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Cholic acid accumulation and its diminution by short-chain fatty acids in bifidobacteria

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Cholic acid (CA) transport was investigated in nine intestinal *Bifidobacterium* strains. Upon energization with glucose, all of the bifidobacteria accumulated CA. The driving force behind CA accumulation was found to be the transmembrane proton gradient (ΔpH , alkaline interior). The levels of accumulated CA generally coincided with the theoretical values, which were calculated by the Henderson–Hasselbalch equation using the measured internal pH values of the bifidobacteria, and a pK_a value of 6.4 for CA. These results suggest that the mechanism of CA accumulation is based on the diffusion of a hydrophobic weak acid across the bacterial cell membrane, and its dissociation according to the ΔpH value. A mixture of short-chain fatty acids (acetate, propionate and butyrate) at the appropriate colonic concentration (117 mM in total) reduced CA accumulation in *Bifidobacterium breve* JCM 1192^T. These short-chain fatty acids, which are weak acids, reduced the ΔpH , thereby decreasing CA accumulation in a dose-dependent manner. The bifidobacteria did not alter or modify the CA molecule. The probiotic potential of CA accumulation *in vivo* is discussed in relation to human bile acid metabolism.

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INTRODUCTION

Bile, which is produced by liver cells, is composed mainly of bile salts, and is secreted into the duodenum via the bile duct. Bile salts are glycine and taurine conjugates of bile acids, and act as natural ionic detergents. In the intestine, the bile salts play an essential role in emulsifying lipids, which enables intra-luminal lipolysis and the absorption of lipolytic products by enterocytes. Cholic acid (CA) is one of the most common free bile acids in the intestine, and is produced mostly by the deconjugation of bile salts, such as taurocholic acid and glycocholic acid. Deconjugation is carried out by the bile salt hydrolases (BSHs) of the indigenous members of the genera *Bacteroides* (Masuda, 1981),

Bifidobacterium (Tanaka *et al.*, 1999), *Clostridium* (Masuda, 1981) and *Lactobacillus* (Tanaka *et al.*, 1999). The free bile acids are further modified by various intestinal microorganisms to produce secondary bile acids, such as deoxycholic acid and lithocholic acid (Baron & Hylemon, 1997; Kitahara *et al.*, 2000).

Within the human intestinal microbiota, the lactobacilli and bifidobacteria have attracted much attention with regard to their potential probiotic effects. Although many *Lactobacillus* and *Bifidobacterium* species have been associated with various health-promoting and beneficial properties (Ouwehand *et al.*, 2002), their interactions with free bile acids are not well characterized. The growth inhibition of intestinal bacteria by free bile acids has been demonstrated (Binder *et al.*, 1975), but the effects of free bile acids on the physiology of intestinal bacteria have not been elucidated. In our previous reports (Kurdi *et al.*, 2000; Yokota *et al.*, 2000), we showed that *Lactococcus lactis* actively extrudes CA from the cell in an ATP-dependent manner, whereas various *Lactobacillus* species from the intestine, dairy products and other environments are

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Abbreviations: BSH, bile salt hydrolase; CA, cholic acid; cFSE, carboxy-fluorescein succinimidyl ester; DiSC₃(5), 3,3'-dipropylthiadicarbocyanine iodide; SCFA, short-chain fatty acid.

capable of accumulating CA when they are energized by glucose. The mechanism underlying CA accumulation seems to be not transporter-mediated, but depends on the diffusion of hydrophobic CA across the bacterial cell membrane according to the transmembrane proton gradient (ΔpH , alkaline interior), which is formed upon energization with glucose. These findings led us to investigate the interactions of CA with bifidobacteria in the intestines of infants and healthy adults. In addition, we studied the effects on CA accumulation of short-chain fatty acids (SCFAs), which are normally present in the human large intestine as a mixture of acetate, propionate and butyrate.

METHODS

Bacterial strains. The *Bifidobacterium* strains used in this study (Table 1) were obtained from the Japan Collection of Microorganisms (JCM, Wako, Japan) and Snow Brand Milk Products Co. Ltd (SBT, Kawagoe, Japan). The *Eubacterium lentum*-like strain c-25 was kindly provided by Professor Dr Hiroshi Oda (Department of Bacteriology, Faculty of Medicine, Kagoshima University, Kagoshima, Japan).

CA transport in *Bifidobacterium* spp.

CA transport experiment. The bacteria were grown until mid-exponential phase in half-strength MRS ($\frac{1}{2}$ MRS) broth (Difco) that was supplemented with 0.25 g L-cysteine/HCl l^{-1} under anaerobic conditions at 37 °C, using a mixed gas ($\text{N}_2/\text{CO}_2/\text{H}_2$; 8:1:1). Preparation of the de-energized, washed cell suspension and the CA transport experiments were carried out essentially as described previously (Kurdi *et al.*, 2000). The cells were harvested, washed with 50 mM potassium phosphate (pH 7.0), 1 mM MgSO_4 and 0.1 U horseradish peroxidase ml^{-1} (Buffer 1), and de-energized with 10 mM of 2-deoxyglucose in Buffer 1 that was supplemented with

1.0 U horseradish peroxidase ml^{-1} (Buffer 2). The cells were washed three times with Buffer 1 to completely remove the 2-deoxyglucose and resuspended at 3 mg protein ml^{-1} in 150 mM potassium phosphate (pH 7.0), 1 mM MgSO_4 and 1.0 U horseradish peroxidase ml^{-1} (Buffer 3), to an OD_{660} value of approximately 10. Buffer 3 was used to prevent substantial extracellular acidification, which would greatly affect the results of the transport experiment. It was confirmed that this buffer allowed a drop of only 0.1 of a pH unit in these experiments. The resulting cell suspension was equilibrated by stirring for 10 min from time 0 in 0.116 mM (16 mCi mmol^{-1} , 592 MBq mmol^{-1}) [carboxyl- ^{14}C]CA (Perkin Elmer) at 37 °C under anaerobic conditions (mixed gas). Under these conditions, once the cells were energized by 10 mM (final concentration) glucose, 100 μl samples were mixed with 3 ml of Stop Buffer (Kurdi *et al.*, 2000) and the samples were filtered quickly through 0.45 μm cellulose acetate filters (Schleicher & Schuell). The cells on the filters were immediately washed with 3 ml of Stop Buffer using filtration. The membranes were placed into Eppendorf tubes, and the levels of radioactivity were counted in a scintillation counter after the addition of 1.4 ml of the scintillation cocktail (Emulsifier Scintillator Plus; Perkin Elmer). The control cells (no added glucose) were treated with 2 μM valinomycin and 1 μM nigericin at time 0, to ensure complete de-energization. A sample of the reaction mixture without cells was also measured as the background level, which was subtracted from each of the test readings.

When the effects of the SCFAs were examined, the sodium salts were added to a final concentration of 117 mM, i.e. 66 mM acetate, 26 mM propionate and 25 mM butyrate. These concentrations approximate the respective levels of the acids in the ascending colon (Cummings, 1997). The effect of 39 mM SCFAs with the same component ratio was also investigated, to check the dose response. The SCFA mixtures were added 8 min after energization with glucose; the addition of the SCFA mixtures did not change the pH of the medium.

Calculation of the accumulated CA. The absolute amount of CA that was associated with the cells was expressed in nmol (mg

Table 1. CA transport in *Bifidobacterium* strains

Strain	Origin	CA amount [nmol (mg protein) $^{-1}$]* in		Accumulation factor†		Internal pH§
		De-energized cells	Energized cells	Measured*	Predicted‡	
<i>B. breve</i> JCM 1192 ^T	Intestine of infant	1.00 ± 0.08	3.6 ± 0.66	8.5 ± 1.82	3.33	7.51
<i>B. catenulatum</i> JCM 1194 ^T	Human faeces	0.83 ± 0.08	2.2 ± 0.25	4.9 ± 0.76	3.06	7.47
<i>B. pseudocatenulatum</i> JCM 1200 ^T	Faeces of infant	0.72 ± 0.34	1.4 ± 0.35	2.8 ± 0.14	1.75	7.20
<i>B. longum</i> SBT-2928	Human intestine	0.81 ± 0.25	2.1 ± 1.13	4.7 ± 2.47	1.75	7.20
<i>B. longum</i> JCM 1217 ^T	Intestine of adult	0.67 ± 0.23	1.5 ± 0.42	3.4 ± 0.71	1.59	7.15
<i>B. infantis</i> JCM 1222 ^T	Intestine of infant	0.83 ± 0.50	2.3 ± 0.21	5.1 ± 0.71	1.79	7.21
<i>B. bifidum</i> JCM 1254	Intestine of adult	0.45 ± 0.21	1.5 ± 0.71	4.0 ± 1.56	1.94	7.25
<i>B. bifidum</i> JCM 1255 ^T	Faeces of infant	0.53 ± 0.01	1.5 ± 0.21	3.6 ± 0.64	1.94	7.25
<i>B. adolescentis</i> JCM 7046	Intestine of adult	0.55 ± 0.18	1.2 ± 0.14	2.7 ± 0.07	ND	ND

ND, Not determined.

*Results are shown as means ± SD where $n=2$, except for JCM 1194^T ($n=3$) and JCM 1192^T ($n=7$).

†The ratio of the internal CA concentration to the external CA concentration. The external CA concentration was 0.116 mM. The internal CA concentration was calculated on the assumption of 3 μl (mg protein) $^{-1}$ internal volume (Poolman *et al.*, 1987). CA amounts exceeding 0.35 nmol (mg protein) $^{-1}$ (corresponding to an accumulation factor of 1.0) in the de-energized (valinomycin- and nigericin-treated) cells were assumed to be due to non-specific binding, and these values were subtracted from the CA levels in the energized cells when calculating the accumulation factors (measured values).

‡Calculated using the Henderson-Hasselbalch equation and the measured internal pH values.

§Measured with the intracellularly conjugated fluorescent pH probe cFSE.

protein)⁻¹. The CA accumulation factor, which is defined as the ratio of the internal CA concentration to the external CA concentration, was also calculated. Calculation of the internal CA concentration was based on the assumption of an internal cell volume of 3 µl (mg protein)⁻¹ (Poolman *et al.*, 1987). Non-specific binding of CA to the cell surface and/or to the cell membrane was estimated from the positive deviation of the calculated internal CA concentration of the control series (no glucose added; de-energized with ionophores) from the extracellular CA concentration of 0.116 mM. The non-specific binding value estimated in this way was subtracted from the calculated internal CA concentration values in the energized series. Thus, the accumulation factor was obtained from these corrected intracellular CA concentrations, where the accumulation factor for the control series was set at 1.0.

The protein content of the cell suspensions was determined using the DC Protein Assay Kit (Bio-Rad) according to the manufacturer's instructions and BSA as the standard. The cell suspensions were boiled for 5 min in 1 M NaOH and then centrifuged; the resulting supernatants were used in the assays.

Measurement of intracellular pH. Internal pH measurements were performed as described previously (Kurdi *et al.*, 2000), using the internally conjugated fluorescent pH probe carboxyfluorescein succinimidyl ester (cFSE; Molecular Probes) (Breeuwer *et al.*, 1996). Briefly, the cells were cultured until mid-exponential phase, harvested and washed twice in Buffer 1. The cells were resuspended to an OD₆₆₀ value of approximately 0.5 in Buffer 3 and incubated at 37 °C for 30 min in the presence of the precursor probe carboxyfluorescein diacetate succinimidyl ester. To eliminate unbound probe, the cells were incubated with glucose for 1 h and then washed once in Buffer 3. The cells were subsequently resuspended in Buffer 3, and the intracellular pH measurements were carried out. The effects of SCFAs on the internal pH were examined by the addition of SCFA mixtures at final total concentrations of 117 or 39 mM.

Transmembrane electrical potential (ΔΨ) measurements. Changes in ΔΨ during energization were monitored using the fluorescent dye 3,3'-dipropylthiadicarbocyanine iodide [DiSC₃(5); Molecular Probes], which is a cationic probe that crosses the cell membrane, and the fluorescence of which is quenched as the membrane potential develops (negative interior). The harvested cells were washed twice with ice-cold Buffer 4 (Buffer 1 that contained 65 U catalase ml⁻¹ instead of peroxidase), then resuspended in Buffer 5 (Buffer 3 with 65 U catalase ml⁻¹ in place of peroxidase), to an OD₆₆₀ value of approximately 10, and stored on ice. The replacement of peroxidase with catalase was important for reproducible measurements of ΔΨ in the bifidobacteria because (i) peroxidase quenched the fluorescence of the DiSC₃(5) probe, even before energization of the cells (see below), while catalase did not have this effect, and (ii) the addition of peroxidase or catalase was critical for bifidobacterial metabolism of glucose under experimental anaerobic conditions. The cells were added to a stirred cuvette that contained Buffer 5 (final OD₆₆₀ value of 0.05) and DiSC₃(5) (final concentration of 0.5 µM). Glucose (10 mM final concentration) was then added under anaerobic conditions (mixed gas was introduced into the cuvette headspace), to energize the cells. Fluorescence measurements were performed with an LS50B fluorimeter (Perkin Elmer) with excitation and emission wavelengths of 651 and 675 nm, respectively (slit widths of 4.0 nm).

CA metabolism by bifidobacteria. The *Bifidobacterium* strains were cultured in 3 ml of ½ MRS broth, as described in the transport experiment section, while the positive control, *E. lentum*-like strain c-25, was grown in GAM Broth 'Nissui' (Nissui Pharmaceutical). Both of these media contained 0.15 mM sodium cholate; the cultures were incubated for 48 h under anaerobic conditions (mixed

gas). The culture broths were acidified with concentrated HCl to pH 2, and the bile acids were extracted with ethyl acetate. The bile acids were separated by TLC using Silica gel 60 (Merck) and cyclohexane/ethyl acetate/acetic acid (7:23:3, v/v; Eneroth, 1963) as the solvent. The bile acid spots on the TLC plate were visualized by spraying with the colouring reagent 5% (w/v) phosphomolybdic acid {H₃[P(Mo₃O₁₀)₄].nH₂O}, which was dissolved in ethanol, and then heated in an oven at 110 °C for 10 min.

RESULTS

CA accumulation in *Bifidobacterium* cells

The CA levels of *Bifidobacterium breve* JCM 1192^T cells equilibrated between the external medium and the de-energized cells (Fig. 1a), since the hydrophobic protonated CA was able to diffuse freely across the cell membrane (Kamp & Hamilton, 1993). The addition of glucose as a fermentable substrate energized the cells, after which the amount of CA in the cells started to increase, as compared to control cells that did not receive glucose (Fig. 1a). All of the tested bifidobacteria accumulated CA [1.2–3.6 nmol (mg protein)⁻¹], and none of the strains showed detectable CA extrusion when energized with glucose (Table 1). The apparent (i.e. measured) CA accumulation factor, which was defined as the ratio of the internal CA concentration to the external concentration, was between 2.7 and 8.5 (Table 1). These values were similar to those observed for lactobacilli (Kurdi *et al.*, 2000). In addition, we measured the CA transport in *B. breve* JCM 1192^T at 1.0 and 2.0 mM external CA concentrations, and observed CA accumulation factors of about 5 and 4, respectively (data not shown).

Bioenergetics of CA accumulation

The observation that CA accumulation is an energy-dependent process led us to investigate the contribution of the components of the proton motive force, ΔΨ and ΔpH, to CA accumulation. Therefore, we studied the effects of valinomycin (dissipates ΔΨ) and nigericin (abolishes ΔpH) on CA accumulation in bifidobacteria. The results of these experiments using *B. breve* JCM 1192^T and *Bifidobacterium bifidum* JCM 1255^T (data not shown) revealed that the addition of 2 µM valinomycin increased the amount of accumulated CA (Fig. 1a), as compared to the control energized cells that were not treated with valinomycin. However, the addition of 1 µM nigericin reduced the amount of accumulated CA in the energized cells to the equilibration level (Fig. 1a). These observations indicate that the pH gradient (ΔpH) is the driving force behind CA accumulation.

To further confirm the involvement of ΔpH in the accumulation process, the internal pH of the JCM 1192^T cells was measured with the fluorescence probe cFSE (Fig. 1b). The internal pH started to increase (from pH 7.15 to around pH 7.5) 5 min after energization by glucose. This pH level was maintained until the addition of ionophores. The formation of the ΔpH coincided with CA accumulation

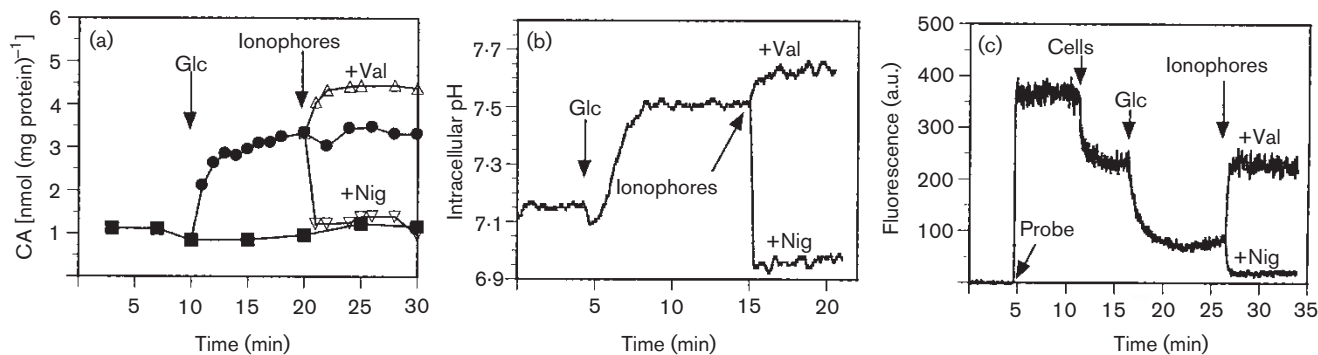


Fig. 1. CA transport (a) follows the kinetics of the internal pH changes (b), but not those of the membrane potential (c) when ionophores are added to energized *B. breve* JCM 1192^T cells. (a) De-energized, washed cells were incubated with 0.116 mM [carboxyl-¹⁴C]CA in the absence (■) or in the presence of 10 mM glucose (Glc, ●). Ionophores, valinomycin (Val, △) and nigericin (Nig, ▽) were added to the energized cells at final concentrations of 2 and 1 μM, respectively. (b) The cells (OD₆₆₀ ~ 0.5) were pre-loaded with cFSE and energized with 10 mM Glc for approximately 10 min, before the addition of Val or Nig at final concentrations of 5 or 200 nM, respectively. (c) Washed cells were added (OD₆₆₀ ~ 0.05) to the cuvette that contained the DiSC₃(5) probe (0.5 μM). The cells were energized with Glc (10 mM) for about 10 min, followed by the addition of Val (5 nM) or Nig (200 nM). The fluorescence intensity is expressed in arbitrary units (a.u.). All the experiments were carried out at an external pH of 7.0. The data shown are representative of at least three experiments that gave similar results.

(Fig. 1a). The addition of valinomycin resulted in an increase in the internal pH, while the addition of nigericin abolished Δ pH (Fig. 1b). These changes in internal pH and Δ pH may be responsible for the alteration of accumulated CA levels in the JCM 1192^T cells upon the addition of ionophores (Fig. 1a).

Monitoring of the changes of $\Delta\Psi$ using DiSC₃(5) (Fig. 1c) revealed that the development of $\Delta\Psi$ upon energization with glucose and CA accumulation occurred simultaneously. However, nigericin addition increased the $\Delta\Psi$, while valinomycin addition totally abolished it. These results clearly demonstrate that the Δ pH component and not the $\Delta\Psi$ component of the proton motive force is the driving force behind the CA accumulation process.

Measurements of the internal pH values of eight *Bifidobacterium* strains with cFSE revealed a positive correlation between the CA accumulation factors and the internal pH values of the respective strains (Table 1). The higher the pH gradient (i.e. Δ pH), the higher the accumulation factor in most cases, which confirms that Δ pH is the driving force behind CA accumulation. The theoretical accumulation factors, which were calculated from the measured internal pH values using the Henderson–Hasselbalch equation ($\text{pH} = \text{p}K_a + \log[A^-]/[HA]$, where the $\text{p}K_a$ of CA was 6.4), were lower than the actual accumulation factors (Table 1). It is possible that active CA transporters contributed to the CA accumulation in certain strains (e.g. JCM 1192^T) that had large differences between their measured and predicted accumulation factors. However, in strains with smaller differences between their predicted and measured accumulation factors, CA may have accumulated

solely as the result of diffusion through the membrane, followed by Δ pH-dependent dissociation.

Effect of SCFAs on CA accumulation

Various mixtures of sodium acetate, sodium propionate and sodium butyrate, at final total concentrations of 117 mM (which corresponds to the concentration of these SCFAs in the ascending colon) or 39 mM, were used to test the effect of SCFAs on CA accumulation. CA accumulation in JCM 1192^T cells was reduced by at least 50% in the presence of the 117 mM SCFA mixture (Fig. 2a), as compared to cells that were incubated in the absence of SCFAs, while the 39 mM SCFA mixture produced a less pronounced reduction (~20%) in CA accumulation. The addition of nigericin further decreased CA accumulation, which suggests that a certain Δ pH level was maintained in the JCM 1192^T cells in the presence of 117 mM SCFAs. These SCFAs are weak acids with $\text{p}K_a$ values of 4.75, 4.87 and 4.81 for acetic, propionic and butyric acid, respectively. These weak acids, as is the case with CA, can be accumulated in bacterial cells (Russell, 1991), and can theoretically reduce the internal pH of bacterial cells (Diez-Gonzalez & Russell, 1997). As expected, measurements of the internal pH changes of energized JCM 1192^T cells upon the addition of the SCFA mixtures revealed decreases in the internal pH (Fig. 2b, c). The Δ pH levels were reduced by about 60 and 22% by the addition of SCFA mixtures at 117 mM (Fig. 2b) and 39 mM (Fig. 2c), respectively. These reductions correspond to the reductions in the amounts of accumulated CA in JCM 1192^T cells following the treatment with the SCFA mixtures. These results indicate that in the presence of SCFAs, acidification

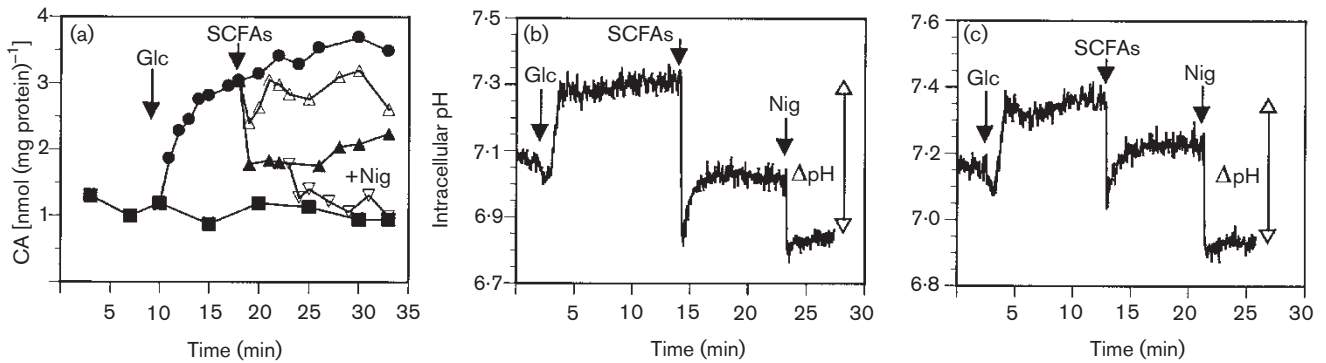


Fig. 2. SCFA mixtures impair CA transport and reduce the internal pH of *B. breve* JCM 1192^T. (a) De-energized, washed cells were incubated with 0.116 mM [carboxyl-¹⁴C]CA (■), and then energized with 10 mM glucose (Glc) in the absence (●) or in the presence of the SCFA mixtures. The SCFAs were applied at total concentrations of 117 mM (▲; acetate 66 mM, propionate 26 mM, butyrate 25 mM) and 39 mM (△; with the same component ratio). Nigericin (Nig, ▽) was added at a final concentration of 1 μM. (b, c) The internal pH measurements were performed as described in the legend to Fig. 1. SCFAs at concentrations of 117 and 39 mM were used in (b) and (c), respectively. Nig was added at a final concentration of 200 nM. The magnitude of ΔpH is indicated. All of the experiments were conducted at an external pH of 7.0. The data shown are representative of at least three experiments that gave similar results.

of the intracellular environment and the subsequent decrease in ΔpH reduce CA accumulation.

CA metabolism by bifidobacteria

Although our experiments demonstrate that CA is accumulated spontaneously in energized bifidobacterial cells, the possibility exists that bifidobacteria metabolize CA. To test this hypothesis, bile acids were extracted from whole culture broths, in which bifidobacteria had been incubated with CA for 48 h, and analysed using the TLC method. As shown in Fig. 3, none of the *Bifidobacterium* strains used in the transport experiments produced deoxycholic acid or any other metabolite of CA, while deoxycholic acid formation was confirmed in the *E. lentum*-like strain c-25.

DISCUSSION

To date, there has been little information on the interactions between free bile acids and bifidobacteria, which are considered to be the most important indigenous bacteria in the large intestine, in terms of their abundance and health-promotion properties (Mitsuoka, 2002). Although one might anticipate that intestinal bacteria have active mechanisms for the extrusion of growth inhibitory compounds, such as bile acids, this notion is not consistent with our observations. We found that bifidobacteria do not have apparent bile acid extrusion activities. On the contrary, energized cells accumulate CA (Fig. 1a, Table 1). This activity is very similar to that described in lactobacilli (Kurdi *et al.*, 2000). Bile acid transport was studied in *Lactococcus lactis*, which can actively export CA via a multi-specific organic anion transporter, which is driven by ATP (Yokota *et al.*, 2000), while *Escherichia coli* utilizes the energy of the

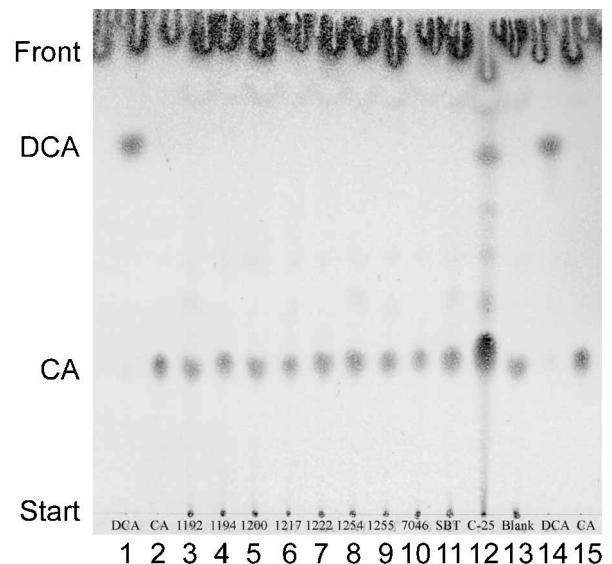


Fig. 3. Bifidobacteria do not metabolize CA. Thin-layer chromatogram of whole-cell extracts from 48 h-old cultures of various *Bifidobacterium* strains. The strains were cultured in the presence of 0.15 mM sodium cholate. Lanes: 1 and 14, deoxycholic acid (DCA); 2 and 15, CA; 3, *B. breve* JCM 1192^T; 4, *B. catenulatum* JCM 1194^T; 5, *B. pseudocatenulatum* JCM 1200^T; 6, *B. longum* JCM 1217^T; 7, *B. infantis* JCM 1222^T; 8, *B. bifidum* JCM 1254; 9, *B. bifidum* JCM 1255^T; 10, *B. adolescentis* JCM 7046; 11, *B. longum* SBT-2928; 12, *Eubacterium lentum*-like strain c-25 (positive control); 13, blank (no strain was inoculated into the medium). The results shown are representative of at least three experiments that gave similar results.

proton motive force to extrude chenodeoxycholic acid and taurocholate (Thanassi *et al.*, 1997). Another intestinal microbe, *Clostridium scindens* (formerly known as *Eubacterium* sp. VPI 12708; Kitahara *et al.*, 2000), takes up CA using the proton motive force (Mallonee & Hylemon, 1996) for the dehydroxylation of the CA molecule at the seventh carbon atom (White *et al.*, 1980). A gene from *Lactobacillus johnsonii* 100-100 was identified that encodes an importer that takes up taurocholate for the intracellular BSH reaction (Elkins & Savage, 1998). Moreover, this strain appeared to contain another gene for a putative conjugated bile acid transporter (Elkins *et al.*, 2001).

As is the case in lactobacilli (Kurdi *et al.*, 2000), the results presented here indicate that the driving force for CA accumulation in bifidobacteria is the ΔpH component of the proton motive force, and not the $\Delta\Psi$ component (Fig. 1). We hypothesize that CA accumulation in bifidobacteria occurs in the following way. (i) The hydrophobic CA molecules diffuse into the cytoplasm of the energized bifidobacterial cell and dissociate according to the ratio given by the Henderson–Hasselbalch equation under the given cytoplasmic pH value. (ii) Since the cytoplasmic pH value is higher than that outside (i.e. ΔpH), more dissociation takes place in the cytoplasm than in the external medium. The resulting negatively charged cholate anions cannot pass the membrane due to their polarity, and thus they are trapped inside the cell. (iii) CA influx continues until the concentration of the protonated CA molecule equilibrates on both sides of the cell membrane. In this situation, the total amount of CA (the sum of the protonated and dissociated species) is higher in the cytoplasm than in the external medium. (iv) The intracellular concentration of CA remains higher than that of the external environment, until the cells are energized and the CA concentration gradient across the membrane disappears, along with the disappearance of ΔpH . It is worth mentioning that substantial discrepancies were observed in some strains between the values for the measured CA accumulation factors and the values that were predicted by the above mechanism, i.e. the latter values were lower than the former values. Therefore, we cannot discount the possibility that an active CA uptake mechanism (e.g. driven by ΔpH) exists.

SCFA production by intestinal bacteria is a very important process in the large intestine, and provides energy for enterocytes (Cummings & Macfarlane, 1997). One of the major benefits of the fermentation of prebiotics is that it yields SCFAs (Cummings *et al.*, 2001), which in turn decrease the intestinal pH. CA accumulation was impaired when mixtures of SCFAs (117 or 39 mM) were added to the experimental reaction mixture (Fig. 2), although the residual amounts of accumulated CA in these cells were clearly higher than those found in the non-energized control cells. The presence of SCFAs at the physiological concentrations found in the colon appears to exert a severe environmental stress, from the bioenergetic standpoint, on bifidobacterial cells. Even in the presence of SCFAs, CA

accumulation may occur in energized *Bifidobacterium* cells, and this process may operate *in vivo*. This hypothesis was strengthened by our results which indicate that *B. breve* JCM 1192^T accumulated CA even when external CA concentrations were 1.0 and 2.0 mM (around its physiological concentrations; estimated from Ewe & Karbach, 1989). Moreover, CA accumulation at 1.0 mM external CA concentration was observed in JCM 1192^T cells even in the presence of 117 mM SCFAs by a factor of 2.4 (data not shown).

Our experiments on CA metabolism by bifidobacteria revealed that *Bifidobacterium* strains were unable to chemically modify the CA molecule (Fig. 3), which is in agreement with a previous report (Takahashi & Morotomi, 1994). This absence of any chemical modification of CA concurs with our hypothetical mechanism for CA accumulation, which suggests that CA accumulation by bifidobacteria results from the co-existence of a membrane ΔpH and a weak acid in the same environment. The participation of a putative active CA uptake system appears to be unlikely, since the tested bifidobacteria did not utilize CA. Therefore, CA accumulation appears to be the result of energization.

The conjugated bile acid taurocholic acid is not accumulated in the BSH-negative *Lactobacillus salivarius* subsp. *salicinius* strain JCM 1044 (Kurdi *et al.*, 2000) due to its hydrophilicity ($\text{p}K_{\text{a}}=1.4$). Thus, only unconjugated free bile acids are accumulated in lactobacilli and bifidobacteria. According to the published distributions of BSH activities, most of the bifidobacterial strains are BSH-positive (Tanaka *et al.*, 1999). Therefore, in bifidobacteria, conjugated bile acids seem to be the source of free bile acids, which are supposed to be formed inside the cells from conjugated bile acids by BSH activities (Tanaka *et al.*, 2000). It is possible that the CA that is formed from taurocholic acid and glycocholic acid is kept inside bifidobacterial cells in the intestine, for as long as the bacteria are energized.

One possible consequence of CA entrapment in bifidobacterial cells would be the decreased formation of deoxycholic acid in the large intestine. The bifidobacteria do not metabolize CA (Fig. 3; Takahashi & Morotomi, 1994), and thus are unable to produce deoxycholic acid following CA accumulation. Deoxycholic acid and lithocholic acid, which are formed via 7α -dehydroxylation from CA and chenodeoxycholic acid, respectively, by certain intestinal *Clostridium* and *Eubacterium* species (Baron & Hylemon, 1997), are cytotoxic and possible tumour promoters (Reddy *et al.*, 1976; Reddy & Watanabe, 1979). Thus, the accumulation of CA may contribute to the decreased occurrence of colon carcinogenesis. Another possible impact of CA accumulation is a decrease in recycled CA during enterohepatic circulation due to the enhanced excretion of CA from the human host via the faeces. Under these conditions, the synthesis of bile acids from blood cholesterol increases, to compensate for the lost amounts of bile acids, thereby decreasing the blood cholesterol level. Although these features appear quite attractive, experimental evidence for

the probiotic relevance of CA accumulation in bifidobacterial cells is lacking. Therefore, *in vivo* experiments that evaluate these possibilities are urgently needed.

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