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Characterization, Identification, and Cloning of the S-Layer Protein from *Cytophaga* sp.

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Abstracts We characterized, identified, and cloned a major protein which comprised 16% of the total proteins from *Cytophaga* sp. cell lysate. After French pressing, the fraction of cell envelope was treated with 0.2% Triton X-100 to remove cell membranes. Subsequent SDS-PAGE analysis of the Triton X-100-insoluble cell wall revealed a protein of 120 kDa with a pI of 5.4, which was identified by gold immunostaining as the surface (S)-layer protein of this soil bacterium. The nucleotide sequence of the cloned S-layer protein gene (*slp*) encoding this protein consisted of 3144 nucleotides with an ORF for 1047 amino acids, which included a typical 32-amino acid leader peptide sequence. Amino acid sequence alignment revealed 29–48% similarity between this protein and the S-layer proteins from other prokaryotic organisms. The 120-kDa protein from the *Cytophaga* sp. cell lysate has been characterized as a member of the S-layer proteins, and the *slp* gene was cloned and expressed in *Escherichia coli*. *E. coli* harboring the plasmid containing the 600- or 800-bp DNA fragment upstream of the initiation codon of the *slp* gene, in the presence of the reporter gene *rsda* (raw starch digesting amylase), showed amylase activity in starch containing plate. The putative promoter region of *slp* located 600 bp upstream of the initiation codon might be used for foreign gene expression.

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Introduction

The bacteria *Cytophaga* usually secrete hydrolytic enzymes which digest biopolymers, such as xylan, cellulose, and collagen [5, 11, 19]. A *Cytophaga* sp. isolated from the soil of a corn field in Taichung (Taiwan), which produces enzymes digesting raw starch, is a fast-growing bacterium, with a generation time of ca. 20 min at 37°C [2]. A major protein with a molecular weight of 120 kDa was stained on the electrophoresis gel of the *Cytophaga* sp. cell lysate. In view of its large quantity, it is reasonable to assume that this protein must have some important physiological functions for this bacterium.

A surface (S)-layer protein with an apparent molecular weight of 40 to 200 kDa has been identified in many species of all major groups of bacteria. Constituting between 5% and 15% of the total proteins of cells, it serves as an interface between the bacteria and the environment. Proposed functions of these S-layer proteins for bacteria vary considerably, such as serving as molecular sieves, protective coats, virulence factors [17, 18], a toxic protein to a coleopteran pest [14], agents helping cell adhesion [23] and cell wall attachment of extracellular enzymes [4,17], and mediators of attachment to host tissues [20]. In recent years *slp* genes of many bacterial species have been successfully cloned and sequenced [1, 6, 10, 13, 18]. In the current study, the 120 kDa protein from the *Cytophaga* sp. cell lysate was characterized and identified as the S-layer protein, and subsequently the *slp* gene was cloned.

Materials and Methods

SDS-PAGE and 2-D PAGE *Cytophaga* sp., isolated from soil and characterized in previous studies [2], was

cultivated and harvested by centrifugation. The cell lysate was analyzed by 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and two-dimensional (2-D) PAGE, respectively. After electrophoresis, the gel was stained with Coomassie Blue, and the protein of interest of 2-D PAGE was estimated using a Bioimage Analyzer (Bio-Rad, U.S.A.).

Purification and characterization of the 120-kDa protein The native 120-kDa protein of *Cytophaga* sp. was isolated by the method of Boot et al. [1]. After dialysis of the 4.0 M guanidine hydrochloride-soluble fraction against RO water at 4°C, the supernatant was loaded onto a Polybuffer Exchanger PBE-94 column (1 × 15 cm; Pharmacia). Fractions containing the 120-kDa protein were confirmed with the antiserum and pooled for study of the self-assembly of S-layer protein subunits from *Cytophaga* sp.

Preparation of antibodies against the 120-kDa protein A portion of the 120-kDa protein in the dialyzed supernatant described above was subjected to preparative SDS-PAGE electrophoresis, and the major protein band was excised and eluted from the gel with an electroeluter. Antiserum of the 120-kDa protein was prepared and purified by the method of Jeang et al. [7].

Cell fractionation Washed cells were resuspended in HEPES-Ca^{2+} buffer and passed through a French pressure cell (SLM; Amicon, Rochester, NY, USA) at 16,000 lb/in^2. The Triton X-100-insoluble cell wall was resuspended in electrophoresis sample buffer and analyzed by SDS-PAGE [9].

Gold immunoelectron microscopy Cells of *Cytophaga* sp. growing to log phase were absorbed onto a copper grid previously made hydrophilic by glow discharge. After blocking with 1% BSA in PBS, diluted antiserum of the 120-kDa protein was added to the grid and then reacted with gold-labeled goat anti-rat IgG for 30 min in the dark [8]. The sample was stained with uranyl acetate and examined with an electron microscope operating at 90 kV.

Specimen preparation and transmission electron microscopy Methods of specimen preparation and electron microscopy of the S-layer protein developed by Lupas et al. [12] were followed. The sample (6–10 μg) purified with Polybuffer exchanger PBE 94 was adsorbed onto a glow-discharged, carbon-coated copper grid, stained with 2% uranyl acetate (pH 4.0), and examined with a transmission electron microscope.

Cloning and expression of the slp gene Cloning procedures were performed according to Stratagene protocols using the predigested λZap II/*Eco*RI/CIAP cloning kit and Gigapack II Gold Packaging Extract (Stratagene).The λ library of the *Cytophaga* sp. genome was screened according to Sambrook et al. [15] with antiserum of the S-layer protein as the first antiserum and anti-rat immunoglobulin G with alkaline phosphatase conjugate (Promega) as the second antiserum. The S-layer protein of *Cytophaga* sp. expressed in recombinant *Escherichia coli* XL1-Blue was detected using the same procedure. For expression in *E. coli*, the *slp* gene was generated by PCR using *Cytophaga* sp. chromosomal DNA as template, the forward primer 5′-ATATATGGATCCAGGAGGCATC ATTTATGGCATCTGATGCTAC-3′ (*Bam*HI), and the backward primer 5′-ATATATCCGCGGTCTCTATT ATTTAGATACAGATAC-3′ (*Sac*II). After the selected clones were verified by DNA sequencing, *E. coli* XL1-Blue harboring pKS*slp* were cultivated at 37°C in LB medium and the cell lysate were analyzed by 8% SDS-PAGE and Western blotting.

N-Terminal amino acid sequencing The N-terminal amino acid sequences of purified S-layer protein of *Cytophaga* sp. were determined with a protein sequencer (Model 476A, Applied Biosystems).

Nucleotide sequence analysis Nucleotide sequencing of the *slp* gene was performed by the dideoxy chain termination method of Sanger et al. [16], using an S2 Bio-Rad gel electrophoresis system with Sequenase Version 2.0 kit (United States Biochemical) and [α-^{35}S]dATP. Both recombinant plasmids and PCR products were used as templates in sequencing.

Southern hybridization and inverse PCR Inverse PCR was used to determine the unknown sequence of 5′-end nucleotides of the *slp* gene. Hybridization was carried out with 3′-end Dig-labeled oligonucleotide probe 5′-TCGCACCGCTTGCAATGTCG-3′. Positive fragments were self-ligated into circular DNA and then PCR amplified with the upstream and downstream primers 5′-TCG CACCGCTTGCAATGTCG-3′ (located at 1328–1309) and 5′-ATATGCTTTAGATTCAAAAGG-3′ (located at 3142–3163), respectively, to obtain the flanking sequences of the PCR products.

Putative promoter region of the slp gene In order to search the putative promoter region, three different DNA fragments, i.e., 200, 600, and 800 bp upstream of the initiation codon of *slp*, were ligated with the reporter gene *rsda* from *Cytophaga* sp. [7], and their expression in *E. coli* XL1-Blue was detected by iodine staining for amylase activity. The 800-bp DNA fragment was generated by PCR using the following upstream and downstream primers: 5′-AACTGAATTCAAAAGGGGCTTTATGAAA-3′ (*Eco*RI) and 5′-TAATAAAGAGCTCCCGTTTTGTACAAAGT-3′ (*Sac*I). The PCR product was digested with *Eco*RI and *Sac*I. The reporter gene *rsda* was generated by PCR using the upstream and downstream primers 5′-CACGAG-CTCAGGAGGCGAAAATGGCTGCAACAAATG-3′

(*Sac*I) and 5′-GCTGGATCCAAATTTACTGCTGAACCC-3′ (*Bam*HI), respectively, and *Cytophaga* sp. chromosomal DNA as template. These two PCR products were ligated with the plasmid pBR322 cut with *Eco*RI and *Bam*HI, giving rise to plasmids pBRS8*rsda*. The product amplified by PCR using pBRS8*rsda* as template and primers 5′-AACCGAATTCAATAAAAGCAGACAAGC-3′ (*Eco*RI) and 5′-GCTGGATCCAAATTTACTGCTGAACCC-3′ (*Bam*HI) consisted of the 600-bp DNA fragment and the reporter gene *rsda*. This PCR product was then ligated with the plasmid pBR322 cut with *Eco*RI and *Bam*HI, giving rise to plasmids pBRS6*rsda*. The plasmid pBRS8*rsda* was digested with *Eco*RI and *Hind*III to delete a fragment of ca. 600 bp, filled in with Klenow fragment, and self-ligated to give rise to pBRS2*rsda*, which contained the 200-bp DNA fragment. pBR*rsda*, as control, was constructed by inserting the *rsda* gene into pBR322. Another plasmid, pBRS8, was constructed by ligating the 800-bp DNA fragment into pBR322. These plasmids were transformed to competent cells of *E. coli* XL1-Blue by heat shock and the clones were cultivated in an LSA plate (LB agar plate containing 0.5% soluble starch) at 37°C. After 12 h of cultivation, 5 ml of iodine solution (containing 3% KI and 1.3% I_2) was added to detect the expression of amylase.

Results

Purification and Identification of the S-Layer Protein of *Cytophaga* sp.

The whole-cell lysate of *Cytophaga* sp. and culture medium were shown to have a major protein with a molecular weight of 120 kDa on SDS-PAGE (Fig. 1A). Both 4 M guanidine hydrochloride and 9 M urea were effective in the extraction of this 120-kDa protein (Fig. 1B). When cells of *Cytophaga* sp. were passed through a French press cell and the precipitate following centrifugation was treated with 0.2% Triton X-100 to remove cell membranes, this 120-kDa protein remained in the Triton X-100-insoluble cell wall (Fig. 1C). This protein from *Cytophaga* sp. cell lysate estimated after 2-D PAGE constituted 16% of the total cell proteins and had a pI of 5.4 (Fig. 2). The 120-kDa protein excised, electroeluted from the preparative SDS-PAGE gel, and subsequently resolved by analytical SDS-PAGE appeared as a homologous protein, which was subsequently used to raise antibodies.

Furthermore, gold immunoelectron microscopy indicated that this 120-kDa protein was located on the surface of *Cytophaga* sp. cells (Fig. 3). Based on its high content (16% of total cell lysate proteins), its association with the cell wall in cell fractionation, and the gold immunoelectron microscopic results, the 120-kDa protein was suggested to

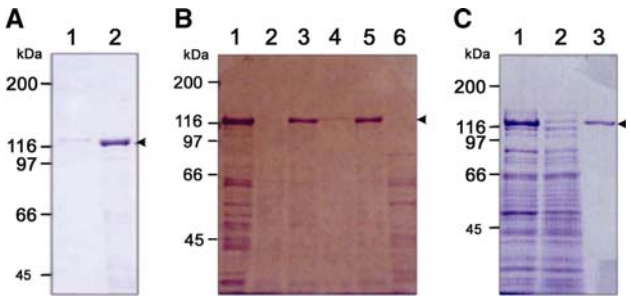

Fig. 1 SDS-PAGE analysis of the 120-kDa protein of *Cytophaga* sp. proteins. **A** Lane 1, medium; lane 2, cell lysate. **B** Cell extracts of *Cytophaga* sp. treated with different chaotropic reagents. Lane 1, cell lysate; lanes 2–6, cells treated with 2 M guanidine-HCl, 4 M guanidine-HCl, 6 M urea, 9 M urea, and 0.2% Triton X-100, respectively. **C** Cell fractionation of *Cytophaga* sp. Lane 1, cell lysate; lane 2, cytoplasmic fraction; lane 3, Triton X-100 insoluble cell wall

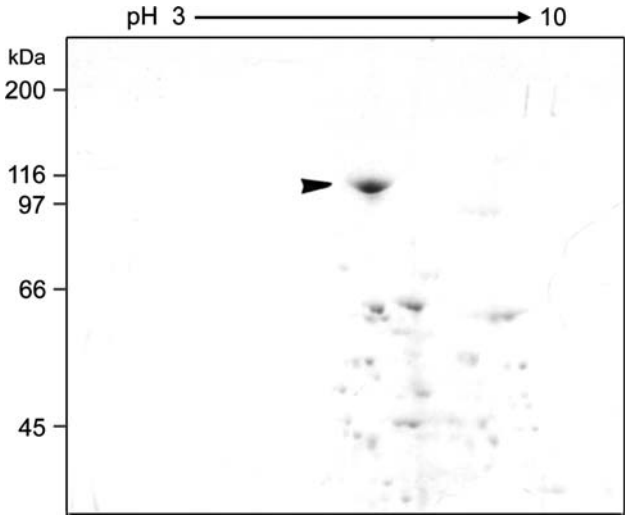

Fig. 2 Two-dimensional electrophoresis pattern of the cell lysate of *Cytophaga* sp

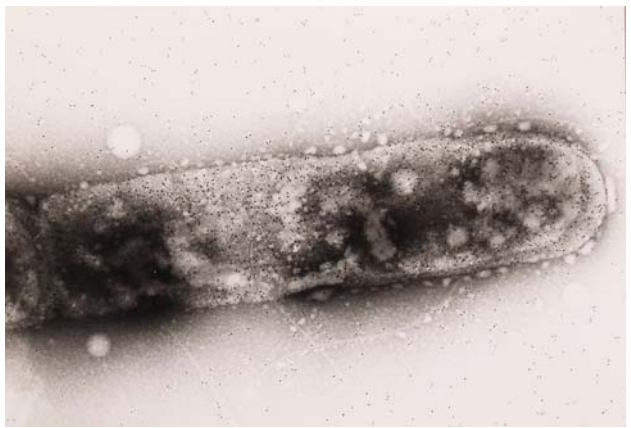

Fig. 3 Electron micrograph of *Cytophaga* sp. pretreated with antiserum specific for the 120-kDa protein and subsequently labeled with goat anti-rat IgG-colloidal gold (18 nm)

be the S-layer protein of *Cytophaga* sp. Isolated S-layer protein subunits from *Cytophaga* sp. have been found to maintain an important feature of this group of proteins, i.e., these protein subunits assembled into lattices of oblique (p2) symmetry (data not shown). Although hexagonal symmetry (p3, p6) of the S-layer is predominant among the archaeobacteria [21], the S-layer protein lattices can be of oblique (p1, p2), square (p4), or hexagonal (p3, p6) symmetry.

Cloning and Expression of the S-Layer Protein Gene *slp* from *Cytophaga* sp.

From the *Cytophaga* sp. genome library, six positive recombinant phages were obtained among 3×10^4 phages screened with antiserum against S-layer protein. Since the protein subsequently expressed in *E. coli* XL1-Blue had a molecular weight of 110 kDa (Fig. 4, lane 4), instead of 120 kDa, we presumed that the clone might contain a truncated version of the S-layer protein gene. A comparison of the N-terminal amino acid sequences of the recombinant protein and the S-layer protein from *Cytophaga* sp. revealed that the clone lacked the 5′-end nucleotide region. The sequence of this missing region was obtained by inverse PCR to give the complete *slp* sequence of 3144 nucleotides with an ORF encoding a 1047-amino acid protein. The sequence reported in this paper has been deposited in the GenBank database (accession number AF068060).

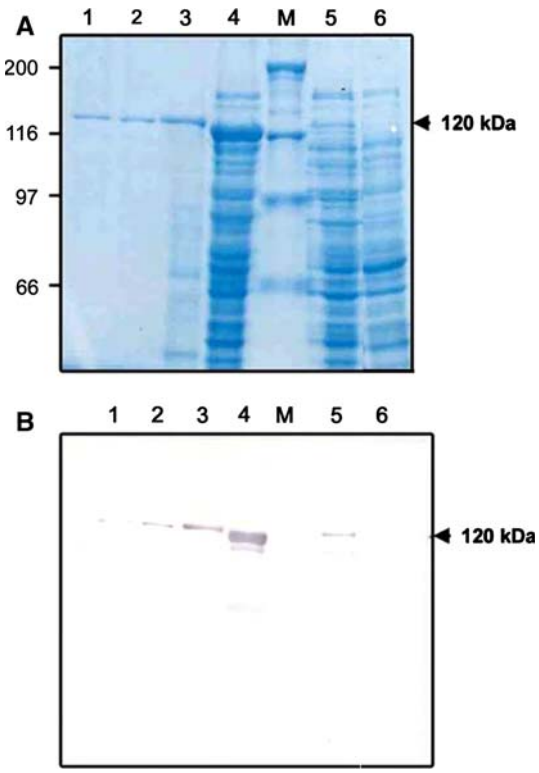

Fig. 4 SDS-PAGE and immunoblot analysis of S-layer protein of *Cytophaga* sp. and *E. coli* XL1-Blue harboring pKS*slp*. (**A**) Coomassie blue staining; (**B**) western blotting. Lane 1, S-layer protein of *Cytophaga* sp. purified by preparative SDS-PAGE; lane 2, S-layer protein of *Cytophaga* sp. purified with a chromatofocusing pBE 94 column; lane 3, S-layer protein of *Cytophaga* sp. extracted with 4 M guanidine hydrochloride; lane 4, cell lysate of *E. coli* XL1-Blue harboring a clone lacking 270 bp at the 5′-end of the *slp* gene; lane 5, pKS*slp*; lane 6, pKS. M: protein marker. Arrow indicates the position of the S-layer protein

For expression of the *Cytophaga* sp. *slp* gene in *E. coli* XL1-Blue, the PCR-amplified DNA fragment was digested with *Bam*HI and *Sac*II and inserted into the expression vector; and in order to carry out the translation, a methionine was added at the N-terminus of the recombinant S-layer protein without signal peptide. After SDS-PAGE and immunoblotting of the cell lysate of *E. coli* XL1-Blue harboring the plasmid pKS*slp*, a positive band with an apparent M_r of ~120 kDa appeared, corresponding to the purified S-layer protein from *Cytophaga* sp. (Fig. 4).

Nucleotide Sequence Analysis of the S-Layer Protein Gene of *Cytophaga* sp.

A putative ribosome binding site, GGGGAGGA, was located 9 bases upstream of the ATG start codon of this ORF. The deduced protein had a characteristic prokaryotic 32-amino acid signal sequence, consisting of a positive region ($MAYQPK^+SYR^+K^+$) followed by a hydrophobic region of 17 amino acids; and the latter is predicted to fold into an α-helix [3]ending with a proline. Following the fifth amino acid after proline, which was predicted as the cleavage site [24], there was a 20-amino acid sequence coinciding with the determined N-terminal sequence of purified S-layer protein from *Cytophaga* sp., i.e., ASDATGIYKDAVNYLIEKGI.

Putative Promoter Region of the *slp* Gene

E. coli XL1-Blue harboring the plasmid pBRS2*rsda* containing the 200-bp DNA fragment upstream of the initiation codon of the *slp* gene and the reporter gene *rsda* did not show any amylase activity in the LSA plate. Yet considerable amylase activity was observed for *E. coli* XL1-Blue with the plasmid containing either the 600- or the 800-bp DNA fragment, with the former showing somewhat higher expression of amylase (Fig. 5). However, the DNA fragment 200 bp upstream of the *slp* initiation codon could be the promoter region, but contained a repressor binding element, and that between 200 and 600 bp upstream of the *slp* initiation codon could be the regulatory region, which contained the enhancer binding element from a different angle. The 600-bp DNA fragment upstream of the initiation codon of *slp* gene could be used

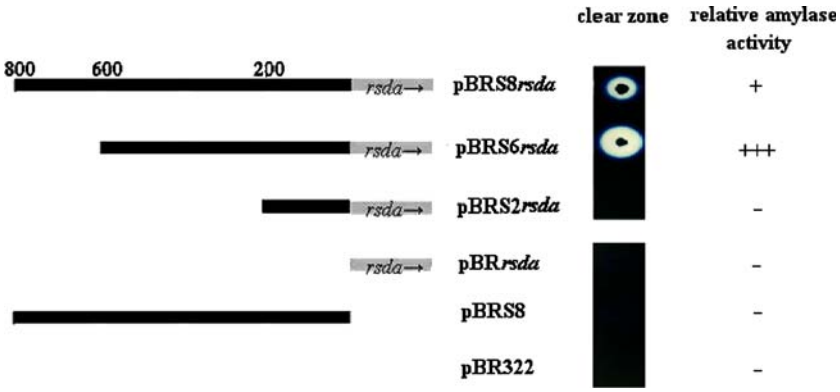

Fig. 5 Expression of several putative promoter regions of *slp* with reporter gene *rsda* during *E. coli* XL1-Blue growth. The size of the clear zone represents the relative amylase activity detected with iodine staining

as the potential promoter for foreign gene expression in *E. coli*.

Discussion

The content, molecular weight, and pI of the S-layer protein of *Cytophaga* sp. agreed in general with those of other S-layer proteins. The deduced *Cytophaga* sp. S-layer protein contained 41% hydrophobic amino acids, while the content of these amino acids in other S-layer proteins was between 40% and 60% [18]. Amino acid sequence alignment between this and other S-layer proteins revealed 29%–48% similarity, which is comparatively low for proteins grouped according to their physiological function. This low level of conservation of the primary structure of S-layer proteins could be related to their diverse functions [4, 14, 17, 18, 20, 23].

Motif scanning by Interpro SCanProsite (at the ExPASY Web site) revealed several unique features in the S-layer protein of *Cytophaga* sp. S-layer homologous motifs (SLH; Pfams accession no. PF00395) have been identified at amino acids 31–73, 92–132, and 151–191, which are known to play a role in the interaction of the S-layer protein with cell wall components. Two bacterial Ig-like groups are suggested to be located separately at amino acids 382–456 (Smart accession no. SM00635) and 505–595 (Pfams accession no. PF02368). In addition, an invasin/intimin cell-adhesion type signature (superfamily accession no. SSF49373) may be located at amino acids 506–599.

The 120-kDa protein from the *Cytophaga* sp. cell lysate has been characterized as a member of the S-layer proteins, which have been demonstrated in many micro-organisms with multiple biological functions; and the *slp* gene encoding this protein was cloned and expressed in *E. coli*.

Since *Cytophaga* sp. is a fast-growing bacterium, the S-layer protein must be synthesized in large quantity by the ribosome, translocated across the cytoplasmic membrane, periplasm, and outer membrane, and assembled into an array that becomes anchored to the cell surface of these Gram-negative bacteria [22]. In view of the fact that *Cytophaga* sp. has spent energy to produce such a large amount of S-layer protein (16% of total protein), the physiological functions of this protein must be of great importance and deserve detailed investigation.

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