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Multidrug resistance in *Lactococcus lactis*: evidence for ATP-dependent drug extrusion from the inner leaflet of the cytoplasmic membrane

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Lactococcus lactis **possesses an ATP-dependent drug extrusion system which shares functional properties with the mammalian multidrug resistance (MDR) transporter P-glycoprotein. One of the intriguing aspects of both transporters is their ability to interact with a broad range of structurally unrelated amphiphilic compounds. It has been suggested that P-glycoprotein removes drugs directly from the membrane. Evidence is presented that this model is correct for the lactococcal multidrug transporter through studies of the extrusion mechanism of BCECF-AM and cationic diphenylhexatriene (DPH) derivatives from the membrane. The non-fluorescent probe BCECF-AM can be converted intracellularly into its fluorescent derivative, BCECF, by non-specific esterase activities. The development of fluorescence was decreased upon energization of the cells. These and kinetic studies showed that BCECF-AM is actively extruded from the membrane before it can be hydrolysed intracellularly. The increase in fluorescence intensity due to the distribution of TMA-DPH into the phospholipid bilayer is a biphasic process. This behaviour reflects the fast entry of TMA-DPH into the outer leaflet followed by a slower transbilayer movement to the inner leaflet of the membrane. The initial rate of TMA-DPH extrusion correlates with the amount of probe associated with the inner leaflet. Taken together, these results demonstrate that the lactococcal MDR transporter functions as a 'hydrophobic vacuum cleaner', expelling drugs from the inner leaflet of the lipid bilayer. Thus, the ability of amphiphilic substrates to partition in the inner leaflet of the membrane is a prerequisite for recognition by multidrug transporters.**

Keywords: BCECF-AM/*Lactococcus*/multidrug resistance/P-glycoprotein/TMA-DPH

Introduction

The development of multidrug resistance (MDR) in human tumour cells is due to the overexpression of the membrane-associated P-glycoprotein (P-gp), encoded by the *MDR*1 gene (Gottesman and Pastan, 1993). P-gp mediates the active extrusion of a number of unrelated toxic compounds resulting in a decrease in intracellular drug concentration, and hence an increase in drug resistance (Horio *et al.*, 1988). Currently, multidrug transporters have been characterized from mammalian cells, lower eukaryotes (Foote *et al.*, 1989), bacteria (Nikaido, 1994) and archaea (Miyauchi *et al.*, 1992). Most of the eukaryotic multidrug transporters belong to the ATP-binding cassette (ABC) family of transport proteins (Higgins, 1992), while the prokaryotic systems are mainly secondary transporters.

Recently, we identified the first structural and functional prokaryotic homologue of P-gp, designated LmrA, in *Lactococcus lactis* strain MG1363 (H.W.van Veen *et al.*, manuscript submitted). Functional evidence for the existence of this system was obtained previously from the analysis of MDR mutants of *L.lactis* (Bolhuis *et al.*, 1994), some of which also express the secondary MDR transporter LmrP (Bolhuis *et al.*, 1995). The substrate 'specificity' of the lactococcal MDR transporters is very similar to that of P-gp. It is most intriguing how these transporters recognize, bind and transport such a broad range of structurally and functionally unrelated compounds. Insight into this phenomenon is of major importance for the rational design of alternative drugs which could block or circumvent drug pumping by multidrug transporters. Many MDR substrates are cationic, lipophilic and planar molecules. On theoretical grounds it can be predicted that hydrophobic ions or hydrophobic compounds with polarizable groups will be stabilized and concentrated at the membrane–water interface (Läuger and Neumcke, 1973; Andersen, 1978). This is due to opposing hydrophobic and hydrophilic interactions which pull the ion into both phases. For example, anthracyclines interact with phospholipid bilayers in such a way that these amphipathic compounds have their hydrophobic parts buried between the hydrocarbon chains, and the positively charged amino-sugar group in the polar headgroup region of the lipids (De Wolf *et al.*, 1991). Ion pairing of the positively charged amino-sugar group of anthracyclines with acidic phospholipids also seems to be of importance (Speelmans *et al.*, 1994).

Based on the above-mentioned characteristics of MDR substrates, an alternative model for drug extrusion was proposed which differs from that of a 'classical' transporter in which the substrate is recognized and bound from the aqueous phase before it is transported across the membrane. In contrast, this model suggests that the hydrophobic compounds are removed from the membrane, thereby functioning as a 'hydrophobic vacuum cleaner' (Raviv *et al.*, 1990). A mechanism via which substrates are expelled from the membrane was proposed by the 'flippase' model (Higgins and Gottesman, 1992; Higgins, 1994), in which the transporter binds substrates located in the inner leaflet of the membrane. Subsequently, sub-

strates are translocated (flipped) from the inner to the outer leaflet of the lipid bilayer from where they can diffuse into the external medium, or alternatively, are directly expelled from the inner leaflet into the external medium. The flippase model is supported by the ability of the mouse P-gp homologue mdr2 to catalyse the ATP-dependent transbilayer movement of a fluorescent phosphatidylcholine (PC) analogue (Ruetz and Gros, 1994), and the inability of an mdr2 knock-out mouse to secrete PC into bile (Smit *et al.*, 1993).

However, the exact molecular mechanism by which P-gp mediates drug efflux remains unclear. Evidence for pumping of colchicine and vinblastine (Stein *et al.*, 1994) from the membrane as well as for pumping of daunomycin and rhodamine 6G (Mülder *et al.*, 1993; Altenberg *et al.*, 1994) from the cytoplasm has been presented. In fact, evidence presented for both models is based on the assumption that the initial rate of passive drug influx is unaffected by P-gp if substrates are pumped from the cytoplasm, while the initial influx rate will decrease when substrates are actively extruded from the membrane. Additional evidence for drug pumping from the membrane was based on experiments using BCECF-AM [2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester] and other acetoxymethyl (AM) esters of fluorescent intracellular indicators (Homolya *et al.*, 1993; Holló *et al.*, 1994).

In order to unravel further the molecular mechanism of substrate binding by P-gp and its homologues, the effect of drug partitioning in the membrane on the drug pumping action of the lactococcal ATP-dependent MDR transporter was studied. Our data revealed that amphiphilic drugs are transported from the inner leaflet of the phospholipid bilayer.

Results

BCECF-AM extrusion is mediated by the lactococcal ATP-dependent MDR transporter

The hydrophobic acetoxymethyl derivative (BCECF-AM) can be used to load cells with the fluorescent pH indicator BCECF. BCECF-AM is non-fluorescent and diffuses across the cytoplasmic membrane. Once inside the cell, BCECF-AM is rapidly hydrolysed by non-specific esterases, thereby trapping the non-permeant hydrophilic free acid BCECF within the cell. In eukaryotic cells, the human P-gp is able to reduce the accumulation of BCECF (Homolya *et al.*, 1993).

Transport experiments with BCECF-AM as substrate confirm the functional similarity between P-gp and the ATP-dependent MDR transporter of *L.lactis*. Figure 1 shows that BCECF accumulates after the addition of BCECF-AM to pre-energized wild-type cells and cells of a mutant which has an increased resistance to ethidium bromide (Eth^R). This mutant predominantly expresses the lactococcal P-gp homologue (Bolhuis *et al.*, 1994). Strikingly, intracellular trapping of the fluorescent probe is strongly reduced in the Eth^R mutant as compared with the wild-type, when the cells are energized. In contrast, BCECF-AM hydrolysis rates were identical for both cell types in the absence of an energy source and in cell-free extracts of both cell types (data not shown). These data show that the rate of BCECF-AM hydrolysis is the same

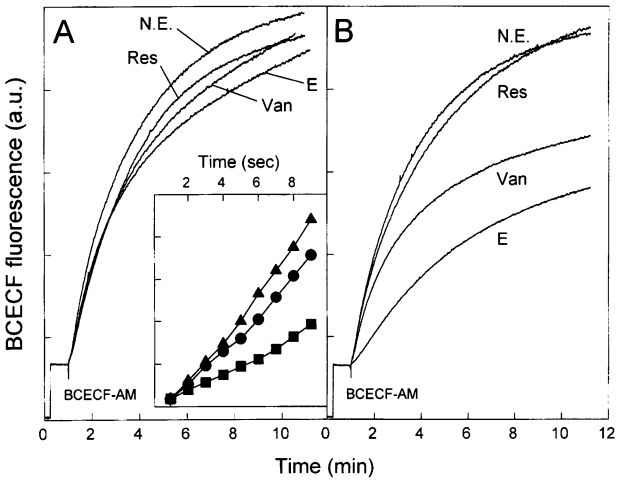

Fig. 1. BCECF-AM extrusion from *L.lactis*. Washed cell suspensions (0.1 mg protein/ml) of either *L.lactis* MG1363 (**A**) or the drug-resistant mutant Eth^R (**B**), were incubated without (N.E.) or with (E) 10 mM arginine for 5 min in the presence of nigericin (1 μM). *Ortho*-vanadate (0.5 mM; Van) or reserpine (10 μg/ml; Res) were added before energization. After the addition of 1 μM of BCECF-AM, the BCECF fluorescence (arbitrary units, a.u.) caused by intracellular hydrolysis of BCECF-AM by non-specific esterases, was followed in time. The inset depicts a detail of the initial BCECF accumulation over the first 9 s in the Eth^R strain in the presence (■) and absence (●) of reserpine and in the wild-type strain (▲).

in the wild-type and Eth^R strain and suggest that the observed difference in BCECF accumulation between the Eth^R mutant and wild-type cells is the result of ATP-dependent extrusion of BCECF-AM in Eth^R. This notion was confirmed by using reserpine, a known inhibitor of several MDR transporters, and the ABC transporter and P-type ATPase inhibitor *ortho*-vanadate to inhibit the lactococcal MDR transporter. Both inhibitors enhanced BCECF accumulation in the Eth^R strain but only slightly affected BCECF accumulation in wild-type cells. Dissipation of the proton motive force upon addition of valinomycin plus nigericin did not affect the BCECF fluorescence in either strain (data not shown). The effect of the ATP-dependent BCECF-AM extrusion on the initial rate of BCECF accumulation is shown in the inset of Figure 1. The initial rate of BCECF accumulation is reduced in the Eth^R strain as compared with the wild-type strain, and is enhanced upon addition of reserpine. The decreased initial rate of BCECF-AM uptake in the Eth^R strain is consistent with substrate pumping from the membrane as was observed for the P-gp-mediated transport of colchicine and vinblastine (Stein *et al.*, 1994). Although *L.lactis* possesses an ATP-dependent BCECF extrusion system (Molenaar *et al.*, 1992), the observed BCECF fluorescence development in the wild-type and Eth^R strains cannot be explained by this system. BCECF itself is not a substrate of the ATP-dependent drug transporter since: (i) a BCECF extrusion mutant, with a 5-fold reduced rate of BCECF extrusion (Molenaar *et al.*, 1992) is not affected in drug sensitivity or drug transport; (ii) BCECF efflux from the wild-type strain is insensitive to reserpine; and (iii) Eth^R cells catalyse BCECF efflux with characteristics comparable with those of the wild-type strain (data not shown).

P-gp-mediated BCECF-AM extrusion was concluded to occur from the membrane based on the assumption that the limiting step in BCECF accumulation is BCECF-

AM diffusion over the membrane and not BCECF-AM hydrolysis (Homolya *et al.*, 1993). If this assumption is correct, the apparent affinity constant (K_m) for BCECF-AM hydrolysis should be higher in whole cells than in permeabilized cells, since a diffusion-limited process leads to a cytoplasmic BCECF-AM concentration for the esterases that is lower than the concentration added to the cell suspension. To test whether BCECF-AM hydrolysis in intact cells is indeed limited by the diffusion of the probe across the membrane, the kinetics of BCECF fluorescence development was determined in non energized wild-type cells and cells permeabilized with nisin, a pore-forming lantibiotic peptide which allows the free entrance of molecules like BCECF-AM (Driessen *et al.*, 1995), and a sonicated cell suspension. The apparent K_m measured was a factor of 2 higher in whole cells (K_m 1.43 ± 0.21 μM) than in permeabilized cells (K_m 0.67 ± 0.07) or a sonicated cell suspension (K_m 0.70 ± 0.10 μM). The V_{max} values in intact and permeabilized cells were identical. Taken together, these results show that the hydrolysis of BCECF-AM is limited by diffusion and suggest that BCECF-AM can be excreted by the ATP-dependent drug transporter of *L.lactis* and that the transporter accepts the substrate from the membrane rather than from the cytoplasm.

Molecular mechanism of TMA-DPH partitioning into the phospholipid bilayer

To strengthen the conclusion that drug extrusion occurs from the membrane, an alternative assay was used which is based on the active efflux of cationic diphenylhexatriene (DPH) derivatives. The amphiphilic character and the high lipid–water partition coefficients results in partitioning of the probe into the phospholipid bilayer, with the charged headgroup positioned between the lipid headgroups and the DPH moiety buried in the non-polar hydrocarbon phase of the membrane (Prendergast *et al.*, 1981). The hydrophobic compounds are only fluorescent when partitioned into the membrane (Prendergast *et al.*, 1981), and the fluorescence reflects the concentration of the probe in the membrane (Kuhry *et al.*, 1985). From TMA-DPH {1-[4-(trimethylamino) phenyl]-6-phenylhexa-1,3,5-triene} binding experiments under conditions of equilibrium we could calculate a cell membrane-associated probe concentration of 5 mM while the extracellular free concentration was only 30 nM (data not shown). This yields a lipid over medium partition coefficient of 10^5, which is in agreement with the octanol/water K_p for TMA-DPH of 2×10^5 (Haugland, 1992). Addition of TMA-DPH to *L.lactis* cells resulted in a biphasic increase in fluorescence due to the association of TMA-DPH with the cytoplasmic membrane (Figure 2; phase A and B). A rapid (1–2 s) initial fluorescence increase, resulting from the immediate insertion of TMA-DPH into the lipid bilayer (phase A), is followed by a slower fluorescence increase (phase B) which reaches its maximum after several minutes. A similar biphasic increase in fluorescence was also observed when TMA-DPH was added to liposomes (not shown).

Fluorescence resonance energy transfer (FRET) measurements in liposomes were carried out to investigate this biphasic behaviour in greater detail. Energy transfer between two fluorescent molecules is possible if the emission wavelength of the donor molecule and the

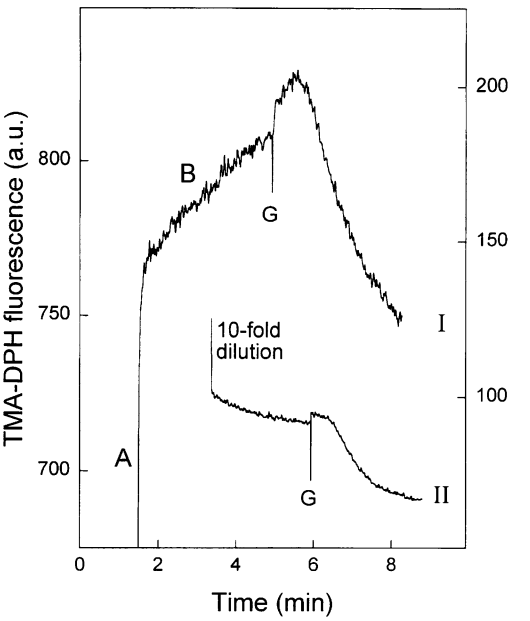

Fig. 2. Kinetics of TMA-DPH association with the cytoplasmic membrane. The time-course of fluorescence development upon addition of 200 nM of TMA-DPH to a washed cell suspension (0.4 mg protein/ml) of *L.lactis* Eth^R, reveals two distinct fluorescent phases (A and B). Subsequently (Trace I), cells were energized with 25 mM of glucose (G; left vertical axis), or (Trace II) cells were diluted 10-fold before energization (G; right vertical-axis).

excitation wavelength of the acceptor molecule overlap, provided that the donor and acceptor molecule are in close proximity (for a review, see Mátyus, 1992). In this assay, the donor and acceptor molecules were TMA-DPH and the fluorescent phospholipid derivative NBD-PE [*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine], respectively. An emission spectrum of liposomes containing 0.5 mol% NBD-PE at the excitation wavelength of TMA-DPH (350 nm) revealed no NBD-PE fluorescence (540 nm) (data not shown), whereas liposomes equilibrated with TMA-DPH but lacking NBD-PE yielded a typical TMA-DPH emission spectrum (maximum at 425 nm). However, liposomes containing both NBD-PE and TMA-DPH gave a clear signal at the emission maximum of NBD-PE, whereas the fluorescence at the emission maximum of TMA-DPH was low. These results show that efficient energy transfer occurs from TMA-DPH to the NBD moiety of NBD-PE.

To discriminate between the fluorescence of NBD-PE located in the outer and inner leaflet of the phospholipid bilayer, the membrane impermeable quencher DPX (*p*-xylene-bis-pyridinium bromide) was used. DPX added to the external medium solely quenches the fluorescence of NBD-PE molecules located in the outer leaflet. Fluorescence resonance energy transfer between TMA-DPH and NBD-PE in liposomes displayed biphasic kinetics (Figure 3). In the presence of DPX, however, the total fluorescence decreased to ~50%, due to a decrease in phase A but not in phase B fluorescence. The NBD-PE fluorescence was completely quenched when DPX was allowed to enter the liposomes by sonication. The results show that ~50% of the TMA-DPH is present in the outer leaflet of the liposomal membrane. These experiments suggest that the initial TMA-DPH fluorescence is due to the fast parti-

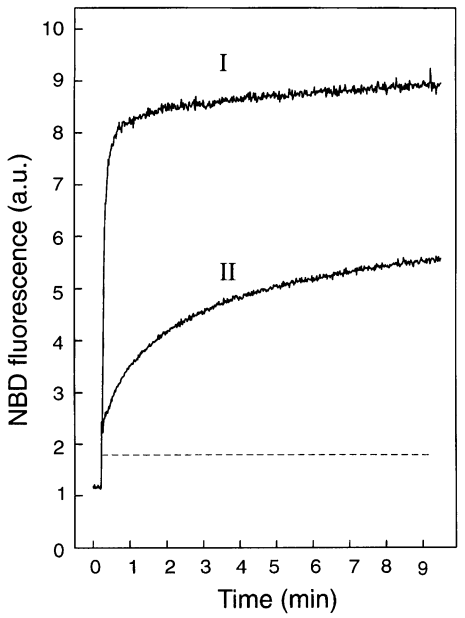

Fig. 3. Fluorescent behaviour of TMA-DPH in phospholipid bilayers. Time-dependent FRET from TMA-DPH to NBD-PE. Energy transfer was followed in time upon addition of TMA-DPH (150 nM) to an NBD-PE containing DOPG/DOPC liposomes (3/1) suspension. The NBD-PE fluorescence was followed in the absence (Trace I) or presence (Trace II) of 200 mM DPX. The NBD-PE fluorescence when DPX was present both intra- and extracellularly was measured after sonication of the suspension (dotted line).

tioning of the probe in the outer leaflet of the phospholipid bilayer (phase A), followed by a slower transbilayer movement (flip-flop) of the probe from the outer to the inner leaflet of the bilayer (phase B). The insertion of TMA-DPH into the liposomes is similar to the biphasic fluorescent behaviour of TMA-DPH observed in intact cells (Figure 2B) and indicates a similar mechanism of partitioning.

DPH derivatives are substrates of the lactococcal ATP-dependent MDR transporter

Energy-dependent TMA-DPH extrusion from the membrane of *L.lactis* Eth^R is shown in Figure 2. TMA-DPH extrusion in the direction of the partitioning equilibrium was assayed after a 10-fold dilution of cells preincubated with TMA-DPH, i.e. 90% of the cells (volume) was removed and replaced by an equal volume of buffer (Figure 2; trace II). Dilution of cells loaded with TMA-DPH resulted in a rapid fluorescence decrease from 809 units to 102 units, which is less than expected when all TMA-DPH would redistribute instantaneously over the membrane and external medium compartments. Subsequently, a slow additional fluorescence decrease was observed which reflects the actual release of TMA-DPH from the outer leaflet of the membrane into the medium until a new equilibrium between membrane-associated and unbound TMA-DPH was reached. The subsequent rapid fluorescence decrease upon energization of the cells by the addition of glucose points to the active extrusion of TMA-DPH from the membrane (Figure 2; trace II). A decrease in TMA-DPH fluorescence was also observed when cells were preincubated with TMA-DPH and glucose was added after 5 min (Figure 2; trace I). Apparently,

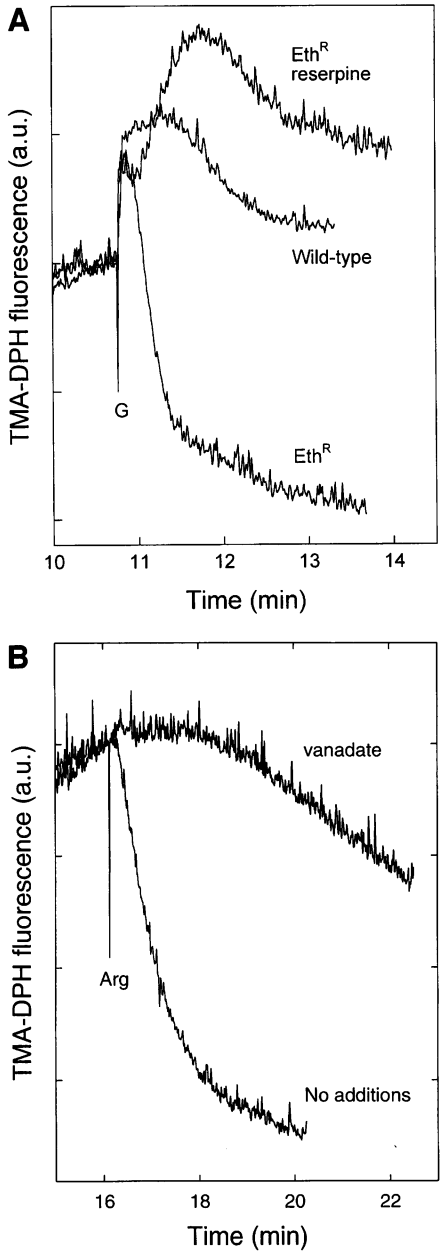

Fig. 4. TMA-DPH fluorescence in *L.lactis*. (**A**) Washed cell suspensions (0.1 mg protein/ml) of *L.lactis* MG1363 (wild-type) or the drug-resistant mutant (Eth^R) were incubated for 10 min with 100 nM of TMA-DPH before energization with 25 mM glucose (G). Reserpine was added to a final concentration of 10 μg/ml. (**B**) The effect of *ortho*-vanadate on TMA-DPH extrusion was investigated in Eth^R cells pre-incubated for 10 min with or without *ortho*-vanadate (0.5 mM). The cells were subsequently de-energized for 30 min with 10 mM of 2-deoxyglucose, washed and resuspended in a fresh buffer. The decrease in TMA-DPH fluorescence was followed upon energization with arginine (10 mM), 15 min after the addition of 100 nM TMA-DPH.

TMA-DPH can be actively extruded against the direction of the partitioning equilibrium.

The energy-dependent fluorescence decrease of TMA-DPH was more extensive in Eth^R than in wild-type cells and could be reversed by reserpine (Figure 4A). *Ortho*-vanadate inhibited the TMA-DPH fluorescence decrease in arginine-energized cells (Figure 4B), while dissipation of the proton motive force upon addition of valinomycin

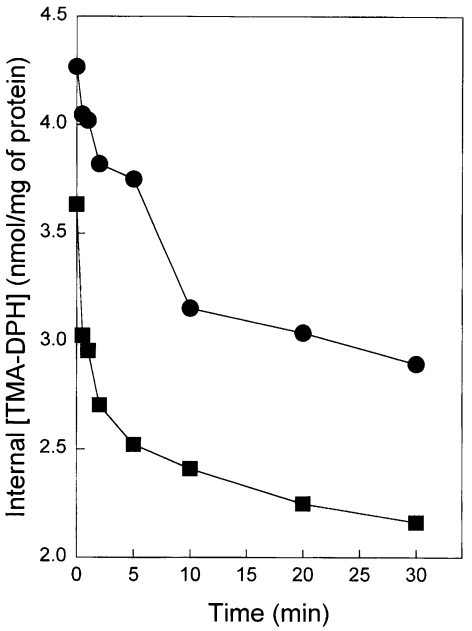

Fig. 5. TMA-DPH efflux from *L.lactis*. Cells of the wild-type *L.lactis* strain MG1363 (●) and the drug-resistant strain Eth^R (■) were de-energized for 30 min with 10 mM 2-deoxyglucose and subsequently preincubated for 1 h with 1 μM TMA-DPH. After the addition of 25 mM glucose, the concentration of cell-associated TMA-DPH was calculated from the amount of TMA-DPH in the external medium.

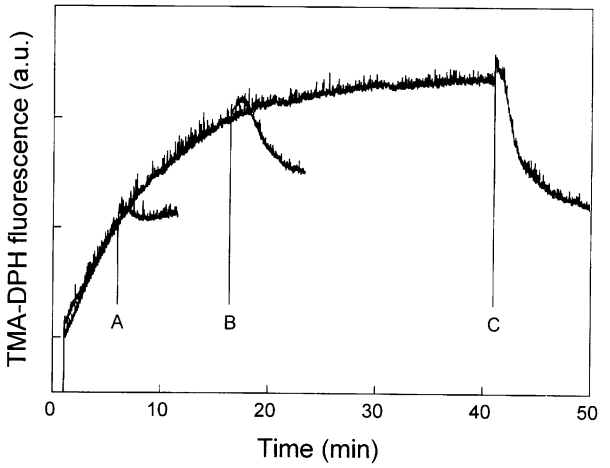

Fig. 6. Time-course of the rate of energy-dependent TMA-DPH extrusion. A washed cell suspension of *L.lactis* Eth^R was incubated with 100 nM TMA-DPH. Subsequently, cells were energized with 25 mM glucose, at 5 (A), 15 (B) and 40 min (C) after the addition of TMA-DPH.

plus nigericin did not significantly affect TMA-DPH fluorescence (data not shown). These observations verify the involvement of the ATP-dependent drug transporter in TMA-DPH transport. To confirm that the decrease in TMA-DPH fluorescence is the result of active extrusion, the amount of free TMA-DPH in the external medium was measured. For this purpose, the external medium was rapidly separated from the cells by centrifugation and the free TMA-DPH in the supernatant was measured fluorimetrically after the addition of SDS. From these data the amount of cell-associated TMA-DPH was calculated (Figure 5). The excretion of TMA-DPH into the medium as determined by this indirect assay was faster with the Eth^R strain than with the wild-type strain, which is entirely consistent with the data obtained in the direct fluorescence assay (Figure 4A). Taken together, these results show that TMA-DPH, a probe that is predominantly associated with the membrane, is a substrate of the ATP-dependent drug transporter of *L.lactis*.

Like TMA-DPH, the cationic DPH derivative TMAP-DPH [*N-p*-(6-phenyl-{1,3,5 hexatrienyl[phenyl-(propyl)]}) trimethyl-ammonium] was transported by the lactococcal MDR transporter (data not shown). In contrast, no extrusion was observed for the neutral DMA-DPH {1-[4-(dimethylamino)phenyl]-6-phenylhexa-1,3,5-triene} or the anionic DPH-CA (1,6-diphenylhexa-1,3,5-triene carboxylic acid). Apparently, the drug extrusion system shows a preference for positively charged DPH derivatives.

TMA-DPH is not expelled from the outer leaflet

To determine whether TMA-DPH can be extruded from the outer leaflet of the cytoplasmic membrane, the dependency of the TMA-DPH efflux rate on the partitioning of TMA-DPH in the membrane was studied. As depicted in Figure 6, the initial rate of active TMA-DPH extrusion increased in time, i.e. in the course of flipping of TMA-DPH from the outer to the inner leaflet. The steady-state levels obtained were identical to that observed for pre-energized cells (data not shown). These data show that the rate of TMA-DPH extrusion increases with the concentration of TMA-DPH in the inner leaflet of the cytoplasmic membrane.

Discussion

The mechanism of extrusion of hydrophobic compounds by the lactococcal ATP-dependent drug extrusion system was studied. The evidence presented is consistent with a model for drug pumping, in which drugs are recognized as substrates after partitioning into the inner leaflet of the membrane. We have shown that BCECF-AM is excreted by the ATP-dependent drug efflux system of *L.lactis*, before hydrolysis by intracellular esterases. Moreover, cationic DPH derivatives such as TMA-DPH, but not the anionic or neutral derivatives, are actively extruded by Eth^R cells. Direct fluorescence measurements revealed the involvement of the ATP-dependent MDR transporter in TMA-DPH extrusion (Figure 4), while the indirect assay confirmed that TMA-DPH indeed appears in the external medium (Figure 5). Furthermore, it was shown that the initial rate of TMA-DPH extrusion increased when more of the probe had accumulated in the inner leaflet of the membrane (Figure 6).

The rate-determining steps in the extrusion of amphiphilic, cationic drugs which readily partition into the membrane, are the transbilayer movement (from the inner to the outer leaflet) and the diffusion from the outer leaflet to the extracellular water phase. This was confirmed by the observation that the rate of passive release of TMA-DPH from the membrane after dilution is slow compared with the rate of active extrusion (Figure 2). This is in accordance with observations made by Kuhry *et al.* (1985), who found a similar slow redistribution after diluting TMA-DPH-loaded mouse fibroblasts. In fact, these researchers did not observe intracellular appearance of

TMA-DPH within 30 min after addition of the probe. In liposomes, TMA-DPH was found to remain membrane associated for at least 3 h, before any internal appearance could be measured (Cranney *et al.*, 1983).

The partitioning of TMA-DPH into the outer leaflet of the lipid bilayer is a fast process which is followed by a slower translocation to the inner leaflet. This was shown by studies in liposomes using FRET between TMA-DPH and NBD-PE in the presence and absence of the membrane impermeable quencher DPX (Figure 3). In the absence of DPX, the fluorescence development resulting from molecules partitioning in the inner leaflet is less apparent than when DPX is present (compare slow phases in Figure 3; traces I and II). The reason is that molecules that flip from the outer to the inner leaflet only contribute to the total fluorescence intensity when the outer leaflet is immediately refilled from the external free pool. Therefore, at limiting external TMA-DPH concentrations, most of the TMA-DPH is present in the membrane and the fluorescence development in the second phase of the experiment shown in Figure 3 is partially masked. Biphasic kinetics were also observed for the partitioning of TMA-DPH in the cytoplasmic membrane of *L.lactis* cells (Figure 2), and in the lipid bilayer of blood platelets (Kitagawa *et al.*, 1991). Assuming normal Michaelis–Menten-like transport kinetics, the initial efflux rates are determined by the local substrate concentrations. Since the occupation of the outer leaflet with TMA-DPH is fast and completed within seconds after addition of the probe, the observed time and hence concentration dependency of TMA-DPH efflux rules out the outer leaflet as the possible site of substrate binding and extrusion (Figure 6).

The lactococcal MDR transporter is able to actively extrude BCECF-AM from cells of *L.lactis*. Active extrusion of BCECF-AM was also observed for P-gp and this was used as an argument for substrate pumping from the membrane rather than from the cytoplasm under the assumption that the esterase activity is not limiting (Homolya *et al.*, 1993; Holló *et al.*, 1994). Since the observed BCECF-AM extrusion might also be explained by competition between the transporter and the esterases for the cytoplasmic pool of BCECF-AM, it is essential to show that diffusion of BCECF-AM and not hydrolysis is the limiting step. Evidence that indeed BCECF-AM diffusion, rather than esterase activity, is limiting the BCECF accumulation has been presented in this study. Since activation of the ATP-dependent drug extrusion system of *L.lactis* lowers the intracellular BCECF concentration, the results are most consistent with an extrusion of BCECF-AM from the membrane.

Taken together, the observed characteristics of TMA-DPH and BCECF-AM transport by the ATP-dependent drug transporter of *L.lactis* are most consistent with a mechanism where drugs are bound and expelled from the membrane rather than from the cytoplasm. Furthermore, the TMA-DPH transport studies suggest that the inner leaflet of the lipid bilayer is the site from which the transporter gains access to its substrates. These results are in agreement with results reported for the human multidrug transporter P-glycoprotein (Stein *et al.*, 1994; Shapiro and Ling, 1995) and for the MDR-related protein MRP (Mülder *et al.*, 1993). Therefore, MDR transporters may function as 'hydrophobic vacuum cleaners' which remove toxic compounds from the inner leaflet of the lipid bilayer in order to preserve the membrane integrity and functioning.

Materials and methods

Growth and preparation of organisms

L.lactis MG1363 and the multidrug resistant mutant, Eth^R (Bolhuis *et al.*, 1994) were grown at 30°C on M17 medium (Difco), supplemented with 25 mM glucose or with 25 mM galactose plus 10 mM arginine.

BCECF-AM transport

Transport and intracellular trapping of the non-fluorescent probe BCECF-AM was determined by continuous recording of the BCECF fluorescence development. Exponentially growing cells were harvested and washed three times in 50 mM potassium–HEPES, pH 7.0, containing 25 mM K_2SO_4 plus 5 mM $MgSO_4$. Subsequently, the cells were resuspended in buffer to a final concentration of 0.1 mg protein/ml. BCECF-AM (1 mM stock solution in dimethylsulfoxide) was added to the cell suspension to a final concentration of 0.5 μM. BCECF fluorescence (arbitrary units) was measured at excitation and emission wavelengths of 502 and 525 nm, using slit widths of 5 and 10 nm, respectively. Fluorescence experiments were performed with a Perkin-Elmer LS 50B fluorometer, equipped with a thermostatically controlled, magnetically stirred cuvette holder.

Esterase activity measurements

The esterase activity in intact cells, cells permeabilized with nisin (5 μg/ml), and sonicated cell suspensions, was measured at various concentrations of BCECF-AM by following the BCECF fluorescence development in time. The initial BCECF-AM conversion rates (in arbitrary units/s) were determined by linear regression of the fluorescence data over the first 5 s after addition of the probe.

TMA-DPH efflux measurements in whole cells

TMA-DPH from cells was measured via two methods: (i) direct measurement of the amount of membrane-associated TMA-DPH; (ii) indirect measurement of TMA-DPH in a cell-free supernatant. In the direct efflux method, the fluorescence development upon addition of TMA-DPH (100 nM final concentration) to a washed cell suspension (0.1 mg protein/ml) was followed in time. The fluorescence intensity was recorded using excitation and emission wavelengths of 350 and 425 nm, and slit widths of 5 and 10 nm, respectively. The indirect TMA-DPH efflux experiment is based on the rapid separation of the cells from the supernatant. A cell suspension (0.2 mg protein/ml) in 50 mM Tris–HCl, pH 6.5, containing 20 mM KCl plus 5 mM $MgSO_4$ was incubated for 1 h with 1 μM TMA-DPH at room temperature. Samples (750 μl) were taken from the suspension at times indicated, and cells removed by centrifugation. From the cleared supernatant, 450 μl was carefully pipetted and mixed with 50 μl of a 10% SDS solution. The TMA-DPH fluorescence in this solution is linear with the probe concentration within the range of 0 to 2 μM TMA-DPH (data not shown). The TMA-DPH concentration was estimated from a linear calibration curve. The cell-associated amount of TMA-DPH concentration was calculated from the difference between the total amount of TMA-DPH added, and the actual external TMA-DPH concentration, assuming a specific internal volume of 3.6 μl/mg of cell protein (Poolman *et al.*, 1987). For the calculation of probe partitioning in the membrane, a 5 nm thickness of the cytoplasmic membrane and a 50% occupation with proteins was assumed.

Fluorescence energy transfer experiments in liposomes

The fluorescence properties of TMA-DPH were studied in liposomes. To identify the location of TMA-DPH during the partitioning into the phospholipid bilayer, a model system was used consisting of DOPG/DOPC liposomes (3/1) containing 0.5 mol% of the fluorescent phospholipid NBD-PE. The fluorescence resonance energy transfer between TMA-DPH and NBD-PE was measured using excitation and emission wavelengths of 350 nm and 540 nm, respectively.

Materials

BCECF-AM, TMA-DPH, TMAP-DPH, DMA-DPH, DPH-CA and DPX were obtained from Molecular Probes Inc., Eugene, OR, USA. Stock solutions of the fluorescence probes were prepared in dimethyl formamide or in dimethylsulfoxide. NBD-PE was obtained from Avanti Polar Lipids, Inc., Birmingham, AL, USA.

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