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Functional characterization of a microbial aquaglyceroporin

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The major intrinsic proteins (MIPs) constitute a widespread membrane channel family essential for osmotic cell equilibrium. The MIPs can be classified into three functional subgroups: aquaporins, glycerol facilitators and aquaglyceroporins. Bacterial MIP genes have been identified in archaea as well as in Gram-positive and Gram-negative eubacteria. However, with the exception of *Escherichia coli*, most bacterial MIPs have been analysed by sequence homology. Since no MIP has yet been functionally characterized in Gram-positive bacteria, we have studied one of these members from *Lactococcus lactis*. This MIP is shown to be permeable to glycerol, like *E. coli* GlpF, and to water, like *E. coli* AqpZ. This is the first characterization of a microbial MIP that has a mixed function. This result provides important insights to reconstruct the evolutionary history of the MIP family and to elucidate the molecular pathway of water and other solutes in these channels.

Keywords: glycerol transport, water transport, *Lactococcus lactis*

INTRODUCTION

The major intrinsic protein (MIPs) constitute a widespread membrane channel family, essential for osmotic cell equilibrium, that has been identified in bacteria, fungi, protozoa, insects, plants and mammals (Preston *et al*., 1992; Maurel, 1997; Froger *et al*., 1998; Agre *et al*., 1998; Borgnia *et al*., 1999a). The MIPs can be classified into three functional subgroups: aquaporins (AQPs), glycerol facilitators (GlpFs) and aquaglyceroporins.

Aquaporins are highly specific for water. The most studied aquaporin, AQP1, has been analysed by electron crystallography and a three-dimensional reconstruction at 0·38–0·4 nm resolution has been obtained (Ren *et al*., 2000; Murata *et al*., 2000). AQP1 is a homotetramer of 28 kDa subunits, each containing six transmembrane helices.

Glycerol facilitators are permeable to glycerol or small uncharged molecules. The crystal structure of the *E. coli* glycerol facilitator (GlpF) has been resolved at 0·22 nm by X-ray crystallography (Fu *et al*., 2000). GlpF crystallizes as a symmetric arrangement of four channels with three glycerol molecules in each.

Abbreviation: MIP, major intrinsic protein.

As expected from their sequence similarities, AQPs and GlpFs exhibit a similar structural organization. However, differences in the channel-lining side chains and the residues at the narrowest parts of the channels create two different environments which should be responsible for the channel selectivity.

Aquaglyceroporins, such as AQP3, AQP7and AQP9, describe a new class of water channels which are also permeable to glycerol, but to a lesser degree than GlpF (Echevarria *et al*., 1994; Ishibashi *et al*., 1994; Ma *et al*., 1994; Ishibashi *et al*., 1997; Kuriyama *et al*., 1997; Ishibashi & Sasaki, 1998; Tsukagushi *et al*., 1998). Aquaglyceroporins are of particular interest for the investigation of the molecular basis of selectivity for both water and solutes and to address the question of a distinct molecular mechanism for such mixed channels.

Using statistical sequence analysis we have pointed out that only few key residues could distinguish aquaporins from glycerol facilitators and thus could contribute to their functional properties (Froger *et al*., 1998; Delamarche, 2000). This finding was supported by an experimental approach where a substitution of two key residues in an aquaporin abolished water transfer and conferred selectivity to glycerol associated with monomerization of the protein (Lagrée *et al*., 1999).

To bring new insights to elucidating the determination

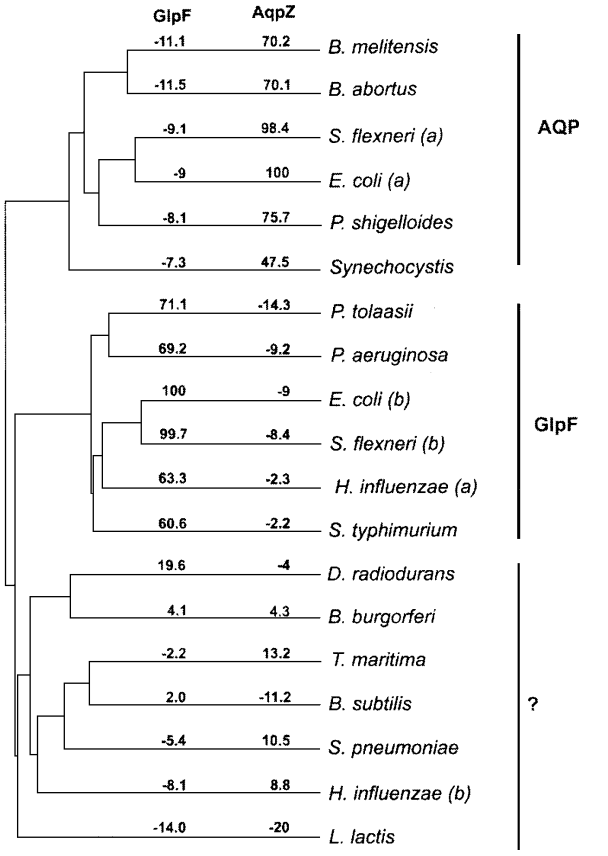

Fig. 1. Sequence comparison analysis. Sequences were extracted from the EMBL and SWISS-PROT databases. *Brucella melitensis* (accession no. AAF73105), *Brucella abortus* (AAF36396), *Shigella flexneri* (O68874, a; P31140, b), *E. coli** (P48838, a; P11244, b), *Plesiomonas shigelloides* (Baa85015), *Synechocystis* sp.* (P73809), *Pseudomonas tolaasii* (O83003), *Pseudomonas aeruginosa* (Q51389), *Haemophilus influenzae** (P44826, a; O86231, b), *Salmonella typhimurium* (P37451), *Deinococcus radiodurans** (Aaf11483), *Borrelia burgorferi* (O51256), *Thermotoga maritima* (AE001795), *Bacillus subtilis** (P18156), *Streptococcus pneumoniae* (P52281) and *L. lactis* (P22094). Asterisks indicate genomes that have been entirely sequenced. The similarity scores with *E. coli* GlpF and AqpZ are presented above each branch.

of MIP specificity, it is of primary importance to analyse the structural and functional properties of homologues, chimaeras, mutants and particularly members bearing unconventional functional properties. Presently, only a few microbial MIPs have been studied functionally (Maurel *et al*., 1994; Calamita *et al*., 1995, 1998; Delamarche *et al*., 1999; Borgnia *et al*., 1999b, Calamita, 2000) and most microbial members of the MIP family have been functionally classified by sequence homology (Hohmann *et al*., 2000). Thus, the physiological roles of prokaryote MIP channels are still largely undefined.

A multiple sequence alignment analysis conducted between bacterial members of the MIP family separates the sequences into three major clusters, one corresponding to aquaporins, one to glycerol facilitators and a third to a subgroup not yet correlated to a defined function, suggesting that some microbial MIPs bear unorthodox functional properties (Fig. 1).

A MIP gene (accession no. P22094) from the Gram-positive bacterium, *Lactococcus lactis*, has been cloned (Nardi *et al*., 1991; Mayo *et al*., 1991). Here we have studied glycerol and water transport properties of this *L. lactis* MIP in two heterologous expression systems: a bacterial one, an *E. coli aqpZ*$^-$ *glpF*$^-$ strain, and a eukaryotic one, *Xenopus laevis* oocytes. We demonstrate that the *L. lactis* MIP transports both glycerol and water and, thus, is the first microbial MIP described that has a mixed function.

METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. The ORF1 coding region of *L. lactis* was amplified by PCR from plasmid pTIL2 (Nardi *et al*., 1991). The resulting PCR band (952 bp) was subcloned into the *Bam*HI and *Bgl*II sites of pUC18 and pXβG-ev1 to give pUC-Llac and pSP-Llac, respectively. In the constructs pUC-glpF and pUC-Llac (Table 1), the expression of the cloned genes is under the control of the lactose promoter.

Growth analysis. Bacterial strains were routinely plated on LB or M9 agar medium in the presence of 50 μg ampicillin ml^{-1} (Sambrook *et al*., 1989). For overnight liquid cultures, bacterial strains were grown aerobically at 30 °C in M9 modified minimal medium (M9: 0·2% Casamino acids, 100 μg ampicillin ml^{-1}) supplemented with maltose (10 mM). Growth was monitored by measuring the OD_{600} of the cultures in M9 modified medium supplemented with glycerol (2 mM).

Glycerol transport assays in *E. coli*. Glycerol transport assays were performed as described by Sweet *et al*. (1990) with the following modifications. Bacteria were grown in M9 modified medium containing maltose (10 mM) at 30 °C to an OD_{600} of 0·3. Cells were harvested, pelleted, washed twice with M9 and then resuspended in M9. Assays were performed at room temperature with 6×10^8 cells at a final volume of 500 μl M9 containing 0·3 μM [U-^{14}C]glycerol (final activity 5·92 Gbq mmol^{-1}; Amersham). After 1 min of incubation, cells were vacuum-filtered through 0·45 μm cellulose nitrate membrane filters (Whatman), washed with 2 ml cold M9 and the radioactivity was counted.

Glycerol transport assays in *Xenopus* oocytes. Plasmids pSP-glpF, pSP-aqpZ and pSP-Llac were linearized with *Xba*I and transcribed with T3 RNA polymerase by means of the mCAP cRNA capping kit (Stratagene). Stage VI *Xenopus* oocytes were microinjected with 40 nl water for controls or with *in vitro* mRNA transcripts (1 μg μl^{-1}) and incubated in OR2 buffer (Le Cahérec *et al*., 1996) for 48–72 h at 16–18 °C.

At 48–72 h after microinjection, the oocytes were incubated in OR2/2 supplemented with 85 mM glycerol to adjust the osmolarity to 176 mosM and with [U-^{14}C]glycerol (final activity 0·3 Mbq ml^{-1}). After 10 min, the oocytes were rapidly rinsed four times in 2 ml ice-cold solution (half strength solution of OR2 supplemented with 85 mM glycerol) and lysed in 10% SDS at room temperature. Radioactivity was measured by using a liquid scintillation counter.

Swelling of *Xenopus* oocytes. Osmotic water permeability (P_f) was measured from the time course of oocytes swelling in

Table 1. Terminology of bacterial strains and plasmids used in this paper

Strain or plasmid	Description	Source or reference
***E. coli* K-12 derivatives**		
JM103	Δ(*lac-pro*) *thi strA supE endA sbcB*15 *hsdR*4 (F′ *traD36 proAB lacI*^{q} Δ*lacZ*M15)	Messing *et al.* (1981)
GD236	JM103 *glpF*$^-$	Sweet *et al.* (1990)
MM294	F$^-$ *endA1 hsdR17* (r_K^-, m_K^-) *supE44 thi-1*	Meselson & Yuan (1968)
SK46	MM294 *glpF*$^-$ *aqpZ*$^-$; Km^r Sm^r	G. Calamita, unpublished
Plasmids		
pUC18	Cloning vector; Ap^r	Yanisch-Perron *et al.* (1985)
pUC-glpF	*glpF* in pUC18	Lagrée *et al.* (1998)
pUC-Llac	*L. lactis* MIP in pUC18	This study
pX*β*G-ev1	Cloning vector; Ap^r	Preston *et al.* (1992)
pSP-glpF	*glpF* in pX*β*G-ev1	Lagrée *et al.* (1998)
pSP-Llac	*L. lactis* MIP in pX*β*G-ev1	This study
SP-aqpZ	pX*β*G-aqpZ = *aqpZ* in pX*β*G-ev1	Calamita *et al.* (1995)

response to a threefold dilution of extracellular OR2. To calculate the activation energy (E_a) the P_f was measured at three different temperatures, 10, 20 and 30 °C, as described previously (Le Cahérec *et al.*, 1996).

Cryoelectron microscopy. *E. coli* SK46, either with or without the plasmid pUC-Llac, was grown overnight in M9 modified minimal medium supplemented with maltose (10 mM). These cultures were diluted and grown at 37 °C until the exponential phase (OD_{600} = 0·8) in M9 containing maltose. The bacteria were then pelleted rapidly and resuspended in M9 (240 mosM) at room temperature. A 2·5 μl drop of the cell suspension was placed directly on a copper grid coated with a thin carbon film, upon which osmotic challenges were performed. Osmotic upshocks were induced by rapidly mixing 2·5 μl of a 1·2 M sucrose-M9 solution with the cell suspension on the grid (final osmolarity 1000 mosM). After 10 s the grid was briefly blotted with filter paper and plunged into liquid ethane held at liquid nitrogen temperature. Specimens were examined at −170 °C in a Philips CM12 microscope with a Gatan model 626 cryoholder (Delamarche *et al.*, 1999). Micrographs were recorded on Kodak SO 163 film under low-dose conditions at a nominal magnification of 6300.

Sequence analysis. The MIP sequences, retrieved from the EMBL and SWISS-PROT databases, were aligned with PILEUP or CLUSTAL W (Devereux *et al.*, 1984; Thompson *et al.*, 1994). Computing was performed using Infobiogen resources (http://www.infobiogen.fr). A score (*i*, *j*) is the sum of the elementary scores between two aligned sequences *i* and *j*, using the BLOSUM matrix (Henikoff & Henikoff, 1992). By default, a score of 8 is attributed for the gap insertions. The similarity scores presented in Fig. 1 were calculated at Infobiogen with the program EDTALN: percentage score (*i*, *j*) = 100*{score (*i*, *j*)/max [score (*i*, *i*), score (*j*, *j*)]}.

RESULTS

Expression of *L. lactis* MIP in *E. coli* KO strains

Different glycerol concentrations between 0·5 and 5 mM were used to determine the optimal concentration of glycerol required to distinguish the growth rates of strain GD236 (*glpF*$^-$) expressing a glycerol channel or not. A concentration of 2 mM glycerol increased the growth rate of the mutant strain transformed with pUC-glpF (Fig. 2a). A similar result was obtained with the mutant strain transformed with pUC-Llac. These

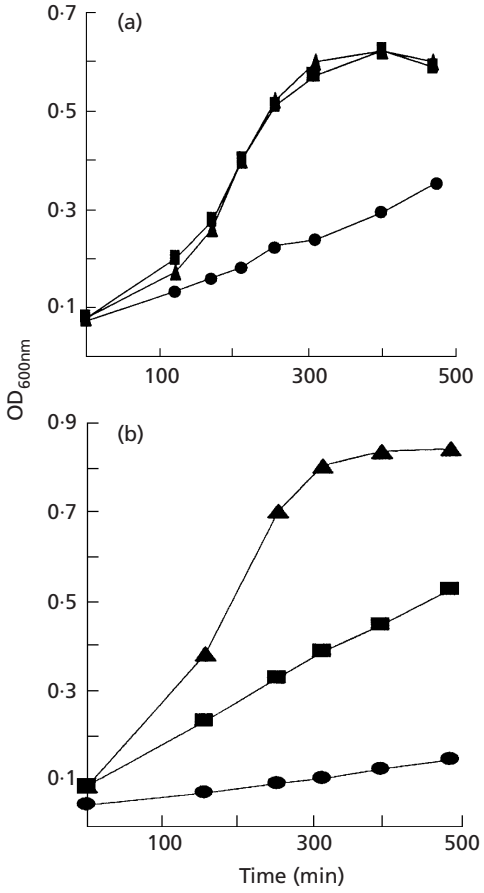

Fig. 2. Complementation experiments in M9 modified medium supplemented with glycerol (2 mM). Growth curve of *E. coli* strains GD236 (*glpF*$^-$) (a) and SK46 (*glpF*$^-$ *aqpZ*$^-$) (b), transformed with pUC18 (●), pUC-glpF (■) or pUC-Llac (▲).

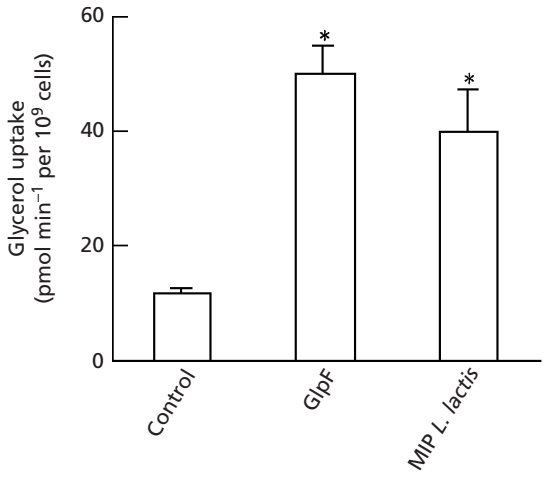

Fig. 3. Glycerol uptake in *E. coli* strain GD236 (*glpF*$^-$) transformed with pUC18 (control), pUC-glpF (GlpF) and pUC-Llac (MIP *L. lactis*). The values represent a mean of three measurements for three independent experiments (±SD) (*$P <$ 0·001 versus control).

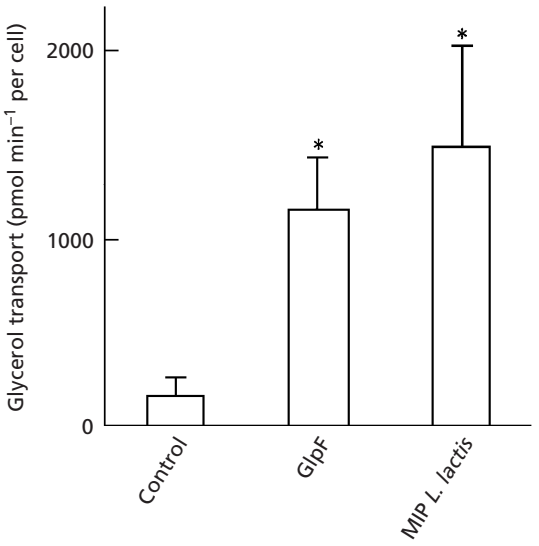

Fig. 4. Glycerol transport in *Xenopus* oocytes. [^{14}C]Glycerol uptake in oocytes injected with water (control), *E. coli* GlpF and *L. lactis* MIP cRNAs (20 measurements±SD) (*$P < 0.01$ versus control).

growth curves are similar to those obtained with the wild-type strain JM103 in the same medium (data not shown). As expected, the control cells, strain GD236 (*glpF*$^-$), reached the same final density as the cells expressing a glycerol channel, but at a slower rate. Strain SK46 (*glpF*$^-$ *aqpZ*$^-$) was not able to grow using only 2 mM glycerol as carbon source (Fig. 2b). The expression of *E. coli* GlpF, from pUC-glpF, stimulated the growth of this strain, but did not restore the growth level obtained when expressed in strain GD236. Comparatively, the expression of *L. lactis* MIP had the same effect on the growth of SK46 as did pUC-Llac or pUC-glpF on GD236 (Fig. 2a). Thus, pUC-Llac complements the double mutation (*glpF*$^-$ *aqpZ*$^-$) in *E. coli* and we suggest that *L. lactis* MIP is involved in glycerol transport as well as water transport.

L. lactis MIP is a glycerol facilitator

E. coli strain GD236 (*glpF*$^-$) transformed with vector pUC18 or with pUC-glpF was used in assays of glycerol uptake under steady-state conditions. In the absence of GlpF there was little uptake of glycerol (Fig. 3). pUC-glpF complemented the transport defect and resulted in a final glycerol uptake level four- to fivefold higher than the control level. pUC-Llac had the same effect on glycerol uptake. The K_m was found to be 20 μM glycerol for strain GD236 expressing the *L. lactis* MIP, a value close to 22·3 μM for the wild-type strain JM103 (data not shown). This is consistent with glycerol transport catalysed by *L. lactis* MIP expressed in *E. coli*.

Expression of heterologous proteins into *Xenopus* oocytes was used as a reference system for the functional studies of MIPs. cRNAs corresponding to *L. lactis* MIP and *E. coli* GlpF were injected into *Xenopus* oocytes and the cells were assessed for permeability to glycerol. The glycerol uptake (P_{gly}) of oocytes was measured in the presence of [^{14}C]glycerol. Oocytes injected with *L. lactis* and *E. coli* MIP cRNA showed a large increase in [^{14}C]glycerol uptake compared to control oocytes. The glycerol uptake of oocytes expressing *L. lactis* MIP was 1484 pmol per min per cell and 1149 pmol per min per cell for oocytes expressing *E. coli* GlpF (Fig. 4). The corresponding calculated P_{gly} values were $6{\cdot}2 \times 10^{-6}$ cm s^{-1} for oocytes expressing *L. lactis* MIP and $4{\cdot}8 \times 10^{-6}$ cm s^{-1} for oocytes expressing *E. coli* GlpF. Thus, the *L. lactis* MIP is a glycerol facilitator imparting permeability to glycerol equivalent to *E. coli* GlpF.

L. lactis MIP is a water channel

E. coli strain SK46 (*glpF*$^-$ *aqpZ*$^-$) containing plasmid pUC-Llac was subjected to a hyperosmotic shock and observed by cryoelectron microscopy. *E. coli* cells expressing the *L. lactis* MIP showed retraction of the cytoplasm, thus forming plasmolysis spaces (Fig. 5b). Under the same conditions no shrinkage was observed in control cells (SK46) lacking the MIP plasmid (Fig. 5a). These results demonstrate that *L. lactis* MIP is responsible for the outward direct water flux and, thus, constitutes an efficient water channel.

The permeability to water of *L. lactis* MIP was calculated by measuring the swelling of cRNA-injected oocytes submitted to hypotonic shock. The P_f value of *L. lactis* MIP cRNA-injected oocytes was 10-fold higher than the P_f of water-injected oocytes or oocytes expressing *E. coli* GlpF and had the same magnitude as

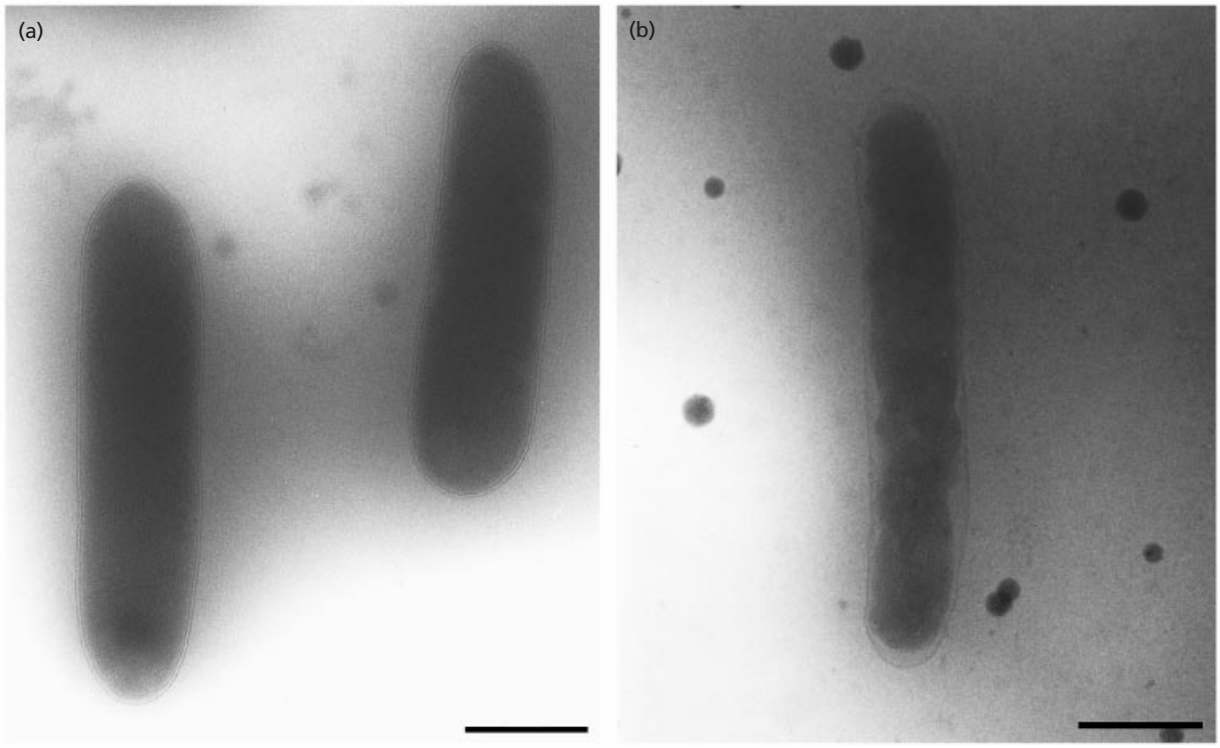

Fig. 5. Cryoelectron micrographs of *E. coli* SK46 following a 10 s hyperosmotic shock. (a) *E. coli* SK46, an AqpZ$^-$ and GlpF$^-$ null mutant. (b) *E. coli* strain SK46 expressing *L. lactis* MIP. Bars, 1 μm.

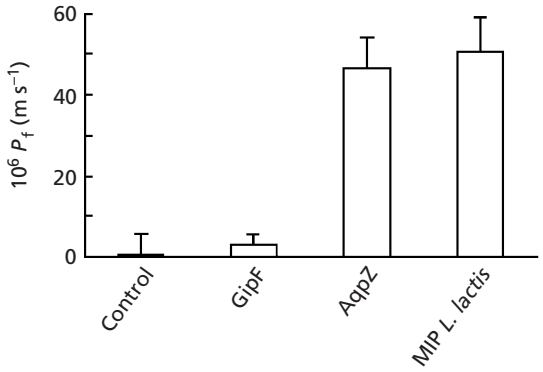

Fig. 6. Osmotic water permeability of *Xenopus* oocytes injected with water (control), *E. coli* GlpF, *E. coli* AqpZ and *L. lactis* MIP cRNAs (20 measurements ± SD).

oocytes expressing *E. coli* AqpZ (Fig. 6). Incubation of cRNA-injected oocytes in $HgCl_2$ did not lower the P_f value (data not shown), a result consistent with the absence of cysteine in *L. lactis* MIP. The Arrhenius activation energy (E_a) was calculated from P_f values obtained by monitoring oocyte swelling at 10, 20 and 30 °C (data not shown). The E_a was 4·76 kcal mol^{-1}, a value within the range expected for a typical aquaporin and higher than that of control oocytes.

DISCUSSION

One way to understand the structural/functional relationships in MIPs resides in the resolution of their three-dimensional structure at the atomic level. However, such structural studies are limited by the need to produce significant amounts of purified wild-type and mutant proteins. A promising alternative resides in gathering knowledge about the MIP family by searching for new members, analysing their functions and the effect of selected mutations. In this paper we present the functional characterization of a novel microbial MIP possessing permeability to both glycerol and water. We have named the protein Gla_{Llac}, for glycerol facilitator-aquaporin of *L. lactis*.

E. coli uses glycerol as a carbon source for glycolysis and for lipid biogenesis. Glycerol enters the cytoplasm by passive diffusion across the lipid bilayer (Sweet *et al.*, 1990) or by facilitative diffusion mediated by GlpF (Heller *et al.*, 1980). The *E. coli* glycerol facilitator, GlpF, has been shown to selectively transport glycerol and not water or ions (Maurel *et al.*, 1994). GlpF contributes directly to bacterial growth as illustrated by the complementation experiments presented in this paper. When expressed in *E. coli*, Gla_{Llac} can play a role in bacterial growth like *E. coli* GlpF. We have therefore analysed the transport of glycerol mediated by Gla_{Llac} both in bacteria and in *Xenopus* oocytes. We found that Gla_{Llac} displays the same characteristics as *E. coli* GlpF

for glycerol transport, allowing us to conclude that Gla_{Llac} is a glycerol facilitator.

So far, AqpZ, the aquaporin of *E. coli*, is the only bacterial water channel which has been extensively functionally studied (Calamita *et al*., 1995, 1998; Delamarche *et al*., 1999; Borgnia *et al*., 1999b; Scheuring *et al*., 1999; Ringler *et al*., 1999; Calamita, 2000). Although puzzling questions on the physiological necessity of fast water transport in bacteria remain, it appears that aquaporins could be directly involved in cell proliferation (Calamita *et al*., 1998). This is supported by our observations in *E. coli*, in which two null mutations in *glpF* and *aqpZ* obviated growth. Growth was partly restored when *E. coli* GlpF was expressed and was completely restored with the expression of Gla_{Llac}. This suggests that Gla_{Llac} can mimic AqpZ function to restore growth in bacteria. Such complementation experiments using *E. coli* strain SK46 can be used to test the function of bacterial MIPs. We previously characterized bacterial aquaporins using cryoelectron microscopy and *E. coli* as an expression system (Delamarche *et al*., 1999; Rodriguez *et al*., 2000). In the present study we show that Gla_{Llac} significantly mediates water fluxes. Moreover, the water channel properties of Gla_{Llac} were demonstrated when the protein was expressed in *Xenopus* oocytes. The calculated P_f for Gla_{Llac} has the same magnitude as AqpZ. Moreover the low activation energy calculated for oocytes expressing Gla_{Llac} corresponds to that of a water channel. Therefore Gla_{Llac} is a mixed channel, like aquaglyceroporins described in mammals, and the first one to be characterized in bacteria. Unlike mammalian aquaglyceroporins (Kuriyama *et al*., 1997; Tsukagushi *et al*., 1998; Echevarria *et al*., 1996), Gla_{Llac} imparts a high permeability to glycerol to the cell membrane.

Protein sequence alignments can be used to predict the function of an MIP (Froger *et al*., 1998; Delamarche, 2000). In Fig. 1, a high score between two sequences suggests that the two corresponding proteins have a similar function. For Gram-negative bacteria, the scores suggest that glycerol and water transport are assumed to occur independently by two distinct channels. We propose that Gram-positive bacteria contain a single MIP that possesses the two functions of glycerol facilitator and water channel. Recent studies on *Bacillus subtilis*, another Gram-positive bacterium, have confirmed this prediction (A. Froger & C. Delamarche, unpublished). According to the key residues predicted to distinguish the functional subgroups of MIP (Froger *et al*., 1998), it can be noted that the sequences of the third group bears the signature of glycerol channels. Thus, it would be interesting to define other key residues or motifs that could determine the properties of mixed MIPs. For example, among residues that interact with glycerol in the GlpF channel (Fu *et al*., 2000), the proline residue at position 246 of *E. coli* GlpF is found in all the glycerol facilitator group defined in Fig. 1. Intriguingly, this proline is substituted by a glycine residue in all the sequences of the third group of putative mixed channels. In mammalian aquaglyceroporins, this proline is also substituted by a leucine, alanine, methionine or phenylalanine.

The determination of the structure of GlpF has been a major factor in the elucidation of the mechanism of selective permeability for glycerol. However, the proposed mechanism for water transport by AQP1 still requires a higher resolution structure. Moreover, the molecular mechanism for mixed channels has still to be cleared up. Analysis of factors affecting the specificity of mixed channels conducted together with high resolution structural studies should provide some key answers to this phenomenon. In that way Gla_{Llac} from *L. lactis* should be an interesting tool for solving this problem.

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