Collagen binding by vaginal aggregative lactobacilli

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ABSTRACT: Ten autoaggregating vaginal Lactobacillus strains (five of these strains were selected among isolates from sows’ vaginal swabs and the other five among isolates from cows’ vaginal swabs) were investigated for their ability to bind type I collagen (Cn-I). All 10 autoaggregating strains in the range of A595 readings 0.118–1.806 bound to immobilised Cn-I (at concentration of 100 μg/ml) in wells of microtitre plates, however, Lactobacillus acidophilus SV31 was much more adherent than the rest of the tested strains. The influence of culture medium on Cn-I binding was confirmed only in 50% of the tested strains when agar-grown cells bound significantly more Cn-I than broth-grown cells. The specificity of the binding was confirmed since the Cn-I binding by lactobacilli was abolished after their preincubation with this protein. The effect of heparan sulphate and hyaluronic acid was tested on 5 vaginal strains displaying the best Cn-I binding in microtitre plates after their cultivation on MRS agar plates. Both selected inhibitors significantly (P < 0.001 or P < 0.01) reduced Cn-I binding by the majority of strains. The presence of the gene coding APF (aggregation-promoting factor) was detected in seven strains (all five sows’ and two cows’ Lactobacillus strains) by PCR.

Keywords: vaginal Lactobacillus; collagen; aggregation; extracellular matrix; probiotic use

INTRODUCTION

The presence of lactobacilli as a constituent of normal microflora of the reproductive tract is generally considered to be beneficial to their host. It is known that autoaggregation is one of the important lactobacillar activities in the human vagina (Kmet’ and Lucchini, 1997). It can also be connected with the ability of lactobacilli to exclude intestinal or urogenital pathogens, e.g. E. coli (Puzová et al., 1994) by the mechanism of coaggregation (Kmet’ et al., 1995; Kmet’ and Lucchini, 1999). That is why Lactobacillus strains with autoaggregation ability were selected for Cn-I binding experiments. One of the factors mediating autoaggregation is APF (aggregation-promoting factor) described earlier by Reniero et al. (1992) which was also screened in our selected vaginal Lactobacillus strains.

It is known that ECM (extracellular matrix) may serve as a substrate not only for the adhesion of the cells of the host organism but also for the attachment of colonizing microorganisms. Many microorganisms express cell surface adhesins that mediate microbial adhesion to the ECM of host tissues (Ljungh and Wadström, 1995). Bacterial binding to ECM proteins is also considered as a virulence factor in pathogens, however, only a few reports offer an evidence of this fact. For example, the binding of S. aureus to collagen was described as the virulence factor in experimental endocarditis (Hienz et al., 1996).

One third of the animal protein is collagen, but different tissues contain different types of collagen. For instance, type II collagen is located primarily in the cartilage while type IV collagen occurs exclusively in basement membranes (Patti et al., 1994). Type I collagen (Cn-I) is the most abundant collagen molecule in soft body tissues (Aleljung, 1994). Lactobacilli were shown to interact only with three types of collagens (type I, II and IV), however, the binding of collagen types II and IV was generally lower than the binding to Cn-I, when 80% of 110 strains bound only Cn-I (Aleljung et al., 1991; Aleljung, 1994).

Collagen binding was described in intestinal (Aleljung et al., 1991; Štyriak et al., 1999b) and oral lactobacilli (McGrady et al., 1995), however, such information should be useful also for lactobacilli considered for probiotic use in the vaginal ecosystem. From our point of view, specific strains may be selected that confer health-promoting effects with the capability to colonise the site threatened by infection. That is why we decided to investigate aggregative vaginal isolates for binding to Cn-I which is often bound by several pathogens.

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MATERIAL AND METHODS

Sources and cultivation of strains

A number of Lactobacillus strains were initially isolated from sows’ and cows’ vaginal swabs using Rogosa agar (Oxoid, Basingstoke, England) plates.

The strains of two phenotypes with different auto-aggregation abilities were isolated from these vaginal swabs – autoaggregating ones, forming a precipitate resulting in a clear solution, and non-autoaggregating ones, producing constant turbidity in a tube.

Five autoaggregating Lactobacillus strains (L. acidophilus SV22, L. acidophilus SV31, L. gasseri SV42, L. reuteri SV81 and L. reuteri SV82) were selected among isolates from sows’ vaginal swabs and the other five autoaggregating strains among isolates from cows’ vaginal swabs (signed CV1 – CV5) for our experiments. The species identification of the strains was carried out by phenotypic methods (growth at defined temperatures, carbohydrate fermentation patterns, etc.) using Anaero-23 test kits (Lachema, Brno, Czech Republic). The human intestinal strain of Lactobacillus gasseri 4B2 with a strong autoaggregative phenotype mediated by APF (aggregation-promoting factor; Lucchini et al., 1998) was obtained from the Instituto di Microbiologia stock collection as a control strain for PCR. Two growth media were examined for their influence on the expression of the surface receptors of all tested strains. Lactobacilli were grown overnight in Man-Rogosa-Sharpe (MRS) broth (Difco Laboratories, Detroit, MI, USA) and on MRS agar plates (Difco) at 37°C in 5% CO2 atmosphere.

Chemicals

Bovine serum albumin (BSA), heparan sulphate and fucoidan were purchased from Sigma Chemicals Co., St Louis (Missouri, USA). Collagen (Cn-I) was purchased from Serva (Heidelberg, Germany), crystal violet from Loba (Austria) and Nunc-Immuno microtitre plates with Maxi Sorp surface (96 wells) from Nunc International (Roskilde, Denmark). All buffers and chemicals were of analytical grade.

Microtitre plate binding assays

Nunc-Immuno microtitre 96-well plates were coated with type I collagen solution (100 µl) at a concentration of 100 µg per ml and subsequently incubated overnight at 4°C. Protein solutions were removed and plates were washed three times with PBS. Then PBS with BSA (200 µl of a 2% solution in PBS) was added to each well to prevent non-specific bacterial binding. After 2 hours incubation at 25°C, BSA was removed and wells were washed twice with PBS. Finally, bacterial suspensions (100 µl; 107 cfu per ml) of individual strains were added and the plates were incubated on an orbital platform shaker for 2 hours at 37°C. All unbound bacteria were removed by washing the wells three times with PBS. Bacteria in the wells were then fixed at 60°C for 20 minutes and stained with crystal violet (95 µl per well) for 45 minutes. Wells were subsequently washed six times with PBS to remove excessive stain. After adding 100 µl of citrate buffer (pH 4.3) to each well and 45 min incubation at room temperature to release the stain bound to bacteria, the absorbance values (A570) were determined in a Multiscan enzyme-linked immuno-sorbent assay reader and the averages of ten absorbance values were calculated. Each batch of assays also included one or more control strains with known Cn-I binding level as well as blank wells. Two Staphylococcus aureus strains (ISP 546 and Cowan 1) and Staphylococcus haemolyticus SM 131 (Paulsson and Wadström, 1990) were used as positive controls. Lactobacilli were classified as strongly adherent (A570 > 0.3), weakly adherent (0.1 < A570 < 0.3), or nonadherent (A570 < 0.1).

The specificity of binding was tested by 1-hour preincubation of bacteria with an equal volume of type I collagen solution at concentration of 100 µg per ml and subsequent washing followed by examination of bacterial binding to the same protein.

Inhibition of bacterial binding in microtitre plates

Bacterial suspensions (100 µl; 107 cfu per ml) of the selected Lactobacillus strains with the highest percentage of collagen binding (SV22, SV31, SV42, CV4 and CV5) were preincubated with an equal volume of heparan sulphate or hyaluronic acid (both inhibitors at a concentration of 1 mg/ml) for 1 hour at room temperature and incubated in microtitre plates for 2 hours at 37°C parallely with non-treated bacteria of these strains. Binding in microtitre plates was assayed as above and the averages of ten absorbance values were compared with those of the same non-treated strains.

PCR amplification

The specific PCR amplification protocol was used for chromosomal DNA extracted from overnight broth-cultures of all 10 investigated autoaggregative Lactobacillus strains according to the method described by Kmet and Lucchini (1999). Specific primers APF3 and APF4 were used as described previously by Lucchini et al. (1998) for the demonstration of apf gene. PCR reaction mixture (25 µl) contained Lactobacillus DNA (10 ng), Taq buffer 1×, Taq polymerase 1.0 U (Promega), MgCl2;
1.5 mM, dNTPs 200 μM each, primers 0.5 μM each. The human intestinal strain of Lactobacillus gasseri 4B2 (Lucchini et al., 1998) was used as positive control strain for PCR. PCR reaction mixture without template DNA was used as negative control.

PCR amplifications were carried out in a Progene (Techne, Cambridge) thermocycler. The tubes were subjected to the following thermal cycling conditions: 5 min at 94°C for one cycle, then 60 s at 94°C, 60 s at 55°C and 60 s at 72°C for 35 cycles. After cycling 10 μl of each reaction was analysed by electrophoresis on 1.5% agarose gel at 7 V/cm.

Table 1. Collagen (Cn-I) binding by 10 Lactobacillus (isolates from sows signed as SV and cows’ isolates as CV) strains cultivated on MRS agar and in MRS broth

<table>
<thead>
<tr>
<th>Strains</th>
<th>MRS agar</th>
<th>MRS broth</th>
<th>apf gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>SV 22</td>
<td>0.313 ± 0.046</td>
<td>0.234 ± 0.035</td>
<td>+</td>
</tr>
<tr>
<td>SV 31***</td>
<td>1.806 ± 0.223</td>
<td>0.669 ± 0.072</td>
<td>+</td>
</tr>
<tr>
<td>SV 42</td>
<td>0.263 ± 0.035</td>
<td>0.233 ± 0.046</td>
<td>+</td>
</tr>
<tr>
<td>SV 81</td>
<td>0.066 ± 0.026</td>
<td>0.137 ± 0.039</td>
<td>+</td>
</tr>
<tr>
<td>CV 1*</td>
<td>0.128 ± 0.015</td>
<td>0.078 ± 0.035</td>
<td>–</td>
</tr>
<tr>
<td>CV 2**</td>
<td>0.118 ± 0.023</td>
<td>0.055 ± 0.030</td>
<td>+</td>
</tr>
<tr>
<td>CV 3***</td>
<td>0.195 ± 0.050</td>
<td>0.092 ± 0.032</td>
<td>+</td>
</tr>
<tr>
<td>CV 4</td>
<td>0.165 ± 0.043</td>
<td>0.140 ± 0.033</td>
<td>–</td>
</tr>
<tr>
<td>CV 5***</td>
<td>0.213 ± 0.030</td>
<td>0.124 ± 0.036</td>
<td>–</td>
</tr>
</tbody>
</table>

Table 2. Inhibition effect of heparan sulphate and hyaluronic acid on Cn-I binding by 5 Lactobacillus strains in microtitre plates

Statistical evaluation of the results was done by one-way analysis of variance (ANOVA). Significance of differences between means was calculated by Tukey’s test.

RESULTS

Ten autoaggregating strains bound to immobilised Cn-I (at a concentration of 100 μg/ml) in the range of A_570nm readings 0.118–1.806 (Table 1). The majority of strains displayed A_570nm readings maximally up to the value 0.313. However, Lactobacillus acidophilus SV31 was much more adherent after its cultivation on MRS agar (A_570nm = 1.806) as well as in MRS broth (A_570nm = 0.669) in comparison with the rest of the tested strains.

The influence of culture medium on Cn-I binding was confirmed only in 50% of the tested strains (Table 1) when agar-grown cells bound significantly more Cn-I than broth-grown cells. Significantly higher binding of Cn-I was observed in 5 vaginal strains (SV31, CV1, CV2, CV3 and CV5) grown on MRS agar than in MRS broth-grown cells. The other 5 vaginal strains showed no significant influence of culture medium on their Cn-I binding.

The specificity of lactobacilli binding to the Cn-I was confirmed by their preincubation with this protein. Such pretreatment abolished the adhesion (Table 2).

The effect of heparan sulphate and hyaluronic acid on Cn-I binding in microtitre plates was tested on 5 selected vaginal strains with the highest percentage of collagen binding after their cultivation on MRS agar plates. Both selected inhibitors significantly (P < 0.001 or P < 0.01) reduced Cn-I binding by these strains with two exceptions (strain CV5 after heparan sulphate pretreatment and SV42 strain after its preincubation with hyaluronic acid) as shown in Table 2.
Using primers APF3 and APF4, a single product of the expected 183 bp size was obtained from the amplification of all five sows’ and two cows’ *Lactobacillus* strains (Figure 1).

**DISCUSSION**

Adherence of pathogens to the extracellular matrix of various host tissues has often been investigated, demonstrating the important role of these interactions in the establishment of many infections (Westerlund and Korhonen, 1993; Ljungh and Wadström, 1995). However, little is known about members of the indigenous microflora, including lactobacilli, and their ability to colonize epithelial lesions and to bind to ECM proteins (Aleljung et al., 1991).

The major constituents of the ECM are collagenous proteins and that is why they may represent a major target site for many microorganisms (Patti et al., 1994). The idea that also non-pathogenic bacteria might utilize an analogous strategy for colonization is especially attractive in the context of probiotic bacteria, where competitive exclusion of pathogens through occupation of common binding sites on mucosal surfaces was discussed (Reid et al., 1990). In fact, a number of interactions exists between non-pathogenic bacteria and host components. The Cn-I binding ability is also one feature of autoaggregating lactobacilli which could be considered in the selection of strains for the preparation of therapeutic products able to antagonize the colonization of Cn-I binding sites by vaginal pathogens.

Bacterial binding to collagen is affected by several factors such as culture medium, presence of different compounds, etc. The influence of culture medium on Cn-I binding was confirmed in 50% of our tested strains when agar-grown cells bound significantly more Cn-I than broth-grown cells. These results are similar to the observations of Aleljung et al. (1991) that agar-grown cells usually bind around 15–25% more Cn-I than broth-grown cells. Some earlier studies also showed that the cell envelope is dependent on the composition and form (solid or liquid) of the culture medium (Al-Hiti and Gilbert, 1983). Moreover, the expression of high molecular-weight proteins by *Staphylococcus aureus* was shown to be enhanced when the bacterium was grown on a solid medium compared with growth on the same medium in the liquid form (Cheung and Fischetti, 1988).

It was earlier confirmed that heparin and other sulphated glycosaminoglycans, for example dextran sulphate and fucoian, strongly inhibit ECM proteins to bind cells of some pathogenic strains like staphylococci (Štyriak et al., 1999a; Pascu et al., 1996), however, non-sulphated compounds do not have such effects (Štyriak et al., 1999a; Pascu et al., 1996). It indicates that the oligosaccharide structures are not as important as the density of sulphate groups on these polymers. However, hyaluronic acid, a nonsulphated compound, also significantly inhibited the binding of Cn-I to gut lactobacilli (Štyriak et al., 1999b) as well as to vaginal *Lactobacillus* strains presented here. It suggests that the sulphation of polymers is much less important for an inhibition effect on lactobacilli in comparison with pathogens. This fact should also be a promising advantage for possible use of sulphated inhibitors for a reduction of ECM binding by pathogens, however, their inhibition effects on ECM binding by beneficial microorganisms complicate these considerations. On the other hand, it should be possible to find by screening of *Lactobacillus* strains a suitable strain with high Cn-I binding ability also in the presence of inhibitors. Our hypothesis is based on the fact that large differences between individual lactobacilli were observed in the present study as far as the influence of culture medium and the effect of tested inhibitors are concerned. It suggests the occurrence of different molecules on individual strain surfac-
es. Some of such structures, for example a collagen-binding S-layer protein in Lactobacillus crispatus (Toha et al., 1995) or a 29 kDa collagen-binding protein from Lactobacillus reuteri (Roos et al., 1996) as well as other glycoprotein molecules were described previously. APF coded by apf gene is also one of such surface molecules. This gene was detected in 7 of the 10 vaginal Lactobacillus strains in our study, however, its role in Cn-I binding was not ascertained. The presence of an APF was detected in 5 vaginal Lactobacillus strains from sows and two from cows (Figure 1). However, there were also reported other aggregation-promoting factors such as a 2 kDa hydrophilic peptide produced by a Lactobacillus gasseri isolate (Boris et al., 1997) or a putative DEAD-box helicase (a 56 kDa protein) produced by a Lactobacillus reuteri strain 1063 (Roos et al., 1999). It means that various factors are responsible for aggregation of lactobacilli which should be important in the vaginal ecosystem. It is probable that the presence and expression of aggregation-promoting factor have no coherence with Cn-I binding by Lactobacillus strains, however, from our point of view both properties are useful for probiotic strains.

We would like to note in conclusion that the L. acidophilus SV31 with the highest percentage of collagen binding was chosen for a field experiment and it is nowadays applied to sows’ vaginas with the aim to decrease the number of pathogens in their reproductive tracts. This experiment should confirm the ability of the L. acidophilus SV31 to antagonize colonization of Cn-I binding sites by vaginal pathogens also in vivo and to support our contention about the convenience of Cn-I binding lactobacilli in probiotic preparations.

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