Assessment of Bile Salt Effects on S-Layer Production, slp Gene Expression and, Some Physicochemical Properties of *Lactobacillus acidophilus* ATCC 4356

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Received: June 21, 2009 / Revised: November 14, 2009 / Accepted: December 6, 2009

In many conditions, bacterial surface properties are changed as a result of variation in the growth medium and conditions. This study examined the influence of bile salt concentrations (0–0.1%) on colony morphotype, hydrophobicity, \( \text{H}_2\text{O}_2 \) concentration, S-layer protein production, and slpA gene expression in *Lactobacillus acidophilus* ATCC 4356. It was observed that two types of colonies (R and S) were in the control group and the stress condition. When the bile level increased in the medium, the amount of S type was more than the R type. A stepwise increment in the bile concentration resulted in a stepwise decline in the maximum growth rate. The results showed that hydrophobicity was increased in 0.01%–0.02% bile, but it was decreased in 0.1% bile. Treatment by bile (0.01%–0.1%) profoundly decreased \( \text{H}_2\text{O}_2 \) formation. S-Layer protein and slpA gene expression were also altered by the stress condition. S-Protein expression was increased in the stress condition. The slpA gene expression increased in 0.01%–0.05% bile and it decreased in 0.1% bile. However, we found that different bile salt concentrations influenced the morphology and some surface properties of *L. acidophilus* ATCC 4356. These changes were very different in the 0.1% bile. It appears that the bacteria respond abruptly to 0.1% bile.

**Keywords:** *Lactobacillus acidophilus*, S-layer, hydrophobicity, slpA gene

A number of clinical studies have been performed on the ability of probiotic strains to prevent or treat gastrointestinal infections (such as diarrhea) and certain types of cancer [12, 16, 30, 32]. Moreover, they are accredited as being immune response modulators and cholesterol-lowering factors [1, 12, 16, 32]. The most common probiotic strains belong to two genera, *Lactobacillus* and *Bifidobacterium* [32, 37]. Adhesion to intestinal epithelial cells is an important prerequisite for colonization of probiotic strains in the gastrointestinal tract [1, 23]. External structures of bacterial cells such as the S-layer, fibronectin, and mucin-binding proteins may play a role in bacterial cell adhesion [1, 2, 15, 23, 39].

Many species of the genus *Lactobacillus* possess as S-layer [2]. Several reports have appeared in which functions of the S-layer are described or assumed [7, 18]; examples of these functions include it being a protective sheath against hostile environmental agents and having an important role in the establishment of *Lactobacillus acidophilus* in the gastrointestinal tract [2, 15, 23]. There is also increasing evidence that S-layer-carrying bacteria may upraise S-layer variations [5, 15, 18, 34]. The presence of multiple S-layer protein genes seems to be quite common for lactobacilli such as *Lactobacillus acidophilus* ATCC 4356, *Lactobacillus brevis* ATCC 14869, and *Lactobacillus crispatus* ICM 5810 [2, 4, 18]. Boot *et al.* [6, 8] identified two S-layer protein-encoding genes, slpA and slpB, where slpA is active and slpB is silent in normal growth conditions. The slpA gene is interchanged with the slpB gene through inversion of a chromosomal fragment in a fraction of a culture (0.3% of the cells growth under laboratory conditions) [5, 8]. The inversion may happen for adaptation to different stress factors such as the immune response of the host for pathogens and drastic changes in the environmental conditions for nonpathogens [15, 18]. They also can produce \( \text{H}_2\text{O}_2 \). The produced \( \text{H}_2\text{O}_2 \) may inhibit or kill other microbiota and pathogens, particularly those that lack or have low levels of \( \text{H}_2\text{O}_2 \)-scavenging enzymes [13]. To dominate the vaginal

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ecosystem, recent data suggest that H$_2$O$_2$ production by lactobacilli may be more important than lactic acid production [31]. Microorganisms must often cope with hostile environmental conditions [4, 40]. Phenotypically, lactobacilli respond to altered growth conditions by morphological changes that become apparent microscopically or colonially on solid media [1, 22]. In addition, the surface properties of microorganisms are dependent on the medium and the growth conditions [4, 22, 35]. Previous studies show the influence of variation of media and growth conditions on the physicochemical surface properties of several *Lactobacillus* species [10, 16, 22, 23, 27, 35], *Bifidobacterium bifidum* [16], and *Pseudomonas aeruginosa* [21].

Probiotic bacteria face several stresses (such as bile) during passage through the gastrointestinal tract [1, 23, 25]. In these conditions, the bacteria have to survive and adhere to intestinal epithelial cells [1]. Kim et al. [20] studied the stress response of *L. acidophilus* in different concentrations of bile salt (0–1%) and the CFU at each level was determined. They reported that bile at 0.05% was chosen as the sublethal level, since cells were still growing slowly at this level. There was a decrease in the CFU at 0.1%–0.9% bile and all cells were killed at 1.0% bile. Moreover, bile at 0.5% was chosen as the lethal level because the number of CFU was significantly reduced [20].

The aims of this study were to investigate the effects of bile salt on the morphotype of the bacteria and colony, production of the S-layer, expression of the *slp*A gene, and also some physicochemical characteristics of *Lactobacillus acidophilus* ATCC 4356.

### MATERIALS AND METHODS

**Bacteria Strain, Medium, Growth, and Storage**

*L. acidophilus* ATCC 4356 was obtained from the Germany Type Culture Collection and was cultivated in MRS broth (Scharlau, Spain) as described previously [7, 14]. The bacteria were stored according to a method described previously [15].

**Bile Salt Stress Conditions**

For studying the effect of bile salt stress, bile (Sigma) composed of sodium cholate and sodium deoxycholate (1:1) was used. MRS broth and MRS agar (Merk, Germany) (pH 6) containing 0.01%, 0.02%, 0.05%, or 0.1% (w/v) bile (as the stress conditions) were prepared. MRS broth and agar without bile salt were used as the control medium. For comparison of bacteria growth in the stress and control conditions, the bacteria were cultured overnight under anaerobic condition in a Jar with Anaerocult A-strip (Merk) at 37°C for 18 h, and then inoculated into fresh 1% (v/v) medium. They were counted according to a method described previously [10, 16].

**Determination of Colony Morphotype**

The colony morphotype of the *L. acidophilus* ATCC 4356 was determined by stereomicroscopy (ZEISS/Stemi SVII) after culturing on MRS agar plate (in the stress and control conditions) in anaerobic condition with a Jar (Anaerocult A-strip; Merck) at 37°C for 48–72 h. The bacterial cell morphology was investigated by Gram staining.

**Bacterial Hydrophobicity Through Interfacial Adhesion**

The MATH (microbial adhesion to hydrocarbons) test was carried out as described previously [23, 29, 35].

The percentage of bacterial adhesion to solvent was calculated as

\[
\% \text{Hydrophobicity} = \left( \frac{A_n - A_s}{A_n} \right) \times 100
\]

where

- $A_n$= the absorbance before adding solvent at 600 nm
- $A_s$= the absorbance of the aqueous phase after adding solvent at 600 nm

The value of MATS (microbial adhesion to solvents) was obtained with two other solvents, chloroform (Merk) and ethyl acetate (Merk) [23, 29].

**Biofilm Formation Assessment**

Glass slides assays were carried out according to Jagnow and Clegg [17] and Welin et al. [41].

**Microtiter Plate Adhesion Assay**

Biofilm formation was examined by a method reported earlier [26]. *Staphylococcus aureus* PTCC 1431 was used as the positive control and MRS broth without bacteria was used as the negative control.

**Determination of H$_2$O$_2$ Production**

H$_2$O$_2$ concentrations were measured as described before [28].

**Isolation of S-Layer Protein**

For isolation of S-layer and total RNA, the recommended optical density is 0.7 at 695 nm and 0.2–0.4 at 600 nm, respectively [7, 8, 36]. However, in this study, we compared *slp*A gene expression and S-layer production at the same time, so the S-layer protein and total RNA were isolated in exponentially growing cells (OD$_{600}$=0.4). The S-layer protein was isolated according to a method described previously [7].

$L. casei$ ATCC 393 was used as the negative control for isolation of the S-layer.

**SDS–PAGE Analysis**

SDS–PAGE of protein samples was carried out using Precision Plus Protein Standard [low molecular mass marker (10–250 kDa); BioRad]. The samples were run on 12% polyacrylamide gel at 100 V. Protein bands were visualized by Coomassie blue staining.

**Measurement of Total Protein**

After isolation of S-protein by 4.0 M guanidine hydrochloride, the supernatant was dialyzed against water at 4°C and its protein concentration was determined according to Bradford’s method [9].

**Isolation of Total RNA**

*L. acidophilus* ATCC 4356 cells were grown in MRS broth (containing 0–0.1% bile salt) at 37°C under anaerobic condition (OD$_{600}$=0.4). Cells were harvested by centrifugation (5,000 *g* for 10 min at 4°C) [8], and washed with ice-cold TE buffer. Total RNA was isolated with the protective RNeasy Minikit (Qiagen), according to the manufacturer’s recommendations.
The prepared RNA was treated with deoxyribonuclease I (DNase I, RNase-free; Fermentas) at 37°C for 30 min according to the manufacturer’s recommendations.

**RT-PCR**

Reverse transcription (RT) of RNA samples was done with 150 ng of template RNA and 0.5 µg of Oligo dT primer using a First Strand cDNA Synthesis kit (Fermentas) at 42°C for 60 min as recommended by the manufacturer.

Forward and reverse primers designed for the slpA gene of *L. acidophilus* ATCC 4356 were as follows: slpA forward (5'-TGG CCG TTC TTG AAT GTG TA-3') and slpA reverse (5'-ACA TCA ACG CTG CAA ACA TC-3'). These primers generated a 154-bp PCR product in the RT-PCR reaction.

16S rRNA was used as the internal control gene using previously reported primers [38] that generate a 370-bp PCR product.

The final volume of PCR reaction was 25 µl with the following components: 1 µl of cDNA (≈7.5 ng), 1 µl (100 pmol/µl) of each primer, 0.5 µl of dNTPs mix, 0.5 µl of MgCl₂, and 0.25 µl (5 U/µl) of *Taq* DNA polymerase (Fermentas).

The Mastercycler (Eppendorf) was programmed as follows: initial denaturation of 5 min at 94°C; 30 cycles of 94°C for 45 s, 54°C for 30 s, 72°C for 30 s, and final extension of 72°C for 8 min. The PCR products (and 50-bp DNA ladder; Fermentas) were separated on 1% agarose gel and visualized by ethidium bromide staining.

**Statistical Assessment**

All tests were replicated at least three times. All statistical analyses of the results were done with the SPSS and Excel 2003 softwares. All experimental results were analyzed by employing mean descriptive statistics, correlation coefficient, and single-factorial analysis of variance for *P*<0.05.

**RESULTS AND DISCUSSION**

**Colony and Cell Morphotypes of *L. acidophilus* in the Stress Conditions and the Control**

After incubation of *L. acidophilus* ATCC 4356 in MRS agar (under stress and control conditions) for 72 h under anaerobic condition with the Jar at 37°C, heterogeneous colony types were revealed on the surfaces of MRS agar plates. In the control plates, there were two morphotypes of colonies, R (rough) and S (smooth), whereas the R types were approximately 2-fold more than the S types. In the surface colony, the R types were large, irregular, and flat to umbonate, with a matte surface and mottled opacity. In contrast, the S types were smaller, circular with a smooth edge, and convex with a glistening translucent appearance (Fig. 1A).

Gram staining showed that the R-type colonies were composed of a mixture of long and short Gram-positive rods in long chains and filaments in tangled masses of cells (Fig. 1B). The S-type colonies were composed of a mixture of long and short Gram-positive rods in single cells (Fig. 1C).

In MRS agar containing 0.01% bile, the colonies were smaller than the control and a mixture of R and S types was seen. In this stress condition, the number of S types was approximately 10-fold more than R types. The results of this section showed that with increasing the level of bile salt in the medium, the number of R type is decreased. In medium containing 0.1% bile, there was only S types (Fig. 2A) and the cells were short rods and single (Fig. 2B). Probably the resistance of R and S types to stress is variable. It seems that the S type is more stable in the stress condition and also that this concentration of bile effects the
physicochemical surface properties in *L. acidophilus* ATCC 4356. This finding confirmed previous studies by Klaenhammer and Kleeman [22], Altermann et al. [1], and Bron et al. [10].

**Counting Bacteria in the Stress and Control Conditions**

*L. acidophilus* ATCC 4356 was cultivated for 1 h in MRS broth containing 0–0.1% bile salt and its count measured by a spectrophotometer at 600 nm.

The growth curves of bacteria in the stress and control conditions showed that the growth of bacteria decreased in higher bile salt concentration. In 0.05–0.1% bile salt concentration, the growth of bacteria was slower than in control or low concentration of bile (0.01%, 0.02%) (Fig. 3). The highest cell density was observed in culture without bile salt (control) and the lowest cell density was observed in culture supplemented with 0.1% bile (Table 1). According to Bron’s study [10], the maximal growth rate was found to decrease significantly as the bile concentration increased. Moreover, the final OD$_{600}$ reached by *L. plantarum* growth in control [10]. In addition, Klaenhammer and Kleeman [22] reported that the bile sensitivity of R and S cells in *L. acidophilus* RL8K was different. S cells were resistant to high concentration of bile and maintained the original population level through all tested concentrations. Alternatively, R cells were more sensitive to bile, and a significant reduction in colony-forming ability was observed as the bile concentration increased [22]. Therefore, we suggested that a reason for the stepwise decrease in bacterial count is that the R strain is sensitive and could not tolerate bile and thus dies, whereas the S strain has resistance and endures and remains in the stress condition [10, 22]. Moreover, the cell counts were decreased in the high level of bile. There are some evidences that *Lactobacillus acidophilus* strains have variable tolerance to bile [1, 16, 35]. Probably, many adaptive proteins are involved in the stress situation and their gene expressions differ according to different stresses [19]. Kim *et al.* [20] reported that in *L. acidophilus* the adaptive response to one stress has also conferred increased resistance or cross-protection against other unrelated stresses. De Angelis and Gobbetti [11] found that *L. acidophilus*, which was adapted to NaCl, showed an increased resistance to bile salt. The two-dimensional SDS–polyacrylamide gel electrophoresis studies also showed that some adaptive

**Table 1.** Viable cell counts of *L. acidophilus* ATCC 4356 cultured in five media.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Cell count (log CFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.53×10$^7$</td>
</tr>
<tr>
<td>bile 0.01</td>
<td>2×10$^6$</td>
</tr>
<tr>
<td>bile 0.02</td>
<td>1.85×10$^6$</td>
</tr>
<tr>
<td>bile 0.05</td>
<td>5.75×10$^5$</td>
</tr>
<tr>
<td>bile 0.1</td>
<td>5.25×10$^5$</td>
</tr>
</tbody>
</table>

$^a$Media without bile salt (control); $^b$media with 0.01% (w/v) bile salt; $^c$media with 0.02% (w/v) bile salt; $^d$media with 0.05% (w/v) bile salt; $^e$media with 0.1% (w/v) bile salt.

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**Fig. 2.** Appearance of *L. acidophilus* ATCC 4356 colonies.

A. S type on MRS agar containing 0.1% bile. Objective magnification, ×3.2. Photomicrograph of *L. acidophilus* ATCC 4356. B. S cells in MRS agar (0.1% bile). Scale bar=20 µm.

**Fig. 3.** Growth rates of *L. acidophilus* ATCC 4356 in the presence of the control and increasing concentrations of bile salts.

A full-grown culture was in the control (●), 0.01% bile salt (■), 0.02% bile salt (▲), 0.05% bile salt (♦), and 0.1% bile salt (χ). Growth was monitored by measuring the OD$_{600}$. 

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proteins (such as GroES, GroEL, and DnaK) were induced in both acid and heat stresses in *L. delbrueckii* subsp. *bulgaricus* [11, 24]. It was observed that stress-induced gene expression can be very complex as regulation can take place at the level of transcription, translation, or mRNA stability [33]. De Angelis and Gobbetti [11] described that the organization of the *groE* operon is highly conserved. Maximum *groESL* transcription activity in *L. johnsonii* was found after a shift to 55°C for 15 to 30 min. Heat shock induction of the *groESL* operon in *L. johnsonii* also provided protection against other stresses such as freezing. The organization of the *dnaK* operon changed during evolutionary time. Moreover, analysis of mRNA in *L. sakei*, *L. johnsonii*, and *L. acidophilus* showed that the *dnaK* operon is regulated at the transcriptional level and that transcription is induced by heat as well as salt and ethanol stresses [11].

**Hydrophobic Characteristics of *L. acidophilus* ATCC 4356**

To define hydrophobic characteristics, the bacteria (in stress and control conditions) were divided into 3 categories: bacteria with high (71-100%), medium (36-70%), and low (0-35%) hydrophobicities [29].

The results of MATH test by xylene indicated that *L. acidophilus* ATCC 4356 was hydrophobic and the hydrophobicity of the bacteria vigorously decreased at 0.1% bile (Fig. 4). The bacterial hydrophobicity was very high in the control group and 0.01%, 0.02% bile salt; medium in 0.05% bile; and low in 0.1% bile salt concentration (Table 2). Our results showed that the surface properties of *L. acidophilus* ATCC 4356 are affected by adding a compound (such as bile) with different concentrations in the growth medium. It was determined that the presence of (glycol-) pertinacious material at the cell surface results in higher hydrophobicity, whereas hydrophilic surfaces are associated with the presence of polysaccharides [23]. The experiments of Schär-Zammaretti *et al.* [35] and Kos *et al.* [23] demonstrated that the hydrophobicity value is different in the species of lactobacilli and growth medium conditions can affect it.

Bacterial adhesion to chloroform and ethyl acetate was tested to assess the Lewis acid–base characteristics of the bacterial cell surfaces. *L. acidophilus* ATCC 4356 showed stronger affinity for chloroform, which is an acidic solvent and electron acceptor, than for ethyl acetate, which is a basic solvent and electron donor (Table 2). It means that the surface has strong electron donors.

The variations were very noticeable (*p* value=0.000).

**Biofilm Formation**

Biofilm formation of the *L. acidophilus* ATCC 4356 was investigated by adhesion to glass slides and microtiter plates. The results indicated that the bacteria produced biofilm neither on the glass slide nor in the microtiter plate after 72 h (data not shown). Therefore, *L. acidophilus* ATCC 4356 could not produce biofilm in spite of modifying and inspecting the different conditions of growth. Thus, modification of the colony and cell morphology is not depended on biofilm formation.

**Production of H₂O₂ by *L. acidophilus* ATCC 4356**

In order to investigate the stress (bile salt) condition effects on *H₂O₂* production in the bacteria, we measured *H₂O₂* concentration in the medium containing bile salt and in

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### Table 2. Adhesion of *L. acidophilus* ATCC 4356 in the stress conditions and the control.

<table>
<thead>
<tr>
<th>Media</th>
<th>Xylene (%)</th>
<th>Chloroform (%)</th>
<th>Ethyl acetate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>85.98</td>
<td>37.38</td>
<td>0</td>
</tr>
<tr>
<td>0.01% bile</td>
<td>125.5</td>
<td>82.07</td>
<td>-46.22</td>
</tr>
<tr>
<td>0.02% bile</td>
<td>120.58</td>
<td>81</td>
<td>-7.7</td>
</tr>
<tr>
<td>0.05% bile</td>
<td>53.92</td>
<td>72.27</td>
<td>-43.56</td>
</tr>
<tr>
<td>0.1% bile</td>
<td>2.85</td>
<td>77</td>
<td>-73</td>
</tr>
</tbody>
</table>

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![Fig. 4. Assessment of *L. acidophilus* ATCC 4356 hydrophobicity in the control and different bile salt concentrations in MRS broth. Error bars represent standard deviations of the mean values of results from three replicate experiments.](image-url)

![Fig. 5. Effects of different bile salt concentrations on the *H₂O₂* formation in MRS broth. Error bars represent standard deviations of the mean values of results from three replicate experiments.](image-url)
the control medium. The results indicated that \( \text{H}_2\text{O}_2 \) concentration was highest in the control medium (Fig. 5) and the stress conditions (bile salt) decreased \( \text{H}_2\text{O}_2 \) production \((p\text{ value}=0.000)\). Barnard and Stinson [3] found that different situations of growth medium influence hydrogen peroxide formation [3].

**Extraction of S-Protein and Measurement of Total Protein**

Surface proteins of *L. acidophilus* ATCC 4356 were extracted by treatment of whole cells with 4 M guanidine hydrochloride, and analyzed by SDS–PAGE. One dominant band of 43–46 kDa, which is known as the S-protein [7, 36], and a few faint bands were visible on the gel (Fig. 6A). In the mid-log phase \((\text{OD}_{600}=0.4)\), S-protein production was low in the control group so the 43-46 kDa band was not clearly seen on the gel (Fig. 6B, lane S). However, in the control group with \( \text{OD}_{600}=0.7 \), a 43–46 kDa band was visible on the SDS–PAGE gel (Fig. 6A, lane S).

In the stress conditions \((\text{OD}_{600}=0.4)\), the S-protein band was visible and the band become sharper, whereas the bile concentration was increased (Fig. 6B).

For determination of total proteins, the Bradford method was used. Total proteins (extracted in mid-log phase) were compared in the control and stress conditions. In the stress conditions, total protein was more than in the control, and it augmented when the bile concentration was increased (Fig. 7). Therefore, it seems that the S-protein is preferentially expressed under unfavorable growth conditions. As one of the various functions that have been assigned for the S-layer in bacteria is as a protective sheath against hostile environmental agents [7, 35], consequently, S-layer protein expressed in the stress conditions more than in the control condition.

**Expression of *slpA* Gene**

*L. acidophilus* ATCC 4356 was cultured to mid-log phase \((\text{OD}_{600}=0.4)\) in MRS broth containing 0.01–0.1% bile salt and without bile salt (control). Then, total RNA was isolated, reverse transcribed to cDNA, and amplified by PCR with specific primers for the *slpA* gene. The results indicated that the *slpA* gene expression increased in the stress conditions. In the 0.1% bile salt, the *slpA* gene expression was lower than in the 0.02% and 0.05% bile concentrations (Fig. 8). It is not known why the *slpA* gene expression was different in 0.1% bile but S-protein
expression was not. Probably, some genes are expressed in stress conditions. It was confirmed by Lorca and Valdez [27], Kim et al. [20], Altermann et al. [1], and Kristoffersen et al. [25]. These stress proteins may influence on slpA gene expression, or slpA expression may be blocked and slpB is expressed in unfavorable growth conditions instead of slpA [5,8]. The S-layer is a protective sheath against hostile environmental agents and its mRNA has a relatively long half-life of 15 min [6], and it can be translated repeatedly.

In conclusion, we found that different bile concentrations influence the colony and bacterial morphotypes, surface properties, S-layer protein, and slpA gene expression in *L. acidophilus* ATCC 3456. These changes occurred progressively from 0.01% to 0.05% bile. However, the changes were very different in the 0.1% bile. It appears that the bacteria respond abruptly to 0.1% bile. Analyzing the other genes that may be involved in response to bile stress such as slpB can improve our knowledge on it. Moreover, whether this bacterium has the same response to bile in the gastrointestinal tract or not remains to be clarified.

**Acknowledgments**

We acknowledge the financial support of the University of Isfahan and the International Center for Science, High Technology and Environmental Sciences.

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