

# Lactobacilli express cell surface proteins which mediate binding of immobilized collagen and fibronectin

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## Abstract

Binding of immobilized collagen-I (Cn-I) and fibronectin (Fn) by *Lactobacillus acidophilus* CRL 639 depends on cell-surface proteins. Capsule formation during the stationary growth phase has a negative effect on adherence of Cn-I and Fn. However, cells from the exponential growth phase, which produce no capsule, exhibit maximal binding. Binding is sensitive to trypsin, proteinase K, pronase E, and heat. Gelatin and soluble Cn-I partially inhibit binding of Cn-I although various proteins, sugars and amino acids do not affect binding to Fn. These results indicate that protein–protein interactions mediate adhesion to extracellular matrix proteins. SDS–PAGE and Western blot analyses of surface proteins revealed that several proteins including the major 43-kDa protein of the S-layer are expressed. Monoclonal antibodies showed that Fn binds to a 15-kDa protein, while Cn-I binds to proteins of 45 and 58 kDa. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Microbiological Societies.

**Keywords:** *Lactobacillus*; Collagen binding; Fibronectin binding; Polysaccharide; Immunoblot; Protein–protein interaction

## 1. Introduction

Fibronectin (Fn) and various types of collagen are high-molecular-mass glycoproteins involved in eukaryotic cell adhesion and spreading [1]. Collagenous proteins are the major constituents of the extracellular matrix (ECM) and represent a common target for pathogenic bacteria. Because bacterial adherence is the critical first step in the development of most infections, it is an attractive target for the development of novel therapeutics, e.g., functional foods containing probiotics.

*Lactobacillus acidophilus* is a common inhabitant of the intestinal tract of humans and animals, and it plays a role in gut microecology, preventing intestinal infections [2,3]. The mechanism of adhesion in lactobacilli involves non-specific factors, particularly hydrophobicity and surface charge [4], as well as specific components such as carbohydrates and proteins on the cell surface. Surface proteins include the S-layers which commonly constitute the major cellular protein species surrounding the cell [5]. Collagen

binding by *Lactobacillus* strains has been proposed to provide a means for colonization of the gut epithelium [5,6]. In *Lactobacillus crispatus* a 43-kDa Cn binding S-layer protein has been identified [7]. Adhesion of *Lactobacillus* to intestinal 407 cells correlates with Fn binding [8]. Studies of intestinal colonization with animal models have identified acidic mucopolysaccharides mediating epithelial cell adhesion to chicken crop [9]. Different results have been reported regarding the role of surface carbohydrates in adhesion. Wadström et al. [10] suggested a role of capsule carbohydrate polymers in colonization of the intestine of pigs by lactobacilli, while Conway and Adams [11] showed that adhesion of lactobacilli to the mouse stomach is mediated by a protein of 13 kDa rather than by extracellular polysaccharide (EPS).

Although efficient adhesion of prokaryotic organisms to tissue surfaces is considered critical for the probiotic effect of lactic acid bacteria, the adhesive surface molecules of lactobacilli and their tissue receptors remain poorly characterized. We here present evidence that the specific binding of immobilized Fn and collagen (Cn-I) by *L. acidophilus* CRL 639 is mediated by a cell-surface protein of 15 kDa and two proteins of 45 and 58 kDa, respectively. Our results suggest that the low adhesiveness of stationary-phase cells is due to capsular polysaccharide formation.

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## 2. Materials and methods

### 2.1. Bacterial strains and culture conditions

The strains used are listed in Table 1. All strains were cultured at 37°C in MRS broth (Difco, Detroit, MI, USA) for 16 h in microaerophilic atmosphere.

### 2.2. Solid-phase Fn and collagen binding assays

Proteins were immobilized in 96-well microtiter plates (8 × 12 wells, Maxisorp Nunc Immuno plates, Nunc, Roskilde, Denmark) at a final concentration of 15 µg ml<sup>-1</sup>, and serial dilutions were made in phosphate-buffered saline (PBS) (0.8% NaCl, 0.02% KCl, 0.14% NaHPO<sub>4</sub> and 0.024% KH<sub>2</sub>PO<sub>4</sub>) buffers (pH 7.2 for Fn and pH 5.5 for collagen). Binding was performed as previously described by Wei and Ljungh [12]. The color was developed by adding a solution of 0.1 M Na-citrate, plus 30% (v/v) ethanol (96%), pH 4.2, 100 µl per well at 25°C for 45 min. The absorbance was measured at 570 nm in an ELISA reader. The OD value of four wells either blocked (without Fn or Cn-I) or incubated with PBS was deduced from the data presented. From the results obtained, *L. reuteri* CRL 1098 was included as a positive control and *L. helveticus* ATCC 15009 as a negative control.

### 2.3. Soluble Fn and collagen binding assays

Cn-I and Fn were labelled with Na<sup>125</sup>I (13–17 mCi µg<sup>-1</sup> I) to a specific activity of 3 × 10<sup>5</sup> cpm µg<sup>-1</sup> protein with Iodo-beads<sup>®</sup> (Pierce Chemical, Richmond, VA, USA) [13], and binding was measured as described by Ljungh et al. [14]. The radioactivity of the pellets was measured in a LKB 1272 gamma counter (LKB Wallac, Turku, Finland).

### 2.4. Effect of the growth phase on Cn-I and Fn binding

*L. acidophilus* CRL 639 was cultured in 2 l of MRS broth using a 1% inoculum from a 16-h culture. At indicated time intervals, bacterial growth, culture pH, and Cn-I and Fn binding were evaluated. The optimal pH for binding was determined in a pH range of 3.0–8.0. Buffers were prepared using PBS adjusted with 1 M HCl or NaOH.

### 2.5. Treatment of bacteria

#### 2.5.1. Protease treatment

Proteases (pronase E and proteinase K), diluted in PBS, pH 7.2 (40 µg ml<sup>-1</sup>, final concentration), were added to 100 µl of the bacterial cell suspension [12]. Digestion was performed at 37°C for 40 min. The reaction was stopped by adding iodoacetamide and benzamidine hydrochloride

to a final concentration of 20 mM. Bacterial cells (5 × 10<sup>7</sup> cfu ml<sup>-1</sup>) were incubated with trypsin (25 µg ml<sup>-1</sup>; 0.04 M Tris-HCl with 1 mM CaCl<sub>2</sub>, pH 8.1) at 37°C for 1 h. The reaction was stopped by adding soybean trypsin inhibitor to a final concentration of 50 µg ml<sup>-1</sup>. After protease treatment, bacterial cells were washed twice with PBS and tested for binding of Cn-I and Fn. Bacterial cells without protease treatment served as negative controls.

#### 2.5.2. Periodate treatment

Bacterial cell suspensions (500 µl, 10<sup>8</sup> cfu ml<sup>-1</sup>) were pre-incubated overnight with an equal volume of 100 mM sodium periodate in 0.1 M NaAc, pH 5.5, at 4°C in the dark. After washing three times with PBS, bacterial cells were further incubated with 100 mM NaBH<sub>4</sub> at 4°C for 1 h, washed twice with PBS and finally resuspended in PBS at the original volume [15].

#### 2.5.3. Tunicamycin treatment

*Lactobacillus* strains were grown at 37°C in MRS broth with tunicamycin (0, 3.1, 6.3, 12.5, 30 and 50 µg ml<sup>-1</sup>) [16]. The cells were subcultured three times in tunicamycin broth before binding experiments were performed.

#### 2.5.4. Inhibition of Cn-I or Fn binding

For inhibition studies, 10 µg of various proteins and carbohydrates (Table 2) were incubated with bacterial suspensions at 25°C for 30 min before conducting the binding assays.

### 2.6. Treatment of immobilized Cn-I and Fn

Microtiter plates coated with 15 µg ml<sup>-1</sup> of Cn-I or Fn were incubated at 4°C for 16 h with 100 µl of 100 mM sodium periodate in 0.1 M NaAc, pH 5.5. After washing three times with PBS, the plates were further incubated with 100 mM NaBH<sub>4</sub> at 4°C for 1 h to prevent non-specific binding. The treated plates were washed twice within PBS, and assayed for binding. Proteins treated without sodium metaperiodate were used as negative controls.

### 2.7. EPS isolation

The slime EPS (EPSs) was isolated from 24-h (stationary phase) cultures of lactobacilli grown in MRS at 37°C. EPS production by *L. acidophilus* CRL 639 was also evaluated during the exponential phase (3-h culture). After removal of cells by centrifugation, the EPS was recovered by ethanol precipitation at 4°C for 24 h and dialyzed against distilled water at 4°C for 72 h. Total EPS was estimated by the phenol sulfuric acid method [17] using glucose as the standard. Total EPS production was expressed as mg l<sup>-1</sup>, and the specific EPS production was expressed as mg EPS cfu<sup>-1</sup>.

## 2.8. Determination of EPS

The negative stain principle with Indian ink was employed to determine the presence of EPS that remained bound to the cells (EPS<sub>c</sub>). EPS<sub>c</sub> was observed microscopically as a clear area around the cells. Cultures were concentrated by centrifugation, and the pellet was negatively stained [18].

## 2.9. SDS-PAGE and immunoblot assays

SDS-PAGE was performed under reducing conditions by using mini-Protean II cell (Bio-Rad, Richmond, CA, USA). Bacterial surface proteins were extracted with 1 M LiCl (pH 5.0) with proteinase inhibitor (Complete<sup>®</sup> mini, Boehringer Mannheim, Mannheim, Germany) at 37°C for 2 h. Fifteen micrograms of protein extract were loaded into each well and separated in a homogeneous gel (12%) for 16 h at 80 V. Molecular mass standards (Promega, Madison, WI, USA), including proteins ranging from 14.3 to 97.4 kDa in size, were treated identically. The proteins were transferred to Immobilon polyvinylidene fluoride (PVDF) membranes (0.45 µm, Micron Separations, Westborough, MA, USA) using a semi-dry electroblot equipment (Ancos, Vig, Denmark) for 1.5 h at a constant current of 0.8 mA cm<sup>-2</sup>.

To detect Fn binding proteins, the PVDF membranes were saturated by incubation twice for 15 min in blocking buffers I [50% EG 2× (1.2% ethanolamine and 1.8% glycine, pH 9.6), 1% polyvinylpyrrolidone, and 25% methanol] and II (50% EG 2× pH 9.6, 0.125% Tween 20, 0.5% gelatin hydrolysate, and 25% methanol) [19]. To detect Cn-I binding proteins, only buffer I was used. Saturated membranes were rinsed once with the washing buffer for 10 min and cut into strips. The washing solutions used were NN<sup>8</sup> pH 7.8 (0.25% gelatin hydrolysate, 0.05% Tween 20, 1.02% NaCl, and 0.012% Tris) for Fn, and

PBS (pH 5.5) with 0.1% Tween 20 for Cn-I. The strips were overlaid with the protein solutions (Cn-I or Fn, 2 µg ml<sup>-1</sup>) in the washing buffer at 4°C for 16 h with gentle shaking. After three washes, primary antibodies for Cn-I or Fn, diluted 1:1000 in washing buffer, were added and incubated for 2 h at 20°C. The membranes were washed three times and incubated with the secondary antibody conjugated with horseradish peroxidase at a dilution of 1:2000 and incubated at 20°C for 2 h. After repeated washing, bound antibodies were detected by reaction in 50 mM sodium acetate buffer (pH 5.0) added with 0.04% 3-amino-9-ethylcarbazole and 0.015% H<sub>2</sub>O<sub>2</sub>.

## 2.10. Statistical analyses

All experiments were performed in triplicate. The mean values of the data and standard deviations are presented.

## 3. Results and discussion

### 3.1. Cn-I and Fn binding, and polysaccharide production

Binding of Cn-I and Fn was restricted neither to the source of the *Lactobacillus* strain nor to the species (Table 1). Only *L. acidophilus* CRL 639, *L. reuteri* CRL 1098, and *L. helveticus* ATCC 15807 gave significant binding (over OD<sub>570</sub> = 0.25) corresponding to > 5% [12] of Cn-I or Fn bound. *L. helveticus* ATCC 15009, *L. fermentum* ATCC 14931, *L. casei* subsp. *rhamnosus* CNRZ 212, *L. casei* subsp. *casei* NCDO 161, *L. rhamnosus* ATCC 7469, *L. delbrueckii* subsp. *delbrueckii* ATCC 9649 and *L. paracasei* subsp. *paracasei* CRL 686 expressed little or no Fn or Cn-I binding activities.

Many of the strains tested produced EPS during the stationary phase which was either bound to the cells forming a capsule (EPS<sub>c</sub>) (*L. acidophilus* ATCC 4356 and CRL

Table 1  
Characteristics of *Lactobacillus* strains

Strain	Collection number <sup>a</sup>	Origin	Polysaccharides		Cn binding <sup>c</sup> (OD <sub>570</sub> )	Fn binding <sup>c</sup> (OD <sub>570</sub> )
			EPS <sub>c</sub>	EPS <sup>b</sup>		
<i>L. acidophilus</i>	ATCC 4356	Human	+	–	0.27 ± 0.03	0.23 ± 0.02
<i>L. acidophilus</i>	CRL 639	Dairy product	+	2.0 × 10 <sup>-9</sup>	0.35 ± 0.02	0.26 ± 0.01
<i>L. helveticus</i>	ATCC 15807	Emmental cheese	–	1.13 × 10 <sup>-9</sup>	0.40 ± 0.01	0.29 ± 0.01
<i>L. helveticus</i>	ATCC 15009	Emmental cheese	–	–	0.01 ± 0.01	0.09 ± 0.01
<i>L. reuteri</i>	CRL 1098	Sourdough	–	–	0.29 ± 0.01	0.29 ± 0.01
<i>L. gasseri</i>	DSM 20243	Human	–	1.0 × 10 <sup>-9</sup>	0.21 ± 0.03	0.35 ± 0.01
<i>L. delbrueckii</i> subsp. <i>delbrueckii</i>	ATCC 9649	Fermented seeds	–	9.0 × 10 <sup>-9</sup>	0.23 ± 0.02	0.16 ± 0.02
<i>L. fermentum</i>	ATCC 14931	Fermented beet	–	–	0.14 ± 0.03	0.09 ± 0.02
<i>L. casei</i> subsp. <i>rhamnosus</i>	CNRZ 212	Cheese	+	–	0.13 ± 0.01	0.07 ± 0.01
<i>L. casei</i> subsp. <i>casei</i>	NCDO 161	Cheese	–	–	0.22 ± 0.01	0.12 ± 0.01
<i>L. rhamnosus</i>	ATCC 7469		+	–	0.12 ± 0.01	0.09 ± 0.01
<i>L. paracasei</i> subsp. <i>paracasei</i>	CRL 686	Sausage	+	–	0.09 ± 0.01	0.08 ± 0.01

<sup>a</sup>ATCC: American Type Culture Collection; DSM: Deutsche Sammlung für Mikroorganismen; CNRZ: Centre National de la Recherche Zootechnique; NCDO: National Collection of Dairy Organisms; CRL: Centro de Referencia para Lactobacilos.

<sup>b</sup>All determinations were made at the stationary growth phase. The specific EPS production is expressed as mg EPS cfu<sup>-1</sup>.

<sup>c</sup>The value for phosphate-buffered saline (PBS) (negative control) has been subtracted.

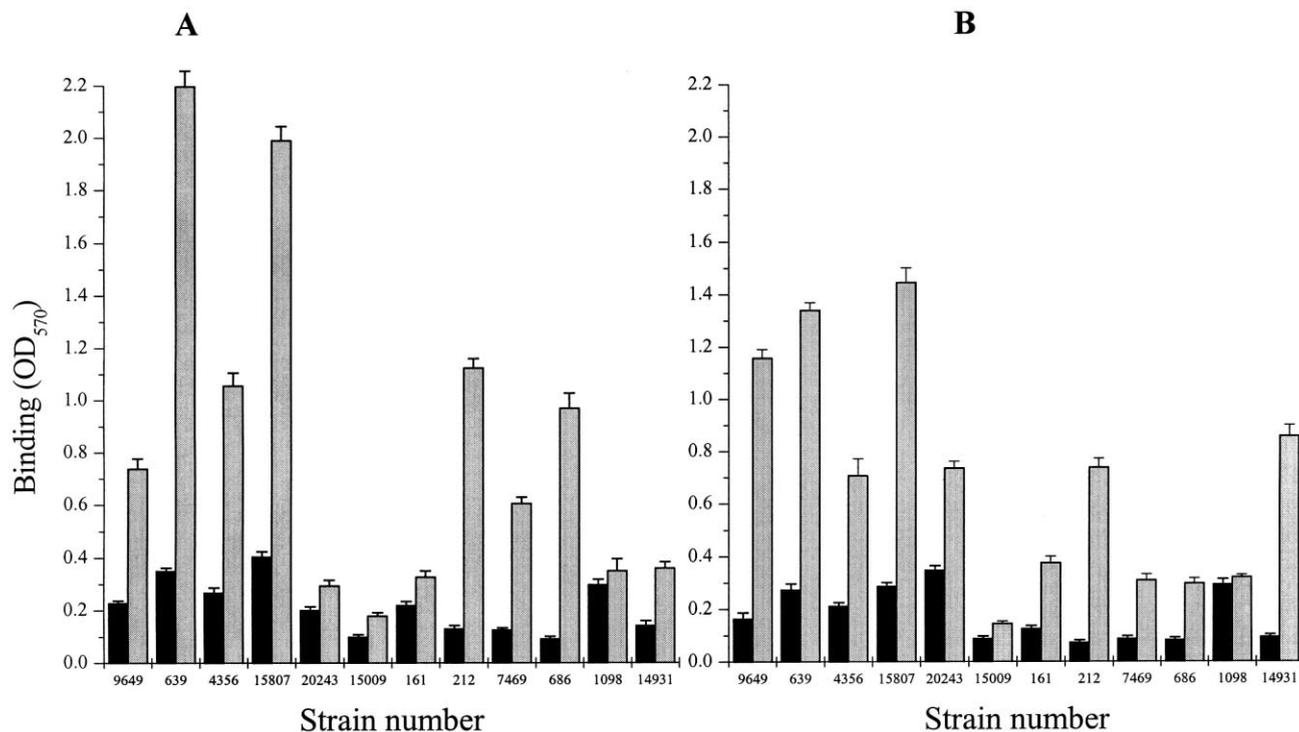


Fig. 1. Effect of periodate treatment on Cn-I (A) and Fn (B) binding by *Lactobacillus*. Non-treated (black bars) or treated cells (gray bars). The mean values of the data and the S.D. values are presented here and in Figs. 2 and 3.

639, *L. casei* subsp. *rhamnosus* CNRZ 212 and ATCC 7469, and *L. paracasei* subsp. *paracasei* CRL 686) or released into the culture media as slime (EPSs) (*L. acidophilus* CRL 639, *L. gasseri* DSM 20243, *L. delbrueckii* subsp. *delbrueckii* ATCC 9649 and *L. helveticus* ATCC 15807).

In order to investigate the role that both kinds of polymer (EPSc and EPSs) might play in the binding of ECM proteins, all the strains tested (Table 1) were treated with periodate at 4°C for 16 h to oxidize the carbohydrates in the cell surface. Fig. 1 shows a 3–10-fold increase in binding ability of the cells after treatment. According to Roberts [20], EPS promotes adhesion of bacteria to various surfaces, thereby facilitating biofilm formation. This apparent disagreement with our results might be due to the

complexity of bacterial adhesion to protein surfaces and to mucus-covered tissues such as the intestine.

To determine whether or not the decrease in binding observed during the stationary phase is related to a different degree of protein glycosylation, the *Lactobacillus* strains were grown in MRS broth with tunicamycin which blocks surface protein glycosylation [16]. Expression of binding was not affected by the antibiotic, indicating that glycoproteins are not involved in the binding of Fn or Cn-I (data not shown).

Further characterization of Fn and Cn-I binding was performed with *L. acidophilus* CRL 639. This strain binds immobilized Cn-I and Fn but not the soluble forms (data not shown). The optimal temperature for adherence was

Table 2  
Effect of various potential inhibitors on adherence of immobilized Cn-I or Fn by *L. acidophilus* CRL 639

Compound	Concentration <sup>a</sup>	Cn-I OD <sub>570</sub> <sup>b</sup> (mean ± S.D.)	Fn OD <sub>570</sub> <sup>b</sup> (mean ± S.D.)
None (control)		0.39 ± 0.05	0.29 ± 0.06
Collagen type I	10 µg	0.21 ± 0.03*	0.28 ± 0.03
Fn	10 µg	0.29 ± 0.01	0.27 ± 0.03
BSA	10 µg	0.31 ± 0.02	0.27 ± 0.02
Gelatin	10 µg	0.23 ± 0.01*	0.31 ± 0.03
D-Galactose	100 mM	0.33 ± 0.02	0.30 ± 0.02
D-Mannose	100 mM	0.31 ± 0.03	0.27 ± 0.03
D-Fucose	100 mM	0.29 ± 0.01	0.29 ± 0.01
Proline	100 mM	0.33 ± 0.14	0.31 ± 0.10
Glycine	100 mM	0.31 ± 0.01	0.29 ± 0.01

\*Significantly different from control ( $P < 0.005$ ).

<sup>a</sup>Final concentration in the assay.

<sup>b</sup>The value for PBS (negative control) has been subtracted.

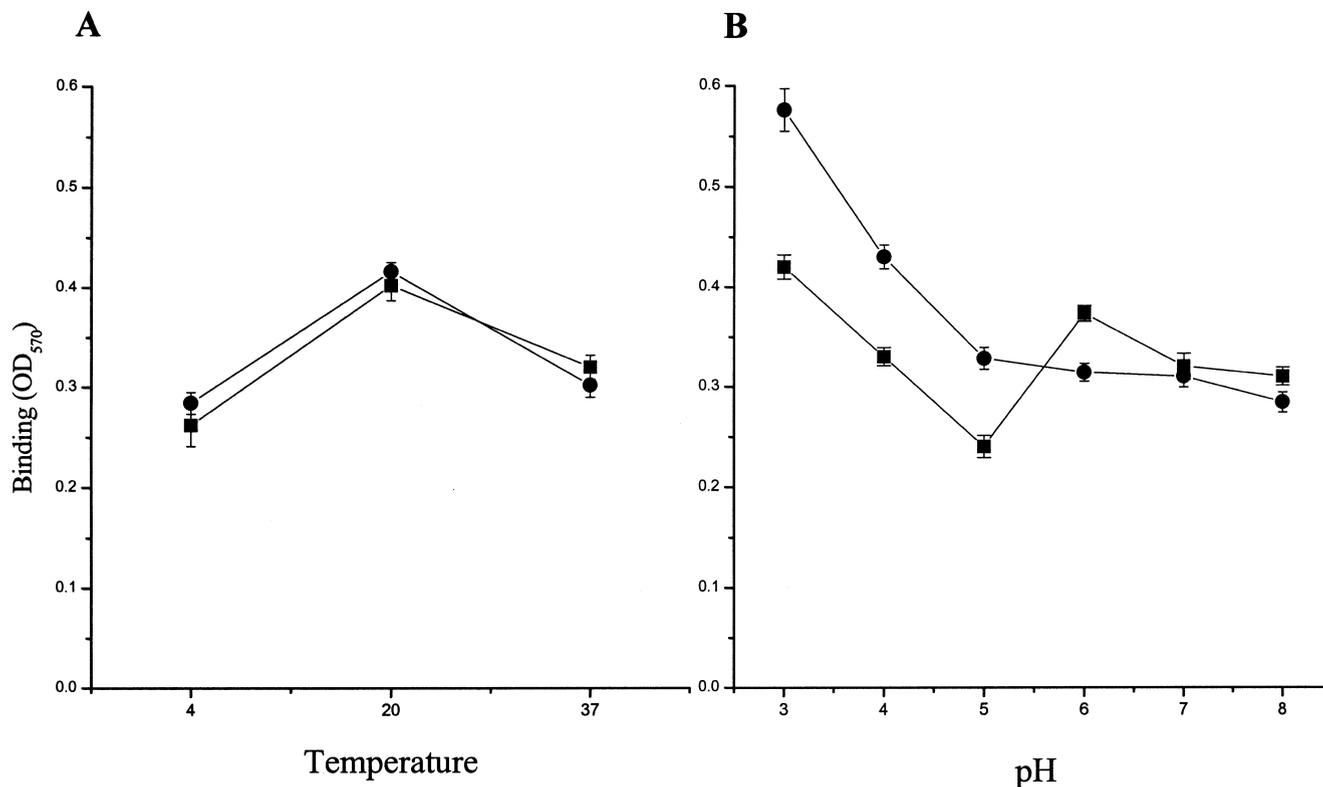


Fig. 2. Influence of pH (A) and temperature (B) on binding of Cn-I (■) and Fn (●) by *L. acidophilus* CRL 639.

found to be 25°C (Fig. 2A), but the optimum pH varied according to the coated proteins used. Best results were obtained at pH 3.0 for Fn and Cn-I although significant Cn-I binding was also observed at higher pH (Fig. 2B).

The effect of cellular physiological state on adhesion of ECM proteins was further studied. Maximal binding for Cn-I (OD<sub>570</sub> = 0.65) and Fn (OD<sub>570</sub> = 0.40) corresponded to exponentially growing cells, and the minimal binding corresponded to stationary-phase cells (OD<sub>570</sub> = 0.35 and 0.29, respectively) (Fig. 3). This latter observation is probably related to formation of EPSs. In fact, *L. acidophilus* CRL 639 produces EPSs ( $1.8 \times 10^{-7}$  mg EPS cfu<sup>-1</sup>) during the exponential phase and EPSs in the stationary phase. When cultures at the latter growth phase were treated with periodate, the binding capacity of Cn-I and Fn was improved from 4.6- to 6.3-fold (Fig. 3). These data show that the formation of capsule (EPSs) rather than the production of slime (EPSs) is the main factor affecting adhesion of ECM proteins.

Table 2 shows the effect of various potential inhibitors such as proteins, amino acids and sugars on binding of Cn-I and Fn by *L. acidophilus* CRL 639. On the whole, they had no effect on Fn binding, but they exhibited a 35% and 40% reduction of Cn-I binding in the presence of gelatin and Cn-I, respectively. Numerous protein–protein interactions among ECM proteins have been described which may prevent visualization of specific inhibition. The finding that *L. acidophilus* CRL 639 expressed binding activity only of the immobilized form of the proteins may

explain the slight inhibition obtained after preincubation of the cells with the soluble forms of the proteins.

The activities of proteases upon cell-surface proteins were dependent on the growth phase, exponentially growing cells being very sensitive to protease treatment compared to stationary-phase cells (Fig. 3). Trypsin treatment resulted in a 65% decrease in binding of Cn-I followed by proteinase K (43%) and pronase E (26%) while adherence of Fn was inhibited by 58–62%. Heat treatment (100°C) also reduced binding of Cn-I (57%) and of Fn (45%) (Fig. 3).

Several publications reported the involvement of carbohydrate-specific molecules (lectins) in *L. acidophilus* adherence [21–23]. However, our data showed that binding by *L. acidophilus* CRL 639 is likely to be mediated by protein–protein interactions since the cells lost their binding properties after treatment with proteases and the presence of monosaccharides did not affect activity.

### 3.2. SDS-PAGE and immunoblot assays

Surface proteins extracted with 1 M LiCl were subjected to SDS-PAGE. Several proteins with relative molecular masses ( $M_r$ ) from 15 to 70 kDa, including the major S-layer protein of 43 kDa, were visualized. After transferring to PVDF membranes, Western blot analysis was performed. Fn bound a 15-kDa protein while Cn-I bound proteins with  $M_r$  45 kDa and 58 kDa (Fig. 4). Identical reactivity was observed with antigens extracted from cells

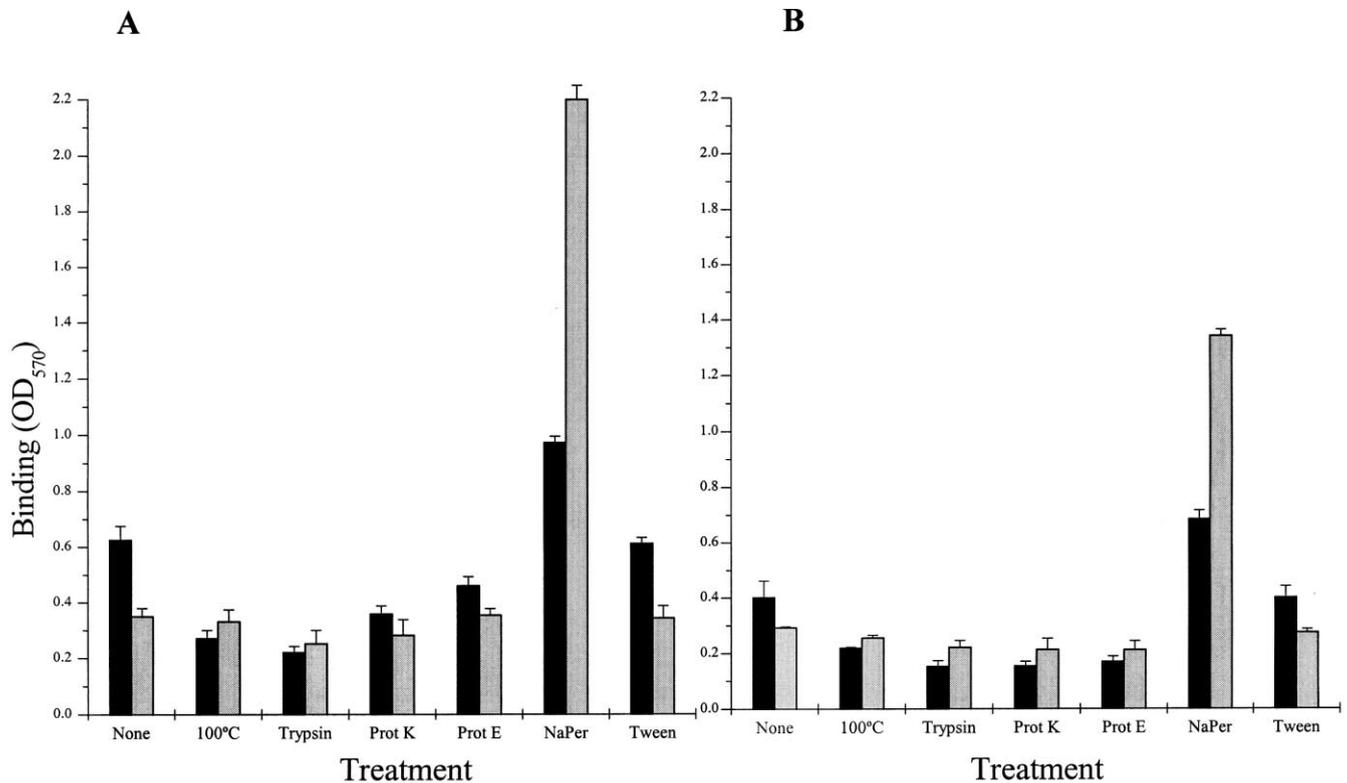


Fig. 3. Effect of various treatments of *L. acidophilus* CRL 639 on adherence to Cn-I (A) and Fn (B) immobilized onto wells of microtiter plates. Effect on cells from the exponential (black bars) and stationary growth phases (gray bars).

at different growth phases or with periodate-treated surface proteins after they were transferred to PVDF membranes (data not shown).

The S-layer proteins are potential mediators of the initial steps in bacterial adhesion. These proteins have been detected in many Gram-negative and several Gram-positive organisms, including lactobacilli. *L. acidophilus* CRL 639 exhibits both capsule and S-layer, but the structural relationship between them is not understood. Although these structures can be synthesized independently, functional interactions are not excluded. Thus, the fine structure of the capsule may rely on the presence of the underlying S-layer [24]. Despite the role ascribed to the S-layer in ECM protein binding [5] it does not appear to be involved in the binding of Cn-I and Fn by *L. acidophilus* CRL 639. In *L. reuteri* NCIB 1195 [6], binding of Cn was reported to depend on two lectin-like proteins (29 and 31 kDa), one of them being identified as a part of an ABC transporter [25].

Results obtained in *L. acidophilus* CRL 639 may contribute to the initial establishment of the normal bacterial flora in the intestine. Adhesion of lactobacilli to human subintestinal ECM proteins may thus have a direct implication in their probiotic function, e.g., in preventing adhesion to and colonization of damaged intestinal tissue sites by invading pathogens. Results presented for *L. acidophilus* 639 support this hypothesis since this microorganism only recognizes the immobilized, but not the soluble, forms of Cn-I and Fn, i.e., the forms which are likely to

be exposed in the intestinal epithelium during shedding in the mucus.

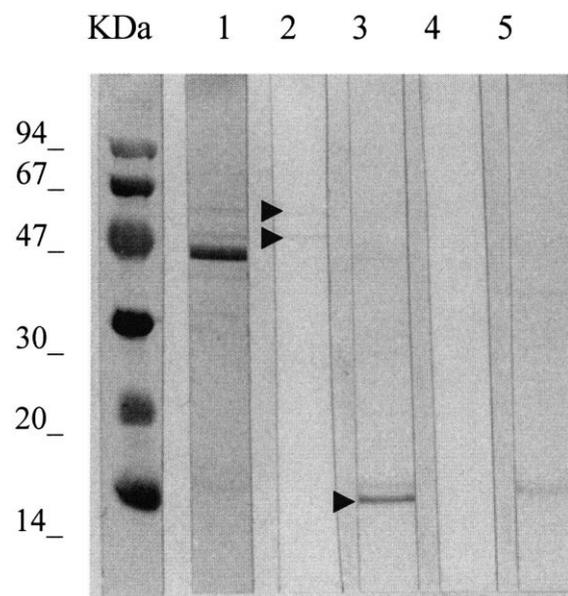


Fig. 4. SDS-PAGE and immunoblot analysis of *L. acidophilus* CRL 639 surface proteins. Lane 1: SDS-PAGE profile of surface proteins transferred to a PVDF membrane and stained with amido black; 2: probed for binding to Cn-I, or 3: probed for binding to Fn. To evaluate the specificity of the antibody, the experiment was performed in the absence of antibodies (lane 4) for Cn-I and (lane 5) for Fn. Left lane: molecular mass standards.

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