Extracellular proteins of *Lactobacillus crispatus* enhance activation of human plasminogen

Veera Hurmalainen,^{1†} Sanna Edelman,^{1†} Jenni Antikainen,¹
Marc Baumann,² Kaarina Lähteenmäki¹ and Timo K. Korhonen¹

Correspondence

Timo K. Korhonen

timo.korhonen@helsinki.fi

¹General Microbiology, Faculty of Biosciences, PO Box 56, FIN00014 University of Helsinki, Finland

²Protein Chemistry Unit, Institute of Biomedicine/Anatomy, PO Box 63, FIN00014 University of Helsinki, Finland

The abundant proteolytic plasminogen (Plg)/plasmin system is important in several physiological functions in mammals and also engaged by a number of pathogenic microbial species to increase tissue invasiveness or to obtain nutrients. This paper reports that a commensal bacterium, *Lactobacillus crispatus*, interacts with the Plg system. Strain ST1 of *L. crispatus* enhanced activation of human Plg by the tissue-type Plg activator (tPA), whereas enhancement of the urokinase-mediated Plg activation was lower. ST1 cells bound Plg, plasmin and tPA only poorly, and the Plg-binding and activation-enhancing capacities were associated with extracellular material released from the bacteria into buffer. The extracellular proteome of *L. crispatus* ST1 contained enolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as major components. The enolase and the GAPDH genes of ST1 were cloned, sequenced and expressed in recombinant *Escherichia coli* as His₆-fusion proteins, which bound Plg and enhanced its activation by tPA. Variable levels of secretion of enolase and GAPDH proteins as well as of the Plg activation cofactor function were detected in strains representing major taxonomic groups of the genus *Lactobacillus*. So far, interference with the Plg system has been addressed with pathogenic microbes. The results reported here demonstrate a novel interaction between a member of the microbiota and a major proteolytic system in humans.

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INTRODUCTION

The plasminogen (Plg)/plasmin system is important in a wealth of physiological and pathological processes in mammals, and is also utilized by several microbial pathogens for migration within the host and to fulfil nutritional demands (reviewed by Castellino & Ploplis, 2005; Lähteenmäki *et al.*, 2001, 2005; Myöhänen & Vaheri, 2004; Plow *et al.*, 1995). Plg circulates at a high concentration, around 180–200 µg ml⁻¹, in human plasma, and is also present in milk and other secretions (Myöhänen & Vaheri, 2004; Wang *et al.*, 2006). The liver is the primary tissue that synthesizes the proenzyme Plg. However, other identified tissue sources for Plg synthesis are numerous and include the intestine (Zhang *et al.*, 2002). Plg activators

(PAs) convert Plg into plasmin, which is a powerful serine protease whose major biological function is to dissolve fibrin clots. Plasmin is also involved in remodelling of vascular tissue, enhancement of cellular migration and damage of tissue barriers, initiation of autoimmune diseases, as well as in processes affecting pathogen susceptibility and inflammation, wound healing and neurologically related processes (Myöhänen & Vaheri, 2004; Plow *et al.*, 1995). In accordance with the central role of localized plasmin activity in the metastasis of tumour cells through basement membranes into secondary tissue sites, the research on microbe–Plg interactions has exclusively been done in connection with invasive bacterial infections. Indeed, the Plg system has been found to be critical for tissue and organ invasion by several severe pathogens (Coleman & Benach, 1999; Lähteenmäki *et al.*, 2001, 2005).

The Plg/plasmin system is tightly controlled under normal physiological conditions (Longstaff & Thelwell, 2005; Myöhänen & Vaheri, 2004). Mammals have two PAs, tissue-type Plg activator (tPA) and urokinase (uPA), which cleave Plg at a single site, thus forming the two-chain plasmin molecule joined via two disulfide bonds. Plg is hardly susceptible to activation without conformational or

†These authors contributed equally to this work.

Abbreviations: α₂AP, α₂-antiplasmin; EACA, ε-aminocaproic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IEM, immunoelectron microscopy; PA, Plg activator; Plg, plasminogen; tPA, tissue-type Plg activator; uPA, urokinase.

The GenBank/EMBL/DDBJ accession numbers for the nucleotide sequences of *L. crispatus* ST1 *gap* and *eno* determined in this paper are AJ849470 and AJ849471.

proteolytic modification. Plg is immobilized onto lysine-containing Plg/plasmin receptors on bacterial and mammalian cell surfaces and onto lysine-containing cofactors such as fibrin, components of the extracellular matrix, denatured mammalian proteins and small molecule ligands (Castellino & Ploplis, 2005; Longstaff & Thelwell, 2005; Lhtenmki *et al.*, 2001). Immobilization is mediated by five so-called kringle domains of Plg and alters the conformation of Plg so that it becomes more susceptible to activation, in particular by tPA (Mangel *et al.*, 1990). The serine protease domain is responsible for the proteolytic activity of plasmin (Longstaff & Thelwell, 2005). The primary circulating inhibitor of plasmin is the antiprotease α_2 -antiplasmin (α_2 AP), which binds to the kringle domains and effectively inactivates soluble plasmin. When Plg/plasmin is bound to the cell surface or fibrin, its lysine-binding sites are occupied and α_2 AP acts more slowly. Microbial pathogens that utilize the Plg system for migration in the host overcome the control by cleaving α_2 AP or by immobilizing Plg on bacterial surface receptors (Lhtenmki *et al.*, 2005). Bacteria can also produce PAs; this has been detected in *Yersinia pestis* and *Salmonella enterica*, which express surface-bound proteolytic activators, as well as in staphylococci and streptococci, which produce secreted non-proteolytic activators called staphylokinase and streptokinase (Coleman & Benach, 1999; Lhtenmki *et al.*, 2001, 2005).

The identified bacterial Plg receptors are multifunctional surface proteins (Lhtenmki *et al.*, 2001) and include cell-wall-associated proteins previously assigned to metabolic functions only. The glycolytic enzymes enolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are cytoplasmic proteins that are also expressed on the surface of several, mainly Gram-positive, bacterial pathogens, as well as on fungal cells, parasites and mammalian cells. Surface-associated forms of bacterial enolase and GAPDH immobilize Plg and in this way enhance its activation; they also exhibit adhesive functions that may have a role in bacteria–host interactions (Bergmann *et al.*, 2001, 2003; Broeseker *et al.*, 1988; Chhatwal, 2002; Coleman & Benach, 1999; Lhtenmki *et al.*, 2001, 2005).

Species of the genus *Lactobacillus* are important members of the indigenous microbiota in the human intestine and genitourinary tract, which has aroused interest in their role as health-promoting, probiotic organisms (Mercenier *et al.*, 2003). The presence of a biologically functional extracellular proteome in commensal lactobacilli has been indicated by recent findings showing that proteins in the conditioned, cell-free culture medium of *Lactobacillus rhamnosus* GG modulate signal transduction pathways and prevent cytokine-induced apoptosis and heat-shock protein expression in human and mouse intestinal cell lines (Tao *et al.*, 2006) as well as decrease chemokine production in mouse macrophages (Pea & Versalovic, 2003). Recently, GroEL, a cytoplasmic heat-shock protein, was also shown to be present on the surface and in the cell-free culture medium of

Lactobacillus johnsonii NCC 533 and found to have adhesive and immunomodulatory effects in human and murine cells (Bergonzelli *et al.*, 2006). A recent *in silico* analysis of the genome of *Lactobacillus plantarum* predicted the existence of 57 proteins that are secreted into the medium or remain associated with the cell wall by unknown anchoring mechanisms (Boekhorst *et al.*, 2006). Here, we describe the presence of extracellular forms of the glycolytic enzymes enolase and GAPDH in *Lactobacillus crispatus* as well as their functions as Plg activation cofactors. These results further indicate the importance of extracellular proteins in the cross-talk between lactobacilli and their hosts, as well as demonstrating a novel variant in bacteria–Plg interaction mechanisms.

METHODS

Bacteria. Strain ST1 of *L. crispatus* was isolated from chicken faeces and shows adhesiveness to chicken and human cells (Edelman, 2005; Edelman *et al.*, 2002). *Lactobacillus acidophilus* E507 (Miettinen *et al.*, 1996), *Lactobacillus amylovorus* JCM 5807 (Mitsuoka, 1969), *Lactobacillus gallinarum* T-50, *Lactobacillus gasseri* JCM 1130/ATCC 19992 and *Lactobacillus johnsonii* F133 (Fujisawa *et al.*, 1992), *Lactobacillus rhamnosus* GG (ATCC 53103; Miettinen *et al.*, 1996; Pea & Versalovic, 2003; Tao *et al.*, 2006), *Lactobacillus paracasei* E506 and *Lactococcus lactis* subsp. *cremoris* E523 (Miettinen *et al.*, 1996) have been described. The bacteria were cultivated overnight at 37 °C in static MRS broth (Difco; BD Biosciences). After cultivation, the bacteria were collected, washed with phosphate buffered saline (PBS), pH 7.1, and used for the assays. For analysing lactobacillar cell-free extracellular material, washed cells were incubated in PBS at 37 °C for 1–5 h. After incubation, cells were removed by centrifuging, and the PBS supernatant was filtered through a 0.45 µm membrane.

Cloning, overproduction and purification of recombinant GAPDH and enolase. The primers for amplification of internal sequences of *L. crispatus* ST1 *gap* and *eno* genes were designed on the basis of the *gap* gene in *Lactococcus lactis* subsp. *lactis* II1403 (Bolotin *et al.*, 2001) and the *eno* gene in *L. johnsonii* NCC 533 (Pridmore *et al.*, 2004; locus tag LJ1416). The amplicons were sequenced by using an ABI PRISM 310 Genetic Analyser (Perkin Elmer Life and Analytical Sciences), and the information was then used in sequencing the entire *eno* and *gap* genes from the chromosomal DNA of *L. crispatus* ST1. Genes encoding *L. crispatus* ST1 GAPDH and enolase were cloned into the pQE30 expression system (Qiagen) for expression as N-terminal His₆-fusions, which were purified by the Qiaexpress Protein Purification System (Qiagen) under non-denaturing conditions. The purified proteins were extensively dialysed against PBS before use.

Subcellular localization of enolase and GAPDH. Antisera against the purified His₆-GAPDH and His₆-enolase of ST1 were raised in rabbits using routine immunization procedures (Medprobe, Viikki Laboratory Animal Center, University of Helsinki). Preimmunization serum was collected before primary immunization. For immunoelectron microscopy (IEM), IgG molecules were purified from the preimmune and the hyperimmune sera by affinity chromatography using Protein A Sepharose CL-4B (Pharmacia LKB Biotechnology). For studying the extracellular GAPDH and enolase, the cell-free extracellular material from 3×10^8 bacteria was precipitated with trichloroacetic acid after incubating cells at 37 °C for 5 h; the proteins were then separated by electrophoresis in 12% (w/v) SDS-PAGE gels and transferred onto 0.2 µm nitrocellulose membrane for Western blotting with the anti-His₆-enolase and the

anti-His₆-GAPDH antibodies. As a marker for a cytoplasmic peptide (Alvarez *et al.*, 2003), IgG specific for the RNA polymerase β 1-subunit (NeoClone) was used. For cell lysis, ST1 cells in 1 ml PBS were treated with mutanolysin (50 U ml⁻¹; Sigma Aldrich) and lysozyme (20 mg ml⁻¹; Roche Diagnostics) at 37 °C for 2 h; cells were then lysed by boiling for 20 min. Lysate from 3×10^8 cells was used for Western blotting, and the relative amounts of enolase and GAPDH in the cell and supernatant samples were assessed using the TINA 2.0 program (Isotopenmessgeräte). Localization of GAPDH and enolase on the surface of *L. crispatus* ST1 was studied by routine post-embedding IEM labelling methods. The washed bacteria were immediately fixed in 0.1 mM sodium acetate buffer (pH 5.0) containing 4% (w/v) paraformaldehyde and 0.1% glutaraldehyde for 2 h at room temperature and washed with sodium acetate buffer prior to embedding into LR-White resin. After polymerization, ultrathin sections were cut and collected onto carbon-coated 200-mesh nickel grids. Sections were incubated on drops of diluted (1:30 in PBS) anti-His₆-GAPDH-IgG and anti-His₆-enolase-IgG containing 2% (w/v) bovine serum albumin (BSA; Sigma, Aldrich), 0.1% Tween 20 and 0.1% fish skin gelatin (Sigma Aldrich) in 0.1 M sodium phosphate buffer (pH 7.1) for 3.5 h at room temperature, and were then washed five times in sodium phosphate buffer prior to incubation on drops of 1:80 diluted protein A colloidal gold (10 nm in size) for 30 min. After washing, the sections were post-stained in uranyl acetate and lead citrate before examination in a JEOL EXII transmission electron microscope. For enzyme activity measurements, extracellular material from 5×10^9 bacteria was used after incubating cells in 50 mM Tris/HCl (pH 8.0) at 37 °C for 2 h. The GAPDH activity was measured as described by Pancholi & Fischetti (1992). The enolase activity was measured by coupled assay (Pancholi & Fischetti, 1998) using 3-phosphoglycerate (Fluka Chemie) as substrate and phosphoglycerate mutase for converting substrate to 2-phosphoglycerate in 50 mM Tris/HCl (pH 8.0). The reactions were allowed to occur for 15 min.

Binding and activation of plasminogen. The tPA- and uPA-mediated activation of Plg in the presence of bacteria or bacterial components was measured essentially as described by Lähteenmäki *et al.* (1995). Briefly, cell-free extracellular material from 3×10^8 bacteria, bacterial cells, or recombinant His₆-GAPDH and His₆-enolase (both 137 nM) were incubated with 4 µg human or bovine Glu-Plg (American Diagnostica), 2 ng tPA (Biopool) or uPA (American Diagnostica) and 0.45 mM chromogenic substrate of plasmin S-2251 (Kabivitrüm) in a final volume of 200 µl, and increase in plasmin activity was assessed at intervals by measuring absorbance at 405 nm. In inhibition tests, 4 µg α_2 AP (Calbiochem), 0.09 TIU apro-tinin (Sigma Aldrich) or 1 mM ϵ -aminocaproic acid (EACA; Sigma Aldrich) were included. The proportions of bacterium-bound plasmin activity and plasmin activity in the supernatant were determined by incubating 3×10^8 bacteria in PBS with Plg and tPA for 5 h at 37 °C in the presence or absence of α_2 AP. After incubation, the cells and the supernatant were separated, the cells were washed once with PBS, and both fractions were incubated for 3 h in a final volume of 200 µl PBS containing 0.45 mM S-2251. For analysing protection from α_2 AP, plasmin (109 nM; Fluka Chemie) was incubated with the ST1 extracellular material for 15 min at room temperature prior to adding S-2251 and α_2 AP in increasing concentrations. Plasmin activity was measured after 1 h incubation at 37 °C.

Binding of ¹²⁵I-labelled Plg, plasmin and tPA on the bacterial surface was tested essentially as described by Kukkonen *et al.* (1998). After labelling with ¹²⁵I (Amersham Biosciences), the specific activities obtained were 3.5×10^6 c.p.m. µg⁻¹ for Glu-Plg, 1.0×10^6 c.p.m. µg⁻¹ for plasmin and 2.6×10^6 c.p.m. µg⁻¹ for tPA. Bacteria (2×10^9 cells) were incubated for 2 h at 37 °C with 20 ng ¹²⁵I-labelled Plg, plasmin, or tPA in the presence or absence of 4 mM EACA in a volume of 500 µl. Bacteria were collected, washed twice with PBS and the bound

radioactivity was measured. For analysing binding of Plg and plasmin as well as conversion of Plg to plasmin on the surface of ST1, bacteria (1.6×10^8 cells in 100 µl PBS) were incubated for 5 h at 37 °C with 10 µg Plg in the presence or absence of 5 ng tPA, or with 10 µg plasmin. After incubation, the cells and the supernatant were separated, the cells were washed once, and both fractions were analysed by Western blotting with anti-Plg IgG (American Diagnostica) and secondary antibodies.

The binding of Plg and plasmin to extracellular material from 3×10^8 bacteria as well as to purified His₆-GAPDH and His₆-enolase (tested at 180 nM) was measured by time-resolved fluorometry as described by Kukkonen *et al.* (1998). Briefly, polystyrene microtitre plates were coated in PBS with extracellular material, enolase, GAPDH, or the S-layer protein from *L. crispatus* ST1; the latter was purified with guanidine hydrochloride as described by Antikainen *et al.* (2002). Laminin-coated surface was used as a positive control for Plg binding. One microgram of Plg or plasmin was added in 100 µl PBS/0.1% Tween 20, in the presence or absence of 10 mM EACA. For detection of the binding, anti-Plg IgG (720 ng per well) and Eu³⁺-labelled anti-rabbit IgG (80 ng per well; PerkinElmer Life and Analytical Sciences) were used. For analysing proteolytic cleavage and release of enolase and GAPDH from the cell surface, ST1 cells (2×10^9 ml⁻¹) were incubated with 20 µg plasmin ml⁻¹ for 5 h at 37 °C. The cells were removed and the supernatant was analysed by Western blotting with anti-His₆-enolase and anti-His₆-GAPDH IgGs.

RESULTS

Cellular location of enolase and GAPDH in *L. crispatus* ST1

To detect the extracellular proteome in *L. crispatus* ST1, peptides released from ST1 cells into PBS were analysed by SDS-PAGE and Western blotting. Washed ST1 cells were suspended into PBS and incubated for 5 h at 37 °C, the cells were pelleted, and the supernatant was filtered to completely remove any remaining cells. The major extracellular peptides detectable by Coomassie blue staining (Fig. 1a) included a peptide of 47 kDa in apparent molecular mass and a peptide of 38 kDa. The 38 kDa peptide was excised from the gel and its N-terminal amino acid sequence was determined. The sequence obtained, TVKIGINGFGRI-GRLAFRRRL, has 85–100% identity with the amino-terminal sequence of GAPDHs sequenced from species of *Lactobacillus* and *Lactococcus* (Altermann *et al.*, 2005; Bolotin *et al.*, 2001; Kleerebezem *et al.*, 2003; Pridmore *et al.*, 2004). In Western blotting, this peptide reacted with the antiserum raised against His₆-GAPDH cloned from ST1 and purified from recombinant *E. coli* (Fig. 1a; see below), giving further proof that the peptide indeed was GAPDH. Enolase is another glycolytic enzyme detected on the bacterial surface, and we indeed identified the 47 kDa peptide as enolase by immunoblotting with specific antisera raised against enolase from *Streptococcus pneumoniae* (Bergmann *et al.*, 2001) as well as against the His₆-enolase cloned from ST1 and purified from recombinant *E. coli* in this study (Fig. 1a; see below for cloning details).

Western blotting analysis of enolase and GAPDH in the supernatant over time revealed a gradual increase in their amounts (Fig. 1b). After 5 h incubation, the amounts of

Fig. 1. Subcellular localization of GAPDH and enolase in *L. crispatus* ST1. (a) Extracellular proteins of *L. crispatus* ST1 cells incubated in PBS for 5 h. Peptides released from 3×10^8 cells were precipitated with trichloroacetic acid and detected in Coomassie-stained SDS-PAGE as well as by Western blotting with IgGs raised against enolase, GAPDH or RNA-polymerase β -subunit; the latter was used to detect possible cell lysis. (b) Time-course of the release of enolase and GAPDH into PBS. Washed *L. crispatus* ST1 cells were suspended into PBS, and the 0 h sample of the buffer for Western blotting was taken immediately. The other time points are indicated. For comparison, detection of enolase and GAPDH in bacterial cells lysed after the 5 h incubation are shown on the right. (c) IEM images of localization of GAPDH and enolase in washed ST1 cells. The detection was post-cutting using anti-His₆-GAPDH and anti-His₆-enolase IgGs and protein A-gold particles. The arrows indicate binding to the cell wall. Reactivity of IgGs from the hyperimmune and the preimmune sera is shown. Bars, 100 nm.

enolase and GAPDH in the supernatant were 22 % and 20 % of their amounts in lysed cell samples. During the 5 h incubation, the pH of the buffer changed from 7.1 to 6.8, indicating that the bacteria had a weak metabolic activity in PBS, and the number of viable cells slightly decreased from 5×10^8 to 3×10^8 ml⁻¹. No viable cells were recovered upon cultivation of 100 μ l samples from the filtered buffers. Microscopic examination of the cell suspensions did not reveal detectable cell lysis or cell damage after the 5 h incubation, neither did we detect DNA in the cell-free buffer (data not shown). Furthermore, the cytoplasmic marker

protein RNA polymerase β 1-subunit (Alvarez *et al.*, 2003) was detectable in the lysed cell sample but not in the cell-free buffer (Fig. 1a). Enolase and GAPDH enzymic activities were detected in the cell-free extracellular material (data not shown). Next, we used IgGs raised against His₆-enolase and His₆-GAPDH (see below) in IEM of washed ST1 cells. Both IgGs bound to antigens in the cytoplasm as well as in the cell wall, and the binding of IgGs from the preimmune sera was significantly poorer (Fig. 1c). In particular, IEM revealed that a fraction of enolase and GAPDH antigens are within or in close proximity to the cell membrane (Fig. 1c). It was

concluded that enolase and GAPDH of *L. crispatus* ST1 are present in the cellular cytoplasm and in the cell wall and also represent major components in the extracellular proteome of *L. crispatus* ST1.

Interaction of Plg by the extracellular material from *L. crispatus* ST1

Considering that streptococcal enolase and GAPDH bind Plg, we next assessed the Plg receptor function in ST1 cells and in the material released into PBS. Washed ST1 cells

Fig. 2. Binding and conversion of Plg and plasmin by *L. crispatus* ST1 cells. (a) Kinetic measurement of Plg activation in the presence of ST1 cell suspension (4×10^7 cells). Plg with tPA ($\blacktriangle$) or uPA ($\triangle$) was incubated with ST1 cells, and the formation of plasmin activity was measured using the chromogenic substrate of plasmin. (b–d) Binding of (b) 125 I-plasmin, (c) 125 I-Plg and (d) 125 I-tPA to ST1 cells. Bacteria were incubated with 125 I-labelled protein in the absence (grey columns) or presence (open columns) of the lysine analogue EACA. (e) The conversion of the single-chain Plg to the two-chain plasmin in the presence or absence of ST1 cells and tPA. The bacteria (1.6×10^8 cells) were incubated with Plg in the presence or absence of tPA, or with plasmin for 5 h. The cells and supernatant were separated and Plg and plasmin were analysed from both fractions by Western blotting using anti-Plg antibodies. (f) Distribution of plasmin activity between cells and buffer. Bacteria (3×10^8 cells) were incubated in PBS with Plg and tPA for 5 h, the cells and the supernatant were separated, and both fractions were incubated with chromogenic substrate of plasmin. Plasmin activity was measured at 3 h. The means and standard deviations from two independent assays with quadruplicate samples in (a) and (f) and with triplicate samples in (b), (c) and (d) are shown.

enhanced activation of human Plg by both tPA and uPA (Fig. 2a); the effect on tPA-mediated activation was clearer than that on uPA-mediated catalysis, and no Plg activation by tPA was seen in the absence of ST1 cells (Fig. 2a). The enhancement of tPA-mediated activation of bovine Plg was similar to that of human Plg (data not shown). The surface association of the plasmin generated in the presence of ST1 cells was then assessed by three approaches: (i) by analysing the binding of radiolabelled Plg, plasmin and tPA onto ST1 cells, (ii) by analysing the conversion of the single-chain Plg molecule into the two-chain plasmin, and (iii) by determining the distribution of plasmin enzymic activity between cells and buffer.

The binding of 125 I-plasminogen, 125 I-plasmin, and 125 I-tPA onto the surface of washed *L. crispatus* ST1 cells was assessed using conditions commonly used with pathogenic bacteria by us and others (Bergmann *et al.*, 2001; Kukkonen *et al.*, 1998; Kuusela & Saksela, 1990; Lhtenmki *et al.*, 1995; Pancholi & Fischetti, 1992). The level of Plg binding onto ST1 cells was low, in independent assays corresponding to 1–3% of the added amount of Plg (Fig. 2c), whereas the binding of plasmin was approximately two- to threefold higher (Fig. 2b). Radiolabelled tPA showed only negligible binding onto ST1 cells (Fig. 2d). The binding of both plasmin and Plg onto ST1 cells was significantly inhibited by EACA (Fig. 2b, c). EACA is a lysine analogue and an inhibitor of kringle-mediated binding of Plg/plasmin onto receptor or cofactor molecules. These results indicated that Plg binds to ST1 cells in a lysine-dependent manner, which indicates involvement of the kringle domains. However, the observed binding level is low, and a better binding occurs with plasmin, which indeed is known to have a higher affinity than Plg to lysine-containing targets (Kuusela & Saksela, 1990).

The conversion of the single-chain plasminogen into the two-chain plasmin in the presence or absence of ST1 cells and tPA was then assessed. The suspensions were incubated for 5 h, the cells and the buffer were separated, and Plg and plasmin were analysed from both fractions by Western blotting. The assay showed that the presence of ST1 cell suspension enhanced the tPA-catalysed conversion of Plg into plasmin (Fig. 2e). In assays lacking tPA, no binding of Plg onto ST1 cells was observed, and Plg was detected only in the buffer (Fig. 2e). When tPA was added with Plg and ST1 cells, generation of plasmin as well as its partial immobilization onto the bacterial cells were observed; however, most of the plasmin formed was present in the cell-free supernatant (Fig. 2e). In accordance with this observation, a small amount of exogenously added plasmin was immobilized onto the ST1 cells (Fig. 2e). To confirm the results on Plg conversion, we assessed the relative amounts of plasmin enzymic activity on bacterial surface and in the buffer after a 5 h incubation of washed ST1 cells with Plg and tPA. The bacterial suspension enhanced plasmin formation, and after fractionation of the suspension into the cells and the buffer, 92 % of the formed plasmin activity was detected in the cell-free supernatant (Fig. 2f). The plasmin formed was nearly completely inhibited by α_2 AP (Fig. 2f). Similar fractionation after shorter incubation times (1, 2 and 3 h) in PBS revealed a gradual enrichment of plasmin formation in the buffer and a decrease in cell-associated plasmin (data not shown), which is in agreement with the gradual release of the extracellular material from ST1 cell surface (Fig. 1b).

We next measured binding of Plg and plasmin onto the extracellular material and, secondly, its capacity to enhance Plg activation. The extracellular proteome of ST1 efficiently bound plasminogen and plasmin as well as enhancing tPA- and uPA-catalysed plasminogen activation (Fig. 3a, b). EACA reduced the observed binding of plasminogen by 93 % and plasmin by 80 %, and the plasmin activity formed was also undetectable in the presence of EACA (data not shown). These observations indicate that the extracellular material of ST1 cells expresses Plg activation cofactor function, i.e. it binds Plg in a lysine-sensitive manner and enhances plasmin formation by tPA. An important property of Plg receptors or cofactors is that binding to them protects the forming plasmin from α_2 AP. Plasmin (109 nM) was incubated for 15 min with the ST1 supernatant and its activity was subsequently assessed in the presence of increasing amounts of α_2 AP (Fig. 3c). A low level of protection was seen; at α_2 AP concentrations of 72 nM and 144 nM, plasmin activity with the extracellular material was 18 % and 44 % higher than in PBS alone ($P < 0.05$), whereas at a lower concentration of α_2 AP the difference between the samples was not statistically significant.

Characterization of enolase and GAPDH of *L. crispatus* ST1

The published genomic sequences of lactococci and lactobacilli each contain a *gap* gene encoding GAPDH proteins sharing 80–90 % amino acid sequence identity.

Fig. 3. Interaction of extracellular material from *L. crispatus* ST1 with Plg and plasmin. (a) The binding of Plg and plasmin to the cell-free extracellular material from 3×10^8 cells in the absence (grey columns) or presence (open columns) of EACA was measured by time-resolved fluorometry with anti-Plg IgG and Eu^{3+} -labelled secondary immunoglobulins. The means and standard deviations from two independent assays with quadruplicate samples are shown. (b) Enhancement of Plg activation. Plg with tPA (▲) or uPA (△) was incubated with the extracellular material from 3×10^8 cells. The means and standard deviations from two independent assays with quadruplicate samples are shown. (c) Protection of plasmin from α_2 AP. Plasmin was incubated in the absence (open columns) or presence (grey columns) of extracellular material from 1.5×10^8 ST1 cells prior to adding the α_2 AP and a chromogenic substrate of plasmin. Means and standard deviations from two independent assays with triplicate samples are shown. An asterisk indicates that the difference between the two test samples was statistically significant ($P < 0.05$; Student's two-tailed *t*-test).

Fig. 4. Interaction of *L. crispatus* ST1 His₆-enolase and His₆-GAPDH with human Plg. The binding of Plg (a) and plasmin (b) to the purified recombinant proteins in the absence (grey columns) or in the presence (open columns) of EACA was measured by time-resolved fluorometry using anti-Plg IgG and Eu³⁺-labelled secondary antibodies. The binding of Plg and plasmin to their known protein target laminin as well as to the S-layer protein of *L. crispatus* ST1 is also shown. The means and standard deviations from two independent assays with quadruplicate samples are shown. (c) Enhancement of tPA-catalysed plasmin formation by His₆-enolase and His₆-GAPDH. Plg and tPA were incubated with the recombinant proteins, and plasmin activity was measured using a chromogenic substrate of plasmin. The plasmin formation was tested in the absence (▲) or presence (●) of the serine protease inhibitor aprotinin. As a control, plasmin formation with laminin as well as with the S-layer protein isolated from *L. crispatus* ST1 are shown. The means and standard deviations from two independent assays with quadruplicate samples are shown. (d) Release of enolase and GAPDH from cells in the absence or presence of plasmin during a 5 h incubation. Enolase and GAPDH were detected by Western blotting with specific antibodies. The minor degradation product of enolase is marked with an arrow on the left.

(Altermann *et al.*, 2005). Both genes were cloned from the ST1 genome into the vector pQE30 and expressed in *E. coli* M15. The N-terminal His₆-tagged fusion proteins were purified from *E. coli* M15 under non-denaturing conditions and tested for plasminogen receptor activity. Both proteins exhibited an EACA-inhibitable binding of Plg (Fig. 4a) and bound plasmin (Fig. 4b), as well as enhanced tPA- (Fig. 4c) and uPA- (data not shown) catalysed Plg activation. Binding of plasmin to enolase and to the control protein laminin was inhibited by EACA, which, however, did not diminish binding to GAPDH (Fig. 4b). A major surface protein of *L. crispatus* ST1, the S-layer, showed only poor binding of Plg and enhancement of its activation (Fig. 4). The serine protease inhibitor aprotinin inhibited the activity, indicating that the substrate cleavage was by plasmin and not by enolase or GAPDH. The observed release of plasmin from the ST1 cell surface might involve plasmin proteolysis, which could cleave or release Plg receptor molecules from the ST1 surface, or alternatively, cryptic terminal lysines may become exposed after cleavage. We therefore analysed the release of enolase and GAPDH from ST1 cells in the absence and presence of added plasmin. Presence of active plasmin did not affect the amounts of enolase or GAPDH in the buffer; a limited degradation of enolase, however, was detected (Fig. 4d). This indicates that plasmin proteolysis does not affect the detachment of enolase and GAPDH from the *L. crispatus* ST1 cell surface and that these proteins are poor substrates for plasmin proteolysis.

Enhancement of Plg activation by extracellular material from other lactic acid bacteria

The available genomic sequences of lactobacilli and lactococci reveal the presence of enolase and GAPDH

Enolases in lactobacilli are more variable; *eno* genes per strain vary between 1 and 3 in number and the overall sequence identity of the proteins is between 47 and 93 %. We amplified internal sequences of *gap* and *eno* of ST1 using primers designed according to the sequences of enolase genes in *L. johnsonii* NCC 533 (Pridmore *et al.*, 2004) and *gap* of *Lactococcus lactis* subsp. *lactis* Il1403 (Bolotin *et al.*, 2001). The internal DNA amplicons were sequenced, and to obtain the correct 5' and 3' termini of the ORFs, the complete ORFs of *eno* and *gap* were sequenced from the ST1 genome. The ORF of ST1 *gap* (EMBL AJ849470) encodes a protein of 338 amino acids with 88 % sequence identity to GAPDHs of *L. johnsonii* (Pridmore *et al.*, 2004) and *L. acidophilus* (Altermann *et al.*, 2005). The predicted sequence of ST1 enolase (EMBL AJ849471) is 428 amino acids long and 94 % identical to the enolase of *L. acidophilus*

genes that are variably related to those of *L. crispatus* ST1. *L. crispatus* is of the DNA homology group A2 in the *Acidophilus* group of lactobacilli (Johnson *et al.*, 1980). Extracellular proteins in PBS were prepared from five strains of *Lactobacillus* representing the DNA homology groups (Johnson *et al.*, 1980) A1 (*L. acidophilus* E507), A3 (*L. amylovorus* JCM 5807), A4 (*L. gallinarum* T-50), B1 (*L. gasseri* JCM 1130/ATCC 19992) and B2 (*L. johnsonii* F133) as well as three strains in probiotic or dairy use (*L. rhamnosus* GG, *L. paracasei* E506 and *Lactococcus lactis* E523). The extracellular proteins were screened for enhancement of tPA- and uPA-catalysed Plg activation and analysed by Western blotting for possible presence of enolase and GAPDH (Fig. 5). The extracellular preparations exhibited variable capacity to enhance tPA- and uPA-catalysed Plg activation, with *L. gallinarum* T-50 (A4) and *L. johnsonii* F133 (B2) showing the highest and *L. amylovorus* JCM 5807 (A3), *L. gasseri* JCM 1130/ATCC 19992 (B1) and *L. rhamnosus* GG the lowest activity (Fig. 5a). The preparations from *L. acidophilus* E507, *L. paracasei* E506 and *Lactococcus lactis* E523 gave similar activity as the preparation from *L. crispatus* ST1. No plasmin activity was detected in absence of tPA or uPA, and a highly similar

pattern of Plg activation was seen when bacterial cell suspensions were tested (data not shown). Anti-His₆-enolase and anti-His₆-GAPDH antibodies detected cross-reactive peptides in five and seven, respectively, of the protein preparations (Fig. 5b). SDS-PAGE analysis of the proteins revealed variable amounts of peptides with apparent molecular masses of enolase and GAPDH in all preparations (Fig. 5c).

DISCUSSION

The interaction of *L. crispatus* with human/mammalian Plg here described represents a novel host interaction for *Lactobacillus*, and our results give further evidence for a role of extracellular proteins in *Lactobacillus*-host interactions. The intestinal commensal and opportunistic pathogen *Bacteroides fragilis* was recently shown to bind Plg (Sijbrandi *et al.*, 2005), and our results extend Plg interaction to commensal lactic acid bacteria as well. These results suggest that Plg interaction with bacteria is more common than so far expected (Lähtenmäki *et al.*, 2005). The enhancement of Plg activation by *L. crispatus* ST1 was associated mainly with the extracellular proteins

Fig. 5. Extracellular Plg cofactor function, enolase and GAPDH in lactic acid bacteria. (a) Enhancement of tPA- and uPA-catalysed Plg activation by the extracellular material from 3×10^8 cells analysed, at 5 h after adding the chromogenic substrate. (b) Identification of enolase and GAPDH in the extracellular material by Western blotting with specific antibodies; in (c), the corresponding Coomassie blue-stained SDS-PAGE gel is shown. The migration distances of enolase and GAPDH are marked with arrows on the left, and the bacterial strains are given at the bottom.

gradually released from the bacterial surface. This is in sharp contrast to Plg activation by Gram-positive bacterial pathogens which immobilize Plg/plasmin on their surface (Lähtenmäki *et al.*, 2001, 2005). The system here described is close to the one shown by *Streptococcus pyogenes*, whose surface enolase, in a purified form, enhances Plg activation by tPA but protects the plasmin formed only partially from inhibition by α_2 AP. By contrast, plasmin generated by streptokinase is fully protected from inhibition (Derbise *et al.*, 2004; Pancholi & Fischetti, 1998). In this respect, the main differences between streptococci and *L. crispatus* are the extracellular location of enolase and lack of streptokinase-like PA activity in *L. crispatus*. It is likely that lactobacillus-mediated Plg activation results in locally restricted plasmin proteolysis. So far, plasmin activity in relation to lactobacilli has been studied for fermentation quality in cow milk (Bastian & Brown, 1996); in this context it is noteworthy that *L. crispatus* ST1 also enhanced activation of bovine Plg.

Several lines of evidence indicate that enhancement of Plg activation by *L. crispatus* involves mainly extracellular proteins. (i) Binding of plasmin and in particular of Plg onto ST1 cells was poor; we detected levels of 2–2.5 % in plasminogen binding and 5.5–6.3 % in plasmin binding, which under similar assay conditions with pathogens have been up to 78 % (Ullberg *et al.*, 1990, 1992). (ii) Although the conversion of Plg into the two-chain plasmin by tPA was accelerated in the presence of ST1 cells, the resulting plasmin molecules were almost exclusively in the extracellular environment. In similar assays with *Staphylococcus aureus* and *Salmonella enterica*, which have cell-bound Plg receptors, the conversion of Plg to plasmin is seen on the bacterial surface only (Kuusela & Saksela, 1990; Lähtenmäki *et al.*, 1995). (iii) In a 5 h incubation of ST1 cell suspension with Plg and tPA, over 90 % of the enzymic activity generated was associated with the cell-free material. (iv) The extracellular material released from ST1 cells bound Plg and plasmin and also enhanced tPA- and uPA-catalysed Plg activation. (v) Plasmin formed by tPA was only weakly protected from α_2 AP, which inhibits soluble plasmin with unoccupied kringle domains. However, the presence of enolase and GAPDH as well as Plg/plasmin binding were detected both on the surface of washed ST1 cells and in the extracellular proteome. Our hypothesis is that enolase and GAPDH interact with Plg in the cell wall, as well as after their release from the bacterial surface, and also in the extracellular phase. Enolase and GAPDH are enriched over time in the extracellular environment and are able to quickly react with Plg and its activators.

Enolase and GAPDH were major peptides in the extracellular proteome; they were also detected by IEM in cytoplasm and in the cell wall of *L. crispatus* ST1. These enzymes have been detected on the cell surface of various organisms, where they also function as Plg receptors and cellular adhesins (Pancholi & Chhatwal, 2003). The predicted sequence of ST1 GAPDH exhibits high sequence identity, 80–90 %, to the orthologues in lactobacilli. On the

other hand, the enolases are more diverse in *Lactobacillus*; the published sequences show 47–93 % sequence identity and *L. johnsonii* contains three copies of the *eno* gene (Pridmore *et al.*, 2004). The closest homologue to the ST1 enolase is in *L. acidophilus* (Altermann *et al.*, 2005); the predicted sequences are 93 % identical, which is in accordance with the close phylogenetic association of *L. crispatus* and *L. acidophilus* as species (Fujisawa *et al.*, 1992; Johnson *et al.*, 1980). Only one copy of *eno* is present in the genomic sequence of *L. acidophilus* (Altermann *et al.*, 2005), making the gene essential for survival, and our ongoing genetic analyses have failed to produce evidence for more than one *eno* gene in *L. crispatus* ST1 (V. Hurmalainen, unpublished data). The mechanisms of secretion of enolase and GAPDH to the bacterial surface or exterior remain unknown. The presence of an auxiliary *secA2* gene has been detected in *Listeria*, where it is involved in the secretion of various peptides, including enolase (Lenz *et al.*, 2003). Two *sec* genes are also present in *L. johnsonii*; thus *secA2* could affect secretion of one or more of the enolases of this bacterium (Pridmore *et al.*, 2004). However, only a single *secA* gene is present in the genome of *L. acidophilus* (Altermann *et al.*, 2005) as well as in other *Lactobacillus* genomes available in the databases. Few reports have described a transient release of enolase and GAPDH proteins from the bacterial surface. Release and reassociation of enolase was reported in the cell surface of *Strep. pneumoniae* D39 (Bergmann *et al.*, 2001), whereas in *Strep. pyogenes* D471 enolase remains surface-bound (Pancholi & Fischetti, 1998), but GAPDH was released from the cell surface during iron starvation (Eichenbaum *et al.*, 1996). Change of pH affects the release of enolase from the surface of *Streptococcus gordonii* (Nelson *et al.*, 2001). These findings suggest that cell-wall association of enolase and GAPDH is reversible, and our hypothesis is that their efficient release from the ST1 surface results from loose interactions with other cell-wall structures rather than, for example, from more active secretion through the cytoplasmic membrane. We have also detected extracellular enolase and GAPDH in the culture medium of *L. crispatus* (V. Hurmalainen & J. Antikainen, unpublished), and recent studies have demonstrated biologically active proteins in conditioned culture medium of *L. rhamnosus* (Peña & Versalovic, 2003; Tao *et al.*, 2006); this indicates that the presence of extracellular proteins in *Lactobacillus* is not a response to the PBS environment.

The number of Plg-binding proteins in the extracellular proteome of *L. crispatus* remains unresolved. We identified the presence of enolase and GAPDH in the ST1 extracellular proteome and showed that their His₆-fusion forms from *E. coli* expressed Plg cofactor activity. His₆-GAPDH of *L. crispatus* ST1 was more efficient than His₆-enolase in Plg binding, and our ongoing research (V. Hurmalainen, unpublished) has shown that GAPDH isolated from ST1 supernatant also expresses Plg cofactor activity. Many of the Plg/plasmin-binding proteins, including enolases and GAPDH, from prokaryotes and eukaryotes harbour C-terminal lysines that are recognized by the kringle domains

of Plg (Derbise *et al.*, 2004; Winram & Lottenberg, 1998). On the other hand, Bergmann *et al.* (2003) recently described an internal nine-residue Plg-binding motif in the enolase of *Strep. pneumoniae*. The sequence ²⁴⁸FYDKERKVVY is located on the surface of the octameric pneumococcal enolase, whereas the C-terminal lysines are located in the inter-dimer groove and apparently not accessible to Plg binding (Ehinger *et al.*, 2004). The predicted ST1 enolase as well as the GAPDH sequence lack C-terminal lysines, and the ST1 enolase resembles pneumococcal enolase in having the sequence FYNKDDHKY starting at position 248. We are currently analysing which regions in ST1 enolase and GAPDH proteins are involved in Plg cofactor activity.

Our results show that extracellular enolase and GAPDH as well as the Plg activation cofactor function are common in species of *Lactobacillus*. We detected extracellular GAPDH in eight of the nine strains analysed in this study. Extracellular enolase was detected in six of the strains but not in *L. paracasei*, *L. rhamnosus* or *Lactococcus lactis*, which are phylogenetically distant from *L. crispatus*. Failure to detect extracellular enolase in the organisms may thus be due to lack of serological cross-reactivity, as the enolase sequences in lactic acid bacteria are variable. Species of *Lactobacillus* are members of the human microbiota and regarded as health promoting; however, isolates of *L. acidophilus*, *L. rhamnosus* and *L. paracasei* – species studied here – as well as some other species of *Lactobacillus*, are associated with the rare but dangerous opportunistic disease infective endocarditis (IE; Salvana & Frank, 2006). The primary event in IE is bacterial adherence to damaged heart valves where the extracellular matrix is exposed. Isolates of *Lactobacillus* frequently adhere to extracellular matrices (Aleljung *et al.*, 1991; McGrady *et al.*, 1995), which contain components of the Plg system (Farina *et al.*, 1996) and offer an environment where local Plg activation by lactobacilli could be functional and enhance tissue damage. On the other hand, a possible probiotic function could be interference with the exploitation of Plg by gastrointestinal pathogens that express Plg receptors or activators, such as *Helicobacter pylori* (Jönsson *et al.*, 2004) or *Salmonella* (Lähtenmäki *et al.*, 2005). The resolution of these questions is a challenging topic, and the lactobacilli–Plg interaction here described offers an interesting model to compare the Plg/plasmin system in bacterial commensalism and pathogenicity.

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