Functional characterization of a microbial aquaglyceroporin

Alexandrine Froger, Jean-Paul Rolland, Patrick Bron, Valérie Lagrée, François Le Cahérec, Stéphane Deschamps, Jean-François Hubert, Isabelle Pellerin, Daniel Thomas and Christian Delamarche

Author for correspondence: Christian Delamarche. Tel.: +33 2 99 28 61 22. Fax: +33 2 99 28 14 77. e-mail: christian.delamarche@univ-rennes1.fr

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.

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, AQP7 and 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
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 pTII2 (Nardi et al., 1991). The resulting PCR band (952 bp) was subcloned into the Bam HI and Bgl II sites of pUC18 and pXGl-G-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 OD₆₀₀ 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 OD₆₀₀ of 0·3. Cells were harvested, pelleted, washed twice with M9 and then resuspended in M9. Assays were performed at room temperature with 6 × 10⁶ cells at a final volume of 500 µl M9 containing 0·3 µM [U⁻¹³C]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 XbaI 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 Cañére 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 osmolality to 176 mosM and with [U⁻¹³C]glycerol (final activity 0·3 Mbq ml⁻¹). After 10, 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) was measured from the time course of oocytes swelling in...
Table 1. Terminology of bacterial strains and plasmids used in this paper

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Source or reference</th>
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<tr>
<td><strong>E. coli</strong> K-12 derivatives</td>
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<tr>
<td>JM103</td>
<td>Δ(lac-pro) thi strA supE endA sbcB15 hsdR4 (F’ traD36 proAB lacF ΔlacZM15)</td>
<td>Messing et al. (1981)</td>
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<tr>
<td>GD236</td>
<td>JM103 glpF-</td>
<td>Sweet et al. (1990)</td>
</tr>
<tr>
<td>MM294</td>
<td>F- endA1 hsdR17 (rK, mC) supE44 thi-1</td>
<td>Meselson &amp; Yuan (1968)</td>
</tr>
<tr>
<td>SK46</td>
<td>MM294 glpF- aqpZ- ; Km' Sm'</td>
<td>G. Calamita, unpublished</td>
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<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>pUC18</td>
<td>Cloning vector; Ap'</td>
<td>Yanisch-Perron et al. (1985)</td>
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<tr>
<td>pUC-glpF</td>
<td>glpF in pUC18</td>
<td>Lagree et al. (1998)</td>
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<td>pUC-Llac</td>
<td>L. lactis MIP in pUC18</td>
<td>This study</td>
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<tr>
<td>pXP-gfp</td>
<td>Cloning vector; Ap'</td>
<td>Preston et al. (1992)</td>
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<td>pSP-glpF</td>
<td>glpF in pXP-G-ev1</td>
<td>Lagree et al. (1998)</td>
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<td>pSP-Llac</td>
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<td>This study</td>
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response to a threefold dilution of extracellular OR2. To calculate the activation energy (Ea) the P, E was measured at three different temperatures, 10, 20 and 30 °C, as described previously (Le Cahérez 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 (OD600 nm = 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, 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 EDITALN: 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
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 Km 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 (Pgly) of oocytes was measured in the presence of [14C]glycerol. Oocytes injected with L. lactis and E. coli MIP cRNA showed a large increase in [14C]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 Pgly values were 6.2 × 10−6 cm s−1 for oocytes expressing L. lactis MIP and 4.8 × 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 Pw value of L. lactis MIP cRNA-injected oocytes was 10-fold higher than the Pw of water-injected oocytes or oocytes expressing E. coli GlpF and had the same magnitude as
oocytes expressing *E. coli* AqpZ (Fig. 6). Incubation of cRNA-injected oocytes in HgCl₂ 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 476 kcal mol⁻¹, a value within the range expected for a typical aquaporin and higher than that of control oocytes.

**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).

**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 Glα$_{Llac}$, 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*, Glα$_{Llac}$ can play a role in bacterial growth like *E. coli* GlpF. We have therefore analysed the transport of glycerol mediated by Glα$_{Llac}$ both in bacteria and in *Xenopus* oocytes. We found that Glα$_{Llac}$ displays the same characteristics as *E. coli* GlpF.
for glycerol transport, allowing us to conclude that \( \text{Gla}_{\text{Llac}} \) is a glycerol facilitator.

So far, AqpZ, the aquaporin of \( \text{E. coli} \), is the only bacterial water channel which has been extensively functionally studied (Calamita et al., 1995, 1998; Delamarche et al., 1999; Borgia et al., 1999b; Scheurig 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 \( \text{E. coli} \), in which two null mutations in \( \text{glpF} \) and \( \text{aqpZ} \) obviated growth. Growth was partly restored when \( \text{E. coli} \) GlpF was expressed and was completely restored with the expression of \( \text{Gla}_{\text{Llac}} \) This suggests that \( \text{Gla}_{\text{Llac}} \) can mimic AqpZ function to restore growth in bacteria. Such complementation experiments using \( \text{E. coli} \) strain SK46 can be used to test the function of bacterial MIPs. We previously characterized bacterial aquaporins using cryoelectron microscopy and \( \text{E. coli} \) as an expression system (Delamarche et al., 1999; Rodriguez et al., 2000).

In the present study we show that \( \text{Gla}_{\text{Llac}} \) significantly mediates water fluxes. Moreover, the water channel properties of \( \text{Gla}_{\text{Llac}} \) were demonstrated when the protein was expressed in \( \text{Xenopus} \) oocytes. The calculated \( P_l \) for \( \text{Gla}_{\text{Llac}} \) has the same magnitude as AqpZ. Moreover the low activation energy calculated for oocytes expressing \( \text{Gla}_{\text{Llac}} \) corresponds to that of a water channel. Therefore \( \text{Gla}_{\text{Llac}} \) 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), \( \text{Gla}_{\text{Llac}} \) 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 \( \text{Bacillus subtilis} \), 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 bear 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 \( \text{E. coli} \) 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 \( \text{Gla}_{\text{Llac}} \) from \( \text{L. lactis} \) should be an interesting tool for solving this problem.

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