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ScienceDirect
Research in Microbiology 157 (2006) 720–725
Research in Microbiology
www.elsevier.com/locate/resmic

Effect of bile acid on the cell membrane functionality of lactic acid bacteria for oral administration

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Received 10 January 2006; accepted 11 April 2006

Available online 2 May 2006

Abstract

Lactic acid bacteria and other species dwelling in the gut must be tolerant to bile salts. This study sought to determine the effects of the bile salts taurodeoxycholate (TDCA) and deoxycholate (DCA) on *Lactobacillus reuteri* CRL 1098, a strain of likely probiotic value. When compared to other lactobacilli, *L. reuteri* showed the highest survival rate but remained sensitive to high (>3 mM) DCA concentrations. DCA produced complete permeabilization of cells, abolished glucose uptake and severely distorted the cell envelope, as shown by electron microscopy. Detailed analytical studies revealed a change in the phospholipid to glycolipid ratio, and also in lipid proportions. The C18:1 W9C form remarkably increased, possibly following a rapid adaptive response during DCA treatment. This study provides the first solid evidence of the mechanism of DCA toxicity in a lactic acid bacterium.

Keywords: Deoxycholic acid; Cell membrane; Lactic acid bacteria

1. Introduction

Indigenous microbiota of the intestinal tract is exposed to bile acids, which are products of cholesterol metabolism in the liver and play an important role in the digestive process due to their amphipathic nature. When selecting lactic acid bacteria for use as dietary adjuncts, a number of factors should be considered. While the functionality of probiotics depends on their ability to survive and colonize the gastrointestinal tract, resistance of cells to bile acids is a strictly necessary property. Natural tolerance to bile salts was initially associated with the presence of bile salt hydrolase activity [10,26]; however, different research works showed that, at least in lactobacilli, bile salt resistance could not be correlated with the presence of this enzyme [16,26,28]. Furthermore, conjugated bile acids are less inhibitory than free bile acids (cholic and deoxycholic) toward intestinal aerobic and anaerobic bacteria [15]. Significant variations in bile tolerance have been reported among lactobacillus species and strains [6,8,16]. In Gram-positive bacteria, the toxicity pattern of bile acids resembles that of detergents such as SDS [2,14,28]; however, the actual effects of bile acids on the bacterial cell, and consequently the mechanisms of tolerance/resistance, have not been clearly established. Through isolation of bile-salt-sensitive mutants in *Enterococcus faecalis* and *Listeria monocytogenes,* it was shown that survival in a bile-rich environment relies on multiple factors. Different loci related to this effect were identified, such as stress response systems and transcriptional regulators, elements involved in the maintenance of the cell envelope, energy metabolism, amino acid transport (putative role in pH homeostasis) and fatty acid or isoprenoid biosynthesis [2,4].

In the present work, we sought to determine the mechanisms of toxicity of deoxycholic acid (DCA) and taurine-conjugated (TDCA) on *Lactobacillus reuteri* CRL 1098, a strain with probiotic potential [30].

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0923-2508/$ – see front matter
doi:10.1016/j.resmic.2006.04.002

2. Materials and methods

2.1. Microorganisms and culture conditions

Lactobacillus strains used in this study were: *L. reuteri* CRL 1098 (from CERELA collection, Tucumán, Argentina), *L. casei* CECT 5275 (formerly strain ATCC393 [pLZ15$^-$]); *L. acidophilus* ATCC 4356^T; and *L. delbrueckii* subsp. *bulgaricus* ATCC 11842^T. Cultures were grown twice in MRS broth [9] at 37 °C for 16 h before use. For transport experiments, strains was cultured at 37 °C under static conditions in MRS.

2.2. Viability assay

An overnight (16-h-old) culture of each strain (approximately 10^9 CFU/ml) was plated in mass in MRS agar and MRS agar supplemented with different concentrations (1, 3, 5, 10 mM) of taurodeoxycholic (TDCA) or deoxycholic acid (DCA). After incubation of plates at 37 °C for 48 h, colonies were counted and results expressed as percentage of inhibition with respect to the cell count in the absence of the bile salts.

2.3. β-Galactosidase activity

Cells from overnight cultures were collected by centrifugation, washed twice with buffer Tris–HCl (50 mM, pH 7.0) and suspended in the same buffer to a final concentration of 10^9 CFU/ml (resting cell system). One ml of the cell suspension ($A_{560} = 1$) was incubated at 37 °C in the presence of TDCA and DCA at different concentrations (1, 3, 5, 10, 12, and 15 mM). Fifty-µl samples were taken every 15 min for 120 min, and β-galactosidase activity determined according to Chassy and Thompson [5] using *o*-nitrophenol-β-D-galoctopyranoside as substrate. Activity was expressed as nmol of *o*-nitrophenol released per mg of wet cell biomass per min. Cells permeabilized by the addition of 100 µl of toluene-acetone (1:9, v/v) per ml of cell suspension under agitation for 5 min were used as reference controls for total permeabilization.

2.4. Glucose transport assays with and without bile acids

The effect of DCA and TDCA on glucose transport was determined in 15 ml of 0.05 M phosphate (7.0) buffer (PMB) containing 100 µg of wet cell weight. The bile acids were added separately at final concentrations of 1, 3, 5 and 10 mM. Cell suspensions were incubated at 37 °C for 5 min before D-[^{14}C]glucose (0.6 mCi mmol^{-1} 11.1 MBq mmol^{-1}) was added. At various intervals (0, 15, 30, 60, and 120 s) 1 ml aliquots were withdrawn and quickly cooled by mixing with 10 ml ice-cold PMB. Samples were filtered through 0.45 µm membrane filters (HAWP 02500, Millipore) and washed with 10 ml ice-cold PMB. Filters were dried and dissolved in a scintillation cocktail (Hisafe3, LKB-Pharmacia, Uppsala, Sweden). Radioactivity was quantified with a scintillation counter (Wallac, LKB-Pharmacia, Uppsala, Sweden). A transport assay without bile acids was used as control.

2.5. Transmission electron microscopy

Cells treated with 5 mM DCA or TDCA for 20 min at 37 °C were harvested by centrifugation, washed with 0.05 M Tris–HCl buffer (pH 7.0) and suspended (1:1, vol/vol) in 0.05 M Tris–HCl buffer (pH 7.0) for 2 h at room temperature (20 °C), followed by dehydration in ethanol and embedding in Durcupan. Staining was performed as described previously [27]. Thin sections were examined in an electron microscope (EM 109 Zeiss, Germany).

2.6. Lipid profile

Cell lipids were extracted by the method of Bleigh and Dyer [3] with the following modification: To 1 ml suspensions in Milli-Q water containing about 40–50 mg wet cell biomass (obtained from the different culture media with and without 5 mM of TDCA and DCA) was added 3.75 ml of a methanol:chloroform (2:1, v/v) mixture to obtain a monophasic system. Samples were kept at room temperature for 2 h under agitation, left to stand at 4 °C for 24 h, and centrifuged (3000 *g*, 15 min, 4 °C). The supernatant fluids were collected and pellets washed with 4.75 ml of a methanol:chloroform: water (2:1:0.8, v/v) mixture followed by centrifugation. To turn the system into a diphasic state, 2.5 ml water and 2.5 ml chloroform were added to each tube. The chloroform-rich (bottom) phase containing the cell lipids was quantitatively removed with a Pasteur pipette and the solvent was flash-evaporated. The lipid residue was immediately dissolved with a mixture of chloroform:methanol (1:1, v/v) and stored at −20 °C under nitrogen atmosphere.

The total amount of phosphorous and sugar was determined by the modified technique of Allen and Dubois [23,24] and results were expressed as mg (P or sugar) mg^{-1} dry weight. Total fatty acids were extracted and methyl-esterified from 60–80 mg (wet weight) of cell pellets treated or not (control) with 7 mM of DCA and TDCA for 30 min at 37 °C as previously described [12]. Analytical gas-liquid chromatography was carried out on a Hewlett-Packard 7890 gas-liquid chromatograph (Hewlett-Packard, Avondale, Pa) interfaced with a mass spectrometer (Trio-2 VG; Masslab, Manchester, UK) equipped with a split–splitless injector, flame ionization detector and integrator.

3. Results

Fig. 1 shows the effect of conjugated and non-conjugated bile acids on microorganism survival. TDCA was less toxic than DCA, with differences among strains. *L. reuteri* CRL 1098 proved to be resistant to all concentrations of TDCA tested, while *L. delbrueckii* subsp. *bulgaricus* ATCC 11842^T, *L. acidophilus* ATCC 4356^T and *L. casei* CECT 5237 were very sensitive (62–87% cell inhibition) ($P < 0.001$). Similarly, 1 mM DCA (Fig. 1b) caused significant ($P < 0.001$) growth inhibition (80–100%) in the latter strains, while *L. reuteri* CRL 1098 was slightly inhibited (12%).

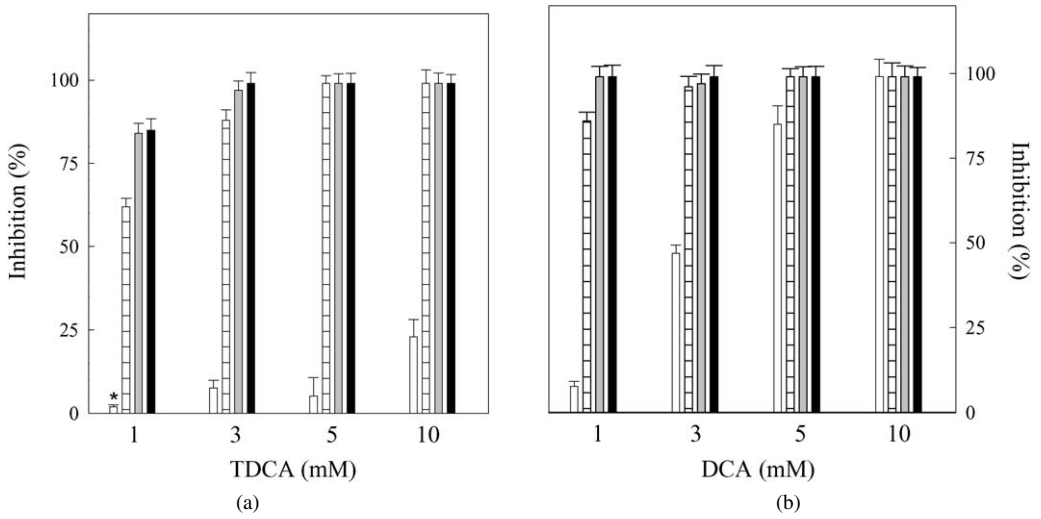

Fig. 1. Effect of TDCA (a) and DCA (b) on the viability of *Lactobacillus* strains. (□) *L. reuteri* CRL 1098; (▤) *L. casei* CECT 5275; (▩) *L. acidophilus* ATCC 4356^T; (■) *L. delbrueckii* subsp. *bulgaricus* ATCC 11842^T.

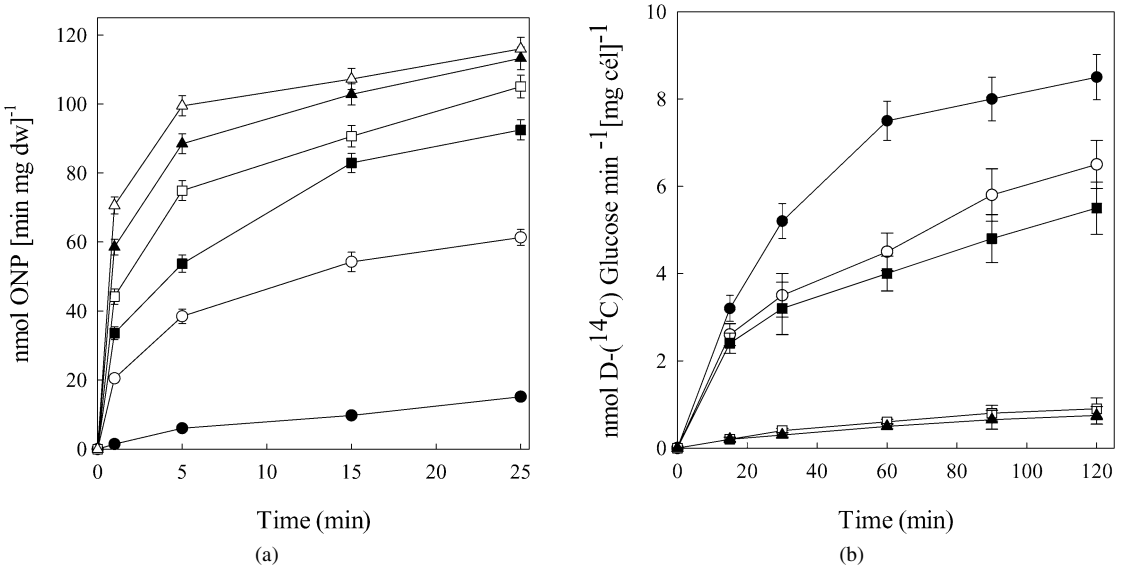

Fig. 2. Effect of different concentrations of DCA on the permeabilization of *L. reuteri* CRL 1098 cells, measured as: (a) release of β-galactosidase activity at different times, in 1 (●), 3 (○), 5 (■), 10 (□), 12 (▲) and 15 (△) mM of DCA; (b) glucose uptake in (●) control, (○) 1, (■) 3, (□) 5, (▲) 10 mM of DCA.

In order to test the documented effect of bile acid on cell membrane permeabilization [1,14], β-galactosidase activity [17] was used as an intracellular marker when *L. reuteri* CRL 1098 cells were treated with increasing concentrations of DCA and TDCA. β-Gal activity increased with increasing DCA concentrations (Fig. 2a). Values obtained with 10–15 mM DCA were similar ($P > 0.05$) to those of control cells permeabilized with toluene: acetone mix (9:1, v/v) (108.2 nmol ONP min^{-1} mg^{-1} dry weight) [5]. Similar results were obtained for *L. casei* CECT 5275 with maximal release of β-gal activity at 5 mM of DCA, after 15 min of permeabilization (data not shown). No enzyme activity was detected after TDCA treatment at any of the concentrations tested (data not shown).

To confirm the effect of bile acids on the cell membrane, glucose uptake in the presence of DCA and TDCA by *L. reuteri* CRL 1098 was also measured (Fig. 2b). This functional marker was more sensitive at detecting changes in the structural organization of the cell membrane. A 25 and 50% decrease in glucose transport was observed in cells treated with 1 and 3 mM DCA, respectively, while no effect at all was observed with TDCA (data not shown). At higher concentrations of DCA glucose incorporation was completely inhibited.

Cell changes occurring in *L. reuteri* treated with DCA or TDCA were examined under an electron microscope. TDCA-cells (Fig. 3b) had the same appearance as untreated cells (control), i.e., a clearly defined cell wall and cell membrane (Fig. 3a). In contrast, DCA-treated cells displayed alterations in the cell surface structure such as pocket granules and low electron density vesicles. Moreover, abnormal proximity of the plasma membrane to the cell wall, or else pronounced dissociation from it, was observed (Figs. 3c–3d).

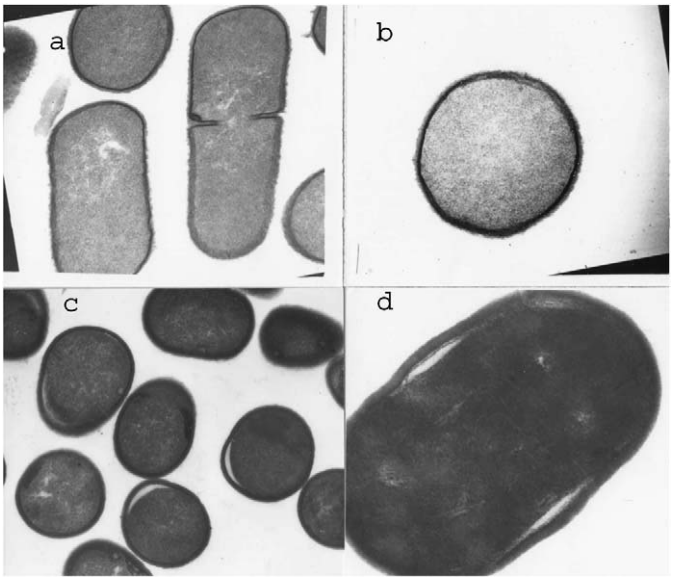

Fig. 3. Transmission electron microscopy examination of cells of *L. reuteri* CRL 1098 treated with TDCA (b), and DCA (c, d) and untreated (a). Magnification: (a–c) ×50 080; (d) ×82 640.

Table 1
Content in fatty acid methyl esters (FAMEs) in *L. reuteri* CRL 1098 after TDCA and DCA treatments

Lipids	Control	TDCA	DCA
FAMEs[a]			
14:0	6.1 ± 0.001[b]	4.01 ± 0.001	4.12 ± 0.005
16:0	57.7 ± 0.007	30.18 ± 0.007	21.77 ± 0.003
18:0	3.86 ± 0.001	4.86 ± 0.002	3.6 ± 0.001
19:0 Cyc	7.55 ± 0.002	9.17 ± 0.003	7.44 ± 0.001
18:1 W9C	11.8 ± 0.007	27.31 ± 0.006	42.84 ± 0.007
18:1 W7C	16.54 ± 0.006	15.68 ± 0.005	11.8 ± 0.002

[a] Percent of total (%).
[b] Standard deviation. All values are the average of triplicate determinations.

Table 2
Glycophospholipids in *L. reuteri* CRL 1098 after TDCA and DCA treatments

Lipids	Control	TDCA	DCA
Glycophospholipids[a]			
Glycolipids (G)	230 ± 3.7[b]	152 ± 3.2	190 ± 2.2
Phospholipids (P)	166 ± 3.1	92 ± 2.3	50.1 ± 1.9
Glyco/phospholipids	1.38	1.65	3.79

[a] mg (P or sugar) [mg dry weight]$^{-1}$.
[b] Standard deviation. All values are the average of triplicate determinations.

Tables 1 and 2 show fatty acid methyl esters (FAMEs) and glycophospholipids of *L. reuteri* CRL 1098 cells after TDCA and DCA treatments, respectively. The major fatty acids (accounting for over 80% of total fatty acids) corresponded to C14:0, C16:0, C18:0, C18:1 W7C, C18:1 W9C, and C19-Cyc. An important increase (2.51 and 5.65 fold) in C18:1 W9C was found in cells treated with TDCA and DCA, respectively, while the concentration of the other fatty acids was quite similar, with a slight increase in C19-Cyc and a decrease in C16:0 and 18:1 W7C. Moreover, in the presence of DCA cells showed a 3.79-fold increase in the glycolipid to phospholipid ratio compared to untreated cells, mainly due to a decrease in phospholipids.

4. Discussion

In a previous study, the effect of bile acids throughout cell growth was determined in *L. reuteri* [29]. Results showed a decrease in membrane phospholipids and a lower ratio of saturated *vs*. unsaturated fatty acids as metabolic adaptation of bacterial cells to bile salts. The present study addresses the toxic effect of different concentrations of unconjugated and taurine-conjugated bile acids on the viability of *L. reuteri* CRL 1098, and the mode of action. Survival of *L. reuteri* CRL 1098 on these bile acids was compared with that of three other *Lactobacillus* strains belonging to species of probiotic interest including *L. casei* CECT 5275, *L. acidophilus* ATCC 4356^{T} and *L. delbrueckii* subsp. *bulgaricus* ATCC 11842^{T}. TDCA was less toxic than DCA, with differences among strains. *L. reuteri* CRL 1098 proved to be resistant to all concentrations of TDCA tested, while the other strains were very sensitive (62–87% cell inhibition). The presence of 1 mM DCA caused significant growth inhibition (80–100%) in all strains except *L. reuteri* CRL 1098 that was only slightly inhibited (12%). The high tolerance of *L. reuteri* CRL 1098 to DCA may be a critical property that determines an effective adaptation to the gut environment [11]. In fact, *L reuteri* has a broad range of hosts [25]. Moreover, the release of free bile acids by the bile salt hydrolase of *L. reuteri* CRL 1098 [30] would be an additional selective advantage for gut colonization with respect to indigenous microbiota.

It has been suggested that the major effect of bile acids would be the disaggregation of the lipid bilayer structure of the cell membrane [1,14]. In fact, glucose uptake by *L. reuteri* CRL 1098 decreased up to 50% after 3 mM DCA treatment, and was completely abolished at higher concentrations. These results are in concordance with electron microscopy observations that provided evidence of structural distortions of the cell surface in DCA-treated cells. Changes in the lipid profile of these cells also supported the fact that the cell surface may be the target of the unconjugated bile acid action. In the presence of DCA, cells showed a 3.79-fold increase in the glycolipid to phospholipid ratio compared to untreated cells, mainly due to a decrease in phospholipids, the main structural component of the cell membrane (Table 2). This decrease may be due to phospholipid solubilization by DCA [22] or to rapid interconversion between glycolipids and phospholipids [31]. The relative increase in glycolipids—also reported for bifidobacteria [18]—could enhance the stability of the cell membrane when confronted with environmental changes due to the ability of these compounds to undergo interlipid hydrogen bonding via glycosyl headgroups [7].

Six major fatty acids (accounting for over 80% of total fatty acids) were found, corresponding to C14:0, C16:0, C18:0, C18:1 W7C, C18:1 W9C and C19-Cyc. Interestingly, C18:1 W9C increased 2.51- and 5.65-fold in cells treated with TDCA and DCA, respectively (Table 1). The concentrations of the other fatty acids were quite similar, with a slight increase in C19-Cyc and a decrease in C16:0 and 18:1 W7C. A similar pattern of saturated vs. unsaturated fatty acids was found when lengthening the time of adaptation to bile acids in

L. reuteri [29], *Bifidobacterium* [18] and *L. helveticus* as a response to heat and oxidative stress [20]. It has been proposed that certain octadecenoic (18:1) acids may play a key role in thwarting the bactericidal effects of bile acids, as suggested by their differential presence in intestinal and gastric isolates of *Helicobacter* [21].

Some of the changes detected in the lipid content and profile during this work may be due to partial or selective lipid solubilization by bile acids. However, changes were detected in both treatments, DCA and TDCA, which had very different effects on permeabilization. Alternatively, *L. reuteri* could have reacted to the bile treatment during the incubation time through a primary physiological response leading to the incorporation of low-melting-point unsaturated fatty acids and glycolipids to the membrane, in an attempt to reinforce its stability [19]. This could be related to the quick adaptative response, independently of protein synthesis, found in *L. monocytogenes* and *E. faecalis*, in which previous exposure to sublethal bile concentrations resulted in a significant increase in tolerance to toxic bile acid concentrations [2,14].

In conclusion, results obtained in this work provide solid evidence that in *L. reuteri* CRL 1098, the toxic effect of DCA may be explained by its interaction with structural lipids of the membrane bilayer, probably as a consequence of its hydrophobic character [13,17]; in fact, DCA is more hydrophobic and more toxic than TDCA. After treatment, significant changes in lipid composition of the membrane were detected. This could be explained by a quick adaptive response to bile, while cells were still viable, that improved the physico-chemical properties of the membrane. However, harsh bile acid treatments introduced severe changes in cell envelope integrity affecting essential cellular functions, as revealed by sugar transport and permeability assays, that led to cell death.

Acknowledgements

This project was supported by the Agencia Española de Cooperación Internacional of the Spanish Ministry of Science and Technology, and grants from CONICET, FONCyT and CIUNT from Argentina.

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