR ribotype 012 (Fig. 3b). Only a very weak signal for peptide 2 was observed with both the rabbit pre-immune sera and a commercial human serum used as negative controls (data not shown). Recognition of peptides by a polyclonal serum raised against SlpA of PCR ribotype 012, whose sequence contains only stretches of low similarity, cannot easily be explained without further characterization. It is possible that this behaviour was simply due to the presence of antibodies that show low-specificity binding. It is noteworthy that peptides 1, 10 and 11 were not recognized by any of the sera. The former two peptides may not be accessible to the immune system when assembled to form the S layer, as it has been shown that the regions comprising these peptides are involved in the interaction between the LMW and HMW SLPs (Fagan *et al.*, 2009). Similarly, peptide 11, which represented a region of 100% sequence identity among all PCR ribotypes, may be assigned to a domain of SlpA hidden within the S-layer structure and therefore not exposed to the immune system of the host. Alternatively, peptide 11 may belong to a region of the LMW protein showing low antigenicity. The reaction of both human sera from patients infected by *C. difficile* strains of PCR ribotypes 027 and 001 with the LMW proteins from strains of PCR ribotypes 027 and 001 confirmed the antigenic role of this

Fig. 3. Dot blot of synthetic peptides designed based on the LMW SLP regions with sequence identity between *C. difficile* PCR ribotypes 027 and 001. Peptides are indicated by the numbers 1–11 (see Table 3). (a) Peptides recognized by human serum obtained from a patient infected with *C. difficile* of PCR ribotype 027 (upper filter) or from a patient infected with *C. difficile* of PCR ribotype 001 (lower filter). (b) Peptides recognized by rabbit polyclonal serum against *C. difficile* PCR ribotype 027 SLPs (upper filter) or by rabbit polyclonal serum against *C. difficile* PCR ribotype 012 SLPs (lower filter).

protein. In addition, three regions of this protein, corresponding to residues 67–79, 174–186 and 216–229 of the SlpA sequence of strain R20291, were found to be immunodominant and recognized by both human sera against *C. difficile* PCR ribotype 027 or 001 and rabbit polyclonal serum against the SLPs of PCR ribotype 027. As antigenic variation of surface proteins is assumed to provide pathogenic bacteria the means to escape recognition by the immune system of the host, these results suggest that the variable regions of SlpA may have a role in evasion of the host immune response.

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