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Collagen Binding by Lactobacilli

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Abstract. ^{125}I-labeled type I collagen (Cn-I) binding of 92 fresh isolates and 18 type culture collection strains of lactobacilli was tested. More than 75% of the strains bound Cn-I. The binding was inhibited by excess of unlabeled Cn-I, gelatin, and was sensitive to proteinase K. Other proteins such as fibronectin and albumin and various carbohydrates such as D-galactose, D-fucose, and D-mannose did not inhibit the binding. Therefore, we propose binding of Cn-I to lactobacilli involving specific surface protein(s).

Lactobacilli are members of the indigenous gastrointestinal and genital microflora of animals and man. It has been proposed that bifidobacteria and lactobacilli play an important role in the host intestinal microecology, especially in prevention of intestinal infections during the weaning period [16]. A number of enteropathogenic bacteria have been shown to colonize the intestinal mucosa by specific surface proteins called adhesins or colonizing factor antigens [4, 10]. Little attention has focused on how members of the indigenous flora, including lactobacilli, colonize the gut [21]. Enteropathogenic *Escherichia coli* adhere and colonize intestinal mucosal surfaces by both fimbrial and non-fimbrial adhesins [10, 22]. A number of tissue-invasive pathogens such as *Staphylococcus aureus* and other staphylococci bind to various tissue collagens [8, 18], fibronectin, and vitronectin in the extracellular matrix or in damaged tissue [9, 11, 15]. However, connective tissue protein binding has been found also in the noninvasive enteric pathogens such as *Vibrio cholerae* [23] and enterotoxigenic *E. coli* [6, 20].

Lactobacilli colonize the intestinal mucous membranes and antagonize gut colonization by various enteropathogens [17]. Therefore, studies on the possible interactions of lactobacilli with proteins of the mammalian host cells are important. It is of interest to determine whether such commensal bacteria, which rarely cause infections and then only in the immune compromised host, share colonization strategies with pathogens.

We now describe collagen type I (Cn-I) binding to 110 strains from different species of lactobacilli, including both fresh isolates and type culture collection strains. Cn-I binding properties of a few selected strains were further characterized for binding specificity and for growth influence on binding properties.

Materials and Methods

Strains. Type culture collection strains were obtained from Deutsche Sammlung von Mikroorganismen (DSM) (Braunschweig, Germany) and from the National Collection of Industrial and Marine Bacteria Ltd (NCIMB) (Aberdeen, Scotland) (Table 1). *Staphylococcus aureus* strain Cowan I was used as a reference strain in the growth experiment [8, 15]. Fresh lactobacilli isolates from human, porcine, calf, and chicken were obtained from a culture collection at Medipharm (Ängelholm, Sweden) [7, 21]. These isolates were biotyped by a commercial kit, API 20 CH (Analytab, Montalieu, Vercieu, France).

Chemicals. Purified collagen type 1 (Cn-I) preparations (Vitrogen 100, 95% collagen type I and 5% collagen type III, lot nos. 87H183 and 88K283) were purchased from Collagen Corporation (Palo Alto, California, USA). Porcine fibronectin was a kind gift from BioInvent AB International (Lund, Sweden). Gelatin, D-mannose, D-fucose, eggwhite lysozyme, pepsin, trypsin, papain, and proteinase K were purchased from Sigma Chemicals (St. Louis, Missouri, USA) and D-galactose from Merck (Darmstadt, Germany). Human IgG was obtained from Kabi (Stockholm, Sweden), BSA from Boehringer Mannheim GmbH (Mannheim, Germany), and Tween 20 from Kebo Lab AB (Stockholm, Sweden). Iodobeads were purchased from Pierce Chemical Company (Rockford, Illinois, USA) and isotopes from Amersham (Solna, Sweden). All chemicals used in the preparation of buffers and solutions were of analytical grade.

Bacteriological media and culture conditions. Lactobacilli were grown in the Man-Rogosa-Sharpe-medium (MRS Oxoid Ltd, Basingstoke, England), in Lactobacillus Carrying Medium (LCM) [2], and on Rogosa agar (Oxoid). Bacto agar (1.5%, Difco Labora-

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Table 1. Reference strains and their collagen type I binding profiles[a]

Strains	Number	Origin	% Cn-I binding
Lactobacillus acidophilus	DSM 20356	Turkey feces	7
L. acidophilus	DSM 20552	Human infection	19
L. brevis	DSM 20485	Kefir grains	8
L. casei subsp *casei*	DSM 20244	Milk	11
L. casei subsp *rhamnosus*	DSM 20023	?	11
L. casei subsp *tolerans*	DSM 20012	Pasteurized milk	6
L. cellobiosus	DSM 20055	Saliva	7
L. coryniformis	DSM 20007	Silage	11
L. fruktosus	DSM 20394	Flowers	5
L. gasseri	DSM 20077	Human feces	18
L. minutus	DSM 20586	Abscess	29
L. reuteri	DSM 20016	Human intestine	20
L. reuteri	DSM 20053	Human feces	7
L. reuteri	NCIMB 11951	Human intestine	40
L. sp	DSM 20182	Marinated meat	11
L. sp.	DSM 20452	Rat intestine	21
L. vaccinostercus	NCIMB 11808	Bovine feces	7
L. gasseri	DSM 20604	[b]	8

[a] Lactobacilli reference strains, their sources, origin of isolation, and their collagen type-I binding values. The strain NCIMB 11951 was chosen for further characterization.
[b] Human septic urinary infection.

tories, Detroit, Michigan, USA) was added to the broth prior to autoclaving for the preparation of solid media. Agar cultures were incubated in a CO_2 incubator (Assab AB, Stockholm, Sweden) with 5% CO_2. All broth cultures were grown with slow agitation (50 rpm) in 50 ml of medium in 200-ml flasks at 37°C for 16 h unless otherwise stated. *Staphylococcus aureus*, strain Cowan-1, was grown in Trypticase Soy Broth (TSB, Difco). Cells were harvested, after 16 h, in the stationary phase of growth and washed twice in 0.02 *M* of potassium phosphate-buffered saline (KPB), pH 6.8. The cell density was adjusted to 5×10^9 organisms/ml in a Linson 3 photometer (Stockholm, Sweden).

Protein binding assays. Cn-I was labeled with ^{125}Iodine to a specific activity of $1–3 \times 10^5$ cpm/μg protein according to a modified Chloramine-T method using Iodobeads [6, 12]. Labeled Cn-I was stored at 4°C in 1% BSA. Labeled Cn-I was diluted in PBS (0.02 *M* sodium phosphate buffer, 0.15 *M* NaCl, pH 7.2) with 0.1% BSA, to an activity of approximately 2.5×10^4 cpm/50 μl; 100 μl of bacterial suspension was mixed with 50 μl of labeled protein and incubated for 1 h at 20°C. After this incubation, 2 ml of ice-cold KPB containing 0.1% Tween 20 was added. Supernatants were aspirated after centrifugation (1760 *g* for 15 min), and the radioactivity of cell pellets was measured in a Multigamma Counter (1260 LKB-Wallac, Turku, Finland).

Cn-I binding to lactobacilli during various phases of growth. The influence of growth phase on collagen binding of *Lactobacillus sp*, strain PF67, and *Staphylococcus aureus*, strain Cowan-1, was investigated. Strains were cultured in 2 L of medium (LCM for PF67 and TSB for *S. aureus* Cowan-1) in 5-L flasks with a 1% inoculum from overnight grown broth cultures. At indicated time intervals bacterial growth, culture pH, and collagen binding were determined.

Bacteria from various broth and agar cultures of strains B27, PF67, and *Lactobacillus reuteri* NCIMB 11951 were tested for Cn-I binding. Agar-grown bacteria were suspended from plates in KPB and washed twice with the same buffer. Cells of broth-grown strains were washed twice in KPB. The pH optimum for binding was determined for 10 strains in a pH range of 4.0–8.0. Buffers were prepared from 0.02 *M* KPB adjusted with 1 *M* of HCl or NaOH. Both experiments and washings were performed in such pH-adjusted buffers.

Protease treatments. Proteinase K (buffer pH 8.0), lysozyme (pH 5.0), pepsin (pH 4.0), trypsin (pH 8.0), and papain (pH 6.8) were added to a final concentration of 60 μg/ml to 5×10^9 cells/ml. Buffers were prepared from 0.02 *M* KPB adjusted with 1 *M* HCl or NaOH to the appropriate pH. Bacteria were washed twice with KPB to stop protease treatment and were resuspended in KPB before the binding assay was performed. Control bacterial suspensions were treated in the same way except that no enzyme was added.

Saturation and inhibition of collagen type I binding. For the saturation experiments increasing amounts of unlabeled Cn-I was added to a constant amount (2.5×10^4 cpm, approximately 10 ng of protein) of labeled Cn-I to investigate when the unlabeled protein or carbohydrate blocked binding. For inhibition studies, 100 μg of various proteins and carbohydrates were added to bacterial suspensions. Fibronectin, BSA, IgG, gelatin, D-galactose, D-mannose, and D-fucose were added as potential inhibitors to standard cell suspensions just before the labeled protein, and the mixtures were incubated at 20°C for 60 min.

Results

Collagen-I binding of fresh isolates and type culture collection strains. Binding of collagen-I is a common property among both fresh isolates and type culture collection strains of lactobacilli grown in MRS medium (Table 1 and Fig. 1). Binding properties were not restricted to any special origin of the lactobacillus strains. More than 75% of the strains proved to be collagen type I binders. The cut-off value was set at 7%.

The influence of growth conditions on collagen-I binding. Growth-phase-related binding is shown for lactobacillus strain PF67 (Fig. 2a), a fresh isolate exhibiting 25% Cn-I binding in the protein-binding assay after 16 h of growth. The result is presented in comparison with the *S. aureus* strain Cowan 1 (Fig. 2b). In both strains, pF67 and *S. aureus* Cowan I, Cn-I binding increased during the logarithmic

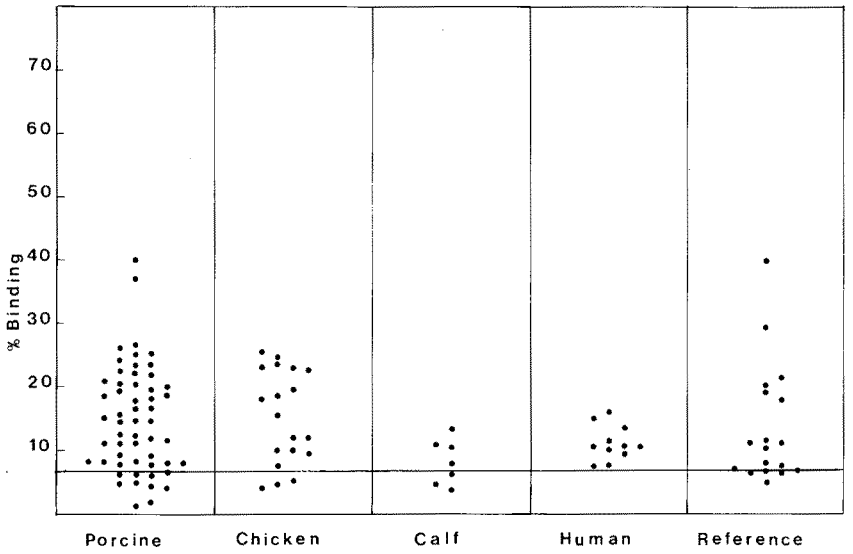

Fig. 1. The incidence of binding to Cn-I by lactobacilli isolated from different sources. Strains on or below the horizontal line are negative binders. (0–7% negative binders, 7–15% low binders, 15–25% medium binders, and <25% high binders). All fresh isolates were isolated from the small intestine of the origin species.

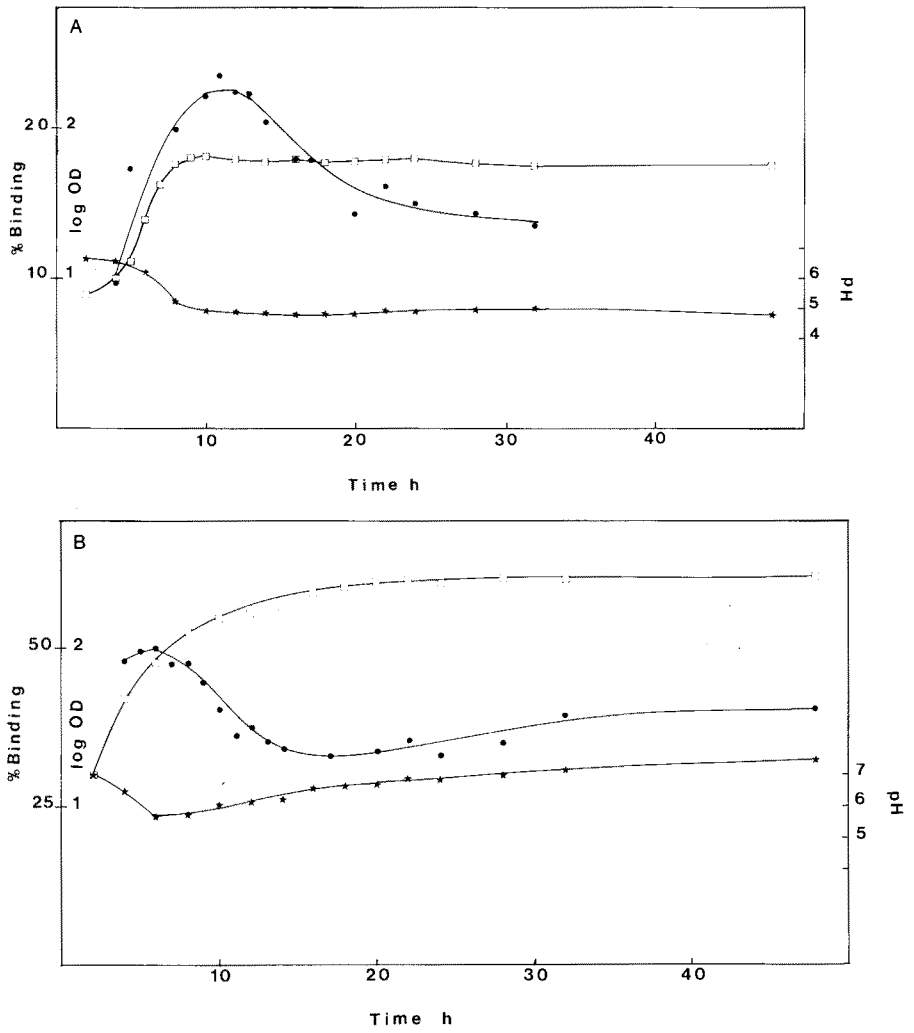

Fig. 2. Influence of growth on binding to Cn-I for *Lactobacillus sp.* PF67 (a) and *Staphylococcus aureus* Cowan-1 (b). □ indicates the optical density in log OD, ★ gives the pH curve, and ● shows the binding values in percentage binding of added labeled protein.

Table 2. The influence of media on collagen I binding of strains PF67, B27, and NCIMB 11951[a]

	Binding (%)					
	MRS		LCM			MRS
Strains	Broth	Agar	Broth	Agar	Rogosa agar	broth (48 h)
PF67	36	31	45	13	36	37
B27	37	53	31	48	44	35
L. reuteri NCIMB 11951	43	52	26	51	75	47

[a] Media-related binding for three strains, B27, PF67, and *L. reuteri* NCIMB 11951. The binding capacity of the agar-grown strains was higher for B27 and NCIMB 11951, but lower for PF67.

phase of growth. Maximum binding was observed with cells harvested in the early stationary phase for strain PF67, and in the late log phase for strain Cowan 1. Both strains showed maximum binding when the pH of the culture reached a minimum value.

To investigate the influence of culture media on the expression of Cn-I binding, we studied two types of broth and agar media (Table 2). Cn-I binding for two fresh isolates (PF67 and B27) and one reference strain *Lactobacillus reuteri* (NCIMB 11951) was compared. Binding values of broth- and agar-grown cells of MRS and LCM cultures were compared. Agar-grown bacteria showed higher binding capacity compared with broth-grown cells (there are some individual differences between the strains when the optimal binding capacity is expressed). Prolonged incubation times of broth cultures, up to 48 h, had no significant effect on Cn-I binding.

pH optimum for collagen-I binding. Since *Lactobacillus* strain PF67 showed maximum Cn-I binding when harvested in a growth phase with decreased pH value, it seemed relevant to study how pH of the assay buffer affected binding (Fig. 2a). For this purpose Cn-I binding of 10 strains was tested in assay buffers adjusted to various pH values. The binding pattern of *L. reuteri* strain NCIMB 11951 is shown as a representative for all the 10 strains (Fig. 3). All strains showed the same type of binding profile as NCIMB 11951 except that the pH optimum was different for different strains. The pH optimum for Cn-I binding for NCIMB 11951 was pH 6.8. A sharp decrease was observed above pH 7, and a more moderate decrease in binding capacity occurred below pH 7 (down to pH 4).

Other binding characteristics. Proteinase K-treated cells (strain PF67, B27 and NCIMB11951) showed a decrease in the Cn-I binding, but the binding was not affected by the other proteases after incubation at 37°C for 1 h (Table 3).

Gelatin blocked the binding completely, whereas unlabeled Cn-1 blocked up to 60% (Table 4). No sugars tested had any effect on the binding of Cn-1.

Discussion

A number of pathogens colonize mucosal surfaces, and wounded mucosal tissues have been shown to possess specific cell surface structures (usually proteins) binding fibronectin [3, 6, 9] and various types of collagens [1, 13, 15]. Recent studies by Naito and Gibbons [13] showed that certain oral bacterioides bind specifically to collagen-like, proline-rich proteins in human saliva. Our findings indicate that nonpathogenic lactobacilli, similar to tissue-invasive pathogens such as *Staphylococcus aureus* [8, 14], *Escherichia coli* [6], or noninvasive pathogens as enterotoxigenic and uropathogenic *E. coli* [22] and *Vibrio cholerae* [23], interact with specific connective tissue proteins of the host. Collagen type I binding to lactobacilli seems to be a common property in comparison with fibronectin, where we found no binding strains. The high number of collagen binders among type culture collection strains indicates that Cn-I binding is a stable genetic property.

Binding of Cn-I was specific, since only an excess of native or denatured homologous protein blocked Cn-I binding while heterologous proteins, various carbohydrates, or the detergent Tween 20 failed to inhibit binding. Blocking by denatured collagen (i.e., gelatin) has also been shown for *S. aureus* [18]. Various culture conditions drastically alter the expression of collagen binding. Agar-grown cells usually bound around 15–25% more Cn-I than broth-grown cells.

Though maximum binding occurs in the late logarithmic to early stationary phase of growth, more than half of the binding capacity is relatively stable over a period of 30 h in broth culture (Fig. 2a). Collagen-binding, proteinase K-sensitive surface components are expressed under various growth conditions and over a wide pH range. Maximum binding is expressed when pH drops below 5. However, a neutral pH value is preferable for maximum binding (pH 6.8 for *L. reuteri* NCIMB 11951). It seems as if the optimum pH for expression of the interaction differs from the optimum for binding ac-

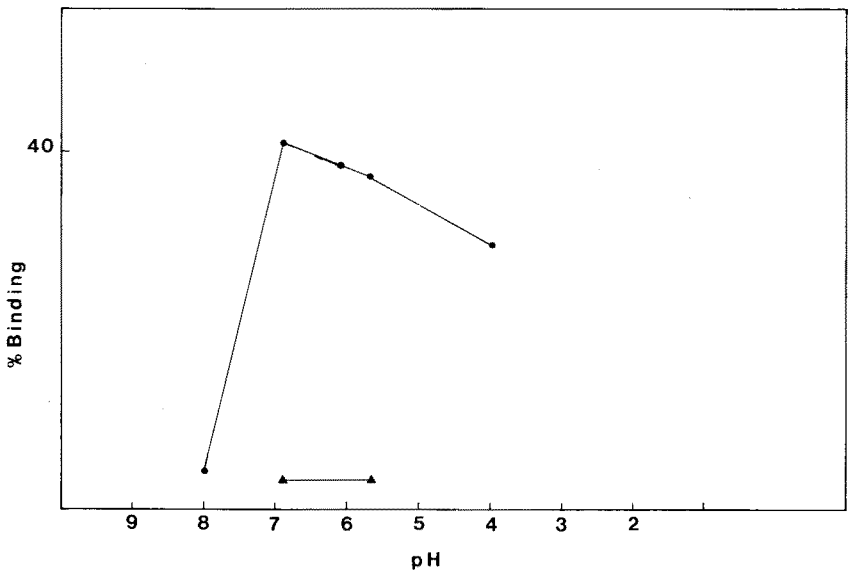

Fig. 3. Influence of pH on binding of Cn-I to cells of *L. reuteri* NCIMB 11951. The ▲—▲ indicates the interval where the other strains had their pH maximum for binding collagen type-I. Except for the optimum, all the curves had a similar appearance.

Table 3. Effect of protease and lysozyme treatments on PF67, B27, and *L. reuteri* NCIMB 11951[a]

Strains	% Relative binding		
	B27	PF67	NCIMB 11951
Control	100	100	100
Proteinase K	14	31	26
Trypsin	99	110	111
Pepsin	104	120	106
Papain	104	116	99
Lysozyme	96	101	98

[a] Effect of protease-treated lactobacilli (B27, PF67, and NCIMB 11951) and their ability to bind Cn-I. Proteases and lysozyme were added to a final concentration of 60 μg/ml. Proteinase K was the only protease that decreased the Cn-I binding abilities.

Table 4. The blocking effect of some proteins and sugars on strain PF67, B27, and *L. reuteri* NCIMB 11951[a]

	Amount of added agent	Relative binding of strains (%)		
		B27	PF76	NCIMB 11951
Control		100	100	100
Cn-I	100 μg	84	58	73
Gelatin	100 μg	10	29	41
BSA	10 μg	98	102	113
Fibronectin	100 μg	113	118	109
IgG	100 μg	109	NT	NT
D-galactose	100 μg	102	96	105
D-mannose	100 μg	110	96	100
D-fucose	100 μg	91	98	96
Tween 20	1%	92	104	98

Cn-I, Collagen type I; NT, not tested.

[a] Effect of various proteins and sugars on binding of Cn-I for three strains B27, PF67 and *L. reuteri* NCIMB 11951. The proteins or sugars were added at the same time as the radiolabeled Cn-I.

tivity. Gullmar et al. [7] and Dickson and Koohmaraie [5] studied cell surface hydrophobicity and charge and the possible relationship of these properties in the colonization of the intestinal tract of animals and attachment to meat surfaces. Our finding that specific collagen binding is common among lactobacilli of various origins may suggest that these microbes are adapted to colonize various tissues by interacting with collagens or collagen-like, proline-rich proteins in the mucin layer in a similar manner as certain oral bacterioides interact with salivary proteins in the oral cavity [13, 19].

Further work on the isolation and characterization of the collagen-binding, proteinase K-sensitive surface component of *Lactobacillus reuteri* NCIMB 11951 is now in progress in our laboratory.

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Literature Cited

1. Ascencio F, Aleljung P, Olusanya O, Wadström T (1990) Type I and IV collagen and fibrinogen binding to aeromonas

species isolated from various infections. Zbl Bakt 273:186–194
2. Axelsson L, Chung TC, Dobrogosz WJ, Lindgren SE (1989) Discovery of a broad spectrum antimicrobial substance produced by *Lactobacillus reuteri*. Microb Ecol Health Dis 2:131–136
3. Baloda SB, Faris A, Fröman G, Wadström T (1985) Fibronectin binding to Salmonella strains. FEMS Microbiol Lett 28:1–5
4. Borriello SP, Welch AR, Barclay FE, Davies HA (1988) Mucosal association by *Clostridium difficile* in the hamster gastrointestinal tract. J Med Microbiol 25:191–196
5. Dickson JS, Koohmaraie M (1989) Cell surface charge characteristics and their relationship to bacterial attachment to meat surfaces. Appl Environ Microbiol 55:832–836
6. Fröman G, Switalski LM, Faris A, Wadström T, Höök M (1984) Binding of *Escherichia coli* to fibronectin; a mechanism of tissue adherence. J Biol Chem 259:14899–14905
7. Gullmar B, Hjerten S, Wadström T (1988) Cell surface charge of lactobacilli and enterococci isolated from pig small intestine as studied by free zone electrophoresis: a methodological study. Microbios 55:183–192
8. Holderbaum D, Hall GS, Ehrhart LA (1986) Collagen binding to *Staphylococcus aureus*. Infect Immun 54:359–364
9. Höök M, Switalski LM, Wadström T, Lindberg M (1989) Interactions of pathogenic microorganisms with fibronectin. In: Mosher DF (ed) Biology of extracellular matrix: fibronectin. New York: Academic Press, pp. 295–308
10. Jones GW, Rutter JM (1974) The association of K88 antigen with haemagglutinating activity in porcine strains of *Escherichia coli*. J Gen Microbiol 84:135–144
11. Jonsson P, Lindberg M, Haraldsson I, Wadström T (1985) Virulence of *Staphylococcus aureus* in a mastitis model: studies of alpha hemolysin, coagulase and protein A as possible virulence determinants with protoplast fusion and gene cloning. Infect Immun 49:765–769
12. Markwell MAK (1982) A new solid state reagent to iodinate proteins. Anal Biochem 125:427–432
13. Naito Y, Gibbons RJ (1988) Attachment of *Bacterioides gingivalis* to collagenous substrata. J Dent Res 67:1075–1080
14. Ohtomo T, Yoshida K (1988) Adhesion of *Staphylococcus aureus* to fibrinogen, collagen and lectin in relation to cell surface structure. Zbl Bakt Hyg 268:325–340
15. Paulsson M, Wadström T (1990) Vitronectin and type-I collagen binding by *Staphylococcus aureus* and coagulase-negative staphylococci. FEMS Microbiol Immunol 65:55–62
16. Pedersen K, Tannock GW (1989) Colonization of the gastrointestinal tract by lactobacilli. Appl Environ Microbiol 55:279–283
17. Silva M, Jacobus NV, Deneke C, Gorbach SL (1987) Antimicrobial substance from a human *Lactobacillus* strain. Antimicrob Agents Chemother 31:1231–1233
18. Switalski LM, Speziale P, Höök M (1989) Isolation and characterization of a putative collagen receptor from *Staphylococcus aureus* strain Cowan I. J Biol Chem 35:21080–21086
19. Thiel S, Reid KBM (1989) Structures and functions associated with the group of mammalian lectins containing collagen-like sequences. FEBS 250(1):77–78
20. Visai L, Speciale P, Bozzini S (1990) Binding of collagens to an enterotoxigenic strain of *Escherichia coli*. Infect Immun 58:449–455
21. Wadström T, Andersson K, Sydow M, Axelsson L, Lindgren S, Gullmar B (1987) Surface properties of lactobacilli isolated from the small intestine of pigs. J Appl Bacteriol 62:513–520
22. Westerlund B, Kuusela P, Risteli J, Vartio T, Rauvala H, Virkola R, Korhonen T (1989) The O75X adhesin of uropathogenic *Escherichia coli* is a type IV collagen-binding protein. Mol Microbiol 3:329–337
23. Wiersma EJ, Fröman G, Johansson S, Wadström T (1987) Carbohydrate specific binding of fibronectin to *Vibrio cholerae* cells. FEMS Microbiol Lett 44:365–369