The *Bifidobacterium longum* NCIMB 702259<sup>T</sup> *ctr* Gene Codes for a Novel Cholate Transporter

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Preexposure of *Bifidobacterium longum* NCIMB 702259<sup>T</sup> to cholate caused increased resistance to cholate, chloramphenicol, and erythromycin. The *B. longum* *ctr* gene, encoding a cholate efflux transporter, was transformed into the efflux-negative mutant *Escherichia coli* KAM3, conferring resistance to bile salts and other antimicrobial compounds and causing the efflux of [14C]cholate.

Bifidobacteria are major components of the human intestinal microflora (13) and are widely used as probiotics in food supplements. Probiotic survival depends on resistance to antibiotics and to inhibitory host-produced substances, such as bile salts (9). Bifidobacteria are resistant to a range of antibiotic compounds (5), which could allow them to withstand concurrent antibiotic administration. This study aimed to identify and prove the functionality of a possible efflux system encoded by the *ctr* gene in *Bifidobacterium longum* which may contribute to bile and antibiotic resistance.

**Adaptation to sodium glycocholate and antibiotics.** To determine the intrinsic MICs for *B. longum* NCIMB 702259<sup>T</sup> (NCIMB, United Kingdom) of antimicrobial agents, 10 μl of a standard cell suspension (optical density at 600 nm, 0.5) of a culture grown anaerobically on BYG agar (14) was spotted onto BYG plates containing a twofold dilution range of sodium glycocholate, ampicillin, chloramphenicol, erythromycin, or tetracycline. Adaptation to the antibiotics was tested using a method modified from the work of Carsenti-Etesse et al. (4). Mid-exponential-phase *B. longum* cells grown in BYG broth were streaked for four passages onto sodium glycocholate gradient plates, and the MICs for these cells were tested as described above. Adapted *B. longum* showed an increase in resistance to sodium glycocholate, chloramphenicol, and erythromycin but not to ampicillin and tetracycline (Table 1). This indicated that *B. longum* may possess multidrug transporters, since these are often regulated by the compounds that they transport but may confer resistance to structurally unrelated antimicrobial agents (3).

**Cloning and antimicrobial characterization of the *ctr* gene.** Open reading frame BL1102 (*B. longum* NCC 2705, GenBank accession number AE014295) was identified as a possible sodium-dependent bile acid transporter. The BL1102 orthologue was isolated from *B. longum* NCIMB 702259<sup>T</sup> genomic DNA (14), using standard PCR protocols and the primers ctrans-F (5′-AGCTGAATTCCGGCAACAGG-3′) and ctrans-R (5′-ACGCCGGGTACCTCATAATCG-3′). EcoRI and KpnI restriction enzyme sites (underlined) were introduced to ctrans-F and ctrans-R, respectively, to assist subcloning into pBluescriptSK. The nucleotide sequence of the insert in the recombinant plasmid pCtr was determined (14), and nucleotide and amino acid homology searches were performed using the BLAST algorithm and NCBI databases (1). The deduced amino acid sequence of Ctr was 100% identical to that of *BL1102*. Plasmid pCtr was transformed into competent (*Escherichia coli*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Antimicrobial MIC a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amp (μg/ml)</td>
</tr>
<tr>
<td>Control</td>
<td>1.6</td>
</tr>
<tr>
<td>Cholate preexposure</td>
<td>1.6</td>
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</table>

ª MICs of antimicrobial agents tested against *B. longum* NCIMB 702259, without (control) and following preexposure to cholate, as determined by plating cells onto BYG plates containing a twofold dilution range of each antibiotic. The antimicrobial agents used were ampicillin (Amp), chloramphenicol (Chl), erythromycin (Em), tetracycline (Tet), and sodium glycocholate (cholate). The experiments were done in triplicate.

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<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Acr (μg/ml)</th>
<th>Chl (μg/ml)</th>
<th>Em (μg/ml)</th>
<th>EtBr (μg/ml)</th>
<th>Cholate (%)</th>
<th>SDS (%)</th>
<th>Tet (μg/ml)</th>
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<tbody>
<tr>
<td>pBluescriptSK</td>
<td>6.25</td>
<td>0.4</td>
<td>1.25</td>
<td>12.5</td>
<td>0.5</td>
<td>0.01</td>
<td>0.4</td>
</tr>
<tr>
<td>pCtr</td>
<td>25</td>
<td>1.6</td>
<td>2.5</td>
<td>50</td>
<td>8</td>
<td>0.02</td>
<td>1.6</td>
</tr>
</tbody>
</table>

ª The antimicrobial agents used were acriflavine (Acr), ampicillin (Amp), chloramphenicol (Chl), erythromycin (Em), ethidium bromide (EtBr), sodium dodecyl sulfate (SDS), sodium glycocholate (cholate), and tetracycline (Tet). The experiments were done in triplicate.

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**TABLE 1. MICs of antimicrobial agents tested against *B. longum* NCIMB 702259**

<table>
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<tr>
<th>Treatment</th>
<th>Amp (μg/ml)</th>
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<th>Tet (μg/ml)</th>
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<tbody>
<tr>
<td>Control</td>
<td>1.6</td>
<td>1.6</td>
<td>0.8</td>
<td>0.4</td>
<td>1.6</td>
</tr>
<tr>
<td>Cholate preexposure</td>
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<td>3.2</td>
<td>3.2</td>
<td>0.8</td>
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ª MICs of antimicrobial agents tested against *B. longum* NCIMB 702259, without (control) and following preexposure to cholate, as determined by plating cells onto BYG plates containing a twofold dilution range of each antibiotic. The antimicrobial agents used were ampicillin (Amp), chloramphenicol (Chl), erythromycin (Em), tetracycline (Tet), and sodium glycocholate (cholate). The experiments were done in triplicate.

**TABLE 2. MICs for *E. coli* KAM3 harboring pCtr and the control vector pBluescriptSK as determined by the broth dilution method**

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ª The antimicrobial agents used were acriflavine (Acr), ampicillin (Amp), chloramphenicol (Chl), erythromycin (Em), ethidium bromide (EtBr), sodium dodecyl sulfate (SDS), sodium glycocholate (cholate), and tetracycline (Tet). The experiments were done in triplicate.
KAM3 (11), a K-12 derivative lacking the multidrug transporter AcrAB. The MICs of acriflavine, sodium dodecyl sulfate (Merck), chloramphenicol, erythromycin, ethidium bromide, tetracycline (Sigma), and sodium glycocholate (Difco) were determined using the broth dilution method (8). Plasmid pCtr conferred cholate resistance on E. coli KAM3, increasing the MIC of sodium glycocholate by 16-fold (Table 2). Resistance to the antimicrobial agents was increased by two- to fourfold.

Efflux of $[14C]$cholate. To determine whether pCtr conferred resistance to bile through the active efflux of the compound, de-energized washed cell suspensions of E. coli KAM3 (pCtr or pBluescriptSK) that had been grown to mid-exponen-

**FIG. 1.** Energy-dependent extrusion of $[14C]$cholate in E. coli KAM3 harboring (A) pCtr or (B) pBluescriptSK. Cells were preloaded with cholate, and the amount of cell-associated cholate was subsequently measured over time with the addition of glucose (dotted lines) or without glucose (solid lines) Glucose was added to a final concentration of 10 mM at 35 min as indicated by the arrow. Each experiment was performed in triplicate. Error bars indicate the deviation from the mean.

**FIG. 2.** Multiple sequence alignment of the significant regions of the putative B. longum bile transporter Ctr (GenBank accession no. DQ017587) with closely related bacterial sodium/bile acid transporters. Sequences included are from the following organisms: B. longum (Ctr) (NP_696274), Streptococcus thermophilus (S.the) (YP_141686), Streptococcus mutans (S.mut) (NP_721034), Leucconostoc mesenteroides (L.mes) (ZP_00065561), Oceanobacillus iheyensis (O.ihe) (NP_691915), Bacillus subtilis (B.sub) (CAB13827.1), Pseudomonas fluorescens (P.flu) (ZP_00262238.1), and Neisseria meningitides (N.men) (NP_273747). Amino acids conserved in all sequences are shaded in dark gray, and amino acids conserved in over 75% of the sequences are shaded in light gray. The conserved proline residue is indicated by an asterisk, and the SBF signature motif is underlined.
the presence of nine transmembrane segments as well as a highly conserved proline residue, corresponding to P290 in the human bile transporter (Fig. 2), which is an essential residue for bile acid transport (15). The phylogenetic relationship of various SBF proteins from different taxa was determined using the neighbor-joining method of ClustalW (Fig. 3). The Ctr protein is closely related to a number of sodium bile acid cotransporter proteins from bacteria, including two Streptococcus species and Leuconostoc mesenteroides. Prior to this study, the members of the SBF family with proved function were all in eukaryotes, and in mammals, these transmembrane proteins are responsible for the cotransport of sodium and bile acids across the plasma membrane in the liver and ileum (6, 7). SBF transporters from plants, namely, Arabidopsis thaliana and Oryza sativa (12), form a separate distinct cluster. These are inducible during growth, but neither their efflux function nor their substrates have been established. The ACR3 protein from Saccharomyces cerevisiae is an efflux transmembrane protein involved in resistance to arsenic compounds (16).

In this study we have confirmed that the ctr gene of B. longum encodes a cholate efflux transporter which is responsible for the efflux of cholate from E. coli and confers resistance to a number of structurally unrelated antimicrobial compounds. This is the first characterization of a bile acid transporter of the SBF family in bacteria and the first multidrug transporter to be characterized from a Bifidobacterium species.

**Nucleotide sequence accession number.** The GenBank accession number for the putative B. longum bile transporter Ctr is DQ017587.

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REFERENCES


