Characterizing the osmosensitive phenotypes of assorted knockout mutants of the filamentous fungi *Neurospora crassa*[1].

Maryam Rasti-Talkhoncheh[2] and Roger R. Lew, Biology Department, York University

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OBJECTIVE

To determine the osmosensitivity of different mutants of *Neurospora crassa* to either sugar (sucrose) or salt (NaCl or KCl) osmotic shocks, with emphasis on transporter mutants, and mutations in transport regulators.

[2] RAY (Research at York) Research Assistant. Experiments were performed 01MAY2008 through 31AUG2008 in the Lew Laboratory (RRL email: planters@yorku.ca) and were funded in part by NSERC (Natural Sciences and Engineering Research Council)

INTRODUCTION

Fungi are mostly filamentous growing by polarized extension of the hyphae in a process known as tip growth, which is essential to their saprophytic life style, as a means to explore new territory for nutrients (Griffin, 1981). The extension of the hyphae is normally driven by an internal hydrostatic pressure (turgor) (Lew et al., 2004). Plant and fungal walled cells must be able to adapt to the varied changes that can occur in the terrestrial environment. Drought or rain cause dramatic changes in the external osmolarity. Thus, turgor regulation or other osmoadaptations are crucial to the survival of the cell. Ion transport plays a key role in osmoadaptation (Lew et al., 2006).

The objective of our research was to explore the ability of *Neurospora crassa* to survive high osmotic conditions, and the possible role of ion transporters and their regulators in osmoadaptation. Their roles were explored by using knockout mutants (Colot et al., 2006).

There are at least four known K^+ transporters in *N. crassa* (Kiranmayi & Mohan, 2006): *trk* (Haro et al., 1999), *trm–8* (Kiranmayi & Mohan, 2006), *hak–1* (Haro et al., 1999), and *tok* (Roberts, 2002).

TOK was identified to encode a voltage-gated K^+ efflux channel in *N. crassa* by patch clamp measurements after heterologous expression in yeast (*Saccharomyces cerevisiae*). Although the channel is an outward rectifier, it may play a role in K^+ uptake, based upon survival of yeast cells lacking other K^+ transporters (Δ*trk1* Δ*trk2*) but expressing the *N. crassa* TOK gene at 10 mM KCl (Roberts, 2002).

Hak–1 is believed to be a H^+/K^+ symporter, and *trk* a K^+ uniporter (Haro et al., 1999). Since there is a higher concentration of K^+ inside the cell in comparison to the outside, these transporters have to work against the concentration gradient, and rely upon the negative-inside electrical potential and $\Delta[H^+]$ gradient as driving forces for net influx. The TRK protein is considered be the dominant transporter since *hak-1* is only expressed when extracellular K^+ is very low (0.25 mM). HAK–1 depletes extracellular K^+ (due to K^+ uptake) more efficiently than *trk* (Haro et al., 1999) and thus may play a central role under conditions of K^+ depletion.

The *trm–8* gene was identified as a potential K^+ transporter candidate on the basis of comparative genomics (Kiranmayi & Mohan, 2006). It was one of a total of 65 open reading frames for putative cation transporter genes that were identified in the *N. crassa* genome sequence using BLAST comparisons with motifs in a transport database (Kiranmayi & Mohan, 2006). The transport of metals–8 (*trm–8*) gene sequence shows strong similarity to members of voltage gated ion channels (VIC) family of K^+ transporters (Kiranmayi & Mohan, 2006).

A putative chloride transporter was identified on the basis of BLAST comparisons. The putative *clc–3* gene in the *N. crassa* genome has high similarity to CLC-3 voltage gated chloride channels in mammals (38% identity and 57% positive).

In all living organisms aquaporin provides a channel for water transport at the plasma membrane and intracellular membranes (Tajkhorshid et al., 2002). It may play a role in water availability under osmotic stress.

In addition to transport mutants, we also examined putative regulators of osmoresponses in *N. crassa*. The response regulator RRG-1 is a two-component signal transduction pathway that is located upstream of the mitogen-activated protein kinase pathway which controls responses to osmotic stress (Jones et al., 2007). A two-component sensor consists of a histidine kinase domain and a response regulator domain. The kinase domain autophosphorylates its own histidine residue and then transfers the phosphoryl group to an asparate residue on the response regulator domain. The eventual result is activation of the downstream osmotic MAP kinase pathway (Jones et al., 2007). The *rrg–1* mutant has a similar growth rate as wildtype under standard conditions, but exhibit other developmental defects, and is sensitive to hyperosmotic stress.

The 14-3-3 proteins belong to a family of proteins that are expressed in eukaryotic cells (Fu et al., 2000). These proteins bind to different signaling proteins such as phosphatases, kinases and transmembrane receptors in order to regulate a wide range of biological processes such as cell growth control, neuronal development and viral and bacterial pathogenesis (Fu et al., 2000). The 14-3-3 protein knockout mutant of *N. crassa* was used in this experiment to examine the role of this regulatory protein in osmoadaptation in conditions of high salt or sugar in order to maintain the optimal growth rate.

A net negative electrical membrane potential is an important property of living organisms, used as a driving force for ion movement across the plasma membrane. In fungi, the plasma membrane H^+-ATPase is the major cause of the net negative-inside potential (Goossens et. al., 2000). Ptk2p is a protein kinase in *Saccharomyces cerevisiae* which activates the H^+-ATPase in response to glucose, to effect uptake of glucose through a H^+/glucose symporter to support celluar metabolism (Goossens et. al., 2000). There is a homolog of the yeast Ptk2 gene in *N. crassa* (NCU01940) based on BLAST comparisons (27% identity, 45% positive).

All of the above transporters and transport regulators were examined for their role in osmoadaptation by examining the osmosensitivity of the knockout mutants (Colot et al., 2006).

MATERIALS AND METHODS

The strains used in this project are listed in Table I. They were all obtained from the Fungal Genetics Stock Center (School of Biological Sciences, University of Missouri, Kansas City, Missouri, USA)(McCluskey, 2003). Both wildtype and the mutant strains were grown on Vogel's Minimal medium plates (VM) (with 1.5% w/v sucrose and 2% w/v agar).

Table I: Listing of strains used in osmosensitivity assays.

Strain	NCU locus number	FGSC
Wildtype		2489
aqp	NCU08052	12017
clc–3 homolog	NCU06624	14929
14–3–3 protein homolog	NCU 02806	14662
hak–1	NCU00790	13816
rrg–1	NCU01895	13363
tok	NCU04065	12045
trk	NCU06449	12678 (mating type a)
trm–8	NCU02456	16162 (mating type A)
trk & trm–8		12678 X 16162
Ptk2 homolog	NCU01940	17932

Osmosensitivity phenotype of the mutants. Growth sensitivity to salts was examined by measuring growth in VM (with 1.5% w/v glucose and 2% w/v agar) plus NaCl at 0.02, 0.01, 0.4, and 1.2 M. IN some experiments, higher concentrations of NaCl (0.7, 1.2 and 1.5 M). Growth sensitivity to sugar was examined by measuring growth in VM (with 1.5% w/v glucose and 2% w/v agar) plus sucrose at 0.0, 0.04, 0.2, 0.6 and 1.5 M. Care was taken to assure that the final volume of the sucrose solution was brought to 150 ml (the large amount of sucrose had a significant effect on the volume of the solution). The solutions were autoclaved, then dispensed in the Petri dishes (25 ml per dish). The dishes were inoculated in the center of the dish with small blocks of agar (0.3–0.4 cm square) from plates of wildtype or the mutant strains freshly prepared 1–2 days beforehand. The edges of the Petri dishes were wrapped with stretched Parafilm (a 1 cm X 10 cm strip) to minimize evaporation over the course of the experiment. The plates were incubated at 28ºC. Periodically, the colony perimeter was outlined with a permanent ink pen until the colony had reached the edges of the 90 mm diameter Petri dishes. Measurements of the colony diameter were made by first drawing a cross on the dish through the block at the center of the dish. The colony diameters were measured where the cross marks intersected the previously drawn colony perimeters. The two diameters were averaged. The increase in the diameter of the colony with respect to time was linear. The growth rate was calculated from the slope of the diameter *versus* time (cm h^{-1}).

In all cases, control dishes with wildtype were run along with the mutant strain, with three replicates for each treatment for wildtype and the mutant strain.

Potassium requirement for growth of the mutants: The K^+ requirement for growth of strains with deleted K^+ transporters (*trk*, *trk & trm–8*) was examined by measuring the growth on VM (with1.5% w/v glucose and 2% w/v agar) plus KCl at 0 and 0.04 M. The solutions were prepared in batches of 270 ml sufficient for 9 race tubes (32 cm X 1.5 cm X 1.50 cm) capped with cotton to maintain sterility. The solutions were autoclaved, then dispensed in the race tubes (30 ml per race tube). The race tubes were inoculated at one end with small blocks of agar (0.3–0.4 cm square) from plates of wildtype or the mutant strains prepared 1–2 days beforehand. The race tubes were incubated at 28ºC. Periodically, the colony edge was marked with a permanent ink pen. Once the colony had reached the other end of the race tube, measurements of the colony length were made. First a line was drawn at the edge of the block on the race tube. The length was measured for each growth mark from the edge of the block. The growth rate was calculated from the slope of the length *versus* time (cm h^{-1}). In all cases, control race tubes with wildtype were run along with the mutant strain, with three replicates for each treatment for wildtype and the mutant strain.

pH sensitivity phenotype of Ptk2 homolog. Growth sensitivity of Ptk2 homolog to pH was examined in this experiment to test for its role in pH regulation. Stock solutions of BTP and MES (50 mM) were prepared and mixed together to reach the desired pH. The ratios used to bring the solution to the desired pH are shown in Table II. For each pH, 150 ml of solution was prepared. Glucose (1.5% w/v), 2% w/v agar and 0.15 ml of biotin were added to the solution, and the final pH measured. The solutions were autoclaved, then dispensed in the Petri dishes (25 ml per dish). Inoculations and measurements of growth rates were performed as described above. In all cases, controls with wildtype were run along with the mutant strain, with three replicates for each treatment.

Table II: Ratios of BTP/MES required to reach the specified pH.

pH	BTP/MES
4	0.035
5	0.116
6	0.5
7	0.89
8	1.87

RESULTS

Characterization of the K^+ Transporter Mutant Phenotypes: The phenotype of different strains *Neurospora crassa* mutants were compared to wildtype for osmosensitivity to either sugar (sucrose) or salt (NaCl) osmoticum. Of the potassium transport mutants, the *trk* mutant exhibited the wildtype morphology when it was treated with sucrose (the mutant growth rate was 114% of wildtype for 1.5 M sucrose) but for NaCl treatment the mutant shows a partial sensitivity compared to wildtype (51% of wildtype in 1.2 M NaCl). The *trm–8* mutant had the same growth rate as wildtype for both NaCl and sucrose treatment (102% of wildtype for NaCl and 99% of wildtype for sucrose). The *trk & trm–8* mutant growth rate was similar to wildtype for the sucrose treatment (114% of the wildtype at 1.5 M sucrose) but was sensitive when treated with NaCl as the osmoticum (51% of the wildtype at 1.2 M NaCl), similarly to *trk*. Compiled data are shown in Fig. 1.

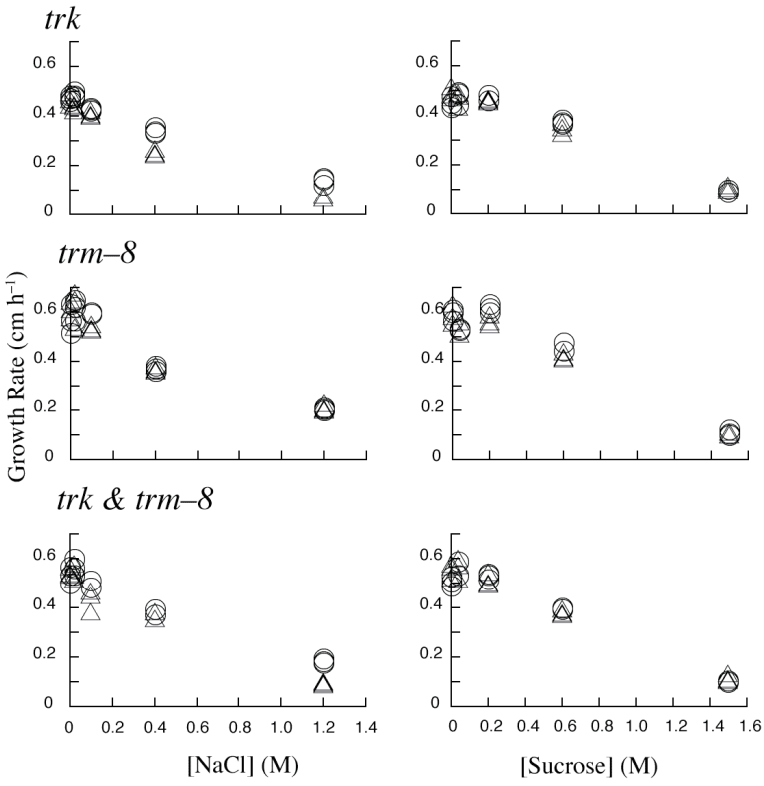

Figure 1. The effect of osmoticum (NaCl or sucrose) on growth of the *trk*, *trm–8*, and *trk & trm–8* mutants (triangles) and wildtype (circles) of *Neurospora crassa*. Treatments with NaCl are shown in the left panels; treatments with sucrose in the right panels

At higher NaCl concentrations, the *trk* mutant was osmosensitive. The growth rate was 52% of wildtype at 0.7 M NaCl and 39% at 1.2 M. Both the mutant and wildtype grew poorly at the highest NaCl concentration used (1.5 M). The *trk & trm–8* exhibit a similar osmosensitivity as *trk*: 66% of wildtype at 0.7 M NaCl, 42% of wildtype at 1.20 M NaCl and 57% of wildtype at 1.50 M NaCl. Compiled data are shown in Fig. 2.

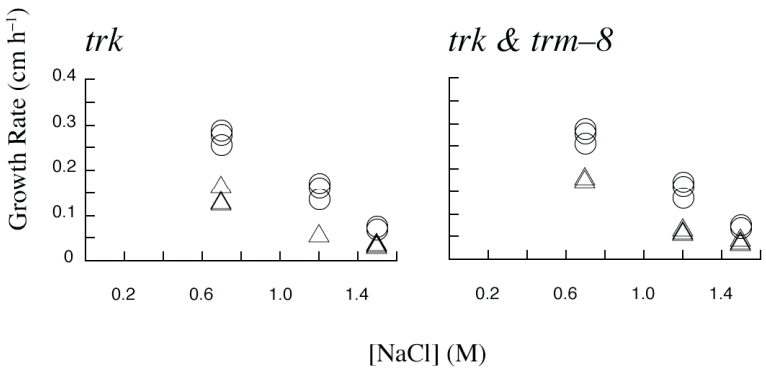

Figure 2. The effect of osmoticum (high NaCl concentration) on growth of the *trk* and *trk & trm–8* mutants (triangles) and wildtype (circles) of *Neurospora crassa*.

To determine whether the K^+ transporters were required for growth under K^+ deficient conditions, its effect on growth of the mutants was examined. Both *trk* and *trk & trm–8* mutants had the same growth rate as wildtype. The *trk* mutant growth was 96% of the wildtype and *trk & trm–8* wass 105% of the wildtype (Fig. 3).

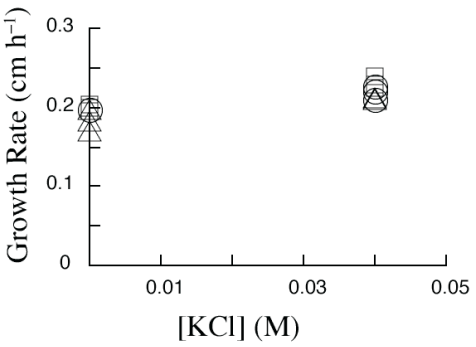

Figure 3. The effect of KCl on growth of wildtype (circles), and the *trk* (triangles), *trk & trm–8* (squares) mutants of *Neurospora crassa*.

The K^+ channel mutant, *tok*, exhibited no significant osmosensitivity compared to wildtype for either NaCl (97% of wildtype at 1.2 M NaCl) or sucrose (87% of wildtype at 1.5 M sucrose). The high affinity K^+ transporter mutant *hak–1* was slightly sensitive compared to wildtype for both NaCl (78% of wildtype at 1.2 M) and sucrose (90% of wildtype at 1.5 M) (Fig. 4).

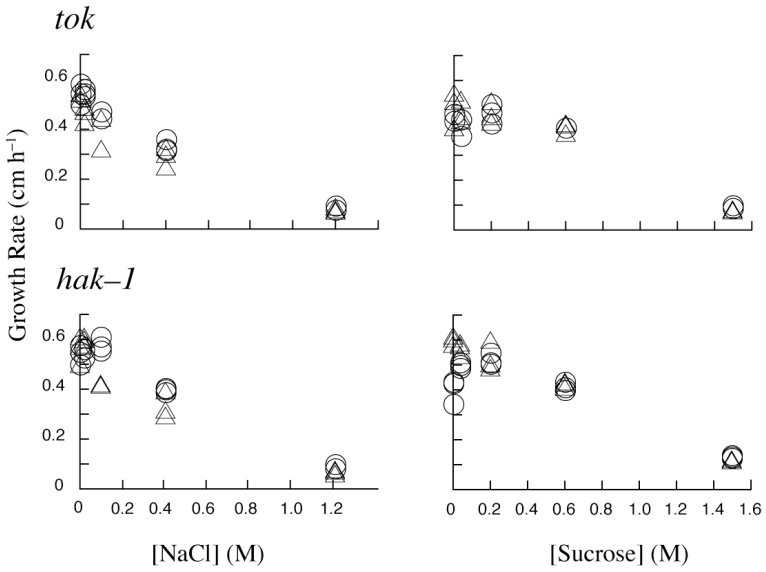

Figure 4. The effect of osmoticum (NaCl or sucrose) on growth of the *tok*, and *hak–1* mutants (triangles) and wildtype (circles) of *Neurospora crassa*. Treatments with NaCl are shown in the left panels; treatments with sucrose in the right panels

The *aqp* mutant had a similar growth rate as the wildtype (112.5% of the wildtype in 1.2 M of NaCl and 111% of the wildtype at 1.5 M of sucrose). The *clc–3* mutant also had a similar growth rate as the wildtype (88% of wildtype at 1.2 M of NaCl and 106% of wildtype at 1.5 M of sucrose) (Fig. 5).

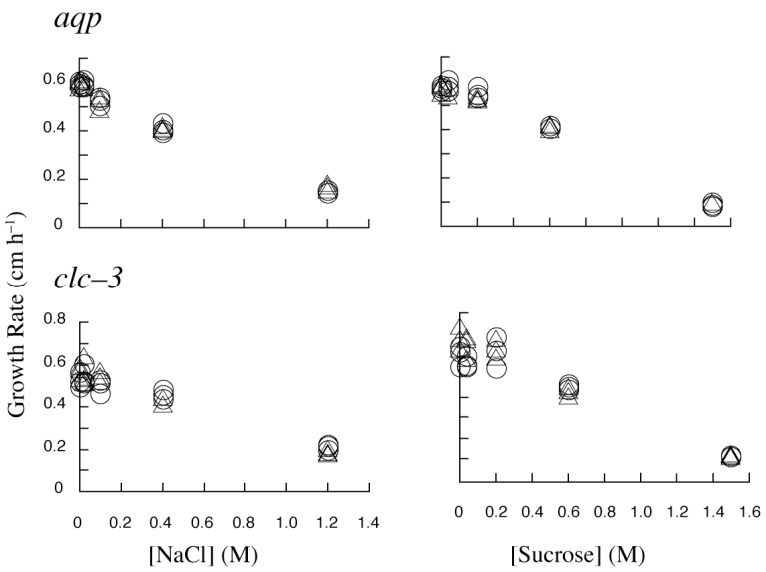

Figure 5. The effect of osmoticum (NaCl or sucrose) on growth of the *aqp* and *clc–3* mutant of *Neurospora crassa*. Treatments with NaCl are shown in the left panels; treatments with sucrose in the right panels.

The knockout of the downstream regulator of the osmotic MAP kinase pathway, *rrg–1* was very osmosensitive to both NaCl (0% of wildtype at 1.2 M) and sucrose (0% of wildtype at 0.6 M) condition. The knockout of a 14-3-3 protein (FGSC#14662) was generally a slow grower compared to wildtype even in the absence of hyperosmotic stress (69% of wildtype). The 14-3-3 protein growth rate was 60% of the wildtype at 1.2 M

NaCl and 74% of wildtype at 1.5 M of sucrose. The ptk2 homolog exhibit a high osmosensitivity to 1.2 M NaCl (26% of wildtype) but it was not sensitive to sucrose (119% of wildtype at 1.5 M sucrose) (Fig. 6).

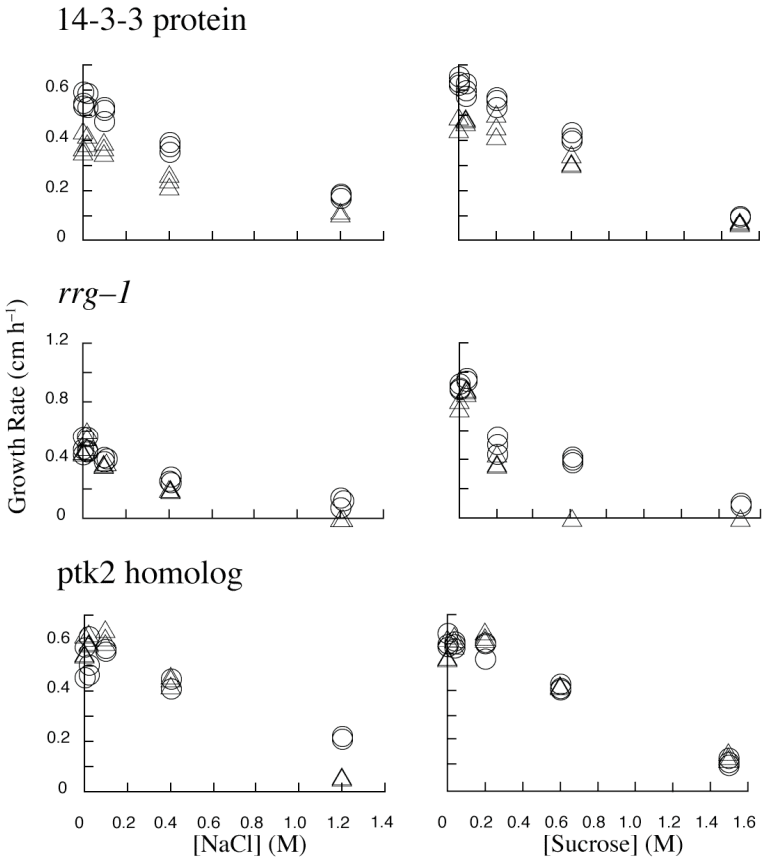

Figure 6. The effect of osmoticum (NaCl or sucrose) on growth of a 14-3-3 protein homolog, *rrg–1*, and the Ptk2 homolog mutants (triangles) and wildtype (circles) of *Neurospora crassa*. Treatments with NaCl are shown in the left panels; treatments with sucrose in the right panels.

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At higher NaCl concentrations, the *rrg–1* was highly osmosensitive: the growth rate was 28% of wildtype at 0.7 M NaCl and 0% at 1.2 M. The Ptk2 homolog mutant had a growth rate similar to wildtype at 0.7 M NaCl (96% of wildtype) but was osmosensitive at 1.2 M of NaCl (36% of wildtype). Both the mutant and wildtype grew poorly at the highest NaCl concentration used (1.5 M) (Fig. 7).

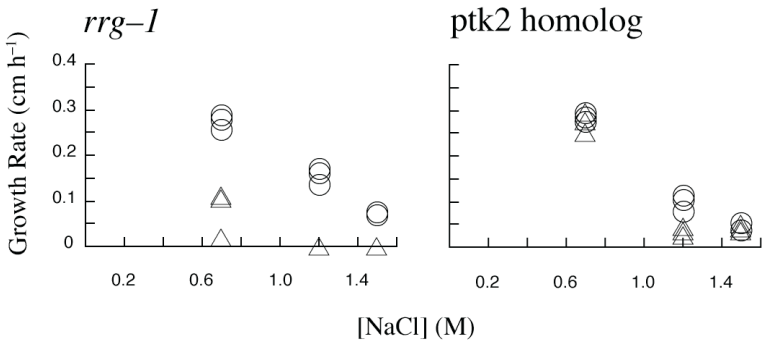

Figure 7. The effect of osmoticum (high NaCl concentration) on growth of the *rrg–1*, ptk2 homolog mutants (triangles) and wildtype (circles) of *Neurospora crassa*.

The effect of pH was examined for the Ptk2 homolog mutant, since it may regulate the plasma membrane H^+-ATPase and thereby play a role in pH regulation. At all pH tested, the Ptk2 homolog mutant had a similar growth rate as the wildtype (pH 4.38, 94%; pH 5.21, 105%; pH 6.02, 101%; pH 6.99, 115%; pH 8.04, 122% of wildtype) (Fig. 8).

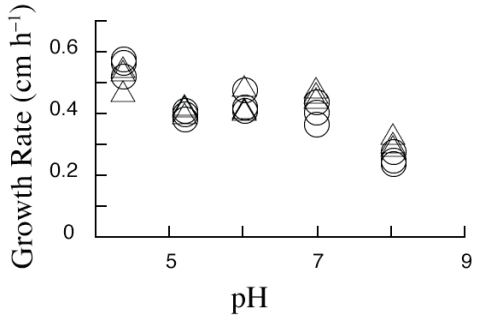

Figure 8. The effect of pH on growth of the Ptk2 homolog mutant (triangles) and wildtype (circles) of *Neurospora crassa*. To test whether Ptk2 functions in pH regulation, the growth of the mutant (triangles) was compared to that of wildtype (circles) at a range of pH, as shown

DISCUSSION

In the real world, *Neurospora crassa* must regulate its osmolarity to survive (Jones et al., 2007). To explore the mechanisms underlying osmoadaptation, we examined the possible roles of transporters and transport regulators. This objective was made easier by the recent creation of knockout mutants for almost all genes in the *N. crassa* genome (Colot et al., 2006). As an osmotic challenge, salt (NaCl) has two impacts on growth and survival of the cell. One is salinity. Na^+ and Cl^- ions, at a high enough intracellular concentration, have a deleterious effect on cellular function. Secondly, NaCl has an osmotic effect. The osmotic effect is greater than the salt concentration because when the salt dissociates to the Na^+ and Cl^- ions, both are osmotically active. Sucrose also has an osmotic effect, but would not have an effect as a dissociated ion. For the experiments performed here, both salt and sugar osmotica were tested. It is important to note that the osmolarity of sucrose treatments was lower than NaCl treatments, due to their differing solubilities, thus direct comparisons cannot be made, although 1.5 M sucrose would be approximately equivalent to 0.75 M NaCl.

The first set of transport mutants examined were those that might function in K^+ transport. The *N. crassa trm–8* gene probably encodes a K^+ transporter (Kiranmayi & Mohan, 2006), but it has no effect on the osmoregulation of the *N. crassa* in either NaCl or sucrose hyperomotic treatments. K^+ ions are maintained at a high concentration inside the cell and are required for the survival and growth of the fungi (Haro et al. 1999). It has to actively be accumulated into the cell (Haro et al. 1999). To test the role of the *TRM–8* protein in K^+ accumulation, the mutant was grown at low [K^+], but showed no difference in growth rate compared to the wildtype. This could be because other K^+ transporters can transport K^+. For example, the *trk* channel is an inward K^+ uniporter (Haro et al., 1999). The *trk* mutant is partially osmosensitive to NaCl, so it may play a role in osmoregulation due to K^+ uptake. The *trk* mutant grows the same as wildtype under K^+ deficient conditions so alternative K^+ transporters may be sufficient to accumulate K^+. The double mutant, *trk & trm*, grew similarly to the *trk* mutant under all conditions tested (NaCl, sucrose and low KCl), suggesting a role for *trk* in osmoregulation, but not for *trm–8*. The HAK–1 protein is a H^+/K^+ symporter which is expressed when extracellur K^+ is very low (0.25 mM) (Haro et al., 1999). It has only a slight effect on the osmoregulation of the *N. crassa* in NaCl but not sucrose hyperomotic treatments. The *tok* gene encodes a K^+ efflux channel, based on patch clamp measurements (Roberts, 2003), who reported that it may function in K^+ uptake. The *tok* knockout mutant growth rate is similar to wildtype in either NaCl or sucrose hyperosmotic treatment.

Besides K^+ transporters, other transport mutants were also examined. The *aqp* gene encodes a water channel (Tajkhorshid et al., 2002). Both wildtype and the mutant have a similar growth rate in NaCl and sucrose hyperosmotic treatment. The result indicates that this channel does not play a significant role in conditions of high osmotic stress caused by NaCl or sucrose. The *clc–3* gene encodes a putative chloride transporter (Henchenberger et al. 1996). The *clc–3* mutant and the wildtype have similar growth rates at NaCl and sucrose hyperosmotic condition. The result indicates that this transporter does not play a significant role in osmoadaptation.

Regulators of transport were also examined. The gene for *rrg–1* encodes a putative response regulator of osmotic stress (Jones et al., 2007). The mutated *rrg–1* is very sensitive to high concentration of NaCl and sucrose (Jones et al., 2007). The results confirm the osmosensitivity of *rrg–1* mutant to NaCl and sucrose hyperosmotic conditions, and are good evidence for the critical role of this response regulator plays in response to osmotic stress.

14-3-3 proteins belong to a family of proteins that bind to their effecter proteins such as kinases, phosphatases in order to regulate different biological processes such as cell growth (Fu et al. 2000). The knockout mutant was examined for its role in osmoadaptation in NaCl or sucrose hyperosmotic conditions. The 14-3-3 protein knockout mutant was generally a slow grower compared to wildtype even in the absence of hyperosmotic stress. In NaCl and sucrose hyperosmotic condition the growth rate was only partially different from the wildtype.

Ptk2 is a protein kinase in *Saccharomyces cerevisiae* which activates the H^+-ATPase in response to glucose, to effect uptake of glucose through a H^+/glucose symporter to support cellular metabolism (Goossens et.al., 2000). The Ptk2 homolog mutant in *N. crassa* is very sensitive to high concentration of NaCl. The growth rate of the Ptk2 homolog mutant in different pH mediums has been examined to check for the role of Ptk2 in regulating the H^+ concentration inside the cell to maintain the optimal growth of the fungi at different extracellular pH. The Ptk2 homolog mutant had the same growth rate as wildtype at different pH, suggesting it plays no role in regulating cytoplasmic pH.

In summary, some of the K^+ transporter mutants exhibit a slight osmosensitivity such as *trk, trk& trm–8* and *hak–1*. The *rrg–1* mutant was confirmed to have a significant effect on osmoregulation. The transport regulator, the 14-3-3 protein knockout mutant, was found to be a slow grower at all concentrations, and the Ptk2 homolog mutant was very osmosensitive. Thus, some, but not all of the mutants, examined do play a role in osmoadaptation.

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