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Deconjugation and bile salts hydrolase activity by *Bifidobacterium* strains with acquired resistance to bile

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Abstract

Deconjugation by bile salts hydrolases in probiotics has been related to reduction of serum cholesterol levels in mammals. We compared the susceptibility to conjugated primary (glycocholate and taurocholate) and secondary (glycodeoxycholate and taurodeoxycholate) salts and the level of hydrolase activity of *Bifidobacterium* strains with acquired resistance to bile and of their more sensitive original strains. Minimum inhibitory concentrations against conjugated salts of the resistant strains were higher than that of the corresponding originals. None of the strains displayed deconjugation against primary salts, whereas most of them deconjugated secondary salts. Salts of cholic acid were more toxic than that of deoxycholic acid. Derivatives showed higher hydrolase activity than their originals. These results suggested a relationship between bile resistance and deconjugation. Finally, the resistance of bifidobacteria against glycodeoxycholate increased in the presence of maltose and cellobiose as compared with glucose, which could be related to a more efficient energy procurement from disaccharides.

Keywords: *Bifidobacterium*; Deconjugation; Bile salts hydrolase; Bile salts resistance

1. Introduction

Bile salts are synthesised in the liver from cholesterol and secreted as conjugates of either glycine or taurine into the duodenum, where they facilitate fat absorption and undergo enterohepatic circulation (Hofmann, 1984). During this process, bile salts can undergo two major modifications by the intestinal microbiota. Deconjugation of primary bile salts by bile salts hydrolases (BSH) results in the liberation of the amino acid residue and the formation of deconjugated bile acids (mainly cholic and quenodeoxycholic). These primary bile acids may subsequently be 7α-dehydroxylated into secondary bile acids (deoxycholic and lithocholic) (Baron & Hylemon, 1997).

Deconjugation has been recently included by World Health Organisation (WHO) experts as one of the main activities of intestinal microbiota for them to be considered as probiotic microorganisms (FAO/WHO, 2002). Enzymatic deconjugation of bile salts has been related to a reduction of serum cholesterol levels in mammals (Klaver & van der Meer, 1993; Pereira, McCartney, & Gibson, 2003). In contrast, excessive deconjugation and 7α-dehydroxylation plays a role in gall stone formation (Dowling & Murphy, 1990) and enhances the risk of colon cancer (Marteau et al., 1995). Apart from these effects on the host's health, the mechanism of how BSH activity contributes to the functions of the producer microorganisms in the gastrointestinal tract (GIT) is still under discussion. It has been suggested that deconjugation may decrease the toxicity of conjugated bile salts against the producer bacteria, therefore increasing its bile salts resistance (De Smet, van Hoorde, van de Woestyne, Cristiaens, & Verstraete, 1995; Grill, Cayuela, Antoine, & Schneider, 2000a; Savage, 1992). Since deconjugated molecules can still be harmful, microorganisms having 7α-dehydroxylating activity can lower the toxicity of these compounds at moderately acidic pH by dehydroxylation of the deconjugated bile salts and subsequent precipitation of the secondary bile salts formed (De Boever & Verstraete,

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1999). In spite of these hypotheses, some other authors did not report any relation between BSH activity and resistance to toxicity of conjugated bile salts (Ahn, Kim, Lim, Baek, & Kim, 2003; Moser & Savage, 2001; Taranto, Magni, de Mendoza, & de Valdez, 2001; Usman & Hosono, 1999).

BSH are widely present in GIT species of the genera *Lactobacillus* and *Bifidobacterium*, amongst others (Knarreborg, Engberg, Jensen, & Jensen, 2002; Tanaka, Doesburg, Iwasaki, & Mierau, 1999; Vinderola & Reinheimer, 2003). However, the 7α-dehydroxylase activity has not been detected in lactobacilli and bifidobacteria (Ahn et al., 2003; Ferrari, Pacini, & Canzi, 1980; Gilliland & Speck, 1977). In order to exert health-promoting effects attributed to *Bifidobacterium*, these microorganisms must overcome biological barriers of the GIT such as acid in the stomach and bile in the intestine (Gilliland, 1978; Kanbe, 1992; Lankaputhra & Shah, 1995). The adaptation to high bile salt concentrations would be a valuable tool for increasing the ability of probiotic microorganims to survive in the GIT. We recently obtained *Bifidobacterium* derivatives with enhanced resistance to ox gall or cholate by progressive adaptation of more sensitive original strains to gradually increasing concentrations of these compounds (Margolles, García, Sánchez, Gueimonde, & de los Reyes-Gavilán, 2003; Noriega, Gueimonde, Sánchez, Margolles, & de los Reyes-Gavilán, 2004). Acquisition of resistance against a given bile salt also conferred cross-resistance to other deconjugated bile salts, promoted an increase in the survival of these microorganisms at low pH (Noriega et al., 2004) and enhanced the growth in the presence of bile (Margolles et al., 2003). In this context, the aim of the present work was to evaluate how the acquisition of resistance to bile by *Bifidobacterium* affected BSH activity and resistance to conjugated bile salts by these microorganisms.

2. Material and methods

2.1. Bacterial strains

Original *Bifidobacterium* strains and their bile resistant derivatives used in this study were obtained in previous works (Gueimonde, Noriega, Margolles, de los Reyes-Gavilán, & Salminen, 2005; Margolles et al., 2003; Noriega et al., 2004). Strains were cultured under anaerobic conditions (Anaerocult A System, Merck, Darmstadt, Germany) at 37 °C in MRS broth (Merck) supplemented with 0.05% w/v L-cysteine (Merck) (MRSC).

2.2. Sensitivity of Bifidobacterium strains to conjugated bile salts

Minimum inhibitory concentration (MIC) determinations of original strains and bile resistant derivatives were carried out on MRSC agar supplemented with two-fold serial dilutions from 0.125% to 10% (w/v) of salts of cholic and deoxycholic acids: glycocholate (GC), taurocholate (TC), glycodeoxycholate (GDC) and taurodeoxycholate (TDC) (Sigma, St. Louis, MO, USA). Inocula from overnight cultures were spotted on agar plates by means of a Steers replicator and the growth was tested after 48 h of incubation.

The influence of glucose disaccharides maltose and cellobiose on bile resistance levels was also evaluated. For this purpose, MICs of GDC were determined as indicated above in a basal solid medium (peptone 10 g L^{-1}, yeast extract 4 g L^{-1}, sodium acetate 5 g L^{-1}, diammonium citrate 2 g L^{-1}, L-cysteine 0.05 g L^{-1}, magnesium sulphate 0.2 g L^{-1}, manganesium sulphate 0.5 g L^{-1}, pH 6.4) supplemented with 2% (w/v) glucose, maltose or cellobiose (Sigma).

Experiments were made in duplicate, using independent inocula each time. Variability between both measures did not exceed one order of dilution.

2.3. Screening of cultures for bile salts deconjugating activity

The ability of strains to deconjugate primary and secondary bile salts was determined according to Taranto, de Ruiz Holgado, and de Valdez (1995). Bile salt plates were prepared by adding 0.5% (w/v) sodium salts of TC, TDC, GC and GDC to MRSC agar. Overnight liquid cultures of strains (10 μL) were spotted on agar plates and incubated for 72 h. The presence of precipitated bile acid around colonies (opaque halo) was considered a positive result. The strains *Bifidobacterium longum* NIZO B667, *B. animalis* IPLA 4549, *B. bifidum* A1, *B. bifidum* M6 and their corresponding bile resistant derivatives were selected for the subsequent quantification of BSH activity.

2.4. Quantitative determination of bile salts hydrolases activity

Cells were grown overnight (around 18 h incubation) in 10 mL MRSC, washed twice in 0.1 M pH 6.8 sodium-phosphate buffer containing 10 mM dithiothreitol (DTT), and re-suspended in the same buffer to give a final optical absorbance ($A_{600\,nm}$) of approximately 3. For obtaining cell-free extract, cells were sonicated for 60 s while cooling on ice with two cycles of 16 μm, using a CV17 sonicator (VibraCell, Sonics and Materials Inc., Newtown, CT, USA). Cell-free extracts were centrifuged to remove cell debris.

Determination of BSH activity was performed using a two-step procedure by quantification of the amount of liberated amino acids from the conjugated bile salts as indicated by Tanaka et al. (1999), with minor modifications. The appropriate conjugated bile salt (10 μL of a 200 mM solution) and 10 mM DTT were added to 180 μL of 0.1 M pH 6.0 sodium phosphate buffer. To this mixture 10 μL of cell-free extract were added and the reaction was carried out at 37 °C. Samples (35 μL) were taken after 10