TITLE

Exploring the Phenotype of an Aquaporin Knockout Mutant in the Filamentous Fungi *Neurospora crassa*[1].

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ABSTRACT

Aquaporins are water channels in cell membranes that have been extensively studied for many years in diverse organisms ranging from bacteria, lower eukaryotes, plants, animals, and mammals (where they have been studied for their potential medical significance). Are they required for the growth and development of cells? Such a question is best addressed through the study of simple organisms such as the fungus *Neurospora crassa*. A single gene with homology to aquaporins in other organisms is found in the genome of *N. crassa*. The phenotype of the knockout mutant of this gene has not been examined in *N. crassa*. Growth experiments were performed with varying concentrations of ammonium chloride (since ammonium/ammonia may permeate aquaporins), glycerol (since certain aquaporins are permeable to glycerol), or sucrose (at high concentrations to induce hyperosmotic stress, affecting water availability). No difference was seen between the growth rates of the wildtype and aquaporin mutant. We explored water permeability by applying hypoosmotic treatment, inducing lysis, and hyperosmotic treatment inducing hyphal shrinkage. Wildtype lysed more easily than the aquaporin mutant at low osmotic gradients, but similarly at high osmotic gradients, consistent with a lower water permeability in the absence of a water channel. Hyphal shrinkage was the same in wildtype and the aquaporin mutant at high osmotic gradients while experiments done at low osmotic gradients were inconclusive. We conclude that the putative aquaporin gene in *N. crassa* does encode an aquaporin which functions as a water channel in this lower eukaryote. The phenotype is revealed at low osmotic gradients, where hydraulic conductivity of the plasma membrane may be limiting

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OBJECTIVE

To determine the phenotype of a model organism, *Neurospora crassa*, when a putative water channel (*aqp*) is absent.

INTRODUCTION

It is well documented that aquaporins are primarily water channels that play a role in water transport (Tajkhorshid *et al*., 2002). Several studies have suggested that certain aquaporins have the ability to transport small, uncharged molecules such as glycerol, ammonia and certain gases such as carbon dioxide (Yu *et al*., 2006). Aquaporin channels have been found to be impermeable to charged molecules of any size including protons (Chakrabarti *et al*., 2004)(de Groot *et al*., 2003), which is surprising since those same aquaporin channels are permeable to relatively large uncharged molecules such as glycerol (Henin *et al*., 2007). The fact that the individual aquaporin channels form tetramers is also interesting and it has been suggested that ions could be made permeable through the resulting synergetic effects (Wang *et al*., 2007). The water transport mechanism that has been proposed is that water molecules passing through the channel change their orientation in the middle of the channel due to hydrogen bonding with a conserved NPA (asparagine-proline-alanine) motif (Tajkhorshid *et al*., 2002)(Chakrabarti *et al*., 2004)(de Groot *et al*., 2003). The aquaporin-like gene (NCU08052.3) in *N. crassa* shows homology to known aquaporins in other organisms and contains the highly conserved NPA motif but has not otherwise been shown to be an aquaporin. The gene is present as a single copy, and a deletion mutant is available (Colot et al., 2006). We explored the phenotype of this putative aquaporin in *N. crassa*, to determine the role it plays in the growth and morphogenesis of a relatively simple model organism.

Three phenotypes were explored. One was permeability of the aquaporin to NH_4^+/NH_3 by testing for the effect on growth of varying concentrations of NH_4^+/NH_3. Similar growth experiments were performed to test for glycerol transport through the aquaporin. Glycerol transport plays a role in osmoregulation in the yeast *Saccharomyces cerevisiae* (Nevoigt & Stahl., 1997) where aquaglyceroporins are regulated by osmotic pressure to minimize osmotic stress (Carbrey *et al*., 2001), (Hohmann., 2002) and might play a similar role in the related Ascomycete *N. crassa*. The transport of water was tested by comparing growth under varying conditions of water availability (by varying the external osmolyte, sucrose, concentrations) and by assay of water transport under conditions causing water uptake and lysis of the fungus, and under hyperosmotic conditions resulting in net water efflux and shrinkage of the hyphae.

MATERIALS AND METHODS

Strains. Stock cultures of wildtype (St. Lawrence 74–OR23–1VA, FGSC 2489) and *aqp*$^-$ (FGSC 12017, deletion mutant of NCU08021 [Colot et al., 2006]) were obtained from the

Fungal Genetics Stock Center (School of Biological Sciences, University of Missouri, Kansas City, Missouri, USA) (McCluskey, 2003) and maintained on slants of Vogel's Minimal Medium (Vogel, 1956) plus 1.5% (w/v) sucrose and 2.0% (w/v) agar.

Ammonium Growth Experiments. Agar (2% w/v) plates were prepared without Vogel's minimal medium, but containing a carbon source (1.5% w/v sucrose), biotin (50 ng ml^{-1}) and ammonium chloride at 0, 1, 3, 5 or 10 mM. The plates were inoculated by placing a small block of agar (*ca* 2 X 2 mm, with mycelium) from a previously prepared plate of either wildtype or the *aqp* mutant in the center of the Petri dish. The dishes were incubated at 28 ºC overnight. The next day, colony diameters were measured every hour at two compass points and averaged. The increase in colony diameter was linear over time; growth rates were calculated as mean ± SD (cm h^{-1}) for three replicates.

Sucrose Growth Experiments. To test the ability of the *aqp* mutant to grow under conditions of low water availability, agar plates were prepared with Vogel's minimal medium containing biotin (50 ng ml^{-1}) plus glucose (0.6 M) or sucrose (0.6, 0.8, 1.0 or 1.2 M) as both a carbon source and an osmoticum. The plates were inoculated with agar blocks as described above and incubated at 28 ºC overnight. The next day, colony diameters were measured every hour at two compass points and averaged. The increase in colony diameter was linear over time; growth rates were calculated as mean ± SD (cm h^{-1}) for three replicates.

Glycerol Growth Experiments. To test whether the aquaporin played a role in glycerol permeation, growth of wildtype and the *aqp* mutant was assayed with glycerol as the sole carbon source. For growth on agar, the medium consisted of biotin (50 ng ml^{-1}), and 0.0, 0.5, 2.0, 5.0 or 10% w/v glycerol. The Petri dishes were incubated overnight at 28°C. The next day, colony diameters were measured every hour at two compass points and averaged. The increase in colony diameter was linear over time; growth rates were calculated as mean ± SD (cm h^{-1}) for three replicates.

To force conditions where glycerol was the sole carbon source (without the complication of agar), growth in liquid media was also measured. Conidia were harvested from slants by washing in sterile dH_2O, and filtering through 4 layers of sterile cheesecloth. Ehrlenmeyer flasks (125 ml) containing 50 ml Vogel's minimal medium, biotin (50 ng ml^{-1}), and 100 mM glycerol were inoculated with 10^6 conidia. The flasks were harvested after 1–3 days of growth at 28 ºC in a reciprocating shaker (100 rpm) by vacuum filtration using No. 1 filter paper, transferred to aluminum weighing dishes, and dried at 95 ºC for at least 4 hours. Growth was measured as dry weight (mean ± SD, mg h^{-1}) for three replicates.

Lysis Experiments. In preliminary experiments, wildtype and *aqp* mutant colonies were grown (28 ºC overnight) sandwiched between two layers of dialysis tubing (molecular weight cutoff of 14000) overlaying media in Petri dishes (90 mm diameter) containing Vogel's minimal medium, 2% (w/v) agar, biotin (50 ng ml^{-1}). After overnight growth, the layers of dialysis tubing (with the sandwiched mycelium) was transferred to the lid of a Petri dish (90 mm diameter), taped down with masking tape, and flooded with BS

solution (10 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM MES, pH adjusted to 5.8 with KOH (ca 1 μl/ml) containing 683 mM sucrose. After five min, the hyperosmotic solution was decanted and the lid flooded with BS solution alone. This experimental protocol was problematic. Transfer of mycelium appears to cause the hyphae to become deformed, as if compressed between the two layers of dialysis tubing. The hyphal tips resumed growth after flooding with BS plus osmoticum, but replacement with BS solution alone caused no visible lysis. Further trials used mycelium growing on top of dialysis tubing overlaying agar, and transferred as described above. However, the hyphal tips tended to float, even more so after replacing the solution with BS alone, so that lysis could not be scored easily.

An alternative protocol was devised to ensure good anchoring of the mycelium by eliminating the need for solution replacement and maximizing the induction of lysis.

Mycelial colonies were grown on Vogel's minimal medium (with 50 ng ml^{-1} biotin) plus 2% w/v agar and 0.8 M sucrose. The inoculations were made with long agar blocks (about 2 mm by 20 mm) along one side of the Petri dish (55 mm diameter) so that the colony edge was straight and near the center of the dish after incubation overnight at 28 °C. The dishes were treated with solutions of varying osmolarity: Vogel's minimal medium plus 0.2 M, 0.3 M, 0.4 M, 0.5 M or 0.6 M sucrose. The growing edge was scanned under a microscope (X10 objective) and the number of lysed and the total number of tips were scored thrice (once while moving along the edge towards the left and twice towards the right). Lysis was scored only along the hyphal tips, where a tip is considered an individual if it is the only tip within a 0.15 mm radius. If a collection of tips within 0.15 mm of each other was found, they were collectively considered as one tip, and the lysis of any one of them was considered a lysis for the whole. Lysis was deemed to have taken place if intracellular material is found in areas outside the cell; or if the cell had lost enough of its intracellular material (in cases where intracellular material expelled from the cell floated away from the site of lysis) so as to be strikingly less dense than a full cell (eg: a clear colour density difference across septa).

The above scoring protocol was used for most experiments, but a subset of experiments used a double blind protocol (for treatments with 0.6 M sucrose plus Vogel's minimal medium), where the wildtype and *aqp* plates were inoculated by an independent colleague who coded the plates using random numbers, while the second experimenter did the scoring for lysis.

Hyphal volume Shrinkage Experiments. As an alternative method to measure water fluxes, water efflux from the hyphae was measured using hyperosmotic treatment and measuring the decrease in hyphal volume. Conidia were inoculated on a single layer of dialysis tubing (14000 MW cutoff) overlaying Vogel's minimal medium plus 2% w/v agar, 1.5% w/v sucrose and biotin, and incubated overnight at 28 °C. A section of the dialysis tubing that included the growing edge of the colony was cut out, placed in the lid of a Petri dish and taped down with masking tape. Care was taken during the transfer to avoid damage to the mycelial colony. BS solution (10 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 133 mM sucrose, 10 mM MES, pH adjusted to 5.8 using KOH) (3 ml) was added

to the dish and the sample was left for at least 5 minutes to confirm resumption of growth at the colony edge. A hyphal trunk was selected and imaged with a X63 water immersion objective at a septum. Images were captured before and after perfusion with BS solution plus 0.55 M sucrose using an Orca C-4742-95 camera (Hamamatsu) controlled with OpenLab software (version 3.1.7, Improvision). The images were analyzed in ImageJ (http://rsb.info.nih.gov/ij). The hyphal diameter 10 μm above and below the septa were measured, averaged, then squared (to be proportional to hyphal volume). The squared diameters were normalized to those prior to the onset of hyperosmotic perfusion (V/V_i).

The perfusion system supplied the hyperosmotic BS solution (10 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 500 mM sucrose, 10 mM MES, pH adjusted to 5.8 with KOH) at a rate of 6.4 ml sec^{-1}. The intake and outflow were positioned close to and on either side of the objective to assure rapid arrival of the perfusate at the hypha.

The above procedure was repeated using BS solution plus 0.2M sucrose as the hyperosmotic "shock" solution.

RESULTS

The *aqp* mutant is unaffected by NH_4^+/NH_3, hyperosmotic conditions, and glycerol carbon source. For both wildtype and the *aqp* mutant, the growth rates went up with the concentration of ammonium ions. However, there was no appreciable difference in growth rates between wildtype and the knockout mutant (Fig. 1).

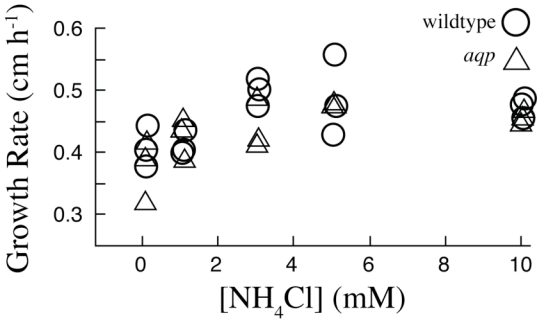


Figure 1: Wildtype and *aqp* mutant growth rates on an ammonium chloride concentration gradient.

Growth rate increased with increasing [NH_4Cl], saturating by 10 mM, but there was no difference between wildtype and the *aqp* mutant at any of the NH_4Cl concentrations.

For both wildtype and knockout mutant, growth rate went down with the concentration of glucose/sucrose due to high osmolarity. However, there was no appreciable difference in growth rates between wildtype and knockout mutant (Fig. 2).

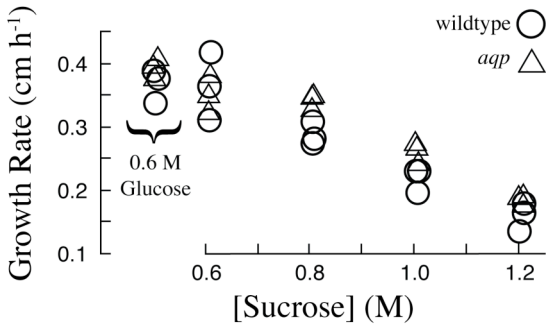


Figure 2: Wildtype and *aqp* mutant growth: Effect of hyperosmotic glucose/sucrose.

Growth of both wildtype and the *aqp* mutant are lower at high osmolarity, but exhibit no difference between each other.

For both wildtype and the *aqp* mutant, growth rates decreased as glycerol concentration increased when solid (agar) growth media is used, but growth was not significantly different (Fig. 3). Glycerol was not the sole carbon source as both strains grew well even at 0% glycerol, so the agar itself must provide alternative carbon sources. When glycerol dependence is forced by growing the cultures in liquid media, there was no clear difference between wildtype and knockout mutant growth rates (Fig. 4).

Thus, it is unlikely that the AQP protein functions as a permease of NH_3, or glycerol, or plays a role in the long-term challenges of water availability under hyperosmotic stress. The alternative is that it has a primary role in rapid water fluxes into and out of the cell.

Figure 3: Wildtype and *aqp* mutant growth rates with glycerol carbon source: on agar medium.

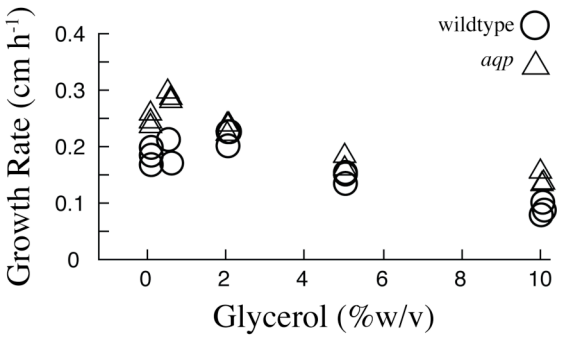
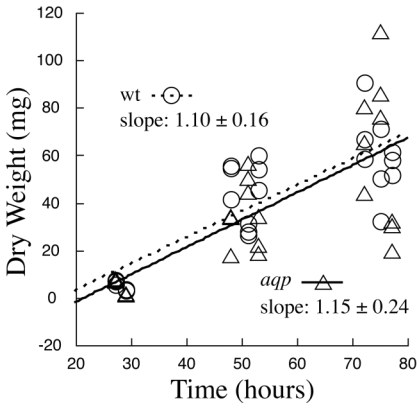


Figure 4: Wildtype and *aqp* mutant growth rates with glycerol carbon source: in liquid medium.



The *aqp* mutant has a lower water permeability than wildtype. Water influx, into the hypha, was tested by treatment in hypoosmotic solutions of various sucrose concentrations. When the extracellular solution is hypoosmotic to the intracellular solution, water influx into the cell will cause an increase in the internal hydrostatic pressure (turgor), which, at a high enough pressure can cause the cell wall to fail structurally and the hypha to lyse (Walker et al., 2006). For both wildtype and the *aqp* mutant, the percentage of hyphal tips that lysed increased as the sucrose concentration of the hypoosmotic solution decreased. The difference between wildtype and knockout

mutant were minimal or nonexistent when treated with very hypotonic solutions (0.2 M sucrose compared to 0.8 M sucrose in the growth media) but a clear pronounced difference ($p = 0.001$) was seen when a slightly hypotonic solution (0.6 M sucrose compared to 0.8 M sucrose of growth media) was used. 50% lysis was achieved at a higher sucrose concentration (0.35 M) in wildtype or a lower osmotic concentration difference (0.45 M less sucrose than growth media) than the *aqp* mutant (0.31 M sucrose, or 0.49 M less sucrose than the sucrose concentration in the growth media)(Fig. 5a). Thus, wildtype lysed more readily.

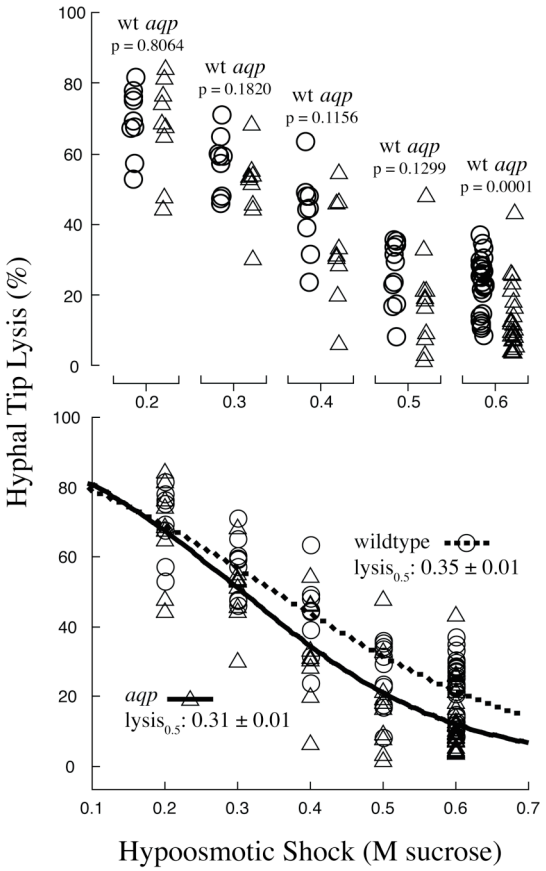
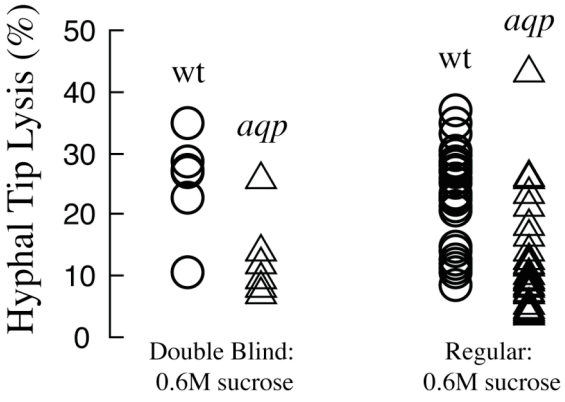


Figure 5a (Left panel): Hypotonic lysis of wildtype (circles) and the *aqp* mutant (triangles) when treated with hypotonic shock solutions. The data are shown as percent lysis. The total number of hyphal tips assayed per datum ranged from 100 to 250.

Figure 5b (Lower panel): Double blind Test of lysis scoring compared to all experimental measurements at 0.6 M sucrose.



To assure that the lysis scoring was objective, a double blind experiment was done for a subset of the experiments at 0.6 M sucrose. The results from a double blind experiment confirm our results ($p = 0.02$, Fig. 5b)**.**

Water efflux out of the hypha was tested by using rapid perfusion with hyperosmotic BS. For both wildtype and knockout mutant, hyphal volume decreases sharply within the first 2 minutes before reaching its limit (between 60%-70% initial hyphal volume). The time constants (± standard error) for the two strains were similar (wildtype: 1.74 ± 0.25 min; *aqp*: 1.31 ± 0.22, n=8). Although the *aqp* time constant was faster, standard errors for the two strains overlapped, so differences are not significant (Fig. 6).

When the experiment was repeated using hyperosmotic BS solution containing just 0.2M sucrose, the time constant (± standard error) for wildtype was 1.09 ± 0.53 min and for the *aqp* mutant 0.78 ± 0.25 min (n=5). Variability was high, so the differences were not significant due to large standard errors.

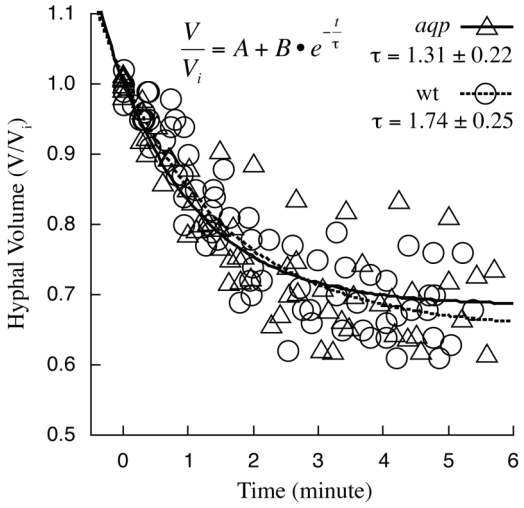


Figure 6: Wildtype and *aqp* mutant hyphal volumes decrease over time when treated with hyperosmotic (0.5 M sucrose) BS solution. Best fits to exponential declines are shown, along with the time constant (± standard error). Data are shown for 8 experiments for each strain.

DISCUSSION

Results from the ammonium chloride growth experiment suggest that the putative aquaporin channel does not transport ammonia. However, it is important to note that the high pK of ammonia (~9) (Perrin & Dempsey., 1974) will result in most of the ammonium being charged ions (NH_4^+) rather than uncharged (NH_3) and thus unable to pass through the channel. Also, the lack of a difference between the two could also be due to the presence of other ammonium transporters in *N. crassa* (Galagan *et al*., 2003).

Results from the sucrose growth experiments suggest that there is no difference between the wildtype and knockout. Aquaporins are passive water channels that allow water flow via diffusion (Agre *et al*., 1998). The plasma membrane alone is also permeant to water. The presence of aquaporins might decrease the time needed for equilibrium to be reached, but the equilibrium poise for both wildtype and the *aqp* mutant are the same. Thus growth conditions after the initial hyperosmotic shock will be the same for both wildtype and *aqp* mutant. Moreover, after initial hyperosmotic shock, *N. crassa* will recover by increasing osmolyte concentrations within its cells to match the osmolarity outside, thus negating the difference in osmolarity across the membrane over time (Lew and Levina, 2007). Thus, the AQP protein does not appear to play a role in long-term osmoadaptation.

Glycerol growth experiments suggest that this aquaporin has no function in the transport of glycerol. At this stage of our understanding of aquaporins, we only know that for some organisms, determination of whether an aquaporin can conduct glycerol or not is dependent on the diameter of the narrowest point in its channel; in other words,

substrate permeation is sterically controlled (Wang *et al*., 2005). A study of motif homology between the NCU08052.3 gene and other aquaporins and glyceroaquaporins and pure glycerol channels were inconclusive as all three possess structural homology, being major intrinsic proteins. One thing all aquaporins have in common is two highly conserved NPA (asparagine-proline-alanine) motifs near the middle of the channel that allow for their distinct function of transporting water molecules without transporting protons (de Groot *et al*., 2003). Interestingly, the *N. crassa aqp* gene has only one such motif, the other being an NPV (asparagine-proline-valine). The human aquaporin-1 gene also has only one NPA motif, so the second motif might not be required for function (Fig. 7 and 8).

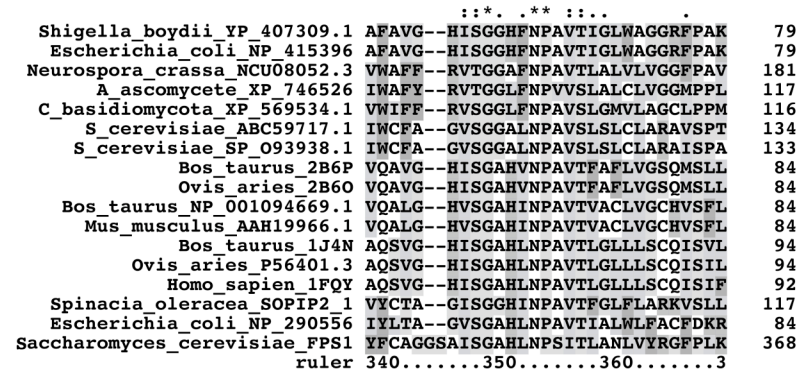


Figure 7: Aligned sequences of various aquaporins across kingdoms and species. The first NPA motif is highly conserved with both the N and P being present in all species while the A was conserved in 15 out of 17 species.

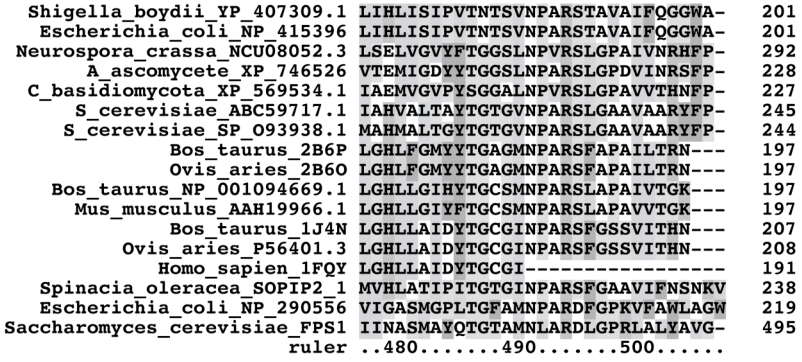


Figure 8: Aligned sequences of various aquaporins across kingdoms and species. The second NPA motif is highly conserved with both the N and P being present in all species except that of *H. sapien* 1FQY, while the A was conserved in 14 out of 17 species.

Results from the lysis experiment suggest that wildtype lyses more readily than aquaporin, although the difference is slight. Interestingly, there is no difference when the osmotic shock is greatest. It was assumed that with a functional aquaporin gene, wildtype would lyse much more than the knockout mutant under these conditions as many more water channels are open for water flow into the cell. However, we need to consider the driving forces for water flow: $J_V = L_p \bullet [P(t)+\pi^o+\Delta\pi^o-\pi^i(t)]$ where J_V is water flow, L_p the hydraulic conductivity, $P(t)$ the change in pressure over time, π^o the external osmotic

pressure, $\Delta\pi^o$ the change in external osmotic pressure and $\pi^i(t)$ the change in internal osmotic pressure (Philip., 1958). L_p is the sum of the hydraulic conductivities due to aquaporins, and the plasma membrane. Provided that the contribution to the total hydraulic conductivity by aquaporin is not too large, a sufficiently large external osmotic pressure $\Delta\pi^o$ would be sufficient to generate a large enough water flow into the cell to cause lysis regardless of whether aquaporins were present. At low osmotic gradients, the effect of L_p is relatively large and thus the effect of the aquaporin becomes apparent. The differences between wildtype and knockout mutant did not seem obvious for hypoosmotic concentrations lower than 0.6 M sucrose ($0.1 < p < 0.2$). This could be explained due to a small sample size. A larger sample size, keeping the same trend, might result in statistically significant differences. With that said, it appears that the gene product is present and is a water channel, but its effects are neither large nor critical to the survival of the fungus as the fungus thrives in a variety of growth conditions.

Shrinkage experiments suggest that there is no difference between the water outflow rates of wildtype and the *aqp* mutant. This is surprising because the lysis experiment suggested that the gene product was a water channel that influenced water inflow rate. This could be because the osmotic gradient is just too high (0.5 M sucrose difference in this case) for the effect of aquaporin channels to be seen. When shrinkage experiments were done at a lower osmotic gradient (0.2 M sucrose difference), results suggest that the *aqp* mutant lost water more rapidly compared to the wildtype. However, greatly overlapping error values greatly reduces our confidence in these results. Error values were very high in part due to a relatively small sample size used (compared to the sample size used for the lysis experiments), and also because the low osmotic difference resulted in a smaller osmotic shock, causing the amount of shrinkage to be much less (90% of initial volume compared to 60% of initial volume when 0.5 M hyperosmotic BS was used). This smaller osmotic shock also led to the fungus recovering sooner, resulting in a gradual return to original volume after about 2 min, causing additional scatter in the data (results not shown). Another possibility is that *N. crassa aqp* water channel is a unidirectional channel, or gated and thus regulated. While more research has to be done to confirm this, it is plausible, since research has shown that water movement through aquaporin channels is a dynamic process involving many interactions between amino acid residues within the channel and research has also suggested mechanisms for the gating and regulation of aquaporin function, especially in higher eukaryotes (Tornroth-Horsefield *et al.*, 2006)(Yu *et al.*, 2006).

CONCLUSION

We conclude that the *aqp* homolog in *N. crassa* is an aquaporin channel enhancing water permeability of the plasma membrane.

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