Biotechnol Lett (2011) 33:2265–2269
DOI 10.1007/s10529-011-0696-3

ORIGINAL RESEARCH PAPER

Efflux of bile acids in *Lactobacillus reuteri* is mediated by ATP

Ana Yanina Bustos · Raúl Raya · Graciela Font de Valdez · María Pía Taranto

Received: 24 May 2011 / Accepted: 1 July 2011 / Published online: 10 July 2011

Abstract
Purpose of work To study whether an active bile acid (BA) efflux occurs in *Lactobacillus reuteri* CRL 1098 as well as the nature (ATP or proton motive force [PMF] mediated primary transport) of the BA extrusion mechanism.
BAs are powerful detergents which disorganize the lipid bilayer structure of cellular membranes. Specific bile resistance mechanisms (bile efflux, bile salt hydrolysis, and intrinsic architecture and composition of cell membrane the most prevalent) have been described in intestinal bacteria. *L. reuteri*, showed a significant degree of resistance to the toxic action of BA and the presence of an active efflux ATP-dependent of conjugated (taurocholic [TCA]) and free (cholic [CA]) BA in the CRL 1098 strain is now reported. However, due the high p*K*a (5.5) of cholic acid (CA) compared with the conjugated species, a significant fraction (between 35 and 50% at pH 6.5 and 5.2, respectively) of free BA also diffused passively, even in the absence of ATP. To our knowledge, our results represent the first evidence of ATP as the energy source involved in the BA extrusion in *L. reuteri*.

Keywords Active efflux · Bile acid resistance · *Lactobacillus*

A. Y. Bustos · R. Raya · G. F. de Valdez · M. P. Taranto (✉)
Centro de Referencia para Lactobacilos (CERELA-CONICET)—Chacabuco 145 (T4000ILC), San Miguel de Tucumán, Argentina
e-mail: ptaranto@cerela.org.ar

G. F. de Valdez
Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Ayacucho 491 (T4000INI), San Miguel de Tucumán, Argentina

Introduction

Bile acids (BAs) are made by the liver and secreted into the bile to facilitate the enzymatic digestion of fats in the small intestine. In humans, they are synthesized as primary BA, mainly cholic (CA) and chenodeoxycholic (CDCA) acids, and secreted as conjugated species via amide bonds to either glycine or taurine. BA are toxic for all cells and their detersive character resembles the toxicity pattern of SDS (Begley et al. 2005; Schmidt et al. 2001). These compounds dissipate the membrane potential ($\Delta\Psi$) of most bacteria, with the resulting loss of membrane integrity and cell death (Kurdi et al. 2006).

Specific bile resistance mechanisms have been described in intestinal bacteria, bile efflux and bile salt hydrolysis being the most prevalent (Piddock 2006). Intrinsic architecture and composition of cell membrane are also important. Gram-negative

bacteria are inherently more resistant to bile than Gram-positive bacteria. Furthermore, multidrug resistance (MDR) proteins seem to play a crucial role in conferring bile resistance phenotype in Gram-negative bacteria (Lin et al. 2003; Prouty et al. 2002; Thanassi et al. 1997).

Although several MDR systems have also been identified in Gram-positive bacteria (Florez et al. 2006; Mazurkiewicz et al. 2005) the relationship of these transporters with BA extrusion has not received much scientific attention so far.

Transporters able to extrude BA have been described in *Lactococcus lactis* (Yokota et al. 2000), *Lactobacillus johnsonii* (Elkins and Savage 2003) and *Bifidobacterium longum* (Gueimonde et al. 2009).

Lactobacillus reuteri is the most widely distributed *Lactobacillus* species in mammals. Its ability to survive the passage through the intestinal tract, and hence to resist the toxic effect of BA, is a key factor related to its probiotic function. Whitehead et al. (2008) identified two MDR transporters (lr1265 and lr1584) in *L. reuteri* ATCC 55730 that were significantly overexpressed during bile exposure. Interestingly, two other MDR transporters (lr0018 and lr1519) were found in the genome sequence of *L. reuteri* DSM 20016; however, the link between them and BA extrusion was not established.

L. reuteri CRL 1098, a probiotic bacterium with a proven hypocholesterolemic effect (Taranto et al. 2000), shows a significant degree of resistance to BAs. This strain displays high levels of bile salt hydrolase (BSH) activity and its membrane potential is not significantly modified as a consequence of BA exposition (Bustos et al. 2011; Taranto et al. 1999). Previous reports (Taranto et al. 2001) have shown that a conjugated BA (taurocholic acid [TCA]) is incorporated in the cells by an active and saturable transport. The non-conjugated BA, released by the action of the intracellular BSH outside the cell was visualized by a dense/diffuse granular precipitate around the colonies grown in agar medium. Nevertheless, the mechanism involved in BA extrusion in this strain was not studied in detail yet.

In this work, the occurrence of an active BA efflux in *L. reuteri* CRL 1098 through a primary ATP mediated transport is reported. To our knowledge, this is the first evidence of an ATP-mediated BA extrusion in a *L. reuteri* strain.

Materials and methods

Microorganisms and chemicals

L. reuteri CRL 1098, from the CERELA Culture Collection (San Miguel de Tucumán, Argentina), was grown in MRS broth at 37°C for 16 h.

Preparation of everted membrane vesicles

Inside-out membrane vesicles were obtained from *L. reuteri* CRL 1098 as described by Minahk et al. (2004). Cells in stationary phase were harvested and washed twice with 50 mM HEPES buffer pH 7.5. The cell suspension was divided in two fractions, harvested again and resuspended either in 50 mM acetate buffer pH 5.2 (buffer A) or 50 mM MOPS pH 6.8 (buffer B). Then, they were passed through a French press cell at 1,200 psi. After centrifugation ($14{,}000\times g$ for 15 min), cell debris and unbroken cells were discarded. Everted membranes were collected from the supernatants by ultracentrifugation (at $150{,}000\times g$ for 1 h at 4°C) and resuspended in the corresponding buffer supplemented with 10% (v/v) glycerol. The vesicles were stored at −70°C until use.

Accumulation of BA in everted membrane vesicles by PMF

Everted vesicles were thawed on ice and diluted to 0.8 mg protein/ml using the opposite buffer, that is, those vesicles prepared in buffer A were resuspended in buffer B, and vice versa; the difference between the internal and external pHs generates a proton gradient through the vesicles membranes. Vesicles were then incubated for 20 min at 37°C and immediately 2 mM TCA was added (final concentration). At several times, samples were removed and were immediately cooled to discontinue the transport. After thawing, supernatants were collected by ultracentrifugation ($150{,}000\times g$ for 1 h at 4°C) and stored at −20°C until analysis. In independent experiments, the pH_{in} and pH_{out} were equilibrated by addition of 2 μM nigericin (final concentration).

Accumulation of BA in everted membrane vesicles by primary transport mediated by ATP

Everted vesicles were thawed on ice and diluted to 0.8 mg protein/ml using the same buffer in which they were prepared. Vesicles were incubated for 20 min a 37°C and immediately TCA and CA were added to give 2 mM. After 5 min, incubation vesicles were energized by adding 5 mM (final concentration) ATP. Samples were removed at different times and processed as described above. In independent control experiments, vesicles were not energized with ATP or were de-energized with 1 mM sodium vanadate.

Analysis of BA concentrations using HPLC

Supernatant samples were prepared for HPLC analysis using the procedure described by Jones et al. (2004) with some modifications. Briefly, 250 µl supernatants were acidified with 2.5 µl 6 M HCl and supplemented with 250 µl methanol. Samples were mixed in vortex for 10 min and centrifuged (1,000×*g* for 20 min at 10°C); the supernatants were filtered through a 0.22 µm PVDF filter (Millipore). Analyses were performed on a RP C18 column (250 × 4.6 mm, 5 µm). The solvents used were 0.05 M sodium acetate buffer adjusted to pH 4.3 with *o*-phosphoric acid and filtered through a 0.22 µm filter (Solvent A) and HPLC-grade methanol (Solvent B). An isocratic elution was applied, consisting of 30% Solvent A and 70% Solvent B at 1 ml/min. Standards for calibration containing 0, 0.5, 1, 1.5 and 2 mM TCA and CA were treated as above.

Reproducibility

All results presented in this paper are the means of three independent assays. The variations were less than 5%.

Results and discussion

Accumulation of conjugated BA (TCA) by everted membrane vesicles

Strains of *L. reuteri* are members of the intestinal microbiota and are currently used as probiotics (Liu et al. 2010; Taranto et al. 2000). *L. reuteri* CRL 1098 can resist the toxic action of BA (Bustos et al. 2011; Taranto et al. 2006).

To study the mechanism involved in the BA efflux in this strain, a widely-described system used to study bacterial transport mechanism (Minahk et al. 2004; Thanassi et al. 1997; Kodama et al. 1998) was employed. If BA are actively pumped out by intact cells, this same process should produce an active accumulation of these acids in everted (inside-out) membrane vesicles. We therefore investigated if the conjugated BA TCA is accumulated by everted membrane vesicles of *L. reuteri* CRL 1098 by using PMF or a chemical energy source such ATP.

The assays of accumulation of TCA by everted membrane vesicles were carried at pH values of 5.2 and 6.5 (Fig. 1a, b, respectively). To study the effect of the PMF, two types of everted vesicles were used; one displaying an electrochemical gradient generated through the membranes with internal and external pH values of 5.2 and 6.5, respectively; and the second one with an electrochemical gradient generated with reverse pH values. In contrast, the effect of a chemical energy source (ATP) was studied in the absence of an electrochemical gradient using everted vesicles with the same external and internal pH values (either 5.2 or 6.5).

No incorporation of TCA was observed in the absence of a chemical energy source or in the presence of a PMF, while after addition of ATP, the TCA accumulation by the everted membranes of *L. reuteri* CRL 1098 strain reached the highest values after 15 min (about 3 and 3.5 mmol/mg protein at pH 6.5 and 5.2, respectively) and then decreased. The TCA transport mediated by ATP was inhibited when the vesicles were de-energized with sodium vanadate (Fig. 1a, b) and nigericin (data not shown). Active accumulation of TCA in everted membrane vesicles without electrochemical proton gradient, as well as the immediate inhibition of the transport process by sodium vanadate, strongly suggests that this conjugated BA excretion system in *L. reuteri* CRL 1098 is driven by ATP instead PMF.

Accumulation of free BA (CA) by everted membrane vesicles

L. reuteri CRL 1098 possesses an intracellular BSH that deconjugates the incorporated BA (i.e., TCA) to free BA (i.e., CA) plus amino acids in the cytoplasm.

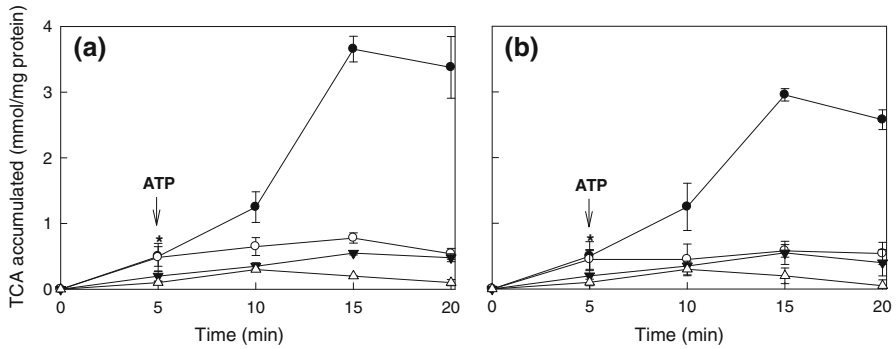

Fig. 1 TCA accumulation by everted membrane vesicles of *L. reuteri* CRL 1098 at pH 5.2 (**a**) and 6.5 (**b**). Everted membrane vesicles of *L. reuteri* CRL 1098 strain were incubated in the absence (*open circles*) or in the presence (*closed circles*) of 5 mM ATP. In separate experiments, the vesicles were incubated with 1 mM sodium vanadate plus ATP (*closed inverted triangles*) and in presence of an electrochemical gradient (*filled inverted triangles*). (*Filled circles*) with ATP; (*open circles*) without ATP; (*filled inverted triangles*) vanadate plus ATP; (*open triangles*) PMF. *Standard deviation

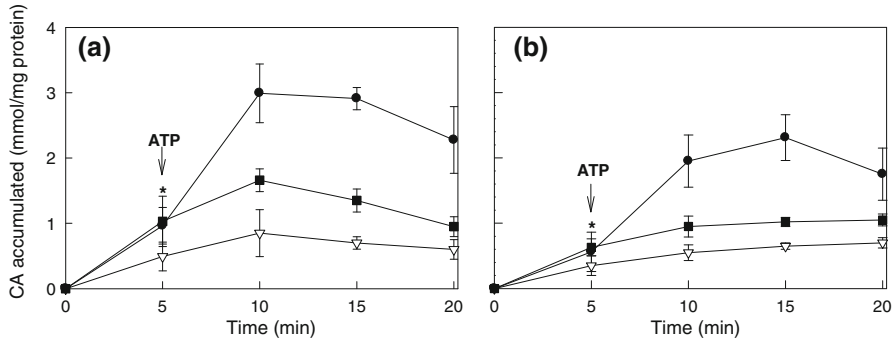

Fig. 2 CA accumulation by everted membrane vesicles of *L. reuteri* CRL 1098 at pH 5.2 (**a**) and 6.5 (**b**). Everted membrane vesicles of *L. reuteri* CRL 1098 strain were incubated in the absence (*open inverted triangles*) or presence (*filled squares*) of 5 mM ATP. In separate experiments, the vesicles were incubated with 1 mM sodium vanadate plus ATP (*open inverted triangles*). (*Filled circles*) with ATP; (*filled squares*) without ATP; (*filled inverted triangles*) vanadate plus ATP. *Standard deviation

To study whether the CA could be accumulated in everted vesicles using ATP, similar assays as described above were carried out. As shown in Fig. 2a, b, the CA was actively incorporated into the vesicles at both pH values after addition of ATP. However, incorporation of free BA was also observed in absence of ATP; this passive transport was higher at pH 5.2 than at pH 6.5. In fact, at pH 5.2, 50% of CA passively diffused through the membranes of everted vesicles while the remaining free bile was incorporated by active transport. On the contrary, at pH 6.5 most of CA used was accumulated by active transport (around 65%). These results could be explained by the p*K*a value (5.5) of CA; at an external pH value of 5.2, 50% of CA is in its protonated form enabling it to cross the lipid bilayer by passive diffusion. However, at pH 6.5, the CA is mainly in its ionized form preventing the passive diffusion through the cell and allowing only the membrane passage by active transport. On the contrary, tauro-conjugates BA are stronger acids (TCA p*K*a = 1.5) than free BA and consequently, their non-dissociated (acid) forms are not able to passively leave the cell at both pH values tested.

L. reuteri CRL 1098 showed a significant degree of resistance to bile (Bustos et al. 2011; Taranto et al. 2006). Our results suggest that the CRL 1098 strain extrudes both TCA and CA by an active transport system mediated by ATP. Both, the presence of BSH activity and the active efflux of BA in this microorganism could contribute to the greater bile tolerance of this strain. Thus, the protonated conjugated

BA that enters the cell by active transport can be converted into their weaker deconjugated counterparts by the BSH enzyme. The latter compounds may then recapture the cotransported proton, preventing the excessive expenditure of ATP to maintain pH homeostasis. According to the external pH conditions, the protonated deconjugated BA may be then detoxified by being passively transported outside the cell or excreted from the cell by an additional active transport system. BA efflux was not observed in everted vesicles obtained from the strain *L. delbrueckii* CRL 494, a BA sensitive and BSH negative strain (Bustos et al. 2011) when PMF or ATP was used as energy source (unpublished data).

In summary, the presence of an active efflux for both conjugated and free BA ATP-mediated primary transport in *L. reuteri* CRL 1098 has been demonstrated. To our knowledge, this is the first evidence to show that ATP is the energy source involved in BA extrusion in a LAB strain. The relationship between this system and any MDR remains to be elucidated.

Acknowledgments This work was supported by grants from CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas), ANPCyT (Agencia Nacional de Promoción Científica y Tecnológica), and CIUNT (Universidad Nacional de Tucumán) from Argentina.

References

Begley M, Gahan CG, Hill C (2005) The interaction between bacteria and bile. FEMS Microbiol Rev 29:625–651

Bustos AY, Raya R, Bru E, de Valdez GF, Taranto MP (2011) Application of fluorescent techniques to evaluate the survival of probiotic lactobacilli to bile acid. Biotechnol Lett 33:1389–1394

Elkins CA, Savage DC (2003) CbsT2 from *Lactobacillus johnsonii* 100-100 is a transport protein of the major facilitator superfamily that facilitates bile acid antiport. J Mol Microbiol Biotechnol 6:76–87

Florez AB, de Los Reyes-Gavilan CG, Wind A, Mayo B, Margolles A (2006) Ubiquity and diversity of multidrug resistance genes in *Lactococcus lactis* strains isolated between 1936 and 1995. FEMS Microbiol Lett 263:21–25

Gueimonde M, Garrigues C, van Sinderen D, de los Reyes-Gavilan CG, Margolles A (2009) Bile-inducible efflux transporter from *Bifidobacterium longum* NCC2705, conferring bile resistance. Appl Environ Microbiol 75:3153–3160

Jones ML, Chen H, Ouyang W, Metz T, Prakash S (2004) Microencapsulated genetically engineered *Lactobacillus plantarum* 80 (pCBH1) for bile acid deconjugation and its implication in lowering cholesterol. J Biomed Biotechnol 2004:61–69

Kodama K, Hashimoto A, Morita Y, Tomochika K, Tsuchiya T (1998) Preparation and characterization of everted membrane vesicles from cells of *Staphylococcus aureus*. Biol Pharm Bull 21:5

Kurdi P, Kawanishi K, Mizutani K, Yokota A (2006) Mechanism of growth inhibition by free bile acids in lactobacilli and bifidobacteria. J Bacteriol 188:1979–1986

Lin J, Sahin O, Michel LO, Zhang Q (2003) Critical role of multidrug efflux pump CmeABC in bile resistance and in vivo colonization of *Campylobacter jejuni*. Infect Immun 71:4250–4259

Liu Y, Fatheree NY, Mangalat N, Rhoads JM (2010) Human-derived probiotic *Lactobacillus reuteri* strains differentially reduce intestinal inflammation. Am J Physiol Gastrointest Liver Physiol 299:G1087

Mazurkiewicz P, Sakamoto K, Poelarends GJ, Konings WN (2005) Multidrug transporters in lactic acid bacteria. Mini Rev Med Chem 5:173–181

Minahk CJ, Dupuy F, Morero RD (2004) Enhancement of antibiotic activity by sub-lethal concentrations of enterocin CRL35. J Antimicrob Chemother 53:240–246

Piddock LJ (2006) Multidrug-resistance efflux pumps—not just for resistance. Nat Rev Microbiol 4:629–636

Prouty AM, Van Velkinburgh JC, Gunn JS (2002) *Salmonella enterica* serovar typhimurium resistance to bile: identification and characterization of the tolQRA cluster. J Bacteriol 184:1270–1276

Schmidt EJ et al (2001) Activities of cholic acid-derived antimicrobial agents against multidrug-resistant bacteria. J Antimicrob Chemother 47:671–674

Taranto MP, Sesma F, Font de Valdez G (1999) Localization and primary characterization of bile salt hydrolase from *Lactobacillus reuteri*. Biotechnol Lett 21:935–938

Taranto MP, Medici M, Perdigon G, Ruiz Holgado AP, Valdez GF (2000) Effect of *Lactobacillus reuteri* on the prevention of hypercholesterolemia in mice. J Dairy Sci 83:401–403

Taranto MP, Magni C, de Mendoza D, Valdez GF (2001) Kinetics characterization of taurocholic transport in *Lactobacillus reuteri*. Curr Microbiol 42:18–20

Taranto MP, Perez-Martinez G, Font de Valdez G (2006) Effect of bile acid on the cell membrane functionality of lactic acid bacteria for oral administration. Res Microbiol 157:720–725

Thanassi DG, Cheng LW, Nikaido H (1997) Active efflux of bile salts by *Escherichia coli*. J Bacteriol 179:2512–2518

Whitehead K, Versalovic J, Roos S, Britton RA (2008) Genomic and genetic characterization of the bile stress response of probiotic *Lactobacillus reuteri* ATCC 55730. Appl Environ Microbiol 74:1812–1819

Yokota A, Veenstra M, Kurdi P, van Veen HW, Konings WN (2000) Cholate resistance in *Lactococcus lactis* is mediated by an ATP-dependent multispecific organic anion transporter. J Bacteriol 182:5196–5201