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S-layer proteins of *Lactobacillus acidophilus* inhibits JUNV infection

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ARTICLE INFO

Article history:

Received 19 April 2012

Available online 14 May 2012

Keywords:

Surface layer

Lactobacillus

Antiviral

ABSTRACT

It has been previously described that S-layer binds to the C-type lectin DC-specific ICAM-3-grabbing non-integrin (DC-SIGN, CD209). It was also shown that DC-SIGN is a cell-surface adhesion factor that enhances viral entry of several virus families. Among those, Junin virus (JUNV) entry is enhanced in cells expressing DC-SIGN and for that reason surface-layer protein (S-layer) of *Lactobacillus acidophilus* ATCC 4365 was evaluated as a possible JUNV inhibitor. Experiments using 3T3 cells stably expressing DC-SIGN, showed an almost complete inhibition of JUNV infection when they were treated with S-layer in a similar extend as the inhibition shown by mannan. However no inhibition effect was observed in 3T3 wild type cells or in 3T3 cells expressing liver/lymph node-specific ICAM-3 grabbing nonintegrin (L-SIGN or DC-SIGNR or CD209L).

Treatments with S-layer during different times in the infection demonstrated that inhibition was only observed when S-layer was presented in early stages of the viral infection. This inhibition does not involve the classic recognition of mannose by this C-type lectin as the S-layer showed no evidence to be glycosylated. In fact, the highly basic nature of the S-layer (pI > 9.5) seems to be involved in electrostatic interactions between DC-SIGN and S-layer, since high pH abolished the inhibitory effect on infection cause by the S-layer. *In silico* analysis predicts a Ca²⁺-dependant carbohydrate recognition domain in the SlpA protein.

This novel characteristic of the S-layer, a GRAS status protein, contribute to the pathogen exclusion reported for this probiotic strain and may be applied as an antiviral agent to inhibit several kinds of viruses.

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1. Introduction

Many species of the genus *Lactobacillus* possess surface layer (S-layer) proteins which are arrays of a single protein noncovalently bound that constitute the outermost cell envelope also found in several members of Bacteria and Archaea. One of the main characteristics of *Lactobacillus* S-layer proteins is their high isoelectric point (pI) showing them as highly basic [1]. *Lactobacillus acidophilus* is one of the major species found in human intestines and some strains have probiotic characteristics making them GRAS (generally recognized as safe). Probiotics are live microorganisms, usually contained in food, traditionally regarded as safe for human consume that, when ingested in sufficient number, play an important role in control of the host intestinal microbiota and in the modulation of the host immune response. *L. acidophilus* ATCC4356 has been described as a probiotic strain [2] with a 45 kDa S-layer protein highly basic with a calculated pI of 9.59, (Expasy) that was recently showed to have an antibacterial endopeptidase

activity [3,4]. S-layers have also been considered to function as protective coats, maintenance of cell shape and ion exchangers and be involved in adhesion to biotic and abiotic surfaces [5,6]. Although many studies focus on the structure of the S-layer, their biological functions remains poorly understood. S-layer would account for the probiotic properties of some strains [7] being capable of influencing the immune response [8] and favoring cell adhesion [9–12]. Several reports have involved the S-layer proteins in the enteric pathogen exclusion phenomena [13,14].

In the gastro-intestinal tract (GIT) *L. acidophilus* regularly encounters many antigens presenting cell, such as dendritic cells (DCs) [15]. These cells express DC-specific ICAM-3-grabbing nonintegrin protein (DC-SIGN), which is a cell-surface receptor, that is express mainly in DCs and recognize mannose and fructose glycans that are present on microbial and viral surfaces. DCs play a very important role in the innate and adaptive immune response [16]. It has been shown that DC-SIGN can act enhancing viral entry of different viruses such as HIV type 1, hepatitis C, Ebola, Dengue and SARS [17–19]. It was also shown that DCs interact with *L. acidophilus*; this contact involve the DC-SIGN and the S-layer presented on the bacterial cell-envelope, and regulates the induction of a number of cytokines involved in cellular immune regulation [8,20].

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JUNV, a New World arenavirus, is the etiological agent of the Argentine Hemorrhagic Fever (AHF) [21], and endemo-epidemic disease that causes hemorrhagic and neurological complications. AHF presents mortality rates from 20–30% and mainly affects population on the fertile farming land of Argentina [22]. It has been shown that JUNV uses the human transferrin receptor 1 (TfR1) to infect human cells [23]. In addition to its primary receptor (TfR1), JUNV virus infection was shown to be enhanced by C-type lectins (Martinez & Candurra, unpublished results). Among the C-type lectins DC-SIGN and the Liver/lymph node-specific ICAM-3-grabbing nonintegrin (L-SIGN) were studied. DC-SIGN and L-SIGN are human homologs, share 77% of the amino-acid sequence and has function similarities. The role of these receptors (DC- and L-SIGN) in the infection of several major importance viruses placed them as a target for antiviral agents. The most common compounds used so far are carbohydrate-binding agents that can interact with the glycoprotein present on the virus surface and prevent the infection [24].

Lactobacilli have been described to have antiviral activity [25] however the nature of this effect has not been clearly established. In this work we evaluated the effect of the purified S-layer protein of *L. acidophilus* ATCC 4353 on JUNV infection to provide evidence on its role in pathogen exclusion and in the search for new antiviral agents with GRAS status. We used 3T3 cells stably expressing DC-SIGN, or its homolog L-SIGN or Vero cells for transient expression as *in vitro* models. Since 3T3 cells are poorly infect by JUNV in the absence of expression of C-type lectins this model provides a really strong tool to study the effect of S-layer protein on the infection enhanced by DC-SIGN or L-SIGN.

2. Materials and methods

2.1. Isolation of S-layer proteins

The S-layer proteins were extracted from overnight *L. acidophilus* ATCC 4356 cells grown in MRS medium at 37 °C, by using 6 M LiCl, extensively dialyzed against distilled water overnight at 4 °C and after centrifugation (10,000g 20 min) suspended in sterile PBS and store at –20 °C [3].

2.2. Cell culture

Monolayers of Vero cells (ATCC CCL 81) were grown in MEM containing 5% heat-inactivated fetal bovine serum (FBS) and supplemented with 50 µg/ml gentamycin. Maintenance medium (MM) consisted of MEM containing 1.5% fetal calf serum, and titration medium consisted of MEM 2X, 2% FBS and methyl cellulose (0.7%). 3T3 cells (ATCC CCL 1658) and 3T3-derived DC-SIGN and L-SIGN cells (NIAID AIDS Research and Reference Reagent Program) were grown in MEM supplemented with 10% FBS. In all cases, cultures were grown in 5% CO₂ and were supplemented with HEPES (20 µM).

2.3. Viruses

The naturally attenuated Junin IV₄₄₅₄ strain was used in all experiments with live virus, and propagated in Vero cells. Virus yields were then determined by plaque formation (PFU) assays in Vero cells. The titers of the JUNV stock suspensions were 2–8 × 10⁶ PFU/ml.

2.4. Reagents and antibodies

FITC-goat anti mouse and mannan were purchased from Sigma. Anti-JUNV monoclonal antibodies NA05-AG12 were kindly do-

nated by Dr. A. Sanchez (Centers for Disease Control, Atlanta GA, USA). Anti-hDC-SIGN and anti-hL-SIGN MAbs (120526, 9E9A8, 14EG7) were obtained from the NIAID AIDS Research and Reference Reagent Program.

2.5. Inhibition of JUNV infectivity

Confluent 3T3 DC-SIGN cells grown in 24-well plates were treated with different concentrations of S-layer for 1 h. After the treatment period, the medium was removed and the cells were infected in the presence of the protein. After infection, the inoculum was removed and fresh MM was added. The viral supernatants were harvested after 24 h and JUNV yields were determined by PFU assays. Viability of cell cultures under each treatment condition was determined by the MTT assay.

2.6. Immunofluorescence assays

3T3 WT, DC-SIGN or L-SIGN expressing cells were grown on glass coverslips for 24 h. After this period cells were treated with the appropriate concentration of S-layer for 60 min and then infected with JUNV (m.o.i. = 1) or in other cases first infected with JUNV (m.o.i. = 1) and then treated with S-layer protein. After a 24 h incubation period, cells were rinsed three times with PBS, fixed with 4% formaldehyde, permeabilized with 0.2% Triton X-100 and incubated with a MAb specific for JUNV NP (NA05-AG12). After washing, anti-JUNV bound antibody was detected by incubation with FITC-goat anti-mouse antibody. Samples were examined using an Olympus BX51 microscope and the average number of positive and non infected cells on each coverslip was calculated as the average of 20 optical fields chosen randomly. Data is expressed as percentage of virus positive cells over the number of total cells.

When pH treatment was performed 3T3 DC-SIGN or L-SIGN cells were treated with S-layer protein (400 µg/ml) during 60 min. After this period control cell were rinsed during one min with PBS or with basic pH medium. Then the cells were infected with JUNV (m.o.i. = 1). After 60 min of infection the virus inoculum was removed and fresh MM was added. 24 h after the infection cells were fixed with 4% formaldehyde and proceed for IF as described in the previous paragraph.

2.7. Transfection

Transfection was performed according to the Lipofectamine2000 manufacturer (Invitrogen).

2.8. Glycoprotein staining

Glycosylation detection was performed with periodic acid-Schiff's reagent (PAS) according to the procedure of Segrest et al. [26] and Pro-Q_Emerald 300 Glycoprotein Gel Stain Kit (Invitrogen) on SDS-polyacrylamide gels. This fluorescence-based staining method allows the detection of less than 0.5 ng glycoprotein which is 50 times more sensitive than PAS staining.

2.9. Sequence-based analysis

Free access sites were used to predict protein structure and function for the S-layer protein SlpA CAA61560.1 [*L. acidophilus* ATCC 4356] and compared to possible orthologous proteins.

2.10. URL links

ProtParam (<http://web.expasy.org/cgi-bin/protparam>)
CDART (<http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi>)

Clustal-W (<http://www.ch.embnet.org/software/ClustalW.html>)
 I-TASSER 3D structure and function prediction platform (<http://zhanglab.ccmb.med.umich.edu/I-TASSER>)
 SMART, Simple modular architecture research tool (http://smart.embl-heidelberg.de/smart/do_annotation.pl?DOMAIN=CLECT)

3. Results

3.1. S-layer treatment blocks JUNV infection by C-type lectins

We infected 3T3 cells wt or stably expressing C-type lectins with JUNV. As it can be seen in Fig. 1A 3T3 cells are poorly infected with JUNV in the absence of DC-sign or L-sign expression. To study the effect of S-layer protein on JUNV infection 3T3 cells and 3T3-derived stably expressing DC-SIGN and L-SIGN cells were incubated with different concentrations of S-layer protein, infected with JUNV and then cells expressing viral nucleoprotein (NP) were quantified. The results showed reduction in the infection of JUNV in 3T3DC-SIGN cells, but none inhibition was shown in 3T3 L-SIGN cell (Fig. 1A). When we performed a similar experiment but adding the S-layer protein after the infection with JUNV, there was almost no effect of this treatment in cell expressing JUNV NP. This suggests that the effect of the S-layer treatment in JUNV infection is only in an early step of the replication cycle, not affecting later stages (Fig. 1B).

To provide further evidence that blocking in the infection with S-layer was due to the presence of DC-SIGN but not L-SIGN, we performed transient transfection of Vero cells with plasmids expressing hDC-SIGN or hL-SIGN. After 24 h of the transfection the cells were treated with the S-layer protein and then infected with JUNV (Table 1). This confirms that the S-layer inhibitory effect was exclusive to cells expressing DC-SIGN but not L-SIGN.

To further investigate the potential of the S-layer protein to act as an inhibitor for JUNV infection 3T3 DC-SIGN cells were incubated with different concentrations of S-layer for 1 h and then infected with JUNV. Then, 24 h post infection we measure viral production by PFU assay and quantified viral NP by IF (Fig. 2). As it is shown in Fig. 2A when we increased the concentration of S-layer we observe less JUNV infection. However when we exceed 500 $\mu\text{g/ml}$, where it is likely to have auto-assembly of a crystalline S-layer array, the inhibitory effect was suppressed. Virucidal effect on JUNV was assayed by treatment with S-layer during 90 min at 37 °C. No difference with the untreated control was observed by infectivity assay (Fig. 2C). This indicates that the S-layer protein have no effect on the viral particles. Cytotoxicity effect of the protein S-layer protein in 3T3 cells was also studied: no toxicity was found in the concentration of S-layer needed for the inhibition (Fig. 2B).

3.2. Effect of the pH in the S-layer treatment to block JUNV infection cycle

Previous reports suggested that DC-SIGN and the S-layer protein interact [8]. To determine glycosylation state of the S-layer preparation, two assays were performed as shown in methods (Fig. 3). Results showed no glycosylation therefore S-layer protein of *L. acidophilus* recognition of DC-SIGN does not involve the classic mannose interaction.

To verify if the highly basic nature of the S-layer ($\text{pI} > 9.5$) was responsible for electrostatic interactions that could be responsible for the binding with DC-SIGN, interfering with JUNV interaction with the lectin, basic pH treatments were assayed. Increasing the pH in the medium reduced the inhibition of infection by JUNV in DC-SIGN expressing cells, due to a change in S-layer net charge

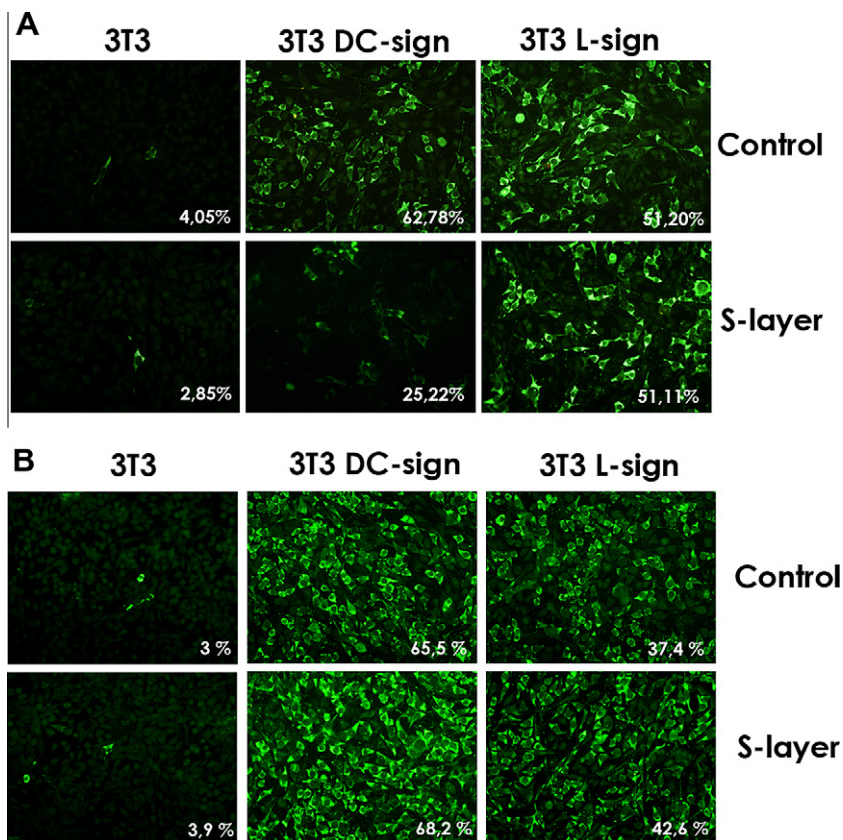


Fig. 1. S-layer blocks virus infection at early stages. 3T3, DC-sign 3T3 or L-sign 3T3 cells treated with S-layer (400 $\mu\text{g/ml}$) for 60 min prior to infection (A) and then infected with JUNV at a m.o.i. = 1 or cells were infected (B) and then treated with S-layer. Fixed cells (24 h p.i.) were stained for IF microscopy. Images from three independent experiments were captured and quantified. Data are indicated in each figure as a percentage of virus positive cells over the number of total cells. Magnifications 400 \times .

Table 1

Transient expression of lectins on Vero cells. Vero cells were transfected with plasmids encoding hDC-SIGN or hL-SIGN. 24 h post-transfection, cells were treated with S-layer (400 µg/ml), mannan (100 µg/ml) or left untreated (control) and then infected with JUNV (m.o.i. = 1). Cells were fixed at 24 h p.i. and stained for IF microscopy. Lectin expression was verified using anti hDC-/hL-sign antibodies provided by NIH (11423). Data is indicated as a mean of the percentage of inhibition relative to each control. Results from three independent experiments were captured and quantified and are shown.

	% of Inhibition	
	L-sign	DC-sign
Mannan	10.9 ± 1.1	13.6 ± 1.2
S-layer	2.8 ± 0.2	26.3 ± 2.5

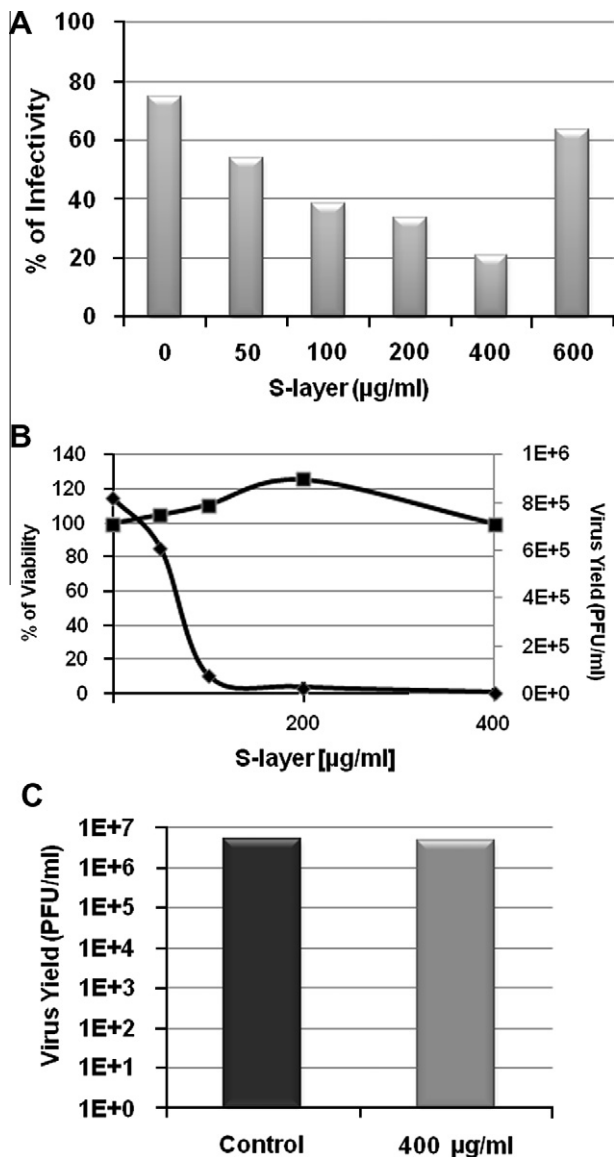


Fig. 2. Inhibitory effect of S-layer on virus infection, dose response curves, cell viability analysis and virucidal activity. 3T3 DC-sign cells were treated with S-layer during 60 min and infected with JUNV (m.o.i. = 1). 24 h post infection supernatants were collected and cells were fixed for IF. (A) JUNV NP was detected by IF using anti-NP monoclonal antibodies and detected by IF. Data was expressed as percentage of positive cells in 3 independent experiments. (B) Cell viability was determined by MTT assay after a 24 h treatment. Supernatants from IF (A) were titrated by PFU assay. (C) Viral stocks were treated with S-layer protein, or only with medium (control), during 90 min at 37 °C. Then infectivity of the treated virus was studied by PFU assay in Vero cells.

(pI 9.59) (Fig. 4A). This effect was not observed in treatments of L-SIGN expressing cells, where no virus inhibition was observed (Fig. 4B), indicating that there was not modification of the infectivity due to basic pH. Chelating effects were also verified to influence the interaction, reducing the inhibitory effect on virus infection (not shown).

3.3. Bioinformatics analysis

We presumed that the C-terminal portion of the S-layer would be responsible of the interaction with the glycan strand of DC-SIGN, since we have already shown it to interact with cell wall peptidoglycan [3] and it has been reported, that two repeats sequences in the C-terminal portion of the protein showed homology to carbohydrate binding domains (CBD) of *Clostridium difficile* S-layer/toxin [27,28]. The repeated sequences are a general theme for protein-carbohydrate interaction described for extracellular virulence factors of Gram positive bacteria [29]. SlpA protein sequence launched in CDART showed that the C-terminal part, Domain SLAP pfam03217, aligned with structural homology with putative CBD of a subgroup of bacterial protein homologous C-type lectin-like (CLECT or CTLD domain), protein domains homologous to the carbohydrate-recognition domains (CRDs) of the C-type lectins (CLECT cd03603 and CLECT_VCBS cd00037) however bacterial CTLDs within this group are functionally uncharacterized. When only the amino-acid sequence of SLAP pfam03217 domain (127 aa C-terminal portion) was used as query for SMART analysis two internal repeats not previously revealed with the whole protein sequence, because they overlap with pfam domain, were actually found with good accuracy (E-value $3.43e^{-05}$). The two internal repeats showed 33% identity (Table 2). Total number of positively charged residues (Arg + Lys) within the repeats gives a predicted pI of 9.26 and 9.90 for each sequence and supports the carbohydrate binding capacity suggested (Table 2). When using I-TASSER for 3D structure and function prediction, result obtained showed similarity to Ca^{2+} binding protein. The Consensus Prediction of Gene Ontology terms obtain include functions of cell adhesion, protein binding and surface expression (<http://zhanglab.cmb.med.umich.edu/I-TASSER/output/S97651>). In summary these analyses results suggest that the S-layer of *L. acidophilus* is predicted to have a carbohydrate binding capacity.

4. Discussion

Cells stably expressing DC-SIGN can be infected by JUNV, but an almost complete inhibition of JUNV infection was shown when they were treated with purified S-layer protein from *L. acidophilus* ATCC 4365 prior to infection (Fig. 1). This effect was not observed in cell lines that do not express this C-type lectin. Post infection treatments with S-layer did not have an effect in viral infection, leading to the idea that the inhibition caused by S-layer was due to an early effect during viral replication cycle. Since no virucidal effect was observed for the S-layer, it seems that the target is not the viral particle but the direct interaction with the DC-SIGN. This inhibitory effect is a novel characteristic of the S-layer protein of *L. acidophilus*, which could account for the pathogen exclusion effect described for this probiotic bacterium. The S-layer is a highly stable protein with no toxicity to cells as cell viability was higher than 95% (Fig. 2).

Differences in binding properties between DC-SIGN and L-SIGN to its target have been reported [30]. Therefore it is not surprising the lack of an inhibitory effect in cell expressing L-SIGN (Fig. 1 and Table 1). The mechanism of interaction involved it is not expected to be the classic mannose recognition, known for this kind of lectins. In fact no glycosylation was observed in the S-layer

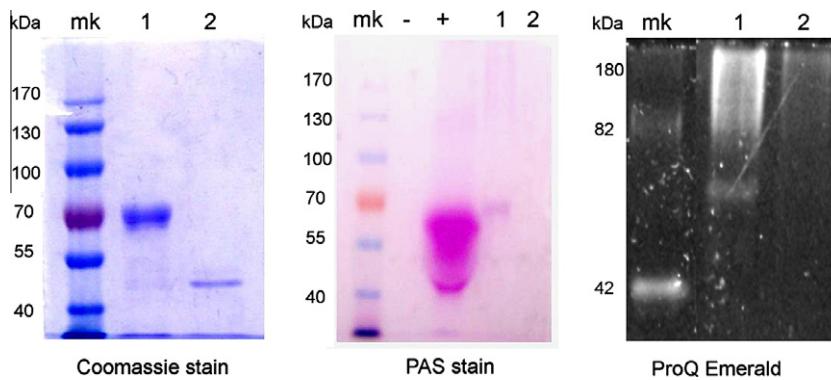


Fig. 3. Absence of glycosylation in *Lactobacillus acidophilus* ATCC 4356 S-layer protein. Periodic acid-Schiff detection (PAS) and ProQ Emerald 330 fluorescent detection were performed to detect glycosylation. For PAS assay Bovine serum albumin served as negative control (-) and TNF α was used as positive control (+). In ProQ Emerald the molecular weight standard CandyCane was used both as molecular marker and glycosylation control. S-layer protein from *Lactobacillus kefir* strains JCM 5818 is in Lane 1. S-layer protein from *Lactobacillus acidophilus* ATCC 4356 is in Lane 2.

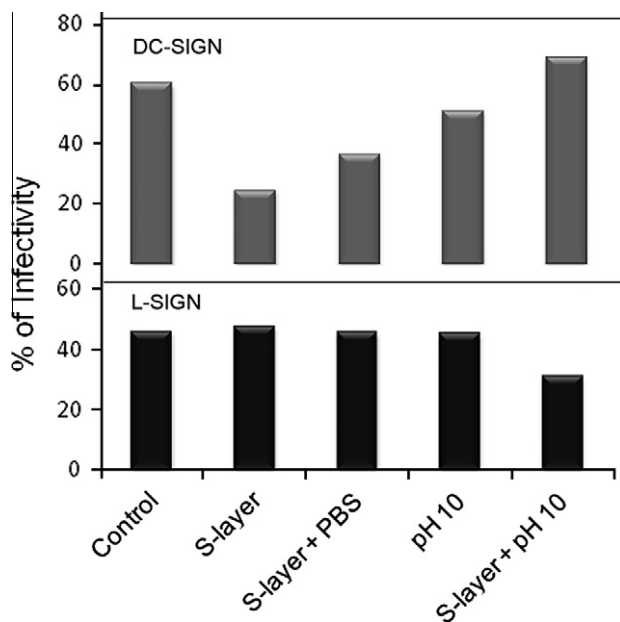


Fig. 4. 3T3 DC-SIGN (A) and 3T3 L-SIGN (B) cells were treated with S-layer (400 μ g/ml) for 60 min. After treatment cells were rinsed during 1 min with PBS as control or with a basic pH buffer (pH 10). As control we also used non S-layer treated cells without pH treatment and with the pH treatment. Then cell cultures were infected with JUNV at a m.o.i. of 1. After 60 min of infection the virus was removed and cells were fixed at 24 h postinfection. Fixed cells were stained for IF microscopy with the anti-JUNV NP (NA05AG12). Images from three independent experiments were captured and quantified. Data is indicated as a percentage of virus positive cells over the number of total cells.

preparation (Fig. 3). It has been previously described that DC-SIGN forms a tetramer that enables multivalent interaction with pathogens. DC-SIGN-pathogen complex can be internalized into compartments with acid pH, and it was shown that there is a pH sensitive control of the oligomerization state of this complex [31]. Therefore we believe that the highly basic nature (pI > 9.5) of this particular S-layer protein might be responsible of interfering with the calcium-dependent viral interaction by structural and electrostatic forces (Fig. 4).

The interaction of the S-layer protein that lacks glycan strands with the DC-SIGN was predicted by bioinformatics methods. It is interesting to remark that this novel mechanism of interaction between SlpA that lacks glycan strands and the DC-SIGN resembles the interaction between non glycosylated C-type animal lectins with their ligands, for example galectin-1 protein [32]. This family of soluble proteins displays conserved CRD that recognizes glycans in its glycoprotein ligand. The repeating sequences described in SlpA (Table 2) are a general theme for protein-carbohydrate interaction [29] and the S-layer protein is proposed to interact with carbohydrate moieties in a calcium dependant manner.

DC-SIGN has been shown to determine susceptibility to HIV type 1, hepatitis C virus, Ebola virus, cytomegalovirus, Dengue virus and SARS coronavirus [17–19]. The results obtained in this *in vitro* model, suggest that S-layer could also inhibit these viruses that use DC-SIGN for viral entry.

Although a great deal of work needs to be done to address the type of interaction predicted between the S-layer protein with the DC-SIGN, this is the first report of a direct viral inhibition by the purified S-layer protein that support the probiotic status of the strain.

Table 2

Internal repeats obtained with SMART within SLAP domain (pfam03217) and Clustal W alignment with Consensus Von Eichel-Streiber Carbohydrate binding sites [29]. pI was predicted with ProtParam (Expasy). Consensuses are in italic or bold letters. (K + R) indicate positively charged residues (Arg + Lys), N° for number of residues and position within SlpA protein. Symbols: “|” identical aminoacid, “:” indicates group similarity, “.” indicates low group similarity. Amino acid notation according to IUPAC-IUB-CBN.

SMART SLAP Domain Internal Repeat alignment and identity				identity= 33%	
Internal Repeats SlpA,					
pI	(K+R)	N°	position		
9.90	12	56	387	KHNAVYVYASSKKRANKVVLKKGVEVVTYGASYTFKNGQKYYKIGDNTDKTYVKVAN	
9.26	8	54	321	MHNAYYYDKDAKRVGTDVSKRYNSVSVLPNTTTI.NGKTYQVVENG.KAVDDKYIN	
	HNAY Y	KR	K V	T NG YY	N K K N
Von Eichel-Streiber Consensus				<i>LKA</i>	<i>VTGWx</i>
				<i>TIxxGkwYYFxxxNPN-T</i>	

Acknowledgments

This work was supported by research Grants from the Consejo Nacional de Investigaciones Científicas y Técnicas from Argentina (CONICET) and Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT-MINCYT).

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