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A microarray-based transcriptomic time-course of hyper- and hypo-osmotic stress signaling events in the euryhaline fish *Gillichthys mirabilis*: osmosensors to effectors

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SUMMARY

Cells respond to changes in osmolality with compensatory adaptations that re-establish ion homeostasis and repair disturbed aspects of cell structure and function. These physiological processes are highly complex, and require the coordinated activities of osmosensing, signal transducing and effector molecules. Although the critical role of effector proteins such as Na^+, K^+-ATPases and $Na^+/K^+/Cl^-$ co-transporters during osmotic stress are well established, comparatively little information is available regarding the identity or expression of the osmosensing and signal transduction genes that may govern their activities. To better resolve this issue, a cDNA microarray consisting of 9207 cDNA clones was used to monitor gene expression changes in the gill of the euryhaline fish *Gillichthys mirabilis* exposed to hyper- and hypo-osmotic stress. We successfully annotated 168 transcripts differentially expressed during the first 12 h of osmotic stress exposure. Functional classifications of genes encoding these transcripts reveal that a variety of biological processes are affected. However, genes participating in cell signaling events were the dominant class of genes differentially expressed during both hyper- and hypo-osmotic stress. Many of these genes have had no previously reported role in osmotic stress adaptation. Subsequent analyses used the novel expression patterns generated in this study to place genes within the context of osmotic stress sensing, signaling and effector events. Our data indicate multiple major signaling pathways work in concert to modify diverse effectors, and that these molecules operate within a framework of regulatory proteins.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/211/22/3636/DC1

Key words: fish, genomics, *Gillichthys mirabilis*, microarray, osmotic, salinity, signaling, stress, transcriptome.

INTRODUCTION

Osmolality is a pervasive abiotic factor that has a strong influence on cellular and organismal function (Somero and Yancey, 1997; Kültz and Burg, 1998). Consequently, regulation of intracellular and extracellular solute and water balance has become a fundamental requirement for metazoans. Fish and other aquatic or semi-aquatic organisms, which remain in direct contact with environmental water, are constantly challenged to maintain ion concentrations within the defined range necessary for proper cell function. Most fish species rely upon stable environmental salinities to preserve osmolality in their body fluids. These stenohaline species can only endure relatively minor changes in environmental salinity and are adapted to inhabit either seawater or freshwater. However, there are numerous fish species that can tolerate dramatic variations in environmental salinity. These euryhaline species have evolved exceptional osmoregulatory capabilities and some can survive salinities ranging from freshwater to four times that of seawater (Fiol and Kültz, 2007).

The gills of fish are exposed directly to the external aqueous environment and are the dominant site for the balance of ion movement between gains and losses (Evans et al., 2005). In freshwater teleosts, salts are imported from the aqueous environment through the gills. Conversely, saltwater teleosts excrete excess salts through the gills. Euryhaline species adapt to either freshwater or saltwater by efficiently switching these systems (Kato et al., 2005; Tang and Lee, 2007). The particular range of salinities that a given fish species can adapt to is dependent on the strength of its osmoregulatory mechanisms. These physiological processes are highly complex and extend across all levels of biological organization from behavior to molecules (Kültz et al., 2007). At the molecular level, achieving ion homeostasis during osmotic stress is contingent upon the cell's ability to recognize and quantify environmental osmolality and arrange an appropriate response. Integral to this process are the coordinated activities of osmosensors, which activate appropriate signal transduction pathways; signal transducers, which relay molecular messages to specific target molecules; and effectors, which work in concert to actively restore homeostasis (Fiol and Kültz, 2007). Effector mechanisms involved in osmotic acclimation of a great many fish species have been identified and characterized in detail. These studies have demonstrated that ion homeostasis is restored through extensive cell remodeling, including volume regulatory changes, altered patterns of cell differentiation, and modulation of expression and activity of many proteins, such as ion transporting Na^+, K^+-ATPases and $Na^+/K^+/Cl^-$ co-transporters, and water transporting aquaporins (Evans, 2002; Fiol et al., 2006b). However, only modest attention has been directed towards identifying the molecular osmosensing and signal transduction events leading up to the activation of these effector proteins.

In the present study, we have utilized a cDNA microarray-based transcriptomic profiling approach to identify the molecular constituents of early osmoregulatory processes in the gill tissue of a euryhaline estuarine goby, *Gillichthys mirabilis*. This species tolerates broad spatial and temporal variations in salinity within its

natural habitat, and can encounter salinities of over 100 parts per thousand (p.p.t.) in the wild (www.elkhornslough.org). The application of our 9207 feature *G. mirabilis* cDNA microarray (Gracey et al., 2001; Buckley et al., 2006; Gracey, 2008) allows the expression of several thousand genes to be monitored simultaneously, providing a broad and integrated picture of the way an organism's messenger RNA (mRNA) pool, the transcriptome, responds to a changing environment. Because altering the transcriptome is one of the most rapid and versatile responses available to organisms experiencing environmental stress, transcriptomic analysis allows one to capture early or transient changes in gene expression that may occur at the initial stages in responses to osmotic stress. Importantly, osmotic stress relevant molecules such as subunits of Na^+, K^+-ATPases, $Na^+/K^+/Cl^-$ co-transporters (Tipsmark et al., 2002), urea transporters (Mistry et al., 2001), taurine transporters (Takeuchi et al., 2000) and aquaporins (Cutler and Cramb, 2002) are regulated by osmolality at the mRNA level (Burg et al., 1996). However, because changes in abundance of these effector proteins are not generally detected until 12–18 h of osmotic stress exposure, it is likely that they are regulated by earlier upstream events that remain to be characterized (Fiol and Kültz, 2005). To complement the transcriptomic analysis, we monitored the abundance and/or activity of key proteins in order to quantify the net effects of gene expression on specific cellular processes at the protein level.

MATERIALS AND METHODS

Animal collection and maintenance

Specimens of *G. mirabilis* (Cooper) were collected from Elkhorn Slough National Estuarine Research Reserve, CA, USA (121.7W, 36.8N). Fish were maintained at Hopkins Marine Station in a 200 l aquarium containing recirculating seawater pumped from Monterey Bay at a salinity of 31 p.p.t. Fish were acclimated in these tanks for at least 14 days, and were fed trout pellets (Bio-Oregon, Warrenton, OR, USA) daily. Fish were not fed during the final 10 days of acclimation to normalize nutritional state. Mortality during acclimation was 0%. Salinity did not change during the acclimation period. Water temperature was stable at 11.5°C during acclimation. The genders of the specimens were not determined because this species does not display external sexual dimorphism.

Osmotic stress exposure protocol

Hyper- and hypo-osmotic solutions were prepared by dissolving Instant Ocean pre-mixed aquarium salts (Aquarium Systems, Mentor, OH, USA) into 15 l aquaria containing double distilled water to a final concentration of 70 p.p.t. and 10 p.p.t., respectively. Hyper- and hypo-osmotic solutions were recirculated during exposures using a standard aquarium pump (Millennium 2000, Aquarium Systems, Mentor, OH, USA). Because fluctuating ambient temperatures can cause osmoregulatory disturbances in fish (Sardella et al., 2008), temperature was held stable at 11.5°C by placing hyper- and hypo-osmotic aquaria into large water baths containing recirculating seawater.

A total of 39 individuals were used for osmotic stress exposures. Fish (N=3 for each exposure condition at each time point) were transferred by net from the 200 l holding tank and placed in 15 l exposure aquaria containing either hyper- or hypo-osmotic solutions. Three control fish were simultaneously transferred from the 200 l holding aquarium into a 15 l aquarium containing recirculating seawater pumped from Monterey Bay (31 p.p.t.). Thus, a total of nine fish were used at each time point. Following exposure, fish were killed by cervical transection and flash frozen in liquid nitrogen. This procedure was repeated for each of the four exposure lengths of 1, 2, 4 and 12 h in succession. Salinity was monitored by refractometry and remained stable at 70, 10 or 31 p.p.t. throughout all exposures in the hyper-, hypo-osmotic and control aquaria, respectively. Three individuals from the 200 l holding aquarium were killed immediately before osmotic stress exposures to represent time zero. The same 39 individuals were used to harvest gill tissue used in microarray, western blotting and enzyme activity experiments performed in this study.

cDNA microarray preparation

A description of the construction of the *G. mirabilis* microarray used in these experiments is reported elsewhere (Gracey et al., 2001; Buckley et al., 2006; Gracey, 2008).

RNA extraction for hybridization

Gill tissue was dissected from partially thawed fish and placed immediately into 2 ml plastic tubes containing 500 µl ice-cold TRIzol solution (38% acid phenol, 800 mmol l^{-1} guanidine thiocyanate, 400 mmol l^{-1} ammonium thiocyanate, 100 mmol l^{-1} sodium acetate pH 5.0, 5% glycerol). Tissue was homogenized using a TissueLyzer (Qiagen, Valencia, CA, USA) for 2 min at 25 strokes s^{-1}. Homogenized tissues were centrifuged for 10 min at 12,500 ***g*** at 4°C using a Hermle refrigerated centrifuge (Labnet, Woodbridge, NJ, USA). The resulting supernatant was extracted with chloroform and centrifuged for 15 min at 12,500 ***g*** at 4°C. The resulting supernatant was precipitated in 100% isopropanol and centrifuged for 15 min at 12,500 ***g*** at 4°C. The pelleted total RNA was washed with 75% ethanol and dissolved into 100 µl nuclease free water (Growell's, Irvine, CA, USA). 1 µl of sample was loaded onto a 1% agarose in TAE buffer [40 mmol l^{-1} Tris, 5.7% acetic acid, 8 mmol l^{-1} ethylenediaminetetraacetic acid (EDTA) pH 8.0] gel to ascertain that an appropriate amount of RNA had been extracted. Total RNA was cleaned using Ambion 10051 G filter cartridges (Applied Biosystems, Foster City, CA, USA/Ambion, Austin, TX, USA) according to manufacturers' instructions. Total cleaned RNA was dissolved in 20 µl nuclease free water and a 1 µl sample loaded onto a 1% agarose in TAE buffer gel to ensure that RNA was free of impurities. The remaining 19 µl was stored at –80°C.

Profiling gene expression with cDNA microarrays

A total of 39 arrays were used to generate our dataset, corresponding to individual hybridizations of all three fish from each treatment group (control, hyper- and hypo-osmotic stress) and time point (0, 1, 2, 4, 12 h). Total, cleaned RNA was also collected from gill tissues of 10 reference individuals, which were acclimated to ambient Monterey Bay seawater salinity (31 p.p.t.) and temperature (11.5°C) for at least 14 days. It was against this reference sample that the values from the experimental (both control and osmotic-stressed) samples were normalized (Podrabsky and Somero, 2004; Buckley et al., 2006). The concentration of total clean RNA was determined by A_{260} absorbance using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). 20 µg of total cleaned RNA were reverse transcribed, labeled and purified as described elsewhere (Podrabsky and Somero, 2004; Buckley et al., 2006). Samples were diluted to a final volume of 41.3 µl using 20 mmol l^{-1} N-2-Hydroxyethylpiperazine-N′-2 ethanesulfonic acid (HEPES) pH 7.0, 3×SSC (450 mmol l^{-1} NaCl, 50 mmol l^{-1} sodium citrate), 0.2% sodium dodecylsulfate (SDS) and 0.4 mg ml^{-1} poly[A] blocker (Invitrogen, Carlsbad, CA, USA). Samples were denatured for 2 min at 95°C and allowed to cool for 5 min at room temperature

before being applied to the slides. Hybridizations were performed at 65°C overnight in Genomic Solutions (Ann Arbor, MI, USA) hybridization chambers. Following hybridizations, the arrays were gently washed in a warmed solution (~35°C) of 0.5×SSC and 0.01% SDS for 2 min. Slides were then transferred to a wash of 0.06×SSC and gently washed for 15 min, followed by an immersion rinse in deionized water. Slides were dried by low speed centrifugation and scanned on an AXON GenePix 4000B microarray scanner (Axon Instruments, Molecular Devices, Sunnyvale, CA, USA).

Analysis of microarray data

Data from the 39 arrays were extracted using GenePix 4.0 software. Gene expression levels were determined at each time point by comparing the amount of mRNA transcript present in the experimental samples (osmotic stress and control) relative to a reference sample. The use of a reference sample allowed the comparison of the relative amount of each transcript at each time point and salinity with a common sample. The ratios of fluorescence intensities at a given salinity time point were first Lowess normalized and then normalized against the mean ratio for that spot for the four control (1, 2, 4 and 12 h) time points. This method of normalizing fluorescence values in time course experiments conforms to that of previously published studies on time course transcriptomic profiling in fish (Podrabsky and Somero, 2004; Buckley et al., 2006). The resulting ratio then represents Cy5 fluorescence from an osmotic stress time point to Cy3 from the reference divided by the mean ratio of Cy5 from all control time points to Cy3 reference. Microarray data were submitted to the Gene Expression Omnibus (GSE11700).

Statistical analyses of microarray data

One-way analysis of variance (ANOVA) was used to identify genes for which the expression patterns showed a significant effect of treatment using GeneSpring 7.0. To remove any bias associated with natural cycling of gene expression that may have occurred during the duration of each exposure, control hybridizations for each individual at all four time points were treated as 'replicates'. The three time zero individuals were not included with controls and were considered a separate treatment group. A Tukey's *post hoc* test was subsequently employed to isolate genes whose expression differed significantly at each time point during hyper- and hypo-osmotic stress. Importantly, we did not impose an arbitrary minimal fold-change threshold on our data set as is common practice in microarray-based studies (Gracey et al., 2001; Podrabsky and Somero, 2004; Buckley et al., 2006; Aluru and Vijayan, 2007; Kassahn et al., 2007). Therefore, all genes deemed significant using the methodology described above were considered in subsequent analyses (see Table S2 in supplementary material).

DNA sequencing

Sequencing was attempted on all genes deemed significant by ANOVA that were not annotated in previous versions of the array (Buckley et al., 2006). BlastX queries were performed against NCBI public databases to identify sequenced genes. The BlastX result with the highest homology to the *G. mirabilis* sequence was used to annotate the clones. A minimum e-value of $e=1.0\times10^{-6}$ was imposed as the requirement for annotation, although the vast majority of annotated clones had considerably more significant e values (median e-value of sequenced clones=1.0×10^{-41}). All sequences have been entered into the GenBank database (see Table S2 in supplementary material).

Protein extraction

Partially thawed gill tissue was dissected and placed into ice-cold homogenization buffer [32 mmol l^{-1} Tris-Cl pH 6.8, 2% SDS, 1 mmol l^{-1} EDTA with protease inhibitors (complete, Mini, Roche Applied Science, Indianapolis, IN, USA)]. Dissected tissue was homogenized using a TissueLyzer for 2 min at 25 strokes s^{-1}. Homogenates were then heated for 5 min at 100°C and centrifuged at 8700 ***g*** for 10 min. Pellets were discarded and the total protein content in the soluble fraction determined by Pierce BCA protein assay (Pierce, Rockford, IL, USA).

Antibodies

A total of nine different primary antibodies were employed in this study. In order to maximize the signal-to-noise ratio, dilutions and incubation times of primary antibodies as well as the amount of total protein loaded per lane varied among individual antibodies. The optimized condition for each antibody is detailed below. Phospho-(Ser/Thr) PKA substrate antibody (#9621), Phospho-(Ser/Thr) AKT substrate antibody (#9611) and Phospho-(Ser) PKC substrate antibody (#2261) (Cell Signaling Technologies, Danver, MA, USA) were all diluted to 1:1000, incubated overnight at 4°C with gentle agitation and reacted against 15 µg total gill protein. The ubiquitin-protein conjugate antibody (UG 9510, Biomol International, Plymouth Meeting, PA, USA) was also diluted to 1:1000 and reacted against 15 µg total protein but was incubated at room temperature for 90 min with gentle agitation. The phospho-p44/42 (Erk1/Erk2) MAP kinase (Thr202/Tyr204) (#9101, Cell Signaling Technologies) and α-tubulin (sc-5546, Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies were both diluted to 1:500, incubated overnight at 4°C with gentle agitation and reacted against 30 µg total gill protein. Antibodies directed against proliferating cell nuclear antigen (PCNA, sc-7907) and phospho-Histone H3 (Ser 10) (sc-8656-R) (Santa Cruz Biotechnology) were diluted 1:200, incubated for 90 min at room temperature and reacted against 15 µg total gill protein. Finally, the actin antibody (JLA20) developed by Jim Jung-Ching Lin was obtained from the Developmental Studies Hybridoma Bank under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA, USA. This antibody was diluted 1:100, incubated overnight at 4°C with gentle agitation and reacted against 10 µg total protein.

Western blot analysis

Total protein was diluted in 1×Laemmli sample buffer (Bio-Rad, Hercules, CA, USA), heated for 5 min at 100°C and loaded onto pre-cast 10% Tris-HCl polyacrylamide gels (Bio-Rad). Electrophoretically separated proteins were wet transferred to nitrocellulose membranes for 2 h at 4°C. Resulting blots were blocked for 1 h in 5% blocking grade non-fat dried milk (Bio-Rad) dissolved in Tris-buffered saline (250 mmol l^{-1} Tris-Cl pH 7.5, 1.5 mol l^{-1} NaCl) containing 0.1% Tween-20 (TBST), washed twice for 5 min in TBST, and incubated in the primary antibody diluted in 5% bovine serum albumin (BSA) in TBST. Following three 5 min washes in TBST, blots were incubated in the secondary antibody (either goat anti-rabbit (sc-2004) or goat anti-mouse (sc-2055), Santa Cruz Biotechnology). All secondary antibodies were diluted 1:5000 in 5% BSA in TBST and incubated for 60 min at room temperature with gentle agitation. Following six 5 min washes in TBST, blots were treated with enhanced chemiluminescent reagent (Amersham, Piscataway, NJ, USA) for 2 min. Finally, blots were exposed to film (Blue Lite Auto Rad F-9024, ISC BioExpress, Kaysville, UT, USA) and developed. Relative intensity values were calculated by

normalizing intensities against a standard protein loaded on all gels. Densitometric analysis was performed using ImageJ (http://rsb.info.nih.gov/ij/). Statistical significance of protein quantification data was determined by one-way ANOVA.

Enzyme assays

Gill tissue was dissected from partially thawed individuals, weighed and placed in ice-cold homogenization buffer [50 mmol l^{-1} KPO_4 pH 6.8 at 20°C, 1 mmol l^{-1} EDTA, 0.1 mmol l^{-1} phenylmethanesulfonyl fluoride (PMSF)]. Tissue was homogenized using a Janke and Kunkel Labortechnik drill for 10 s. Homogenates were then centrifuged at 12,500 ***g*** for 5 min at 4°C to remove any debris. The soluble protein fraction was transferred to a new tube and stored on ice for the remainder of the experiment.

All enzyme assays were performed at 20±0.1°C. Malate dehydrogenase (MDH) activity was determined as described by Fields and colleagues (Fields et al., 2006) with minor modifications to the enzyme cocktail. Specifically, 25 µl of crude homogenate was added to 2 ml MDH enzyme cocktail (200 mmol l^{-1} imidazole-HCl pH 7.0 at 20°C, 0.15 mmol l^{-1} NADH, 0.2 mmol l^{-1} oxaloacetate). Lactate dehydrogenase (LDH) activity was determined as described by Fields and colleagues (Fields et al., 2008) with minor modifications to the enzyme cocktail. Specifically, 25 µl of crude homogenate was added to 2 ml LDH enzyme cocktail (80 mmol l^{-1} imidazole-HCl pH 7.5 at 20°C, 4 mmol l^{-1} pyruvate, 0.15 mmol l^{-1} NADH). Citrate synthase (CS) activity was determined as described by Fields and colleagues (Fields et al., 2008) with minor modifications to the enzyme cocktail. Specifically, 25 µl of crude homogenate was added to CS enzyme cocktail (50 mmol l^{-1} imidazole-HCl pH 8.2 at 20°C, 1.5 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} DTNB, 0.15 mmol l^{-1} acetyl CoA) and activity recorded for 200 s. The subsequent addition of oxaloacetate (0.2 mmol l^{-1}) initiated the true CS reaction, yielding a second rate from which the background rate was subtracted to yield net CS activity.

Assays were performed for each individual fish from 12 h hyperosmotic (N=3), hypo-osmotic (N=3) and control (N=1) groups. MDH and LDH assays were performed in triplicate. CS assays were performed in duplicate. Enzyme activities from each individual fish were converted to international units (I.U.), and statistical analyses were performed on individual I.U. values using a one-way ANOVA.

RESULTS AND DISCUSSION

Information regarding the osmosensing and signal transduction pathways governing the activity of osmoregulatory effector molecules is limited. We have addressed this issue through time course transcriptomic profiling of gill tissue harvested from *G. mirabilis* during the first 12 h after abrupt transfer to either hyper- or hypo-osmotic solutions. Following normalization of the microarray data, a one-way ANOVA with a Benjamini and Hochberg false discovery rate of P<0.05 (Benjamini and Hochberg, 1995) revealed 1057 significantly differentially expressed cDNAs. Given the relatively brief exposure lengths used in this time course, the dual function of cortisol as a rapidly acting stress hormone and in facilitating osmotic stress adaptation, and the likelihood of handling stress to influence the transcriptome of fish (Krasnov et al., 2005; Fast et al., 2008), we considered it imperative to eliminate genes whose expression was potentially affected by handling stress. For this reason, a Tukey's *post hoc* test was used to exclude those genes that differed exclusively between time=0 and controls. Following this filtering step, 187 and 173 cDNAs differed significantly from control fish during hyper- and hypo-osmotic stress, respectively. Only these genes were considered osmotically regulated. These clones were sequenced, and comparison of the generated sequences with those in public databases successfully identified 92 hyperosmotically expressed genes and 76 hypo-osmotically expressed genes (see Table S2 in supplementary material).

Osmotically regulated genes were subsequently assigned to broad functional categories in an effort to determine gross biological processes potentially affected by osmotic stress (Fig. 1A; Table S1 in supplementary material). Designations were based upon information contained in the Gene Ontology database (Harris, 2004), Uniprot (www.uniprot.org) and the primary literature. Functional classifications revealed that genes regulating cell signaling were the dominant class of molecules differentially expressed during the early response of *G. mirabilis* gill tissue to both hyper- (25 of 92 or 27% of total genes) and hypo-osmotic (20 of 76 or 26% of total genes) stresses. Such a result provided an important proof of concept regarding our experimental design and objectives.

Knowledge concerning the extent to which osmoregulatory mechanisms are shared during hyper- and hypo-osmotic adaptation is limited. During *G. mirabilis* osmotic stress, 18 genes (14%) were differentially expressed during both hyper- and hypo-osmotic stress in the same direction, indicating that some osmoregulatory processes may be common to both types of stress. To gain additional insight, we performed a principal components analysis (PCA) on all osmotically regulated genes. PCA on conditions demonstrated that expression profiles were divergent between hyper- and hypo-osmotic stressed fish. This divergence was most visible during hours four and 12 of exposure (Fig. 1B). We attributed this pattern to the expression of effector proteins required to meet the specific demands of each individual stress. To further investigate this issue, we performed a PCA on genes and identified those that clustered tightly to principal component 2 (PC2). Four of the top five genes correlated to PC2 regulate the production of organic osmolytes, critical effectors in osmotic stress adaptation and some of the most highly expressed genes observed in this study (data not shown). We conclude that although a considerable proportion of differentially expressed genes are shared between both stresses, the majority of the variation in gene expression during osmotic stress is attributed to genes expressed exclusively during hyper- or hypo-osmotic stress.

Novel putative osmosensors

Knowledge of molecular osmosensors that monitor and quantify environmental and extracellular osmolality in fishes is minimal. Potential osmosensors include membrane proteins influenced by stretching and compacting, molecular chaperones that monitor the degree of protein unfolding, DNA damage sensors and proteins associated with cytoskeletal organization (Fiol and Kültz, 2007). It is likely that multiple osmosensors act synergistically to control osmoregulatory signal transduction networks. In the present study, genes encoding several reputed osmosensors were significantly differentially expressed during osmotic stress, including mucin-4 (see Table S1F in supplementary material) (de Nadal et al., 2007) and casein kinase 2 (see Table S1B in supplementary material) (Poole et al., 2005). In addition, we have identified two strong candidates for novel putative osmosensors during osmotic stress (Fig. 2A,B). FK506-binding protein 51 (FKBP-51) is a molecular chaperone comprising one component of a heterocomplex of proteins maintaining the glucocorticoid receptor (GR) in a constitutively inactive state (Westberry et al., 2006; Zhang et al., 2008). During osmotic stress in *G. mirabilis*, unfolded proteins may recruit the chaperone activity of FKBP-51 away from the GR, relieving its inhibitory functions and promoting GR ligand binding

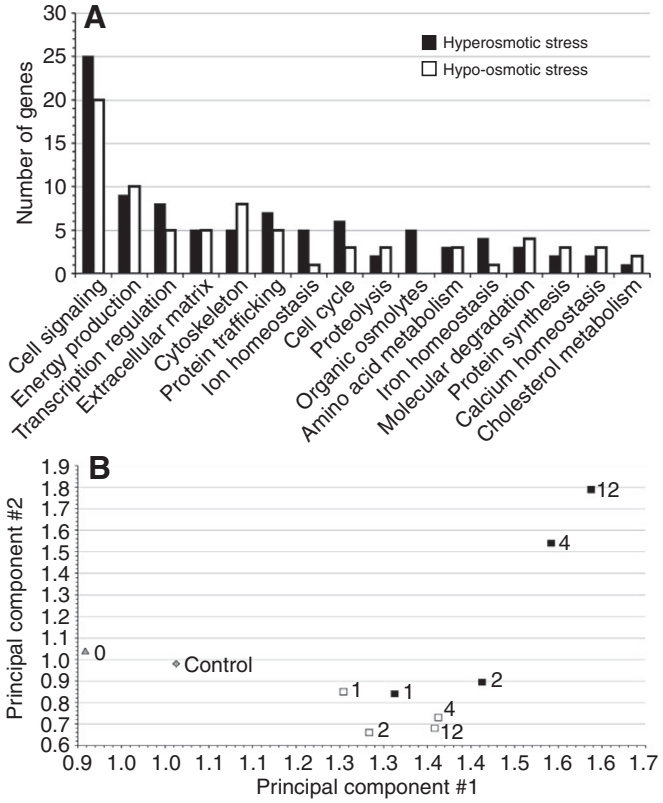

Fig. 1. (A) Functional classification of all annotated genes that differed significantly from control values (N=168; ANOVA P<0.05) during hyper- (filled bars) and hypo-osmotic stress (open bars) in *G. mirabilis* gill tissue. Designations were based upon an assigned putative role during osmotic stress as determined by data contained within the Gene Ontology database, Uniprot and the primary literature. (B) Principal components analysis (PCA) illustrating gene expression differences between hyper- and hypo-osmotic stressed *G. mirabilis* gill tissue. PCA was performed on conditions (hyperosmotic stress shown as filled symbols; hypo-osmotic stress shown as open symbols; time=0 and controls shown as gray symbols) using all genes that differed significantly from control values (ANOVA P<0.05) during osmotic stress. Clustering indicates similar expression profiles among conditions (*x*-axis PCA component 1, 49.81% of variance; *y*-axis PCA component 2, 18.73% of variance).

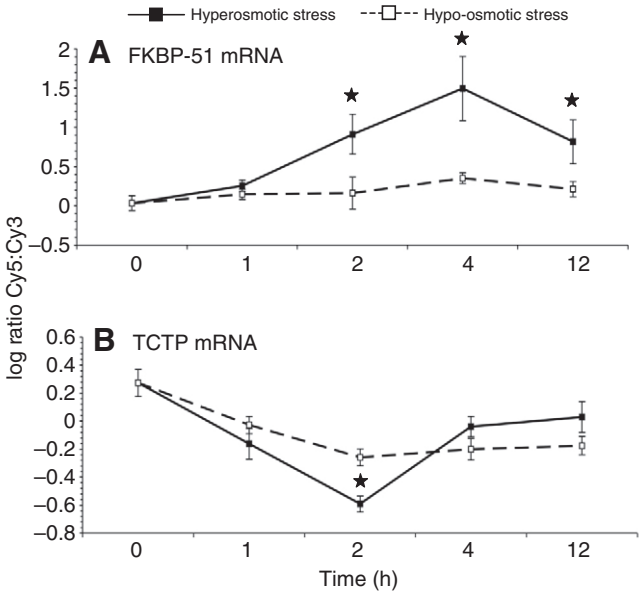

Fig. 2. Expression profiles of possible novel osmosensors in gill tissue of *G. mirabilis*. (A) FK506 binding protein 51 (FKBP-51) mRNA. (B) Translationally controlled tumor protein (TCTP) mRNA. Data points were plotted as the natural log of the ratio of Cy5 (experimental and control) to Cy3 (reference) divided by the mean ratio of Cy5 (control) to Cy3 (reference) for all control time points. The natural log of the ratio generated for control fish was then subtracted from all hyperosmotic (filled symbols, solid line), hypo-osmotic (open symbols, broken line) and control values at each time point (0, 1, 2, 4, 12 h post-exposure). Therefore, the expression of the control data point used in the microarray ANOVA is represented as 0 at each time point, and time=0 values have a degree of handling stress incorporated. A Tukey's *post hoc* test was performed to determine at which time points each gene differed significantly from controls. Filled stars denote significant expression during hyperosmotic stress. Open stars denote significant expression during hypo-osmotic stress. Values are shown ± s.e.m. P<0.05.

and subsequent signaling events. Such a scenario is especially appealing given the prominent role for GR-mediated processes during osmoregulation. Cortisol, the major corticosteroid in fish, is considered a master regulator of chloride cell differentiation and function during osmoregulation, and operates in synergy with both prolactin and insulin-like growth factor 1 (IGF-1) (McCormick, 2001). The physiological actions of cortisol are exerted through the GR, which acts as a ligand-dependent transcription factor to control the expression of specific genes (Cato et al., 2002). These actions are relatively rapid and take approximately 30–60 min. We observed a dramatic 4.6-fold upregulation of FKBP-51 mRNA beginning at 2 h post-exposure during hyperosmotic stress in *G. mirabilis* gill tissue (Fig. 2A). Given the rapid actions of the activated GR, it is plausible that FKBP-51 was recruited and the GR activated by cortisol during the first 60 min of hyperosmotic stress. GR activity may become subsequently repressed as FKBP-51 mRNA levels increase through to hour 12. This mechanism of GR regulation is consistent with current knowledge regarding the regulation of the GR by chaperones (Grad and Picard, 2007) and reminiscent of heat shock factor regulation, an environmental stress-induced transcription factor responsible for the rapid synthesis of heat shock proteins (Voellmy, 2004).

Translationally controlled tumor protein (TCTP) is a highly conserved, transcriptionally regulated protein widely expressed in eukaryotic cells and implicated in a variety of cellular processes (Bommer and Thiele, 2004). Interestingly, the protein structure of TCTP in yeast reveals homology with a family of small chaperone proteins involved in mediating cell signaling (Thaw et al., 2001). Recent data have shown that TCTP interacts with the cytoplasmic domain of the catalytic α-subunit of Na^+, K^+-ATPase, acting as a repressor of activity (Kim et al., 2008). We speculate that TCTP may be a potential osmosensor in *G. mirabilis*. Protein damage incurred by osmotic stress or required signaling events may recruit TCTP away from the Na^+, K^+-ATPase, relieving inhibition and promoting Na^+, K^+-ATPase activity along with other osmoregulatory processes. Indeed, this role agrees with the observed decrease in TCTP mRNA and subsequent increase in Na^+, K^+-ATPase α-subunit mRNA during hyperosmotic stress (Fig. 2B; Fig. 6A).

Upstream osmotic signaling events – receptors, ligands and hormones

The identification of upstream signaling molecules related to the activation, abundance or presentation of ligands and receptors allows for an inference into which specific pathways may be affected during

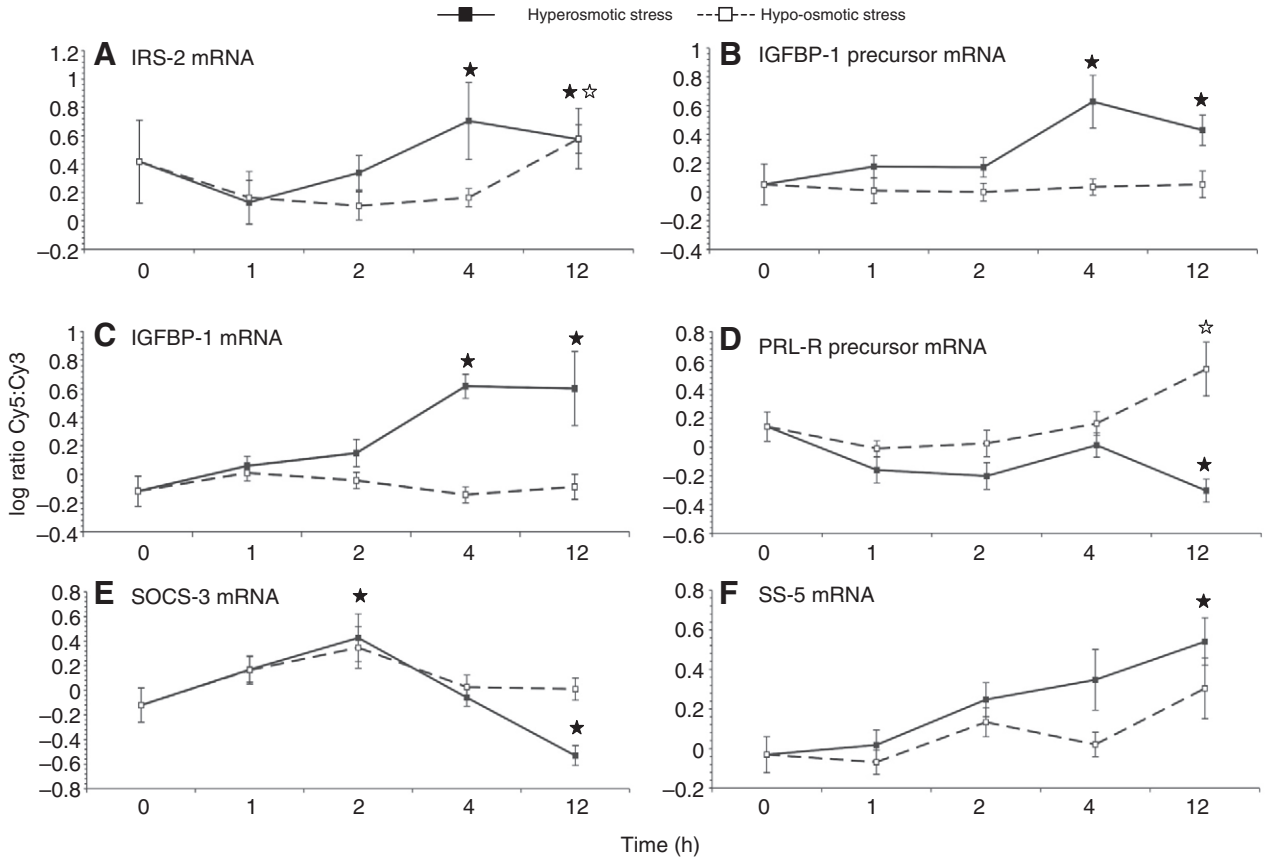

Fig. 3. Expression profiles of genes participating in insulin-like growth factor signaling cascades identified during osmotic stress in *G. mirabilis* gill tissue. (A) Insulin receptor substrate 2 mRNA (IRS-2; Gm_43o05). (B) Insulin-like growth factor binding protein 1 precursor mRNA (IGFBP-1 precursor; Gm_CC11). (C) Insulin-like growth factor binding protein 1 mRNA (IGFBP-1; Gm_CF01). (D) Prolactin receptor precursor mRNA (PRL-R precursor). (E) Suppressor of cytokine signaling 3 mRNA (SOSC-3). (F) Somatostatin receptor 5 mRNA (SS-5). Data points were plotted and labeled as described previously. In cases where a single gene was represented by multiple significant features (those proceeded by a clone ID above), the feature with the strongest *P*-value was illustrated. Filled stars denote significant expression during hyperosmotic stress. Open stars denote significant expression during hypo-osmotic stress. Values are shown ± s.e.m. $P<0.05$.

osmotic stress. Growth hormone (GH) is a pituitary polypeptide hormone that has been shown to affect osmoregulatory processes during hyperosmotic adaptation (Sakamoto and McCormick, 2006). This effect is due to the capacity of GH to increase the number and size of gill chloride cells, and the abundance of Na^+, K^+-ATPases and $Na^+/K^+/Cl^-$ co-transporters (McCormick, 2001; Pelis and McCormick, 2001). Some of the actions of GH are integrated through IGF-1. Exogenous application of IGF-1 increases salinity tolerance in rainbow trout, Atlantic salmon, killifish and brown trout (Mancera and McCormick, 1998; Seidelin et al., 1999). Upon ligand binding, IGF-1 receptors engage insulin receptor substrate (IRS) proteins, which in turn activate various downstream signaling pathways, including mitogen-activated protein kinases (MAPK) and protein kinase B (AKT) (Rui et al., 2001). In accordance with these data, expression of the gene encoding insulin receptor substrate-2 (IRS-2) increased 2-fold in hyperosmotic stressed *G. mirabilis* gill tissue by 4 h (Fig. 3A), and probably increased to accommodate an IGF-1 initiated adaptive response. Interestingly, IRS-2 mRNA expression also significantly increased 1.6-fold in hypo-osmotically stressed fish by 12 h. A role for IGF-1 signaling during freshwater acclimation has not been established despite its well-documented role in seawater adaptive responses. Although data presented in the current study do not exclude this possibility, we suggest an alternative centered upon the regulation of IGF-1 activity by binding proteins. The majority of IGFs in extracellular fluids are found complexed with specific high affinity binding proteins (IGFBPs). IGFBPs have been suggested to mediate the efflux of IGFs from the vascular space to the cell surface, thereby modulating interactions between the ligands and receptors, and to prevent proteolytic degradation of IGFs, prolonging their half-lives (Wood et al., 2005). In the present study, IGFBP-1 mRNA increased in expression coordinately with IRS-2 in hyperosmotically stressed gill tissue but IGFBP-1 expression was absent from hypo-osmotically stressed individuals (Fig. 3B,C). Therefore, these data suggest that although IRS-2 mRNA did increase during hypo-osmotic stress, IGF-1-generated signaling may not be efficiently transduced in the absence of IGFBPs and may not be a critical component of hypo-osmotic stress responses.

Prolactin is a pituitary polypeptide hormone that mediates cellular effects through the single transmembrane domain prolactin receptor (PRL-R). One of the earliest known functions of prolactin in teleost fish was its role in ion uptake as a freshwater adapting hormone (Sakamoto and McCormick, 2006). Gene expression, synthesis, secretion and plasma levels of prolactin increase following freshwater acclimation (Manzon, 2002), and prolactin regulates osmotic balance by decreasing water permeability and increasing ion retention on osmoregulatory surfaces. PRL-R mRNA expression differed significantly from control fish during both hypo- and

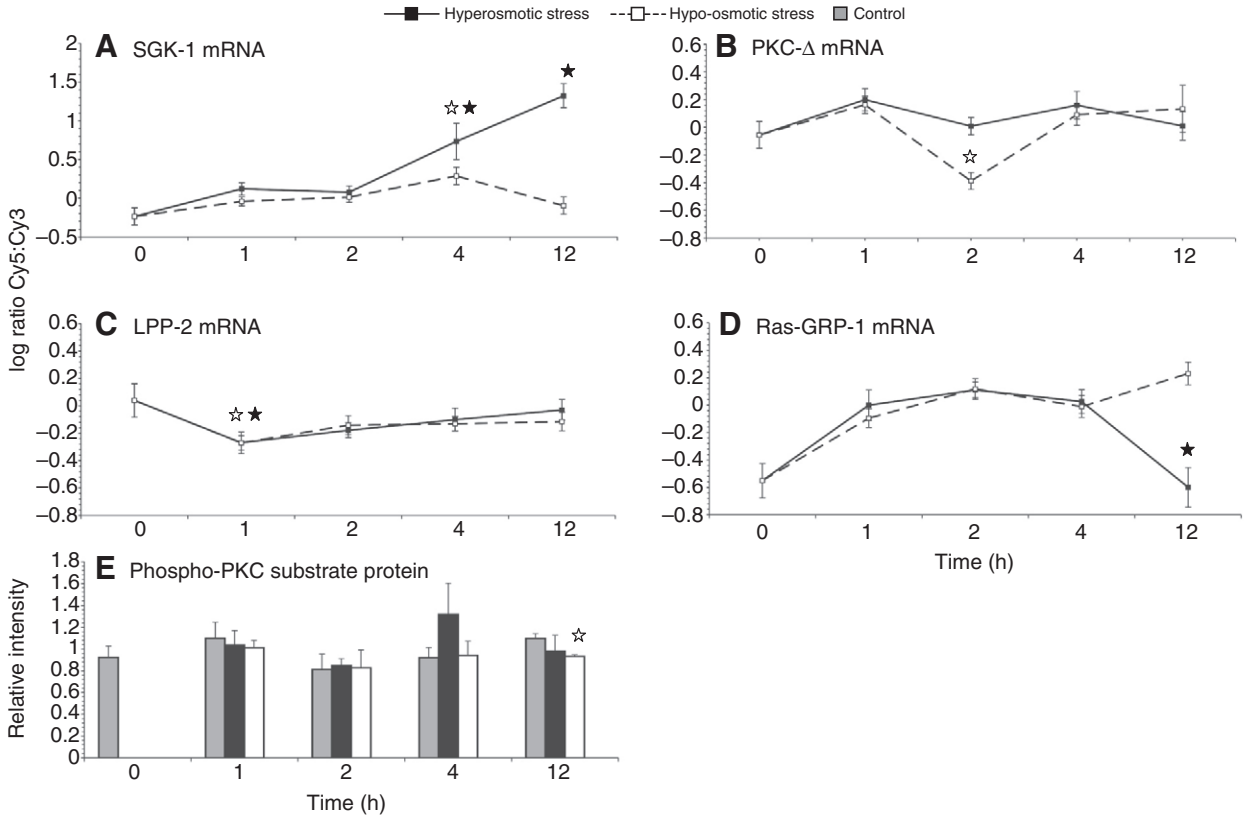

Fig. 4. Expression profiles of signal transduction genes expressed during osmotic stress in *G. mirabilis* gill tissue. (A) Serum and glucocorticoid regulated kinase isoform 1 mRNA (SGK-1; Gm_11g08). (B) Protein kinase C Δ mRNA (PKC-Δ). (C) Lipid phosphate phosphatase 2 mRNA (LPP-2). (D) Ras-guanyl releasing protein 1 mRNA (Ras-GRP-1). Data points were plotted and labeled as described previously. (E) Phosphorylated protein kinase C (PKC) substrate protein expression as determined by western blot analysis. Relative intensity was calculated by normalizing intensities against a standard protein loaded on each gel. The resulting value was then averaged from each of the three fish at each treatment (hyperosmotic stress shown as filled bars; hypo-osmotic stress shown as open bars; control and time=0 shown as gray bars) and time point (1, 2, 4, 12 h post-exposure) to yield the mean relative intensity values illustrated. Filled stars denote significant expression during hyperosmotic stress. Open stars denote significant expression during hypo-osmotic stress. Values are shown ± s.e.m. $P<0.05$.

hyperosmotic stresses, increasing 1.8-fold during hypo-osmotic stress and decreasing 1.4-fold during hyperosmotic conditions (Fig. 3D). PRL-R expression during hypo-osmotic stress probably reflects an accretionary demand for receptors to transduce prolactin signals to downstream effectors.

Prolactin and IGF belong to a large family of molecules called cytokines. One of the major classes of molecules responsible for inhibiting cytokine signaling is the suppressor of cytokine signaling (SOCS) proteins. Transcripts for SOCS genes are rapidly induced by cytokines *in vitro* and *in vivo* (Krebs and Hilton, 2001), and SOSC-3 has been previously implicated in the inhibition of IGF-1 signaling (Zong et al., 2000). In this study, SOSC-3 mRNA expression initially increased 1.6-fold but was rapidly reduced 1.7-fold by hour 12 during hyperosmotic stress (Fig. 3E). The reduction of SOSC-3 mRNA is coordinate with the increase in IGFBP-1 mRNA expression, suggesting SOSC-3 expression was reduced to accommodate IGF-1 mediated signaling events and may be an important regulator of IGF-1 activity during osmotic stress.

Somatostatins (SS) are physiological inhibitors of GH that act through G-protein signaling cascades, which inhibit the release of GH (Nelson and Sheridan, 2005; Lin et al., 2000). SS receptor 5 mRNA was gradually upregulated to 1.8-fold by hour 12 during hyperosmotic stress, reflecting a requirement for increased SS-mediated signaling events (Fig. 3F). These data suggest that GH secretion is transient and tightly regulated during osmotic stress, and that this regulation is, at least in part, mediated by SS signaling. However, because SS acts by inhibiting GH secretion, endogenous levels of GH would remain unaffected and may still be executing significant biological actions.

Prostaglandins are fatty acid-derived signaling molecules that exert diverse biological effects through G-protein coupled receptor signaling, including roles in osmoregulatory processes. Decreases in prostaglandin E2 (PGE2) activity are associated with increased water permeability in trout and frog urinary bladders (Parnova et al., 1997; Natochin et al., 1998). PGE2 is enzymatically converted to its active form by prostaglandin E2 synthase. A small but significant reduction in prostaglandin E2 synthase mRNA was observed in hyperosmotic stressed fish (see Table S1A in supplementary material). We hypothesize that water exiting gill cells *via* passive osmosis immediately following immersion in hyperosmotic solutions may be, in part, actively counteracted by promoting the movement of water into the cell through the inhibition of PGE2 signaling cascades.

Estradiol 17 β dehydrogenase (17βEDH) enzymatically converts estrone to a biologically active estrogen, estradiol. Although fundamentally important to the completion of certain life stages in anadromous fish such as salmon, estradiol can interfere with osmoregulatory processes (McCormick et al., 2005). Several studies

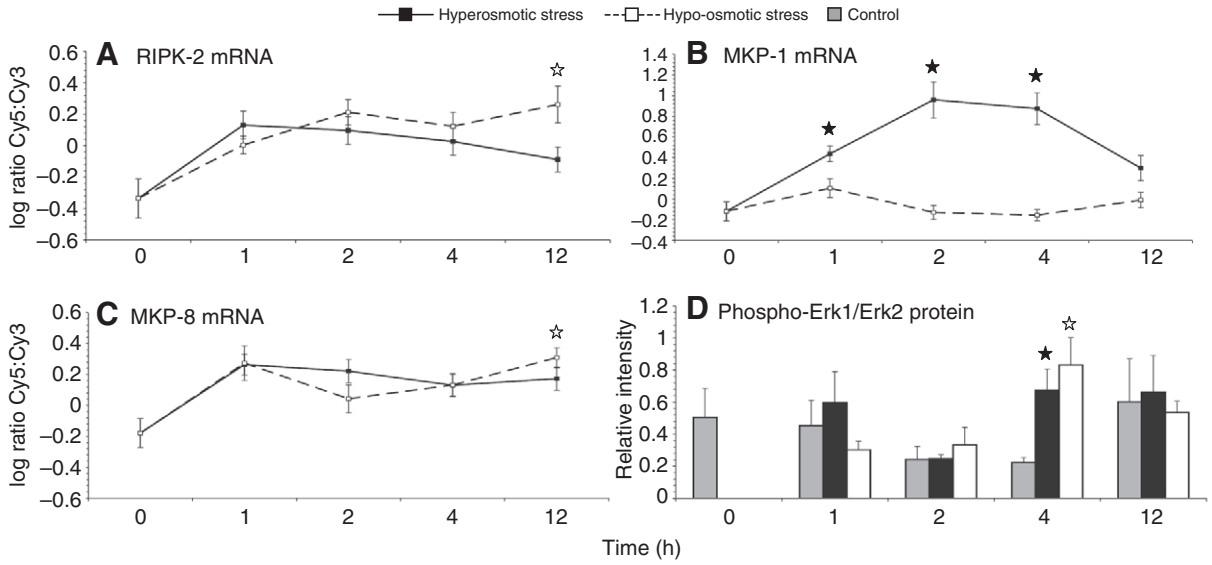

Fig. 5. Expression profiles of genes participating in mitogen activated protein kinase (MAPK) signaling cascades during osmotic stress in *G. mirabilis* gill tissue. (A) Receptor interacting serine/threonine protein kinase 2 mRNA (RIPK-2). (B) MAPK phosphatase 1 mRNA (MKP-1). (C) MAPK phosphatase 8 mRNA (MKP-8). Data points were plotted and labeled as described previously. (D) Phosphorylated Erk1/Erk 2 protein expression as determined by western blot analysis. Relative intensity and significance values were calculated as described previously. Filled stars denote significant expression during hyperosmotic stress. Open stars denote significant expression during hypo-osmotic stress. Values are shown ± s.e.m. $P<0.05$.

have implicated estradiol as particularly detrimental during freshwater adaptation (Madsen and Korsgaard, 1991; Guzman et al., 2004; McCormick et al., 2005). In agreement with these earlier data, 17βEDH mRNA decreased 1.6-fold during hypo-osmotic stress, suggesting suppression in estradiol production during *G. mirabilis* gill freshwater acclimation (see Table S1A in supplementary material).

The data generated in this study regarding the activity of upstream molecules suggest that both hyperosmotic and hypo-osmotic osmoregulatory events are likely to be complex cellular processes involving the coordinated function of multiple major signaling pathways (see also Table S1A in supplementary material). Our data corroborate the established critical role of IGF-1 and PRL-R signaling cascades during osmotic adaptation but also introduce novel regulatory roles for molecules such as IGFBP-1, FKBP-51, SS-5 and SOCS-3 during osmoregulation. Coordination of an appropriate response may depend largely upon the activities of these imperative accessory molecules. We also strengthen emerging evidence regarding the detrimental effects of estradiol during hypo-osmotic adaptation, the role of prostaglandins in regulating water permeability and the possibility of TGF-β signaling being important to osmotic homeostasis (see Table S1A in supplementary material) (Fiol et al., 2006a).

Osmotic signal transduction events – kinases and phosphatases

Signaling events initiated upstream through ligand-receptor binding are propagated and diversified through the action of signal transducers, which ultimately regulate effector mechanisms responsible for acclimation to changes in osmolality (Fiol and Kültz, 2007). Of particular importance are kinases and phosphatases, which post-translationally modify proteins through phosphorylation and dephosphorylation, in order to amplify or repress their activities, respectively. Expression changes in kinases and phosphatases may be especially telling given that their target proteins are likely to undergo changes in activity without a corresponding change in abundance and would, therefore, be undetectable by microarray analysis.

Data generated in this study suggest the activity of kinases and phosphatases are integral to transducing osmotic-sensitive signals. Of the 11 kinases and phosphatases to undergo significant changes in expression, serum and glucocorticoid regulated kinase isoform 1 (SGK-1) has the most established role in ion homeostasis, and was significantly altered during both hyper-and hypo-osmotic stresses (Fig. 4A). SGK-1 is strongly upregulated by osmotic stress, corticosteroids (i.e. cortisol), TGF-β, cell shrinkage and cell swelling (Loffing et al., 2006). The foremost function of SGK-1 is a stimulatory effect on sodium transport *via* the epithelial sodium channel (Pearce, 2001), where it serves as a convergence point for multiple regulators of sodium transport. Recent evidence also suggests that SGK-1 may influence the activities of other ion-transporters such as the K^+ channel (see Table S1C in supplementary material) and $Na^+/K^+/Cl^-$ co-transporters. These properties allow SGK-1 to integrate numerous signaling inputs into varied cellular responses (Lang and Cohen, 2001; Pearce and Kleyman, 2007).

Protein kinase C (PKC) is a major cell signaling intersection with an established role during osmotic stress, including promoting cell volume regulation during hypo-osmotic stress (Ollivier et al., 2006) and activating $Na^+/K^+/Cl^-$ co-transporters during hyperosmotic stress (Lionetto et al., 2002). During hypo-osmotic stress in *G. mirabilis* gill tissue, PKC Δ mRNA expression was downregulated 1.7-fold during the second hour of exposure (Fig. 4B). The observed decrease in PKC expression was backed by a coincident decrease of other PKC relevant genes. Phosphatidic acid phosphatase 2, also called lipid phosphate phosphatase-2 (LPP-2), catalyzes the dephosphorylation of phosphatidic acid yielding diacylglycerol (DAG) and inorganic phosphate (Sciorra and Morris, 2002; Carman and Han, 2006). DAG is an important lipid second messenger that participates in the activation of PKC. LPP-2 mRNA was downregulated during both hyper-and hypo-osmotic stress in *G. mirabilis* gill tissue (Fig. 4C). We also observed a sharp decline in Ras-guanyl releasing protein-1 (Ras-GRP-1) mRNA (Fig. 4D). Ras-

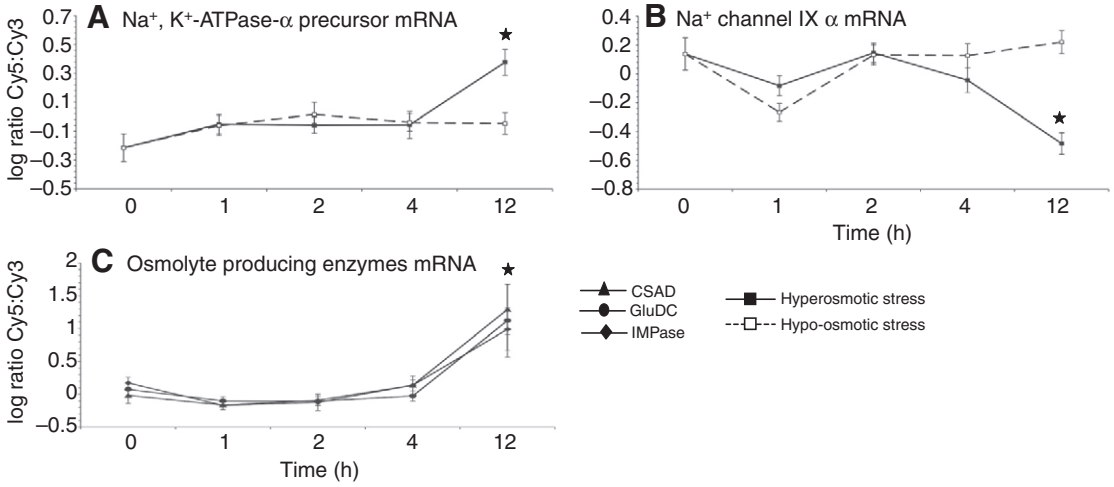

Fig. 6. Expression profiles of genes regulating ion homeostasis (A,B) and organic osmolyte production (C) during osmotic stress in *G. mirabilis* gill tissue. (A) Sodium/potassium transporting ATPase α-1 chain precursor mRNA (Na^+, K^+-ATPase-α precursor). (B) Sodium channel protein type IX α subunit mRNA (Na^+ channel XI α). (C) Osmolyte producing enzymes during hyperosmotic stress. Cysteine sulfinic acid decarboxylase mRNA (CSAD; Gm_49c21) catalyzes the rate limiting step in the formation of taurine (triangular symbols). Glutamate decarboxylase mRNA (GluDC) catalyzes the formation of GABA (circular symbols). Inositol monophosphatase mRNA (IMPase) catalyzes the formation of inositol (diamond-shaped symbols). Data points were plotted and labeled as described previously. Filled stars denote significant expression during hyperosmotic stress. Open stars denote significant expression during hypo-osmotic stress. Values are shown ± s.e.m. $P<0.05$. The expressions of ion homeostasis and organic osmolyte genes not illustrated are described in Table S1C,D, respectively, in supplementary material.

GRP-1 is a DAG binding protein whose activity increases in the presence of DAG (Ebinu et al., 1998). Collectively, these data strongly indicate a reduction in PKC signaling cascades. We addressed the collective effect of these transcriptomic changes at the protein level through the use of a PKC-substrate antibody that reacts specifically to PKC phosphorylated residues on target proteins and provides a proxy for PKC activity. The relative abundances of these proteins were quantified using western blotting. A statistically significant decrease in the abundance of PKC phosphorylated target proteins was detected at hour 12 during hypo-osmotic stress (Fig. 4E). These data support our microarray-based expression patterns and suggest that PKC-mediated signaling events are not major components of the hypo-osmotic stress response in *G. mirabilis* under these experimental conditions.

Lyn kinase belongs to the src-family of protein tyrosine kinases that have been implicated in contributing to an array of signaling networks regulating cellular metabolism, viability, proliferation, differentiation and migration (Ingley, 2008). One particularly well established role for Lyn kinase is during IGF-1 signaling events, where Lyn kinase appears to be critical for IGF-1 dependent activation of phosphatidylinositol-3-kinase and subsequent AKT cascades (Cui et al., 2005; Cui and Almazan, 2007). These data support the 1.3-fold upregulation of the Lyn kinase gene observed during the first hour of *G. mirabilis* hyperosmotic stress (see Table S1B in supplementary material). Furthermore, the gene encoding receptor-type tyrosine protein phosphatase γ (RPTPγ), responsible for mitigating receptor tyrosine kinase signaling *via* dephosphorylation was significantly downregulated during this same period of hyperosmotic stress (see Table S1B in supplementary material) (Wang et al., 2003). These opposing expression patterns imply receptor tyrosine kinase signaling is active very early during hyperosmotic stress, and the most parsimonious candidate is IGF-1.

A key target of IGF-1 signaling cascades is AKT, a major crossroad for signals governing cell survival. Given the preponderance of evidence for IGF-1 contributing to osmoregulatory events in this study, we investigated whether increased IGF-1 signaling was reflected in AKT activity using an AKT substrate-specific antibody and western blotting. A statistically significant difference in the abundance of phosphorylated AKT target proteins was not observed during hyper- or hypo-osmotic stress (data not shown). These data indicate IGF-1 signaling events may not be transduced through AKT during osmotic stress in *G. mirabilis* or utilized in only a minor capacity.

Cyclic AMP (cAMP) is a major second messenger in hormonal signaling and may contribute to osmosensory signal transduction in fish (Borksi et al., 2002). Most of the cellular effects of cAMP are mediated through protein kinase A (PKA). To determine whether osmotic stress resulted in changes in PKA activity, we utilized a PKA substrate-specific antibody. Significant differences were not observed in the abundances of phosphorylated PKA target proteins during osmotic stress in *G. mirabilis* (data not shown). PKA signaling may not play a major role in osmoregulatory signaling in *G. mirabilis* under these conditions. These data agree with those reported by Marshall and colleagues, where PKA inhibitors did not alter Cl^- secretion or retention in killifish opercular epithelium (Marshall et al., 2005).

Receptor-interacting serine/threonine kinase-2 (RIPK-2) is best characterized for its role in mammalian cell death. However, the function of its kinase domain, which is not required to integrate cell death related signals, appears to be as an activator of MAPK signaling (Navas et al., 1999). As it is possible that RIPK-2 was functioning within cell death pathways in this study, we consider it unlikely given the absence of expression changes in other apoptotic related molecules or molecular indicators of severe stress, such as heat shock proteins (Fig. 5A). Conversely, a role for MAPK activity during osmoregulation was vigorously supported in this study. Dual specificity protein phosphatases [MAP kinase phosphatases (MKP)] are osmotically regulated enzymes that dephosphorylate MAPK at threonine and tyrosine residues critical for activation, thereby suppressing their activity

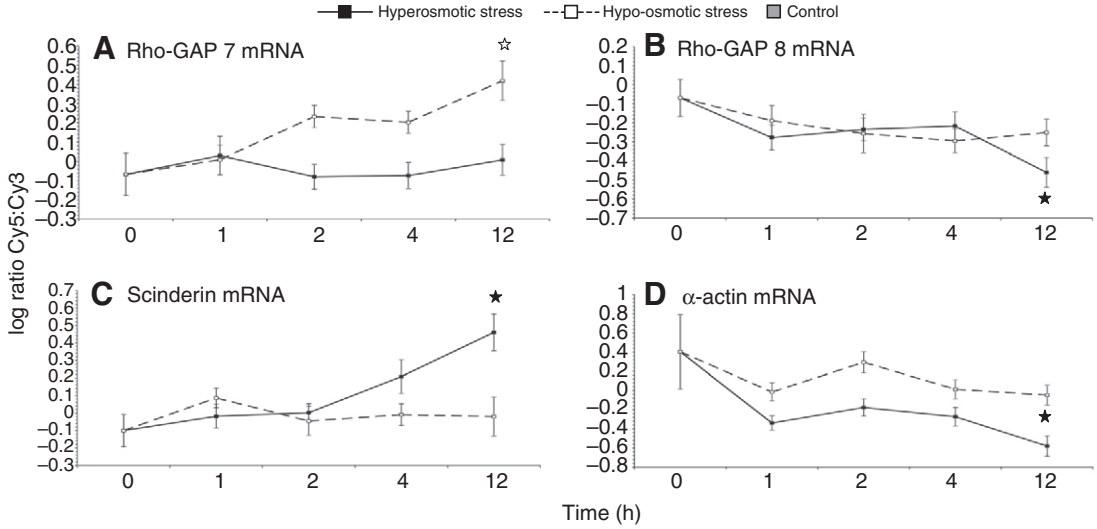

Fig. 7. Expression profiles of cytoskeleton associated genes during osmotic stress in *G. mirabilis* gill tissue. (A) Rho-GTPase activating protein 7 mRNA (Rho-GAP 7). (B) Rho-GTPase activating protein 8 mRNA (Rho-GAP 8). (C) Scinderin mRNA. (D) α-actin mRNA (α-actin). Data points were plotted and labeled as described previously. Filled stars denote significant expression during hyperosmotic stress. Open stars denote significant expression during hypo-osmotic stress. Values are shown ± s.e.m. $P<0.05$. The expressions of cytoskeleton genes not illustrated are described in Table S1E in supplementary material.

(Schliess et al., 1998; Camps et al., 2000; Lornejad-Schafer et al., 2003; Vasudevan et al., 2005). We observed an upregulation of MKP-1 and MKP-8 under hyper- and hypo-osmotic stresses in *G. mirabilis* gill tissue, respectively (Fig. 5B,C). As expected, MKP-8 was not significant until hour 12 of hypo-osmotic stress, suggesting a role in attenuating MAPK activity (Fig. 5C). However, MKP-1 was significantly upregulated at hour one during hyperosmotic stress and remained elevated in excess of 2.5-fold through hours two and four, indicating an extremely rapid suppression of downstream signaling (Fig. 5B). To clarify these confounding results, we quantified the abundances of activated MAPK Erk1 and Erk2 using phospho-specific antibodies. These data reveal that activated Erk1 and Erk2 proteins are significantly elevated by hour four during both hyper- and hypo-osmotic stresses (Fig. 5D). These results corroborate data reported by Kültz and Avila, which demonstrate an increase in the abundance of phosphorylated Erk1 during the early stages of hyper-and hypo-osmotic stress in the gill of the euryhaline teleost *Fundulus heteroclitus* (Kültz and Avila, 2001). We propose a delay between MKP-1 mRNA induction and suppression of MAPK activity during hyperosmotic stress in *G. mirabilis*. Lornejad-Schafer and colleagues (Lornejad-Schafer et al., 2003) and Schliess and colleagues (Schliess et al.1998) both document a delay in the translation of MKP-1 protein as a function of hyperosmotic stress.

Osmotic effector mechanisms

Osmotically regulated signaling cascades terminate by altering the activity and/or abundance of effector molecules, which function to restore cellular homeostasis. Our data imply osmotic effector molecules are diverse and not limited to proteins involved in shuttling ions either intra- or extracellularly. Data generated in this study indicate that osmotic stress affects a large number of cellular components and that restoring normal cellular function requires the rapid (<12 h) and coordinated activity of a varied network of effectors (see Table S1C–Q in supplementary material).

Ion homeostasis

Adaptive responses of ion shuttling proteins such as Na^+, K^+-ATPases and $Na^+/K^+/Cl^-$ co-transporters are fundamental to osmoregulatory processes in fish. In this study, a 1.6-fold increase in mRNA of the α subunit of Na^+, K^+-ATPase was observed during hour 12 of hyperosmotic stress (Fig. 6A). The increase in Na^+, K^+-ATPase was mirrored by a 1.7-fold decrease in sodium channel type IX α subunit mRNA expression (Fig. 6B). Altered expressions of genes encoding ion-transporting proteins illustrate that our osmotic stress exposures were sufficient to induce a typical adaptive response (see also Table S1C in supplementary material). Furthermore, because the expression of these important effector molecules represents a termination point for osmotic based signaling cascades, it is likely that we captured the majority of osmotically relevant signaling processes occurring at earlier time points.

Volume regulatory processes

Acute exposure of cells to hyper- or hypo-osmotic solutions induces rapid changes in cell volume, resulting in cell shrinkage and cell swelling, respectively. A critical phase of compensatory action involves genetic changes that impact the synthesis and transport of compatible osmolytes. Cells experiencing hyperosmotic stress accumulate compatible organic osmolytes in an attempt to equalize intra- and extracellular tonicity, prevent the passive movement of water extracellularly and regulate increases in cell volume (Yancey, 2005). The expressions of genes encoding several osmolyte-producing enzymes increased during hyperosmotic stress in *G. mirabilis*, representing some of the greatest fold expression changes observed in this study (Fig. 6C; Table S1D in supplementary material). These expression changes suggest that cell volume regulation through the accumulation of organic osmolytes is central to the hyperosmotic stress response in *G. mirabilis*.

Cytoskeletal structure and organization

Cytoskeletal elements provide critical structural integrity to cells, and cytoskeletal organization is markedly affected by cell volume perturbations (Pedersen et al., 2001). The expressions of numerous

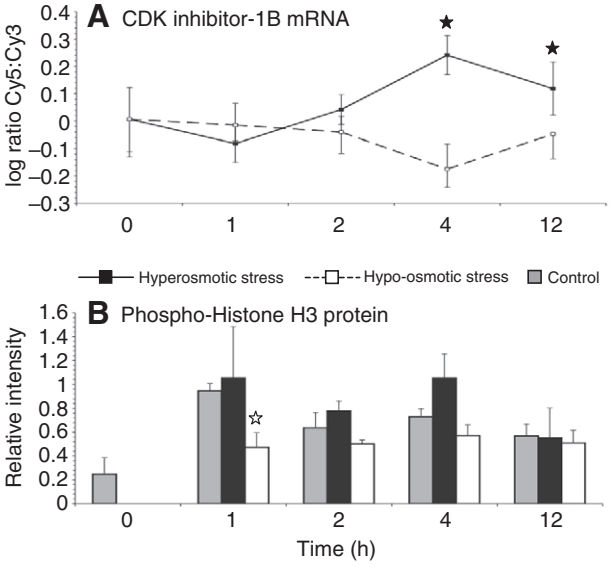

Fig. 8. Expression profiles of cell cycle associated genes during osmotic stress in *G. mirabilis* gill tissue. (A) Cyclin dependent kinase inhibitor 1B mRNA (CDK inhibitor-1B; Gm_39c13). Data points were plotted and labeled as described previously. The expressions of cell cycle genes not illustrated are described in Table S1I in supplementary material. (B) Phosphorylated (Phospho) histone H3 protein expression as determined by western blot analysis. Relative intensity and significance values were calculated as described previously. Filled stars denote significant expression during hyperosmotic stress. Open stars denote significant expression during hypo-osmotic stress. Values are shown ± s.e.m. $P<0.05$.

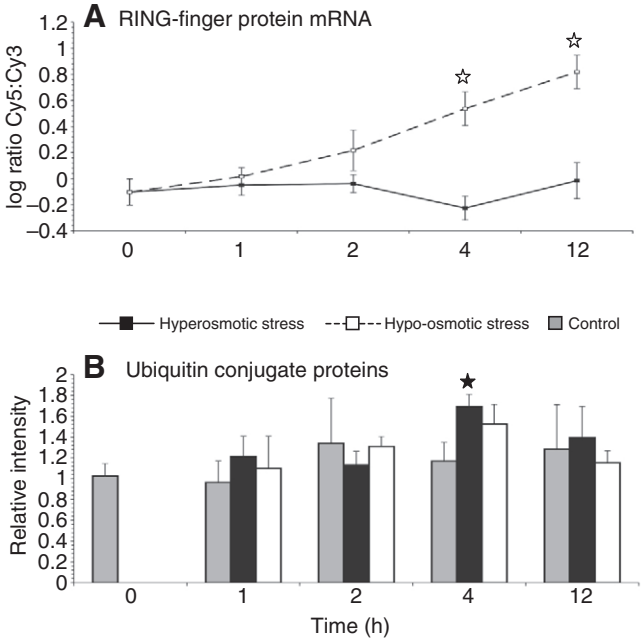

Fig. 9. Expression profiles of proteolysis associated genes during osmotic stress in *G. mirabilis* gill tissue. (A) RING-finger protein mRNA (Gm_44a16). Data points were plotted and labeled as described previously. The expressions of proteolysis genes not illustrated are described in Table S1N in supplementary material. (B) Ubiquitin conjugated protein expression as determined by western blot analysis. Relative intensity and significance values were calculated as described previously. Filled stars denote significant expression during hyperosmotic stress. Open stars denote significant expression during hypo-osmotic stress. Values are shown ± s.e.m. $P<0.05$.

cytoskeletal related genes were modified during both hyper- and hypo-osmotic stress in this study (Fig. 7; Table S1E in supplementary material). These data are best explained *via* a remodeling of cytoskeletal elements required to accommodate volume regulatory processes. Major players during cytoskeletal remodeling are Rho-GTPases, upstream molecular switches triggering signaling cascades that target cytoskeletal effector proteins to induce morphological change (Di Ciano-Oliceira, 2006). In the present study, Rho-GTPase activating protein 7 mRNA increased whereas Rho-GTPase activating protein 8 decreased during hypo- and hyperosmotic stress, respectively (Fig. 7A,B). Additionally, the gene encoding scinderin, a protein involved in severing actin polymers leading to actin depolymerization, was upregulated during hyperosmotic stress (Fig. 7C) (Dermitzaki et al., 2001). Altered expression of these master regulators of cytoskeletal organization strongly suggests cytoskeletal modulation is a component of osmoregulatory processes in *G. mirabilis*. These data were supported by the expression of principal cytoskeletal genes, such as the increase in myosin light chain mRNA during hypo-osmotic stress (see Table S1E in supplementary material) and the decrease in α actin mRNA during hyperosmotic stress (Fig. 7D). In order to relate these changes to the protein level and better approximate net changes in the cytoskeleton, we quantified the abundances of actin and α tubulin proteins during osmotic stress in *G. mirabilis*. We did not observe any significant differences in either of these proteins across all time points relative to controls (data not shown). We speculate that given the large abundances of these proteins normally present in cells, the relatively small changes observed at the mRNA level in this study may not affect overall abundance at the protein level. However, cytoskeletal restructuring in the absence of large changes in protein abundance may still be occurring. All of the studies we encountered detailing a significant effect of osmotic stress on the cytoskeleton employed a combination of fluorescent staining and confocal microscopy to quantify organization *in situ* (e.g. Pedersen et al., 1999; Ebner et al., 2005).

Cell cycle

Hormones and growth factors (such as prolactin and IGF-1) are potent mitogenic stimulators, and cell proliferation may play a role in osmoregulation. For example, GH and IGF-1 can increase gill chloride cell numbers during seawater adaptation (Sakamoto et al., 2001). Several cell-cycle related genes were differentially expressed during *G. mirabilis* osmotic stress (Fig. 8; Table S1I in supplementary material) including cyclin dependent kinase inhibitor 1B (Fig. 8A), Histone H2B (see Table S1I in supplementary material), and growth arrest and DNA damage inducible protein 45 (GADD45) (see Table S1I in supplementary material). Collectively, our microarray-based data hint that the cell cycle may be divergently affected by hyper- and hypo-osmotic stress. To better clarify this issue, we monitored the protein-level expression of phosphorylated-histone H3 (pH3) and proliferating cell nuclear antigen (PCNA), two commonly employed markers of cell division (e.g. Dmitrieva et al., 2001). A significant reduction in pH3 was observed during hour one of hypo-osmotic stress but this difference was not maintained at additional time points (Fig. 8B). No significant difference was observed in the abundances of PCNA protein (data not shown). Based on these data, cell division is not radically affected in *G. mirabilis* during osmotic stress under the given experimental conditions. One possible explanation is that the short duration of

our time course limited our opportunity to observe significant changes in cell division.

Energy metabolism

A key aspect of the cell stress response is modulation of major pathways of energy metabolism (Kültz, 2005). Induction of these enzymes may generate reducing equivalents for antioxidant systems or promote ATP production to fuel adaptive responses such as increased protein synthesis. Metabolically relevant genes where highly represented during osmotic stress in *G. mirabilis* (see Table S1Q in supplementary material). The number and diversity of metabolically relevant genes showing changed expression suggests regulating metabolic processes at various stages in energy production may be a component of the *G. mirabilis* osmotic stress response.

To determine if these changes in RNA abundance were manifested through shifts in enzyme activity, we monitored the activity of key metabolic enzymes at the 12 h time point under hyper-, hypo-osmotic and control conditions. No significant differences were observed in the activities of lactate dehydrogenase, citrate synthase or malate dehydrogenase (data not shown). Therefore, the functional significance of modulating metabolically relevant gene expression during osmotic stress in this study remains unresolved.

Proteolysis/molecular degradation

Environmental stress can have an adverse affect on cellular proteins, possibly causing denaturation and loss of function (Hofmann and Somero, 1995). Denatured proteins may be rescued through the action of molecular chaperones, such as heat shock proteins. However, in cases of irreversible damage, proteins will be eliminated. This process is mediated by ubiquitin ligation, which targets these proteins for degradation by cytoplasmic proteases. The 2.5-fold increase in RING finger protein mRNA during hypo-osmotic stress (Fig. 9A), an immediate-early gene involved in mediating the transfer of ubiquitin to target proteins (Joazeiro and Weissman, 2000), suggests that osmotic stress may be causing irreversible protein damage (Pan et al., 2002; Fiol et al., 2006b). However, the absence of inducible heat shock proteins from our analysis implies that ubiquitin ligation may not be a function of protein damage but may instead regulate the activity and turnover of signaling molecules (Haglund et al., 2003; Haglund and Dikic, 2005). To better distinguish these two possibilities, we quantified the abundance of ubiquitin-conjugated proteins during osmotic stress in *G. mirabilis* using western blotting. A significant increase in ubiquitin-conjugates was observed during a single time point, hour four of hyperosmotic stress (Fig. 9B). Ubiquitination in response to irreversible protein damage would probably be manifested across multiple time points and be coordinately expressed with heat shock proteins (Hofmann and Somero, 1995). Therefore, we conclude that this transient increase in ubiquitin-conjugated proteins more likely reflects a mechanism of cell signaling regulation than the degradation of stress-damaged proteins.

Conclusion

The main objective of this study was to identify early cell-signaling events underlying the function of well-characterized effector proteins during osmotic stress adaptation in *G. mirabilis*. Our results strongly suggest that the microarray-based time-course approach used in this study was effective in capturing many of these events from osmosensors (e.g. FKBP-51) to effectors (Na^+, K^+-ATPase). We demonstrate that multiple major signaling cascades operate within a framework of accessory molecules, many not previously recognized as relevant to osmotic stress (e.g. IRS-2, IGFBP-1, SS-5, SOSC-3). Subsequent protein level analyses shed light on how these changes in gene expression influence the outcome of specific signaling pathways and affect fundamental cellular processes. Whereas our transcriptomic analysis illustrates the power of microarray experimentation to reveal patterns of gene expression likely to be critical in adaptive responses, deep understanding of the significance of these transcriptional changes can only be attained through complementary proteomic and/or metabolomic analysis.

LIST OF ABBREVIATIONS

17βEDH	Estradiol 17 β dehydrogenase
a.a	amino acid
AKT	protein kinase B
ANOVA	analysis of variance
ATP	adenosine triphosphate
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CDNA	cloned deoxyribonucleic acid
CoA	coenzyme A
CS	citrate synthase
DAG	diacylglycerol
DMSO	dimethylsulfoxide
DTNB	dithionitrobenzoic acid
dUTP	deoxyuridine triphosphate
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
Erk	extracellular signal regulated kinase
FKBP-51	FK506-binding protein 51
GABA	γ-aminobutyric acid
GADD45	growth arrest and DNA damage inducible protein 45
GH	growth hormone
GR	glucocorticoid receptor
GTPase	guanosine triphosphate synthase
HEPES	hydroxyethylpiperazine-N′-2 ethanesulfonic acid
hyper	hyperosmotic
hypo	hypo-osmotic
I.U.	international units
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IMPase	inositol monophosphatase
IRS	insulin receptor substrate
JAK/STAT	Janus kinase/signal tranducers and activators of transcription
LDH	lactate dehydrogenase
LPP	lipid phosphate phosphatase
MAPK	mitogen activated protein kinase
MDH	malate dehydrogenase
MKP	MAP kinase phosphatase
mRNA	messenger ribonucleic acid
NADH	nicotinamide adenine dinucleotide plus hydrogen
NCBI	National Center for Biotechnology Information
NICHD	National Institute of Child Health and Human Development
NOR	neural derived orphan receptor
PC	principle component
PCA	principal components analysis
PCNA	proliferating cell nuclear antigen
PGE2	prostaglandin E2
pH3	phosphorylated histone H3
PKA	protein kinase A
PKC	protein kinase C
PMSF	phenylmethanesulfonyl fluoride
PNRC	proline-rich nuclear receptor coactivator
p.p.t.	parts per thousand
PRL-R	prolactin receptor
RAS-GRP	Ras-guanyl releasing protein
RIPK	Receptor-interacting serine/threonine kinase
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid

RPTPγ	receptor-type tyrosine protein phosphatase γ
SDS	sodium dodecylsulfate
SGK	serum and glucocorticoid regulated kinase
SNAT	system N amino acid transporter
SOCS	suppressor of cytokine signaling
SS	somatostatin
SSC	sodium chloride and sodium citrate
TAE	tris acetate EDTA
TBST	tris-buffered saline containing 0.1% Tween-20
TCTP	translationally controlled tumor protein
TGF-β	transforming growth factor β
α	alpha
β	beta
Δ	delta
γ	gamma

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