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Role of Transporter Proteins in Bile Tolerance of *Lactobacillus acidophilus*▽†

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***Lactobacillus acidophilus* NCFM derivatives containing deletion mutations in the transporter genes LBA0552, LBA1429, LBA1446, and LBA1679 exhibited increased sensitivity to bile. These strains showed unique patterns of sensitivity to a variety of inhibitory compounds, as well as differential accumulations of ciprofloxacin and taurocholate.**

Lactobacillus acidophilus NCFM is a probiotic strain that is widely used in yogurt formulations and dietary supplements (17). Analysis of its genomic sequence has facilitated functional characterization of many of its probiotic features (1, 3–6, 10). The global transcriptional response of this strain to bile was previously characterized and showed the induction of 78 genes (12). Among these were two transporters of the major facilitator superfamily (MFS) (LBA1429 and LBA1446) and the permease and ATPase subunits of an ABC transporter (LBA1679 and LBA1680). Each of these genes was annotated as a member of the family of multidrug resistance (MDR) transporters, a class of transporters that can act as a defense mechanism against inhibitory compounds by extruding a wide variety of structurally dissimilar substrates from the cytoplasm, including antibiotics, bile salts, and peptides. MDR transporters can belong to different classes of transporters, including those of the MFS and ABC transporter families (13, 15).

Of the 10 genes most highly induced by bile in *L. acidophilus* NCFM, two encode MDR transporters (LBA1446 and LBA1679), which suggests that MDR transport systems may be important in achieving bile tolerance in this species. Additionally, these transporters have been shown to play a role in bile tolerance in other species, notably, *Listeria monocytogenes* and *Lactobacillus reuteri* (18, 21). This study investigated the role of transporter genes in bile tolerance in *L. acidophilus* NCFM. It also examined the role of these versatile transporters in tolerance to other compounds whose presence is detrimental to the cell.

Previous microarray analysis of *L. acidophilus* NCFM (12) indicated the induction of three transporter genes, LBA1429 (MFS transporter), LBA1446 (MFS transporter), and LBA1679 (ABC transporter [permease component]), in the presence of 0.5% oxgall, as well as the slight repression of LBA0552, an MFS transporter also annotated as MDR. BLAST analyses (2) of these proteins indicated that all are widespread among members of the *Firmicutes*, with similar proteins being found in high-GC gram-positive bacteria. LBA1429, however, was more widespread, with similar proteins present in members of the *Bacteroides*, the *Betaproteobacteria*, and the *Deltaproteobacteria*. LBA0552 and LBA1446 showed sequence similarity to EmrB/QacA family drug resistance transporters in *Enterococcus faecalis* and *L. monocytogenes*. This type of transporter was previously shown to be involved in bile efflux and was induced in *E. faecalis* in the presence of bile (19, 20). Additionally, TBLASTN analysis showed similarity (57% identity) between LBA1446 and lr1265, the *L. reuteri* protein which was implicated in bile shock survival in this species (2, 21). LBA1429 shows similarity to the quinolone resistance protein GlpT in *Bacillus cereus* (50% positive results). LBA1679 does not show similarity to any protein encoded by any named gene but shows similarity to other ABC transporter permeases.

Because LBA1429, LBA1446, and LBA1679 were induced in the presence of bile in *L. acidophilus* NCFM, in-frame deletion mutant strains were created as described previously (12, 16); the method used included excising internal fragments from each of these genes in order to examine their role in bile tolerance. Lists of the strains used in this study and of the primers used to generate them can be found in Tables S1 and S2 in the supplemental material. Although LBA0552 was not induced by the presence of bile, a deletion mutation was created in this gene because of its strong annotation as an MFS transporter. Survival of early-log-phase cells (optical density at 600 nm [OD_{600}], 0.2 to 0.3) was assayed by plating cells on MRS agar and MRS agar plus 1% (wt/vol) oxgall. While there was no difference in the results with respect to recovery of the strains on MRS plates, all mutant strains, including ΔLBA0552, were more sensitive to oxgall than the wild-type strain (Fig. 1).

Since transporter proteins of this type typically interact with more than one substrate (13), the mutant strains were examined for growth in a number of compounds, including individual bile salts, detergents, and antibiotics. Early-log-phase cells (OD_{600}, 0.2 to 0.3) were inoculated into 200 μl of MRS broth containing dilutions of the inhibitory compound in 96-well plates. Plates were held anaerobically for 24 h at 37°C, after which the OD_{600} of each strain was measured. These assays

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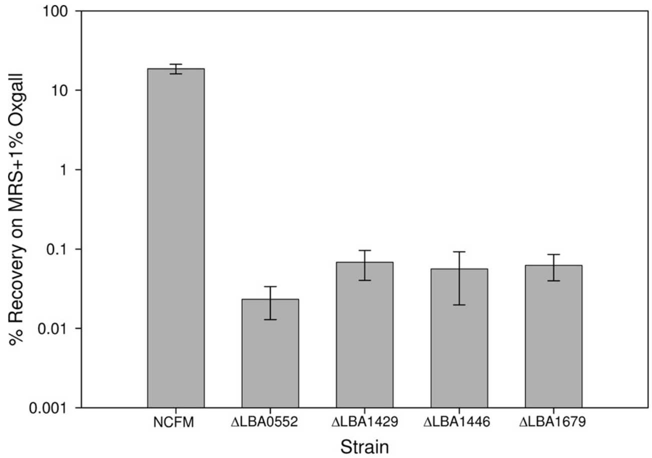

FIG. 1. Recovery of early-log-phase *L. acidophilus* NCFM strains on MRS agar plates containing 1% (wt/vol) oxgall. Error bars represent the standard deviations of the results of three replicate experiments.

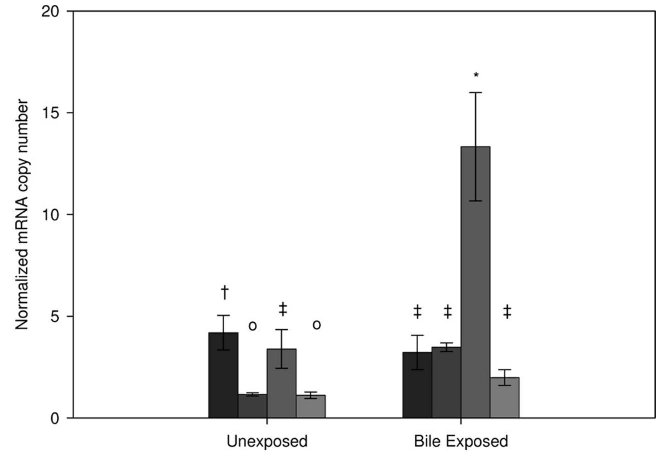

FIG. 2. RTQ-PCR analysis of the expression of (from left to right) LBA0552, LBA1429, LBA1446, and LBA1679 in the presence and absence of 0.5% oxgall. Copy numbers were normalized to the number of mRNA copies of LBA383 (DNA polymerase III [delta subunit]). Error bars represent the standard deviations of the results of four biological replicate experiments. Bars with different symbols represent significant differences, as determined by analysis of variance ($P < 0.05$).

were performed in triplicate, and the results showed the concentration of compound required to inhibit growth of the strain in MRS broth by 50%, as indicated by a 50% decrease in final OD of the cultures (Table 1).

Expression of LBA0552, LBA1429, LBA1446, and LBA1679 in the presence and absence of 0.5% oxgall was analyzed by reverse transcriptase quantitative PCR (RT-QPCR). Expression of LBA1429, LBA1446, and LBA1679 was induced in the presence of bile, and the results with respect to expression of LBA0552 were similar under the two sets of conditions, although the expression level was slightly reduced in bile (Fig. 2). A list of the primers utilized in these experiments can be found in Table S3 in the supplemental material.

Sequence analysis, expression data, and mutant phenotype analysis suggest that these four proteins act to transport bile salts and/or antibiotics from the cellular cytoplasm. In order to confirm this activity, assays were conducted to examine the accumulation of the fluoroquinolone antibiotic ciprofloxacin and the bile salt taurocholate in the wild-type and mutant strains (Table 2).

Because of the sensitivity of the ΔLBA0552 mutant to ciprofloxacin relative to the other strains, the accumulation of this antibiotic was assayed by the method of Chapman and Georgopapadakou (7). ΔLBA0552 was shown to accumulate more ciprofloxacin per OD_{600} unit than the other strains, which correlates with the inhibition of growth that was observed for ΔLBA0552 in the presence of ciprofloxacin. ΔLBA0552 was considerably more sensitive to ciprofloxacin, with sensitivity occurring at a concentration of 50 μg/ml, whereas the rest of the strains did not exhibit sensitivity until a higher concentration (60 to 70 μg/ml) of ciprofloxacin was used. This increased

TABLE 1. Concentration of compound added to MRS broth needed to reduce the OD_{600} of the culture by 50% compared to the results seen with MRS broth alone[a]

Compound	Compound concn range	Inhibitory concn[b]				
		NCFM	ΔLBA0552	ΔLBA1429	ΔLBA1446	ΔLBA1679
Antibiotics						
Ampicillin	0.1–0.5 μg/ml	0.4	0.4	0.4	0.4	0.4
Chloramphenicol	1–5 μg/ml	3.0	3.0	3.0	3.0	3.0
Ciprofloxacin	50–90 μg/ml	70	50	70	60	70
Erythromycin	0.1–0.5 μg/ml	0.3	0.2	0.3	0.3	0.3
Detergents						
Sodium dodecyl sulfate	2–6%	2.0	2.0	2.0	2.0	2.0
Triton X-100	0.2–0.6%	0.2	0.2	0.3	0.2	0.2
Bile salts						
Glycocholate	0.4–1.2%	0.8	0.6	0.4	0.4	0.4
Taurocholate	1.0–1.8%	1.2	1.0	1.0	1.0	1.0
Glycodeoxycholate	0.1–0.5%	0.4	0.4	0.4	0.3	0.4
Taurodeoxycholate	0.6–1.0%	0.9	1.0	0.9	0.6	0.6

[a] Values are representative of the results of three replicate experiments.
[b] Units for antibiotic concentrations are μg/ml; inhibitory concentrations for detergents and bile salts are given as percentages.

TABLE 2. Accumulation of ciprofloxacin and taurocholate in *L. acidophilus* cells[a]

L. acidophilus strain	Compound accumulation	
	Ciprofloxacin (ng/OD_{600} unit)	Taurocholate (pmol/mg total protein)
NCFM	79.0 ± 8.6	3.9 ± 0.17
ΔLBA0552	97.0 ± 1.8*	4.4 ± 0.50
ΔLBA1429	74.0 ± 2.0	4.4 ± 0.38*
ΔLBA1446	73.5 ± 10.9	4.6 ± 0.70*
ΔLBA1679	77.3 ± 10.9	4.3 ± 0.52

[a] Mean values ± standard deviations of the results of three replicate experiments are shown. Values marked with an asterisk are significantly different from *L. acidophilus* NCFM values, as determined by Student's *t* test ($P < 0.05$).

accumulation of ciprofloxacin in ΔLBA0552 correlates with its increased sensitivity to this compound.

Tritium-labeled taurocholate was used to assay accumulation of this bile salt by the use of the method of Sleator et al. (18) with slight modifications. Cells were exposed to 200 pmol of [^{3}H]taurocholate (Perkin Elmer) for 15 min, after which they were collected by centrifugation through silicon oil (70% fluid 550 and 30% fluid 510; Dow Corning). In these assays, the mutant strains accumulated more taurocholate than wild-type NCFM, with strains ΔLBA1429 and ΔLBA1446 accumulating significantly more taurocholate than NCFM, albeit all strains accumulated relatively low amounts of this compound. Compared to other bile salts, taurocholate has a low pK_a (1.5) and the ionized form predominates at the pH of the assay (7.0). Since the ionized form of the bile salt is less likely to passively diffuse into the cell, a relatively small amount of this compound was accumulated in all strains. Other studies that have examined the transport of bile salts used deconjugated bile salts with higher pK_as (11, 14, 18). Taurocholate was selected for this study because all of the mutant strains were sensitive to it.

Each of the genes examined in this study is annotated as an MDR transporter, and each gene makes a contribution to bile tolerance in *L. acidophilus* NCFM, while at the same time each exhibits differences in expression and contributes uniquely to resistance to various compounds. Initially, the dramatic bile sensitivity of ΔLBA0552 was surprising, since expression of this gene was previously shown to be slightly repressed in the presence of bile. However, RT-QPCR analysis indicated that this gene is expressed at a level equal to that of the induced expression of LBA1429 and LBA1679. The ΔLBA0552 mutant showed the most sensitivity to antibiotics due to its reduced growth in the presence of erythromycin and ciprofloxacin. This mutant strain also retained significantly more ciprofloxacin than the other strains when exposed to this compound, correlating with its increased sensitivity and demonstrating a role in ciprofloxacin extrusion for this transporter. This strain was also sensitive to the bile salts glycocholate and taurocholate, contributing to its overall bile sensitivity. Taking these data together, the constitutive expression of this gene, along with the wide range of sensitivities exhibited by the mutant strain, suggests that LBA0552 acts as a true MDR transporter, protecting the cell from a number of toxic substrates that could be encountered in its environment.

The considerable induction of LBA1446 in the presence of bile was confirmed by RT-QPCR, and the sensitivity of ΔLBA1446 to bile was demonstrated by loss of viability on bile plates. This strain was sensitive to all bile salts tested and accumulated significantly more taurocholate than the wild-type strain, indicating a role in the extrusion of bile salts for this species. The high level of gene induction, along with the mutant phenotypes, suggests that LBA1446 may be the most critical transporter contributing to bile tolerance in *L. acidophilus* NCFM.

The ΔLBA1429 and ΔLBA1679 mutants were sensitive to growth on oxgall plates and sensitive to some of the individual bile salts tested. Overall, however, these strains showed sensitivity to the fewest compounds. The ΔLBA1429 mutant showed a slight increase in resistance to the detergent Triton X-100, although the reason for this phenotype is unclear. Despite their being sensitive to fewer compounds than ΔLBA0552 and ΔLBA1446, it is apparent that LBA1429 and LBA1679 also play a critical role in bile tolerance in *L. acidophilus* NCFM. The redundancy of transporters necessary for bile tolerance in the NCFM genome indicates the importance of this trait in this intestinal species.

There is some concern over the potential for exchange of antibiotic resistance genes between microbes in the gastrointestinal environment. Although the genes examined in this study contributed at minor levels to increased resistance to erythromycin and ampicillin, the levels were not physiologically relevant. The level of resistance of ΔLBA0552 to ciprofloxacin was generally similar to levels previously reported for *L. acidophilus* strains (8, 9).

Transcriptional analysis has suggested the importance of transporters in the bile tolerance phenotype of *L. acidophilus* NCFM. The subsequent generation of transporter mutants and the associated phenotypic analyses confirmed the role of these proteins in bile tolerance and indicated that they may transport a wide range of substrates that can be detrimental to the cell.

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