RESEARCH ARTICLE

Suppression subtractive hybridization identifies bacterial genomic regions that are possibly involved in hBD-2 regulation by enterocytes

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Scope: Human β-defensin 2 (hBD-2) is an inducible antimicrobial peptide synthesized by the epithelium to counteract bacterial adherence and invasion. It has been suggested that probiotic bacteria sustain gut barrier function via induction of defensins. The goals of this study were (i) to evaluate the potential immunomodulatory effects of 11 different *Lactobacillus fermentum* strains isolated from *Kimere*, an African fermented pearl millet (*Pennisetum glaucum*) dough, on the hBD-2 secretion by human intestinal CaCo-2 cell line and (ii) to examine genetic differences between two strains of *L. fermentum* (K2-Lb4 and K11-Lb3) which differed in their effect on the production of hBD-2 in this study.

Methods and results: Totally, 46 strains of *L. fermentum* from *Kimere* were isolated and characterized using molecular biology methods including pulsed-field gel electrophoresis patterns. After performing time- and dose-experiments, CaCo-2 cells were incubated with or without bacteria for 12 h. *L. fermentum* PZ1162 was included as the positive control. Cell-free supernatants were analyzed for hBD-2 protein by enzyme-linked immunosorbent assay (ELISA). To identify potential bacterial genes associated with hBD-2 regulation, suppression subtractive hybridization (SSH) was used. Among the 11 strains tested, only two strains of bacteria, K11-Lb3 and K2-Lb6, significantly induced the production of hBD-2 by CaCo-2 cells. This effect was strain-specific, dose-dependent and particularly seems to be bacterial genomic-dependent as manifested by SSH. *L. fermentum* strains with and without hBD-2 inducing effect differed in genes encoding proteins involved in glycosylation of cell-wall proteins e.g. glycosyltransferase, UDP-*N*-acetylglucosamine 2-epimerase, rod shape-determining protein MreC, lipoprotein precursors, sugar ABC transporters, and glutamine ABC transporter ATP-binding protein.

Conclusion: This study implies that certain strains of *L. fermentum* isolated from *Kimere* may stimulate the intestinal innate defense through the induction of hBD-2. The molecular basis of hBD-2 induction by *L. fermentum* strain K11-Lb3 may be based on glycosylated cell-surface structures synthesized with the aid of glycosyltransferase, UDP-*N*-acetylglucosamine 2-epimerase, and rod shape-determining protein MreC.

Keywords:
Human β-defensin-2 / Intestinal epithelial cells / Suppression subtractive hybridization

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Abbreviations: CFU, colony forming unit; **CD,** Crohn's disease; **gDNA,** genomic DNA; **hBD-2,** human β-defensin 2; **IBD,** inflammatory bowel disease; **NP,** nested primer; **SSC,** saline-sodium citrate; **SSH,** suppression subtractive hybridization

1 Introduction

Intestinal epithelium cells (IECs) form a physical barrier to limit the access of enteric microbes to the host and they contribute to innate host defense by producing effector molecules against luminal microbes. Under normal conditions there is a stable balance of luminal bacteria and host immune system to prevent infections or inflammatory bowel diseases (IBD). A complex network of environmental, genetic, and immunoregulatory factors may precipitate the onset of ulcerative colitis (UC) and Crohn's disease (CD), the primary manifestations of IBD [1–5]. Although disruption of mucosal barrier is a multifactor process, defensins appear to be crucial in the pathophysiology of CD and IBD, since defensin induction is impaired in CD and reduced defensin levels are associated with a weakened intestinal barrier function to intestinal microbes [6–9]. Moreover, defective expression of human β-defensin 2 (hBD-2) in the gut mucosa of patients with CD is due to a reduced copy number of the hBD-2 gene [10].

Human β-defensins are small cationic antimicrobial peptides synthesized by the epithelium to counteract bacterial adherence and invasion. There are three main subsets of mammalian defensins; α-defensins, β-defensins, and θ-defensins. The best characterized gut defensin is the hBD-2 which is an inducible antimicrobial peptide synthesized and secreted by the epithelium to counteract bacterial adherence and invasion and to kill bacteria directly. Unlike human β-defensin-1 (hBD-1), which is produced constitutively, hBD-2 is expressed after adequate stimulation by cytokines and/or bacterial components in epithelial tissue and mononuclear phagocytes but may be deficient in patients withCD. Recent evidences suggest that probiotic bacteria may stabilize gut barrier function via induction of antimicrobial peptides such as defensins, in general, and probiotic induction of hBD-2 contributes to an enhanced mucosal barrier to the luminal bacteria, in particular [11].

Suppression subtractive hybridization (SSH) has been successfully used to compare bacterial genomes and particularly to identify strain- or species-specific DNA sequences in a variety of bacteria. SSH is also a powerful method for studying genomic differences between two closely related strains especially where prior knowledge of the sequence dose not exist and can be used to identify sequences that are present in one bacterial genome, but absent in another[12].

Despite the existence of these relevant data, however, the molecular basis on which the induction of the production of hBD-2 is found is unknown. Therefore, in this study we aimed at (i) evaluating the potential immunomodulatory effects on the hBD-2 secretion by human intestinal CaCo-2 cell line of 11 different *Lactobacillus fermentum* strains, which we described recently [13] and (ii) examinating genetic differences between the two *L. fermentum* K11-Lb3 and K2-Lb4 strains, with different hBD-2-inducing properties, in order to identify candidates genes potentially responsible for mediating hBD-2 modulatory effect. Subtracted cDNA libraries for *L. fermentum* strains K2-Lb4 (hBD2-nonstimulating strain) and K11-Lb3 (hBD2-stimulating strain) were constructed using SSH. To obtain a cDNA library specific for strain K11-Lb3, *RsaI*-digested genomic DNA from *L. fermentum* strain K11-Lb3 was used as ''tester'' and *L. fermentum* strain K2-Lb4 DNA as ''driver'' DNA. A reciprocal subtraction was done as a control with DNA of K2-Lb4 as tester and of K11-Lb3 as driver to construct K2-Lb4-specific genes library. Differential cDNA fragments were directly cloned into pSTBlue-1 cloning vector, subjected to blue/white screening and dot blot analysis to identify strain-specific fragments.

2 Materials and methods

2.1 Bacterial strains

Previously, we isolated and characterized 46 strains of *L. fermentum* from various *Kimere* samples, collected from 11 different homesteats in the Mbeere community of Kenya [13] using different molecular biology methods [14]. Briefly; *Lactobacillus* isolates were characterized and identified using biochemical methods such as carbohydrate fermentation patterns, API 50 CHL, growth temperatures, acid and bile salt tolerance, Gram and catalase reaction, and molecular methods such as species-specific polymerase chain reaction (PCR), amplified rDNA restriction analysis (ARDRA), and partial sequencing of the 16S rDNA. To study strain diversity, 46 *L. fermentum* isolates were subjected to pulsed-field gel electrophoresis (PFGE) analysis, using AscI as the restriction enzyme. Analysis of *L. fermentum* strains with PFGE indicated different profiles and relatively large biodiversity within that species for 38 strains; 8 strains were excluded from further evaluation due to unsatisfactory PFGE profiles. Based on bile salt tolerance 11 strains were selected, in which the potency of hBD-2 modulation was tested: *K1-Lb1, K2-Lb4, K3-Lb1, K2-Lb6, K5-Lb2, K6-Lb4, K7-Lb1, K8-Lb3, K9-Lb3, K10-Lb1*, and *K11-Lb3*. *L. fermentum* PZ1161 [15] was included as a control.

2.2 Propagation of bacteria

Bacterial strains were propagated according to previously published procedures [16]. Briefly, using a 0.02% inoculum from stocks stored at −80°C in 30% glycerol, bacteria were grown anaerobically (The Modular Atmosphere Controlled System, MACS-VA500 workstation with airlock, Don Whitley Scientific Limited, UK) in MRS broth medium (Lactobacilli MRS according to de Man, Rogosa, Sharpe; Merck, Darmstadt, Germany), at 37°C for 12 h. Colony forming units (CFU) per millilitre of each bacterial culture was estimated by measuring the optical density at 600 nm (OD_{600}) of a 1:6 dilution and using an experimentally

derived conversion factor for each strain. The conversion factors were determined by plating stationary phase cells and determining the number of CFU/mL per OD_{600} unit for each strain. The bacterial culture media were centrifuged at 4°C at 5000 × *g* for 10 min. After washing three times with endotoxin-free phosphate-buffered saline without Ca^{2+} and Mg^{2} (DPBS; BioWhittaker, Lonza, Basel, Switzerland), the bacterial pellet was diluted to 5×10^6, 1×10^7, 2×10^7, and 4×10^7 CFU in 50 μL of cell culture medium (see below). This was freshly added to cultures of CaCo-2 cells to give bacteria-to-cell ratios of 2.5:1, 5:1, 10:1, and 20:1. Dilutions were also plated on agar plates; after 48 h of incubation colonies were counted to confirm the accuracy of dilution and viability of bacteria.

2.3 Cell culture

The human colon epithelial cell line Caco-2 (ATCC, American Type Culture Collection) was cultured in DMEM (Dulbecco's Modified Eagle's Medium), supplemented with 10% foetal bovine serum (FBS) and 2 mM L-glutamine, and then maintained at 37°C in 5% CO_2 and 95% air.

2.4 Dose-and time course experiments

Caco-2 cells (2×10^6 cells/mL) in 12-well tissue culture plates (Corning-Costar, Cambridge, MA, USA) were incubated for 6 h without (medium as the control) or with 5×10^6, 1×10^7, 2×10^7, and 4×10^7 CFU/mL of bacteria (bacteria-to-cell ratios of 2.5:1, 5:1, 10:1, and 20:1). For time course experiments, Caco-2 cells (2×10^6 cells/mL) were incubated for 6, 12, 24, and 48 h without (medium as the control) or with 2×10^7 CFU/mL of bacteria (bacteria-to-cell ratio of 10:1). In both experiments *L. fermentum* PZ1161 [15] was included as the positive control. After incubation, cell-free supernatants were harvested and stored at −80°C until analysis.

2.5 hBD-2 stimulation and measurement

Caco-2 cells (2×10^6 cells/mL) in 12-well tissue culture plates were incubated for 12 h without (medium as the control) or with 2×10^7 CFU/mL of bacteria (bacteria-to-cell ratios of 10:1). This bacteria-to-cell ratio was chosen, since that dose produced a maximal increase in hBD-2 secretion by the all strains tested. In a subset of experiments, Caco-2 cells were stimulated for 12 h with LPS (10 μg/mL) in the presence or absence of bacteria (2×10^7 CFU/mL). After incubation, cell-free supernatants were harvested, concentrated in the case of hBD-2 as described previously [17], and analyzed for hBD2 by developed ELISA Development Kits (PeproTech GmbH, Hamburg, Germany), according to the manufacturer's instructions. Since the increase in the hBD-2 secretion level was induced 12 h after incubation with *L. fermentum* strains K2-Lb6 and K11-Lb3 in comparison to the control, the 12-h incubation time was chosen in subsequent experiments.

2.6 SSH

2.6.1 Bacterial strains

L. fermentum strains used for SSH were selected according to their influence on the induction of hBD-2 by IECs. Strain K11-Lb3 was able to induce high levels of hBD-2, while strain K2-Lb4 did not induce hBD-2 expression under the tested conditions.

2.6.2 Genomic DNA extraction

For the isolation of genomic DNA, one millilitre of a culture grown overnight was inoculated into 50 mL of fresh MRS broth supplemented with 40 mM DL-threonine and incubated at 37°C to an OD_{600} of ca. 0.6. Cells were harvested by centrifugation at 4500 × *g* for 10 min, washed with 20 mL 1 × saline-sodium citrate (SSC) buffer, resuspended in 5 mL SSC buffer, and held on ice for 15 min. One millilitre 1 × SSC buffer containing 30 mg/mL lysozym (Sigma), 300 U/mL mutanolysin (Sigma), and 60 μL RNase (10 mg/mL, Sigma) were added, and incubation on ice was continued for 30 min. The cell lysate was then further incubated at 37°C for 60 min, after which it was treated with 150 μL 20% sodium dodecyl sulfate (SDS) solution lysis buffer, followed by incubation at 60°C until the lysate turned clear. Nearly, 60 μL proteinase-K (10 mg/mL, Sigma) was then added and cells were incubated at 50°C for 60 min. Chromosomal DNA was sequentially extracted twice with one volume phenol (transferring the upper layer into new a tube) and once with one volume chloroform/isoamylalcohol (24:1). Phase separation was achieved by centrifugation at 10 000 × *g* for 5 min for the first phenol extraction and 12 000 × *g* for 3 min for subsequent extractions. DNA was precipitated by addition of 1/10 volume of 4 M sodium acetate buffer (pH 5.5) and two volumes of ethanol (98%) and incubation at −20°C. It was collected by winding onto a sterile glass rod. The DNA was placed in a 1.5 mL-Eppendorf tube, washed in 80% ethanol, air-dried, and dissolved in 500 μL TE buffer. Concentration and purity of all DNA preparations were determined in triplicate by measuring the optical densities at three different nanometres and calculating the ratios OD_{260}/OD_{280} and OD_{260}/OD_{230}, respectively). Only DNAs with an OD_{260}/OD_{280} ratio > 1.8 and $OD_{260}/OD_{230} \geq 2$ were used. The quality of DNA was further analyzed by conventional gel electrophoresis, which showed only minor fragmentation due to the purification process. DNA was then stored at 4°C for further experiments.

2.6.3 Driver and tester DNA preparations

Subtracted DNA libraries were made by using the PCR-Select Bacterial Genome Subtraction Kit (Clontech). Genomic DNA (gDNA) of *L. fermentum* strain K11-Lb3 was used as a tester and gDNA of *L. fermentum* strain K2-Lb4 as a driver DNA. As a control, the complementary subtraction was made. gDNAs were digested with the restriction enzyme *Rsa*I (FastDigest® enzyme, Fermentas) to obtain short, blunt-ended fragments. In brief, 4 μg of tester or driver gDNA was digested in 400 μL reaction volume at 37°C for 5 min. DNA fragments were then extracted with phenol, precipitated with ethanol, and resuspended in sterile distilled water. *RsaI*-digested tester DNA was ligated to adaptors 1 and 2R overnight at 16°C. The sequences of the adaptors and primers were as follows: adaptor 1: 5′-CTAATACG ACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3′+5′-ACCTGCCCGG-3′; adaptor 2R: 5′-CTAATACGACT CACTATAGGGCAGCGTGGTCGCGGCCGAGGT-3′+5′-AC CTCGGCCG-3′; Primer P1, 5′-CTAATACGACTCACTA-TAGGGC-3′ (primary PCR); Nested Primer 1 (NP1): 5′-TCGAGCGGCCGCCCGGGCAGGT-3′ (secondary PCR); Nested Primer 2 (NP2): 5′-AGCGTGGTCGCGGCCGAG GT-3′ (secondary PCR). Following ligation, a series of two hybridization steps was performed. gDNA was denatured at 98°C for 2 min and hybridization performed at 63°C first for 2 h with each adaptor-ligated tester and driver. A second hybridisation was performed by combining adaptor 1- and adaptor 2R-ligated fractions and an excess of fresh driver DNA, followed by overnight incubation at 63°C. Primary PCR was performed with adaptor-specific primer P1 using the following program: 30 cycles of denaturation 94°C for 30 s, annealing at 68°C for 30 s, and extension at 72°C for 1.5 min, followed by final extension at 72°C for 10 min and holding at 4°C. Secondary PCR was performed with nested primers NP1 and NP2 using the same PCR program as for primary PCR but for 15 cycles only.

2.6.4 Cloning into AccepTorTM Vector

The subtracted libraries were cloned directly into pSTBlue-1 (AccepTorTM Vector Giga Kits, Novagen). Secondary PCR products were purified using PCR purification kits (NucleoSpin®) and ligation was carried out in 10 μL volume consisting of 1 μL (AccepTor Vector50 ng/μL), 4 μL purified PCR product and 5 μL ConablesTM 2X Ligation premix. The ligation mixture was incubated at 16°C for 2 h and stored at −20°C. Transformation into NovaBlue SinglesTM (Novagen) was done according to the manufacturer's instruction. In total, 50 μL of transformed cell suspensions were spread on LB medium agar plates, supplemented with 100 μL/mL carbenicillin, 100 μL/mL X-Gal, and 100 μM IPTG. Plates were incubated at 37°C for 16 h until blue and white colonies were visible. Sixty-three K11-Lb3- and 45 K2-Lb4-specific white colonies were selected randomly and grown in LB-broth supplemented with 100 μL/mL carbenicillin overnight at 37°C in a shaker for plasmid isolation and stock culture.

2.6.5 Screening of clone library by plasmid isolation, subsequent restriction of plasmid

Recombinant white colonies were picked from LB plate, inoculated in 10 mL LB broth supplemented with 100 μL/mL carbenicillin, and incubated in a shaker at 37°C for 16 h. Plasmids were isolated using plasmid a DNA Purification Kit (NucleoSpin® Plasmid Quick Pure; Macherey-Nagel GmbH, Germany). Plasmid DNA was then screened for the presence of inserts by digestion with FastDigest EcoRI restriction enzyme at 37°C for 5 min in the following mixture: 1 μL plasmid DNA, 2 μL EcoRI buffer, 1 μL EcoRI enzyme and 16 μL nuclease free water. Fragments were separated by agarose (1%) gel electrophoresis containing ethidium bromide (10 mM). Positive recombinants were maintained as frozen as stock cultures: 75 μL overnight culture was mixed with 25 μL sterile glycerol in 2 mL-Eppendorf tubes and frozen at −80°C for future reference.

2.6.6 Evaluation of cloning efficiency with PCR amplifications of bacterial colonies

To further confirm with certainty the presence of our subtracted inserts in the recombinants, 17 clones were selected randomly and subjected to PCR using nested primers NP1 and NP2. Briefly, 1 μL of plasmid DNA (diluted 1:50 in water), 18.5 μL nucleic acid-free water, 2.5 μL 10 × PCR Buffer, 1 μL of primers NP1 and NP2 each, 0.5 μL dNTP mix (10 nm), and 0.5 μL 50 × Advantage 2 Polymerase Mix (Clontech) were mixed. PCR program constituted of 30 cycles of denaturation at 94°C for 30 s, annealing at 68°C for 30 s, and extension at 72°C for 1.5 min, followed by final extension at 72°C for 10 min and cooling and holding at 4°C. PCR products were visualized by electrophoresis in 1% agarose gels.

2.6.7 Dot blot hybridisation

Screening for clones containing strain-specific DNA was carried out by dot blot hybridisation. About 5 μL of plasmid DNA from clones was denatured by heating in boiling water for 10 min and immediately transferred onto ice. One microlitre of each denatured sample was spotted onto positively charged nylon membrane in duplicate, using an α numerical matrix for spot identification. DNA was fixed by backing at 120°C for 30 min. Digoxigenin-11-dUTP-labelled *Rsal*-digested DNA from tester and driver, respectively, were used as probes, and dot blot hybridisation was

carried out with the DIG Filter Hybridisation System (Roche Applied Science, Mannheim, Germany). Pre-hybridization was done for 1 h and hybridisation for 16 h with DIG Easy Hyb (Roche Applied Science) at 42°C in a rotary oven. The membranes were washed twice in 2 × SSC+0.1% SDS buffer at room temperature for 5 min, followed by one wash in 0.1 × SSC+0.1% SDS buffer at 68°C for 15 min. After posthybridisation stringently washing, the membranes were equilibrated in wash buffer-1 (0.1 M maleic acid; 0.15 M NaCI; 0.3% Tween 20) for 5 min, followed by incubation in blocking buffer (buffer 2) for 1 h. Anti-DIG-alkaline phosphate was diluted 1:10 000 in buffer 2 (2 μL:20 mL), added, and incubation was continued in sealed transparent pouches at room temperature for 30 min. The membranes were then washed twice in buffer 1 for 15 min with constant shaking, followed by 5 min equilibration in detection buffer 3 (0.1 M Tris-HCI and and 0.1 M NaCI) with 2 mL of 25 nM CSPD (1:100) (chemiluminescent substrate for alkaline phosphatase) in buffer 3 added. The membranes were incubated for 5 min at room temperature, and then dried with Whatman filter paper. Detection substrate was then activated by incubating the membranes at 37°C for 30 min before exposing the membranes to X-ray film (Fuji-Tokyo, Japan) for 1 h for detection of the chemiluminescence signal.

2.6.8 DNA sequencing and analyses

Twenty-six randomly selected strain K11-Lb3-specific and 14 K2-Lb4-specific clones were sequenced by Eurofins MWG-Operone, Martinsried, Germany, using T7 sequencing primer. About 1200 bp of sequence were obtained for each clone, of which ca. 800–900 were of sufficient quality. The sequences obtained were inspected for the following subsequences: nested primers NP1 and NP2 and RsaI restriction site. Only sequences flanked by NP-primer sequences were considered for further analysis. If RsaI sites were detected, the sequences were considered accidentally joined by ligation und thus treated as different sequences. DNA sequences were analysed using the ''blastn'' and ''blastx'' algorithms of the National Centre for Biotechnology Information (NCBI) (http://blast.ncib.nlm.nih.gov/Blast) to determine putative functions of potentially expressed proteins. Similarities between sequences of different clones were analysed by ClustalW at http://align.genome.jp/.

2.7 Statistical analysis

Data are presented as mean ± standard error of the mean. Statistical significance was assessed by a one-way analysis of variance (ANOVA) and differences were pinpointed by a Student–Newman–Keul's multiple range test. A *p*-value of less than 0.05 was taken as indicative of statistical significance.

3 Results

3.1 Time- and dose-dependent bacterial induction of hBD-2 expression

When CaCo-2 cells were exposed to different bacteria-to-cell ratios, a significant dose-dependent increase in hBD-2 secretion was observed (Fig. 1A). Above a bacteria-to-cell ratio of 5:1, the hBD-2 production attained a plateau according to a saturation curve. *L. fermentum* strains K2-Lb6 and K11-Lb3 induced hBD-2 secretion in a time-dependent manner (Fig. 1B).

3.2 Strain-specific modulation of hBD-2 secretion

L. fermentum strains, isolated from *Kimere*, induced hBD-2 secretion in CaCo-2 cells in a strain-specific manner (Fig. 1C). Among 11 strains, only two strains namely K2-Lb6 and K11-Lb3 significantly induced hBD-2 secretion. Our data suggest that *L. fermentum* PZ1161 (positive control) and *L. fermentum* K2-Lb6 and K11-Lb3 isolated from *Kimere*, induce hBD-2 secretion in CaCo-2 cells in the tested conditions, while *L. fermentum* K2-Lb4 beside others had no effect.

3.2.1 SSH *identification of L. fermentum genomic regions* that may be involved in hBD-2 regulation of enterocytes

L. fermentum K11-Lb3 stimulating hBD-2 and strain K2-Lb4 nonstimulating hBD-2 were subjected to SSH as tester and driver, respectively. Figure 2A and B demonstrates the effect of subtraction. While unsubtracted, fragmented DNA appeared as a continuous smear on the agarose gel, distinct products were seen after subtraction.

After cloning of subtracted products, a total of 124 individual clones (white colonies) were obtained from reciprocal subtractions. Digestion of clones with EcoRI confirmed the presence of inserts: cloning vector pSTBlue-1 vector (Novagen) has two EcoRI restriction sites within the *lac*Z α-fragment (91, 106), separated by the EcoRV restriction site used for cloning. The range of fragments was approximately from 150 bp to more than 1000 bp (not shown). To further confirm the presence of subtracted inserts in the recombinants, 18 clones were selected randomly and subjected to PCR using nested PCR primers NP1 and NP2 (not shown). Moreover, strain specificity of the inserts was tested by dot blot hybridization. As shown in Fig. 3, most but not all of the dots appeared to show strain-specific hybridization (intensive signal with homologous DNA, weak signal with heterologous DNA). Based on the results of dot blot hybridization, 24 K11-Lb3- and 14 K2-Lb4-specific clones were randomly selected and sequenced.

Blastx analysis of the inserted cDNA sequences from the recombinant plasmids of 40 clones was carried out to

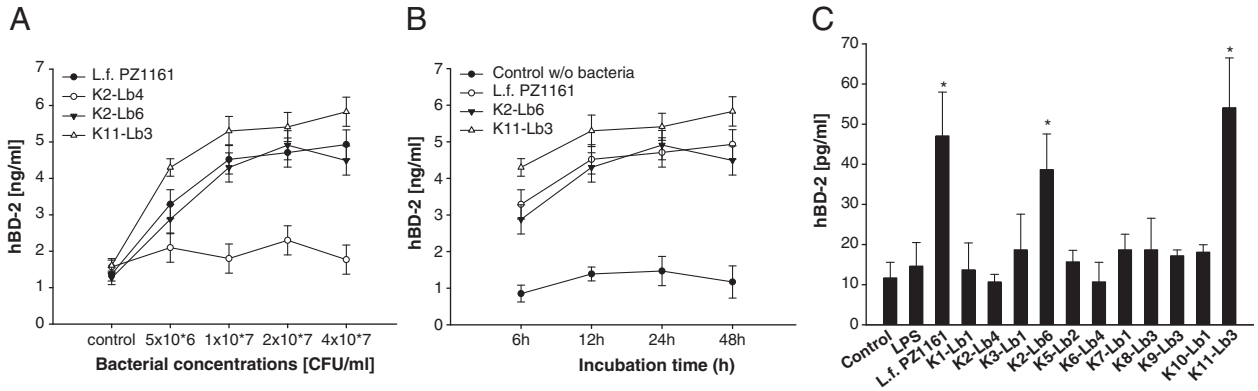

Figure 1. Some strains of potentially probiotic *L. fermentum* strains, isolated from *Kimere,* induce hBD-2 secretion by CaCo-2 cells in time- and strain-specific dose-dependent manner. (A) Dose-response experiment. CaCo-2 cells (2×10^6/mL) were treated without (medium as control) or with *L. fermentum* strains (bacteria-to-cell ratios of 2.5:1, 5:1, 10:1, and 20:1) for 12 h. After 12 h, cell-free supernatants were analyzed for hBD2 levels using ELISA. Experiments were performed twice with $n = 3$ at each concentration. Data represent mean ± SEM for experiments. (B) Time–response experiment. CaCo-2 cells (2×10^6/mL) were treated without (medium, control) or with *L. fermentum* strains (bacteria-to-cell ratio of 10:1) for indicated time. After incubation, cell-free supernatants were analyzed for hBD2 levels using ELISA. Experiments were performed twice with $n = 3$ at each concentration. Data represent mean ± SEM for experiments. (C) Represents the effect of different strains of *L. fermetum* on hBD2 secretion by CaCo-2 cells. Cells (2×10^6/mL) were treated without (medium as control) or with *L. fermentum* strains (bacteria-to-cell ratio of 10:1) for 12 h. After 12h, cell-free supernatants were analyzed for hBD2 levels using ELISA. Experiments were performed twice with $n = 4$–6 at each concentration. Data represent mean ± SEM for experiments. $^*p < 0.05$ versus control.

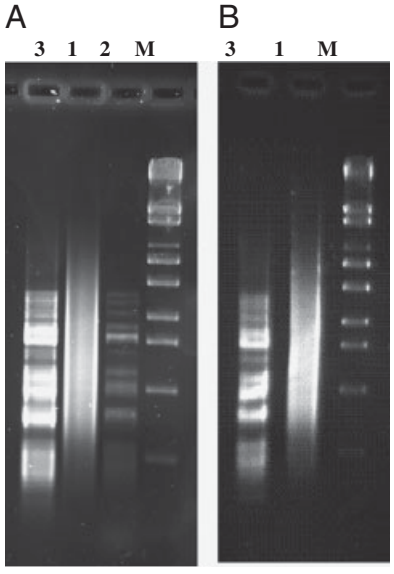

Figure 2. Analysis of two reciprocal (forward and reverse) subtracted secondary PCR products by agarose gel electrophoresis, (1%), stained in ethidium bromide. (A) represents the subtracted secondary PCR product of K11-Lb3 (tester, forward). Lane M, λ-DNA marker; lane 1, unsubtracted K11-Lb3 DNA; lane 2, 3 μL subtracted K11-Lb3 DNA; lane 3, 6 μL subtracted K11-Lb3 DNA. (B) represents the reverse subtracted secondary PCR product of K2-Lb4 (driver). Lane M, λ-DNA marker; lane 1, unsubtracted K2-Lb4 DNA; lane 3, 6 μL subtracted K2-Lb4 DNA.

identify putative functions or proteins encoded by these fragments. In addition, similarity analyses were carried out with the aid of the program ClustalW, as described in Section 2. The ClustalW analyses showed that a considerable number of cloned sequences were identical: one K11-Lb3 RsaI fragment was retrieved five times, another one three times, and two fragments two times each. This indicated that subtraction had worked quite efficiently. However, eleven of the K11-Lb3-specific RsaI fragments were also retrieved in the reciprocal control subtraction, which was supposed to enrich for K2-Lb4-specific fragments. Most of these fragments were shown to putatively encode transposase genes. Transposase genes may be present in higher copy numbers within the genome due to multiple insertions within the host genome. For further analysis, we only considered those RsaI fragments, which were not detected in the reciprocal subtraction. Information about these 18 fragments (it should be noted that one clone contained two different RsaI fragments) is presented in Table 1.

Among the 18 K11-Lb3-specific fragments, three revealed no significant similarity (*e* value > 1e−04) to published nucleotide sequences. Six showed similarity to transposase-encoding regions. They were not considered further, even if other genes of parts of genes were encoded by the fragment, because their identification after subtraction may have just been due to their higher copy numbers. Two fragments appeared to encode integrase genes of bacteriophages. It appears obvious that phage genes may be enriched by subtraction. However, they are most likely only indicative for strain-specific differences in general, but not with respect to a specific potentially probiotic trait. Three fragments encoded hypothetical proteins, for which no putative functions have been proposed. When analysing the genetic context of the genes identified, no further clues could be retrieved. Of the remaining fragments, clones C10a, B3 and C-10

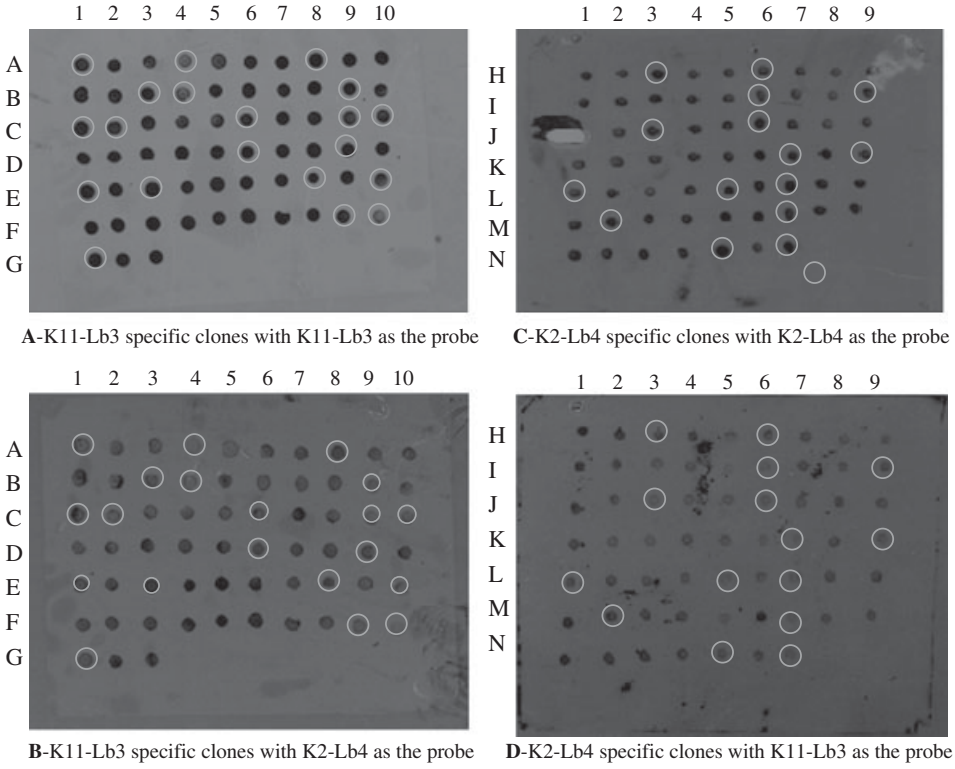

Figure 3. Dot blotting to screen tester-specific inserts using *RsaI*-digested tester and driver DNAs in a reciprocal subtraction. Positively charged nylon membranes were designated by an α numerical system (matrix). Using a soft-lead pencil, the membranes were lightly marked with dots to guide subsequent sample application and 1 μL of each denatured plasmid DNA from clones was spotted on to an appropriate place on the membranes. (A) represents K11-Lb3-specific clones with K11-Lb3 as the probe. (B) represents K11-Lb3-specific clones with K2-Lb4 as the probe. (C) represents K2-Lb4-specific clones with K2-Lb4 as the probe. (D) represents K2-Lb4-specific clones with K11-Lb3 as the probe. The circled dots represent random clones, which were sequenced.

appeared to encode interesting functions: Glycosyltransferase (46%), rod shape-determining protein MreC (97%), and UDP-*N*-acetylglucosamine 2-epimerase (77%) are all proteins, which obviously act at the cell surface of *L. fermentum*. Thus, they are the likely candidates to be involved in shaping the cell surface for the specific interaction of K11-Lb3 with Caco-2 cells.

Of the 14 K2-Lb6-specific fragments obtained by the reciprocal subtraction, only four were specific for K2-Lb6, since they were not obtained by the subtraction enriching for K11-Lb3 fragments (not shown). These four fragments represented just two RsaI fragments, which were both retrieved two times. One fragment carried a transposase gene, which we considered uninteresting for the reasons given above. The other encoded a gene expressing L-lactate-dehydrogenase, one of the two lactate dehydrogenase genes of heterofermentative lactobacilli [18].

4 Discussion

In this study we found that some strains of *L. fermentum*, isolated from Kimere [13], enhanced hBD-2 secretion by intestinal epithelial cell line CaCo-2 in time-, dose-, and particularly strain-specific manner. Among 11 tested strains only two, namely *L. fermentum* strains K2-Lb6 and K11-Lb3, significantly induced hBD-2 secretion. These data suggest that *L. fermentum* strains K2-Lb6 and K11-Lb3 may enhance innate defence by enhancing the secretion of hBD-2, which sustains mucosal barrier function [11]. While *L. fermentum* strains K11-Lb3 induced hBD-2 secretion, *L. fermentum* strains K2-Lb4 and K6-Lb4 did not. Strain specificity as observed here, could be due to differences in molecular patterns that are recognized by the pattern recognition receptors (PRRs) or by products affecting the physiology of the host cells. Thus, identification of genomic differences between strains exhibiting strain-specific hBD-2 modulatory effects would help to understand the mechanism through which these potential probiotics act on the host cells. Genetic differences between strains of one species form the basis for differentiation into probiotic and nonprobiotic strains. Evidence for genetic diversity within the species of *L. fermentum* has been recently demonstrated with respect to reuterin and cobalmin production [19].

Among the putative gene products identified by subtraction to be specific for K11-Lb3, UDP-*N*-acetylglucosamine, rod shape-determining protein MreC, and glycosyltransferases are involved in building cell-surface structures and exopolysaccharides, both of which may be involved in mediating host–microbe interaction. Bacterial UDP-*N*-acetylglucosamine 2-epimerase is essential for the first step of sialic acid (*N*-acetylneuraminic acid) biosynthesis [20]. Rod shape-determining protein MreC is a bacterial cytoskeletal transmembrane protein required for peptidoglycan synthetic activity [21]. Glycosyltransferases catalyze the attachment of sugars (donor) to an aglycone (acceptor) and are involved in modifying several categories of biopolymers (oligosaccharides, proteins, nucleic acids,

Table 1. Genes of *L. fermentum* strains K11-Lb3 and K2-Lb6, respectively, identified by SSH

Strain	Clone no.[a)b)]	Length of homology (bp)	Similarity to protein[a)]	Score (*e*-value)[c)]	Identity (%)	Identity/organism	Accession no.	Remarks
K11-Lb3								
	E-1a	219	Transposase	150 (4e–35)	99	*Lactobacillus helveticus* DPC 4571	YP_001578083	
	E-1b	735	Chromosome segregation protein	438 (4e–121)	97	*L. fermentum* 28-3-CHN	ZP_05864460	
	K11-33a	90	Transposase	48.5 (4e–06)	74	*Lactobacillus casei* Zhang	YP_003788945	
****	K11-33b	390	Conserved hypothetical protein	97.4 (3e–18)	45	*Lactobacillus oris* PB013-T2-3	ZP_07729600	PemK-like
	C-2, C-9	96	Transposase	67.8 (1e–20)	97	*Lactobacillus casei* Zhang	YP_003788406	
		441	Hypothetical protein	277 (2e–72)	95	*Lactobacillus paracasei*	ABA12818	–
		162	DNA-damage-inducible protein	109 (3e –31)	97	*Lactobacillus hilgardii ATCC 8290*	ZP_03954201	
	D-6	729	Integrase	205 (9e–51)	48	*L. oris* PB013-T2-3	ZP_07728837	
	G-1	711	Bacteriophage integrase	323 (2e–86)	70	*Lactobacillus vaginalis* ATCC 49540	ZP_03959000	
	C-10a	270	Shape-determining protein MreC	173 (5e–41)	97	*L. fermentum* CECT 5716	ADJ40989	
	C-10b	549	DNA replication protein, DnaC	123 (6e–26)	37	*Lactobacillus* phage phiPYB5	ADA79921	
**	B-3	777	UDP-*N*-acetylglucosamine 2-epimerase	371 (1e–100)	77	*Lactobacillus rhamnosus Lc 705*	YP_003174688	
***	E-10	345	Glycosyltransferase	64.3 (5e–09)	32	*Enterococcus faecalis* TX0104	ZP_03949866	
	B-4, F-9	192	Conserved hypothetical protein	74.7 (9e-8)	60	*Lactobacillus coleohominis* 101-4-CHN	ZP_05553637	–
*****	K11–55	194	Hypothetical protein	73.9 (7e–12)	56	*L. fermentum* IFO 3956	YP_001843298	gp58-like phage protein
	D-9, K11-8, K11-30	–	–	–	–	–	–	very low similarity to ''sugar ABC transporter, periplasmic component''
K2-Lb6								
	H-3, L-5	684	Transposase	458 (3e–127)	99	*Lactobacillus sakei*	CAA53008	
	M-7, K2-37	684	L-lactate dehydrogenase	396(2e–108)	97	*L. oris* PB013-T2-3	ZP_07730226	

a) If more than one protein is indicated, they are encoded by different regions of the DNA sequences of the clones.
b) In this column the clones are identified by their positions on the dot blots as shown in Fig. 3.
c) The *e* value indicates the probability of the match. An *e* value $< e^{-5}$ of an alignment means that alignment is highly unique, and not due to error or by chance. The score is a measure of the similarity of the query to the sequence shown.

and lipids) plus numerous natural products, with remarkably varied effects [22].

Of the gene products identified in the reciprocal subtraction, only two appear to be specific for strain K2-Lb4: a transposase and L-lactate dehydrogenase, each of which was found in two different clones (Table 1). In lactic acid bacteria such as lactobacilli, lactate dehydrogenases (LDHs) play key roles in lactic acid fermentation [23], so that antimicrobial activity of luminal fluid predominantly depends on its lactic acid content [24].

The reciprocal subtraction with K2-Lb6 DNA as driver, on one hand may serve as a quality control for SSH of K11-Lb3 DNA, since genes, which are present in higher copy numbers, may be identified in both reciprocal subtractions. On the other hand, genes may be identified, which are not present in K11-Lb3 DNA. Absence of genes may also be involved in establishing specific CaCo-2 cell interacting properties. However, the L-lactate-dehydrogenase gene identified is one of the typical ''house-keeping'' genes of lactic acid bacteria, catalyzing the formation of L-lactate by hydrogenation of pyruvate. *L. fermentum*, a heterofermentative lactic acid bacterium, produces a mixture of L- and D-lactate [18]. Inactivation of one of the two *ldh* genes is easily be tolerated by the cells and results in accumulation of one of the optical isomers of lactate [18]. Since *ldhL* produces L-lactate – the metabolically active form of lactate in the human host – an effect of loss of this gene on the interaction with and modulation of human cell cultures is easily envisaged.

These data argue that the strain-specific modulation of the hBD-2 secretion by different strains of *L. fermentum*, isolated from *Kimere*, can be explained, in part, by genetic differences, which may affect hBD-2 secretion in intestinal epithelial cells. For instance, strain K11-Lb3-specific sequence encodes peptidoglycan synthesis-related enzymes and peptidoglycan in turn induces the induction of the antimicrobial peptide hBD-2 via nucleotide-binding oligomerization domain protein 2 (NOD2/CARD15) [25]. Further, strain K11-Lb3-specific sequence encodes UDP-*N*-acetylglucosamine 2-epimerase which contributes to teichoic acid synthesis [26]. Teichoic acid in turn induces hBD-2 expression in epithelial cells via TLR2 [27], which is known to enhance NF-kB, a transcription factor binding to the promoter of hBD-2 and enhancing hBD-2 [11].

Overall, our data indicate that, some strains of *L. fermentum*, isolated from *Kimere*, induce hBD-2 secretion in time, dose-, and strain-dependent manner. Thus, *Kimere* may stimulate the intestinal innate defence through the induction of inducible antimicrobial peptides like hBD-2, presumably, leading to an enhanced mucosal barrier to the luminal bacteria. Differences exist in the genes of different *L. fermentum* strains, isolates from *Kimere*, since our SSH library revealed that strain K11-Lb3 possesses some gDNA sequences encoding for certain enzymes, which catalyze modification of envelope and/or cell-surface structures, which may enhance the hBD-2 secretion, while at the same time these enzymes are missing in strain K2-Lb4. Further comparisons between defensin-inducing and noninducing strains and particularly knock-down or knock-out of these enzymes will have to be done in order to show, which of the enzymes is mediating the hBD-2-inducing effect. Such findings may help to further elucidate the signalling pathway for defensin induction.

K.H. and J.S. applied for a Patent on the strains described. The other authors have declared no conflict of interest.

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