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Macrolide Resistance Mediated by a *Bifidobacterium breve* Membrane Protein

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A gene coding for a hypothetical membrane protein from *Bifidobacterium breve* was expressed in *Lactococcus lactis*. Immunoblotting demonstrated that this protein is located in the membrane. Phenotypical changes in sensitivity towards 21 antibiotics were determined. The membrane protein-expressing cells showed higher levels of resistance to several macrolides.

Bifidobacteria are natural inhabitants of the human gut microbiota, representing up to 91% of the total gut population in breast-fed babies (3). Some strains of the genus *Bifidobacterium* are considered probiotics and can exert several health-promoting effects (11). Among them, *Bifidobacterium breve* is one of the species more often found in infants (9).

The intestinal microbiota is continuously exposed to cytotoxic agents, including antibiotics. Recent evidence indicates that *B. breve* is generally more resistant to antibiotics than other *Bifidobacterium* species (10). It is thus reasonable to assume that this species may have a stronger intrinsic resistance than the other species of this genus. In this context, we investigated *B. breve* genes with a potential role in conferring resistance to cytotoxic compounds, such as antibiotics and bile salts.

Gene selection and protein location. Using a bioinformatics-based analysis with the preliminary genome sequence of *B. breve* UCC2003 (S. Leahy, J. A. Moreno, M. O'Connell-Motherway, H. G. Higgins, G. F. Fitzgerald, and D. Van Sinderen, unpublished data), a 3,301-bp DNA fragment was selected. Its genetic analysis revealed the presence of a 1,074-bp open reading frame encoding a hypothetical 357-amino-acid membrane protein. Two incomplete open reading frames, transcribed in opposite directions, were found, indicating that the gene is located in a monocistronic operon (Fig. 1A). A database enquiry allowed us to determine that the hypothetical protein of 38.6 kDa displayed significant homology to several hypothetical bile and multidrug resistance secondary transporters. Hydropathy profile analysis using the Expasy Proteomic Server predicted a highly hydrophobic protein with eight transmembrane-spanning regions and hydrophilic sequences, at both the N and C termini, located in the cytoplasm (Fig. 1B).

Currently, genetic studies of the genus *Bifidobacterium* are limited by the lack of molecular tools for disrupting genes and expressing proteins. Since previous studies have shown that *Lactococcus lactis* can express large quantities of *Bifidobacterium* proteins (8), *L. lactis* was chosen as the host to analyze the change of phenotype occurring as a consequence of expressing the hypothetical membrane protein. Total DNA was obtained from *B. breve* as described previously (8), and the gene was amplified using the primers 5'-TGCGACCACCATGGAGAAGGTCAAGGCTTTCGC-3' and 5'-GCCGACTCTAGATTATCAGCCTTCGACCTTGGC-3'. The PCR product was digested with NcoI and XbaI and ligated into the pNZ8048 vector (2), resulting in pN38. This nisin-inducible plasmid was transformed into *L. lactis* NZ9000 (7). A variant of the gene was constructed, which contained a set of 3'-tagged histidine codons, using the same forward primer combined with the primer 5'-TGCGATCAAAGCTTTTATCAGTGATGGTGATGGTGATGGCCTTCGACCTTGGCATCAGCG-3'. The resulting PCR product was digested with NcoI and HindIII and ligated into pNZ8048 to yield plasmid pNH38.

Inside-out membrane vesicles from *L. lactis* were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2A) and Western blotting (Fig. 2B), using horseradish peroxidase-conjugated antihistidine antibodies (QIAGEN, Inc., Valencia, CA). Western blots were developed with 4-chloro-1-naphthol. The nisin-induced control cells, harboring the empty vector, and the noninduced pNH38-containing cells did not show any signal on the Western blot (Fig. 2B). In contrast, the addition of nisin to *L. lactis* harboring pNH38 resulted in the synthesis of the histidine-tagged protein, which was located in the membrane and had the expected molecular mass of about 38 kDa.

Antimicrobial susceptibility. For MIC determinations, control cells (harboring pNZ8048) and pN38-containing cells were grown at 30°C to an optical density at 600 nm (OD_{600}) of about 0.4 in GM17 (M17 [Oxoid Limited, Hampshire, United Kingdom] with 0.5% of glucose) containing 5 μg/ml chloramphenicol. At this point, 0.05% of the culture supernatant of the nisin-producing strain *L. lactis* NZ9700 (5) was added to trigger transcription. Subsequently, the cells were incubated for 1 h, and one milliliter of the culture was added to 30 ml of soft (0.7% agar) GM17 at 40°C, containing 0.05% of the *L. lactis* NZ9700 culture supernatant. Then, the mixture was layered on

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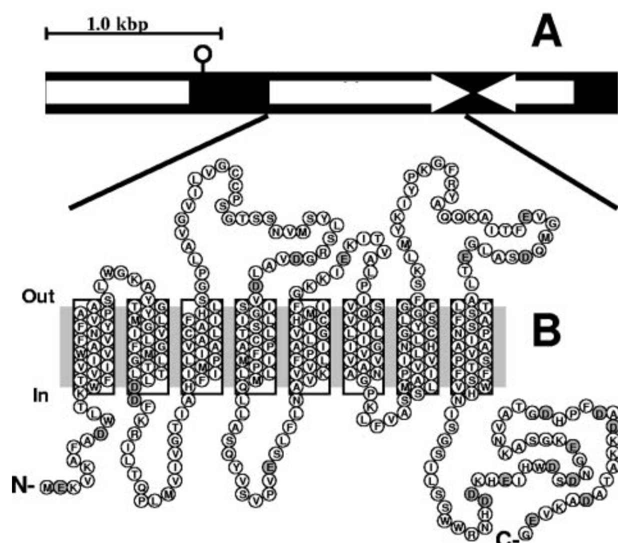

FIG. 1. Organization of the *B. breve* UCC2003 genomic region containing the gene encoding a hypothetical membrane protein (A). White arrows indicate the position and direction of transcription of the open reading frames. The pin-like symbol indicates a predicted palindromic sequence. The hydropathy profile prediction is shown in B.

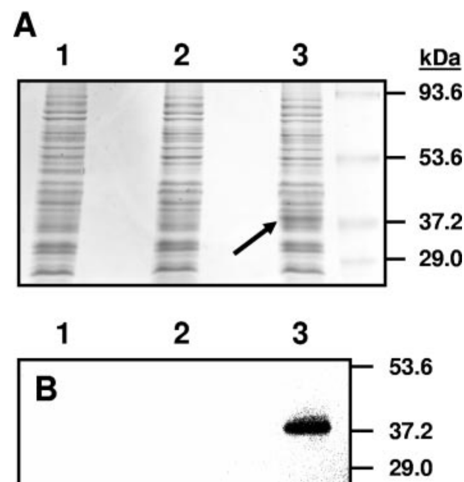

FIG. 2. A, SDS-PAGE gel of inside-out membrane vesicles (30 μg of protein per sample) prepared from nisin-induced control cells harboring the empty vector (lane 1), noninduced pNH38-containing cells (lane 2), or nisin-induced pNH38-containing cells (lane f3). B, Western blot of the SDS-PAGE gel of A. The arrow indicates the position of the expressed protein.

the top of 15-cm petri dishes containing 50 ml of GM17 (2% agar), to which supernatant of the *L. lactis* nisin-producing strain had been added. Etest strips of 21 different antibiotics (AB Biodisk, Solna, Sweden) were applied with an applicator, and MICs were determined after 48 h of incubation. The membrane protein conferred resistance (more than 3.9-fold increase in MIC determinations) to erythromycin, clarithromycin, dirithromycin, and azithromycin (Table 1). A smaller increase of resistance was observed for amynoglycosides, quinupristin-dalfopristin, rifampin, polymyxin, and vancomycin. Additional susceptibility tests were carried out using ethidium bromide, ox bile extract, and several bile salts. However, no differences in growth inhibition between the control (containing pNZ8048) and the cells containing pN38 were found for these compounds.

Growth inhibition assays. Precultures of induced control and pN38-containing cells grown to an OD_{600} of 0.8 were diluted 100-fold in GM17 containing nisin and erythromycin, clarithromycin, dirithromycin, or azithromycin at different concentrations. Cells were grown until they reached the stationary phase (8 to 10 h) and compared by monitoring the growth rate and the optical density. In the absence of antibiotic, the growth rate of control cells was comparable to that of the membrane protein-expressing cells (0.71 $\pm$ 0.053 and 0.77 $\pm$ 0.033, respectively), and the OD_{600}s of the cultures at the stationary phase were very similar (2.31 $\pm$ 0.07 for control cells and 2.37 $\pm$ 0.15 for the membrane protein-expressing cells). However, nisin-induced pN38-containing cells consistently reached higher densities and higher growth rates than the control cells under the same conditions. Figure 3 shows the effect of erythromycin, clarithromycin, and azithromycin on the maximum specific growth rate. Half-maximal inhibitory concentrations for the four macrolides studied were between 1.7 and 2.7 times higher for the membrane protein-expressing cells (erythromycin, 0.22 $\pm$ 0.015 μM; clarithromycin, 0.08 $\pm$ 0.008 μM; dirithromycin, 6.7 $\pm$ 1.8 μM; and azithromycin, 0.64 $\pm$ 0.06 μM) compared to the control cells (erythromycin, 0.13 $\pm$ 0.01 μM; clarithromycin, 0.04 $\pm$ 0.007 μM; dirithromycin, 3.1 $\pm$ 0.1 μM; and azithromycin, 0.24 $\pm$ 0.04 μM).

Concluding remarks. Several studies have dealt with resistance patterns of *Bifidobacterium* (1, 6, 10, 15, 16), but only a

TABLE 1. MICs of several antibiotics for the membrane protein-expressing *L. lactis* cells (pN38) and *L. lactis* cells not expressing the protein (control)[a]

Cell group	MIC (μg/ml) of antimicrobial[b]																				
	β-Lactams					Tetracyclines			Aminoglycosides			Macrolides				Others					
	AMP	PEN	CAZ	CEF	MEM	TET	DOX	MIN	KAN	GEN	STR	ERY	AZM	DTM	CLR	CLI	Q-D	RIF	SXT	POL	VAN
Control	0.125	0.125	3	1.5	0.032	0.19	0.094	0.064	64	12	48	0.19	0.50	4	0.047	0.38	1.5	16	>32	512	1
pN38	0.125	0.125	3	1.5	0.047	0.125	0.094	0.094	128	16	128	0.75	3	16	0.25	0.5	3	>32	>32	>1,024	3

[a] At least two independent experiments were carried out for those antibiotics with a more than two-fold increase of resistance between the control and the membrane protein-expressing cells.

[b] Ampicillin (AMP), azithromycin (AZM), benzylpenicillin (PEN), ceftazidime (CAZ), cephalothin (CEF), clarithromycin (CLR), clindamycin (CLI), dirithromycin (DTM), doxycycline (DOX), erythromycin (ERY), gentamicin (GEN) (high range, 0.064–1024 μg/ml), kanamycin (KAN), meropenem (MEM), minocycline (MIN), polymyxin (POL), quinupristin-dalfopristin (Q-D), rifampicin (RIF) (low range, 0.002 to 32 μg/ml), streptomycin (STR) (high range, 0.064 to 1,024 μg/ml), tetracycline (TET), trimethoprim-sulfamethoxazole (SXT), and vancomycin (VAN) were used.

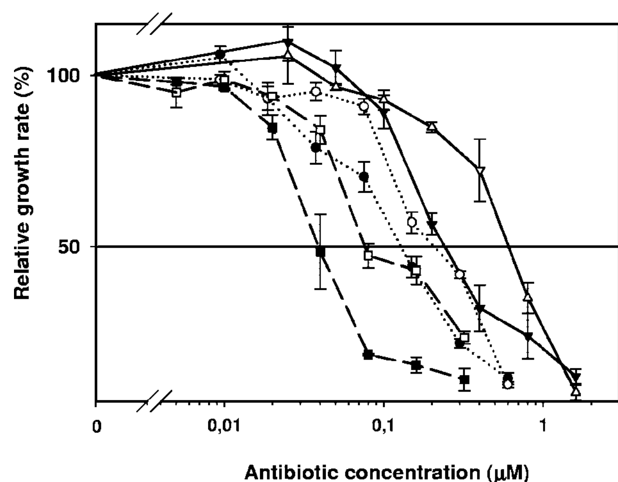

FIG. 3. Effect of erythromycin (circles), clarithromycin (squares), or azithromycin (triangles) on the maximum specific growth rate (μm) of control cells (closed symbols) and the membrane protein-expressing cells (open symbols). The maximum specific growth rates obtained in the absence of antibiotics were set at 100%. The growth rate was estimated from the growth curve by fitting the data to the equation $N_t = N_0 \times e^{\mu m \times t}$, in which N_t and N_0 are the cell densities at time t and time zero in the exponential growth phase, respectively. The half-maximal inhibitory concentration was calculated as the antibiotic concentration that inhibited the maximum specific growth rate by 50%.

single molecular determinant, *tetW*, has so far been identified (14). In this report we show the first evidence for the involvement of a membrane protein from *B. breve* conferring moderate resistance to macrolides when expressed in the heterologous host *L. lactis*. This membrane protein exhibits characteristics reminiscent of multidrug resistance proteins, representing transporters with a broad substrate specificity, which includes bile salts and many antibiotics (4, 12, 13). We therefore propose to name this membrane protein BbmR (*Bi*fidobacterium *b*reve *m*acrolide *R*esistance protein; GenBank accession number DQ115902).

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