

Two membrane proteins from *Bifidobacterium breve* UCC2003 constitute an ABC-type multidrug transporter

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Intrinsic resistance to drugs is one of the main determining factors in bacterial survival in the intestinal ecosystem. This is mediated by, among others, multidrug resistance (MDR) transporters, membrane proteins which extrude noxious compounds with very different chemical structures and cellular targets. Two genes from *Bifidobacterium breve* encoding hypothetical membrane proteins with a high homology with members of the ATP-binding cassette (ABC) family of multidrug efflux transporters, were expressed separately and jointly in *Lactococcus lactis*. Cells co-expressing both proteins exhibited enhanced resistance levels to the antimicrobials nisin and polymyxin B. Furthermore, the drug extrusion activity in membrane vesicles was increased when both proteins were co-expressed, compared to membranes in which the proteins were produced independently. Both proteins were co-purified from the membrane as a stable complex in a 1 : 1 ratio. This is believed to be the first study of a functional ABC-type multidrug transporter in *Bifidobacterium* and contributes to our understanding of the molecular mechanisms underlying the capacity of intestinal bacteria to tolerate cytotoxic compounds.

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INTRODUCTION

Bifidobacteria are important members of the human gut microbiota, in which they can be present at concentrations as high as 10^{11} cells per g faeces, representing up to 91 % of the total microbial population in breast-fed infants (Harmsen *et al.*, 2000). Their presence has been associated with beneficial effects. On the basis of their role in promoting human wellbeing, some species and strains of the genus *Bifidobacterium* are considered to be probiotic and are used as active ingredients in functional foods, mainly dairy-based products (Ouweland *et al.*, 2002). Well-documented clinically established applications of some strains of this genus are the treatment of diarrhoea and the balancing of the intestinal microbiota (Salminen & Gueimonde, 2004), while other health-promoting actions, such as anticarcinogenic activity, immunomodulation and reduction of serum cholesterol levels, have been suggested (Isolauri *et al.*, 2004). *Bifidobacterium breve*, one of the

representative species of its genus, is among the predominant bacteria present in the gastrointestinal tract of infants (Matsuki *et al.*, 1999).

Enteric bacteria have evolved to tolerate inhibitory factors in the intestinal niche. Their survival depends on tolerance to host-produced substances, such as bile (Begley *et al.*, 2005; Yokota *et al.*, 2000), and antimicrobial peptides (Ganzle *et al.*, 1999; Mahida *et al.*, 1997), but they are also conditioned by exposure to exogenous cytotoxic agents, including antibiotics (Vedantam & Hecht, 2003). Nowadays, drug efflux, mediated by MultiDrug Resistance (MDR) transporters, is considered as one of the main mechanisms responsible for these resistances (Grkovic *et al.*, 2002). These proteins can be subdivided into two groups according to structural and bioenergetic criteria. ATP-binding cassette (ABC) transporters power the transport via the hydrolysis of ATP, whereas the activity of secondary transporters is dependent on the transmembrane electrochemical gradient, typically the proton-motive force (Kim *et al.*, 2004; Mazurkiewicz *et al.*, 2005). Most of the bacterial multidrug efflux systems characterized up to now belong to the second class of transporters (Putman *et al.*, 2000), and just a few of them, such as LmrA and LmrCD from *Lactococcus lactis* (Lubelski *et al.*, 2004; van Veen *et al.*, 1996), HorA from

Abbreviations: ABC, ATP-binding cassette; AMP-PNP, 5'-(β,γ -imido) triphosphate; Bbm, *Bifidobacterium breve* multidrug transporter; DDM, *n*-dodecyl β -D-maltoside; MDR, multidrug resistance.

The GenBank/EMBL/DDBJ accession number for the sequence of the *B. breve* gene cluster is DQ486860.

Lactobacillus brevis (Sakamoto *et al.*, 2001), MsbA from *Escherichia coli* (Karow & Georgopoulos, 1993), BmrA from *Bacillus subtilis* (Orelle *et al.*, 2003) and EfrAB from *Enterococcus faecalis* (Lee *et al.*, 2003), belong to the ABC-type family.

Recent evidence indicated that *B. brevis* is more resistant to antibiotics than other *Bifidobacterium* species (Moubarek *et al.*, 2005), suggesting that this species could have a stronger intrinsic resistance. In a previous study, we characterized BbmR, a membrane protein from *B. brevis* which was able to confer resistance to macrolides and exhibited characteristics reminiscent of MDR proteins (Margolles *et al.*, 2005). Its homologue in *Bifidobacterium longum*, named Ctr, was also found to confer resistance to several antibiotics and to transport radioactive cholate (Price *et al.*, 2006). The current study presents work relating to *B. brevis* genes that play a role in its intrinsic resistance to cytotoxic compounds. We describe the gene cloning and functional characterization of a novel bifidobacterial ABC-type multidrug transporter in *L. lactis*, which shares both structural and functional properties with prokaryotic and eukaryotic MDR proteins, being able to confer resistance to several antimicrobials and to transport cytotoxic drugs.

METHODS

Chemicals. DNase A, creatine phosphokinase, phosphocreatine, potassium EDTA, DTT, HEPES, adenosine 5'-(β,γ -imido) triphosphate

(AMP-PNP), chloramphenicol, erythromycin, nisin, polymyxin B, imidazole and *n*-dodecyl β -D-maltoside (DDM) were purchased from Sigma. Takara supplied all restriction enzymes, excluding *Bsp*LU11I (Roche Applied Science). Platinum-*Pfx* DNA polymerase and Hoechst-33342 [2'-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5'-bi-1*H*-benzimidazole] were obtained from Invitrogen, and Ni²⁺ nitriloacetic acid (Ni-NTA) agarose was supplied by Qiagen. E-test strips were from AB Biodisk and ATP was from Amersham Biosciences. All chemicals were reagent grade and all solutions were made with molecular biology reagent water (Sigma).

Bacterial strains, plasmids, and culture conditions. Bacteria and plasmids used in this study are shown in Table 1. *Bifidobacterium breve* UCC2003 was grown at 37 °C in MRS medium (Merck) supplemented with 0.05% (w/v) L-cysteine in an anaerobic chamber (Mac500, Don Whitley Scientific). *Lactococcus lactis* subsp. *lactis* NZ9000 and NZ9700 (Kuipers *et al.*, 1993, 1998) were cultivated at 30 °C in M17 broth (Oxoid) with 0.7% (w/v) glucose (GM17) and 5 μ g chloramphenicol or 5 μ g erythromycin ml⁻¹ when they contained pNZ8048 and its derivatives or pNZE8048 and its derivatives respectively. Cells cotransformed with pNAbcA (or pNHAbcA) and pNAbcB were grown under the same conditions, but in a medium that contained 3 μ g chloramphenicol plus 3 μ g erythromycin ml⁻¹.

DNA and RNA manipulation, cloning of the genes, and sequence analysis. DNA manipulations were carried out as described by Sambrook *et al.* (1989). Total DNA was obtained from *B. brevis* as previously described (Margolles & de los Reyes-Gavilán, 2003). Vector pNZE8048 was constructed by amplifying a DNA fragment from the vector pNG8048 (Zúñiga *et al.*, 2002), with the primers *emr*-f and *emr*-r (Table 1). The resulting PCR product was

Table 1. Bacterial strains, plasmids and primers

Strain or plasmid	Relevant phenotype or genotype	Source or reference
<i>B. brevis</i> UCC2003	Infant isolate	MacConaill <i>et al.</i> (2003)
<i>L. lactis</i> NZ9000	<i>L. lactis</i> MG1363 <i>pepN::nisRK</i>	Kuipers <i>et al.</i> (1998)
<i>L. lactis</i> NZ9700	Nisin-producing strain	Kuipers <i>et al.</i> (1993)
pNZ8048	Gene expression vector, P _{<i>nisA</i>} Cm ^r	de Ruyter <i>et al.</i> (1996)
pNZE8048	Gene expression vector, P _{<i>nisA</i>} Em ^r	This work
pNAbcA	pNZE8048 derivative; <i>abcA</i>	This work
pNAbcB	pNZE8048 derivative; <i>abcB</i>	This work
pNHAbcA	pNZE8048 derivative; His-tag <i>abcA</i>	This work
Primer	Sequence*	
<i>emr</i> -f	5'-AAGGCCAGGAAGATCTCCGATTCACAAAAAATAGGCACACG-3'	This work
<i>emr</i> -r	5'-CGATATCGGATCCGTCGACCCGTGC-3'	This work
<i>abcA</i> -f	5'-TGCGATCACATGTACGTCGATCCGGGAATCAATACCAGCGC-3'	This work
<i>abcA</i> -r	5'-ATACGGAGAATTCTTATTAATAAACCCCTGTGCGGCGCC-3'	This work
<i>abcB</i> -f	5'-TGCGACCACCATGGCACAACGCAATACATTCGCG-3'	This work
<i>abcB</i> -r	5'-GCCGACTCTAGATTACTACTCAGTAGCCCCGGTGG-3'	This work
<i>abcAh</i> -f	5'-GCTGTATCAAGATTTCGCAAGCTGC-3'	This work
<i>abcAh</i> -r	5'-TGCGATCAGAAATCTTATTAGTGATGGTGATGGTGATGAAAC- CCCTGTGCGGCG CCGGACTGG-3'	This work
<i>abcA</i> -fq	5'-ACGACTGGCAGCGATTCAAC-3'	This work
<i>abcA</i> -rq	5'-GACCTGCTCGACGATTGTGA-3'	This work
<i>abcB</i> -fq	5'-TTCTCGGACATCTTCACATTCTTC-3'	This work
<i>abcB</i> -rq	5'-CAGTACGAGGCTCCACAATGC-3'	This work

*Restriction sites are underlined and the codons for the histidine tag are in bold italic.

digested with *Sall* and *Bgl*II, and ligated with *Sall*/*Bgl*II-digested pNZ8048, yielding pNZE8048, in which the chloramphenicol resistance marker is replaced by an erythromycin resistance cassette.

The structural genes *abcA* and *abcB* were amplified from the genome of *B. breve* by PCR using primers *abcA*-f and *abcA*-r (for gene *abcA*), and *abcB*-f and *abcB*-r (for gene *abcB*) (Table 1), respectively. Given that the *abcA* gene contains an internal *Nco*I site, the enzyme *Bsp*LU111, which yields compatible ends with *Nco*I, was used in the cloning procedure. The *abcA* gene was amplified, digested with *Bsp*LU111 and *Eco*RI, and ligated into pNZE8048, previously treated with *Nco*I and *Eco*RI, yielding pNAbcA. For *abcB*, the amplicon was digested with *Nco*I and *Xba*I, and ligated with *Nco*I/*Xba*I-digested pNZ8048, resulting in pNAbcB. Furthermore, in order to introduce a hexahistidine tag at the C-terminus of AbcA, a fragment of *abcA* was amplified using the primers *abcAh*-f and *abcAh*-r (Table 1), digested with *Cl*aI and *Eco*RI, and ligated into pNAbcA, previously cut with the same enzymes. This construction yielded plasmid pNHAbcA. The vectors pNAbcA and pNAbcB were transformed into electrocompetent *L. lactis* NZ9000 cells using previously described methods (de Ruyter *et al.*, 1996), and transformants were screened by restriction analysis of the recovered plasmids. For co-expression of AbcA and AbcB, pNAbcA (or pNHAbcA) was introduced into electrocompetent NZ9000 cells containing pNAbcB. To confirm that no PCR-borne mutations were introduced, the fidelity of the inserts was verified by DNA sequencing of both strands with an ABI Prism 377 sequencer (Applied Biosystems).

Real-time PCR was used to assess the expression levels of *abcA* and *abcB*. Primers *abcA*-fq, *abcA*-rq, *abcB*-fq and *abcB*-rq were chosen to amplify internal fragments of 128 and 84 bp of *abcA* and *abcB*, respectively (Table 1). Four independent cultures of *L. lactis* cells were disrupted with glass beads (0.5 µm) in a FastPrep FP120 Instrument (Thermo Savant). Total RNA was extracted using Tri-Reagent solution according to the manufacturer's instructions (Sigma). Two micrograms of total RNA was treated with 2 units of DNase (Fermentas) for 1 h at 37 °C. Then, cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad). Absence of chromosomal DNA contamination was checked by real-time PCR. Real-time PCR reactions were carried out using an ABI PRISM 7500 with a SYBR green PCR master mix (Applied Biosystems). The efficiency was calculated based on the slope of a standard curve. In all cases, the 16S rRNA level was used as an internal control.

The stability of pNAbcA and pNAbcB co-existing in the same cell was checked by growing the cells for more than 70 generations in GM17 broth containing chloramphenicol and erythromycin (five consecutive cultures inoculated with 0.0037% (v/v) of a culture grown to an OD₆₀₀ of about 1.2). In parallel, cells were plated on GM17 agar with and without antibiotics, to confirm that all cells contained the two antibiotic markers. Subsequently, 24 independent colonies (12 colonies from plates inoculated with cultures grown for 42 generations, and 12 colonies from plates inoculated with cultures grown for 71 generations) were analysed, and plasmids were extracted with the GenElute DNA kit (Sigma) and digested with *Bgl*II to verify that no DNA recombination had occurred. Furthermore, the genes *abcA* and *abcB*, and the upstream region corresponding to the nisin promoter in pNAbcA and pNAbcB, were sequenced after the five consecutive cultures to confirm that no mutations were produced during growth under selective pressure when both plasmids are present in the same cell.

DNA and protein sequences were analysed using the computer program Clone Manager 5 (Scientific and Educational Software). Homology searches and multiple sequence alignments (clustalw) were carried out using the BLAST server of the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>) and the software available on the Web page of the Pôle-Bioinformatique Lyonnais (<http://pbil.univ-lyon1.fr/>). The Neural Network Promoter

Prediction Software (http://www.fruitfly.org/seq_tools/) was used for searching putative promoter regions. Homology trees were constructed with the TreeTop-Phylogenetic Prediction program (<http://www.genebee.msu.su/>).

Preparation of inside-out membrane vesicles. For the isolation of inside-out membrane vesicles of *L. lactis* NZ9000, cells were grown at 30 °C to an OD₆₀₀ of about 0.4. At this point, 0.1% (v/v) of the supernatant of the nisin-producing *L. lactis* strain NZ9700 was added to the culture to trigger transcription of the *abcA* or *abcB* gene, or jointly the *abcA/abcB* genes from the *nisA* promoter. Subsequently, the cells were incubated for 1 h at 30 °C, harvested at an OD₆₀₀ of approximately 0.8 by centrifugation, and membrane vesicles were obtained as previously described (Margolles *et al.*, 1999). The membrane vesicles were stored in small aliquots in liquid nitrogen.

Antimicrobial susceptibility testing. Cells were grown at 30 °C to an OD₆₀₀ of about 0.4 in GM17 broth containing chloramphenicol or erythromycin, or both. To induce gene expression at this point, 0.1% (v/v) of the culture supernatant of *L. lactis* NZ9700 was added to the GM17 broth. Subsequently, the cells were incubated for 1 h. For MIC determinations, 1 ml of the culture was added to 30 ml soft (0.7% agar) GM17 at 40 °C, containing 0.1% of the *L. lactis* NZ9700 culture supernatant. Then, the mixture was layered on the top of 15 cm Petri dishes containing 50 ml GM17 (2% agar), to which supernatant of the *L. lactis* nisin-producing strain had been added. E-test strips of 15 different antibiotics were applied with an applicator, and MICs were determined after 24 h incubation. For resistance assays on agar media, cultures were centrifuged, resuspended in GM17 broth and the OD₆₀₀ was adjusted to 1. Then, serial 10-fold dilutions were performed, and 5 µl of each dilution was spotted on the GM17 agar medium with or without the inhibitory agent (nisin or polymyxin B). The plates were incubated for 24 h and all the experiments were done at least in triplicate.

Hoechst-33342 transport in membrane vesicles. Inside-out membrane vesicles (1 mg of total membrane protein) were diluted in 2 ml ATP regenerating buffer (50 mM potassium HEPES buffer, pH 7.1, containing 5 mM MgSO₄, 8.5 mM NaCl, 0.1 mg creatine kinase ml⁻¹ and 5 mM phosphocreatine) in a 3 ml quartz cuvette. After 1 min incubation at 30 °C, Hoechst-33342 was added to a final concentration of 0.2 µM. Once the signal was stable, Mg²⁺-ATP or Mg²⁺-AMP-PNP was added to a final concentration of 2 mM, and the fluorescence intensity (excitation 355 nm, emission 457 nm) was followed with an Eclipse fluorescence spectrophotometer (Varian) provided with a magnetically stirred holder at 30 °C.

Affinity purification and identification of the purified proteins. Inside-out membrane vesicles from *L. lactis* NZ9000 containing pNHAbcA/pNAbcB (12 mg total membrane protein ml⁻¹) were solubilized in 50 mM potassium phosphate buffer, pH 8.0, containing 10% (v/v) glycerol, 100 mM NaCl and 1% (w/v) DDM. The suspension was mixed and, after 30 min incubation at 4 °C, the insoluble material was removed by centrifugation (250 000 g, 20 min, 4 °C). For purification of histidine-tagged AbcA, 1 ml solubilized membrane proteins was mixed and incubated for 1 h with 200 µl Ni-NTA agarose, which was preequilibrated in buffer A [50 mM potassium phosphate, pH 8.0, 100 mM NaCl, 10% (v/v) glycerol, 0.05% (w/v) DDM] plus 10 mM imidazole. After incubation, the resin was transferred to a Bio-spin column (Bio-Rad) and washed first with 25 column volumes of buffer A containing 10 mM imidazole, and subsequently with 12 column volumes of buffer A (pH 7.0) containing 30 mM imidazole. The protein was eluted with buffer A, pH 7.0, supplemented with 250 mM imidazole. All steps were carried out at 4 °C.

Proteins from the membrane and eluted fractions were checked by SDS-PAGE by using a Mini-Protean II system (Bio-Rad). SDS-PAGE

gels were stained with Coomassie BioSafe (Bio-Rad), and densitometric scanning was carried out by using the Gel Doc 2000 system with the Quantity One software (Bio-Rad). For protein identification, bands were excised from gels and submitted to tryptic digestion, and mass spectrometry analyses were performed at the Servicio de Proteómica of the Centro Nacional de Investigaciones Cardiovasculares. All protein concentrations were determined by the Lowry method.

RESULTS AND DISCUSSION

Identification and sequence analysis of *abcA* and *abcB*

A 7930 bp DNA fragment was selected from the preliminary genome sequence of *B. breve* UCC2003 (S. Leahy, J. A. Moreno, M. O'Connell-Motherway, H. G. Higgins, G. F. Fitzgerald & D. Van Sinderen, unpublished data). Its genetic analysis revealed the presence of two adjacent ORFs displaying significant homology to several hypothetical MDR transporters, named *abcA* and *abcB*, putatively transcribed in the same direction and separated by 202 bp. A putative promoter sequence was found 182 bp upstream of the potential *abcA* start codon, but not upstream of the *abcB* gene. The first gene, *abcA*, possesses a putative ribosome-binding site 8 bp upstream of its start codon (GGTGAT), while the second gene, *abcB*, is followed by a transcription terminator-like (inverted repeat) sequence (Fig. 1a). The *abcA* and *abcB* genes are predicted to encode 636 and 601 aa proteins, respectively, identified by a database enquiry (BLASTP) as putative ABC transporters. Hydropathy profile analysis using the ExPasy Proteomic Server predicted that both proteins possess a transmembrane domain, composed of six putative transmembrane helices, followed by a hydrophilic portion with a putative ATP-binding domain, containing the Walker A and Walker B motifs, and the ABC signature sequence (Schneider & Hunke, 1998; Walker *et al.*, 1982) (Fig. 1b). Since the ABC domain is highly conserved, in order to determine evolutionary relationships with other transporters the predicted permease domains of AbcA (339 N-terminal amino acids) and AbcB (347 N-terminal amino acids) were subjected to BLASTP analysis and compared with homologous sequences. Analysis of a multiple alignment of the primary sequence of these domains showed that they are closely related to a number of ABC transporters from bacteria (Fig. 1c, d). Both protein A and B domains displayed the highest homology scores with proteins that were located in tandem on the genomes. Interestingly, the AbcA permease domain matched with proteins encoded by the upstream genes, whereas the AbcB permease domain matched with proteins encoded by the downstream genes of the tandem-like structures. These results indicated that AbcA/AbcB homologues are widely distributed in bacteria, and most likely they are orthologous systems performing similar physiological functions. This prompted us to study the functionality of both proteins (independently or together) by investigating phenotypic changes of a cell that expresses these proteins, as well as determining their

role in multidrug resistance and their ability to transport drugs.

Production of the ABC transporters and antimicrobial activity profiles

In recent years, molecular techniques for disrupting genes and controlling gene expression have been extensively used to functionally study MDR transporters (Doerrler & Raetz, 2002; Hirata *et al.*, 2004; Lubelski *et al.*, 2004; Ravaut *et al.*, 2006). However, the lack of efficient transformation systems and the paucity of effective molecular tools (e.g. cloning and expression vectors and gene inactivation systems) have so far severely limited functional studies in *Bifidobacterium* (Ventura *et al.*, 2004). Since previous studies from our group have shown that the nisin-inducible system from the Gram-positive bacterium *L. lactis* can generate large quantities of bifidobacterial membrane and cytosolic proteins (Margolles & de los Reyes-Gavilán, 2003; Margolles *et al.*, 2005), we selected this bacterium as the host to produce AbcA and AbcB. We cloned (independently or together) *abcA* and *abcB* by constructing plasmids pNAbcA and pNAbcB, which were then introduced into *L. lactis* cells. Both plasmids could be maintained in the same host cell after more than 70 generations. Total cell counts indicated that all cells contained the two antibiotic markers, and no recombinations or mutations were observed after sequencing and restriction enzyme analysis of the plasmids in any of the tested colonies. This proves that pNAbcA and pNAbcB could co-exist and are stable in the same cell, and shows that they did not segregate and were not maintained in different cell subpopulations.

Gene expression and protein synthesis were investigated by real-time PCR and SDS-PAGE. Only the expression of AbcB was apparent on the protein electrophoresis profiles, probably due to the coincidence of the molecular mass of AbcA with one of the major membrane proteins of *L. lactis* (Fig. 2). However, real-time PCR showed that both *abcA* and *abcB* were transcribed, either when the genes were cloned separately or together. Interestingly, in cells carrying both pNAbcA and pNAbcB, the *abcB* transcript appears to be about two times more abundant than the *abcA* transcript ($2^{\Delta\Delta CT} = 2^{1.04 \pm 0.059}$), whereas when *abcA* and *abcB* were expressed alone, their transcripts were $2^{2.43 \pm 1.17}$ times and $2^{1.49 \pm 1.52}$ times more abundant, respectively, than the corresponding transcripts of the cells containing both plasmids.

We investigated if AbcA and AbcB could be involved in conferring antimicrobial resistance. We determined the MIC of 15 antibiotics using E-test strips, a quantitative method that has emerged in the last few years as an accurate alternative to the traditional methods, such as microdilution and disk diffusion (Turnbull *et al.*, 2004). The antibiotics tested were ampicillin, benzylpenicillin, clindamycin, ciprofloxacin, doxycycline, kanamycin, meropenem, minocycline, polymyxin, quinupristin-dalfopristin, rifampicin (low range, 0.002 to 32 $\mu\text{g ml}^{-1}$), streptomycin (high range,

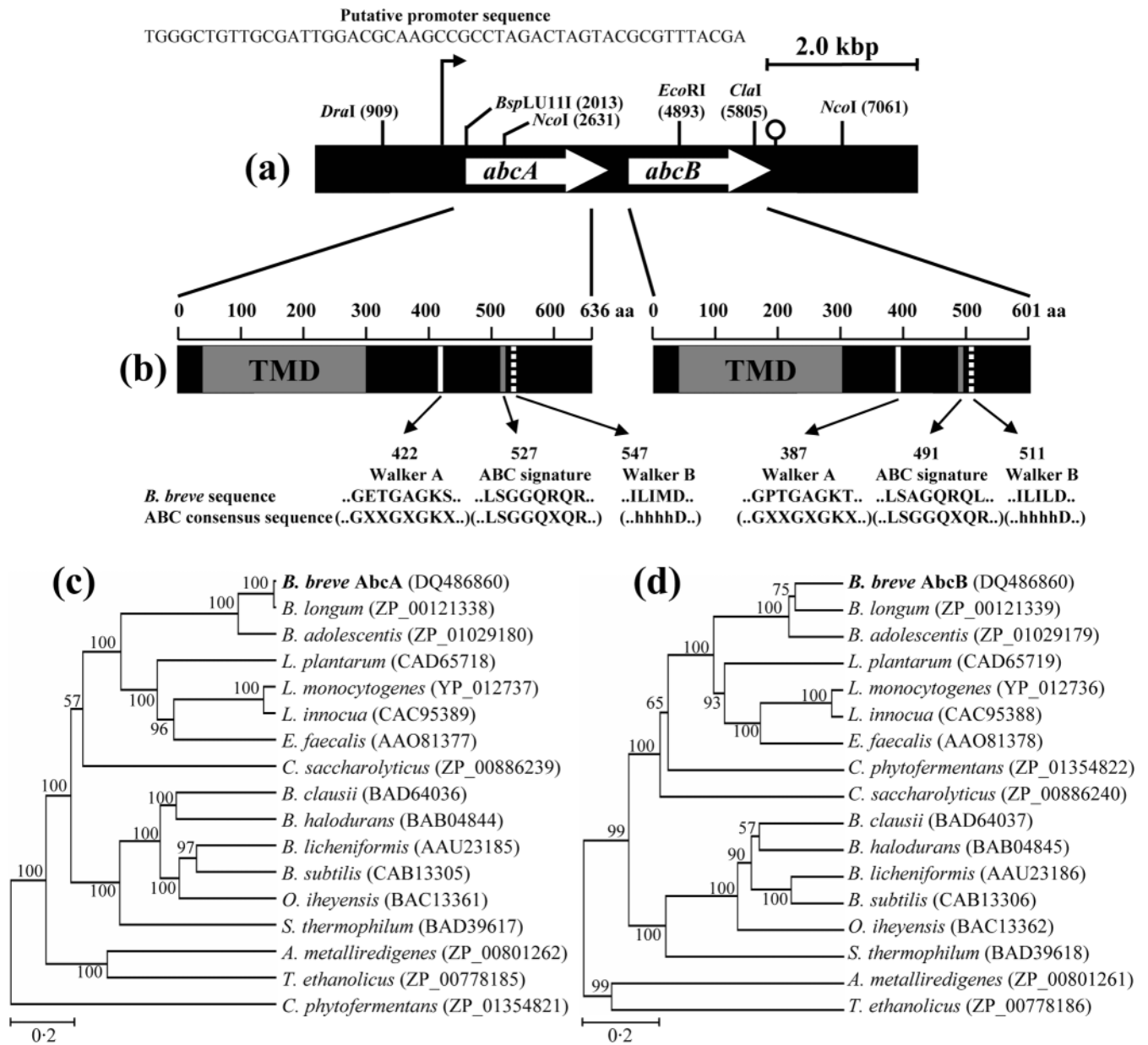


Fig. 1. (a) Organization of the *B. breve* UCC2003 genomic region containing the *abcA* and *abcB* genes. The white arrows indicate the relative positions and direction of transcription of the ORFs. The pin-like symbol indicates a terminator-like sequence. Relevant restriction sites and their locations in the sequence are also indicated. A putative promoter region is indicated with a black arrow upstream of *abcA*. (b) Modular structure of AbcA and AbcB. The amino acid sequences in parentheses are the consensus sequences of the ABC transporter family. TMD, transmembrane domain; X, any amino acid; h, hydrophobic residue. (c, d) Phylogenetic relationship analysis of the permease domains of AbcA (c) and AbcB (d). Database accession numbers are given in parentheses. Trees were constructed with the matrix of pair distances between sequences using the cluster algorithm, and bootstrap values (100 replicates) are given at the branch points.

0.064 to 1,024 $\mu\text{g ml}^{-1}$), tetracycline, trimethoprim-sulfa-methoxazole, and vancomycin. Chloramphenicol and macrolides were not tested since they are the selection markers in the vectors. A small increase in resistance was observed for ciprofloxacin. The MICs for the control strain, harbouring the empty plasmid, and the strain harbouring pNAbcA were $3.3 \pm 0.6 \mu\text{g ml}^{-1}$, whereas those for cells

containing pNAbcB or pNAbcA/pNAbcB were 6.0 ± 0 and $6.7 \pm 1.2 \mu\text{g ml}^{-1}$, respectively. Interestingly, a recent report demonstrated that the increased expression of two multidrug ABC transporter-like genes is associated with ethidium bromide and ciprofloxacin resistance in *Mycoplasma hominis* (Raheison *et al.*, 2005). In our case, the most significant changes were found for polymyxin B,

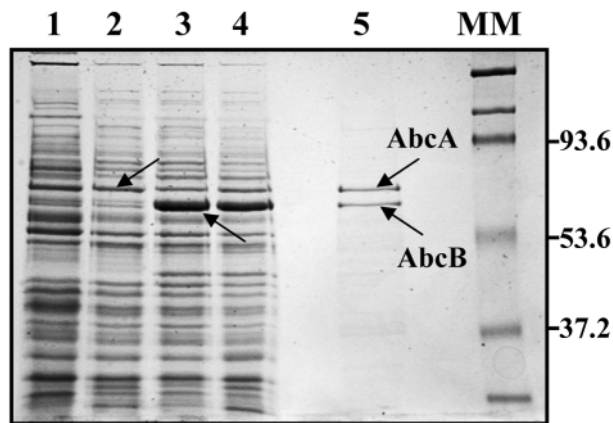


Fig. 2. Expression of AbcA and AbcB and co-purification of the AbcA/AbcB complex. Coomassie BioSafe-stained SDS-PAGE gel of inside-out membrane vesicles (30 μg protein/lane) from cells harbouring the empty vector (pNZ8048, lane 1) or cells expressing AbcA (pNAbcA, lane 2), AbcB (pNAbcB, lane 3), or jointly AbcA and AbcB (pNAbcA/pNAbcB, lane 4). The purified histidine-tagged AbcA from a Ni-NTA column with 250 mM imidazole and the co-eluted protein AbcB are represented in lane 5. MM, protein markers (molecular masses in kDa are indicated on the right). The arrows indicate the positions of AbcA and AbcB.

the resistance increasing more than fourfold for cells expressing AbcA ($\text{MIC } 42.7 \pm 9.2 \mu\text{g ml}^{-1}$, compared to 9.3 ± 2.3 for the control strain), and more than twelve-fold for cells expressing AbcB ($128.0 \pm 0 \mu\text{g ml}^{-1}$) or both proteins ($149.3 \pm 40 \mu\text{g ml}^{-1}$). No significant differences between the control strain and the membrane-protein-expressing cells were found for any other antibiotic using the E-test assay.

Since polymyxin B is a polycationic antimicrobial peptide that acts at the cell surface level, dissipating proton-motive force by making pores in the cell membrane (Hancock & Chapple, 1999), we decided to investigate the resistance-conferring capability of AbcA and AbcB to the antimicrobial

peptide nisin. Therefore, susceptibility differences against nisin and polymyxin B between *L. lactis* cells harbouring pNAbcA, pNAbcB or pNAbcA/pNAbcB, and bacterial cells harbouring the control plasmid, were determined in GM17 agar. The joint expression of AbcA and AbcB resulted in an increased resistance to nisin and polymyxin with respect to the control, which became apparent by colony formation at the highest dilution used. However, considerable nisin resistance was also found under similar conditions for cells expressing only AbcB (Fig. 3). This indicated that the expression of AbcB alone could also reduce the cell susceptibility to nisin, although to a lesser extent than when both proteins are co-expressed. Consistent with the above finding, it has previously been shown that certain ABC transporters can act on bacteriocin-like compounds. For example, the ABC-transporter LmrB from *L. lactis* confers resistance to LsbA and LsbB, two class II bacteriocins (Gajic *et al.*, 2003). It does so, most likely, by removing LsbA and LsbB from the cytoplasmic membrane, which is the target of these antimicrobial peptides. Other studies have suggested that ABC transporters could play a key role in generating resistance to nisin and other antimicrobial peptides in Gram-positive bacteria (Kok *et al.*, 2005).

Transport of Hoechst-33342 in membrane vesicles

Hoechst-33342 is a cytotoxic drug extensively employed to detect the activity of MDR transporters (Lubelski *et al.*, 2004; Margolles *et al.*, 1999; Sakamoto *et al.*, 2001; van Veen *et al.*, 2000; Woebking *et al.*, 2005). This probe has the property of being fluorescent when present in the lipid environment of biomembranes, while being essentially non-fluorescent in the aqueous phase (Shapiro & Ling, 1995). Since MDR transporters are thought to pump out hydrophobic drugs from (or close to) the cytoplasmic membrane (Putman *et al.*, 2000), this compound was used to study the extrusion of drugs directly from the membrane to the aqueous phase, via the decrease of fluorescence observed when extrusion activity is present (Margolles *et al.*, 1999; Shapiro & Ling, 1995; Woebking *et al.*, 2005). Furthermore, the advantage of carrying out functional

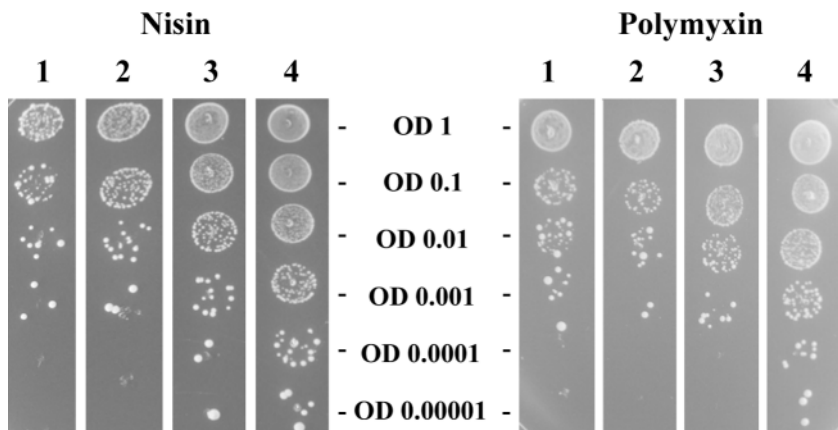


Fig. 3. Effect of AbcA, AbcB, and AbcA/AbcB expression on the susceptibility of *L. lactis* to nisin and polymyxin. Results shown are from cells harbouring pNZ8048 (1), pNAbcA (2), pNAbcB (3), or pNAbcA and pNAbcB (4) and spotted on GM17 plates containing 78 ng nisin ml^{-1} or 62 μg polymyxin B ml^{-1} .

studies in isolated membranes is the avoidance of possible interference caused by other cellular components that may modify the transport activity. Experiments performed in inside-out membrane vesicles of *L. lactis* NZ9000 cells showed the highest transport rate of Hoechst-33342 in membranes harbouring AbcA and AbcB, although significant transport was also detected in AbcB-containing membrane vesicles. AbcA-containing membranes displayed an extremely low transport activity as compared to the other two membrane systems (Fig. 4). In addition, the ATP-dependency of the transport process was demonstrated, since no activity was detected in the presence of the non-hydrolysable ATP analogue AMP-PNP. ATP-dependent transport of Hoechst-33342 was not observed in control membrane vesicles.

AbcA and AbcB form a stable complex in the membrane

The results of the experiments described above point to cooperation between AbcA and AbcB that results in an increased resistance to nisin and polymyxin, and an enhanced ability to extrude Hoechst-33342 from the membrane of *L. lactis*. As suggested for other ABC transporters (Abele & Tampe, 1999; Lee *et al.*, 2003; Lubelski *et al.*, 2004), this is likely to happen through a direct interaction of AbcA and AbcB in the membrane, where these two proteins are assumed to form a complex that represents the functional unit of the transporter. To prove this assumption for the AbcAB proteins, a histidine tag was attached to the C-terminal part of AbcA to facilitate purification of the complex by affinity chromatography. For this purpose, membranes were isolated from cells harbouring pNHAbcA/pNAbcB, solubilized with a DDM-containing buffer and subjected to a single-step Ni-NTA affinity

purification procedure (Fig. 2). When His-tagged AbcA was purified, two protein bands were eluted, with molecular masses that correspond to AbcA and AbcB. The identity of the proteins was confirmed by mass spectrometry, the upper band being identified as AbcA, and the lower as AbcB. Purified proteins were analysed by densitometry of Coomassie BioSafe-stained SDS-PAGE gels, showing that both proteins are present in approximately equal amounts, indicating the existence of a stable membrane-associated complex with a stoichiometry of 1 : 1 (Fig. 2).

It has been experimentally proved that several prokaryotic ABC transporters act as dimers. Homodimerization has been demonstrated for LmrA of *L. lactis* (van Veen *et al.*, 2000), MsbA of *E. coli* (Chang & Roth, 2001) and BmrA of *B. subtilis* (Ravaud *et al.*, 2006), while a heterodimeric complex was found to be the functional unit of the MDR transporter LmrCD from *L. lactis* (Lubelski *et al.*, 2004). However, in our study we have found that AbcB can retain some activity by itself, conferring nisin resistance and extruding Hoechst-33342 from the membrane, although to a lesser extent than when both proteins are present. A similar effect has also been reported for other ABC transporters. In eukaryotic cells, the mammalian proteins ADLP (adrenoleukodystrophy protein), ALDRP (adrenoleukodystrophy-related protein) and PMP70 (70 kDa peroxisomal protein) were found to act as homo- as well as heterodimers, and it was proposed that the different dimer combinations vary in activity and substrate specificity (Liu *et al.*, 1999). Genetic evidence suggests that the substrate specificity of the traffic ATPase transporters involved in the uptake of eye pigment precursors in *Drosophila melanogaster* (the white, scarlet and brown gene products) depends on the dimer formed: white and scarlet together form a tryptophan transporter, while the white and brown gene products form a guanine transporter

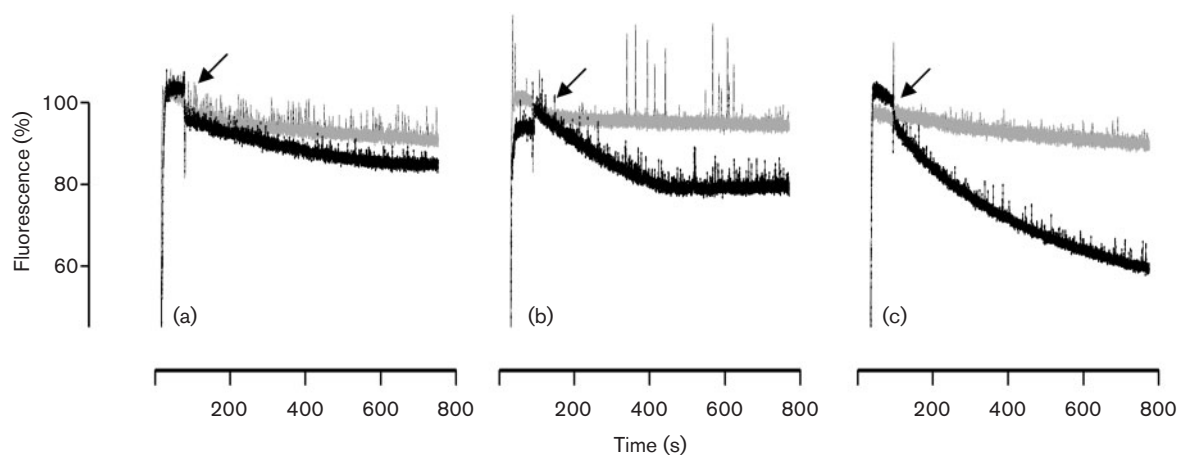


Fig. 4. AbcA-, AbcB- and AbcA/AbcB-mediated extrusion of Hoechst-33342 from inside-out membrane vesicles. Membranes were prepared from NZ9000 cells harbouring pNAbcA (a); pNAbcB (b), or pNHAbcA/pNAbcB (c). The arrow indicates the addition of 2 mM ATP (black lines) or AMP-PNP (grey lines). Hoechst-33342 transport in *L. lactis* NZ9000/pNZ8048 control membranes was not observed under the same assay conditions (data not shown). The graphics shown are representative of three independent experiments with three different batches of vesicles for each strain.

(Mackenzie *et al.*, 1999). In a similar way, we have observed that when AbcA and AbcB are co-expressed in *L. lactis* cells, AbcB is produced about two times more than AbcA. This implies that in pNAbcA/pNAbcB-containing cells there would be a population of free AbcB, not bound to AbcA. Since we have shown that AbcB alone is active under certain conditions, this could partially mask the activity of the heterodimer. Future reconstitution studies could address these questions.

In short, this study has provided evidence that AbcA and AbcB act as a heterodimeric ABC-type multidrug transporter, conferring resistance to nisin and polymyxin and extruding cytotoxic compounds. Therefore, we propose to rename AbcA and AbcB as BbmA (*Bifidobacterium breve* multidrug transporter) and BbmB, respectively. These findings provide the basis for further biochemical studies of BbmAB and the half-size transporters BbmA and BbmB, and open some questions about the physiological role of this *B. breve* transporter in its natural environment, the intestinal niche.

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