A Novel Aquaporin 3 in Killfish (Fundulus heteroclitus) Is Not An Arsenic Channel

Dawoon Jung,* ‡⊥ † Bryce MacIver,§ Brian P. Jackson,¶ Roxanna Barnaby,* † J. Denry Sato,§ Mark L. Zeidel,§ Joseph R. Shaw,‡ || and Bruce A. Stanton* ‡⊥

*Department of Microbiology and Immunology and ‡Department of Physiology, Dartmouth Medical School, Hanover, New Hampshire 03755; †Mount Desert Island Biological Laboratory, Salisbury Cove, Maine 04672; §Nephrology Division, Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts 02215; ¶Department of Earth Sciences and ‖Department of Chemistry, Dartmouth College, Hanover, New Hampshire 03755; and ||The School of Public and Environmental Affairs, Indiana University, Bloomington, Indiana 47405

Received December 20, 2011; accepted February 1, 2012

The Atlantic killfish (Fundulus heteroclitus) is a model environmental organism that has an extremely low assimilation rate of environmental arsenic. As a first step in elucidating the mechanism behind this phenomenon, we used quantitative real-time PCR to identify aquaglyceroporins (AQPs), which are arsenite transporters, in the killifish gill. A novel homolog killifish AQP3 (kfAQP3a) was cloned from the killifish gill, and a second homolog was identified as the consensus from a transcriptome database (kfAQP3b). The two were 99% homologous to each other, 98% homologous to a previously identified killifish AQP3 from embryos (kfAQP3ts), and 78% homologous to hAQP3. Expression of kfAQP3a in Xenopus oocytes significantly enhanced water, glycerol, and urea transport. However, kfAQP3a expressed in HEK293T cells did not transport significant amounts of arsenic. All sequence motifs thought to confer the ability of AQP3 to transport solutes were conserved in kfAQP3a, kfAQP3b, and kfAQP3ts; however, the C-terminal amino acids were different in kfAQP3a versus the other two homologs. Replacement of the three C-terminal amino acids of kfAQP3 (GKS) with the three C-terminal amino acids of kfAQP3b and kfAQP3ts (ANC) was sufficient to enable kfAQP3a to robustly transport arsenic. Thus, the C-terminus of kfAQP3b and kfAQP3ts confers arsenic selectivity in kfAQP3. Moreover, kfAQP3a, the only AQP expressed in killifish gill, is the first aquaglyceroporin identified that does not transport arsenic, which may explain, in part, why killifish poorly assimilate arsenic and are highly tolerant to environmental arsenic.

Key Words: Fundulus heteroclitus; aquaporin 3; arsenic transport.

Arsenic is a toxic metalloid that occurs naturally in the environment and chronic exposure to arsenic has been linked to lung, skin, liver, and bladder cancer, cardiovascular diseases, and type 2 diabetes (Abernathy et al., 2003; Ferreccio et al., 2000). Currently, over 500 million people worldwide are exposed to arsenic in drinking water, and in the United States, 13 million people are exposed to arsenic levels in their drinking water above the Environmental Protection Agency safety standard (10 μg/l) (Heck et al., 2009) (States et al., 2011). Therefore, the World Health Organization considers arsenic the chemical of most concern to human health. In addition, in the United States, arsenic is listed as the number one chemical of concern in the Agency for Toxic Substances and Disease Registry’s list of priority pollutants in the environment (ATSDR, 2011). Exposure to arsenic in food products, notably rice, is also emerging as a health concern, and the Foods Standards Agency in the United Kingdom has advised mothers to avoid rice drinks for toddlers and young children to keep arsenic exposure as low as possible (Meharg and Raab, 2010; Rahman and Hasegawa, 2011).

Aquaglyceroporins (AQP3, AQP7, AQP9, and AQP10) are a family of membrane-bound proteins that mediate the bidirectional movement of water, urea, glycerol, and arsenic across cell membranes (Agre and Kozono, 2003; Bhattacharjee et al., 2008; Lee et al., 2006; Liu et al., 2004; Wu and Beitz, 2007). AQPs are the major transport pathway for arsenite (AsIII) uptake into cells, and zebrafish AQP3 also transports monomethylarsonate into cells (Hamdi et al., 2009). Several studies have examined arsenic assimilation and accumulation in the Atlantic killfish (Fundulus heteroclitus), a widely studied environmental model organism (Burnett et al., 2007; Dutton and Fisher, 2011a; Marshall et al., 2005; Shaw et al., 2010, 2007; Wills et al., 2010), in response to arsenic exposure (Dutton and Fisher, 2011a,b; Gonzalez et al., 2006; Shaw et al., 2010). These studies have noted that assimilation of arsenic from waterborne exposure is extremely low, and therefore, a biological effect of arsenic is seen only in killifish exposed to extremely high levels of arsenic that exceed levels found in the environment (> 1000 ppb). Since gills have a high surface area and are directly exposed to copious amounts of water, gills are considered primary sites for arsenic assimilation (Evans, 1987; Randall et al., 1998). Because the physiological basis for

© The Author 2012. Published by Oxford University Press on behalf of the Society of Toxicology. All rights reserved.
For permissions, please email: journals.permissions@oup.com
low arsenic assimilation has not been examined in killifish, the goal of this study was to identify the AQPs that are expressed in the gill and to test the hypothesis that water-borne arsenic uptake is extremely low in killifish gill, at least in part due to the low arsenic transport activity of killifish AQPs. We report that only AQP3 is expressed in killifish gill and that although this novel killifish homolog (kfAQP3a) transports water, urea, and glycerol, it does not transport arsenic. kfAQP3a is the first aquaglyceroporin identified that does not transport arsenic.

MATERIALS AND METHODS

Animals. Killifish were caught from Northeast Creek, Salisbury Cove, ME. Fish were transferred to Mount Desert Island Biological Laboratory (MDIBL) and kept in 15–17°C natural seawater (SW) under natural light for 1 month to acclimate. Some fish were kept in 10% SW for 2 weeks, then moved to freshwater (FW, 12 g/l NaHCO3, 7.5 g/l CaSO4, 7.5 g/l MgSO4, and 0.5 g/l KCl) (American Society for Testing and Materials [ASTM], 1985) for 1 month before experimental use. Fish were fed commercial flake food daily (Tetracichlid, Tetra, Blacksburg, VA). All experimental protocols were in accordance with the Institutional Animal Care and Use Guidelines of Dartmouth Medical School (10-03-03) and MDIBL (10-01).

Quantitative real-time PCR. Gills of killifish acclimated to SW or FW were isolated, and total RNA was isolated using Qiagen RNaseasy Mini Kit (Valencia, CA). The quality of RNA was determined using the Agilent Bioanalyzer (Agilent Technologies, Wilmington, DE). Only RNA with distinct 18S and 28S peaks and no degradation were processed for analyses. Complementary DNA (cDNA) was synthesized using the Promega Reverse Transcription System (San Luis Obispo, CA). Primers for AQP3, 7, and 9 were designed from a killifish contig dataset that was determined by 454 pyrosequencing of the killifish transcriptome (Shaw, Colbourne, Hampton, Chen, Stanton, in preparation) (Table 1). AQP10 was not identified in a search of the killifish transcriptome (Shaw, Colbourne, Hampton, Chen, Stanton, in preparation) (Table 1). AQP10 was not identified in a search of the killifish transcriptome. Quantitative real-time (Q-RT)-PCR was performed with a ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Known amounts of each primer, 3μM MgCl2, and 10 μl of 1X HRM Master dye, which includes reaction buffer, deoxynucleotide triphosphate mix, FastStart Taq DNA polymerase, and HRM Dye. PCR cycling conditions followed the manufacturer’s recommendations and included an initial denaturation at 95°C followed by 55 cycles of denaturing at 95°C for 10 s, annealing at 59°C for 15 s, and extension at 72°C for 30 s. Following amplification, products were ramped for 1 min each at 95°C and 40°C to achieve heteroduplex formation. To generate melt profiles, the PCR amplicons were gradually increased through a temperature range from 65°C to 95°C at a rate of 0.02°C per sec, whereas acquiring fluorescence measurements 25 times per °C. These data were normalized, temperature shifted, and melting temperature (Tm) calculated as described by Wittwer et al. (2003) using the LightCycler 480 software (v.1.5.0.39).

Mutation of C-terminal amino acids. A mutant AQP3 cDNA (kfAQP3a mut) was created from the cloned kfAQP3a so that the three amino acids in the C-terminus (GKS) would match kfAQP3b and kfAQP3ts (ANC). Mutation analysis was performed by PCR using the 18mer F1 primer (CTCCAAATCTCCACCGCC) and a 44nt reverse primer incorporating three mutations (CCCTTGGCCCTTTTTTTACGTTAGTCCCTTTGGCCCGTTGG). The mutant kfAQP3a cDNA sequence was ligated into the TopoTA pCR2.1 vector, and the sequences of individual clones were verified through Sanger sequencing.

The killifish AQP3s (kfAQP3a and kfAQP3a mut) were cut from the vector TopoTA pCR2.1 by standard molecular cloning techniques using the EcoRI site. Following incubation for 1 h with calf intestinal alkaline phosphatase, sequences were inserted into pC31.1(-) expression vector (Invitrogen). Human AQP3 (hAQP3), in the expression vector PCMV6, was purchased from Origene (Rockville, MD).

Cell arsenic uptake. Arsenic uptake was measured in HEK293T cells transfected with various AQP3 cDNAs. HEK293T cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 1mM sodium pyruvate, 50 μg/ml penicillin, 50 μg/ml streptomycin, and 2mM L-glutamine, and kept in a 5% CO2–95% air incubator at 37°C. Cells were transiently transfected with different amounts (0.3, 0.6, 0.9, and 1.0 μg) of various AQP3 cDNAs using Effectene (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Expression of transfected AQP3 was confirmed by Western blot, as described (Jung et al., 2012). Forty-eight hours after transfection, HEK293T cells were switched to media containing 2-μg/l (2 ppb) sodium arsenite. In preliminary studies, we determined that arsenic uptake was a linear function of time (0–20 min) at 2 ppb. At designated times after exposure to arsenic (15, 30, 60, 90, and 120 min) cells were washed twice with

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’-3’): Reverse primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>kfAQP3</td>
<td>CTCCACCTGGCGGCTCTT; GACAATCCAATGACCAAAAC</td>
</tr>
<tr>
<td>kfAQP7</td>
<td>GGGCAGAAAAGAAAGAAA; AGCCAAAGTTGATGCTAAGGG</td>
</tr>
<tr>
<td>kfAQP9</td>
<td>GAGGGAGGGAGGAGATGAG; AGGGTGAAACCGGATGTTGA</td>
</tr>
</tbody>
</table>
KILLFISH AQUAPORIN 3 IS NOT AN ARSENIC CHANNEL

PBS and pelleted by centrifugation at 600 × g for 10 min. Hundred microliter of optima grade HNO3 (Fisher Scientific, Pittsburg, PA) was added to the cells, which were then placed in a laboratory oven at 70°C for 2 h. The samples were then allowed to cool, and 50 μl of H2O2 was added to each sample, after which samples were exposed to another heating cycle. The digested sample was weighed and analyzed for total arsenic by inductively coupled plasma mass spectrometry (7500cx/7700x, Agilent, Santa Clara, CA) using He as a collision gas (Shaw et al., 2007). Data are reported as milligram of total arsenic per kilogram of dry tissue weight.

Production of aquaporin CRNs for microinjection in Xenopus laevis oocytes. Killifish AQP3s (kfAQP3a and kfAQP3a\textsuperscript{med}) were amplified by PCR from the vector TopoTA pCR2.1 and cloned into the Xenusps expression vector pXT7 by standard molecular cloning techniques using BglII and SpeI sites. Human AQP3 (hAQP3) was amplified by PCR from the clone SC117030 (Origene) and cloned into pXT7 at KpnI and SpeI sites. Primers used for PCR amplification are given in Table 2. Sequence integrity was verified by automated deoxy sequencing.

Plasmid DNA for each aquaporin was cut with restriction enzyme XbaI to linearize the plasmid and prevent run on of transcription. cRNA transcripts were produced using a mMessage mMachine kit (Ambion, Austin, TX) utilizing T7 DNA-dependent RNA Polymerase. The CRNA produced was purified using phenol/chloroform extraction followed by isopropanol precipitation, dissolved in RNase-free water, and quantified using a Biophotometer spectrophotometer (Eppendorf, Westbury, NY).

Preparation of Xenopus laevis oocytes. All experiments were done in accordance with Institutional Animal Care and Use Committee approved protocols at Beth Israel Deaconess Medical Center. Xenopus laevis frogs (Harvard Institute of Medicine, Boston, MA, approval number 043-2009) were anesthetized in 1 1 0.5% (wt/vol) 3-aminobenzoic acid ethyl ester methanesulfonate salt (Tricaine) containing ice for 20 min. Oocytes were removed bilaterally from the abdominal cavity and the egg mass cut into small pieces and placed in calcium-free ND96 buffer (96mM NaCl, 1mM KCl, 1mM MgCl2, 5mM Hepes, pH 7.5). Oocytes were then defolliculated in 2 mg/ml collagenase (Sigma-Aldrich) and 0.2 mg/ml trypsin inhibitor (Sigma-Aldrich) in calcium-free ND96 for 55 min with rotation on an Adams Nutator before washing three times with phosphate buffer (100mM K2HPO4, 0.1% (wt/vol) bovine serum albumin, pH 6.5) and then allowing oocytes to incubate in phosphate buffer for 10 min at room temperature. Oocytes were transferred to calcium-free ND96 and then to modified Barth’s solution (MBS; 88mM NaCl, 1mM KCl, 2.4mM NaHCO3, 0.82mM MgSO4, 0.33mM Ca(NO3)2, 0.41mM CaCl2, 10mM Hepes, pH 7.4, supplemented with 1% vol/vol penicillin/ streptomycin) where they were maintained at 18°C.

cRNA (10 ng) for each of the three AQPs was injected into oocytes using a Nanoject II Auto-Nanoliter Injector (Drummond Scientific Co., Broomall, PA). Control oocytes were injected with water alone. After 3 days incubation at 18°C, oocytes were tested for their ability to transport water, urea, or glycerol. Water transport kinetics was assessed at room temperature by quantitation of water uptake, oocytes were washed six times with ice-cold buffer (20–40 Ci/mmol) and 2mM glycerol was added. At the end of the incubation period for uptake, oocytes were washed six times with ice-cold buffer containing 5mM solute. Individual oocytes were then placed in scintillation vials with 200 μl 20% SDS added, and vortex mixed for 10–15 s before addition of 4 ml Scintisafe scintillation cocktail. Vials were counted for 2 min in a Packard 1500 liquid scintillation analyzer.

Statistics. All statistics were performed with Prism 5 (GraphPad Software, San Diego, CA). Two-way ANOVA was used to analyze water and arsenic transport assays using a Bonferroni-corrected post hoc test for pair-wise comparison. Solute transport assays were analyzed using unpaired and unequal variance t-test. Statistical significance was accepted at p < 0.05. Data are expressed as mean ± standard error of means.

RESULTS

Only kfAQP3 is Expressed in the Killifish Gill

To identify the AQPs expressed in the gill of killifish, Q-RT-PCR studies were performed on total RNA isolated from gills obtained from killifish acclimated to FW or SW. We used both

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5'–3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Killfish AQP3 -5'</td>
<td>GGCAGATCTACAGCCACCACATGGGTGCACAGAAGATTATTTT</td>
</tr>
<tr>
<td>Killfish AQP3a-3'</td>
<td>GGAATACTAGTCTACATTAGAATTTACCTCTTTGCCGTTT</td>
</tr>
<tr>
<td>Killfish AQP3a\textsuperscript{med} -3'</td>
<td>GGCATTACACCGCACCCTGGGTCAGCACAGAAGAGCTTG</td>
</tr>
<tr>
<td>Human AQP3-5'</td>
<td>GGCAGTTACCACCGCACCCTGGGTCAGCACAGAAGAGCTTG</td>
</tr>
<tr>
<td>Human AQP3-3'</td>
<td>GGGAATACATTTCTACGTAGTCATAGACGATGTCCTTTG</td>
</tr>
</tbody>
</table>

Note. Restriction endonuclease sites are underlined.
conditions because AQP3 expression is dependent on environmental salinity (Cutler and Cramb, 2002; Jung et al., 2012; Tse et al., 2006). AQP3 was the only AQP identified in gills of killifish, and the expression was higher in FW acclimated fish compared with SW acclimated fish (Fig. 1). Neither AQP7 nor AQP9 were significantly expressed in gill. Because the primers used in the Q-RT-PCR studies were used to construct a standard curve from killifish AQP7 and AQP9 cloned from liver, the lack of expression on these AQP3 in gill was not due to ineffective primers or PCR cycling conditions. Therefore, all subsequent studies focused on killifish AQP3 (kfAQP3).

**One Homolog of the kfAQP3 Expressed in Gill**

We identified a novel homolog of the kfAQP3. This homolog (kfAQP3a) was identified by PCR from gills of killifish acclimated to SW. Figure 2 shows the alignment of the protein sequence of this new homolog with a second homolog (kfAQP3b) identified as the consensus contig from the killifish transcriptome assembly that was determined by 454 sequencing of pooled killifish tissues, and with a previously published killifish AQP3 sequence (kfAQP3ts) from embryos of *F. heteroclitus* obtained in southern Spain (Tingaud-Sequeira et al., 2009). All killifish AQP3 homologs have the signature six transmembrane regions, four amino acids corresponding to aromatic amino acid/Arg (ar/R) constriction (F63, G202, Y211, and R217), and two Asn-Pro-Ala (NPA) motifs that are common in all aquaporins (Cerdà and Finn, 2010). In addition, we identified the five amino acids conserved in mammalian and teleost AQP: P1 (aromatic, Tyr123), P2 (Asp, Asp218), P3 (Lys or Arg, Arg222), P4 (Pro, Pro245), and P5 (nonaromatic, Leu226) (Cutler and Cramb, 2002; Giffard-Mena et al., 2007; Tingaud-Sequeira et al., 2010; Tingaud-Sequeira et al., 2009).

![Expression of AQP messenger RNA (mRNA) in the killifish gill. Q-RT PCR was performed on total mRNA isolated from gills of killifish acclimated to either FW or SW. The copy number of each amplicon was measured using known copy numbers of full sequences as standards. Only AQP3 was significantly different from background. N = 3 per group.](image)

**Arsenic Uptake by AQP3**

In all studies to date AQP3 expressed in fish and mammals have been shown to transport arsenic (Bhattacharjee et al., 2005; Liu et al., 2005; Wu and Beitz, 2007). To determine if the kfAQP3s transport arsenic, HEK293T cells were transfected with kfAQP3a, kfAQP3amut (derived from replacement of C-term GKS of kfAQP3a to ANC of kfAQP3b and kfAQP3ts), hAQP3 (positive control), or empty vector (negative control). Transfected cells were exposed to 2 ppb (2 μg/l) arsenic in the medium, and cellular arsenic uptake was measured as described in the Materials and Methods section. Arsenic uptake was increased in cells transfected with kfAQP3amut and hAQP3. The cellular arsenic concentration increased as the amount of cDNA transfected was increased (Fig. 3A), and cellular arsenic accumulation increased in a time-dependent manner (Fig. 3B). By contrast, arsenic uptake in cells expressing kfAQP3a was not different from cells transfected with the empty vector. These data reveal that kfAQP3a is the first AQP3 identified that does not transport arsenic and that replacing the C-terminal amino acids with that of kfAQP3b and kfAQP3ts (GKS to ANC) was sufficient to allow arsenic transport.

**kfAQP3a and kfAQP3amut Transport Water, Urea, and Glycerol**

*Xenopus laevis* oocytes injected with either water, kfAQP3a, kfAQP3amut, or hAQP3 cRNA were used to examine their ability to transport water, urea, and glycerol. To measure water
permeability, oocytes were exposed to a hypotonic solution (65% MBS), and osmotic water permeability ($P_f$) of the oocytes was measured as described in the Materials and Methods section. Expression of all AQP3s increased the $P_f$ of oocytes compared with oocytes injected with water (control) (Fig. 4A). Although hAQP3 was not sensitive to changes in pH over a range of values from 6.6 to 8.6, a reduction in pH (pH 7.0) significantly reduced the $P_f$ of kAQP3a and kAQP3amut (Fig. 4B). At pH values above 7.4, the $P_f$ of oocytes expressing kAQP3a, kAQP3amut, and hAQP3 was similar. Mercury, a well-described inhibitor of AQP water channels, reduced $P_f$ in all AQP3s studied (Fig. 4B).

Studies were also conducted to measure the uptake of radiolabeled ([14C]urea and [3H]glycerol). As shown in Fig. 5, kAQP3a and kAQP3amut were effective channels for urea (Fig. 5A) and glycerol (Fig. 5B).

**DISCUSSION**

The major new finding of this study is that kAQP3a does not transport arsenic, which makes it a unique AQP3, because all other AQP3s studied to date transport arsenic (Hamdi et al., 2009; Lee et al., 2006; Naranmandura et al., 2009). Like all other AQP3s, it transports water, urea, and glycerol.

In this study, we cloned a new homolog of kAQP3 from adult killifish gill. This homolog (kAQP3a) and another homolog identified as a consensus contig by a BLAST search of a transcriptome assembly derived from the sequencing of pooled killifish tissues (kAQP3b) were homologous to a previously identified AQP3 (kAQP3ts) from a population of killifish in Spain (Tingaud-Sequeira et al., 2009) (96 and 98% identity, respectively). kAQP3s have all of the sequence motifs characteristic of AQP3s. Thus, examination of the amino acid sequence of the cloned kAQP3a did not suggest that it would functional as a novel AQP3. However, assays revealed that kAQP3a did not transport significant amounts of arsenic. Comparison of the amino acid sequences of regions of AQP3s thought to confer AQ3 channel selectivity including the ar/R constriction region, residues in the NPA region, and amino acids lining the pore (Wu and Beitz, 2007) revealed that there were no differences between the three variants of kAQP3 or with other AQP3 that transport arsenic. Because the most pronounced difference between kAQP3a and the other kAQP3s was in the C-terminus (Gly-Lys-Ser in kAQP3a versus Ala-Asn-Cys in kAQP3b), we tested the hypothesis that the last three amino acids of kAQP3a (GKS) prevented it from transporting arsenic.

**TABLE 3**

<table>
<thead>
<tr>
<th>Fish</th>
<th>Treatment (population)</th>
<th>Melting temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>FW (population 1)</td>
<td>83.27</td>
</tr>
<tr>
<td>2</td>
<td>FW (population 1)</td>
<td>82.56</td>
</tr>
<tr>
<td>3</td>
<td>SW (population 1)</td>
<td>82.81</td>
</tr>
<tr>
<td>4</td>
<td>SW (population 1)</td>
<td>83.33</td>
</tr>
<tr>
<td>5</td>
<td>SW (population 2)</td>
<td>83.05</td>
</tr>
<tr>
<td>6</td>
<td>SW (population 2)</td>
<td>83.07</td>
</tr>
</tbody>
</table>

*Mean values reported ($N = 4$ per fish).
arsenic. To test this hypothesis, we replaced the last three amino acids of kfAQP3a with ANC and found that this change was sufficient to enable kfAQP3amut to transport arsenic robustly. Thus, the C-terminal amino acids of kfAQP3b and kfAQP3ts (ANC), possibly by interacting with loop D in kfAQP3, are essential for arsenic transport. This speculation is supported by a study that identified the importance of the interaction of the C-terminus with loop D in substrate selectivity of AQPs (Törrnroth-Horsefield et al., 2010).

All AQP3s studied to date transport arsenic, including hAQP3 and teleost AQP3 (Hamdi et al., 2009; Lee et al., 2006; Naranmandura et al., 2009). Although one study reports that hAQP3 expressed in oocytes does not transport arsenic (Liu et al., 2004), we and others (Lee et al., 2006; Naranmandura et al., 2009) have demonstrated that this same hAQP3 gene effectively transports arsenic. The reason for this discrepancy is unknown. Zebrafish AQP3 has been shown to transport monomethyl arsonious acid as well as As(OH)3 (Hamdi et al., 2009). Because As(OH)3 is a molecular mimic of glycerol, it has been suggested that As(OH)3 and glycerol traverse the same permeability pathway in AQP3 (Bhattacharjee et al., 2008). However, the data in the present study question this assertion because kfAQP3a transports glycerol but not arsenic. Clearly, additional studies are required to determine why the ANC sequence in C-terminus is essential for arsenic transport in kfAQP3 and to address the issue of how glycerol and arsenic are transported by AQP3.

The inability of kfAQP3a expressed in gill to transport arsenic is beneficial to an organism that is exposed to environmental arsenic. Estuarine waters often have very high levels of arsenic.
from geogenic sources as well as industrial runoff (Grecco et al., 2011; Neff, 1997). Some fish inhabiting estuaries contaminated with arsenic have high levels of tissue arsenic (Fabris et al., 2006). However, in killifish, assimilation of arsenic from water is extremely low, and killifish are extremely tolerant to arsenic (Dutton and Fisher, 2011a,b; Shaw et al., 2007, 2010). Our data suggest that the inability of kfAQP3a in the gill to transport arsenic may be responsible, in part, for low arsenic assimilation. Of course, many other factors may also contribute, including reduced assimilation in the intestine, enhanced metabolism in the liver, and enhanced excretion in the bile and by the kidneys. The low rate of arsenic assimilation by killifish is advantageous for an organism that is exposed to multiple stressors including changes in salinity, temperature, and oxygen (Burnett et al., 2007) because arsenic is notable in that it often exacerbates the untoward effects of environmental stressors.

All other AQPs studied to date transport water, glycerol, urea, and arsenic (Bhattacharjee et al., 2008; Maclver et al., 2009; Wu and Beitz, 2007; Zeuthen and Klaerke, 1999), including both forms of zebrafish AQP3 (Hamdi et al., 2009). Like other AQP3s, the water permeability of kfAQP3s was inhibited by mercury, and water permeability was sensitive to pH, but not to changes in pH within the physiological range for killifish (7.4–7.8) (Maclver et al., 2009; Zeuthen and Klaerke, 1999). Interestingly, phloretin, a chemical inhibitor of aquaporins, can selectively affect AQP9’s glycerol permeability while not affecting uptake of arsenite (Mcdermott et al., 2010). In terms of AQP3, phloretin inhibits the protein’s urea permeability but not glycerol permeability (Tsukaguchi et al., 1998). The mechanism behind this selectivity by phloretin is not yet understood, but these studies, in addition to our results, imply that permeability of AQPs to different solutes are not necessarily controlled by identical mechanisms (Mcdermott et al., 2010).

kfAQP3a and kfAQP3ts were sequenced directly by PCR cloning of gill and embryos, respectively, whereas kfAQP3b was identified computationally from a transcriptome assembly derived from deep sequencing (454) of cDNA from adult and embryonic killifish. Since kfAQP3a was the only killifish AQP3 derived exclusively from adult fish, it is tempting to speculate that kfAQP3 expression is life stage dependent. However, these discussions would be beyond the scope of this paper, given that genome sequence data in this species is not yet available.

In conclusion, we have identified a novel variant of AQP3 expressed in the gill of Atlantic killifish, F. heteroclitus. To our knowledge, this is the first report of an AQP that does not transport arsenic. However, kfAQP3a does maintain the characteristics of AQPs as channels for water as well as other small neutral solutes such as urea and glycerol. Together, these results suggest that uptake of arsenic from surrounding water may not be conducted through this protein in the gills of killifish. In addition, this inability of kfAQP3a to transport arsenic may partly be a reason behind the low accumulation of and high tolerance to arsenic by the killifish.

FIG. 5. kfAQP3 is a urea and glycerol channel. *Xenopus laevis* oocytes were injected with AQP3 cRNAs and urea uptake (A) and glycerol uptake (B) were measured as described in the Materials and Methods section ($p < 0.05$). The uptake of both glycerol and urea is not statistically different between kfAQP3a and kfAQP3a$^{mut}$ ($p < 0.05$). $N = 3$ individual experiments with 10–12 oocytes per aquaporin per experiment. $N = 3$ per group.

KILLIFISH AQUAPORIN 3 IS NOT AN ARSENIC CHANNEL 107

FUNDING

National Institute of Environmental Health Sciences at National Institute of Health (P42 ES007373 to B.A.S.); National Center for Research Resources at National Institute of Health (P20 RR-016463 to P.H.); Department of Defense-Strategic Environmental Research and Development Program (ER1503 to J.R.S.).

ACKNOWLEDGMENTS

We thank Meredith Adamo and Rebecca Lehman for their laboratory support. We also thank Drs Dean Madden and Dean Wilcox for helpful discussions.
REFERENCES


