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Characterization of Surface Layer Proteins in *Lactobacillus crispatus* Isolate ZJ001

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***Lactobacillus crispatus* (*L. crispatus*) ZJ001 is highly adhesive to epithelial cells and expresses S-layer proteins. In this study, S-layer genes were sequenced and expressed in *E. coli* to characterize the function of S-layer proteins with this particular strain. *L. crispatus* ZJ001 harbored two S-layer genes *slpA* and *slpB*, and only *slpA* gene was expressed in the bacterium, as revealed by RT–PCR and immunoassays. The mature SlpA showed 47% amino acid sequence identity to SlpB. The SlpA and SlpB of *L. crispatus* ZJ001 were highly homologous at the C-terminal region to other *Lactobacillus* S-layer proteins, but were substantially variable at N-terminal and middle regions. Electron microscopic analysis indicated that His-slpA expressed in *E. coli* was able to form a sheet-like structure similar to the natural S-layer, but His-slpB formed as disc-like structures. In the cell binding experiments, HeLa cells were able to bind to both recombinant His-slpA and His-slpB proteins to the extent similar to the natural S-layer. The cell binding domains remain mostly in the N-terminal regions in SlpA and SlpB, as shown by high binding of truncated peptides SlpA2-228 and SlpB2-249. Our results indicated that SlpA was active and high binding to HeLa cells, and that the *slpA* gene could be targeted to display foreign proteins on the bacterial surface of ZJ001 as a potential mucosal vaccine vector.**

Keywords: *Lactobacillus crispatus*, S-layer proteins, transcription, expression, binding

The proteinaceous surface layers (S-layers) are composed of subunits of single protein or glycoprotein covering the entire cell as the outermost envelope, with molecular masses ranging from 40 to 200 kDa [14]. The S-layer protein represents 10–15% of the total protein of the bacterial cells. The genes encoding the S-layer proteins are diverse, but their amino acid compositions are similar. S-layers have been considered to act as protective coats, cell shape determinants, traps of other molecules and ions, virulence factor (for pathogenic species), and adhesion sites for exoenzymes and host cells [2].

Lactobacilli, belonging to the commensal gastrointestinal and urogenital microbiota of human and animals, are thought to endow the host with several beneficial health effects. S-layer proteins in several species of the genus *Lactobacillus* were the smallest known proteins of the type ranging from 25 to 71 kDa in size [1]. They are highly basic (pI>9), in contrast with acidic S-layer proteins of other bacterial species [1]. Strains of *Lactobacillus helveticus* (*L. helveticus*) [7], *L. brevis* [12, 19], *L. acidophilus* [3, 4], *L. crispatus* [16], *L. amylovorus* [5], and *L. gallinarum* [10] have been found to possess S-layer proteins, whereas *L. acidophilus* group B strains (*L. johnsonii* and *L. gasseri*) do not appear to have an obvious S-layer [5, 13].

Only a few lactobacilli are known for the primary structures of their S-layer proteins [3, 16, 18, 19]. The predicted S-layer proteins among different lactobacillial species or even within the same lactobacillial species were considerably variable in the two-thirds region of the proteins towards the N-terminus, but conserved in the C-terminal one-third region [3, 14, 16, 19]. The S-layer proteins in several lactobacillial species were found to adhere to epithelial cells [9, 15] and mammalian extracellular matrix [11, 16, 18].

Our previous research showed that the S-layer proteins were involved in adherence of *L. crispatus* ZJ001 to HeLa cells [8]. In this study, we attempted to characterize the S-layer protein genes in this particular strain, and to examine the formation of S-layers and adhesion to HeLa cells of their expression products in *E. coli*.

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MATERIALS AND METHODS

Bacterial Strains and Culture Condition

L. crispatus ZJ001, isolated from pig intestines, was grown under static condition in de Man–Rogosa–Sharp (MRS) broth (Oxoid) at 37°C. *Escherichia coli* (*E. coli*) DH5α and BL21, used as hosts for cloning and expression of S-layer proteins, respectively, were routinely grown in Luria–Bertani medium (LB) (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, pH 7.2) containing ampicillin (50 μg/ml) or kanamycin (50 μg/ml) where appropriate.

Extraction of Chromosomal DNA

Chromosomal DNA was isolated from overnight cultures of *L. crispatus* ZJ001 in 10 ml of MRS broth at 37°C. After washing with 1 ml of 0.02 mol/l Tris-HCl (pH 8.0), the cells were pelleted and resuspended in 150 μl of 0.02 mol/l Tris-HCl (pH 8.0), 100 μl of 50 mg/ml lysozyme, and 250 μl of 24% (w/v) PEG20000, and incubated at 37°C for 2 h under constant shaking. Cells were collected by centrifugation at 6,000 ×*g* for 5 min and the pellet was resuspended in 600 μl of lysis buffer (0.5% sodium dodecyl sulfate, 5 mmol/l EDTA, 10 mmol/l Tris-HCl, pH 8.0). Twenty μl of proteinase K (20 mg/ml) was added and incubated for 3 h at 50°C. The mixture was extracted three times with phenol–chloroform–isoamyl alcohol (25:24:1). After ethanol precipitation, the DNA was dissolved in 100 μl of TE buffer containing 10 mg/ml RNase A.

PCR Amplification and Sequence Analysis of S-Layer Genes

S-layer genes were amplified using primers AB-1 and AB-2 (Table 1) and ligated into pMD18-T vector (Takara Biotechnology Co., Ltd, Dalian, China). The recombinant plasmid was sequenced by Invitrogen Biotechnology Co., Ltd, Shanghai, China. Nucleotide and amino sequences were aligned using a ClustalW algorithm (http://www.ebi.ac.uk/clustalw/). The GenBank accession numbers for S-layer protein genes used for comparison are as follows: AF001313 for *cbsA* and AF079365 for *cbsB* of *L. crispatus* JCM5810; X89375 and X89376 for *slpA* and *slpB* of *L. acidophilus* ATCC 4356; AF253043 and AF253044 for *slpNA* and *slpNB* of *L. crispatus* LMG12003; and AB110090 and AB110091 for *lbsA* and *lbsB* of *L. crispatus* MH315.

Transcription Analysis of *slpA* and *slpB* by Reverse Transcription PCR

Total RNA was isolated from lactobacillial cells using the UNIQ-10 column total RNA purification kit (Sangon Biological Engineering Technology & Services Co., Ltd., Shanghai, China), treated with RNase-free Deoxyribonuclease I (Takara Biotechnology Co., Ltd., Dalian, China), and reverse-transcribed into cDNA with M-MLV Reverse Transcriptase (Promega) using oligonucleotides AB-3 (Table 1), followed by PCR using gene-specific primers A3 and A4 for *slpA*, B3 and B4 for *slpB*, and AB-1 and AB-3 for both *slpA* and *slpB*. The amplicons were analyzed by agarose gel electrophoresis.

Expression and Purification of SlpA and SlpB Peptides in *E. coli* BL21

The mature and truncated protein genes of the S-layer were amplified from ZJ001 chromosomal DNA with the following primers listed in Table 1: A1 and A2 for *slpA*, A3 and A4 for *slpA*2-228, B1 and B2 for *slpB*, and B3 and B4 for *slpB*2-249. The amplified fragments were then cloned into pET30(a) as BamHI/XhoI fragments using the sites added to the 5' ends of the primers (underlined). Recombinant plasmids were transformed into *E. coli* BL21 (DE3) and protein expression was induced with 1 mmol isopropyl-β-D-thiogalactopyranoside (IPTG). The His-tagged S-layer peptides were purified by Ni–NTA agarose (Invitrogen) and quantified by the Bradford method.

Production of the Polyclonal Antibody

The purified proteins were emulsified with equal volume of Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FIA) (Sigma, St. Louis, MO, U.S.A.) and administered to specific pathogen-free (SPF) male rabbits (The Laboratory Animal Center, Zhejiang University of Traditional Chinese Medicine, Hangzhou, China) *via* the subcutaneous route twice with a 2-week interval between immunizations. Blood samples were collected 2 weeks after booster immunization for serum preparation. The antibody titer was tested by ELISA against the immunogen. Normal rabbit serum was obtained from nonimmunized rabbit of the same lot.

Western Blot Analysis

The protein samples were resolved by 12% SDS–PAGE and transferred to nitrocellulose membranes (Waterman). The blot was then blocked with 5% nonfat dry milk and incubated with primary antibodies, followed by incubation with goat anti-rabbit IgG-conjugated horseradish peroxidase (HRP). Immunoreactive bands were visualized and captured using the image analyzer.

Indirect ELISA Assay

Nunc Maxisorb polyvinyl wells (Fisher Scientific AG, Wohlen, Switzerland) were coated overnight at 4°C with 16 μg of purified recombinant proteins. Nonspecific binding was quenched with 5%

Table 1. Primers used in PCR or RT–PCR.

Primers	Nucleotide sequence (5'-3')	Enzyme site
AB-1	CCACATGAAGAAAAATTTAAGAAT	
AB-2	TTACTGTTCGCCTTAACTA	
AB-3	CTTGTTAGCACGCTTCTTTG	
A1	AAGGATCCATGGCAAGCTCAAGTGCTG	BamHI
A2	GCCTCGAGTTAAAAGTTTGAAACCTTTAC	XhoI
A3	AAGGATCCATGAGCTCAAGTGCTGTTCA	BamHI
A4	CGCTCGAGTTAGTTGTTGGTGTATGAA	XhoI
B1	AAGGATCCATGGCTGACTCTACTGCAAC	BamHI
B2	GCCTCGAGTTAAAAGTTTGAAACCTTTAC	XhoI
B3	AAGGATCCATGGACTCTACTGCAACTA	BamHI
B4	CCCTCGAGTTATTCAACATCTGACTTA	XhoI

(w/v) nonfat milk powder, and the plate was washed three times with PBS–0.05% Tween 20, followed by incubation with the first antibody. After the washing and further incubation with goat anti-rabbit IgG conjugated to HRP, the enzymatic activity was revealed with *O*-phenylenediamine at 492 nm with a SpectraMax M2 microplate reader (Molecular Devices Corporation, Sunnyvale, CA, U.S.A.).

Transmission Electron Microscopy

Purified proteins were dialyzed against ddH_2O and negative stained with 1% (w/v) phosphotungstate (pH 7.3). The electron micrographs were obtained with a JEM 1230 transmission electron microscope operating at 80 kV (Japan Electron Optics Laboratory Co., Ltd., Mitaka, Tokyo, Japan).

Assays of HeLa Cells Binding to S-Layer Proteins

A piece of nitrocellulose membrane was dissolved in methanol, spread in wells of 24-well plates, and dried in a sterile cabinet. A 0.5 ml volume of proteins (100 μg/ml) was added and incubated for 2 h at 37°C. Bovine serum albumin (100 μg/ml) was used as negative control. After three washes with PBS, 0.5 ml of HeLa cells (1×10^6/ml) in DMEM was added and incubated at 37°C, 5% CO_2 for 24 h. Wells were then washed three times and the cells detached by repeated pipetting. Cell detachment was verified by microscopic examination. The cell suspensions in PBS were brought to a total volume of 1 ml for enumeration on a hemocytometer chamber. Each protein was tested in triplicate wells. Binding differences among these proteins were analyzed by Tukey's HSD test.

GenBank Accession Number

The GenBank accession number of the *slp* locus containing both *slpA* and *slpB*, reported in this paper, is DQ923618.

Results

Sequence Analysis of *slp* Genes

Two S-layer genes of *L. crispatus* ZJ001, *slpA* and *slpB*, were amplified by PCR using AB-1 and AB-2 as primers. Two genes were distinguished by digestion with PstI, because *slpB* had a PstI enzyme site that was absent in *slpA*. Both genes were cloned and sequenced.

The *slpA* gene is 1,329 bp in length and encodes a polypeptide of 443 amino acids. A signal sequence of 30 amino acids (MKKNLRIVSAAAAALLAVAPVAASAVS VNA) was found at the N-terminus. The molecular mass of the mature SlpA was calculated to be 44,310 Da and the isoelectric point is 9.57 (Table 2). The ORF of *slpB* is 1,386 bp long and encodes a protein of 461 amino acids, and the mature SlpB has a theoretical molecular mass of 45,115 Da and an isoelectric point of 9.57 (Table 2). The *slpB* sequence contains two PstI sites, CTGCAG, at 701–706 bp and 764–769 bp. The amino acid compositions of mature SlpA and SlpB proteins are similar (Table 2). They have typical features of S-layer proteins: a high content of hydrophobic amino acid residues (31.8% in SlpA and 32.0% in SlpB) as well as of charged residues (22.8% in SlpA and 20.4% in SlpB) and polar amino acids (39.1% in SlpA and 41.5% in SlpB), and absence of cysteine residues.

There is 47% similarity between mature SpA and SlpB from *L. crispatus* ZJ001 in their amino acid sequences (Fig. 1), with high identity (86.9%) in the C-terminal region but substantial variability in the N-terminal and middle parts. Of the individual S-layer proteins, the SlpA in *L. crispatus* ZJ001 exhibited highest identity (99%) to LbsB, which was not expressed in *L. crispatus* MH315, and SlpB showed highest identity at 56% to slpA of *L. acidophilus* ATCC 4356.

Only *slpA* is Transcribed in *L. crispatus* ZJ001

To investigate which of the *slp* genes was actively transcribed, total RNA from ZJ001 cells was extracted and the *slpA* and *slpB* transcription was evaluated by RT–PCR. The band with an apparent size of about 1,100 bp was amplified when primers AB-1 and AB-3 were used (Fig. 2, lane 2). The band with an apparent size of 700 bp was

Table 2. Major physicochemical characteristics of *Lactobacillus* S-layer proteins.

	Strains									
	L. crispatus ZJ001		*L. crispatus* JCM5810		*L. crispatus* MH315		*L. crispatus* LMG12003		*L. acidophilus* ATCC 4356	
	SlpA	SlpB	CbsA	CbsB	LbsA	LbsB	SlpNA	SlpNB	SlpA	SlpB
Molecular mass (Da)	44,310	45,115	43,909	45,599	45,889	43,907	42,626	44,235	43,705	44,882
pI	9.57	9.57	9.63	9.43	9.48	9.66	9.40	9.50	9.53	9.44
% Identity (slpA-slpB)	47		44		42		42		54	
	Animo acid composition (%)									
Polar amino acids	39.08	41.53	37.32	38.32	41.28	38.59	38.32	38.63	40.19	39.20
Hydrophobic amino acids	31.80	32.02	31.95	30.77	29.82	31.80	31.07	32.52	34.24	33.33
Charged residues	22.82	20.39	22.93	24.48	25.23	23.06	24.30	23.47	23.49	22.30
Cysteine residues	0	0	0	0	0	0	0	0	0	0

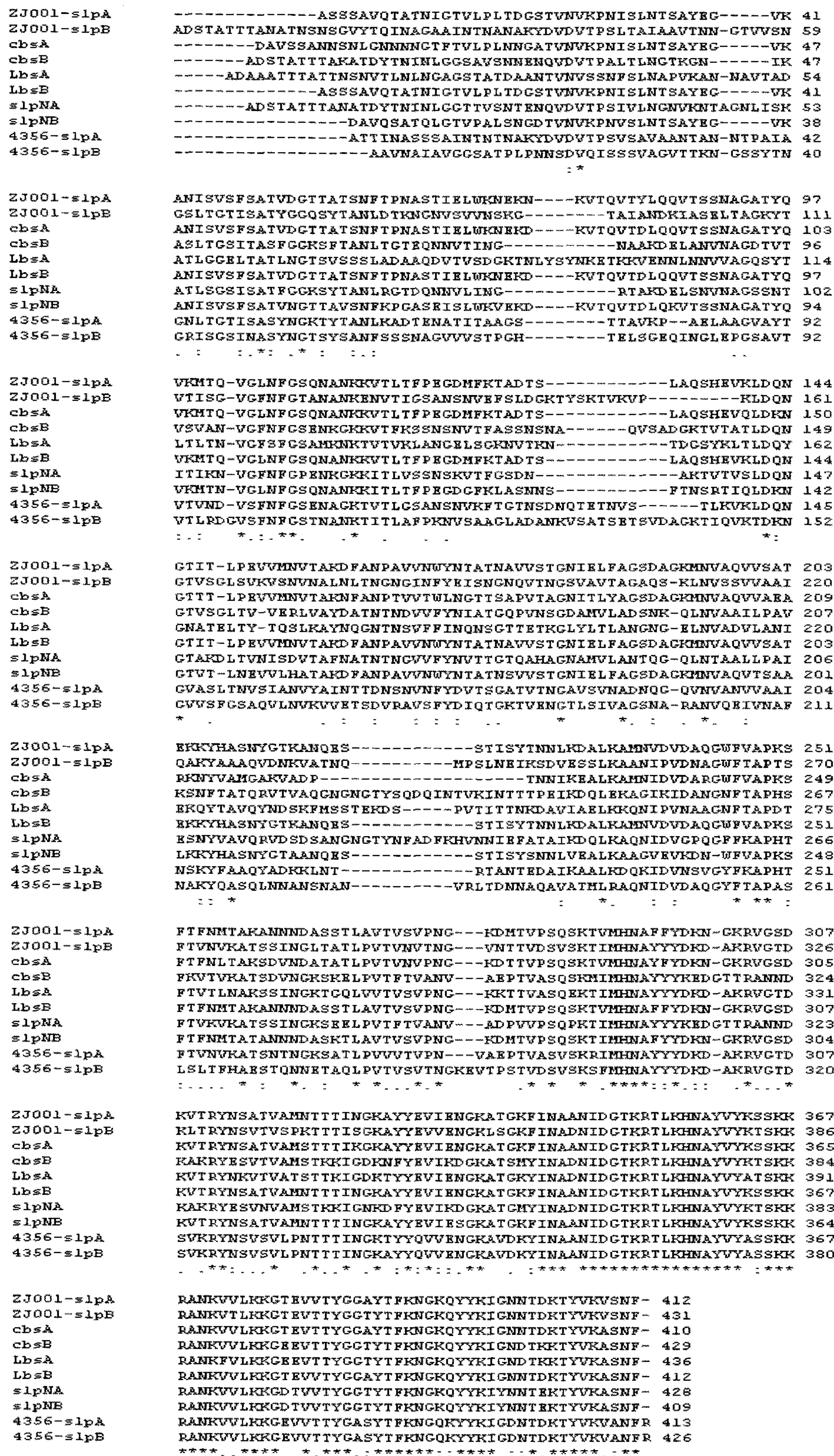

Fig. 1. Comparison of the amino acid sequences of *Lactobacillus* S-layer proteins by sequence alignment: cbsA and cbsB from *L. crispatus* JCM5810, LbsA and LbsB from *L. crispatus* MH315, slpNA and slpNB from *L. crispatus* LMG12003, and 4356-slpA and 4356-slpB from *L. acidophilus* ATCC 4356.

There is significant homology in the C-terminal region and substantial variability in the N-terminal and middle parts. "*" means that the residues are identical in all sequences in the alignment, and ":" and "." mean that conserved and semiconserved substitutions are observed.

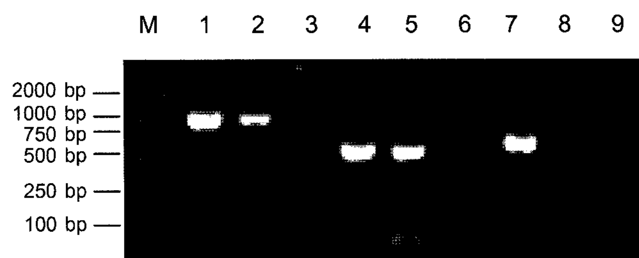

Fig. 2. Only *slpA* was transcribed from total RNA of *in vitro* grown *Lactobacillus crispatus* ZJ001 by RT–PCR.
Three different primer pairs were used: the primers AB-1 and AB-3 (Lanes 1–3), *slpA*-specific primers A3 and A4 (Lanes 4–6), and *slpB*-specific primers B3 and B4 (Lanes 7–9). The amplicons from genomic DNA were used as positive control (Lanes 1, 4, and 7), and from total RNA as negative control (Lanes 3, 6, and 9). M, DNA marker DL2000.

amplified using the *slpA*-specific primers, whereas no signal was amplified with the *slpB*-specific primers (Fig. 2, lanes 5 and 8). PCR reactions were separately performed on genomic DNA as a positive control (Fig. 2, lanes 1, 4, and 7), and on total RNA treated with RNase-free deoxyribonuclease as negative control, using the same primers as for RT–PCR. PCR reaction did not produce any products on total RNA samples (Fig. 2, lanes 3, 6, and 9), indicating no contamination of genomic DNA. These results showed that the *slpA* gene is indeed the structural gene for production of the S-layer protein of *L. crispatus* ZJ001.

Only SlpA is Expressed on the Surface of *L. crispatus* ZJ001

Expression of SlpA on the surface of ZJ001 was proved by using antibodies raised against the purified S-layer protein, His-slpA, and His-slpB, as revealed by Western blot and indirect ELISA assays (Fig. 3). S-layer proteins from ZJ001 could be recognized by both anti-His-slpA and anti-His-slpB polyclonal antibodies (Fig. 3A). Using the antibody against S-layer proteins from *L. crispatus* ZJ001 as the probe, strong signals could be seen with peptides His-slpA

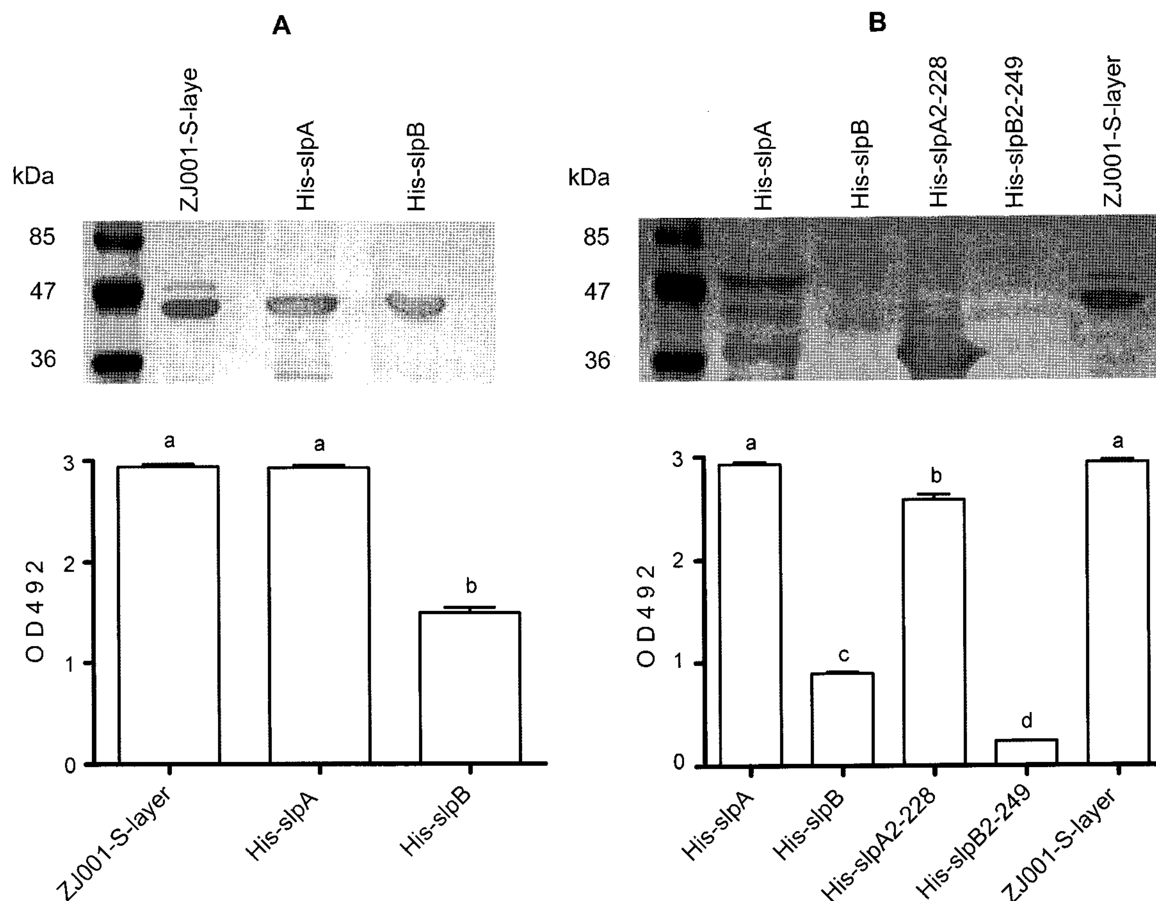

Fig. 3. Analysis of cross-reactivity between SlpA and SlpB as determined by Western blot (top) and indirect ELISA (bottom).
A. The S-layer proteins from *L. crispatus* ZJ001 were blotted onto the nitrocellulose membranes (top) or coated onto microplate wells (bottom), and probed with polyclonal antibodies against homologous S-layer proteins or His-slpA or His-slpB expressed in *E. coli*. **B.** The S-layer proteins from *L. crispatus* ZJ001 as well as mature SlpA or SlpB and their truncated proteins lacking the C-terminal regions expressed in *E. coli* were blotted onto nitrocellulose membranes (top) or coated onto microplate wells (bottom) and probed with the polyclonal antibody against the natural S-layer proteins from *L. crispatus* ZJ001. Error bars represent standard deviations of mean values from three replicate experiments in ELISA. Means with different lowercase letters were significantly different among the different proteins based on Tukey's HSD test ($P<0.05$).

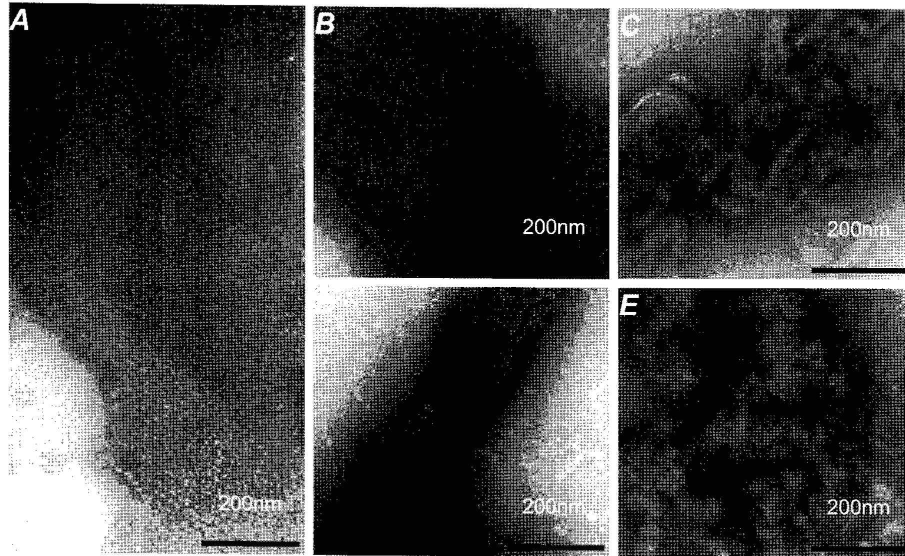

Fig. 4. Transmission electron microscopy of S-layer proteins from *Lactobacillus crispatus* ZJ001 (**A**) and expressed proteins His-slpA (**B**), His-slpB (**C**), His-slpA2-228 (**D**), and His-slpB2-249 (**E**) from *E. coli.*

and His-slpA2-228, faint or lower signals with His-slpB but no signal in His-slpB2-249 (Fig. 3B). These results indicate that the S-layer of ZJ001 is only composed of SlpA.

In vitro Crystallization of SlpA and SlpB and Binding Ability to HeLa Cells

Both mature and truncated proteins purified from *E. coli* became aggregated readily upon removal of the salt by dialysis, forming a white precipitate. Electron microscopic analysis indicated that SlpA from ZJ001 formed a crystalline sheet structure (Fig. 4A) and His-slpA from *E. coli* exhibited sheet-like crystalline layers (Fig. 4B). However, His-slpB was aggregated and formed into disc-like structures (Fig. 4C). The regularly arranged cylindrical polymers were seen with the truncated His-slpA2-228, but His-slpB2-249 did not form regular structures (Figs. 4D and 4E). Fig. 5 shows that HeLa cells could adhere to His-slpA and His-slpB immobilized onto the nitrocellulose membranes, almost in the same degree as to natural SlpA from ZJ001. Their binding was lower with the truncated versions of the proteins than the full-length ones. However, their binding to BSA-treated control membrane was rather low.

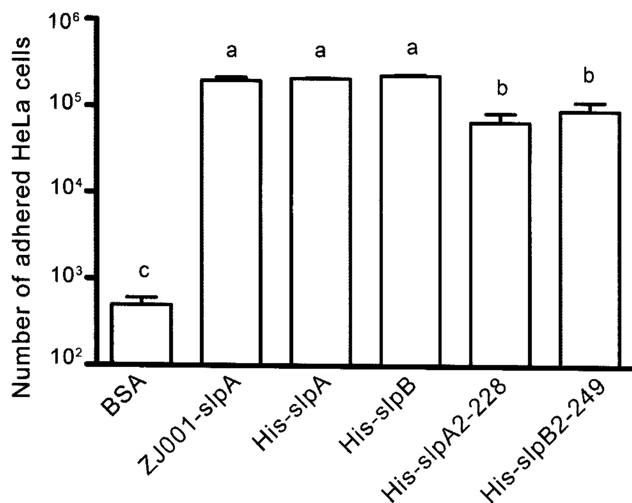

Fig. 5 Binding of HeLa cells to S-layer proteins blotted onto nitrocellulose membranes.

Error bars represent standard deviations of mean values from three replicate experiments. Means with different lowercase letters were significantly different among the different proteins, based on Tukey's HSD test ($P<0.05$).

DISCUSSION

Crystalline bacterial surface layers are found in a broad range of bacteria and archaea, as the outermost cell envelope component. S-layers are common cell surface structures in many *Lactobacillus* species and are involved in adhesion to different host surfaces. We have shown that S-layer proteins are involved in the adhesion of *L. crispatus* ZJ001 to HeLa cells by antibody-mediated inhibition assay [8]. In the present study, we attempted to describe the expression pattern of S-layer genes and cell binding activity of their expression products in *E. coli*.

Lactobacillus species in the *acidophilus* group, such as *L. acidophilus*, *L. crispatus*, *L. amylovorus*, and *L. gallinarum,* harbor two S-protein-encoding genes, one being active for expression and the other silent [5]. We found no evidence of expression of *slpB* of *L. crispatus* ZJ001 under the laboratory conditions, as revealed by Western blot and ELISA (Fig. 3), nor was a *slpB*-specific RNA transcript

detected (Fig. 2), suggesting that *slpB* is a silent gene in the strain. In *L. acidophilus* 4356, the silent gene *slpB* could be translocated to an expression site *via* an inversion of a chromosomal segment by site-specific recombination system at the 5' homologous region [6]. However, *L. brevis* ATCC 14869 was shown to express the *slp* genes differently under different growth conditions [12]. We are not sure if *slpB* expression would be activated in ZJ001 by such inversion or under certain circumstances. *Lactobacillus* S-layer proteins are variable in the N-terminal two-thirds region (SAN), but conserved in the C-terminal one-third region (SAC) [1, 16, 17]. SAN is responsible for crystallization and adherence to host cells, whereas SAC serves as anchoring molecules to the bacterial cells [17]. To obtain more information about the structure and function of SlpA and SlpB of ZJ001, His-slpA and His-slpB and their truncated versions lacking the C-terminus were expressed in *E. coli* and purified. Unlike the natural S-layer proteins from ZJ001, His-slpA exhibited sheet-like crystalline layers (Fig. 4B). The regular cylindrical polymers were formed with truncated His-slpA2-228 (Fig. 4D). This finding is in general agreement with that by Antikainen *et al.* [1], who reported the change of assembly patterns with the deletion of amino acids at the C-terminus of the SAN region from aa269 to aa279. His-slpB and its truncated version were seen as disc-like structures and irregular aggregates, respectively (Figs. 4C and 4E), which were apparently different from those of SlpA proteins, probably due to their differences in amino acid sequences at the N-terminal and middle regions that are involved in the assembly of the sheet-like structure (Fig. 1).

The functions of S-layer proteins in lactobacilli are largely unknown, although their involvement in adhesion has been characterized [9, 11, 15, 16, 18]. In our previous study, the S-layer protein from *L. crispatus* ZJ001 was found to be involved in bacterial adhesion to HeLa cells [8]. Here, we found that the full-length and truncated SlpA and SlpB proteins expressed in *E. coli* were able to bind to HeLa cells at a significantly higher level than the bovine serum albumin control. However, the truncated peptides His-slpA2-228 and His-slpB2-249 had a lower binding ability to HeLa cells than the full-length ones, yet still far higher than the bovine serum albumin control (Fig. 5). This finding is in agreement with the observations of other *Lactobacillus* S-layers exhibiting marked sequence variability, but still with similar adherence to cultured cells [11, 16]. These results suggest that the cell binding domains remain largely in the N-terminal regions of SlpA and SlpB. In *L. crispatus* JCM5810, the prokaryotic expression product of the silent gene *CbsB* polymerized into the crystalline layer as that of *CbsA*, but could not bind to collagen [16]. Hence, the variability of the S-layer protein structure and their adhesive functions among different *Lactobacillus* species should be further investigated.

In conclusion, we have identified the S-layer genes of *L. crispatus* ZJ001 and found that only *slpA* is expressed. When expressed in *E. coli*, both S-layer proteins, His-slpA and His-slpB, were adhesive to cultured cells, although different in the structure of protein polymers. Therefore, it is possible to explore the strain *L. crispatus* ZJ001 as a mucosal vaccine delivery vector, by engineering heterologous protein genes into *slpA,* because of the expression pattern and high cell binding ability of SlpA.

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