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Purification and Characterization of *Campylobacter rectus* Surface Layer Proteins

HIROSHI NITTA,[1] STANLEY C. HOLT,[2] AND JEFFREY L. EBERSOLE[1]*

Department of Periodontics[1] and Department of Microbiology,[2] University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284

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Campylobacter rectus **is a putative periodontopathogen which expresses a proteinaceous surface layer (S-layer) external to the outer membrane. S-layers are considered to play a protective role for the microorganism in hostile environments. The S-layer proteins from six different *C. rectus* strains (five human isolates and a nonhuman primate [NHP] isolate) were isolated, purified, and characterized. The S-layer proteins of these strains varied in molecular mass (ca. 150 to 166 kDa) as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. They all reacted with monospecific rabbit antiserum to the purified S-layer of *C. rectus* 314, but a quantitative enzyme-linked immunosorbent assay demonstrated a strong antigenic relationship among the five human strains, while the NHP strain, 6250, showed weaker reactivity. Amino acid composition analysis showed that the S-layers of four *C. rectus* strains contained large proportions of acidic amino acids (13 to 27%) and that >34% of the amino acid residues were hydrophobic. Amino acid sequence analysis of six S-layer proteins revealed that the first 15 amino-terminal amino acids were identical and showed seven residues of identity with the amino-terminal sequence of the *Campylobacter fetus* S-layer protein SapA1. CNBr peptide profiles of the S-layer proteins from *C. rectus* 314, ATCC 33238, and 6250 confirmed that the S-layer proteins from the human strains were similar to each other and somewhat different from that of the NHP isolate (strain 6250). However, the S-layer proteins from the two human isolates do show some structural heterogeneity. For example, there was a 17-kDa fragment unique to the *C. rectus* 314 S-layer. The amino-terminal sequence of this peptide had homology with the *C. rectus* 51-kDa porin and was composed of nearly 50% hydrophobic residues. Thus, the S-layer protein from *C. rectus* has structural heterogeneity among different human strains and immunoheterogeneity with the NHP strain.**

Surface layers (S-layers) are a common feature on the surfaces of many eubacteria and archaebacteria. The S-layers are composed of regularly arranged protein or glycoprotein subunits ranging in size from 40 kDa to around 200 kDa (36) and cover the entire cell surface. Because of their relative abundance and physical location, S-layers are considered to play essential roles, such as providing microorganisms a protective coating, acting as molecular sieves and ion traps, or promoting cell adhesion and surface recognition (19, 30). Trust and colleagues (39) reported that expression of the S-layer influenced the cell surface hydrophobicity and appeared to significantly increase the virulence of *Aeromonas salmonicida*, an important fish pathogen. Blaser et al. (3) reported that S-layer-positive strains of *Campylobacter fetus*, which causes abortion in sheep and cattle and various systemic infections or acute diarrhea in humans, had increased resistance to complement killing by abrogating C3b binding to the bacterial cell surfaces. Thus, the S-layer proteins of pathogenic microorganisms appear to confer a change in surface characteristics (e.g., hydrophobicity) which seems to enhance the ability of the microorganism to evade host protective responses, for example, by an altered interaction with various host cells.

Campylobacter rectus, a gram-negative, anaerobic, asaccharolytic, motile rod, has been associated with periodontal diseases (35, 38). Electron microscopic studies demonstrated that *C. rectus* has a characteristic S-layer composed of hexagonally arrayed subunits (6, 23, 28), each with a molecular mass of approximately 150 kDa (5, 25). Haapasalo et al. (18) suggested that the S-layer of *C. rectus* is an important determinant of cell surface hydrophobicity and alters the interaction of this microorganism with the subgingival microenvironment. Previous work from our laboratory (5, 24) also showed that the S-layer plays an important role in the interaction between this microorganism and host cells in vitro and in vivo. Strains of *C. rectus* that have lost their S-layers during long-term in vitro subculturing adhere to human gingival fibroblasts twice as well as low-passage *C. rectus* strains which have retained the S-layer (5). Kesavalu et al. (24) have shown that high-passage, S-layer-deficient *C. rectus* strains were significantly less virulent than low-passage, S-layer-positive strains in a murine abscess model. Importantly, these studies indicated that the *C. rectus* S-layer may contribute to the virulence of this microorganism.

To date, there is a limited amount of information available concerning the biochemical and immunological characteristics of the S-layer of *C. rectus*. In this study, we have developed methods for purification of this macromolecular structure from *C. rectus*, with the goal of characterizing the protein and determining its functional contribution to the colonization and virulence of this pathogen.

MATERIALS AND METHODS

Bacteria. *C. rectus* ATCC 33238 and ATCC 33238 (S^-) (a variant of the ATCC strain whose S-layer has been lost by spontaneous mutation during in vitro passage), *C. rectus* 314, 471, 112, 576, and 726 (human isolates), *C. rectus* 6250 (a nonhuman primate [NHP] isolate), *Campylobacter concisus* ATCC 33237, and *Campylobacter curvus* ATCC 35224 were grown in mycoplasma-formate-fumarate broth, supplemented with 5% horse serum, under anaerobic conditions as described previously (17).

Isolation of S-layer protein. Cells were harvested by centrifugation at 10,000 × *g* for 20 min at 4°C. The harvested cells were then washed once by centrifugation with 10 mM HEPES buffer, pH 7.4. The cell pellet was resuspended in 10 mM

* Corresponding author. Mailing address: Department of Periodontics, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Dr., San Antonio, TX 78284. Phone: (210) 567-3591. Fax: (210) 567-6858. E-mail: ebersole@uthscsa.edu.

HEPES buffer, pH 2.5, and incubated for 30 min with gentle stirring at room temperature. Cells were then centrifuged at 10,000 × *g* for 20 min at 4°C and the supernatant was filtered through a cellulose acetate membrane filter (pore size, 0.2 μm; VWR Scientific, West Chester, Pa.). After the pH of the filtrate was adjusted to 7.3 with NaOH, the filtrate was centrifuged at 100,000 × *g* for 2 h to sediment insoluble material. The supernatant was dialyzed against distilled water overnight with constant stirring at 4°C.

The S-layer protein was purified from acid extracts by preparative isoelectric focusing using a Rotofor apparatus (Bio-Rad Laboratories, Richmond, Calif.). For this procedure, 2.6 ml of ampholyte solution (Bio-Lyte; pH ranges, 4 to 6 and 5 to 7; 40% wt/vol; 1.3 ml each; Bio-Rad) was added to the acid extracts, and the sample was diluted to 55 ml with 8 M urea and loaded into the Rotofor cell. The sample was electrofocused at a constant power of 12 W for 4 h at 15°C. Fractions containing the S-layer protein were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were dialyzed sequentially against 1 N NaCl for 4 h and distilled water for 24 h to remove the ampholyte and urea.

SDS-PAGE. A discontinuous gel system of 7.5, 14, or 10 to 20% exponential acrylamide gels was used for resolution, and a 4% acrylamide gel was used as the stacking gel (27). The protein concentration of the samples was determined by the bicinchoninic acid protein assay (Pierce, Rockford, Ill.), with bovine serum albumin (fraction V; Pierce) as the standard. Samples were solubilized by boiling at 100°C for 3 min in treatment buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 5% 2-mercaptoethanol). Electrophoresis was carried out in a model SE 600 vertical slab gel unit (Hoefer, San Francisco, Calif.) at 30 mA/gel until the dye front reached the bottom of the gel. The molecular mass was determined with a calibration curve established with the Perfect protein markers, consisting of seven recombinant proteins of 15, 25, 35, 50, 75, 100, and 150 kDa (Novagen Inc., Madison, Wis.). Gels were stained with 0.025% Coomassie brilliant blue R-250 (Sigma, St. Louis, Mo.) after electrophoresis.

Antiserum preparation. The S-layer protein of *C. rectus* 314, isolated in the Rotofor procedure, was separated by SDS-PAGE, and a 150-kDa protein band was excised from the gel. The excised gel was smashed with a tissue grinder (Weaton, Millville, N.J.) and used as the antigen. A female New Zealand White rabbit (2 to 3 kg; Martha's Farm, Wimberly, Tex.) was immunized with 100 μg of the antigen by intramuscular injection. The rabbit was given a booster of 75 μg of the antigen every 2 weeks for 4 weeks, and blood was obtained 10 days after the final injection. The resulting antiserum was aliquoted and stored at −20°C.

Western immunoblotting. Following separation by SDS-PAGE, 2.5 μg of whole-cell protein was transferred to a polyvinylidene fluoride membrane, Immobilon-P (Millipore, Bedford, Mass.), as described previously (29). The transfer was performed at a constant current of 100 mA overnight. Unoccupied protein sites on the membrane were blocked by incubation with 5% nonfat dry milk in 10 mM Tris-buffered saline containing 0.05% Tween 20 (TBS-T) overnight. The membrane was washed with TBS-T and reacted with a 1:1,000 dilution of rabbit antiserum to S-layer overnight with shaking. After being washed with TBS-T, the membrane was incubated in a 1:1,000 dilution of peroxidase-conjugated goat anti-rabbit immunoglobulin G (Calbiochem, La Jolla, Calif.) for 4 h followed by addition of 4-chloro-1-naphthol (Sigma) as a substrate.

ELISA. To determine the immunoheterogeneities of the S-layer proteins among *C. rectus* strains, an enzyme-linked immunosorbent assay (ELISA) was performed as described previously (11). Formalin-fixed *C. rectus* strains (10^8 cells/well) or isolated S-layer protein (1 μg/well) was used as the antigen, and anti-S-layer antisera which reacted (25°C, 2 h) with the *C. rectus* strains were detected by incubation (25°C, 2 h) with affinity-purified goat anti-rabbit immunoglobulin G conjugated with alkaline phosphatase (Calbiochem).

Amino acid analysis and sequencing of S-layer protein. The Rotofor-purified S-layer was concentrated and dried with a SpeedVac concentrator (Savant Instrument Inc., Farmingdale, N.Y.). Amino acid analysis was performed on a model 7300 analyzer (Beckman Instruments, Fullerton, Calif.). For N-terminal sequencing, the purified S-layer proteins were electrophoresed through an SDS–7.5% acrylamide gel and blotted to an Immobilon-P membrane as described above. The proteins were visualized with amido black staining and excised from the membrane. The sequencing was performed on a gas-liquid automatic sequencer, model 477A (Applied Biosystems, Inc., Foster City, Calif.), coupled to an on-line high-performance liquid chromatography model 120A analyzer.

CNBr cleavage and amino acid sequence of its digested products. Purified S-layer (50 μg) was dried with a SpeedVac concentrator. CNBr (30 μl of a 300-mg/ml solution) in 70% formic acid was added, and the mixture was incubated for 24 h in the dark at room temperature. The sample was dried with a N_2 gas stream, 100 μl of distilled water was added, and finally the sample was dried with a SpeedVac concentrator. The sample was applied to an Applied Biosystems model 130A high-performance liquid chromatography separation system with an RP 300 C_8 column (Applied Biosystems). Fractions containing the peaks were collected and dried with a SpeedVac concentrator. The samples were then electrophoresed through an SDS–14% acrylamide gel and transferred to an Immobilon-P membrane. N-terminal amino acid sequencing was performed on various CNBr-treated peptides as described above.

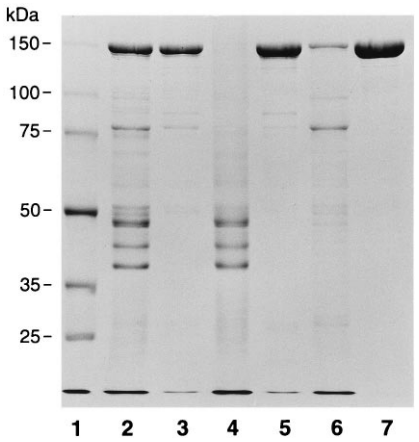

FIG. 1. SDS-PAGE analysis (7.5% acrylamide gel) of *C. rectus* 314 fractions taken during S-layer purification. Lane 1, standard proteins; lane 2, 30 μg of whole cells; lane 3, 10 μg of acid extracts (pH 2.5); lane 4, 30 μg of whole cells after acid extraction; lane 5, 10 μg of acid extracts after adjusting pH to 7; lane 6, 10 μg of precipitants after adjusting pH to 7; lane 7, 10 μg of purified S-layer.

RESULTS AND DISCUSSION

Purification of the S-layer protein of *C. rectus* 314. *C. rectus* S-layer protein was identified previously as a 150-kDa species in strain 314 (5). To isolate this protein, acid extraction of whole cells was used, similar to the methods described for isolation of other S-layer proteins. Acid extraction greatly enhanced isolation of the 150-kDa S-layer protein (Fig. 1, lane 3). An additional isoelectric focusing step was used to obtain pure S-layer protein (Fig. 1, lane 7). Forty milligrams of protein was extracted from 2 g (wet weight) of whole cells by acid extraction, and 9 mg of purified S-layer protein was recovered following isoelectric focusing.

The S-layer interaction with the bacterial outer membrane is mainly ionic, since a low-pH solution disrupts the structural relationship to the cell surface (14). Treatment of *Aeromonas* spp. (7) and *Campylobacter* spp. (30) with a low-pH buffer was reported to be effective in extracting S-layer from whole cells. We noted that the S-layer of *C. rectus* was selectively removed from whole cells with a 10 mM HEPES buffer at pH 2.5. Walker et al. (41) previously reported on a selective extraction of S-layer from whole cells of *Caulobacter crescentus* with a 100 mM HEPES buffer at pH 2.0. Since HEPES generally has a buffering effect under neutral conditions, the mechanism by which it works under acidic conditions is unknown. Nevertheless, this extraction procedure was found to be very efficient at eluting the majority of the S-layer from the surface, with minimal effects on the multitude of other membrane proteins expressed by *C. rectus.*

Comparison of S-layers among *C. rectus* strains. Eight *C. rectus* strains (seven human clinical isolates and one NHP isolate) were examined by SDS-PAGE to determine the presence and relative sizes of the S-layer proteins (Fig. 2A). All of the strains possessed a distinct high-molecular-mass protein similar to the S-layer protein of *C. rectus* 314. These proteins varied in molecular mass, with a range of 150 to 166 kDa. Proteins with similar molecular masses were either absent or present in small amounts in other oral campylobacters, including *C. concisus* and *C. curvus*. The finding that SDS-PAGE and Western immunoblot analysis of eight *C. rectus* strains demonstrated a size heterogeneity of the S-layer proteins (which varied in molecular mass from approximately 150 to 166 kDa) is similar to previous data which demonstrated size heteroge-

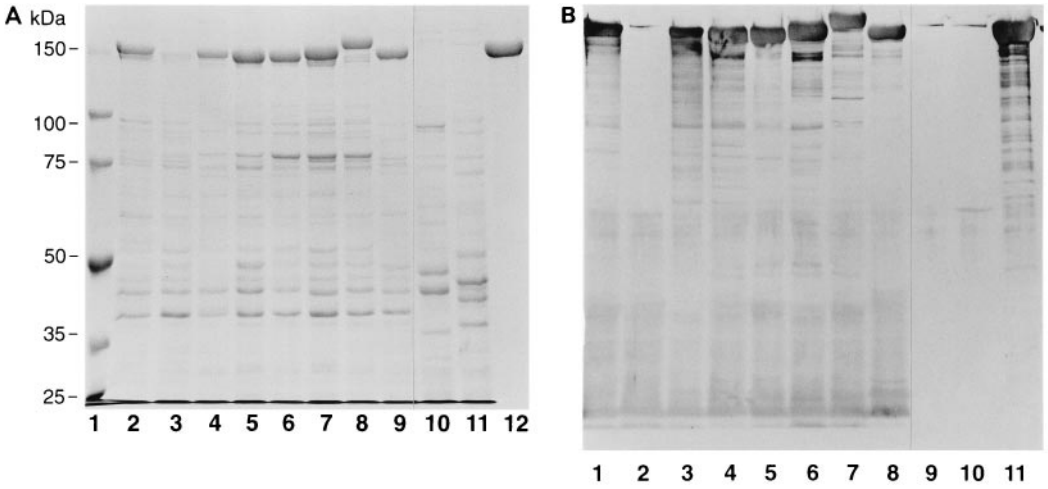

FIG. 2. (A) SDS-PAGE analysis (7.5% acrylamide gel) of 10 different *Campylobacter* species. Lane 1, standard proteins; lane 2, *C. rectus* ATCC 33238; lane 3, *C. rectus* ATCC 33238 (S^-); lane 4, *C. rectus* 314; lane 5, *C. rectus* 471; lane 6, *C. rectus* 112; lane 7, *C. rectus* 576; lane 8, *C. rectus* 726; lane 9, *C. rectus* 6250; lane 10, *C. concisus*; lane 11, *C. curvus*; lane 12, 10 μg of purified S-layer of *C. rectus* 314. Each lane contains 30 μg of protein except for lane 12. (B) Immunoblots of proteins from 10 different *Campylobacter* species probed with rabbit antiserum to purified S-layer of *C. rectus* 314. Lane 1, *C. rectus* ATCC 33238; lane 2, *C. rectus* ATCC 33238 (S^-); lane 3, *C. rectus* 314; lane 4, *C. rectus* 471; lane 5, *C. rectus* 112; lane 6, *C. rectus* 576; lane 7, *C. rectus* 726; lane 8, *C. rectus* 6250; lane 9, *C. concisus*; lane 10, *C. curvus*; lane 11, 1 μg of purified S-layer of *C. rectus* 314. Each lane contains 2.5 μg of protein except for lane 11.

neity of S-layers across genera (36) and among strains of a species of bacteria (31). This heterogeneity may be due to glycosylation (36); however, we have no convincing evidence for glycosylation of the S-layer protein in *C. rectus*. Moreover, what role the variations in the sizes of S-layer proteins play in altering the biological function of the molecule is unclear (4, 13). This alteration in protein size, as well as small amino acid sequence changes in the S-layer protein among strains, could have an effect on the antigenic epitopes of this large protein.

Western immunoblot analysis with rabbit anti-*C. rectus* 314 S-layer revealed strong antigenic reactivity with a large-molecular-mass species in all of the strains tested except *C. concisus* and *C. curvus* (Fig. 2B, lanes 10 and 11) and the S-layer-negative derivative of *C. rectus* ATCC 33238 (Fig. 2B, lane 2), confirming the identification of the 150- to 166-kDa proteins as the S-layer proteins. Importantly, the results also indicate that despite their different molecular masses the S-layer proteins from the different *C. rectus* species appear to be related.

However, while some antigenic relatedness among the S-layer proteins was demonstrated by Western immunoblotting, the human and NHP *C. rectus* strains demonstrated differences in antigenic relatedness as revealed by the ELISA testing for cross-reactivity with the *C. rectus* 314 S-layer antisera (Table 1). Since the SDS-PAGE analyses indicated that similar amounts of S-layer protein were expressed by all of the *C. rectus* strains, an ELISA with polyclonal rabbit antiserum to the S-layer protein of *C. rectus* 314 was used to quantitatively examine antigenic heterogeneity among different *C. rectus* strains. Human clinical isolates exhibiting S-layers with molecular masses different from that of the *C. rectus* 314 S-layer demonstrated a somewhat lower reactivity in the ELISA. Moreover, the NHP strain (*C. rectus* 6250) showed a significantly lower reactivity which approached the activity noted with the S-layer-negative human strain. The S^- strain exhibited some reactivity in the ELISA, which was consistent with the Western immunoblot findings. The results suggested that this strain may produce a low level of antigen related to the S-layer; however, this antigen appeared to have a low molecular mass and not to be capable of forming the three-dimensional surface structure characteristic of the S-layers. Similar results were noted in an ELISA with S-layer proteins isolated from the *C. rectus* strains (Table 1). Again, antigenic relatedness among the human isolates was noted, while the protein from the NHP strain was significantly less reactive. Previous findings with S-layer proteins from other pathogens have demonstrated antigenic similarities or dissimilarities within species (42).

Amino acid compositions of S-layers of *C. rectus*. The amino acid compositions of the purified S-layer proteins from five different *C. rectus* strains (314, 576, 726, 6250, and ATCC 33238) were determined (Table 2). The compositions were similar among the S-layer proteins containing small amounts of proline, methionine, and histidine. These small amounts are similar to those of S-layer proteins from *C. fetus* as well as other bacterial S-layer proteins (16, 20, 32) and may be important factors in the biological function of this molecular structure. All five S-layer proteins showed similar charge characteristics, being weakly acidic, with 22 to 37% acidic residues and only 9 to 12% basic residues. Comparisons of the amino acid

TABLE 1. Reactivity of rabbit antiserum against S-layer protein of *C. rectus* 314 to different *C. rectus* strains

Strain	Host	Mol mass (kDa) of S-layer protein	Reactivity with[a]:	
			C. rectus	S-layer protein
314	Human	150	1.30	1.62
471	Human	150	1.22	1.54
112	Human	150	1.00	1.38
576	Human	156	0.96	1.30
726	Human	166	0.86	1.30
6250	NHP	150	0.55[b]	0.41[b]
ATCC 33238 (S^+)	Human	150	0.96	1.56
ATCC 33238 (S^-)	Human		0.42[b]	NT[c]

[a] Whole cells used as the antigen were reacted with 1:1,000-diluted antiserum to the S-layer of *C. rectus* 314, and a 1:5,000 dilution of the antisera was used with the S-layer protein as the antigen. Values are expressed as optical density at 405 nm as determined by ELISA.

[b] Significantly lower than reactions of antibody with isolated S-layer proteins of other *C. rectus* strains at $P < 0.05$ by a Student *t* test.

[c] NT, not tested since no S-layer was isolated from this strain.

TABLE 2. Relative amino acid compositions of purified S-layer proteins of *C. rectus* strains

Residue type and amino acid	Mol% residues in:					
	C. rectus[a]					*C. fetus*[e]
	314	ATCC 33238	576	726	6250	
Hydrophobic	35.5	34.0	38.7	35.4	34.8	39.3
Alanine	8.9	7.9	7.4	8.1	7.9	11.9 ± 0.7
Valine	7.6	7.1	9.6	8.0	7.8	7.4 ± 1.0
Leucine	7.2	7.3	8.4	7.7	6.7	8.2 ± 0.9
Isoleucine	5.7	5.6	8.2	5.7	5.9	7.2 ± 0.3
Proline	1.7	1.4	0.9	1.5	1.6	0.6 ± 0.2
Phenylalanine	3.4	3.3	3.6	3.1	3.7	2.8 ± 0.6
Methionine	1.1	1.4	0.6	1.4	1.2	1.2 ± 0.5
Neutral	25.1	27.3	34.0	28.8	26.7	31.7
Glycine	8.3	10.1	9.6	11.0	9.8	9.1 ± 1.2
Serine	5.8	5.9	7.8	6.2	6.0	8.9 ± 0.7
Cysteine	ND[b]	ND	ND	ND	ND	0.1 ± 0.1
Tryptophan	ND	ND	ND	ND	ND	0.3 ± 0.1
Tyrosine	1.9	1.7	2.1	1.6	1.8	1.4 ± 0.3
Threonine	9.1	9.5	14.4	10.1	9.1	11.9 ± 1.3
Hydrophilic	39.4	38.8	27.3	35.8	38.5	29.4
Asparagine[c]	18.5	18.6	11.8	19.2	17.1	16.9 ± 1.4
Glutamine[d]	8.1	7.3	1.2	3.8	8.1	4.2 ± 0.6
Lysine	8.7	9.2	9.6	9.5	9.6	7.4 ± 0.5
Arginine	2.7	2.2	2.8	1.9	2.4	0.6 ± 0.5
Histidine	1.6	1.4	1.9	1.4	1.4	0.3 ± 0.3

[a] Values for individual amino acids are averages of two determinations.
[b] ND, not determined.
[c] Combined asparagine and aspartic acid.
[d] Combined glutamine and glutamic acid.
[e] Mean ± standard deviation predicted from nucleotide sequences of S-layer genes from four strains.

residues of the S-layer proteins isolated from microorganisms of different taxonomic origin have shown that the crystalline arrays are usually enriched for acidic amino acids, thus producing a weakly acidic and hydrophobic protein (36). The pI of the protein was determined to be 5.6 to 5.8 by preparative isoelectrofocusing.

The relative hydrophobicities of the *C. rectus* S-layer proteins were between 34 and 46% of the residues. Since hydrophobicity of bacteria has been considered to contribute to bacterial adhesion and inhibition of phagocytic ingestion (1, 39, 40), depending upon the tertiary structure of the molecule, this somewhat hydrophobic nature of the surface structure may contribute to the virulence of *C. rectus.*

Amino acid sequence analysis of S-layers from *C. rectus*. The sequences of the 15 N-terminal amino acids of the S-layer proteins from *C. rectus* 314, 576, 726, 6250, and ATCC 33238 were identical (Table 3). Kay et al. (20) compared the first 27 amino acids of the S-layer proteins from three strains of *A. salmonicida* and showed that 23 were identical. In contrast, Dubreuil et al. (8) reported two different N-terminal amino acid sequences for *C. fetus* S-layers and were able to determine that only 7 of 18 residues were shared. *C. fetus* is classified into two serotypes (A and B) based upon its lipopolysaccharide (34). The first 184 amino acids were conserved in *C. fetus* serotype A strains, while a variable region was located downstream of the conserved region (10). *C. fetus* serotype B strains also demonstrated a conserved N-terminal portion of the S-layer protein and a variable downstream sequence (9). The *C. fetus* S-layer protein binds exclusively to the lipopolysaccharide of homologous cells, and its binding domain exists in the conserved region of the protein (10).

This region is relatively hydrophobic, containing 7 hydrophobic and only 3 charged amino acids within the first 15 residues. In *C. rectus*, a conserved region may also exist in the portion of the S-layer gene encoding the N terminus, since purified S-layer proteins from seven *C. rectus* strains bind to the surface of an S-layer-negative *C. rectus* strain (unpublished observation). This suggests that the domain related to the S-layer binding to the cell surface may comprise a portion of the conserved region of the S-layer protein gene.

While protein homology scans employing the National Center for Biotechnology Information BLAST search system (2) revealed that the *C. fetus* 82-40 surface array protein demonstrated the greatest similarity to the *C. rectus* S-layer protein, with 7 of 13 (53%) identical and 10 of 13 (76%) conservative amino acid matches (8), even *C. fetus* VC119 demonstrated 5 of 13 identical residues. However, CNBr fragmentation resulted in differences in the peptide profiles of three of these strains, suggesting that the S-layer may also exhibit some variability in sequence. Messner and Sleytr (31) compared the amino acid sequences deduced from the S-layer genes of different bacteria and found relatively little overall homology of either primary or secondary structure among these characterized S-layer proteins. However, our analysis indicated that the first 15 N-terminal amino acids of the S-layer of *C. rectus* exhibited homology (7 of 15) with the amino terminus of an S-layer protein of *C. fetus* (33), and this suggests that there may be some structural homology in this protein within the *Campylobacter* genus.

Putative sequences of peptides from CNBr fragments of several S-layer proteins. To further investigate the relatedness of the S-layer proteins from different strains of *C. rectus*, CNBr fragmentation patterns were examined. The SDS-PAGE profiles of the CNBr-digested S-layer proteins revealed that the human isolates had similar fragmentation patterns which differed from that of the NHP isolate. While the amino acid composition data suggested that the S-layer proteins would contain up to 18 methionine residues, this treatment fragmented the protein from the human strains into four major polypeptides, which were identified as having masses of 24, 21, 19, and 17 kDa, and fragmented the NHP strain into three major polypeptides with masses of 19.5, 17.5, and 16 kDa (Fig. 3). N-terminal amino acid sequence analysis of these fragments (Table 4) revealed that the first 27 amino acids of the 24-kDa fragment were identical to those of the 19-kDa fragment in *C. rectus* 314 and ATCC 33238. Moreover, the sequences of both these human strains were identical. No significant homologies between the sequence of this fragment and the protein databases were found. The 21-kDa fragment of *C. rectus* 314 and ATCC 33238 failed to give unique sequences, indicating that this fragment contained multiple peptides (data not shown). Interestingly, in contrast to the 24-kDa fragment, the sequences of the 17-kDa fragments derived from these human

TABLE 3. Homology of *C. rectus* and *C. fetus* S-layers

Strain	Sequence[a]
C. rectus	1 A L T Q T Q V S Q L Y V T L F 15
C. fetus 82-40	1 M L N K T D V S M L Y I T I M 15[b]
C. fetus VC119	1 M I S K S E V S E L F I V L F 15[c]

[a] Shown are the sequences for the 15 N-terminal amino acids of the S-layer proteins. Identical residues are underlined.
[b] Determined by Pei et al. (33).
[c] Determined by Dubreuil et al. (8).

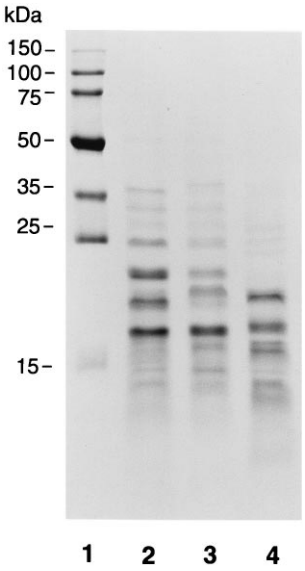

FIG. 3. SDS-PAGE analysis of the products of CNBr treatment of S-layer proteins from three different *C. rectus* strains (10 to 20% acrylamide exponential gel). Lane 1, standard proteins; lane 2, *C. rectus* 314; lane 3, *C. rectus* ATCC 33238; lane 4, *C. rectus* 6250. Each lane contains 50 μg of protein.

strains were different (Table 4), and only the 17-kDa fragment of *C. rectus* 314 showed some significant homology to a protein in the databases, being identical in five amino acids (ITVKK) to the sequence of a *C. rectus* 51-kDa porin (22). Finally, the sequences of the CNBr fragments from the NHP strain (*C. rectus* 6250) did not appear to be related to any of the sequences of either of the human strains. These results indicate some heterogeneity among the S-layer proteins of the human strains, with even greater heterogeneity with the S-layer protein from the NHP strain.

The relationship of the CNBr fragmentation profiles of two human isolates and the NHP strain was also consistent with observed antigenic differences between the human and NHP *C. rectus* strains determined by ELISA. These results suggest that specific host-parasite interactions may have a considerable impact on the structural or antigenic heterogeneity of S-layer proteins of *C. rectus.*

The relationship between outer membrane pores (i.e., porins) and S-layers has been considered to be functionally important in molecular transport processes. Murray et al. (32) have demonstrated that the S-layer of *Spirillum putridiconchylium* consists of 2- to 3-nm-diameter holes, which allow the passage of nutrients and waste products through the layer. Structurally ordered channels involved in outer membrane permeation have also been suggested (15). The S-layers of gram-negative bacteria are usually associated with the outer membrane of the cell wall through weak electrostatic charges, salt bridging, or hydrogen bonding. A tight hydrophobic interaction of the outer membrane with S-layer protein subunits has also been suggested by several investigators (12, 26, 37), although there is no clear evidence for a domain of the S-layer intruding into the outer membrane or strongly interacting with the outer membrane via hydrophobic bonds in a manner that imposes order (15). Amino acid sequence analysis of a 17-kDa fragment of the *C. rectus* 314 S-layer digest revealed that this fragment exhibited some homology to the amino acid sequence of a *C. rectus* 51-kDa porin protein (22). This hydrophobic nature and sequence homology to the porin may indicate that the 17-kDa fragment contains a segment of the polypeptide which is involved in the interactions of the outer membrane and S-layer of *C. rectus*. Further studies of the protein structure and gene sequence should help to elucidate the primary and/or secondary structure of the S-layer that controls its interaction with the bacterial outer membrane.

It is clear from studies of other bacterial pathogens (7, 20, 30), and it has recently been hypothesized for *C. rectus*, that the S-layer is a virulence determinant (5, 24). This surface structure is involved in numerous host-parasite interactions (3, 5, 21, 30) and may be required for *C. rectus* to be able to create and maintain a niche in the complex ecology of the subgingival pocket. Future studies will be designed to explore the functional characteristics of the *C. rectus* S-layer and the domains of the protein which contribute to these functions.

TABLE 4. Amino acid sequences of CNBr-digested products of S-layer proteins of three *C. rectus* strains

Strain	Mol mass(es) (kDa)	N-terminal sequence
314	24 and 19	RLANVAKKDIDVKFEHKKGVLXGFEDK
	17	GNAAFGGVDISKK(K)ITVKK(K)
ATCC 33238	24 and 19	RLANVAKKDIDVKFEHKKGVLXGFEDK
	17	ANAAENIANGIQGXLI
6250	19.5	(E/L)(A/V)(T/K)(S/I)AAKEYFGXAL
	17.5	GNTTDFADLNQI
	16	TEFSANQLRLXLI

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