The aquaporin gene \(aqpX\) of \textit{Brucella abortus} is induced in hyperosmotic conditions

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An aquaporin gene \((aqpX)\) was previously detected in the pathogenic bacterium \textit{Brucella abortus}. Earlier studies showed that AqpX mediated rapid and large water fluxes in both directions in response to sudden osmotic up- or downshifts. Here, to study the role and the expression of the \(aqpX\) gene in \textit{B. abortus}, an \(aqpX\) null mutant was constructed using an \(aqpX::\text{lacZ}\) gene fusion. This mutant showed no significant difference in growth rate compared to the wild-type strain when grown in rich and minimal media, demonstrating that disruption of the \(aqpX\) gene was not lethal for \textit{B. abortus}. The role of the \textit{B. abortus} AqpX water channel was investigated by exposing the cells to hypo- and hyperosmolar conditions. While in hyperosmolar environments the growth rate of the knockout mutant was not affected, in hypo-osmolar conditions this mutant showed reduced viability after 50 h of growth. \(\beta\)-Galactosidase assays and RT-PCR revealed that \(aqpX\) gene expression and the amount of \(aqpX\) mRNA were markedly increased in hyperosmolar conditions. Moreover, \textit{B. abortus} \(aqpX\) expression levels were enhanced during the mid-exponential phase of growth. These results indicated that the expression of \(aqpX\) was regulated during the growth curve and induced in hyperosmolar conditions. This report is believed to be the first example of the induction of a bacterial aquaporin in hypertonic conditions.

INTRODUCTION

The availability of water is one of the most important environmental factors affecting the survival and growth of micro-organisms. Changes in the external osmolarity immediately activate fluxes of solutes and water along the osmotic gradient. If uncontrolled, such fluxes could result in changes in the osmotic conditions of the cytoplasm and in changes in turgor which may end in the swelling and bursting of the cell in hypotonic environments or in plasmolysis and dehydration under hypertonic conditions. Both cytoplasm osmotic condition and cell turgor must remain within strict limits to allow molecular mechanisms to proceed (Wood, 1999). Micro-organisms actively respond to variations in the osmolarity of their habitat to avoid osmotically driven damage. This response, generally referred to as osmoregulation or osmoadaptation, is aimed at maintaining cellular physiology. Micro-organisms have developed different strategies for maintaining turgor under hyperosmotic conditions. The most common response to hyperosmotic stress is the intracellular accumulation of osmotically active compounds that are congruous with cellular functions, the so-called osmoprotectants and compatible solutes. In addition to solutes, water flows through the membranes of all living cells by two distinct mechanisms. Simple diffusion across the lipid bilayer is usually sufficient to balance solute levels, but a more efficient water transit is achieved by diffusion through water-selective channel proteins. These selective channels, named aquaporins, have been identified in a wide variety of organisms, including animals, plants, fungi and bacteria (Calamita \textit{et al.}, 1995; Carrey et al., 2001; Kroger \textit{et al.}, 2001; Preston \textit{et al.}, 1992; Santoni \textit{et al.}, 2000). Aquaporins belong to the major intrinsic protein (MIP) superfamily of membrane proteins (Hohmann \textit{et al.}, 2000; Pao \textit{et al.}, 1991). MIP proteins have been classified into three groups according to their substrate specificity: aquaporins (AQPs), a subset of proteins highly selective for water; glycerol facilitators (GlpFs), possessing a channel permeable to glycerol and small uncharged molecules; and aquaglyceroporins, a group of proteins permeable to both water and glycerol (Heller \textit{et al.}, 1980; Ishibashi \textit{et al.}, 1994; Verkman \textit{et al.}, 1995). Recently, the \textit{Escherichia coli} glycerol facilitator, GlpF, has been shown to be also permeable to water but less so than to glycerol (Borgia & Agre, 2001).

The physiological role of the different mammalian aquaporins has been studied in detail, mostly by study of animal
models deficient in individual aquaporins. These animals showed diverse phenotypes including lens cataract (Shiels & Bassnett, 1996), loss of the red cell Colton blood group antigens (Agre et al., 1995), nephrogenic diabetes insipidus (Deen et al., 1994) and incomplete renal fluid concentration (Agre, 2000). In plants, aquaporins are involved in numerous processes such as transpiration, root water uptake, maintenance of cell turgor and inhibition of self-pollinization (Ikeda et al., 1997; Johansson et al., 2000). In contrast, the physiological role of aquaporins in bacteria is still undefined. Most of the available biological information on bacterial aquaporins derives from the study of the E. coli AqpZ, the first known bacterial aquaporin (Calamita et al., 1995). The functional characterization of AqpZ demonstrated water selectivity without evidence of glycerol transport. Furthermore, cryoelectron microscopy of osmotically shocked cells demonstrated that AqpZ mediated water transport into and out of the cell (Delamarche et al., 1999). In spite of the proven functionality of E. coli aquaporin and the availability of some structural studies on the protein (Borgnia et al., 1999; Calamita et al., 1997), the physiological importance of AqpZ was only apparent when the bacteria were cultured in hypo-osmolar medium and at maximum growth rates (Calamita et al., 1998).

We have previously isolated and characterized the aquaporin of Brucella abortus, a facultative intracellular pathogen causing infertility and abortion in cattle and a debilitating disease in man. Amino acid sequence alignment between B. abortus and E. coli aquaporins is still undefined. Most of the available biological information on bacterial aquaporins derives from the study of the E. coli AqpZ, the first known bacterial aquaporin (Calamita et al., 1995). The functional characterization of AqpZ demonstrated water selectivity without evidence of glycerol transport. Furthermore, cryoelectron microscopy of osmotically shocked cells demonstrated that AqpZ mediated water transport into and out of the cell (Delamarche et al., 1999). In spite of the proven functionality of E. coli aquaporin and the availability of some structural studies on the protein (Borgnia et al., 1999; Calamita et al., 1997), the physiological importance of AqpZ was only apparent when the bacteria were cultured in hypo-osmolar medium and at maximum growth rates (Calamita et al., 1998).

We have previously isolated and characterized the aquaporin of Brucella abortus, a facultative intracellular pathogen causing infertility and abortion in cattle and a debilitating disease in man. Amino acid sequence alignment between the B. abortus AqpX and other MIP family proteins revealed that B. abortus AqpX was 70% identical to E. coli aquaporin AqpZ. AqpX expression in Xenopus oocytes and cryoelectron microscopy of E. coli aqpZ mutants transformed with the B. abortus aqpX gene confirmed that the aquaporin from B. abortus was an efficient water channel. Furthermore, it has been shown that AqpX was not able to transport glycerol (Rodriguez et al., 2000).

The present study was designed to characterize the expression of the B. abortus aqpX gene during the phases of the bacterial growth curve and under different osmotic conditions, and to investigate the physiological function of the AqpX water channel. Our studies showed that expression of the aqpX gene was induced in hypertonic conditions and it was enhanced during the mid-exponential growth phase. Additionally, we demonstrated that aqpX gene was not essential for bacterial survival. To our knowledge, these studies provide the first example of hypertonic induction of a bacterial aquaporin.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** Bacterial strains and plasmids used in this study are listed in Table 1. Brucella strains were grown at 37°C in Brucella broth (BB) or on Brucella agar (BA). Brucella minimal medium (BMM) contained, per litre, 5 ml lactic acid, 5 g l-glutamic acid, 7.5 g NaCl, 10 g K2HPO4, 0.1 g Na2SO4 and 0.5 g yeast extract. Growth was monitored by measuring the OD600 of the cultures. E. coli strains were grown overnight at 37°C in Luria–Bertani (LB) broth with orbital shaking. E. coli S17.1 was used as donor in bacterial conjugations. When necessary the following antibiotics were added to the indicated final concentration: kanamycin (50 µg ml⁻¹), ampicillin (50 µg ml⁻¹), nalidixic acid (10 µg ml⁻¹) and chloramphenicol (25 µg ml⁻¹). Media were made hypertonic by adding NaCl to increase the concentrations up to 125, 250, 500 or 750 mM. Media were made hypotonic by diluting them with water (50% and 33%). Osmalities of the media were measured with an osmometer (Osmo Station OM-6050, Menarini).

**Recombinant DNA techniques.** DNA manipulations were performed by standard procedures (Sambrook et al., 1989). Synthetic oligonucleotides were purchased from Gibco-BRL. PCR reactions were performed using Taq DNA polymerase (OM-6050, Menarini).

**Table 1.** Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Characteristics</th>
<th>Reference</th>
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<td><strong>Strains</strong></td>
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<td>E. coli DH5α</td>
<td>endA1 recA1 gyrA96 thi-1 hsdR17 supE44 relA1 (argF–lacZYA)U169 #80d lacZΔ</td>
<td>Bethesda Research Laboratories</td>
</tr>
<tr>
<td>E. coli S17.1</td>
<td>recA thi pro his hsdR17 supE44 relA1</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td>B. abortus 2308 Nx'</td>
<td>Wild-type, virulent strain</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>aqpX::lacZ-Km</td>
<td>2308 Nx' aqpZ null mutant</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pBluescript SK(+)</td>
<td>Cloning vector, Ap'</td>
<td>Stratagen</td>
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<tr>
<td>pSU4111</td>
<td>Vector carrying a promoterless lacZ-Km fusion</td>
<td>Moncalian et al. (1997)</td>
</tr>
<tr>
<td>pAQPX1</td>
<td>3·6 kb EcoRI fragment containing the B. abortus aqpX gene cloned into pBluescript SK</td>
<td>Rodriguez et al. (2000)</td>
</tr>
<tr>
<td>pAQP2</td>
<td>3·0 kb BamHI/HindIII fragment containing the B. abortus aqpX promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pSKaqpX::lacZ-Km</td>
<td>5·3 kb HindIII fragment containing a promoterless lacZ-Km gene fusion cloned into pAQP2</td>
<td>This study</td>
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<td>pAQPXlacZ-Km</td>
<td>8·2 kb EcoRV fragment containing the aqpX-lacZ-Km gene fusion cloned into pKOK.4</td>
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were performed using *Taq* DNA polymerase (Bioline or Roche Diagnostic). Restriction and modification enzymes (Promega or Roche Diagnostics) were used according to the manufacturer’s instructions.

**Plasmid constructions.** Plasmid pAQPXI contains the *B. abortus* *aqpX* gene cloned in the high-copy-number vector pBluescript SK (Stratagene) (Rodriguez et al., 2000). A 2-3 kb fragment from pAQPXI containing the putative promoter region and the first eight codons of the *B. abortus aqpX* gene was obtained by PCR, using synthetic oligonucleotides T3 (5′-ATTAACCCCTACTAAAGGGA-3′) and ATG1 (5′-GCACCTCCATCCGATATTGTCAAA-3′). A second PCR using synthetic oligonucleotide TAA2 (5′-TGCAAGC-TTTGCAATCATTGAAGGGG-3′) and ATG1 (5′-TAATCGACTCACTATGGG-3′) was carried out to amplify a 0.6-2 kb DNA fragment containing the last 12 codons of the *aqpX* gene and additional downstream sequence. The primers ATG1 and TAA2 both contain HindIII restriction sites. The first PCR product was digested with BamHI/HindIII and the second fragment was digested with EcoRV/HindIII. Both fragments were ligated together into pBluescript SK digested with BamHI/HinII, to generate plasmid pAQP2. This plasmid was linearized with HindIII and ligated with a 5-3 kb HindIII fragment, from pSU4111, containing a promoterless lacZ-Km cassette, to generate pSKapqX::lacZ-Km. The *aqpX::lacZ-Km* fusion was obtained from plasmid pSKapqX::lacZ-Km as an 8.2 kb KpnI/XbaI fragment. This DNA fragment was blunt-ended with Klenow DNA polymerase and ligated into the EcoRV site of pKOK4, a suicide vector mobilizable in *Brucella*. The resulting plasmid was named pAQPXlacZ-Km (Fig. 1).

**Construction of a *B. abortus* aqpX::lacZ-Km chromosomal gene fusion.** Insertional disruption of the *aqpX* gene in *B. abortus* 2308 Nxa was performed by allelic exchange. The plasmid pAQPXlacZ-Km was introduced into *B. abortus* 2308 Nxa from *E. coli* S17.1 by conjugation and the transconjugants were selected on plates containing kanamycin and nalidixic acid. Kanamycin-resistant colonies that were susceptible to chloramphenicol were selected as交换ed candidates. The disruption of the *aqpX* gene was confirmed by Southern blot hybridization.

**Southern blot analysis.** Chromosomal DNA from *B. abortus* 2308 Nxa, and from colonies putatively containing a gene replacement, was separately digested with EcoRI and *Hind*III, electrophoresed in a 0.9 % agarose gel, and transferred to positively charged nylon membranes (Roche Diagnostics) by capillarity. The blots were probed with digoxigenin (Roche Diagnostics). After hybridization, the membranes were washed, incubated with alkaline-phosphatase-labelled anti-digoxigenin antibodies and developed using the luminescent substrate diosodium 3-(4-methoxyxipiro[1,2-]
dioxethane-3,2′-(5′-chloro)tricyclo[3.3.1.1^5^7^9^]decan]-4-yl)phenylphosphate (CSPD, Roche Diagnostics).

**β-Galactosidase assays.** β-Galactosidase assays were carried out with whole cells as described by Miller (1972). Saturated cultures in BB were diluted to reach OD600=0-1 and then grown at 37 °C as appropriate. β-Galactosidase activity was expressed as μmol 2-nitrophenyl β-d-galactopyranoside cleaved min⁻¹ (mg protein)⁻¹ (Miller, 1972). Each assay was performed at least four times independently, and the results were averaged for display as bar graphs.

**Primer extension.** Synthetic oligonucleotide primer E94 (5′-AA-CACCGAGGAAGCCGATACCCAG-3′) was 5′-end labelled with T4 polynucleotide kinase and [-32P]ATP. Total RNA was prepared from *B. abortus* cultures with the High Pure RNA Isolation Kit (Roche Diagnostic). One microgram of RNA was used as template for the synthesis of cDNA with M-MLV (Moloney Murine Leukaemia Virus) reverse transcriptase (Gibco-BRL) from the E94 labelled primer. The products of the extension reactions were analysed in 6 % urea-polyacrylamide gels. A sequencing ladder using the same primer was run in the gel alongside the extension products to map the 5′ end of the mRNA.

**RT-PCR.** The reverse transcription assay was used to quantify the levels of *aqpX* mRNA in different osmolarity conditions. Briefly, 200 ng total RNA was mixed with 10 μl double-strand reverse transcriptase buffer containing 0.8 μM of each dNTP, 4 μM Agpsed. R primer (5′-GGCGAATTCTTCTGATTAATCTCGGCC-3′), starting 6 nt downstream from the *aqpX* stop codon, and 0.2 units μl⁻¹ of RNasin RNase inhibitor (Promega). The samples were incubated for 5 min at room temperature to allow the primer to anneal the template. For cDNA synthesis, 5 units RNasin and 10 units M-MLV reverse transcriptase were added and then the samples were incubated for 60 min at 42 °C. Amplification was performed by adding 5 μl of the reverse transcriptase reaction mixtures containing cDNA to 20 μl amplification buffer containing 0.2 mM of each dNTP, 0.25 μM of the forward and reverse primers and 1 unit *Taq* DNA polymerase (Qiagen). In these assays RT-AMP.R (5′-GGCGAATTCTTCTGATTAATCTCGGCC-3′), starting 10 nt upstream from the *aqpX* termination codon, was used as the reverse primer, and Agpsed.F (5′-GGCG-TATCCCCATGTGGAAGAAAATT-3′) as the forward primer. After 1 min incubation at 94 °C, samples were subjected to 35 amplification cycles (30 s at 94 °C, 30 s at 43 °C and 60 s at 72 °C), followed by a final incubation at 72 °C for 8 min. The reaction products were resolved in 1 % agarose gels run at 5 V cm⁻¹ and quantified using the Molecular Analyst Software (Bio-Rad).
To normalize the aqpX cDNA bands, we used the gene for the translation initiation factor IF-1 of B. abortus, whose expression has recently been demonstrated to be constitutive (Eskra et al., 2001). The RT-PCR assay was performed as described above. The primer RT-IF.R (5'-TGAAGCAGGTAGGATGCGG-3') was used for the reverse transcription assay. For the PCR amplification step we used the primers IF-1.F (5'-ATGGCGAAAGAAGATTCCT-3') and IF-1.R (5'-ACTAGAACCTTGTCACC-3'), giving a specific product of 164 bp (Eskra et al., 2001).

RESULTS

Characterization of the B. abortus aqpX::lacZ-Km chromosomal gene fusion

The aqpX gene in B. abortus 2308 was disrupted by replacing most of the aqpX coding region by a promoterless lacZ-Km cassette, resulting in the aqpX null mutant strain with the lacZ gene under the control of the aqpX promoter (Fig. 1). This construct was made on a plasmid as described in Methods and then introduced into the B. abortus chromosome by standard gene replacement methods. The replacement of the chromosomal region was confirmed by Southern blot hybridization with two probes, a lacZ-Km probe and a 3-6 kbp EcoRI fragment containing the aqpX gene. Southern blot analysis with the lacZ-Km probe produced a 5-3 kbp HindIII band for the B. abortus aqpX::lacZ-Km mutant but not from B. abortus 2308. Additionally, the 3-6 kbp aqpX probe hybridized to a 3-6 kbp band from EcoRI-digested B. abortus 2308 genomic DNA, and to 2-9 and 2-3 kbp fragments from B. abortus aqpX::lacZ-Km as expected (data not shown).

Growth characteristics of the Brucella aqpX::lacZ-Km mutant

B. abortus 2308 and the mutant B. abortus aqpX::lacZ-Km were grown in BB (Fig. 2) and minimal media (data not shown) with different osmolarity conditions. When the optical density was measured, no significant differences were observed between the growth rate of the mutant and the wild-type strain under any condition. However, the number of c.f.u. of the B. abortus aqpX::lacZ-Km strain decreased during growth in hypo-osmolar medium at the late stationary phase (Fig. 2a, right panel). These results demonstrate that disruption of the B. abortus aqpX gene was not lethal and that the production of the aquaporin was apparently necessary for survival at the late stationary phase in hypo-osmolar medium only. On the other hand, no apparent differences in cell morphology were observed under the light microscope between the mutant and the wild-type cells under the different growth conditions (data not shown).

Growth regulation of aqpX expression

β-Galactosidase measurements were performed to evaluate the pattern of aqpX expression along the growth curve (from early exponential to late stationary phase) of B. abortus aqpX::lacZ-Km cultures grown in BB (299 mosmol kg⁻¹). β-Galactosidase activity rose steadily to reach a peak during the mid-exponential phase of growth (OD₆₀₀ ≈ 0.8), then decreased slightly to a level that was maintained up to the late stationary phase (Fig. 3). The same result was obtained in BMM (264 mosmol kg⁻¹) (data not shown).

![Fig. 2. Growth characteristics of the B. abortus aqpX::lacZ-Km mutant under different osmolarity conditions. B. abortus 2308 (△), and B. abortus aqpX::lacZ-Km (■) were grown in BB medium diluted 1/3 with water (a), in BB medium (b), and in BB medium plus 250 mM NaCl (c). Cell viabilities and OD₆₀₀ readings were determined from duplicate cultures. Data are the means of three separate experiments.](image-url)
β-Galactosidase activity was not detected in the wild-type strain Brucella abortus 2308.

**aqpX expression under different osmolarity conditions**

To explore whether a particular osmotic condition could cause induction of the Brucella abortus *aqpX* gene, we compared the expression levels of the *aqpX::lacZ* transcriptional fusion in hypo- and hyperosmolar conditions. *Brucella abortus* *aqpX::lacZ*-Km was incubated in BB medium, BB supplemented with 125 mM NaCl (550 mosmol kg⁻¹), 250 mM NaCl (770 mosmol kg⁻¹), 500 mM NaCl (1200 mosmol kg⁻¹) or 750 mM NaCl (1625 mosmol kg⁻¹), or BB diluted with water to 50% (150 mosmol kg⁻¹) or to 33% (100 mosmol kg⁻¹). β-Galactosidase activity was measured from samples obtained at mid-exponential phase, previously found to be the time of maximal *aqpX* expression. *Brucella abortus* *aqpX* expression levels as assessed by β-galactosidase activity were more elevated when *Brucella abortus* grew in hyperosmolar conditions (125 and 250 mM NaCl added), whereas in hypo-osmolar conditions β-galactosidase activity levels were significantly lower (Fig. 4). The highest level of expression was observed in medium with 125 mM NaCl. β-Galactosidase activity in medium made hyperosmolar by addition of 20% sucrose (815 mosmol kg⁻¹) was also elevated to a level similar to that observed in medium made hypertonic with NaCl (Fig. 4), indicating that the effect was due to the elevated osmolarity and not to the specific action of NaCl.

**Study of aqpX expression by RT-PCR**

To measure the *aqpX* mRNA level we used the RT-PCR assay. We obtained total mRNA from *Brucella abortus* cultures grown in different osmolarity conditions. An overnight culture of *Brucella abortus* 2308 in BB medium was used to inoculate BB medium supplemented with 125, 250 or 750 mM NaCl, and BB diluted 1/2 or 1/3 with water. Total RNA was obtained from each culture at OD₆₀₀=0.4, and the RNA was used to perform the RT-PCR assays. The amount of *aqpX* mRNA found with this method was more elevated when *Brucella abortus* grew in hyperosmolar conditions. The highest level of expression was observed in medium with 125 mM NaCl (Fig. 5). The RT-PCR reaction products of the aquaporin gene were quantified and normalized against those of IF-1, showing that the expression level of *aqpX* increased fourfold when the cultures were grown in BB medium supplemented with 125 mM NaCl relative to the RNA level observed in BB (Fig. 5). These results indicated that *Brucella abortus* *aqpX* expression was increased in cells grown in hyperosmolar media.

**Promoter mapping**

The transcriptional start site of the *Brucella abortus* *aqpX* gene was determined by primer extension using RNA isolated from wild-type cells. Synthetic oligonucleotide E94 (see Methods), complementary to bases 94–117 of the *aqpX* gene sequence, was used to identify the transcriptional start point. The analysis of the primer extension products, the sequence TTAACG-N₁₆-TATCCG, did not reveal a clear-cut promoter sequence. The sequence TTAACG-N₁₆-TATCCG was the closest to the consensus, found 13 nt away from the experimentally determined initiation of transcription. The primer extension assay was also useful as a quantitative method to evaluate *aqpX* expression in *Brucella abortus*. An intense extension product was obtained when the assay was performed on total RNA obtained from...
B. abortus cultures grown under hyperosmolar conditions (BB with 125 mM NaCl), whereas no extension product was observed when the assay was carried out with RNA obtained from cells grown in Brucella broth (Fig. 6a). This result confirmed the stronger expression of the aqpX gene in hyperosmolar conditions.

DISCUSSION

Aquaporins are proteins that allow rapid and massive water flux across biological membranes (Preston et al., 1992). They play important roles in the water exchanges seen in higher organisms. The presence of functional aquaporins in bacteria was recently reported (Calamita et al., 1998; Borgnia et al., 1999). However, the role of these proteins in water homeostasis in bacteria is not well understood. The presence of aquaporins in bacteria suggested that they could be involved in the osmoadaptation responses and at the same time that bacterial mutants lacking these proteins could be impaired in their adaptation to osmotic shifts. The first studies conducted in E. coli revealed that while the E. coli aquaporin was functional, its role was not evident and the mutants defective in the aqpZ gene showed only slight growth defects. Expression of the aqpZ gene was studied and found to be maximal under hypo-osmolar conditions (Calamita et al., 1998).

Recently, we have cloned the gene and characterized functionally an aquaporin water channel in the intracellular facultative bacterium B. abortus (Rodriguez et al., 2000). To determine the function of this channel, an aqpX mutant carrying a single-copy aqpX–lac fusion in the chromosome was constructed by fusing a lacZ-Km cassette with the promoter region of the aqpX gene. No significant differences in growth rates were found between the B. abortus 2308 wild-type strain and the B. abortus aqpX::lacZ-Km mutant when grown in rich and minimal medium.

Fig. 6. Analysis of the B. abortus aqpX promoter region. (a) Primer extension analysis of the aqpX gene. Primer extension products using RNA isolated from bacteria grown in BB medium (1), and BB medium plus 125 mM NaCl (2) are shown alongside a sequencing reaction of the aqpX promoter region. (b) DNA sequence around the transcriptional start site. The ATG start codon of aqpX is shown in bold. The two observed transcriptional start sites are marked with thick and thin arrows. Putative −10 and −35 boxes are underlined.
finding showed that the *aqpX* gene was not essential in *B. abortus* grown in isotonic media. However, the mutant showed decreased viability upon prolonged incubation in hypo-osmolar medium, in line with the observed osmotic regulation of the *E. coli aqpZ* gene (Calamita et al., 1995). In the case of AqpZ, the decrease in cell viability was significant only when the *E. coli AqpZ* strain was co-cultured with the wild-type parental strain in minimal media (Calamita, 2000).

Experiments with the *aqpX: lacZ-Km* transcriptional fusion showed that the *aqpX* gene was active throughout the growth curve, with an activity peak at the mid-exponential growth phase. This result was again similar to that reported for *E. coli* (Calamita, 2000; Calamita et al., 1998), and indicated that the *aqpX* gene was subject to growth phase regulation. A recent report on the regulation of expression of the *E. coli aqpZ* gene using a single-copy chromosomal *aqpZ-lacZ* fusion indicated that expression of *E. coli aqpZ* increased in the stationary phase of growth and that this increase was dependent on the RpoS sigma factor (Soupene et al., 2002).

Expression studies with our *aqpX: lacZ* transcriptional fusion as well as the RT-PCR assays demonstrated that the expression of the *aqpX* gene was increased in hyperosmolar conditions (BB plus 125 mM NaCl). Surprisingly, the *B. abortus* gene showed a behaviour different from that of the *E. coli aqpZ* gene, whose expression was reported to increase significantly in hypo-osmolar conditions, and to decrease in hyperosmolar conditions (Calamita et al., 1998), or found to be unaffected by changes in osmolality (Soupene et al., 2002).

We have also determined the transcriptional initiation point of the *Brucella aqpX* gene and found a nearby sequence with a reasonable similarity with the consensus *E. coli* σ70 promoter sequence. The distance between the −10 box and the start of transcription is longer than usual in this putative promoter. This could be due to degradation of mRNA on its 5′ side as suggested by the two extension products observed. Since the *E. coli aqpZ* gene and some osmotically regulated genes are transcribed from promoters active in the stationary phase, we also analysed the sequence for the presence of alternative promoters such as σ38 (rpoS) (Lee & Gralla, 2002) or gearbox promoters (Ballesteros et al., 1998) with negative results. On the other hand, the translation start of AqpX is 169 bp away from the transcription start site. This long region of untranslated mRNA suggests the existence of some post-transcriptional mechanism of regulation of expression of the AqpX protein.

This study adds to the previous characterization of the *E. coli aqpZ* gene to clarify some aspects of bacterial aquaporin biology. A common finding in the two organisms was that the *aqp* gene was not essential either in *Brucella* or in *E. coli*, and that the aquaporin-defective mutants did not show a major phenotype in either bacterium. This observation is consistent with the irregular distribution of aquaporins in bacteria. Many bacteria do not possess aquaporin genes in their genomes; however, they are able to respond and to adapt to different osmotic conditions. This indicates that bacteria possess mechanisms to respond to water stress that are independent of the production of aquaporins. In spite of their dispensability, some bacteria have acquired aquaporins and evolved mechanisms for their regulated expression. The presence of regulatory mechanisms for the expression of these genes has to be interpreted as the result of some evolutionary advantage conferred by the possession of aquaporins. The differences observed between *E. coli* and *B. abortus* in these regulatory details indicate that the aquaporins play different roles in these bacteria, which have different lifestyles and evolutionary histories.

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