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Expression of the Mucus Adhesion Gene *Mub*, Surface Layer Protein Slp and Adhesion-Like Factor EF-TU of *Lactobacillus acidophilus* ATCC 4356 Under Digestive Stress Conditions, as Monitored with Real-Time PCR

K. Ramiah · C. A. van Reenen · L. M. T. Dicks

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Abstract Expression of the mucus adhesion gene *Mub*, surface layer protein *Slp* and adhesion-like factor *EF-Tu* by *Lactobacillus acidophilus* ATCC 4356 grown in the presence of mucin, bile and pancreatin and at low pH was studied using real-time PCR. None of the genes were up-regulated under increasing concentrations of mucin, while *Slp* and *EF-Tu* were up-regulated in the presence of bile and pancreatin at normal concentrations (0.3%, w/v) and under stress conditions (1.0%, w/v).

Keywords *Lactobacillus acidophilus* · *Mub*, *Slp* and *EF-Tu* expression · Real-time PCR

Introduction

A number of adhesion proteins have been characterized for lactic acid bacteria, e.g. the mucus binding protein (Mub) [23], mucus adhesion promoting protein (MapA) [25], anchorless multifunctional protein elongation factor Tu (EF-Tu) [10] and GroEL [3], surface layer proteins (Slp) [4, 30] and aggregation promoting factors (apf1 and apf2) [13, 29]. The mucus binding protein (Mub) and mucus adhesion promoting protein (MapA) of *Lactobacillus reuteri* 1063 have been described in detail [23, 25]. The *Mub* gene has two possible translation start sites and the membrane anchor sequence at the C terminus contains a cysteine residue. The presence of two types of amino acid residue repeats (MBP-Mub1 and MBP-Mub2) and an N-terminal region of >500 amino acids indicates that Mub is a multifunctional protein. Mub homologues have also been reported for *Lactobacillus gasseri, Lactobacillus johnsonii* and *Lactobacillus plantarum* [1, 22].

Mub and *MapA* of *L. plantarum* 423 were up-regulated in the presence of mucus, proportional to increasing concentrations [22]. Expression of *MapA* was up-regulated in the presence of 0.3% and 1.0% (m/v) bile or pancreatin at pH 6.5. Expression of *Mub*, on the other hand, was down-regulated under these conditions. Expression of *Mub* and *MapA* by strain 423 remained unchanged at pH 4.0.

Surface layer proteins (Slp) are non-covalently attached to each other and form a crystalline structure on the surface of the cell wall [27]. A number of functions have been described for these proteins, including determining cell shape, protection against harsh environmental conditions [17], acting as phage receptor sites [11], adhesion to intestinal cells [7] and entrapment of molecules and ions [24, 27]. S-layer proteins have also been described for *Lactobacillus amylovorus* [5], *Lactobacillus buchneri* [15], *Lactobacillus gallinarum* [16], *Lactobacillus kefir* and *Lactobacillus parakefir* [9]. The adhesive domain of the S-layer protein of *Lactobacillus brevis* ATCC 8287 and *Lactobacillus crispatus* JCM 5810 has been described. Genes encoding the Slp of *L. acidophilus* [4], *L. brevis* [30], *Lactobacillus helveticus* [6] and *L. crispatus* [26] have been characterized. The *Slp* of *L. acidophilus* ATCC 4356 has been expressed in *Escherichia coli* [4].

An adhesion-like protein, classified as elongation factor Tu (EF-Tu), was isolated from *L. johnsonii* NCC 533 (La1) [10]. EF-Tu facilitates the transfer of aminoacyl-tRNA to the A-site of ribosomes during protein synthesis [8] and binds to mucin [10]. The mechanism by which EF-Tu interacts with mucin is not well understood. The importance of the protein in mediating attachment was reflected by the ability of the purified protein to inhibit binding of

K. Ramiah · C. A. van Reenen · L. M. T. Dicks (✉)
Department of Microbiology, Stellenbosch University, Private Bag X1, Matieland 7602, South Africa
e-mail: lmtd@sun.ac.za

strain La1 to mucin by up to 40%. An EF-Tu protein isolated from *L. plantarum* shared 84% homology with the EF-Tu protein of *L. johnsonii* NCC 533 [10]. Expression of *EF-Tu* by *L. plantarum* 423 grown in the presence of bile was up-regulated in the presence of mucus and at pH 4.0, proportional to increasing concentrations, but remained unchanged in the presence of bile and pancreatin [22].

Most studies on adhesion of probiotic bacteria have focused on phenotypic aspects, such as in vitro models with mucus or epithelial cells [2, 19, 28]. The present study looks at the expression of *Mub*, *Slp* and *EF-Tu* of *L. acidophilus* ATCC 4356 cultured under stress, such as in the presence of bile salts, pancreatic juice and low pH. Regulation of the genes in strains cultured in the presence of varying concentrations of mucin was also studied. Real-time PCR has been used to monitor gene expression.

Materials and Methods

Bacterial Strains and Growth Conditions

Lactobacillus acidophilus ATCC 4356 was cultured in De Man Rogosa Sharpe (MRS) broth (Biolab, Biolab Diagnostics, Midrand, South Africa) at 37°C, without aeration. Gene expression studies were conducted in MRS broth (Biolab) supplemented with porcine mucin (Sigma, Mannheim, Germany), bile and pancreatin to simulate normal and stressful gut conditions (Table 1).

Primer Design and PCR

Primers used in this study are listed in Table 2. Primer sequences for the *Mub*, *EF-Tu* and *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) genes of *L. acidophilus* ATCC 4356 were designed from the genome sequence published for *L. acidophilus* NCFM (accession number NC 006814). Primers for the *Slp* gene were designed from the published sequence [4]. Primers were designed with the Primer Designer Program, version 1.01 (Scientific and Educational Software). Primer sets were designed to produce an amplicon of approximately 150 bp. Primer dimer formation was checked by agarose gel electrophoresis (2% agarose) and melting curve analysis. A standard curve consisting of the log template concentration plotted against the Ct value (crossing points of different standard dilutions) was constructed for each primer set. Efficiencies were calculated by $E = 10^{(-1/S)} - 1$, where S = slope of the standard curve.

Table 1 Modification of MRS broth (Biolab) to simulate gastric conditions

Medium[a]	Mucin (%) (w/v)[b]	Bile (%) (w/v)	Pancreatin (%) (w/v)	pH
1	0	0	0	7.0
2	0.01	0	0	7.0
3	0.05	0	0	7.0
4	0.01	0.3	0.3	6.5
5	0.01	1.0	1.0	6.5
6	0.01	0.3	0.3	4.0

[a] Medium 4 simulates normal gut conditions and media 5 and 6 stress conditions

[b] Porcine mucin (Sigma, Mannheim, Germany)

[c] pH adjusted with 0.5 M NaOH or 0.5 M HCl

RNA Isolation and cDNA Synthesis

Cells were grown to mid-log phase (8–18 h-old cultures, depending on the growth conditions), OD_{600} = approximately 4.0. RNA was isolated by using the RNeasy Mini Kit (Qiagen, Valencia, California, USA) and the method described by Ramiah et al. [22]. RNA (0.2 μg) was transcribed into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany) and gene specific primers. The reaction occurred for 30 min at 55°C.

Real-time PCR

Standard curves were constructed by plotting the Ct-value against the log template value of cDNA of each gene. The method described by Ramiah et al. [22] was used. *GADPH* served as endogenous reference. Gene expression levels were calculated by dividing the normalized target concentration by the control sample (cells grown in MRS, medium 1), and vice versa.

Real-time PCR reactions were performed in a LightCycler instrument (Roche), with Relative Quantification Software (version 5.32; Roche) and SYBR Green technology (Sigma). The PCR method of Ramiah et al. [22] was used. Synthesis of cDNA and real-time PCR was performed in triplicate for each gene. A melting curve was performed from 95 to 45°C (0.2°C/s).

Results

All primers and standard curves displayed an efficiency of above 80%. The values recorded for *L. acidophilus* ATCC 4356 were as follows: $y = -3.75x + 33.69$ (*Mub*), $y = -3.68x + 20.93$ (*Slp*), $y = -3.81x + 25.5$ (*EF-Tu*). Standard curves of glyceraldehyde 3-phosphate dehydrogenase for the different genes were $y = -3.65x + 32.34$ (*Mub*), $y = -3.58x + 32.38$ (*Slp*) and $y = -3.71x + 31.09$ (*EF-Tu*). Primers were designed with optimal efficiency, as

Table 2 Primers used in this study

Primer set	Sequence 5′–3′	Size of amplicon (bp)
Mub 4356F RT	GTAATCGTGTTCTACATATACATAG	152
Mub 4356R RT	GGTTATAAAGTTAACAGCATTGTTC	
Slp 4356F RT	GCACAACGCATACTACTACG	150
Slp 4356R RT	CTTGTCAACAGCCTTACCGT	
EFTu 4356F RT	GGTGCTATCTTAGTTGTTGC	150
EFTu 423R RT	CAACCAAGTCGATCAATTCT	
GADPH 4356F RT	CTATCGTTTACTCAGTAAACCAAGA	149
GADPH 4356R RT	CGTGGATAGTAGTCATAGTACCAAC	

indicated by a single product-specific melting curve. *L. acidophilus* ATCC 4356 survived exposure to 0.5 mol l^{-1} HCl at pH 2.0, which reveals that the strain is capable of surviving gastric transit.

Expression of the *Mub* gene of *L. acidophilus* ATCC 4356 remained unchanged in the presence of 0.01% or 0.05% (w/v) mucin and under normal gut conditions (0.3%, w/v, bile; 0.3%, w/v, pancreatin; pH 6.5 or pH 4.0). However, the gene was down-regulated 18 times when cells were cultured under stressful gut conditions (1.0%, w/v, bile; 1.0%, w/v, pancreatin; pH 6.5) (Table 3).

The *Slp* gene of *L. acidophilus* ATCC 4356 does not seem to be stimulated by mucin, as no up-regulation of the gene was monitored when the strain was cultured under varying concentrations of mucin (Table 3). However, the gene expression was induced approximately 30-fold under normal in vitro conditions (0.3%, w/v, bile; 0.3%, w/v, pancreatin; pH 6.5) and 4-fold under stressful conditions (1.0%, w/v, bile; 1.0%, w/v, pancreatin; pH 6.5). No change in gene expression was recorded when cells were grown in the presence of 0.3% (w/v) bile, 0.3% (w/v) pancreatin and pH 4.0 (Table 3).

The *EF-Tu* gene of *L. acidophilus* ATCC 4356 was up-regulated approximately 40-fold under normal gut conditions (0.3%, w/v, bile; 0.3%, w/v, pancreatin; pH 6.5) and 14-fold under stressful conditions (1.0%, w/v, bile; 1.0%, w/v, pancreatin; pH 6.5) (Table 3). No change in expression was monitored in the presence of increasing concentrations of mucin or in the presence of 0.3% (w/v) bile, 0.3% (w/v) pancreatin and pH 4.0 (Table 3).

Discussion

Adhesion of probiotic cells to mucus, especially in the small intestine characterized with high flow rates, may be an important survival strategy [31]. Furthermore, adhesion to mucus may up-regulate mucin-encoding genes such as *muc 3*, thereby stimulating the production of more mucus [14]. Colonization by probiotic cells may also stimulate the immune system [20].

Since the expression of the *Mub* gene of *L. acidophilus* ATCC 4356 remained unchanged in the presence of 0.01% or 0.05% (w/v) mucin, it may be concluded that the Mub

Table 3 Expression of *Mub*, *Slp* and *EF-Tu* of *L. acidophilus* ATCC 4356[a]

Genes	Medium[b]	Av. Ct[c]	Quant.[d]	*GADPH* Av. Ct	Quant.	Normalized	Set to control
Mub	1	30.7	6.2	30.9	2.5	2.5	1
	4	32.3	2.4	32.0	1.3	1.9	1.3
	5	31.5	3.8	27.1	27.5	0.1	0.1
Slp	1	19.8	2.1	30.9	2.6	0.8	1
	4	15.3	34.7	32.0	1.3	26.7	33
	5	15.9	23	29.3	7.2	3.2	4
EF-Tu	1	24.6	1.7	27.0	12.9	0.1	1
	4	21.1	14.1	29.6	2.5	5.7	44
	5	18.4	72.4	25.2	39.8	1.8	14

[a] No change in gene expression was recorded when cells were grown in media 2, 3 and 6 and are thus not listed

[b] See composition in Table 1

[c] *Av. Ct* average crossing point at which a significant increase in fluorescence is detected

[d] *Quant.* quantity

protein does not play an important role in adhesion of strain ATCC 4356 to mucus. Down-regulation of *Mub* at conditions simulating the lower part of the gastro-intestinal tract (1.0%, w/v, bile; 1.0%, w/v, pancreatin; pH 6.5) is further evidence that Mub may not play a significant role in binding to mucus (the lower part of the intestinal tract is usually more rich in mucus). Up-regulation of the *Mub* gene may, however, be controlled by factors not tested for in our study. The opposite was recorded for *L. plantarum* 423 [22]. In the case of strain 423, the *Mub* gene was up-regulated in the presence of mucus and down-regulated in the presence of bile and pancreatin [22]. This suggests that the expression of *Mub* is a characteristic of the species and may be controlled by other yet unknown factors. Although no increase in *Mub* expression could be observed under conditions tested, it does not mean Mub is not required for adhesion of *L. acidophilus* ATCC 4356 to mucus.

Increased expression of *Slp* under normal conditions (0.3%, w/v, bile; 0.3%, w/v, pancreatin) at pH 6.5 as opposed to no change in gene expression under the same conditions at pH 4.0, suggests that strain ATCC 4356 is better adapted to colonize the lower part of the intestinal tract. Changes in mucin levels did not alter the expression of *Slp* in vitro. This suggests that colonization of strain ATCC 4356 in the lower part of the intestinal tract will have to rely on factors other than mucin.

The EF-Tu protein is associated with the membrane of *E. coli* [12] and the periplasm of *Neisseria gonorrhoeae* [21]. In lactobacilli, EF-Tu may function as an 'envelope associated protein', which may be released from the cell when the organism experiences osmotic shock [10, 18]. The *EF-Tu* of *L. acidophilus* ATCC 4356 was not up-regulated in the presence of mucus. However, *EF-Tu* was up-regulated under normal (0.3%, w/v, bile; 0.3%, w/v, pancreatin; pH 6.5) and stressful (1.0%, w/v, bile; 1.0%, w/v, pancreatin; pH 6.5) conditions. These results suggest that the EF-Tu protein may play an important role in the colonization of strain ATCC 4356. Reasons for the up-regulation of the gene encoding EF-Tu under stressful conditions is not known and merits further research.

Conclusion

Lactobacillus acidophilus ATCC 4356 survived exposure to HCl at pH 2.0. The organism may not display strong adhesive properties as none of the adhesion genes were up-regulated in the presence of mucus. However, up-regulation of *Slp* and *EF-Tu* in the presence of bile and pancreatin suggests that the strain has the ability to adapt to physiological conditions, and may be more as in the lower intestinal tract. It is also possible that other adhesion genes may be involved in the colonization of strain ATCC 4356.

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