A functional water channel protein in the pathogenic bacterium *Brucella abortus*

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The gene for a new bacterial aquaporin, AqpX, was cloned from the pathogenic Gram-negative bacterium *Brucella abortus*. The gene was mapped on the large chromosome of *B. abortus*. It is flanked by one upstream and two downstream copies of the *Brucella* repeated sequence Bru-RS. Prediction from the nucleotide sequence indicated that the protein is a member of the MIP family, which comprises channels for water and/or solute transport. Expression in *Xenopus* oocytes and cryoelectron microscopy of *Escherichia coli* cells transformed with the *aqpX* gene confirmed that the protein is an efficient water channel. Glycerol uptake experiments in *E. coli* also showed that the protein is not able to transport glycerol.

**Keywords**: aquaporin, water channel, *Brucella abortus*

INTRODUCTION

Bacterial cells must be able to adapt to rapid changes in their aqueous environments. Numerous mechanisms of adaptation have been studied, and some bacterial responses, such as synthesis and transport of compatible solutes, pH compensating reactions and specific regulatory cascades, are already well characterized. However, water movement across membranes, a phenomenon which should be common to all the above mechanisms, has remained until recently unstudied, on the basis that free water movement through membrane lipid bilayers was the only pathway of water transport in bacteria.

Aquaporins belong to the MIP (major intrinsic protein) superfamily of membrane proteins (Pao et al., 1991). All the members of this group are small proteins of about 250–300 amino acids that share a common topology, consisting of a transmembrane domain formed by six hydrophobic α-helices. These α-helices form a hydrophilic channel through which polar substances can diffuse (Walz et al., 1997). MIP proteins are classified into three groups according to their substrate specificity, namely aquaporins, glycerol facilitators and glycerol–aquaporins. Aquaporins are ubiquitous proteins that transport water but not other small solutes (Verkman & Mitra, 2000). The best known glycerol facilitator is the GlpF protein of *Escherichia coli*. GlpF does not transport water; however it is capable of transporting other small polyalcohols such as erythritol in addition to glycerol (Heller et al., 1980). Glycerol–aquaporins can transport both water and glycerol, and in some cases they may also mediate the transport of small molecules such as urea (Ishibashi et al., 1994). Moreover, the transport of gas molecules (CO₂) through aquaporin channels has also been discussed (Nakhoul et al., 1998).

The genus *Brucella* is composed of several species of Gram-negative animal pathogens differing mainly in their preferred host. They belong to the α-proteobacterial class, whose members often associate both with plants and with animals. *Brucella abortus* preferentially infects cattle and other ungulates. Infection of pregnant animals by *B. abortus* usually results in abortion and one clinical sign of such infection is the presence of erythritol in the placenta (Lowrie & Kennedy, 1972; Smith et al., 1962). This observation was correlated with the reported ability of *E. coli* GlpF to transport erythritol and was one of the reasons which led us to undertake the search for a MIP protein in *Brucella*. *B. abortus* is a facultative intracellular para-
site; this lifestyle is very different from that of E. coli but close to that of Rhizobium, a symbiont of plant root nodules. The presence of the MIP protein nodulin-26 in the peribacteroid membrane of soybean root nodules infected with Rhizobium (Miao & Verma, 1993) suggested a possible role of MIP proteins in the biology of intracellular parasites. Thus we considered that a MIP protein in Brucella could be involved either in erythritol transport or in the growth of the bacterium inside phagocytic vacuoles.

Here, we describe the cloning and characterization of an aquaporin, AqpX, from B. abortus. This finding adds to the previous description of an aquaporin in E. coli (Calamita et al., 1997). Functional studies of the role of AqpX in this different genus will be useful in determining the role of these proteins in bacterial physiology.

METHODS

Bacterial strains and growth conditions. The B. abortus strain 2308 was obtained from the INRA collection (Institut National de la Recherche Agronomique, Noutzilly, France). B. abortus was grown on Brucella agar (BA) or in Brucella broth (BB) (Pronadisa). E. coli K-12 strain DH5α (Life Technologies) was used as recipient in cloning experiments. The host for glycerol uptake experiments was the E. coli K-12 strain OSBR1 (glpF::TnphoA). This strain behaves as a glpFglpK double mutant, probably via the polar effect of a single transposon insertion in the glpF gene (Sanders et al., 1997). The E. coli K-12 glpFapqZ strain SK46 (G. Calamita, unpublished), a derivative of E. coli MM294 (Meselson & Yuan, 1968), was used for cryoelectron microscopy. M9 minimal medium, containing M9 salts with 2 mM MgSO4, 0.1 mM CaCl2 and 0.2% Casamino acids, was used to grow the E. coli strains. M9 medium was supplemented with 0.4% glucose and 10 mM maltose or 2 mM glycerol depending on the experiment. Selective media included ampicillin or kanamycin at final concentrations of 100 and 50 µg ml−1, respectively.

Plasmids. Bluescript SK (Stratagene Cloning Systems) and pUC (Vieira & Messing, 1982) were used as general purpose cloning vectors in E. coli. The pGEM-T vector (Promega) was used for cloning of PCR fragments. The plasmid vector pXPG-ev1 (Preston et al., 1992), which contains the β-globin transcription control region including the 5′ and 3′ untranslated regions, was used for expression studies in Xenopus laevis oocytes, pUCGla, pUCAqpZ and pUCGlPF contained, respectively, the full-length coding sequence of the glycerol-aquaporin from Lactococcus lactis (A. Froger, unpublished), aquaporin AqpZ from E. coli and the glycerol facilitator GlpF from E. coli inserted into pUC19 (Vieira & Messing, 1982).

DNA purification and sequencing, and other DNA manipulations. Standard DNA purification and manipulation methods were performed essentially as described by Sambrook et al. (1989). Purification of genomic DNA was performed by the guanidinium thiocyanate method (Pitcher et al., 1989). Plasmid DNA prepared by standard alkaline lysis was further purified using either the MiniPrep Express Matrix (Bio101) or the Plasmid Midi kit (Qiagen). DNA from agarose gels was purified using the Qiaquick Gel Extraction kit (Qiagen). DNA sequencing was performed on double-strand templates by cycle sequencing using Texas-red labelled primers in an automatic Vistra sequencer.

Southern blot analysis. Chromosomal DNA from B. abortus was partially or totally digested with EcoRI and HindIII, electrophoresed in a 0.9% agarose gel and transferred by capillarity to positively charged nylon membranes (Roche Diagnostics). The blots were hybridized with a 399 bp internal sequence fragment of aqpX labelled with digoxigenin (Roche Diagnostics) and washed three times with 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.2 (SSC) at 42 °C, once with 0.1× SSC at 42 °C, and once more with 0.1× SSC containing 0.1% SDS at 65 °C. Washed blots were incubated with alkaline phosphatase labelled anti-digoxigenin antibody and developed using the luminescent substrate disodium-3-[(4-methoxyspiro[1,2-dioxetane-3,2'-5'-(chboro)tricyclo-[3.3.1.13.7]decane]-4-yl)phenyl phosphate (CSPD; Roche Diagnostics).

For chromosomal mapping of the gene, DNA was isolated and digested in agarose blocks with restriction endonucleases PaeI and SpeI. Fragments were separated by PFGE and hybridized as described by Jumas-Bilak et al. (1997).

Expression and water transport studies in Xenopus oocytes. For expression in oocytes, appropriate inserts were cloned into the plasmid vector pXPG-ev1 (Preston et al., 1992). Capped cRNAs were synthesized in vitro using T3 RNA polymerase after plasmid linearization with XbaI. Defolli- culated X. laevis oocytes (stage V–VI) were injected with 50 nl water or up to 200 ng sample cRNAs and incubated in modified Barth’s solution (osmolality 200 mosM) at 18 °C (Le Caherec et al., 1996). After incubation for 2–5 d, the oocytes were transferred to hypoosmotic modified Barth’s solution (osmolality 70 mosM) at 18 °C. Oocyte swelling was monitored by videomicroscopy and the coefficient of osmotic water permeability (P) was determined (Preston et al., 1992). To calculate the Arrhenius activation energy (Ea), the P was measured for four different temperatures between 16 and 32 °C.

Glycerol transport. The capability of the different gene products to transport glycerol was assayed in the E. coli strain OSBR1 (glpF). Bacterial cultures were grown overnight at 30 °C in M9 medium supplemented with maltose (10 mM). Cultures were then diluted to an OD600 of 0.08 in the same medium and allowed to grow at 30 °C to an OD600 of 0.3. Cells were harvested, pelleted and washed twice with M9 medium.

Assays were performed at room temperature with 6 × 106 cells in a final volume of 500 µl M9 medium containing 1 µM [U-14C]glycerol (Amersham) at a final specific activity of 1.77 GBq mmol−1. After 1 min incubation, cells were vacuum-filtered through 0.45 µm pore diameter cellulose nitrate membrane filters (Whatman), washed with 2 ml cold M9 medium and their radioactivity counted.

Cryoelectron microscopy. Overnight cultures of E. coli SK46 containing the appropriate plasmid in M9 maltose medium were diluted in M9 glucose and grown at 37 °C to the exponential phase of growth (OD600 0.8) in M9 medium supplemented with glucose. Bacteria were then rapidly pelleted and resuspended in M9 medium (osmolality 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 up-shocks were induced by rapidly mixing 2.5 µl 12 M sucrose–M9 solution with the cell suspension on the grid (final osmolality 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).
Aquaporin from *Brucella abortus*

Micrographs were recorded on Kodak SO163 film under low-dose conditions at a nominal magnification of × 6300.

**Computer-assisted sequence analysis and comparison.** We used the BLAST programs (Altschul et al., 1997) for sequence comparison against the databases at the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/). The sequence alignment was done with the CLUSTAL W (Thompson et al., 1994) program at the Pole Bioinformatique Lyonnais website (http://pbil.ibcp.fr). Prediction of protein hydrophobicity and transmembrane regions was performed with the TMPRED program at the swiss EMBlnet web site (http://www.ch.embnet.org/pages/services.html).

**RESULTS**

**Cloning of the aqpX gene**

Chromosomal DNA from *B. abortus* strain 2308 was used as template in a PCR reaction with degenerated primers designed from the two conserved ‘NPA’ sequences of the MIP family (Verkman & Mitra, 2000). The primer F1 (TCN GGC GCS CAY CTN AAY CCN) corresponded to amino acids 58–68 and 182–190, respectively, of the *E. coli* aqpZ sequence. Agarose gel electrophoresis of the PCR products showed a main band of about 400 bp. The total PCR products were cloned into the pGEM-T vector (Promega). Ten colonies containing inserts of 400–600 bp were selected and inserts were sequenced. Four of these clones contained an identical 399 bp cloned sequence. A BLASTN search using this sequence revealed high similarity with the *E. coli* aqpZ gene and with other MIP family members. The 399 bp fragment was used as a probe to detect a genomic fragment containing the complete gene from the *B. abortus* 2308 chromosomal DNA. A single EcoRI hybridization band of 3·6 kb was observed under high-stringency conditions. This fragment was cloned in pBluescript SK (Promega) and the resulting plasmid was named pAQPX1. A restriction map of this plasmid was obtained with different restriction endonucleases and is shown in Fig. 1. Most of the restriction fragments from pAQPX1 were subcloned and sequenced on both strands. A continuous sequence of 2276 bp was determined containing a 687 bp ORF near the left end of the cloned fragment. The predicted protein sequence corresponds to 228 amino acids with a calculated Mr of 23100. The ORF was very similar to members of the MIP family. The highest similarity was nearly 70% with *E. coli* AqpZ. The sequence similarities with AQP1 and with nodulin-26 was 48% and 44%, respectively (Fig. 2). Based on this analysis, we named this new ORF of *B. abortus* aqpX. The GenBank accession number of this sequence is AF148066.

Hydrophobicity analysis and prediction of transmembrane regions and orientation indicated that the
translated protein had six hydrophobic segments that potentially span the cell membrane. By analogy with the structure of AqpZ and other members of the MIP family, these segments may be arranged as a transmembrane domain, with the N- and C termini of the protein located in the cytoplasm (data not shown). Examination of the DNA sequence flanking the aqpX gene did not reveal the presence of any other associated genes. Instead, we found three copies of Bru-RS, a repeated palindromic sequence previously described in Brucella (Halling & Bricker, 1994). The first copy, Bru-RX1, is located at the 5′ side of the gene. It belongs to the Bru-RS class 2 and is truncated at its 5′ end. The second copy, Bru-RX2, is located immediately after the aqpX gene, belongs to the Bru-RS class 1 and is truncated at its 3′ end. The third and only complete copy, Bru-RX3, is located 261 bp downstream of aqpX and also belongs to Bru-RS class 2. The sequence of these repeats aligned with the original Bru-RS1 and Bru-RS2 is shown in Fig. 3. The 3–6 kb insert of plasmid pAQPX1 was used as a probe to locate the aqpX gene in the B. abortus genome. This experiment localized the aqpX gene to a 260 kb SpeI fragment of the large chromosome of B. abortus (data not shown).

**Fig. 3.** Alignment of the three B. abortus repeats flanking the aqpX gene, Bru-RX1 (BRURX1), Bru-RX2 (BRURX2) and Bru-RX3 (BRURX3), with prototype sequences Bru-RS1 (BRURS1) and Bru-RS2 (BRURS2).

**Fig. 4.** Transport of water in oocytes injected with aqpX cRNA. (a) Osmotic water permeability of oocytes expressing B. abortus aquaporin X ( ), or E. coli aquaporin Z ( ), and of control oocytes injected with water ( ) was measured for 5 d following cRNA injection. (b) Swelling of oocytes after 4 d expression of the following aquaporins: L. lactis glyceroaquaaporin (Gla), E. coli aquaporin Z (AqpZ) or B. abortus aquaporin X (AqpX). Swelling of oocytes injected with water was also measured as a negative control. Data shown in (a) and (b) are means of 5 and 18, respectively, independent measurements ± SD.

Water transport in *Xenopus* oocytes

A fragment of pAQPX1 containing the entire aqpX ORF was amplified using primers F2 (GTC AGA TCT GTA TCA CTA CAC GAA) and R2 (GGC GGA TCC TTC TGA TTA ATC TCG), selected from the DNA sequence. The PCR product was digested with BamHI and BglII and ligated into the BglII site of the *Xenopus* expression vector pXβG-ev1. cRNA corresponding to aqpX was injected into *Xenopus* oocytes and assessed for permeability to water 3–5 d later (Fig. 4a). The osmotic water permeability ($P_f$) was calculated after 4 d, a time at which there was a good compromise between transport activity and cell viability. Oocytes injected with aqpX cRNA exhibited a marked increase in $P_f$, as also did oocytes injected with cRNA from the genes aqpZ of *E. coli* and gla from *L. lactis*, whereas control oocytes injected with water did not show any increase in water permeability (Fig. 4b).

AqpX-expressing oocytes exhibited an Arrhenius activation energy of $3.8 ± 1.5$ kcal mol$^{-1}$ ($15.9 ± 6.3$ kJ mol$^{-1}$), a value similar to that obtained with other aquaporins and significantly lower than that of control injected oocytes. Incubation of oocytes expressing AqpX in HgCl$_2$ did not significantly lower $P_f$ (data not shown), a result consistent with the lack of a cysteine residue in the predicted loop regions.
Glycerol transport

The ability of *B. abortus* AqpX to facilitate glycerol uptake was assayed in the *E. coli* glpF strain OSBR1 containing the plasmid pUCAqpX, which contains the *aqpX* ORF cloned into the plasmid pUC18. This strain did not transport any radioactive glycerol, whilst the positive controls, *E. coli* OSBR1 (pUCGlpF) and *E. coli* OSBR1 (pUCGla) incorporated significant amounts of radioactivity under the same assay conditions (Fig. 5).

Cryoelectron microscopy

*E. coli* strain SK46 (*glpFaqpZ*) containing the plasmid pUCaqpX was subjected to an hyperosmotic shock and observed under the electron microscope. *E. coli* cells expressing the *B. abortus* aquaporin AqpX showed dramatic cytoplasm retraction, thus forming plasmolytic spaces. Under the same conditions, no shrinkage was observed in control cells devoid of any aquaporin (Fig. 6). These results demonstrate in a straightforward manner that AqpX is responsible for outwardly directed water flux and thus constitutes an efficient water channel.

DISCUSSION

The discovery of specific water channel proteins as components of biological membranes brings a new insight into problems related to water homeostasis. A bacterial aquaporin has been previously described and analysed in *E. coli* (Calamita et al., 1995). In other bacteria, their presence has been deduced from the available genomic data (Hohmann et al., 2000). Analysis of the available bacterial genomic sequences suggests that many bacteria (*Mycobacterium tuberculosis*, *Bacillus subtilis*, *Mycoplasma genitalium*, etc.) have no chromosomal aquaporin genes. The sequence for an aquaporin from *Brucella melitensis*, nearly identical to *B. abortus* aquaporin AqpX, appeared recently in GenBank (accession no. AF226624). This probably indicates that aquaporins are present in all the species of the genus *Brucella*. This irregular distribution of aquaporins among prokaryotes suggests that these proteins are not essential in bacteria. AqpZ-defective mutants of *E. coli* are viable, corroborating that AqpZ is not essential. However, experimental data available for the

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**Fig. 5.** Glycerol transport in *E. coli* OSBR1 cells containing different MIP genes. Bars show the amount of glycerol incorporated by cells expressing the following MIP proteins: *E. coli* glycerol facilitator (GlpF); *E. coli* aquaporin Z (AqpZ); *L. lactis* glyceroaquaporin (Gla); or *B. abortus* aquaporin X (AqpX). *E. coli* OSBR1 containing plasmid pUC19 was used as a negative control. Data shown are means of three independent measurements ±SD.

**Fig. 6.** Cryoelectron micrographs of *E. coli* SK46 following a 10 s hyperosmotic shock. (a) *E. coli* SK46, an aqpZ and glpF null bacterium. (b) *E. coli* strain SK46 expressing *B. abortus* AqpX. Bar, 1 µm.
E. coli aquaporin AqpZ demonstrate that the gene is better expressed under hypoosmotic conditions. E. coli aqpZ mutants appear to grow poorly under these conditions (Calamita et al., 1998). These results indicate that aquaporins, when present, play a role in bacterial physiology.

The aqpX gene from B. abortus was cloned and its sequence revealed a close homology with aqpZ from E. coli. The gene was flanked by three copies of a palindromic repeated sequence of Brucella (Halling & Bricker, 1994). The function of these sequences, similar to ERIC sequences of E. coli (Hulton et al., 1991), has not yet been determined, and the meaning of this association is also unknown. We have been unable to identify sequences similar to consensus transcription promoters or terminators. However, as previously reported for the E. coli gene, we suspect that the B. abortus aqpX gene forms a single transcriptional unit. The deduced amino acid sequence of AqpX has already suggested that the protein is a water channel belonging to the MIP protein family (Froger et al., 1998). Expression studies in Xenopus oocytes and in an aqpZ null E. coli strain clearly demonstrated that B. abortus AqpX was able to form active water channels in the cell membrane. The specificity for water was confirmed using E. coli mutants defective in glycerol transport containing plasmids expressing AqpX. The primers used in the PCR experiment to clone the aqpX gene were designed to recognize the conserved regions of all MIP proteins, including aquaporins and glycerol facilitators. However, we never obtained any amplification fragment corresponding to a glycerol facilitator using E. coli DNA as the template. Furthermore, secondary hybridization bands were never observed when Brucella chromosomal DNA was hybridized with probes from the B. abortus aqpX gene under low-stringency conditions. Similar hybridization experiments using probes from the E. coli aqpZ and glpF genes were equally negative (data not shown). These negative results seem to indicate that there is no GlpF-like protein in B. abortus, an unexpected finding which leaves unsolved the question relating to the molecular transport pathway for both glycerol and erythritol into B. abortus. The possibility of a phosphotransferase-linked transport system was discarded, at least for erythritol, since it is known that the first step in erythritol catabolism is the phosphorylation of the polyalcohol by a kinase whose gene has been recently identified (Sangari et al., 2000; Sperry & Robertson, 1975).

One of the possible roles for a MIP protein in a bacterium living inside a vacuole is to contribute to the acquisition of nutrients. In the case of B. abortus AqpX, this option should be disregarded given its specificity for the transport of water. Thus, a role specifically linked to water transport, such as adaptation to variation in intravacuolar pH or osmolarity, should be expected for this aquaporin. The construction of aqpX mutants will be of great help to determine the biological role of the aquaporin of Brucella.

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