Arginine-482 is not essential for transport of antibiotics, primary bile acids and unconjugated sterols by the human breast cancer resistance protein (ABCG2)

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The human BCRP (breast cancer resistance protein, also known as ABCG2) is an ABC (ATP-binding cassette) transporter that extrudes various anticancer drugs from cells, causing multidrug resistance. To study the molecular determinants of drug specificity of BCRP in more detail, we have expressed wild-type BCRP (BCRP-R) and the drug-selected cancer cell line-associated R482G (Arg⁴⁸² → Gly) mutant BCRP (BCRP-G) in *Lactococcus lactis*. Drug resistance and the rate of drug efflux in BCRP-expressing cells were proportional to the expression level of the protein and affected by the R482G mutation, pointing to a direct role of BCRP in drug transport in *L. lactis*. In agreement with observations in mammalian cells, the BCRP-R-mediated transport of the cationic substrates rhodamine 123 and tetramethylrosamine was significantly decreased compared with the activity of BCRP-G. In addition, BCRP-R showed an enhanced interaction with the anionic anticancer drug methotrexate when compared with BCRP-G, suggesting that structure/substrate specificity relationships in BCRP, as observed in eukaryotic expression

systems, are maintained in prokaryotic *L. lactis*. Interestingly, BCRP-R exhibited a previously unestablished ability to transport antibiotics, unconjugated sterols and primary bile acids in *L. lactis*, for which the R482G mutation was not critical. Since Arg⁴⁸² is predicted to be present in the intracellular domain of BCRP, close to transmembrane segment 3, our results point to a role of this residue in electrostatic interactions with charged substrates including rhodamine 123 and methotrexate. Since unconjugated sterols are neutral molecules and bile acids and many antibiotics are engaged in protonation/deprotonation equilibria at physiological pH, our observations may point either to a lack of interaction between Arg⁴⁸² and neutral or neutralized moieties in these substrates during transport or to the interaction of these substrates with regions in BCRP not including Arg⁴⁸².

Key words: ATP-binding cassette transporter (ABC transporter), ABCG2, antibiotics, breast cancer resistance protein (BCRP), *Lactococcus lactis*, primary bile acid, unconjugated sterol.

INTRODUCTION

BCRP [breast cancer resistance protein, also termed placenta-specific ABC (ATP-binding cassette) protein, MXR and ABCG2] is a more recently discovered multidrug ABC transporter, which was initially isolated from multidrug-resistant breast cancer cells [1]. Studies on BCRP in non-mammalian cells such as insect cells [2], oocytes [3] and the Gram-positive bacterium *Lactococcus lactis* [4] suggested that the protein is transport-active independent of auxiliary proteins. BCRP is a half-transporter, consisting of an N-terminal nucleotide-binding domain followed by a membrane domain containing six putative transmembrane segments, which is supposed to function as a homodimer or higher oligomer [5]. BCRP belongs to the ABCG subfamily, containing among others, (i) the *Drosophila* white, brown and scarlet proteins, which transport different eye pigment precursors [6], (ii) ABCG1, which is supposed to be a regulator of macrophage cholesterol and phospholipid transport [7] and (iii) the heterodimeric ABCG5/ABCG8 proteins, which have been demonstrated to play a role in the canalicular transport of biliary cholesterol and intestinal secretion of dietary plant sterols [8].

Consistent with the notion that BCRP may play a role in the disposition and pharmacological activity of a broad range of compounds, studies on the tissue distribution of BCRP revealed that the protein is expressed in the apical membrane of cells in tissues with excretory functions, such as the canalicular mem-

brane of hepatocytes, luminal membrane of villous epithelial cells in the small and large intestines, apical membranes of capillary vessels in the blood–brain barrier, ducts and lobules of the breast, and apical pole of the trophoblast cells in the placenta [9]. In addition, studies on BCRP-knockout mice indicated that BCRP plays a role in the transport of dietary toxins such as pheophorbide A and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine, serving to limit the distribution of these toxins and facilitate their elimination [10–12]. BCRP also interacts with plant polyphenols [13,14].

From the earliest studies [2,3,15,16] on BCRP, it has become apparent that some drug-selected mammalian cell lines expressing BCRP display resistance to cationic rhodamine 123 and anthracyclines due to the presence of a neutral G, T, S or M residue at position 482 of BCRP, whereas cell lines expressing wild-type BCRP (with an R residue at position 482) remain sensitive to these substrates. In addition, the mutant BCRP proteins mediate the efflux of anionic methotrexate at significantly decreased rates compared with wild-type BCRP (BCRP-R) [17,18]. Although studies on the substrate specificity of position 482 mutant BCRP proteins may not have a direct physiological relevance, these studies do provide useful insights into the molecular basis of the drug selectivity of the wild-type transporter.

In a previous work [4], the R482G mutant BCRP (BCRP-G) was functionally expressed in *L. lactis* and evidence for the interaction of BCRP-G with sterols was obtained. In the present study,

Abbreviations used: ABC, ATP-binding cassette; BCRP, breast cancer resistance protein; BCRP-G, mutant BCRP with a glycine residue at position 482; BCRP-R, wild-type BCRP with an arginine residue at position 482; MDR, multidrug resistance; SC₅₀, the drug concentration required for half-maximal stimulation; TMR, tetramethylrosamine.

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Table 1 BCRP-G-associated drug resistance and drug-stimulated ATPase activity

	IC ₅₀ (μM)			ATPase activity	
Drug	BCRP-G	Control	Relative resistance*	Maximal stimulation (-fold)	SC ₅₀ (μM)
Antibiotics					
Erythromycin	8.2 ± 1.2	0.1 ± 0.0	82	2.1	1.8 ± 0.0
Tetracycline	18.9 ± 0.2	8.7 ± 0.6	2.2	3.5	1.5 ± 0.9
Dirithromycin	2.8 ± 0.1	0.1 ± 0.0	28	3.1	1.9 ± 0.9
Quinacrine	150 ± 6	62 ± 0	2.4	5.1	1.7 ± 0.5
Ofloxacin	236 ± 4	94 ± 12	2.5	n.d.†	n.d.
Cephalothin	11.1 ± 1.2	4.2 ± 0.2	2.6	2.7	2.9 ± 0.5
Rifampicin	71 ± 8	17 ± 3	4.2	2.3	15 ± 2
Bile acids					
Cholate	4450 ± 210	3090 ± 30	1.4	2.6	3.4 ± 0.3
Deoxycholate	310 ± 5	160 ± 10	1.9	2.1	14 ± 1
Taurocholate	2430 ± 160	1890 ± 10	1.3	1.8	2.3 ± 0.4
Taurodeoxycholate	> 5000	> 5000	—	4.7	4.7 ± 3.0
Glycochenodeoxycholate	3840 ± 108	1150 ± 80	3.3	4.5	4.2 ± 2.4

* Relative resistance was calculated by dividing the IC₅₀ for lactococcal cells expressing BCRP-G by the IC₅₀ obtained for non-expressing control cells.

† n.d., not determined.

investigations on sterol transport were expanded to BCRP-R in *L. lactis*. In addition, the specificities of BCRP-G and -R for antibiotics and primary bile acids were compared in detail to analyse the role of Arg⁴⁸² in the BCRP-mediated transport of these substrates.

MATERIALS AND METHODS

Materials

M17 broth was obtained from Difco (Sparks, MD, U.S.A.). [¹⁴C]-cholic acid (48.6 mCi/mmol) and [2,4,6,7-³H]oestradiol (87 Ci/mmol) were purchased from Amersham Biosciences (Little Chalfont, Bucks., U.K.). *N,N*-dimethyldodecylamine-*N*-oxide was obtained from Fluka Chemika (Gillingham, Dorset, U.K.). All other chemicals and reagents were of analytical grade and obtained from Sigma (Dorset, U.K.).

Genetic manipulations

The *BCRP R482G* (*BCRP-G*) cDNA was subcloned as an *Nco*I–*Xba*I fragment from the lactococcal expression vector pNZ-BCRP-G [4] into the *Escherichia coli* plasmid pGEM, yielding pGEM-BCRP-G. Subsequently, the wild-type *BCRP R482* (*BCRP-R*) gene was created using pGEM-BCRP-G and the Quik Change Site-directed Mutagenesis kit (Stratagene) with the forward and reverse primers 5'-TTACCCATGAGGATGTTACC-AAGTATTATATT-3' and 5'-TTGGTAACATCCTCATGGGTAA-TAAATCAGAT-3' respectively. The *BCRP-R* gene was then cloned back into pNZ8048, giving pNZ-BCRP-R. The intended changes in *BCRP-R* were confirmed by DNA sequencing.

Bacterial strains and growth conditions

L. lactis NZ9000 cells harbouring pNZ8048, pNZ-BCRP-R or pNZ-BCRP-G were grown at 30 °C in M17 medium supplemented with 0.5 % (w/v) glucose and 5 μg/ml chloramphenicol [4].

Preparation of inside-out membrane vesicles

L. lactis cells were grown at 30 °C to an absorbance *A*₆₆₀ of approx. 0.3. Unless stated otherwise, BCRP expression was induced under standard conditions by the addition of 0.1 % (v/v) of the supernatant of the nisin A-producing *L. lactis* strain NZ9700 [31],

Table 2 BCRP-R-associated drug resistance

Drug	IC ₅₀ (μM)		Relative resistance*
	BCRP-R	Control	
Antibiotics			
Erythromycin	7.9 ± 0.8	0.1 ± 0.0	79
Tetracycline	17.0 ± 1.2	8.7 ± 0.6	2.0
Rifampicin	76 ± 61	7 ± 3	4.5
Bile acids			
Cholate	4610 ± 170	3090 ± 30	1.5
Deoxycholate	331 ± 12	160 ± 10	2.1

* Relative resistance was calculated by dividing the IC₅₀ for lactococcal cells expressing BCRP-R by the IC₅₀ obtained for non-expressing control cells.

giving a final concentration of approx. 10 pg/ml nisin A. After incubation for 2 h at 30 °C, cells were harvested by centrifugation at 13 000 *g* for 15 min at 4 °C. Inside-out membrane vesicles were prepared using a cell disruptor (Constant Systems, Northants, U.K.) in 50 mM (K)Hepes buffer (pH 7.0) as described previously [4]. Protein concentrations were estimated by the Bio-Rad protein assay (Bio-Rad Laboratories, Hemel Hempstead, Herts., U.K.), using BSA as a standard.

Cytotoxicity assays

Protein expression in *L. lactis* cells, grown in the wells of a 96-well plate, was induced by the addition of nisin A as described in the previous subsection. For the determination of viable cell counts, cells were incubated at 30 °C for 3 h with various concentrations of antibiotics as indicated in Figure 2(A). The bacterial cultures were then serially diluted up to 10⁹-fold and plated on M17 medium containing 2 % agar. Colonies were counted and the survival ratios were expressed as a percentage of the colony-forming units observed at a given concentration of antibiotics over that observed without added antibiotics. For determining the inhibitory effect of drugs on the growth rate of cells, potential BCRP substrates were added to the cell suspensions at concentrations as indicated in Figure 2(B) and Tables 1 and 2. Water-insoluble compounds were added as solutions in ethanol or methanol to a final solvent concentration below 1 % (v/v). The cell densities were monitored by measuring the *A*₆₆₀ every 10 min for 6–10 h. The maximum

specific growth rate μ_m for each culture was estimated from the growth curve by fitting the data to the equation $N_t = N_0 \exp(\mu_m t)$, where N_t and N_0 are the cell densities at time t and 0 h respectively. Subsequently, the drug concentration that inhibited the maximum specific growth rate by 50 % (IC_{50}) was calculated.

ATPase assays

ATPase activities were measured as described previously [4] by determining the liberation of inorganic phosphate from ATP with a colorimetric ascorbic acid/ammonium molybdate assay. Briefly, inside-out membrane vesicles were diluted to a protein concentration of approx. 1 mg/ml in a buffer containing 20 mM K-Hepes (pH 7.4), 5 mM $MgSO_4$ and 5 mM ATP. ATPase assays were performed at 30 °C in a 96-well plate with different concentrations of drugs, as indicated in Figure 2(C) and Tables 1 and 2. The ATPase reactions were terminated by the addition of 40 μ l of an acidic mixture containing 0.48 % (w/w) ammonium heptamolybdate tetrahydrate, 6.6 % (v/v) concentrated sulphuric acid, 0.01 % (w/w) potassium antimonyltartrate and 0.42 % (w/w) ascorbic acid. After adding 150 μ l of water and incubating for 30 min in the dark, the released phosphate was estimated colorimetrically at 690 nm in a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.). ATPase activity measurements in the presence of 1 mM *o*-vanadate were obtained in parallel and subtracted from the readings.

Transport of fluorescent substrates in intact cells

BCRP-expressing and control *L. lactis* cells were washed three times with 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 [4] and washed three times with 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. For ethidium transport assays, ethidium bromide was added to a final concentration as indicated in the legend to Figure 1(B), and its fluorescence was followed at 30 °C in a PerkinElmer LS 55B fluorimeter using excitation and emission wavelengths of 500 and 580 nm respectively and slit widths of 2.5 nm each. For TMR (tetramethylrosamine) transport assays, TMR was added to a final concentration of 0.2 μ M (Figure 3C) or as indicated in Figure 3(D), and the TMR fluorescence was then measured using excitation and emission wavelengths of 550 and 574 nm respectively and slit widths of 5 nm each.

Transport of radioactive substrates in whole cells

BCRP-expressing and control *L. lactis* cells were treated as described in the previous subsection. For oestradiol and cholate transport experiments, the pellets were then resuspended in KPi buffer supplemented with 1 mg/ml BSA to an A_{660} of 0.5 and kept on ice until use. Aliquots (98 μ l) of cell suspensions were pre-incubated at 30 °C for 5 min in the presence of 2 μ l of 5.8 mM [14 C]cholate (16 mCi/mmol; final concentration at zero time, 116 μ M) or 1 μ l of 50 μ M [2,4,6,7- 3 H]oestradiol (Amersham Biosciences) (8.7 Ci/mmol; final concentration at zero time, 500 nM). For erythromycin transport assays, cells were pre-incubated at 30 °C for 1 h in the presence of 1 μ l of 2 mM [14 C]-erythromycin (3.84 mCi/mmol). Metabolic energy for active transport was then generated in the cells by the addition of 25 mM glucose. After incubation at the times indicated in Figures 2(D), 4(A) and 4(B), the cell suspensions were mixed with 3 ml of ice-cold wash buffer (20 mM Tris/HCl, pH 7.4, containing 5 mM

Figure 1 Expression of BCRP in *L. lactis*

(A) Immunodetection of BCRP-G and -R. Cells harbouring empty pNZ-8048 (control), pNZ-BCRP-R or pNZ-BCRP-G were incubated in the presence of a 1:1000 dilution of 10 ng/ml nisin A stock solution to induce protein expression under standard conditions. Total membrane proteins (50 μ g of protein/lane) in inside-out membrane vesicles prepared from these cells were separated on a 10 % (w/v) SDS/polyacrylamide gel and analysed by Western blotting using the BXP-21 antibody. The migration of molecular-mass standards is indicated. (B) Ethidium transport in cells with a variable expression of BCRP-G. BCRP-G-expressing and control cells were incubated in the presence of a 1:1000 or 1:100,000 dilution of nisin A. Cells were then washed and diluted to an A_{660} of 0.5 in 50 mM KPi (pH 7.0) containing 5 mM $MgSO_4$, and equilibrated in the presence of 2 μ M ethidium bromide for 20 min. Ethidium uptake in the cells was measured by fluorimetry. Approx. 1 min after the fluorescence measurements were initiated, glucose was added to a final concentration of 25 mM (indicated by the arrow). Ethidium transport in control cells lacking the BCRP-G gene was unaffected by incubations with nisin A. The control trace for the 1:1000 dilution is presented. Inset: expression of BCRP-G in *L. lactis* cells at the 1:1000 and 1:100,000 dilution of nisin A. Control cells were incubated in the presence of nisin A at a dilution of 1:1000. Cells were lysed with SDS-loading buffer and sonicated for 2 min. Samples were run on a 10 % SDS/polyacrylamide gel and analysed by immunoblotting.

$MgSO_4$) and rapidly filtered over Whatman GF/G glass fibre filters that were pre-equilibrated overnight at 20 °C in wash buffer. Filters were washed twice with ice-cold Tris buffer, and the radioactivity retained on the filters was measured by liquid-scintillation counting. All data were corrected by subtracting non-specific binding of [14 C]cholate, [3 H]oestradiol or [14 C]erythromycin to the filters, which was usually < 10 % of the total radioactivity.

Hoechst 33342 transport in membrane vesicles

Inside-out membrane vesicles (1 mg of total membrane protein) were diluted in 2 ml of 50 mM KPi (pH 7.4) containing 2 mM $MgSO_4$, 0.1 mg/ml creatine kinase and 5 mM phosphocreatine. After approx. 1 min of incubation at 30 °C, 1 μ M Hoechst 33342 (Molecular Probes, Leiden, The Netherlands) was added and the binding of the dye to the membrane vesicles was monitored by fluorimetry (excitation and emission wavelengths of 355 and 457 nm respectively and a slit width of 2.5 nm each) until a steady state was reached. Subsequently, ATP 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, methotrexate was added to the cuvette at

the concentrations indicated in Figures 3(A) and 3(B) before the addition of Hoechst 33342.

Statistical analyses

Unless indicated otherwise, all statistical analyses were based on four independent observations ($n = 4$) using different batches of cells or membrane vesicles. The statistical analyses were performed using Student's t test with a 95 % confidence interval for the sample mean.

RESULTS

Expression of BCRP-G and -R in lactococcal cells

Our initial BCRP expression studies and measurements of BCRP-mediated substrate transport in *L. lactis* were based on a human BCRP cDNA that was isolated from the mitoxantrone-selected human colon carcinoma cell line S1-M1, in which a guanine residue at position 1648 changed amino acid 482 from R to G (BCRP-G) [19]. To extend our studies on BCRP in *L. lactis* to the wild-type protein, G at position 482 was substituted in this work by R, yielding (wild-type) BCRP-R. As shown in Figure 1(A), BCRP-R and -G were expressed at equal levels in *L. lactis* using the nisin A-dependent expression system that was previously used for the expression of LmrA in this organism [20]. In control experiments, no signal was detected in cells harbouring the empty vector pNZ8048 in the presence of nisin A. BCRP-R and -G were expressed at a level between 0.5 and 1 % of total membrane protein, as determined by densitometric analysis of a Coomassie Brilliant Blue-stained SDS/polyacrylamide gel.

In a previous work [4], BCRP-G was shown to mediate the transport of ethidium in *L. lactis*. To obtain further support for BCRP-G-mediated ethidium transport in this organism, the effect of the expression level of BCRP-G on ethidium transport was tested. For this purpose, BCRP-G expression in *L. lactis* was induced at nisin A concentrations of 10 or 0.1 pmol/ml by the addition of the culture supernatant of the nisin A producer strain *L. lactis* NZ9700 at a dilution of 1:1000 (standard conditions) and 1:100 000 respectively. Differences in the expression levels of BCRP-G in *L. lactis* could be detected by immunoblotting (Figure 1B, inset), and clearly affected the rate of ethidium extrusion in the cells (Figure 1B). The rate of passive transmembrane potential (interior negative)-driven influx of cationic ethidium in the energized non-expressing control cells was not affected by the incubations with nisin A.

BCRP-G and -R are active as antibiotic efflux systems

The interaction of BCRP with antibiotics was tested. When exposed to erythromycin, lactococcal cells expressing BCRP-G or -R exhibited a significantly increased survival ratio (the number of colony-forming units in the presence of erythromycin/number of colony-forming units observed without the antibiotic) at increasing concentrations of erythromycin when compared with the non-expressing control cells. The IC_{50} of the survival ratio increased from $0.10 \pm 0.03 \mu\text{M}$ for the non-expressing control to $1.2 \pm 0.2 \mu\text{M}$ for BCRP-G/R-expressing cells (Figure 2A). When the effect of increasing the concentration of erythromycin on the maximum specific growth rate of cells was studied, BCRP-G-expressing cells exhibited a significant resistance to erythromycin with IC_{50} values of 20.5 ± 2.2 and $2.4 \pm 0.1 \mu\text{M}$ ($n = 5$) for the cells incubated with nisin A at a dilution of 1:1000 and 1:100 000 respectively. For non-BCRP-expressing control cells, IC_{50} values of 0.10 ± 0.02 and $0.08 \pm 0.01 \mu\text{M}$ respectively were obtained under these conditions (Figure 2B). These observations

Figure 2 Erythromycin is a transport substrate of BCRP-G and -R in *L. lactis*

(A) Cell survival in the presence of erythromycin. BCRP-G-expressing (Δ), BCRP-R-expressing ($\square$) and non-expressing control ($\circ$) cells were incubated at 30°C in the presence of the indicated concentrations of erythromycin. After 3 h of incubation, the cell suspensions were serially diluted and plated. Colonies were counted and the survival ratios were calculated as the percentage of colony-forming units observed at a given concentration of erythromycin to that observed without added erythromycin. (B) Effect of erythromycin on the maximum specific growth rate of cells with variable expression of BCRP-G. BCRP-G-expressing cells (open symbols) and control cells (closed symbols) were grown at 30°C in the presence of increasing concentrations of erythromycin and nisin A at dilutions as described in the legend to Figure 1(B). The maximum specific growth rates obtained in the absence of erythromycin were set at 100 %. (C) Erythromycin-stimulated BCRP-associated ATPase activity. Vanadate-sensitive ATPase activity of inside-out membrane vesicles containing BCRP-G (open symbols) and control membrane vesicles (closed symbols) was measured in the presence of increasing concentrations of erythromycin. (D) Energy-dependent transport of [^{14}C]erythromycin. *L. lactis* containing BCRP-G-expressing cells (Δ), BCRP-R-expressing cells ($\square$) or non-expressing control cells ($\circ$) were preincubated at 30°C for 1 h in 50 mM KPi buffer (pH 7.0) containing 5 mM MgSO_4 , 1 mg/ml BSA and [^{14}C]erythromycin at a final concentration of 20 μM . At $t = 0$ min, glucose was added to the cells to a final concentration of 25 mM, allowing the generation of metabolic energy for active transport. The amount of cell-associated radioactivity was measured over time in a rapid filtration assay.

on the BCRP-G-mediated drug resistance in growth experiments supported the BCRP-G-mediated drug resistance in cell survival experiments (Figure 2A), and revealed a relationship between erythromycin resistance and the expression level of BCRP-G in *L. lactis*. BCRP-G-containing inside-out membrane vesicles also displayed a vanadate-sensitive ATPase activity that was stimulated approx. 3.5-fold in the presence of erythromycin with an SC_{50} (the drug concentration required for half-maximal stimulation) of $1.8 \pm 0.1 \mu\text{M}$ ($n = 3$; Figure 2C).

BCRP-R- and -G-mediated efflux of erythromycin in *L. lactis* was measured directly in ATP-depleted cells that were pre-equilibrated in the presence of 20 μM [^{14}C]erythromycin. When metabolic energy was generated in the cells by the addition of 25 mM glucose, BCRP-R- or BCRP-G-expressing cells exhibited a significant decrease in the amount of cell-associated erythromycin, whereas an accumulation of erythromycin was observed in the non-expressing control (Figure 2D).

The specificity of BCRP for other antibiotics was also tested (Tables 1 and 2). The expression of BCRP-R significantly enhanced the growth of *L. lactis* in the presence of tetracycline

Figure 3 BCRP-mediated transport of rhodamine 123 and methotrexate is affected by a G to R substitution at position 482

(A) Effect of methotrexate (MTX) on the BCRP-mediated Hoechst 33342 transport in inside-out membrane vesicles. Inside-out membrane vesicles prepared from BCRP-G- and -R-expressing cells and non-expressing control cells 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

or rifampicin, giving a relative resistance factor (IC_{50} of BCRP-expressing cells/ IC_{50} of control cells) of 2.0 and 4.5 respectively (Table 2). More extensive studies on BCRP-G revealed a high level of BCRP-G-associated resistance in *L. lactis* to the macrolide dirithromycin with a relative resistance factor of 28 and a significant relative resistance of ≥ 2 for (i) tetracycline, (ii) fluoroquinolones such as ofloxacin, (iii) the antiprotozoal drug quinacrine and (iv) other drugs such as cephalothin and rifampicin (Table 1). These drugs also stimulated the BCRP-G-associated ATPase activity by 2–5-fold at SC_{50} values in the lower micromolar concentration range (Table 1). BCRP-G expression did not confer a significant resistance on lactococcal cells to aminoglycosides, β -lactams, lincosamides, sulphacetamine, gramicidin, metronidazole, vancomycin, quinine and phosphomycin as the relative resistance factors remained below 2 for these drugs. However, quinine and phosphomycin inhibited the growth of *L. lactis* with relatively high IC_{50} values of 612 ± 13 and $354 \pm 69 \mu\text{M}$ respectively and, hence, were not very toxic to the cells. Since quinine and phosphomycin stimulated the BCRP-G ATPase activity 4-fold with SC_{50} values of 6.3 ± 2.3 and $47 \pm 3 \mu\text{M}$ respectively, these compounds do appear to interact with BCRP-G. Taken together, our observations suggest that BCRP-G and -R interact with a similar range of antibiotics in *L. lactis*.

Transport of methotrexate and rhodamine 123 is affected by the R to G substitution at position 482 in BCRP

Mammalian cell lines expressing the Arg⁴⁸² mutant BCRP proteins exhibit an enhanced efflux of cationic anthracyclines and rhodamine 123, but a decreased efflux of anionic methotrexate when compared with cell lines expressing BCRP-R [15–18]. Experiments were performed to test whether these differences in drug selectivity of BCRP-G and -R were retained in *L. lactis*. The interaction of BCRP-G/R with methotrexate was studied in competition assays on the basis of the BCRP-mediated transport of the fluorescent substrate Hoechst 33342 [4,21,22]. After the addition of Hoechst 33342 to BCRP-R- and -G-containing inside-out membrane vesicles, a rapid increase in fluorescence was observed due to the diffusion of Hoechst 33342 into the phospholipid bilayer (Figure 3A). In the presence of MgATP, a quenching of the Hoechst 33342 fluorescence was obtained, reflecting the transport of the dye into the lumen of membrane vesicles. Hoechst 33342 transport was not observed after the addition of MgATP to control membrane vesicles without BCRP-G/R. Interestingly, 25 μM methotrexate significantly inhibited the

proteins. On the addition of 1 μM Hoechst 33342 (indicated by the first arrow), the increase in fluorescence of the dye was monitored over time until a steady state was reached. Hoechst 33342 transport was initiated by the addition of 5 mM MgATP (indicated by the second arrow). Before the addition of Hoechst 33342, MTX was added to the assays at a concentration of 25 μM . (B) Effect of the MTX concentration on the rate of BCRP-mediated Hoechst 33342 transport. The rates of Hoechst 33342 transport in the presence of different concentrations of MTX were determined during the first 100 s after the addition of MgATP. Δ , $\square$ or $\circ$, BCRP-G-containing (Δ) or BCRP-R-containing ($\square$) or control ($\circ$) inside-out membrane vesicles respectively. (C) TMR uptake in *L. lactis* cells was measured by fluorimetry. Approx. 1 min after the fluorescence measurements were initiated, TMR was added at the first arrow to a final concentration of 0.2 μM . TMR fluorescence was monitored for approx. 350 s until a steady state was reached. Glucose (25 mM) was added at the second arrow to provide metabolic energy for transport. (D) Concentration dependence of the TMR transport by BCRP-R and -G. Cells were preincubated in the presence of 25 mM glucose for 5 min, after which TMR was added at the concentrations indicated. TMR fluorescence was followed for 20 min, at which a steady state was reached. The steady-state level of fluorescence for control cells at each concentration of TMR was subtracted from the level obtained for cells expressing BCRP-G (open symbols) or BCRP-R (closed symbols).

initial rate of Hoechst 33342 transport by BCRP-R, but exhibited only a modest inhibition of Hoechst 33342 transport by BCRP-G (Figure 3A). A further analysis of this inhibition at a range of methotrexate concentrations revealed that BCRP-R-mediated Hoechst 33342 transport was inhibited by methotrexate with an IC_{50} of $5.0 \pm 0.3 \mu M$, whereas the dose-response curve for inhibition of the BCRP-G-mediated Hoechst 33342 transport by increasing the concentration of methotrexate was right-shifted with an IC_{50} of $26 \pm 3 \mu M$ ($n=3$; Figure 3B). These results suggest the enhanced interaction of methotrexate with BCRP-R compared with BCRP-G.

In subsequent experiments, the specificity of BCRP-G/R for rhodamine 123 was tested. In growth experiments, BCRP-G conferred a significant resistance of > 2 -fold on *L. lactis* to rhodamine 123 ($IC_{50} \geq 250 \mu M$ versus $IC_{50} = 124 \pm 12 \mu M$ in control cells), whereas BCRP-R was unable to do so ($IC_{50} = 125 \pm 6 \mu M$). In transport assays in intact cells, the addition of the rhodamine 123 analogue TMR to the cells resulted in a slow quenching of the TMR fluorescence due to the binding of the dye to chromosomal DNA (Figure 3C). On the addition of glucose, a rapid decrease in the TMR fluorescence intensity was observed in control cells and BCRP-R-expressing cells due to the increase in transmembrane potential (interior negative)-driven passive influx of the dye into the cell. However, the rapid decrease in TMR fluorescence was not observed when glucose was added to BCRP-G-expressing cells, pointing to the active efflux of TMR by this protein (Figure 3C). TMR transport in BCRP-G- and -R-expressing cells was further characterized by using different concentrations of the dye (Figure 3D). The steady-state fluorescence level of TMR in suspensions of BCRP-G-expressing cells increased at increasing concentrations of TMR compared with the level observed in BCRP-G-expressing cells. These results further emphasize the efflux of cationic TMR by BCRP-G, but not by BCRP-R, in *L. lactis*. Together, these observations suggest that the differences in the specificity of BCRP-G and -R for methotrexate and rhodamine 123, as observed in mammalian cells, are at least in part conserved in *L. lactis*.

BCRP-G and -R mediate the transport of oestradiol and bile acids

In a previous work on BCRP-G in *L. lactis*, it was observed that the BCRP-G-associated ATPase was stimulated by (i) sterols including oestradiol and cholesterol, (ii) the natural steroids progesterone and testosterone and (iii) the anti-oestrogen tamoxifen. In addition, BCRP-G mediated the transport of [3H]oestradiol in *L. lactis* [4]. In the present study, the ability of BCRP-R to transport oestradiol was directly assessed by measuring the uptake of [3H]oestradiol in *L. lactis* cells. In the presence of 25 mM glucose, both BCRP-R- and -G-expressing cells exhibited a 4-fold lower uptake of [3H]oestradiol when compared with the control cells or BCRP-R-expressing cells in the absence of glucose (Figure 4A). These results demonstrate a similar ability of BCRP-G and -R to mediate oestradiol transport in *L. lactis*.

Since the primary bile acids are polar derivatives of cholesterol, the specificity of BCRP-G/R for primary bile acids was tested. As shown in Tables 1 and 2, BCRP-G- and -R-expressing cells exhibited a significant 2-fold resistance to deoxycholate, whereas lower relative resistance factors were observed for cholate and taurocholate. However, cholate and taurocholate were not very toxic to *L. lactis* and inhibited the growth of the cells with a relatively high IC_{50} of approx. 2–3 mM. The interaction between BCRP-G and cholate was apparent from the 2.6-fold stimulation of the BCRP-G ATPase activity with an SC_{50} value of $3.4 \pm 0.3 \mu M$. A significant stimulation of the BCRP-G ATPase activity was also observed in the presence of taurocholate (Table 1). In

Figure 4 Transport of sterols and bile acids by BCRP-R and -G

(A) Transport of [3H]oestradiol in BCRP-R-expressing cells ($\square$) or BCRP-G-expressing cells ($\triangle$) or control cells ($\circ$). The cells were preincubated at 30 °C for 5 min in 50 mM KPI buffer (pH 7.0) containing $MgSO_4$, 1 mg/ml BSA and 500 nM [3H]oestradiol. After the preincubation period, 25 mM glucose was added to allow the generation of metabolic energy for active transport. (B) Energy-dependent transport of [^{14}C]cholate in BCRP-R-expressing ($\square$) and BCRP-G-expressing ($\triangle$) cells compared with non-expressing control cells ($\circ$). Cells were preincubated as described in (A) in the presence of 0.116 mM [^{14}C]cholate. Open and closed symbols refer to incubation with BCRP-R-expressing cells in the presence or absence of glucose respectively. The amount of cell-associated radioactivity was measured over time in a rapid filtration assay.

addition, direct evidence for the interaction between BCRP-R/G and cholate was obtained from transport experiments using [^{14}C]cholate. In the presence of 25 mM glucose, both BCRP-R- and -G-expressing cells exhibited an approx. 6-fold lower uptake of 116 μM [^{14}C]cholate when compared with the control cells or BCRP-R-expressing cells without the addition of glucose (Figure 4B).

The interaction of BCRP-G with bile acids was studied more extensively. Interestingly, the BCRP-G ATPase activity was stimulated 2–4-fold in the presence of taurocholate, taurodeoxycholate and glycochenodeoxycholate with SC_{50} values between 2 and 4 μM (Table 1). However, the BCRP-G ATPase activity was not stimulated by glycocholate, glycodeoxycholate and tauro-lithocholate, and BCRP-G expression did not confer resistance on lactococcal cells to these compounds (results not shown). Taken together, the observations of the BCRP-R/G-mediated resistance to bile acids, the bile acid-stimulated BCRP-G-associated ATPase activity, the reduced accumulation of [^{14}C]cholate in BCRP-R/G-expressing cells, and the lack of these phenomena in the non-expressing control demonstrate that BCRP-R and -G both catalyse the transport of primary bile acids in *L. lactis*.

DISCUSSION

In the present study, we have characterized the activity and drug selectivity of wild-type BCRP (BCRP-R) and a mutant BCRP protein, present in drug-selected cancer cell lines, in which an R residue at position 482 is substituted by a G (BCRP-G). Three lines of evidence suggest that BCRP-R and -G were active multi-drug efflux systems in *L. lactis*. First, BCRP-G- and -R-expressing lactococcal cells exhibited a significant resistance to erythromycin compared with non-expressing control cells in both cell survival assays and growth experiments (Figures 2A and 2B). In the growth experiments, high relative resistance factors of 82 and 78 were determined for BCRP-G- and -R-expressing cells respectively. The interaction of erythromycin with BCRP-G stimulated the BCRP-G-associated ATPase activity (Figure 2C). In contrast, erythromycin did not stimulate the vanadate-sensitive ATPase activity in the non-expressing control, suggesting that

endogenous ABC multidrug transporters, which may be expressed at low levels in *L. lactis*, do not contribute to the observed erythromycin resistance and transport in BCRP-R- or BCRP-G-expressing cells. This conclusion is also supported by the lack of active efflux of [¹⁴C]erythromycin in intact control cells compared with BCRP-G/R-expressing cells (Figure 2D), and the lack of a significant ATP-dependent Hoechst 33342 transport in control membrane vesicles compared with BCRP-G/R-containing membrane vesicles (Figure 3A). Secondly, the BCRP-G-associated erythromycin resistance levels and the rate of BCRP-G-mediated ethidium efflux in *L. lactis* were proportional to the expression level of BCRP-G in the cells (Figures 1B and 2B). Thirdly, whereas BCRP-G mediated the transport of rhodamine 123 and the rhodamine analogue TMR, BCRP-R was unable to handle these substrates (Figures 3C and 3D). Conversely, BCRP-R showed an enhanced interaction with methotrexate compared with BCRP-G (Figures 3A and 3B), pointing to different specificities of BCRP-R and -G for charged substrates.

Interestingly, BCRP-R demonstrated a previously unestablished ability to mediate the transport of (i) antibiotics other than erythromycin, (ii) neutral sterols such as oestradiol and (iii) primary bile acids including cholate and deoxycholate, for which Arg⁴⁸² was not crucial. Hence, BCRP-R and -G exhibit overlapping drug specificities. This conclusion was based on a comparison of BCRP-R/G-associated drug resistance (if substrates were cytotoxic) and drug-stimulated ATPase activities (Tables 1 and 2), and the transport of radiolabelled substrates (Figure 4). Although one may expect that drug resistance might correlate directly to the degree of drug stimulation of the ATPase activity, a closer examination of Tables 1 and 2 reveals that, for certain drugs, an inverse correlation exists between these parameters. For example, for cholate, a significantly stimulated ATPase activity was associated with a low relative resistance, whereas, for erythromycin, a relatively modest stimulation of the ATPase activity was associated with a high relative resistance. However, it should be noted that drug resistance will be determined by (i) the relative cytotoxicity of drugs, which is not equal for the drug used in the present study (Tables 1 and 2), (ii) the rate of passive (non-protein-mediated) drug influx into cells, which is affected by the physicochemical properties of drugs, and (iii) the rate of active drug transport out of cells, which is determined by binding affinity and the maximum transport rate. For the ATPase activity, the degree of stimulation by drugs will depend on (a) the affinity of drug binding to the transporter, (b) the rate at which the drug is actively translocated and (c) the rate of passive diffusion of the drug across the membrane, back to the site from which transport occurred. For an amphiphilic drug, the high rate of passive drug influx into the cell could be associated with decreased drug resistance but with increased stimulation of the BCRP-ATPase, compared with a hydrophilic drug exhibiting a low rate of passive drug influx. Hence, although drug resistance and drug-stimulated ATPase activities are useful indicators of drug-protein interactions in ABC multidrug transporters, these parameters cannot always be used to compare directly these interactions in quantitative terms.

Our new observations on the transport of erythromycin and other antibiotics by BCRP are in agreement with reported evidence concerning the interactions of antibiotics with the human MDR (multidrug resistance) P-glycoprotein MDR1 and the mouse orthologue Mdr1a. Fluoroquinolones, acting on bacterial topoisomerases, inhibited the transport of rhodamine-123 across the mouse blood-brain barrier *in vivo*, and in P-glycoprotein MDR1 overexpressing MDR1-LLC-PK1 pig kidney epithelial cells *in vitro* [23]. Rifampin, an inhibitor of bacterial RNA polymerase, modulated the transport activity of the P-glycoprotein

Mdr1a in the mouse blood-brain barrier in a dose- and concentration-dependent manner [24]. Macrolide antibiotics, which inhibit bacterial protein synthesis, were reported to increase the plasma concentrations of the cardiac glycoside digoxin *in vivo* by inhibiting P-glycoprotein-mediated active tubular secretion of digoxin. Macrolides also increased the accumulation of the P-glycoprotein substrates vinblastine and cyclosporin A in P388/ADR cells *in vitro* in a dose-dependent manner. Finally, the transport of erythromycin by human MDR1 and mouse mdr1a P-glycoprotein was also directly demonstrated in LLC-PK1 cell lines using the radiolabelled substrate [25].

Our additional observations on the BCRP-R-mediated transport of unconjugated sterols and primary bile acids are complementary to published results on the BCRP-mediated transport of sulphated conjugates of bile acids and steroids in mouse P388 lymphoma cells [26], and glucuronide conjugates of sterols in HEK-293 cells (human embryonic kidney 293 cells) [27]. Our findings are in apparent contradiction with recent work suggesting that BCRP-R does not interact with free oestrogens in epithelial cells of porcine kidney (LLC-PK1) [28]. However, as discussed previously [4], plasma membranes of mammalian cells contain endogenous levels of sterols of up to 25%, which could make additional interactions between BCRP and exogenously added oestrogens difficult to detect. In contrast, the plasma membrane of *L. lactis* is devoid of human sterols, allowing a direct demonstration of BCRP-R/G-mediated [³H]oestradiol transport and sterol-stimulated BCRP-R/G-ATPase activities. Our observations on BCRP-R/G-mediated [³H]oestradiol transport may relate to the recent identification of a functional oestrogen response element in the human *BCRP* promoter [29]. In addition, they may relate to the identification of a putative START (steroidogenic acute regulatory protein signature) lipid-binding motif in human BCRP and the BCRP homologue NtWBC1 in tobacco reproductive organs [30].

In summary, our results demonstrate that BCRP-R and -G have overlapping specificities for antibiotics, primary bile acids and free sterols, and suggest that Arg⁴⁸² is not critical for the interactions with these substrates. However, Arg⁴⁸² plays an important role in the specificity of BCRP for charged substrates including rhodamine 123, TMR and methotrexate, which may be based on electrostatic interactions between Arg⁴⁸² and charged moieties in these substrates. Since the unconjugated sterols used in our work are neutral molecules, and the primary bile acids and antibiotics contain weakly acidic/basic moieties, our observations may point to a lack of interaction between Arg⁴⁸² and neutral or neutralized moieties in these substrates during transport, or to binding of these substrates to regions in BCRP not including Arg⁴⁸².

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