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Sterol Transport by the Human Breast Cancer Resistance Protein (ABCG2) Expressed in *Lactococcus lactis**

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The human breast cancer resistance protein (BCRP, also know as ABCG2, MXR, or ABCP) is one of the more recently discovered ATP-binding cassette (ABC) transporters that confer resistance on cancer cells by mediating multidrug efflux. In the present study, we have obtained functional expression of human BCRP in the Gram-positive bacterium *Lactococcus lactis*. BCRP expression conferred multidrug resistance on the lactococcal cells, which was based on ATP-dependent drug extrusion. BCRP-mediated ATPase and drug transport activities were inhibited by the BCRP-specific modulator fumitremorgin C. To our knowledge these data represent the first example of the functional expression of a mammalian ABC half-transporter in bacteria. Although members of the ABCG subfamily (such as ABCG1 and ABCG5/8) have been implicated in the transport of sterols, such a role has not yet been established for BCRP. Interestingly, the BCRP-associated ATPase activity in *L. lactis* was significantly stimulated by (i) sterols including cholesterol and estradiol, (ii) natural steroids such as progesterone and testosterone, and (iii) the antiestrogen anticancer drug tamoxifen. In addition, BCRP mediated the efflux of [^{3}H]estradiol from lactococcal cells. Our findings suggest that BCRP may play a role in the transport of sterols in human, in addition to its ability to transport multiple drugs and toxins.

The emergence of multidrug resistant cancer cells is a serious problem in the chemotherapeutic treatment of human tumors. In mammals, multidrug resistance based on the active extrusion of cytotoxic drugs from the cell is mediated by several members of the ATP-binding cassette (ABC)[1] superfamily. These include the multidrug resistance P-glycoprotein MDR1 (also termed ABCB1) and the protein MRP1 (multidrug resistance-associated protein 1, also termed ABCC1) (1). The breast cancer resistance protein (BCRP, also termed MXR, ABCP, or ABCG2) is one of the more recently discovered ABC multidrug transporters in human cancer cells. BCRP confers resistance on cells to (i) toxic ions such as rhodamine 123, (ii) anticancer agents including mitoxantrone and the anthracyclines daunomycin and doxorubicin, and (iii) the camptothecins topotecan and SN-38 (2–7). Overexpression of BCRP has been observed in several human cancer cell lines selected for drug resistance (2, 5, 8) as well as in tumor samples of cancer patients (9–11). Recently, fumitremorgin C (FTC), a novel chemosensitizing agent, was identified and shown to reverse drug resistance in human BCRP-expressing cancer cells by inhibiting BCRP-mediated drug transport (12).

BCRP is a 655-amino acid, 72.1-kDa protein and is the second member of the G subfamily of ABC transporters. Members of the G subfamily are all half-transporters and include among others (i) the *Drosophila* white, brown, and scarlet proteins, which are involved in the transport of eye pigment (13); (ii) ABCG1, which is thought to be involved in the transport of cholesterol and phospholipids (14); and (iii) heterodimeric ABCG5/ABCG8, which has been implicated in the transport of cholesterol and plant sterols (15). In contrast to P-glycoprotein MDR1 and MRP1, which are full size transporters, BCRP most likely functions as a homodimer (16).

In normal tissue, high expression of the BCRP is found in stem cells (17), epithelial cells of small and large intestines, ducts and lobules of the breast, endothelial cells of veins and capillaries (18), and synchitiotrophoblastic cells of the placenta (19). The localization of BCRP suggests that it could have a potential role in protection against toxins. The recent observation in BCRP knock-out mice that BCRP protects against a chlorophyll-derived dietary phototoxin and protoporphyria is consistent with this notion (20).

Previously, we have characterized the molecular basis of the drug specificity of LmrA, a half-transporter homologue of human P-glycoprotein MDR1, in the Gram-positive bacterium *Lactococcus lactis* (21, 22). To allow a detailed comparison of BCRP and LmrA, human BCRP was functionally expressed in *L. lactis* using the nisin A-induced expression system that is used for the expression of LmrA. BCRP was active as an ATP-dependent multidrug transporter in *L. lactis* and was able to interact with sterols. We conclude that the substrate specificity of BCRP partly overlaps with that proposed for ABCG1 and ABCG5/ABCG8. Our observations may suggest a physiological role for BCRP in sterol metabolism in human in addition to its role in mediating resistance to xenobiotics and toxins arising from dietary intake and cellular metabolism.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions

L. lactis strains NZ9000 and NZ9700 (23) were grown at 30 °C in M17 medium (Difco) supplemented with 0.5% glucose. Chloramphenicol (5 μg/ml) was added where appropriate.

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[1] The abbreviations used are: ABC, ATP-binding cassette; BCRP, breast cancer resistance protein; FTC, fumitremorgin C; IC_{50}, drug concentration required for half-maximal inhibition of the cellular growth rate; MDR, multidrug resistance; AMP-PNP, adenosine 5′-(β,γ-imido)triphosphate.

Genetic Manipulations

The human *BCRP* gene was amplified from pcDNA3-BCRP (2) by PCR using the forward primer 5′-GCCGCTAATACCATGGCTTCCAGTAATGTCG-3′ to introduce an *Nco*I site at the 5′ end of *BCRP* and the reverse primer 5′-GCTTGGTACCGAGCTCTCTAGAAATTTAAGAATA-3′ to introduce an *Xba*I site at the 3′ end of *BCRP*. The internal *Nco*I sites in *BCRP* were removed through silent mutations by PCR-based site-directed mutagenesis using two internal complementary primer pairs: the forward primer 5′-CTGTAATCTGATTTATTACCGATGGGGATGTTACC-3′ and the reverse primer 5′-GGTAACATCCCCATCGGTAATAAATCAGATAACAG-3′ for C1440G and the forward primer 5′-GCTTATTCAGCCAGTTCGATGGCACTGGCCATAGCAGCAGG-3′ and the reverse primer 5′-CTGCTGCTATGGCCAGTGCCATCGAACTGGCTGAATAAGC-3′ for C1566G. The final PCR product was digested with *Nco*I and *Xba*I and cloned into the lactococcal pNZ8048 expression vector (23), giving pNZ-BCRP. All PCR-amplified DNA fragments were sequenced to ensure that only the intended changes had been introduced.

Preparation of Inside-out Membrane Vesicles

For the isolation of BCRP-containing and control inside-out membrane vesicles, *L. lactis* NZ9000 cells harboring pNZ-BCRP or pNZ8048, respectively, were grown at 30 °C to an A_{660} of ~0.3. At this density, 0.4% (v/v) of the supernatant of the nisin-producing *L. lactis* strain NZ9700 (containing about 10 ng/ml of nisin A) was added to the culture to induce transcription of the *BCRP* gene under control of the *nisA* promoter. Following the incubation at 30 °C for 2 h, the cells were harvested by centrifugation at an A_{660} of ~0.7–0.9 and washed either with 100 mM KPi (pH 7.0) for transport assays or 50 mM Tris-HCl (pH 7.4) for ATPase assays. The cell pellet was resuspended to an A_{660} of 5 in KPi or Tris buffer supplemented with Complete protease inhibitor mixture (Roche Applied Science). Lysozyme was then added to a final concentration of 2 mg/ml, and the suspension was incubated at 30 °C for 30 min to digest the cell wall. The cells were lysed by three passages through a Basic Z cell disruptor (Constant Systems, Northants, UK) at 20,000 p.s.i. Subsequently, DNase (10 μg/ml), RNase (2 μg/ml), 10 mM $MgSO_4$, and 15 mM K-EDTA (pH 7.0) were added, and the suspension was further incubated for 30 min at 30 °C. Unbroken cells and debris were removed by centrifugation at 13,000 × *g* for 15 min at 4 °C. Inside-out membrane vesicles were collected by centrifugation at 125,000 × *g* for 40 min at 4 °C and resuspended in either 50 mM KPi (pH 7.0) or 50 mM Tris (pH 7.4) supplemented with 10% glycerol. The membrane vesicles were stored in 100-μl aliquots in liquid nitrogen.

Immunoblotting

Inside-out membrane vesicles were subjected to 10% SDS-PAGE. The proteins were electroblotted to Hybond-P membrane (Amersham Biosciences) and were detected in the presence of a 1:1000 dilution of the monoclonal anti-BCRP antibody BXP-21 in accordance with the manufacturer's recommendations (Signet). Detection of the primary antibody was performed using the ECL system (Amersham Biosciences) as suggested by the manufacturer.

Cell Cytotoxicity Assays

L. lactis NZ9000 cells containing pNZ8048 or pNZ-BCRP were grown at 30 °C to an A_{660} of about 0.3 in a 96-well plate. Subsequently, protein expression was induced by the addition of nisin A as described under "Preparation of Inside-out Membrane Vesicles." Cytotoxic drugs were added to the cell suspensions at a concentration ranging from 0 to 200 μM. The A_{660} of the cultures were measured every 10 min for 6 h in a VersaMax plate reader (Molecular Devices, CA). The relative growth rates were determined, and the IC_{50} values were calculated. The data were obtained in duplicate from three independent experiments.

Transport Assays

Ethidium Bromide Transport—L. lactis NZ9000 cells containing pNZ8048 or pNZ-BCRP were grown at 30 °C to an A_{660} of about 0.3. Subsequently, protein expression was induced in the presence of nisin A as described under "Preparation of Inside-out Membrane Vesicles." The cells were washed three times in 50 mM KPi (pH 7.0) containing 5 mM $MgSO_4$. To deprive cells of metabolic energy, the cell suspensions were incubated for 30 min at 30 °C in the presence of 0.5 mM dinitrophenol and washed three times in 50 mM KPi (pH 7.0) containing 5 mM $MgSO_4$. The cell pellet was resuspended to an A_{660} of 0.5 in 2 ml of the KPi buffer and incubated for 5 min at 30 °C in the presence of 25 mM glucose with or without the inhibitor FTC (0.5 μM). Ethidium bromide was added to a final concentration as indicated in the legend to Fig. 2, and its fluorescence was followed at 30 °C in a Perkin-Elmer LS 55B fluorimeter using excitation and emission wavelengths of 500 and 580 nm, respectively, and slit widths of 2.5 nm each.

Hoechst 33342 Transport—Inside-out membrane vesicles (1 mg of total membrane protein) were diluted in 2 ml of 50 mM KPi (pH 7.4) supplemented with 2 mM $MgSO_4$, 0.1 mg/ml creatine kinase, and 5 mM phosphocreatine. For experiments with the inhibitor, 0.5 μM FTC was added to the buffer. After ~1 min of incubation at 30 °C, 1 μM Hoechst 33342 (Molecular Probes, Leiden, The Netherlands) was added, and the binding of the dye to the membrane vesicles was followed by fluorimetry (excitation and emission wavelength of 355 and 457 nm, respectively, and slit widths of 2.5 nm) until a steady state was reached. Subsequently, ATP (or AMP-PNP in the control experiments) was added to a final concentration of 5 mM, and the fluorescence intensity was followed until a new steady state was reached. For the substrate competition studies, estradiol was added to the cuvette at concentrations as indicated in Fig. 6*A* prior to the addition of Hoechst 33342.

ATPase Assays

The ATPase assay in inside-out membrane vesicles of *L. lactis* was based on a colorimetric ascorbic acid/ammonium molybdate assay to measure the liberation of P_i from ATP. Inside-out membrane vesicles prepared from BCRP-expressing and control *L. lactis* cells were diluted to a protein concentration of ~1 mg/ml in a buffer containing 20 mM K-HEPES (pH 7.0), 5 mM $MgSO_4$, and 5 mM ATP. ATPase assays were performed at 30 °C in a 96-well plate in a reaction volume of 10 μl/well. Lipids and water-insoluble drugs were added as solutions in ethanol to a final solvent concentration below 1% (v/v). Following incubation for 10 min, the ATPase reactions were terminated by the addition of 40 μl of a freshly prepared acidic solution consisting of 0.48% (w/w) ammonium heptamolybdate tetrahydrate, 6.6% (v/v) concentrated sulfuric acid, 0.01% (w/w) potassium antimonyl tartrate, and 0.42% (w/w) ascorbic acid. Following the addition of 150 μl of H_2O and 30 min of incubation at 30 °C in the dark, the absorbance of the phosphomolybdate complex formed was measured at 690 nm. ATPase activity measurements in the presence of 1 mM orthovanadate were obtained in parallel and subtracted from the readings.

[^{3}H]Estradiol Uptake in Whole Cells

BCRP-expressing and control *L. lactis* cells were generated as described under "Preparation of Inside-out Membrane Vesicles," washed three times in 50 mM KPi (pH 7.0) containing 5 mM $MgSO_4$, resuspended in KPi buffer supplemented with 1 mg bovine serum albumin/ml to an A_{660} of 0.5, and kept on ice until use. Cell suspensions (100-μl aliquots) were preincubated at 30 °C for 5 min in the presence of [2,4,6,7-^{3}H]estradiol (87 Ci/mmol) (Amersham Biosciences) at a final concentration of 500 nM. Metabolic energy for active transport of estradiol was then generated in the cells through the addition of 25 mM glucose. Following the incubation at the times indicated in Fig. 6*B*, the cell suspensions were mixed with 3 ml of ice-cold 20 mM Tris-HCl (pH 7.4) containing 5 mM $MgSO_4$ and rapidly filtered over Whatman GF/G glass fiber filters that were pre-equilibrated overnight at 20 °C in Tris buffer. The filters were washed twice with ice-cold Tris buffer, and radioactivity retained on the filters was measured by liquid scintillation counting. All of the data were corrected by subtracting nonspecific binding of [^{3}H]estradiol to the filters, which was usually less than 5–10% of the total radioactivity.

RESULTS

Expression of Human BCRP in L. lactis—For expression of human BCRP in *L. lactis* NZ9000, the human *BCRP* gene present in the mammalian expression vector pcDNA3-BCRP was cloned into the lactococcal expression vector pNZ8048 under the control of the nisin A-inducible *nisA* promoter. This expression system had previously been used for the expression in *L. lactis* of LmrA, the lactococcal homologue of the human multidrug resistance P-glycoprotein MDR1 (24). The addition of 40 pg/ml nisin A to exponentially growing *L. lactis* cells harboring pNZ-BCRP resulted in the expression of a 70-kDa polypeptide that could be detected on an immunoblot by using the specific anti-BCRP antibody BXP-21 (Fig. 1). The BXP-21 antibody also showed cross-reactivity with certain constitutively expressed, endogenous membrane proteins in *L. lactis*. The 70-kDa polypeptide was expressed at a level between 0.5

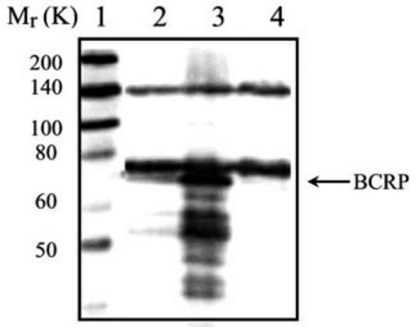

FIG. 1. **Immunodetection of BCRP in the cytoplasmic membrane of *L. lactis*.** Total membrane proteins in inside-out membrane vesicles (50 μg of protein/lane) were separated by 10% SDS-PAGE and analyzed on Western blot using the anti-BCRP BXP-21 antibody. *Lane 1*, biotinylated standard marker; *lane 2*, inside-out membrane vesicles prepared from cells harboring pNZ-BCRP in the absence of nisin A; *lane 3*, inside-out membrane vesicles prepared from nisin A-induced cells harboring pNZ-BCRP; *lane 4*, inside-out membrane vesicle of cells harboring pNZ8048 in the presence of nisin A. The migration of molecular mass markers is indicated.

and 1% of total membrane protein, as determined by densitometric analysis of a Coomassie Brilliant Blue-stained SDS-PAGE gel. In the absence of nisin A, an approximately 10-fold lower expression level of the 70-kDa polypeptide was observed in *L. lactis* cells containing pNZ-BCRP. The 70-kDa polypeptide was undetectable in control cells harboring the pNZ8048 control vector when incubated in the presence of nisin A.

Heterologously Expressed BCRP Is Active as a Multidrug Transporter—The drug resistance of *L. lactis* cells harboring pNZ-BCRP was compared with that of cells harboring the pNZ8048 control vector. The growth rate of the two strains in liquid culture containing 40 pg/ml nisin A was determined at increasing concentrations of ethidium bromide, Hoechst 33342, rhodamine 123, or tetramethylrosamine. The concentrations of drugs necessary to reduce the growth rate of cells by 50% (IC_{50}) are listed in Table I. BCRP expression in *L. lactis* significantly increased the drug resistance of the organism.

To test whether drug extrusion from the cell is the underlying mechanism of drug resistance in *L. lactis* expressing BCRP, ethidium bromide uptake in cells was measured by monitoring the fluorescence of the intracellular ethidium-polynucleotide complex. In the presence of glucose, the uptake of ethidium bromide in cells expressing BCRP was significantly lower than that observed in control cells without BCRP (Fig. 2). This difference in ethidium accumulation between the two cell types reflected a higher ethidium efflux rate in BCRP-expressing cells. Upon the addition of the BCRP-specific inhibitor FTC, the ethidium accumulation in cells expressing BCRP was similar to that observed in control cells in the presence or absence of FTC, pointing to the inhibition of BCRP activity under these conditions.

The fluorescent lipophilic dye Hoechst 33342 is transported by BCRP expressed in mammalian cells (19, 25). To further analyze the activity of BCRP in *L. lactis*, the transport of Hoechst 33342 was studied in *L. lactis*-derived inside-out membrane vesicles in which the nucleotide-binding domain of BCRP was exposed on the outside surface of the membrane. The addition of Hoechst 33342 to the inside-out membrane vesicles resulted in a rapid increase in fluorescence up to a steady state level because of the partitioning of the dye in the hydrophobic environment of the phospholipid bilayer (Fig. 3). The subsequent addition of MgATP resulted in a rapid quenching of the Hoechst 33342 fluorescence in membrane vesicles containing BCRP (Fig. 3*A*) but not in control membrane vesicles without BCRP (Fig 3*B*). FTC strongly inhibited the ATP-dependent

TABLE I
Drug survival characteristics of BCRP-expressing L. lactis cells, compared with cells not expressing BCRP (control)

The IC_{50} values refer to the drug concentration required for half-maximal inhibition of the growth rate.

Drug	IC_{50}[a]	
	BCRP	Control
Ethidium bromide	40 ± 0 μg/ml	8 ± 0 μg/ml
Hoechst 33342	>200 μM	40 ± 0 μM
Rhodamine 123	>200 μM	131 ± 6.6 μM
Tetramethylrosamine	14.4 ± 0.9 μM	8.8 ± 0.8 μM

[a] The values represent the means of three replicate determinations ± S.E.

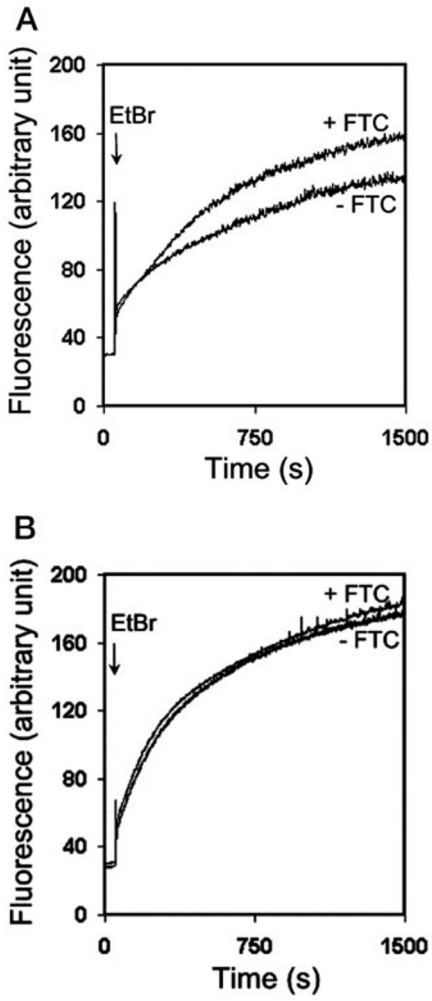

FIG. 2. **Ethidium bromide transport in *L. lactis* cells.** The cells were washed and diluted to an A_{660} of 0.5 in 50 mM KPi (pH 7.0) containing 5 mM $MgSO_4$. Glucose was added to a concentration of 25 mM glucose, and the cells were then preincubated for 5 min in the presence or absence of 0.5 μM FTC. Ethidium bromide uptake in the cells was measured by fluorimetry. Approximately 1 min after the fluorescence measurements were initiated, ethidium bromide was added to a final concentration of 2 μM (indicated by the *arrow*). *A*, BCRP-expressing cells; *B*, control cells without BCRP.

quenching of Hoechst 33342 fluorescence in membrane vesicles containing BCRP. In contrast, no significant changes in the steady state level of Hoechst 33342 fluorescence were observed in BCRP-containing and control membrane vesicles in the presence of AMP-PNP alone or AMP-PNP plus FTC (Fig. 3). These observations point to the BCRP-dependent transport of Hoechst 33342 from the phospholipid bilayer into the aqueous lumen of the membrane vesicles.

Functional studies in membrane vesicles of *L. lactis* also

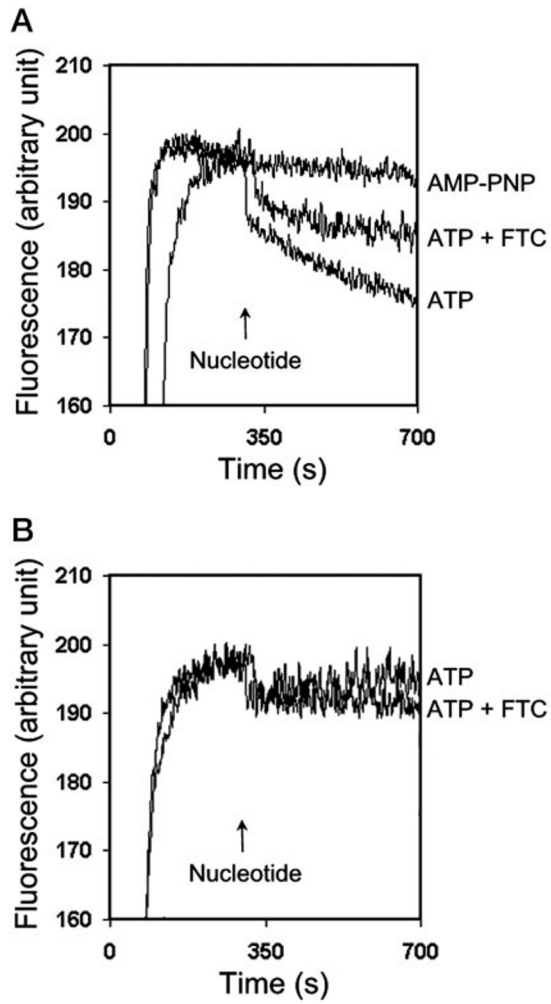

FIG. 3. **Hoechst 33342 transport in membrane vesicles of *L. lactis*.** Inside-out membrane vesicles prepared from BCRP-expressing cells (*A*) and control cells (*B*) were diluted in 50 mM KPi (pH 7.4) containing 2 mM $MgSO_4$ and an ATP regenerating system to a final concentration of 0.5 mg/ml of total membrane proteins. Upon the addition of 1 μM Hoechst 33342, the increase in fluorescence of the dye was followed in time until a steady state was reached. Hoechst 33342 transport was initiated by the addition of 5 mM Mg-ATP or Mg-AMP-PNP (indicated by the *arrow*). FTC (0.5 μM) was used as a specific inhibitor of BCRP activity and was added at the onset of the experiment. The *traces* obtained for BCRP-containing and control membrane vesicles in the presence of AMP-PNP plus FTC were indistinguishable from the one depicted in *A* for BCRP-containing membrane vesicles in the presence of AMP-PNP only.

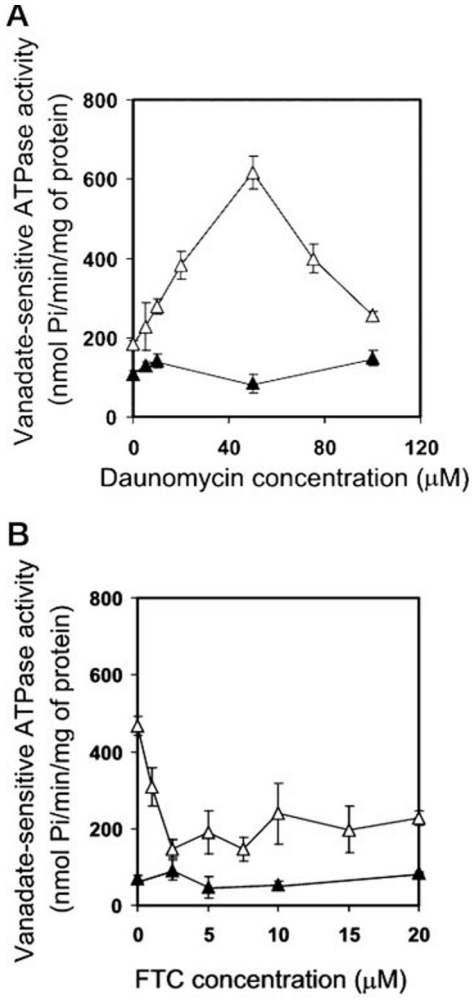

FIG. 4. **Effect of daunomycin and FTC on the BCRP-associated ATPase activity in inside-out membrane vesicles of *L. lactis*.** *A*, vanadate-sensitive ATPase activity of inside-out membrane vesicles containing BCRP (△) and control membrane vesicles (▲) in the presence of increasing concentrations of daunomycin. *B*, effect of increasing concentrations of the BCRP inhibitor FTC on the daunomycin-stimulated vanadate-sensitive ATPase activity of BCRP-containing membrane vesicles (△) and control membrane vesicles (▲) was measured at a daunomycin concentration of 50 μM. The data points represent the means ± S.E. of three independent experiments using different membrane vesicle batches.

aimed at the ATPase activity of BCRP. In contrast to control inside-out membrane vesicles, BCRP-containing inside-out membrane vesicles displayed a significant amount of ortho-vanadate-sensitive ATPase activity that was stimulated up to 5-fold in the presence of daunomycin (Fig. 4*A*), a substrate of BCRP (2). The concentration of daunomycin required for half-maximal stimulation (SC_{50}) of the vanadate-sensitive ATPase activity was about 20 μM. FTC significantly reduced the daunomycin-stimulated vanadate-sensitive ATPase activity, with an IC_{50} concentration below 2.5 μM (Fig. 4*B*). These data strongly suggest that the drug-stimulated vanadate-sensitive ATPase activity is associated with BCRP activity.

Taken together, the observations of (i) multidrug resistance and reduced ethidium bromide accumulation in cells expressing BCRP, (ii) the ATP-dependent Hoechst 33342 transport and the drug-stimulated vanadate-sensitive ATPase activity in inside-out membrane vesicles containing BCRP, and (iii) the inhibition of these drug transport and ATPase activities by the BCRP inhibitor FTC demonstrate the functional expression of human BCRP in *L. lactis*.

Interaction of BCRP with Sterols—Although BCRP (ABCG2) was originally identified as a multidrug transporter, other members in the ABCG subfamily (*e.g.* ABCG1 and ABCG5/G8) have been implicated in the transport of sterols. To explore the interaction of BCRP with sterols, the effect of sterols on the BCRP-associated ATPase activity was examined. Surprisingly, the sterols estradiol and cholesterol both stimulated the BCRP-associated ATPase activity about 4-fold, with SC_{50} values of about 10 and 8 μM, respectively (Fig. 5*A*). In addition, the BCRP-associated ATPase activity was stimulated 4-fold by the natural steroid progesterone and 7-fold by testosterone at SC_{50} values of 5 and 15 μM, respectively, (Fig. 5*B*). Finally, the estrogen receptor modulator tamoxifen (26) stimulated the BCRP-associated ATPase activity almost 3-fold with an SC_{50} of about 50 μM (Fig. 5*C*). None of these sterols significantly affected the low level of vanadate-sensitive ATPase activity observed in control membrane vesicles lacking BCRP (Fig. 5).

The ability of BCRP expressed in *L. lactis* to interact with estradiol was further analyzed in Hoechst 33342 transport

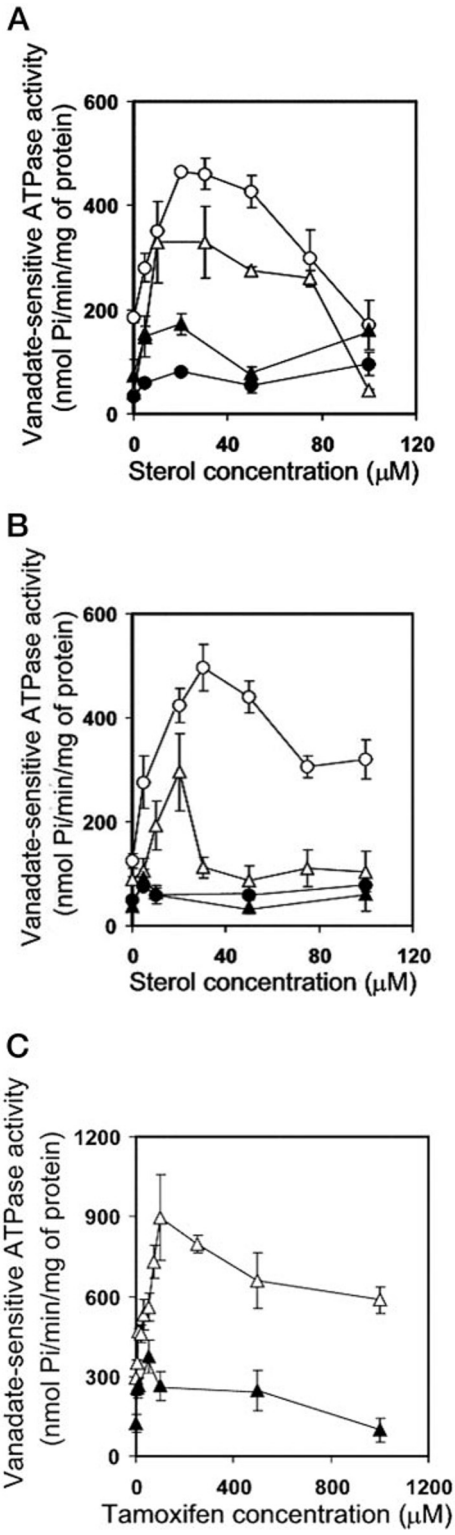

FIG. 5. **Effect of sterols on the BCRP-associated ATPase activity in inside-out membrane vesicles of *L. lactis*.** Vanadate-sensitive ATPase activity of inside-out membrane vesicles containing BCRP (*open symbols*) and control membrane vesicles (*closed symbols*) was determined as described in the legend to Fig. 4 in the presence of increasing concentrations of estradiol (*circles*) or cholesterol (*triangles*) (*A*), testosterone (*circles*) or progesterone (*triangles*) (*B*), or tamoxifen (*C*).

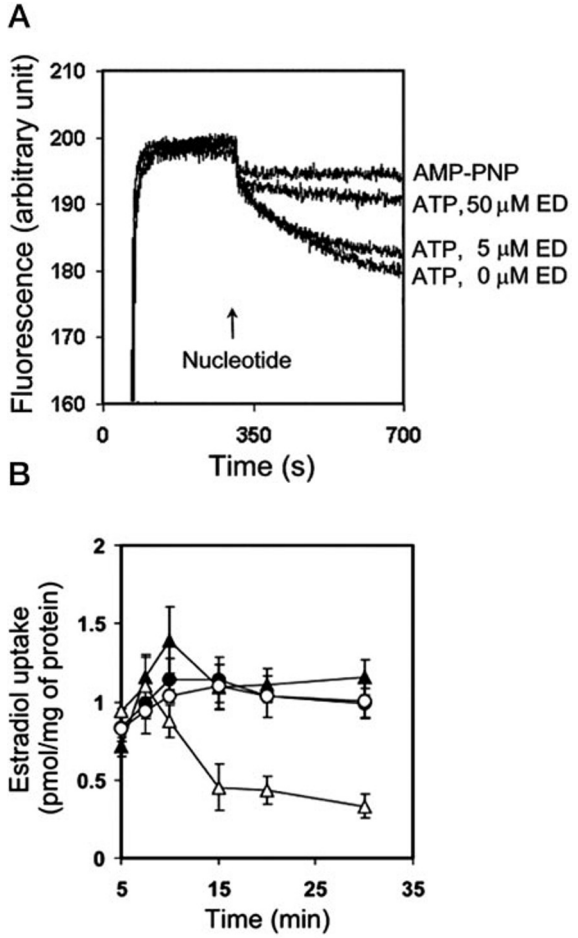

FIG. 6. **Transport of estradiol by BCRP in *L. lactis*.** *A*, effect of estradiol (*ED*) on the BCRP-mediated Hoechst 33342 transport in inside-out membrane vesicles. The ATP-dependent transport of Hoechst 33342 in BCRP-containing inside-out membrane vesicles was measured as described in the legend to Fig. 3. Estradiol was added to the assays at 0, 5, or 50 μM concentrations prior to the addition of Hoechst 33342. *B*, transport of [^{3}H]estradiol in BCRP-expressing *L. lactis* cells (*triangles*) or LmrA-expressing cells (*circles*). The cells were preincubated at 30 °C for 5 min in 50 mM KPi buffer (pH 7.0) containing $MgSO_4$, 1 mg/ml bovine serum albumin, and 500 nM [^{3}H]estradiol. After 5 min, the cells received 25 mM glucose to generate metabolic energy for active transport (*open symbols*) or no addition (*closed symbols*). The cell-associated radioactivity was determined in a rapid filtration assay. The data points represent the means ± S.E. of four measurements.

assays in which estradiol was included as a competing substrate. As shown in Fig. 6*A*, the presence of estradiol significantly inhibited the BCRP-mediated transport of Hoechst 33342 in inside-out membrane vesicles. The degree of inhibition by estradiol was proportional to the concentration of estradiol used, suggesting that estradiol is a potential transport substrate for BCRP. Estradiol did not affect the fluorescence of Hoechst 33342 in control membrane vesicles without BCRP (data not shown). The ability of BCRP to transport estradiol was directly assessed by measuring the uptake of [^{3}H]estradiol in *L. lactis* cells. In the presence of glucose, BCRP-expressing cells exhibited a 4-fold lower uptake of [^{3}H]estradiol than the control cells or BCRP-expressing cells in the absence of glucose (Fig. 6*B*). These results demonstrate the BCRP-mediated transport of estradiol in *L. lactis*. Interestingly, the amount of cell-associated estradiol was not reduced in glucose-energized *L. lactis* cells expressing LmrA, suggesting the lack of a significant LmrA-mediated transport of estradiol under the experimental conditions (Fig. 6*B*).

DISCUSSION

ABC transporters are important contributors to cellular lipid transport and homeostasis, and their dysfunction is often associated with human disease phenotypes. For example, P-gly-

coprotein MDR3 (ABCB4) mediates the transport of phosphatidylcholine across the canalicular membrane during bile formation, and mutations in the *MDR3* gene are a cause of progressive familial intrahepatic cholestasis (27, 28). ABCR (ABCA4) probably transports a complex of retinaldehyde and phosphatidylethanolamine in the retina of the eye, and malfunctioning of this transporter results in Stargardt's macular dystrophy (29). ABCA1 is crucial for the elimination of excess body cholesterol, and mutations in the *ABCA1* gene have been causatively linked to familial high density lipoprotein deficiency and Tangier disease (30). The multidrug resistance P-glycoprotein MDR1 (ABCB1) transports a wide variety of hydrophobic compounds and has been shown to catalyze the transbilayer movement of phospholipid analogues (31), sphingomyelin (32), cholesterol (33), and progesterone (34) but not sitosterol (35). Although members of the ABCG subfamily have been implicated in sterol transport, BCRP (ABCG2) has been characterized as an excretion system for multiple drugs and cellular toxins (2–7, 20). However, in view of its broad substrate specificity and its presence in tissues producing steroid hormones (18, 19), BCRP may also be involved in local lipid transport processes that may have remained undetected in BCRP knock-out mice (20).

To study sterol transport by human BCRP, we have expressed the protein in *L. lactis*, a bacterium that is devoid of mammalian sterols but that synthesizes hopanoids to regulate membrane fluidity (36). This property gives *L. lactis* an advantage over insect cells and mammalian cells where the sterol content of the plasma membranes can vary between 5 and 25%, which may hinder direct measurements of sterol transport and sterol-stimulated ATPase activities in the experimental setting. In addition, the expression of BCRP in *L. lactis* allows a comparison of its substrate specificity with that of the ABC half-transporter LmrA, a lactococcal homologue of P-glycoprotein MDR1. Three lines of experimental evidence suggested that BCRP was functionally expressed in *L. lactis*. Firstly, BCRP expression conferred multidrug resistance on cells. Secondly, BCRP expression enhanced the efflux of ethidium in cells and the transport of Hoechst 33342 in inside-out membrane vesicles. Both activities were inhibited in the presence of the BCRP-specific modulator FTC. Finally, BCRP-containing inside-out membrane vesicles displayed a vanadate and FTC-sensitive ATPase activity, which was stimulated by drugs (such as daunomycin) that are transported by BCRP. The observation of a BCRP-associated ATPase activity in inside-out membrane vesicles of *L. lactis* is consistent with published studies in which a drug-stimulated vanadate-sensitive ATPase activity in isolated membranes of insect cells (37) and mammalian cells (38) was shown to be due to the presence of BCRP.

The BCRP-associated ATPase activity in inside-out membrane vesicles of *L. lactis* was also significantly stimulated in the presence of sterols, including estradiol, cholesterol, progesterone, testosterone, and tamoxifen. It has been shown for P-glycoprotein MDR1 that the lipid environment can significantly influence the characteristics of purified and functionally reconstituted protein (39). Hence, the stimulation of the BCRP-associated ATPase activity in inside-out membrane vesicles of *L. lactis* by mammalian sterols could reflect a requirement of BCRP for the presence of these sterols in its lipid environment. However, the observations of (i) the efflux of [^{3}H]estradiol in BCRP-expressing *L. lactis* cells but not LmrA-expressing cells (Fig. 6*B*) and (ii) the inhibition of BCRP-mediated Hoechst 33342 transport by estradiol (Fig. 6*A*) at concentrations that stimulate the BCRP-associated ATPase activity (Fig. 5*A*) imply competition between sterols and drugs for binding to common binding sites in BCRP. These data would also argue against indirect mechanisms of coupling between the estradiol-induced stimulation of BCRP-associated ATPase activity and [^{3}H]estradiol translocation, in which (i) [^{3}H]estradiol transport would represent a secondary flux associated with the active translocation of an endogenous compound (*e.g.* lipid) by BCRP and (ii) simultaneously, estradiol would interact with an allosteric binding site, rather than a transport site, to enhance the BCRP-associated ATPase activity.

Interestingly, as the concentration of daunomycin or sterols increased beyond that which stimulated the BCRP-associated ATPase maximally, the ATPase activity then decreased (Figs. 4 and 5) similar to observations for the P-glycoprotein MDR1 ATPase (34, 39). The biphasic pattern of stimulation and inhibition of the drug/sterol stimulated BCRP-associated ATPase activity may depend on the saturation state of BCRP transport sites, with enhanced binding of drug/sterol to transport sites at the inside surface of the membrane at low substrate concentrations and reduced dissociation of these substrates from release sites at the outside surface of the membrane at high substrate concentrations. Alternatively, the inhibition of the BCRP-associated ATPase activity at high drug/sterol concentrations may reflect changes in the lipid environment, *i.e.* in lateral pressure, that are less optimal for BCRP activity.

Altogether the data presented in this paper suggest that human BCRP is able to interact with sterols and that BCRP may play a role in the transport of sterols, steroids, and estrogen receptor antagonists used in the treatment of breast tumors, in addition to its ability to transport chemotherapeutic drugs and cellular toxins.

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