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# The S-layer protein from *Campylobacter rectus*: sequence determination and function of the recombinant protein

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**Abstract**

The gene encoding the crystalline surface layer (S-layer) protein from *Campylobacter rectus*, designated *slp*, was sequenced and the recombinant gene product was expressed in *Escherichia coli*. The gene consisted of 4086 nucleotides encoding a protein with 1361 amino acids. The N-terminal amino acid sequence revealed that Slp did not contain a signal sequence, but that the initial methionine residue was processed. The deduced amino acid sequence displayed some common characteristic features of S-layer proteins previously reported. A homology search showed a high similarity to the *Campylobacter fetus* S-layer proteins, especially in their N-terminus. The C-terminal third of Slp exhibited homology with the RTX toxins from Gram-negative bacteria via the region including the glycine-rich repeats. The Slp protein had the same N-terminal sequence as a 104-kDa cytotoxin isolated from the culture supernatants of *C. rectus*. However, neither native nor recombinant Slp showed cytotoxicity against HL-60 cells or human peripheral white blood cells. These data support the idea that the N-terminus acts as an anchor to the cell surface components and that the C-terminus is involved in the assembly and/or transport of the protein. 

*Keywords:* S-layer; *Campylobacter rectus*; DNA sequencing; RTX toxin

## 1. Introduction

*Campylobacter rectus*, a Gram-negative, anaerobic motile rod, is considered one of the pathogens of human periodontal disease because this organism is frequently detected in periodontal lesions using both the conventional [1] and DNA probe detection methods [2]. *C. rectus* possesses a characteristic crystalline surface layer (S-layer) which consists of regularly aligned and closely packed subunits in its outermost cell envelope. The S-layer is one of the most commonly observed cell surface structures among archaeobacteria and eubacteria, and is composed of a single homologous protein or glycoprotein [3]. Electron microscopic analysis has revealed that the S-layer of *C. rectus* is formed in a hexagonal array and is located very close to, but does not extend into, the outer membrane [4]. The major component of the S-layer of *C. rectus* ATCC 33238 (Slp) is a

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non-glycosylated protein with an apparent molecular mass of 150 kDa. Slp is highly immunogenic in that periodontitis patients infected with the corresponding organism show high levels of serum IgG titer against this protein [5]. Clinical isolates of *C. rectus* also contain Slp with a slight variation in molecular mass and in antigenic properties [4]. Very recently Nitta, et al. [6] characterized Slp from a non-human primate isolate. Borinski and Holt [7] reported that long-term in vitro subculture of this organism yielded mutant cells that lacked the S-layer of the cell surface.

The superficial location of the S-layer suggests that this structure has important roles in protection of the cell from natural environmental stresses. This structure may also be involved in cell pathogenesis. Indeed, several studies have indicated a relationship between the presence of the S-layer on *C. rectus* cells and virulence [7,8]. For example, low-passage strains bearing an S-layer induced severe tissue destruction when compared with high-passage, S-layer deficient strains in a murine abscess model [8]. According to the study conducted by Borinski and Holt [7], the S-layer bearing strain showed less adherence to human gingival fibroblast cells than the S-layer deficient strain. They hypothesized that this alternation could represent one way of resisting phagocytic events. Another virulence factor, a 104-kDa extracellular toxin, has been isolated from *C. rectus* culture supernatants and characterized [9,10].

To gain more knowledge about the structure and function of the S-layer of this organism, it is essential to obtain pure polypeptide and to determine its primary sequence. The gene encoding the S-layer, *slp*, was previously cloned from *C. rectus* ATCC 33238 and expressed in *Escherichia coli* [11]. The *slp* gene exists as a single copy on the chromosome and the genes from clinical isolates show minor restriction fragment length polymorphism. The present study describes the complete nucleotide sequence determination for the entire gene and attempts to define the functional regions. We also established an *E. coli* clone expressing the recombinant gene product which can be used for functional studies. By cytotoxicity assay using the recombinant protein, we assessed the relationship with Slp and the extracellular cytotoxin described previously [9,10].

## 2. Materials and methods

### 2.1. Bacterial strains and the plasmids

*C. rectus* ATCC 33238 was cultured in Todd-Hewitt broth supplemented with 0.5% yeast extract, 0.2% ammonium formate and 0.3% sodium fumarate under anaerobic conditions (80% $N_2$, 10% $CO_2$ and 10% $H_2$). *E. coli* XL1-Blue (Stratagene, La Jolla, CA) was used as the host for all cloning and expression procedures and was cultured in LB broth or on LB agar plates. The concentration of antibiotics used was 50 μg $ml^{-1}$ of ampicillin, 25 μg $ml^{-1}$ of tetracycline and 20 μg $ml^{-1}$ of chrolamphenicol. Plasmid pKM201 contains a 4.3-kb *Cla*I fragment encoding the entire *slp* gene ligated into the *Acc*I site of pUC18 [11]. Plasmids pUC18, pUC19 and pHG399 were purchased from Takara Shuzo (Otsu, Shiga, Japan) and were used for subcloning.

### 2.2. Nucleotide sequence analysis

The preparation of plasmid DNA, digestion, ligation, and other necessary procedures were carried out using standard methods [12]. Several deletion mutants were created from pKM201 using the Exo Mung Bean Deletion kit (Stratagene). The nucleotide sequence was determined by the dideoxy chain termination method using a 373A DNA sequencer (Applied Biosystems, Foster City, CA). The nucleotide sequences obtained were compiled and analyzed with the aid of the Genetyx program (Ver. 8, Software Development, Tokyo, Japan). A homology search was performed using the Genetyx homology search program with SWISS-PLOT and NBRF databases. The nucleotide sequence of *slp* has been submitted to DDBJ/EMBL/GenBank DNA database under accession number AB001876.

### 2.3. Expression and purification of recombinant Slp

Previously we reported that *E. coli* containing pKM211, which encoded the C-terminal truncated *slp* in the same direction as the *lac* promoter in plasmid pUC18, produced a high level of truncated protein [11]. Therefore, we subcloned the 4.3-kb pKM201 insert into pHSG399 and constructed plasmid pKM304 which has the *slp* gene downstream

from the *lac* promoter. The *E. coli* containing the constructed plasmid was cultured in LB broth containing chrolamphenicol. When the culture reached an optical density of 0.4 at 660 nm, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce the *lac* promoter. The culture was grown for an additional 3 h at 37°C and the cells were harvested by centrifugation. The cells were suspended in 10 mM phosphate buffer (pH 7.4) containing 0.5 mM phenylmethylsulfonyl fluoride. The cells were then destroyed by sonication using an ultrasonic sonicator (UR-200P; Tomy, Tokyo, Japan) with glass beads. The sonicated extracts were clarified by centrifugation at $100\,000 \times g$ for 30 min. Slp was further purified by ion-exchange chromatography using a DE-52 cellulose (Whatman, Maidestone, UK) column which was equilibrated with 10 mM phosphate buffer (pH 7.4). As reported for the purification of the native Slp [5], the recombinant Slp also was not adsorbed to the column using these conditions. Eluted fractions were pooled and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and Western blot. The pooled fractions containing Slp were dialyzed extensively in phosphate-buffered saline (PBS, pH 7.2) and were confirmed as a single-band on SDS-PAGE. The purified recombinant Slp was also confirmed to be free of lipopolysaccharide (LPS) by the *Limulus* amoebocyte lysate gel test (Limulus J test; Wako Pure Chemical, Osaka, Japan).

### 2.4. Characterization of native Slp and extraction proteins from culture supernatants

Extraction of the native Slp from *C. rectus* cells by acid treatment and the purification procedure was described previously [5]. The purified protein was hydrolyzed in 6 N HCl at 110°C for 14 h, and the amino acid composition was determined by high-performance liquid chromatography (HPLC) (CCP and 8000; Toso, Tokyo, Japan). N-Terminal amino acid sequence analysis was done with a model 477A sequencer (Applied Biosystems) equipped with a model 120A phenylthiohydantoin analyzer. The isoelectric focusing point (p*I*) was determined by gel electrophoresis with the Ampholine PAG plate (pH 3.5–9.5, Pharmacia Biotech., Uppsala, Sweden) following the manufacturer's instructions.

The high-molecular weight proteins were also isolated from the culture supernatants of *C. rectus* ATCC 33238 as follows. Bacteria were cultured in dialysate medium of Todd-Hewitt broth and the culture supernatants were filtered through a 0.2-μm nitrocellulose filter. The proteins in the filtrate were further concentrated by 65% saturated ammonium sulfate precipitation and analyzed by Western blot with polyclonal antiserum to Slp from *C. rectus* ATCC 33238 [5].

### 2.5. Cytotoxicity assay

Cells of the human leukemia cell line HL-60 were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). The culture was maintained at 37°C in a 5% $CO_2$ atmosphere. Human peripheral white blood cells (polymorphonuclear neutrophils (PMNs), monocytes and lymphocytes) were obtained from a healthy volunteer by methods described previously [13]. All cells were suspended in RPMI 1640 medium supplemented with 10% FCS after being washed with PBS (−) (Nissui, Tokyo, Japan). The HL-60 and human peripheral blood cells were seeded in a 96-well plate at concentrations of $1.0 \times 10^6$ and $2.0 \times 10^5$ cells $ml^{-1}$, respectively, and various concentrations of the native or recombinant Slp were added. Control wells contained PBS only. The plates were incubated at 37°C and aliquots of cells were removed at certain intervals up to 60 h. The viability of cells was determined by Trypan blue exclusion with a hemacytometer under a light microscope.

## 3. Results and discussion

### 3.1. Analysis of nucleotide and deduced amino acid sequence

The 3.4-kb pKM201 insert was found to contain only a single large open reading frame (ORF) of 4086 nucleotides coding for 1361 amino acids. The N-terminal sequence of the predicted amino acid sequence perfectly matched that from the N-terminal sequence analysis of the native Slp except for the initial methionine, indicating that Slp was not processed by signal sequence cleavage. The resulting mature protein consisted of 1360 amino acids with a

Table 1
The domains including the glycine-rich repeats from Slp and RTX-family toxins

| | Slp | Hly II | LktA |
|---|---|---|---|
| 1 | ItAGnGKNk | IiGSqFNDi | IiGSnLRDk |
| 2 | ItGSaRDNk | FkGSqFDDv | FyGSkFNDv |
| 3 | IiAGnGGNt | FhGGnGVDt | FhGHdGDDl |
| 4 | IhADaGNNn | IdGNdGDDh | IyGYdGDDr |
| 5 | IkLGnGDDy | LfGGaGDDv | LyGDnGNDe |
| 6 | VtAKdGNNv | IdGGnGNNf | IhGGqGNDk |
| 7 | VeFGnGRDk | LvGGtGNDi | LyGGaGNDr |
| 8 | | IsGGkDNDi | LfGEyGNNy |
| 9 | | | LdGGeGDDh |
| 10 | | | LeGGnGSDi |
| 11 | | | LrGGsGNDk |
| 12 | | | LfGNqGDDl |
| 13 | | | LdGGeGDDq |
| 14 | | | LaGGeGNDi |

Putative glycine-rich repeats in Slp (this study), Hly II [16] and LktA [17] are listed according to the consensus sequence I/L-X-G-G-X-G-N/D-D-X. The arbitrary residues are indicated as small letters and the residues which match the consensus sequence are underlined.

predicted molecular mass of 144 771 Da. This molecular mass was in accord with that of the native Slp which has an apparent size of 150 kDa as determined by SDS-PAGE. The nucleotide sequence upstream from *slp* showed no consensus promoter sequences but a putative ribosomal binding site, AAGGAG, was identified 9 bp upstream from the initial ATG codon. The ORF terminated with two adjacent stop codons, TAA and TGA. At the 3′ non-coding region, a sequence consistent with a rho-independent type transcription terminator was found which included a stem loop of 25 bp with a stem of 11 bp followed by an AU-rich stretch.

The amino acid composition deduced from the nucleotide sequence agreed with that of the purified native Slp (data not shown). The calculated p*I* of 4.85, however, was much lower than those determined from the native Slp; 7.0–7.3 in this study, 6.8–7.2 reported by Kobayashi et al. [4] and 5.6–5.8 reported by Nitta et al. [6]. Nitta et al. [6] purified Slp from several strains of *C. rectus* and determined the N-terminal sequences of cyanogen bromide (CNBr)-treated peptides. Two sequences determined from *C. rectus* ATCC 33238 completely matched the corresponding regions in the deduced amino acid sequence of Slp. Furthermore, one sequence from a clinical isolate and one from a non-human primate isolate also aligned with the Slp sequence with only minor differences.

### 3.2. Comparison of Slp with other bacterial proteins

A protein homology search showed that several bacterial proteins had homology to *C. rectus* Slp. However the *C. fetus* S-layer protein was the only S-layer protein that showed sequence homology to *C. rectus* Slp. This similarity is reasonable considering that both species are classified in the same genus, *Campylobacter*. The lack of N-terminal signal sequence is also a common feature of both S-layer proteins, although the initial methionine is not cleaved in the *C. fetus* S-layer protein. The genes encoding the S-layer proteins of *C. fetus* are multiple homologs. Four genes (*sapA*, *sapA1*, *sapA2* and *sapB*) have been cloned and sequenced from two serotypes, A and B. The amino acid sequence alignment revealed that Slp had 18.1% identity with the *sapA* product and 19.2% identity with the *sapB* product. In the *C. fetus* S-layer proteins, the N-terminal 184 amino acids are specific to the serotype and are thought to be critical for LPS binding specificity [14]. Interestingly, the homology of the *C. rectus* Slp to the *C. fetus* S-layer proteins was greatest at the N-termini. Within the first 184 amino acid residues, the

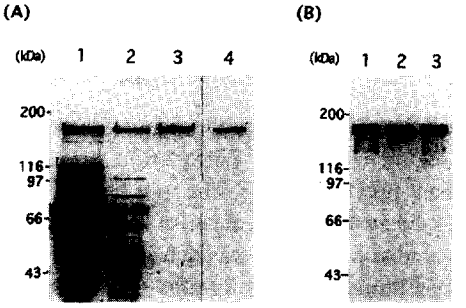


Fig. 1. SDS-PAGE and Western blot profiles of the purified recombinant Slp and the proteins from culture supernatants. (A) Coomassie brilliant blue-stained SDS-PAGE gel of *E. coli* XL1-Blue containing pKM304 lysate after induction with IPTG (lane 1), the supernatant after 100 000 × *g* ultracentrifugation (lane 2), the purified recombinant Slp (lane 3) and Western blot of the purified recombinant Slp (lane 4). (B) Western blot profiles of the native Slp (lane 1), the high-molecular weight proteins isolated from culture supernatants of *C. rectus* ATCC 33238 (lane 2) and the sonicated extracts of *C. rectus* ATCC 33238 (lane 3).

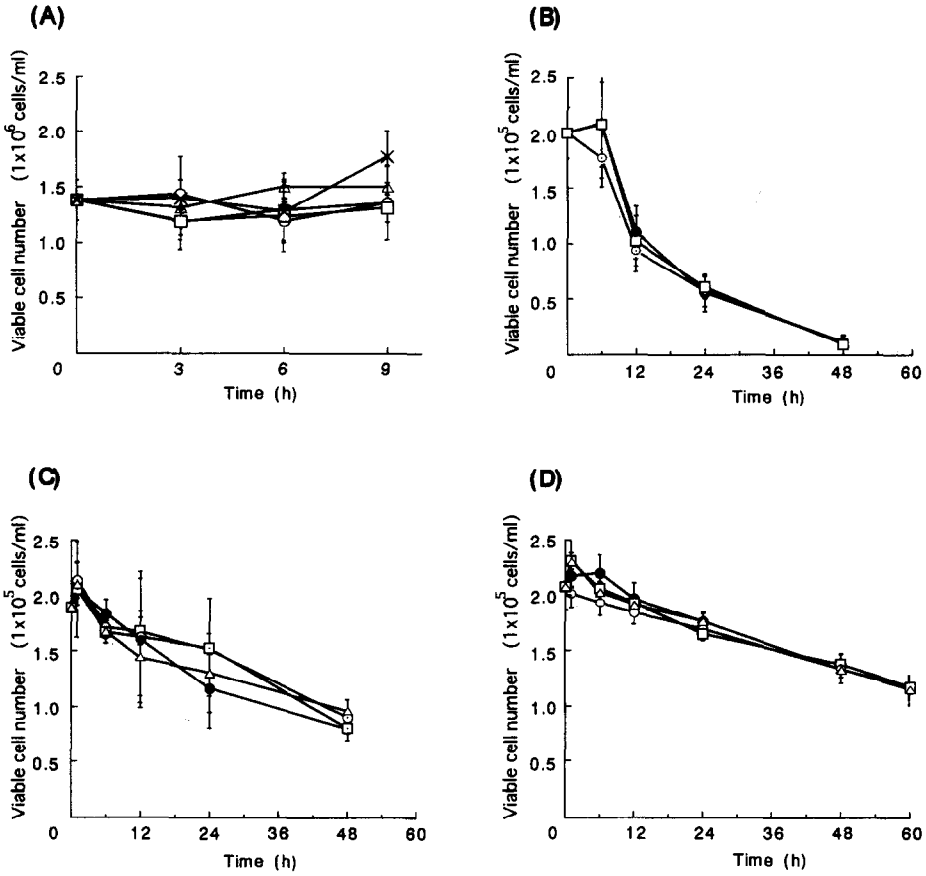


Fig. 2. Time course of viable cell numbers of various cells exposed to native or recombinant Slp. The means and standard deviations of three independent experiments are presented. (A) HL-60: ●, 0 μg; △, 0.26 μg; □, 0.52 μg; ○, 1.04 μg per $1.0\times10^5$ cells of recombinant Slp or (×), 1.04 μg per $1.0\times10^5$ cells of native Slp were used. (B) PMNs: ●, 0 μg; □, 0.5 μg; ○, 10 μg per $1.0\times10^5$ cells of Slp were used. (C) Monocytes and (D) lymphocytes: ●, 0 μg; △, 0.125 μg; □, 1.0 μg; ○, 10 μg per $1.0\times10^5$ cells of Slp were used.

*C. rectus* Slp sequence had 32.2% identity and 43.3% similarity to the sequence of the *sapA* product, and 32.7% identity and 52.2% similarity to that of the *sapB* product. These similarities suggest that the N-terminus of Slp acts as an anchor to the cell surface components such as LPS. This hypothesis is consistent with the three-dimensional structure of the S-layer in that the N-terminal sequence forms the innermost part of the molecule [15].

Several pore-forming exotoxins from Gram-negative bacteria, the RTX toxins, such as *A. pleuropneumoniae* cytotoxin II [16] and *A. actinomycetemcomitans* leukotoxin [17], exhibited homology to one-third of the C-terminus of the Slp sequence, especially in the stretch of amino acids 992 to 1055. This region contained seven glycine-rich repeats, I/L-X-G-G-X-G-N/D-D-X (X represents an arbitrary residue), with minor differences (Table 1). Two other motifs separated by 12 amino acids were also found in the C-terminal part (1165–1194th amino acids). The glycine-rich repeats in the RTX toxins are suspected to be involved in calcium binding [16,17]. Many S-layer proteins are known to require calcium ions for their assembly and attachment to the outer membrane of bacteria [14,18]. Therefore, it is possible that this region in Slp plays a role in calcium binding. Recently Baumann et al. [19] revealed the three-dimensional structure of an alkaline protease from *Pseudomonas aeruginosa* which has eight glycine-rich repeats in the C-terminus. From their data, the glycine-rich repeats are organized in a novel parallel β-roll, which is a new

type of calcium binding structure. Table 1 shows the glycine-rich repeats in Slp and other RTX toxins in the same alignment. Although these regions shared similarity with each other, there were critical differences between the amino acids of Slp and those of other RTX toxins in the repeats. The most important difference is the frequent appearance of Asn at the eighth position in Slp instead of Asp in other RTX toxins. The rate of appearance of Gly at the third position is also low in Slp, which has Ala at the respective position. In the reported three-dimensional structure [20], the calcium ion is bound between a pair of loops in the β-roll, and makes a bridge to the pair of carboxyl side chains of each Asp in the eighth position. The requirement of the Gly at third position is also critical for keeping the calcium ion coordination. In this regard, the glycine-rich repeat-like sequence in Slp may not serve in calcium binding, or the affinity of binding and the number of calcium ions bound may be much smaller than the other RTX toxins. Another possibility is that two other motifs may serve in calcium binding. Further studies are needed to define the function of the motif in Slp.

### 3.3. *Purification of recombinant Slp and its cytotoxicity*

Recombinant Slp was expressed as a soluble protein in *E. coli* cells under the control of the *lac* promoter in the pHSG plasmid. This allowed more mild purification procedures compared with the acid extraction used to treat *C. rectus* cells at pH 2.0. The subsequent purification procedures resulted in a single band of approximately 150 kDa by SDS-PAGE and Western blot analysis (Fig. 1A). Gillespie et al. [9,10] described an extracellular toxin isolated from culture supernatants of *C. rectus*. Its N-terminal sequence matched perfectly with that of Slp described here, and the amino acid compositions resembled each other in the two proteins. The molecular weight reported by Gillespie's study, 104 kDa, could not be evaluated in relation to our data, because they used relatively low molecular weight markers. We detected the 150-kDa protein in the culture supernatants of *C. rectus* ATCC 33238 by Western blot analysis (Fig. 1B). This strongly suggested that the cytotoxin reported by Gillespie et al. was Slp shed from the cell surface into the culture media. Therefore, we evaluated the cytotoxicity of native or recombinant Slp against HL-60 and various human blood cells. Fig. 2A shows the viability of HL-60 cells after exposure to native or recombinant Slp. In contrast to the results of Gillespie et al., the viability of the HL-60 cells did not change during the experimental period. Fig. 2B–D shows the results from PMNs, monocytes and lymphocytes, which were exposed to a higher concentration of recombinant Slp for a longer period. Even under these conditions the viability of the cells did not change compared with the control. These results revealed that neither native nor recombinant Slp showed the cytotoxicity against the eukaryotic cells tested.

Considering that the homology to RTX toxins had been shown in other S-layer proteins [18], it is feasible that the glycine-rich repeats assist in the assembly of the S-layer protein via their calcium binding ability. In addition to the possible role of calcium binding, the glycine-rich repeats may play an important role in protein translocation. In this regard, it is interesting to note that several exported proteins which do not contain the consensus signal sequence of exported proteins of Gram-negative bacteria, including S-layer proteins and RTX toxins, have glycine-rich repeats.

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