Purification and characterization of S layer proteins from *Clostridium difficile* GAI 0714

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The S layer of *Clostridium difficile* GAI 0714 was shown to be composed of two proteins, of 32 kDa and 45 kDa, as determined by SDS-PAGE. The two proteins were extracted with 8 M-urea (pH 8.3) from a cell wall preparation and purified by DEAE-Sepharose CL-6B chromatography followed by HPLC gel filtration. When solubilized in 0.1 M-urea, both proteins appeared to exhibit dimeric forms, with respective molecular masses of about 61 kDa and 99 kDa, upon HPLC. Although the amino acid compositions of the two proteins differed from each other, both proteins had a high content of acidic amino acids, very low contents of histidine and methionine, and no cysteine. The 32 kDa protein exhibited multiple isoelectric forms (pI 3.7–3.9), whereas the 45 kDa protein had a single form (pI 3.3). Radioiodination and immunogold labelling revealed that both proteins were exposed evenly over the entire cell surface. Based on immunodiffusion analysis using monospecific antiserum raised to the individual proteins, there was no antigenic relationship between the two proteins. Furthermore, immunoblot analysis showed that the antigenicity of the 32 kDa protein appeared to be strain specific, whereas that of the 45 kDa protein appeared to be group specific.

Introduction

Crystalline surface layers (S layers) (Sleytr & Messner, 1988) or paracrystalline protein surface arrays (Koval, 1983) are present on the cell surface of various bacteria, including Gram-positive and Gram-negative bacteria and archaeobacteria. Most S layers are composed of a single protein or glycoprotein species of molecular mass about 40–200 kDa (Sleytr & Messner, 1983). The subunit proteins are noncovalently attached either to each other or to underlying cell wall components and they represent the primary barrier between the cells and the environment (Sleytr & Messner, 1983).

Clostridium difficile, an important pathogen causing human pseudomembranous colitis, has a squarely arranged S layer composed of two proteins with different molecular masses (Kawata *et al.*, 1984). On the basis of the molecular mass values of the two proteins, *C. difficile* strains are divided into two groups: group 1, containing about 32 and 45–47 kDa proteins; and group 2, containing about 38 and 42 kDa proteins. The 32 kDa protein of the S layer of *C. difficile* ATCC 11011 (group 1) has been purified and characterized immunochemically; the protein was extracted with phosphate-buffered saline (PBS) from the cell walls without the use of detergents or chaotropic agents such as urea or guanidine hydro-

chloride (Takumi *et al.*, 1987). The 45 kDa protein, however, was not detected in the PBS extract but was solubilized using higher molar concentrations of urea.

The aim of the present work was to gain an understanding of the immunological relationship between the constituent protein subunits (32 kDa and 45 kDa) of the S layer of *C. difficile*. These proteins from *C. difficile* strain GAI 0714 were purified and characterized immunochemically. Immunoelectron microscopy also revealed that both the S layer proteins were evenly exposed on the cell surface.

Methods

Bacterial strains and cultivation. *C. difficile* GAI 0714, which was isolated from a patient with antibiotic-associated pseudomembranous colitis, was the main strain used. Other strains used are listed in the legend to Fig. 8. Bacteria were grown anaerobically in GYPT medium at 37 °C as described previously (Kawata *et al.*, 1984).

Preparation of cell walls. Cell walls were isolated from cells harvested from a 10 h culture (early stationary phase) as described previously (Kawata *et al.*, 1984). Briefly, the cells were washed with cold water and broken in a Braun MSK cell homogenizer. Unbroken cells were removed by low-speed centrifugation, then the wall fragments were separated from the supernatant by centrifugation (18000 g, 15 min) and washed sequentially with 1 M-NaCl, 2% (w/v) Triton X-100 and cold water to remove membranous contaminants. The resulting cell wall pellet was stored at 4 °C in water containing 0.02% (w/v) NaN₃ and 2 mM-phenylmethylsulphonyl fluoride (PMSF, Sigma).

Purification of 32 and 45 kDa proteins. All the buffer solutions used in this procedure contained 2 mM-PMSF. The cell wall preparation was suspended in 50 mM-Tris/HCl buffer (pH 8.3) containing 8 M-urea, stirred at room temperature for 1 h and centrifuged at 100000 *g* for 30 min. This procedure was repeated on the pellet. The combined supernatants were concentrated to about half the original volume by an Amicon diafilter with a YM10 filter. The concentrate was applied to a column (2.5 × 30 cm) of DEAE-Sepharose CL-6B (Pharmacia) equilibrated with the urea buffer. The column was washed with the same buffer, then the bound materials were eluted with a linear gradient of 0–0.3 M-NaCl in the buffer at a flow rate of 20 ml h⁻¹. Fractions of 5 ml were collected, monitored at 280 nm for protein, and analysed by SDS-PAGE. The 32 and 45 kDa protein fractions were pooled and concentrated, and chromatography on the anion-exchange column was repeated under the same conditions as above. The two protein fractions were separately subjected to HPLC with a Hitachi instrument and a TSK G3000SW column (7.5 mm i.d. × 60 cm; Tosoh Co., Tokyo, Japan) equilibrated with 20 mM-PBS containing 0.1 M-urea. The HPLC column was eluted at a flow rate of 0.8 ml min⁻¹ with the PBS. Fractions of 0.3 ml were collected, assayed for protein content, and stored at -20 °C. Each final preparation gave a single protein band on SDS-PAGE.

SDS-PAGE. SDS-PAGE was performed according to the method of Laemmli (1970), using a separating gel of 12% (w/v) acrylamide as described previously (Takumi *et al.*, 1987). Proteins bands were visualized by staining with Coomassie Brilliant Blue R250. Apparent molecular masses were calculated by comparison with standards from a low molecular mass protein kit (Pharmacia).

Chemical analyses. The protein concentration was determined by the Lowry method, with bovine serum albumin as standard. Amino acids were analysed on a Hitachi KLA-5 amino acid analyser after hydrolysis of purified wall proteins in 6 M-HCl at 110 °C for 24 h as described previously (Takumi *et al.*, 1987).

Determination of molecular mass by HPLC. Molecular weights of the undenatured forms of the purified S layer proteins were estimated by HPLC on a G3000SW column, eluted with PBS containing 0.1 M-urea. The molecular masses were determined by comparison with molecular mass standards for gel filtration chromatography (Sigma) including β -galactosidase (116 kDa), phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa) and carbonic anhydrase (30 kDa).

Isoelectric focusing. The isoelectric points (pIs) of the purified S layer proteins were determined with a horizontal electrophoretic apparatus (model AF-3230, Atto Ltd, Tokyo, Japan) on 5% (w/v) acrylamide gels using Pharmalyte 2.5–5 (Pharmacia). Focusing was performed for 2 h at 1500 V and the focused protein bands were revealed with Coomassie Brilliant Blue. The pIs were estimated by determining the relative mobility of the proteins compared with pI marker proteins (LKB Instruments).

Peptide mapping. Peptide mapping of purified S layer proteins was performed after limited proteolysis with 10 ng staphylococcal V8 protease (Miles Laboratories) according to the method of Cleveland *et al.* (1977), using gel pieces containing 30–50 μ g S layer protein, which had been obtained from the gel after SDS-PAGE of the purified proteins, and a separating gel with a 15–20% (w/v) linear gradient of acrylamide.

Iodination with ¹²⁵I-Na. Radioiodination using chloramine T was carried out essentially according to the method of Nesbitt *et al.* (1980). Whole cells were suspended in 6 ml 0.5 M-sodium phosphate buffer (pH 7.5), and mixed with 25 μ l sodium iodate containing ¹²⁵I-Na (50 μ Ci; 1850 kBq) with vigorous stirring for 10 min at room temperature. The reaction mixture was added to 1 ml chloramine T

(2.0 mg ml⁻¹) in 0.5 M-sodium phosphate buffer (pH 7.5) and stirred for 3 min. The iodination was stopped by adding 1 ml sodium metabisulphite (4 mg ml⁻¹). The labelled cells were washed three times with the phosphate buffer, then the cell wall fraction was prepared from the disrupted cells and subjected to SDS-PAGE. The proteins were identified by autoradiography of the gels at 4 °C for 3 d using Kodak X-Omat RP film with Cronex intensifying screens (Dupont de Nemours).

Preparation of antisera and immunological assays. For immunization, purified S layer protein preparations were freed from detergent by ethanol precipitation, dissolved in PBS and emulsified in an equal volume of Freund's complete adjuvant. Rabbits were injected subcutaneously with 1 mg of the protein preparation at several sites on the back. After 2 or 3 weeks, the animals were injected in the same manner with 1 mg of the protein emulsified in incomplete Freund's adjuvant; they were bled 2 weeks later.

Immunodiffusion was carried out with 0.8% (w/v) agarose gels in barbital buffer (pH 8.6) as described previously (Takumi *et al.*, 1983).

Western blotting was done by the electrophoretic transfer procedure of Towbin *et al.* (1979) using a Bio-Rad Immun-Blot assay kit as described previously (Takumi *et al.*, 1987).

Preparation of protein A-colloidal gold conjugate. Colloidal gold particles with an average diameter of 5–7 nm were prepared by the procedure of Mülhpfordt (1982). Chloroauric acid was reduced with a mixture of 1% (w/v) sodium citrate and 1% (w/v) tannic acid. The gold particles were adsorbed with protein A (Pharmacia) to produce a stable colloidal suspension of protein A-gold conjugate according to the method of Roth *et al.* (1978). The conjugate was suspended in a small volume of 20 mM-phosphate buffer (pH 7.4) containing 0.02% (v/v) polyethylene glycol and 0.02% (w/v) sodium azide, and stored at 4 °C.

Immunoelectron microscopy. Whole cells of *C. difficile* GAI 0714 were incubated with a 1:10 dilution of 32 or 45 kDa protein antiserum in PBS containing 1% (w/v) ovalbumin (Sigma) for 1 h at 4 °C. After three washes with PBS, the cells were reincubated with the protein A-gold conjugate for 1 h at 4 °C. The gold-labelled cells were prefixed with glutaraldehyde, fixed with osmium tetroxide, embedded in styrene/methacrylate and cut with glass knives as described previously (Takumi *et al.*, 1979). Thin sections were poststained with 0.2% (w/v) lead citrate and examined with a Hitachi HU-11E electron microscope operating at 75 kV.

Results

Purification of two S layer proteins

To isolate and purify the two S layer proteins from *C. difficile* GAI 0714, cell wall preparations were extracted with Tris buffer containing 8 M-urea; by this method both the 45 kDa protein and the 32 kDa protein could be removed from the cell walls. The urea extract was fractionated on a DEAE-Sepharose CL-6B column with a linear concentration gradient of NaCl (Fig. 1). Fractions containing the 32 and 45 kDa proteins, each showing a single protein band on SDS-PAGE, were eluted around 0.13 and 0.21 M-NaCl, respectively. Further separation of the two proteins was achieved by repeated chromatography on the same anion-exchange resin. Each protein fraction was finally purified by HPLC gel filtration, eluting with 0.1 M-urea in PBS. The 32 and 45 kDa proteins were eluted as single sharp peaks

Fig. 1. Separation of S layer proteins from *C. difficile* GAI 0714 on a DEAE-Sephacrose CL-6B column. Concentrated 8 M-urea-extracted S layer proteins were chromatographed on a 3 cm $\times$ 30 cm column with a linear gradient of 0.0–0.3 M-NaCl in 50 mM-Tris/HCl buffer (pH 8.3) containing 8 M-urea; 5 ml fractions were collected. The bars (I and II) indicate the pooled fractions. Inset: SDS-PAGE profiles of fractions along the column; 50 μ l samples of fractions were applied to the gel; numbers signify the corresponding column fractions.

Table 1. Amino acid composition of 32 kDa and 45 kDa proteins

Constituent	Mol %	
	32 kDa	45 kDa
Asp	13.3	16.0
Thr	6.6	4.0
Ser	8.8	11.2
Glu	10.0	9.0
Pro	3.7	0.5
Gly	7.3	8.0
Ala	9.5	10.2
Val	7.7	7.2
Cys (half)	0	0
Met	1.4	1.5
Ile	4.8	5.2
Leu	7.0	8.9
Tyr	4.5	2.5
Phe	2.7	2.4
Lys	10.0	9.8
His	0.5	0.5
Arg	1.9	2.0
Amino acid type:		
Acidic	23.3	25.0
Basic	12.4	12.5
Hydrophobic	36.8	36.9

at retention times of 12.5 and 16.2 min, respectively (not shown). The purity of the two proteins thus obtained was also estimated to be highly homogeneous by SDS-PAGE and the respective apparent molecular masses of their monomeric forms were confirmed to be 32 and 45 kDa (Fig. 2). When the molecular masses of the 32 and 45 kDa proteins were determined by comparison with marker proteins on HPLC gel filtration, they appeared to be 61 and 99 kDa, respectively, suggesting that the two proteins exhibit dimeric forms in such a low concentration of urea (not shown).

Fig. 2. SDS-PAGE profiles of the 32 and 45 kDa proteins purified by G3000SW HPLC. Each protein (20 μ g) was electrophoresed through a 12% (w/v) polyacrylamide gel. Lanes: 1, molecular mass markers; 2, 8 M-urea extract; 3 and 4, purified 32 and 45 kDa proteins, respectively.

Fig. 3. Peptide maps of the 32 and 45 kDa proteins. Purified proteins were digested with *Staphylococcus* V8 enzyme as described in Methods and run on 15–20% polyacrylamide gradient gels. Lanes 1 and 2 contain 32 and 45 kDa proteins, respectively.

Chemical characterization of the 32 and 45 kDa proteins

Although both proteins had more acidic amino acid residues than basic ones, and also contained similar amounts of hydrophobic amino acids (about 37 mol %), the proteins differed in their contents of individual amino acids (Table 1). Aspartic acid, glutamic acid, serine, lysine, alanine, glycine, and valine were the major

Fig. 4

Fig. 4. Isoelectric focusing of the 32 and 45 kDa proteins. Each purified protein (30 µg) was electrofocused in a 2.5–5 ampholine gradient as described in Methods. Lanes 1 and 2 contain 32 and 45 kDa proteins, respectively. Lane 3 contains pI markers.

Fig. 5

Fig. 5. Autoradiographs of ^{125}I -labelled cell wall proteins from *C. difficile* GAI 0714. The cell walls were prepared from the whole cells labelled with ^{125}I in 0.5 M-sodium phosphate buffer (pH 7.5)/Chloramine T. Proteins were visualized by Coomassie Brilliant Blue (lane 1) or by autoradiography (lane 2).

amino acids for both proteins. However, these amino acids, except glutamic acid, were more predominant in the 45 kDa protein than the 32 kDa protein. The 32 and 45 kDa proteins gave quite different peptide maps after limited proteolysis with *S. aureus* V8 protease (Fig. 3), suggesting marked differences in their primary structures.

When the pIs of the proteins were analysed in polyacrylamide gels using ampholines of pH 2.6–4.0, multiple isoelectric forms of pI 3.7–3.9 were obtained for the 32 kDa protein, while a single form of pI 3.3 was found for the 45 kDa protein (Fig. 4). These pI values were consistent with the acidic nature of the two S layer proteins.

Surface exposure of the 32 and 45 kDa proteins

To determine if the two proteins were exposed on intact whole cells, cells were surface labelled with ^{125}I . The isolated cell walls from the previously labelled cells were analysed by SDS-PAGE followed by autoradiography (Fig. 5). The 32 and 45 kDa protein bands seen on SDS-PAGE were labelled to a very similar extent by iodination, indicating the surface exposure of both proteins. This was further determined by immuno-

electron microscopy using protein A–gold conjugates (Fig. 6). With either anti-32 kDa or anti-45 kDa protein as the first antiserum, the gold particles were evenly distributed on the outermost surface of the cells.

Immunological characterization of the 32 and 45 kDa proteins

Immunological relationships between the two S layer proteins were examined by immunodiffusion tests with polyclonal rabbit antisera raised against the purified protein specimens (Fig. 7). Both proteins produced only one precipitin line with the homologous antiserum; the 32 kDa protein formed no precipitin line with the 45 kDa protein, and vice versa (Fig. 7a). Furthermore, when both antisera were combined in the well, precipitin lines generated with the heterologous proteins were not fused to each other, indicating antigenic heterogeneity between the two proteins (Fig. 7b).

The 8 M-urea extracts from the cell walls of nine strains of *C. difficile* belonging to group 1 were assayed by immunoblotting with either anti-32 kDa or anti-45 kDa protein antiserum. SDS-PAGE profiles of the cell wall proteins revealed that all the strains tested contained two major protein bands corresponding to the 32 and 45 kDa proteins (Fig. 8a). Only strain GAI 0714 had a protein that reacted with the anti-32 kDa protein antiserum, whereas seven of the nine strains had a protein that reacted with the anti-45 kDa protein antiserum (Fig. 8b, c).

Discussion

In our previous work (Takumi *et al.*, 1987), a 32 kDa protein antigen from *C. difficile* ATCC 11011 was solubilized with PBS without the use of detergent or chaotropic agents, but the 45 kDa protein was insoluble in PBS. In the present work, the 45 kDa protein as well as the 32 kDa protein were extracted from the cell walls by treatment with 8 M-urea in Tris buffer. S layer subunits are generally linked to the under-wall layer by differing combinations of hydrophobic bonds, ionic bonds and hydrogen bonds (Sleytr & Messner, 1983). Perhaps the linkage of the 45 kDa protein to the under-wall layer was stronger than, or different from, that of the 32 kDa protein.

On HPLC gel filtration with 0.1 M-urea in PBS, the 32 kDa and 45 kDa proteins were eluted with apparent molecular masses of 61 and 99 kDa, respectively, suggesting that the two proteins maintain a dimeric molecular form under these mild conditions. A similar dimeric form of S layer protein was observed for the A protein of *Aeromonas salmonicida* eluted by HPLC in the

Fig. 6. Electron micrographs of thin sections from cells of *C. difficile* GAI 0714 prepared by reaction with rabbit antisera raised against the 32 kDa (a) or 45 kDa (b) protein followed by protein A-gold conjugate. Bars, 100 nm.

absence of detergent (Phipps *et al.*, 1983). With the A protein, however, only a proportion of the protein was present in dimeric form. In contrast, all the monomeric subunits of each of the two proteins of *C. difficile* were

Fig. 7. Immunodiffusion analysis of the 32 and 45 kDa proteins against their respective antisera. (a) Wells 1 and 3 contain anti-32 and -45 kDa protein antisera, respectively. Wells 2 and 4 contain 32 and 45 kDa proteins, respectively. (b) Well 1 contains 32 kDa protein; wells 2 and 4 contain 45 kDa protein. Well 3 contains the two antisera combined in an equal volume.

Fig. 8. Immunological relationships between the 32 and 45 kDa proteins from group I strains of *C. difficile*. The 8 M-urea extracts from the cell walls were separated by SDS-PAGE (a) and the immunoblots were performed with antiserum raised to the 32 kDa (b) or the 45 kDa (c) protein. Lanes: 1, *C. difficile* GAI 4132; 2, GAI 5415; 3, GAI 5416; 4, GAI 0284; 5, GAI 0714; 6, GAI 0858; 7, ATCC 11011; 8, ATCC 17859; 9, GAI 1140.

apparently converted into dimeric form, suggesting the presence of hydrophobic domains within the individual protein molecules. Since the mobility of the proteins in SDS-PAGE gels did not change when 2-mercaptoethanol was omitted, it is unlikely that the formation of disulphide bonds accounts for the increased molecular mass. Moreover, the amino acid analysis revealed a lack of cysteine residues.

The amino acid compositions of the 32 and 45 kDa proteins were very similar to each other in respect of the distinctive features of S layer proteins (Kandler & König, 1985; Sleytr *et al.*, 1986; Bingle *et al.*, 1986; Koval, 1988), which have a high proportion of nonpolar and acidic amino acids, a low content of sulphur-containing amino acids, and usually a lack of cysteine residues. The acidic nature of the two proteins may contribute to their low isoelectric points, which in the case of the 32 kDa protein was represented by multiple pIs. The appearance of multiple of pI forms is not unusual for cell-surface-localized proteins (Russell, 1979; Phipps *et al.*, 1983; Bingle *et al.*, 1986). Multiple pI forms were also found for the intact cell surface protein of *Micrococcus radiodurans* (Baumeister *et al.*, 1982), suggesting that their appearance is not connected with the purification procedure. The 32 and 45 kDa proteins differed in the peptide maps generated by treatment with the V8 enzyme. No common peptide band was observed in the peptide fragments on SDS-PAGE. This chemical heterogeneity may account for the antigenic dissimilarity between the two antigens: no immunological cross-reaction between them was observed by immunodiffusion tests or immunoblot analyses. Antigenicity of the 32 kDa protein was apparently strain specific, whereas a protein cross-

reacting with anti-45 kDa protein antiserum was rather common among *C. difficile* strains. Sharp & Poxton (1988) reported that the 6 M-urea-soluble antigenic proteins from various strains of *C. difficile* were apparently strain specific, although these antigens were highly variable in the molecular mass. In contrast, we demonstrated that the *C. difficile* strains tested could be divided into two groups on the basis of the molecular masses of the 6M-urea-extractable wall proteins (Kawata *et al.*, 1984). For a solution to this discrepancy, more *C. difficile* strains must be examined.

Daily & Schloemer (1988) reported the cloning and expression of two secreted antigens from *C. difficile* VPI 10463 in *Escherichia coli* RR1 M15. These two antigens were proteins of 32 kDa and 43 kDa, and probably correspond to the two S layer proteins described here.

We confirmed that both the 32 and the 45 kDa proteins were exposed on the cell surface by radioiodination and by immunoelectron microscopy using a protein A-gold conjugate. Since both proteins are essential constituents of the S layer of the organism, it can be said that the S layer is a single macromolecular layer composed of two distinct protein species, which are exposed almost evenly on the outermost cell surface. Most bacterial S layers are composed of a single subunit (Sleytr & Messner, 1983; Koval, 1988). Although the cell surface layer proteins isolated from *Bacillus brevis* 47 possess two protein subunits, this can be attributed to the presence of two distinct superimposed layers (Tsuboi *et al.*, 1982). Thus the S layer of *C. difficile* has a unique surface layer structure and it may therefore represent a model for a new type of morphogenesis or molecular assembly of S layer protein.

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