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The *Saccharomyces cerevisiae* Arr4p is involved in metal and heat tolerance*

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Abstract

Homologues of the bacterial ArsA ATPase are found in nearly every organism. While the enzyme is involved in arsenic detoxification in bacteria, the roles of eukaryotic homologues have not been identified. This article reports the function of the *Saccharomyces cerevisiae* homologue encoded by *ARR4* gene (*YDL100c* ORF). Disruption of *ARR4* was not lethal, but the disrupted strain displayed increased sensitivity to As^{3+}, As^{5+}, Co^{2+}, Cr^{3+}, Cu^{2+} or VO_4^{3-} salts and temperature. A plasmid-encoded copy of a wild-type *ARR4* gene could complement the heat- or metal-related stress responses. Mutation of a codon within the consensus sequence for the nucleotide-binding site resulted in loss of complementation of the disrupted strain and produced a dominant negative phenotype in a wild type strain. Wild type and mutant Arr4p were purified from *Escherichia coli*. The wild type protein exhibited a low level of ATPase activity, and the mutant was inactive. The purified ATPase eluted as a dimer of 80-kDa species. A fusion of *ARR4* and the GFP (green fluorescent protein) gene was constructed. The gene fusion was able to complement stress-related phenotype of the *ARR4* disruption. Under non-stress conditions, GFP fluorescence was found diffusely in the cytosol. Under stress conditions GFP was localized in a few punctate bodies resembling late endosomes. It is proposed that under heat or metal stress, the soluble ATPase becomes membrane-associated, perhaps through interaction with a partner protein, and that this complex is involved in stress tolerance.

Introduction

Bacterial arsenical resistance (*ars*) determinants found in *Escherichia coli* plasmids R773 and R46 (Bruhn *et al.* 1996; Gatti *et al.* 2000) and plasmid pKW301 of *Acidiphilium multivorum* (Suzuki *et al.* 1998) encode an ATP-dependent arsenite extrusion pump that confers resistance to arsenite and antimonite (Dey *et al.* 1994). The 583-residue ArsA ATPase is the catalytic subunit of the pump. ArsA has two homologous halves, A1 and A2, each of which has a consensus sequence for a nucleotide-binding site (NBS) (Walker *et al.* 1982). Also in each half is a signature sequence, DTAPTGHT, which is conserved in homologues from every kingdom, including bacteria, archaea, fungi, plants and animals (Zhou & Rosen 1997). In the R773 ArsA these sequences have been shown to be signal transduction domains that facilitate the flow of conformational information from the NBS to the As^{3+} binding site (Walmsley *et al.* 1999; Zhou *et al.* 2000; Zhou & Rosen 1997).

Members of this ArsA family are widely distributed in nature, with representatives found in members of each domain, prokarya, archaea and eukarya (Bhattacharjee *et al.* 1999). The eukaryotic homologues are approximately 40-kDa and have a single NBS and DTAPTGHT signature sequence (Bhattacharjee *et al.* 2001). Even though only the R733 ArsA has had its function identified, the ubiquity of the homologues

implies a role in the cellular physiology of most cells. Mammalian ArsA homologues have been identified in human (Kurdi-Haidar *et al.* 1996) and mouse (Bhattacharjee *et al.* 2001). The human homologue has been implicated in arsenite resistance (Kurdi-Haidar *et al.* 1998).

The ArsA homologue in *Saccharomyces cerevisiae* encoded by the *YDL100c* ORF is found on chromosome IV and exhibits 46% identity and 65% overall sequence similarity with the human and mouse homologues, which are nearly identical to each other (Bhattacharjee *et al.* 2001). A *YDL100c* null mutant has been reported to have no growth defects on synthetic complete media or on rich media, with glucose or glycerol as carbon source, at 15 °C, 30 °C, or 37 °C, nor in cell morphology, colony size, mating, sporulation or spore germination (Zuniga *et al.* 1999). In that report the null mutant was found to exhibit slight sensitivity to zinc or cobalt, which was more pronounced at 37 °C.

In this report the properties and function of Ydl100p are examined in additional detail. *YDL100c* was disrupted by a one-step gene replacement method (Rothstein 1983). The disrupted strain shows low levels of sensitivity to arsenicals at elevated temperature. In view of this finding and also that the putative polypeptide exhibits significant homology to the bacterial ArsA, we have registered the gene name for the *YDL100c* ORF as *ARR4* in the Saccharomyces Genome Database (SGD). It has earlier been shown that the *ARR1*, *ARR2*, and *ARR3* genes (also called *ACR1*, *ACR2* and *ACR3*) encode for a transcriptional regulator, reductase and a membrane transporter, respectively, that are involved in arsenical detoxification in *S. cerevisiae* (Bobrowicz *et al.* 1997; Ghosh *et al.* 1999; Mukhopadhyay & Rosen 1998).

We report here that the *ARR4* disrupted strain is more sensitive than the wild type to As^{3+}, As^{5+}, Co^{2+}, Cr^{3+}, Cu^{2+} or VO_4^{3-} salts and temperature but not to other metals such as Al^{3+}, Ag^{+}, Cd^{2+}, Fe^{2+}, Hg^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, Pd^{2+}, Pt^{2+}, Rb^{+}, and Zn^{2+}. The disrupted strain could grow very slowly at 40 °C. Sensitivity to Co^{2+} salts could be complemented by expression of *ARR4* on a plasmid. An *arr4* mutant with a mutation in the consensus sequence for the nucleotide binding site was not only unable to complement the null strain but also showed a dominant negative phenotype when expressed in a wild type strain. The *ARR4* gene was fused with the gene for Green Fluorescent Protein (GFP). During growth under non-stress conditions GFP fluorescence exhibited a diffuse cytosolic location. Under stress conditions the majority of the fluorescence was concentrated in a few punctate bodies. Arr4p was expressed and purified in *Escherichia coli* as a six-histidine tagged protein. The purified protein eluted as a homodimer on size exclusion chromatography. Arr4p exhibited Mg^{2+} ATPase activity that was not stimulated by metals including As^{3+}, Sb^{3+}, Cu^{2+}, and Co^{2+}. The results suggest that Arr4p is involved in a general stress response, since the null mutant is sensitive to a variety of stress conditions. The mechanism of Arr4p action is unknown, but the recruitment of the GFP fusion to a small number of punctate bodies under stress conditions suggests that the ATPase forms a complex with a membrane protein, and that the complex is a component of a stress tolerance pathway.

Materials and methods

Strains, plasmids, and media

Strains and plasmids used in this study are listed in Table 1. *E. coli* cells were grown in LB medium (Sambrook & Russell 2001) supplemented with appropriate antibiotics. *S. cerevisiae* strains were grown at 30 °C in complete YPD medium or minimal SD medium as described (Burke *et al.* 2000).

Disruption of the ARR4 gene

Disruption of the *ARR4* gene was carried out by a one-step gene replacement method (Rothstein 1983). A 2.4-kb fragment of yeast genomic DNA containing *ARR4* and flanking sequence was amplified by PCR using a forward primer 5′-AGGCCTGTTGTGCCCGAAGG-3′ that hybridizes with a region 552-bp upstream of *ARR4* and a reverse primer 5′-CAAAGAACGACACAGGCCTTGTC-3′ that hybridizes to a region 920-bp downstream of *ARR4*. The fragment was ligated into vector pGEM-T. The resulting construct pJS-1 was digested with *Msc*I and *Bam*HI, which excised a 180-bp fragment within *ARR4*. The 2.2-kb *Msc*I/*Bam*HI digested, linearized pJS-1 fragment was gel-purified and ligated with a 1.7-kb *HIS3* gene that had been obtained from plasmid pUC18-HIS3-1 as a 1,769-bp *Bam*HI-*Sma*I fragment. The resulting plasmid was digested with *Stu*I, and the 3.9-kb fragment was isolated, purified, and transformed into yeast strain W303-1B, producing the *ARR4*-disrupted strain JSY-1 by homologous recombination. Recombinants were selected for growth in the

Table 1. Strains and plasmids.

Strain/plasmid	Genotype	Reference or source
Bacterial strains		
JM109	*endA*1, *recA*1, *gyrA*96, *thi*, *hsdR*17 (r_k^-, m_k^+), *relA*1, *supE*44, λ^-, Δ(*lac-proAB*), [F′, *traD*36, *proA*$^+$*B*$^+$, *lacI*^{q}ZΔM15]	(Sambrook & Russell 2001)
BL21 (DE3)	F$^-$ *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3)	Novagen
Yeast strains		
W303-1B	*Mat- α ade2-1 his3-11, 15 leu2-3, 112 ura3-1 trp-1*	(Bowman *et al.* 1991)
JSY-1	*Mat- α ade2-1 his3-11, 15 leu2-3, 112 ura3-1 trp-1 ARR4::HIS3*	This study
Plasmids		
pET-28b(+)	*E. coli* cloning and expression vector, Kan^r	Novagen
pGEM-T	Multicopy *E. coli* cloning vector, Ap^r	Promega
pUC18-His3-1	*Bam*HI with *HIS3* ligated to *Bam*HI of pUC18-*Bam*HI site proximal to *Eco*RI site destroyed	S. Ackerman
YEp352	*S. cerevisiae-E. coli* shuttle vector, Ap^r, *URA3*	S. Ackerman
pUG36	*S. cerevisiae-E. coli* shuttle vector, Ap^r, *URA3*, N-fusion *yEGFP3*	J.H. Hegemann

absence of histidine. Verification of *ARR4* disruption was confirmed by PCR using a forward primer 5′-CCTCTACCACTCATAAAGTGG-3′ that hybridizes just after the *ARR4* start codon and a reverse primer 5′-GACTTTGCCATCAGTAATAGGG-3′, which hybridizes just before the stop codon of *ARR4*.

For complementation experiments, a plasmid carrying *ARR4* under control of its endogenous promoter was constructed in the following manner. The *ARR4* gene from *S. cerevisiae* strain W303-1B genomic DNA was amplified by PCR to introduce a *Nco*I site at the 5′ end and a *Xho*I site at the 3′ end. The forward primer was 5′-CCATGGATTTAACCGTGGAAC-3′, and the reverse primer was 5′-CTCGAGTTCCTTATCTTCTAACTC-3′. A 30-cycle PCR (94 °C for 1 min, 55 °C for 0.5 min, and 72 °C for 1 min) was run with yeast genomic DNA. The 1,070-bp amplified product was ligated with p-GEM-T to generate plasmid pJS-2. Plasmid pJS-2 was digested with *Nco*I, blunted using the large fragment of DNA polymerase I, and further digested with *Xho*I. The resulting 1-kb fragment was ligated with the *Sma*I-*Xho*I digested GFP-fusion vector pUG36 (U. Güldener and J. H. Hegemann, *manuscript in preparation*), creating plasmid pUG36-*ARR4*. This construct was further digested with both *Xba*I-*Kpn*I, and the excised 1.3-kb fragment was ligated with *Xba*I-*Kpn*I-digested yeast/*E. coli* shuttle vector YEp352 (Hill *et al.* 1986), creating plasmid pJS-3. The endogenous promoter containing sequence of *ARR4* was PCR-amplified using a forward primer, 5′-CTGCAGGATCTGATTTAACCT-3′, that hybridizes to a region 570-bp upstream of *ARR4* and a reverse primer, 5′-TCTAGATGATCTTCTTGTTCTTGTCGT A-3′, that hybridizes to a region 10-bp upstream of the gene. The PCR product was ligated with pGEM-T vector, creating plasmid pJS-4 that was further digested with *Pst*I and *Xba*I. The excised fragment containing the *ARR4* endogenous promoter was finally ligated to *Pst*I-*Xba*I-digested pJS-3, creating plasmid YEp352-*ARR4*.

Metal ion resistance assays

Strains were grown overnight at 30 °C in liquid SD medium with 2% glucose and appropriate supplements. The cultures were diluted into minimal media to an A_{600} of 0.1 in presence of varying concentrations of the indicated metal ions and incubated for an additional 24 h, after which growth was estimated from A_{600} values.

Purification of the Arr4p-[His]6 protein

Plasmid pJS-2 was digested with both *Nco*I and *Xho*I, and the *Nco*I-*Xho*I fragment containing the complete coding sequence for Arr4p was ligated with the expression vector pET-28b that had been similarly digested with both enzymes. The resulting construct pArr4p-His6 contained the full length *ARR4* gene cloned in frame with the sequence for a C-terminal six-histidine tag followed by a stop codon. The correct reading frame was verified by DNA sequencing.

For expression, cells of *E.coli* strain BL21(DE3) harboring plasmid pArr4p-His6 were grown overnight

in 100 ml of LB medium with 40 μg/ml kanamycin at 37 °C. This culture was then diluted into 2 l of the same medium and incubated with shaking at 37 °C until an A_{600} of 0.5 was reached. Isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 0.1 mM, and the culture was incubated for an additional 3 h. Wild type and mutant Arr4p-[His]6 were purified according to established protocol (Bhattacharjee & Rosen 2000). The concentration of Arr4p-[His]6 in purified preparations was determined from the absorption at 280 nm using a molar extinction coefficient of 19,900. Extinction coefficients were calculated by the method of Gill and von Hippel (1989). ATPase activity was assayed using an NADH–coupled assay method (Hsu & Rosen 1989; Vogel & Steinhart 1976).

Oligonucleotide-directed mutagenesis

To introduce mutations in the nucleotide-binding domain of Arr4p, plasmid pJS-2 was digested with both *Nco*I and *Bam*HI. The *Nco*I-*Bam*HI fragment containing the first 693-bp of the *ARR4* gene was cloned into the *Nco*I-*Bam*HI site of pALTER®-1 mutagenesis vector (Promega). A mutation in the sequence of the *ARR4* gene was introduced by site-directed mutagenesis using the Altered Sites®-II *in vitro* Mutagenesis System (Promega). The mutagenic oligonucleotide, GTGGTGTT**CGT**AAGACTAC was used to introduce the G30R mutation. The mutation was verified by sequencing of the entire insert. The insert was next excised by double digestion with *Nco*I-*Bam*HI and ligated into *Nco*I-*Bam*HI digested plasmid pJS-2. Altered plasmid pJS-2 containing the G30R mutation was double digested with *Nco*I-*Xho*I, and the fragment containing the full length, mutated *arr4* gene was ligated to the pET-28b expression vector, creating plasmid pArr4p$_{G30R}$-His6. Altered *arr4* gene containing the G30R mutation was inserted into the yeast/*E. coli* shuttle vector YEp352 by following the same steps as discussed for the wild type gene, creating plasmid YEp352-*ARR4*[G30R].

Constructs with Green Fluorescent Protein (GFP)

For localization studies, plasmid pUG36-*ARR4* were used to express yeast-enhanced green fluorescence protein (yEGFP3) fused at the N-terminus of Arr4p. The plasmid carries the *S. cerevisiae MET25* promoter and the CEN6/ARSH4 origin for low copy maintenance.

To construct the GFP-*ARR4* gene fusion under the control of endogenous promoter for the *ARR4* gene, plasmid pUG36-*ARR4* was digested with *Xba*I-*Kpn*I, and the excised fragment containing the *ARR4* gene was ligated with *Xba*I-*Kpn*I-digested yeast/*E. coli* shuttle vector YEp352, creating plasmid pJS-5. A fragment containing the *ARR4* endogenous promoter was excised from plasmid pJS-4 by double digestion with *Pst*I-*Xba*I and ligated with similarly digested plasmid pJS-5, generating the plasmid YEp352-GFP-*ARR4*.

Microscopy and imaging analysis

For microscopy analysis, yeast cells grown in selective SD medium were collected by centrifugation and resuspended to an A_{600} of 3.0 in liquid selective medium containing 1% (w/v) LMP agarose. 10 μl of the cell suspension was placed on a glass slide, sealed with a cover slip and the cells examined after 4 h incubation at room temperature. For confocal laser scanning microscopy, adenine was added to the growth media at 100 μg/ml to suppress the endogenous fluorophore produced in *ade2* yeast (Weisman *et al.* 1987). The cells were imaged for green fluorescence by confocal laser scanning microscopy (Fluoview, Olympus) after excitation with the 488-nm line from a Krypton-argon ion laser, and the emission was viewed through a 510–550 nm band pass filter.

Results and discussion

Disruption of the ARR4 gene and phenotypic analyses

The 1,065-bp *ARR4* gene in *S. cerevisiae* strain W303-1B was disrupted by insertion of a *HIS3* gene, creating strain JSY-1. Verification of *ARR4* disruption was confirmed by PCR analysis. A single 1-kb PCR product was observed for the wild type strain upon agarose gel electrophoresis, while the *ARR4* disrupted strain showed the expected 2.7-kb PCR fragment (data not shown), consistent with insertion of the 1.7-kb *HIS3* gene.

Arr4p exhibits significant homology to the *E. coli* ArsA that is known to confer high levels of resistance to arsenic and antimony salts. The sensitivity of *ARR4* disrupted strain to arsenicals was therefore evaluated. Cultures derived from single colony isolates with the *ARR4* disruption, exhibited slightly lower levels of resistance than the wild type in the presence of arsenite at 30 °C (Figure 1A) and even greater sensitivity at

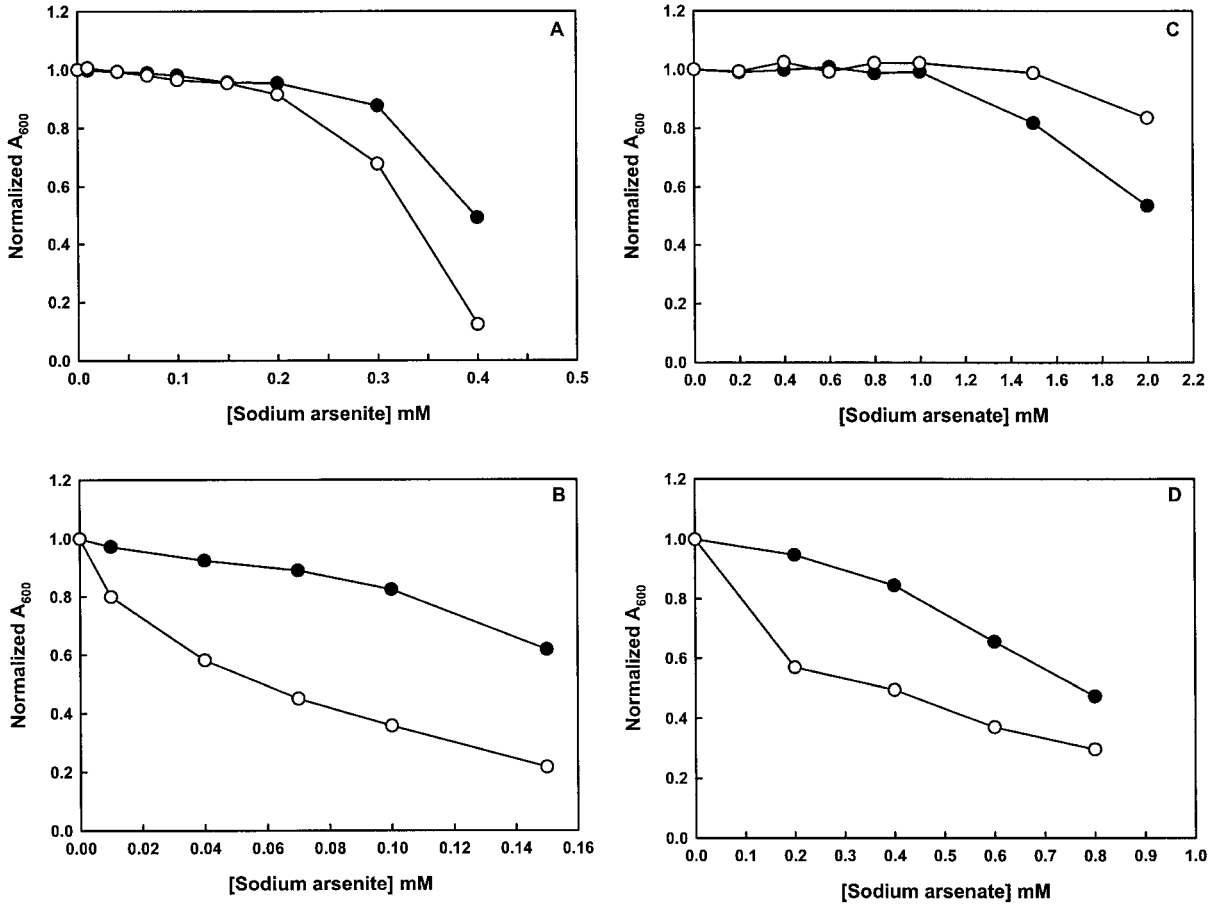

Figure 1. Phenotype of *ARR4* disrupted *S. cerevisiae*. Cells were grown in liquid SD minimal medium with the indicated concentrations of sodium arsenite at 30 °C (A), 37 °C (B), or sodium arsenate at 30 °C (C), 37 °C (D). Strains used: (●), W303-1B (wild type); (○), JSY-1 (*ARR4::HIS3*).

37 °C (Figure 1B). When the phenotypic analysis was conducted in the presence of arsenate, *ARR4* disrupted *S. cerevisiae* showed slightly greater resistance than the wild type cells at 30 °C (Figure 1C) but increased sensitivity at 37 °C (Figure 1D). These results clearly show that the lack of Arr4p increases arsenic sensitivity and suggest that Arr4p participates in low-level resistance to arsenicals in *S. cerevisiae*.

When the growth rates of wild type and JSY-1 strains were investigated as a function of temperature, a significant difference was observed. Growth of the disrupted strain was similar to that of wild-type cells at 30 °C. However, the *ARR4* disrupted strain showed a longer generation time at 37 °C (Figure 2A), and exhibited a profound growth inhibition at 40 °C accompanied by loss of cell viability (Figure 2B). To demonstrate that the temperature-sensitive phenotype was indeed the result of disruption of the *ARR4* gene, strain JSY-1 was transformed either with the multicopy yeast/*E. coli* – shuttle vector YEp352 or with the *ARR4* bearing plasmid, YEp352-*ARR4*. *ARR4* in the multicopy plasmid complemented the temperature sensitivity as depicted in Figure 2A, B. The growth of wild type and the complemented JSY-1 strains were nearly identical.

It has been shown that oxidative stress is involved in heat-induced cell death in *S. cerevisiae* (Davidson *et al.* 1996). However, our experiments indicate that the cellular molecular oxidation levels are similar in both the wild type and *ARR4* disrupted cells. Firstly, the levels of *in vivo* intracellular oxidation was determined for both the wild type and *ARR4* disrupted strain using the oxidant sensitive probe 2′,7′-dichlorodihydrofluorescein diacetate during cell growth at 40 °C (Davidson *et al.* 1996). Cellular oxidant levels were found to be similar in both strains

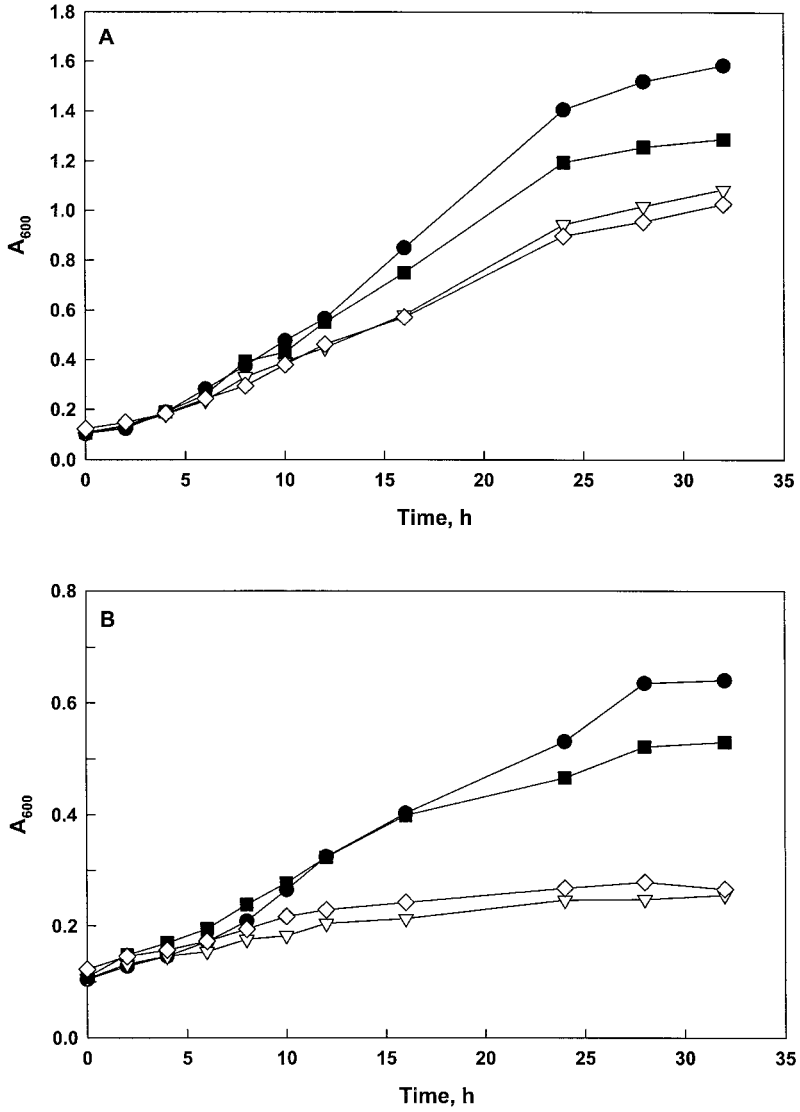

Figure 2. Temperature sensitivity of *S. cerevisiae* strains with disruption of *ARR4*. Cells were grown in liquid SD minimal medium. A_{600} values were determined at the indicated times either 37 °C (A), or 40 °C (B). Strains used: (●), W303-1B (wild type); (▽), JSY-1 (*ARR4::HIS3*); (■), JSY-1 transformed with *ARR4* bearing plasmid, YEp352-*ARR4*; and (◇), JSY-1 transformed with vector plasmid, YEp352.

(data not shown). Secondly, the *ARR4* disrupted strain could grow to same cell density as the wild type in presence of 5 mM H_2O_2 (data not shown). Finally, the *ARR4* disrupted strain showed similar temperature sensitive phenotype under anaerobic conditions (data not shown). These experiments indicate that oxidative stress is most likely not the major cause of temperature sensitivity.

The *ARR4* disrupted strain JSY-1 was also examined for sensitivity to several metal and non-metal ions, including Al^{3+}, Ag^{+}, Co^{2+}, Cr^{3+}, Cu^{2+}, Cd^{2+}, Fe^{2+}, Hg^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, Pd^{2+}, Pt^{2+}, Rb^{+}, Sb^{3+}, VO_4^{3-}, and Zn^{2+}. JSY-1 exhibited slight sensitivity to Co^{2+}, Cr^{3+}, Cu^{2+} and VO_4^{3-} salts at 30 °C that was even more pronounced at 37 °C. An example of the sensitivity of *ARR4* disrupted strain in the presence of Co^{2+} at 37 °C is depicted in Figure 3. The observed sensitivity could be complemented by expression of *ARR4* on a plasmid. These results indi-

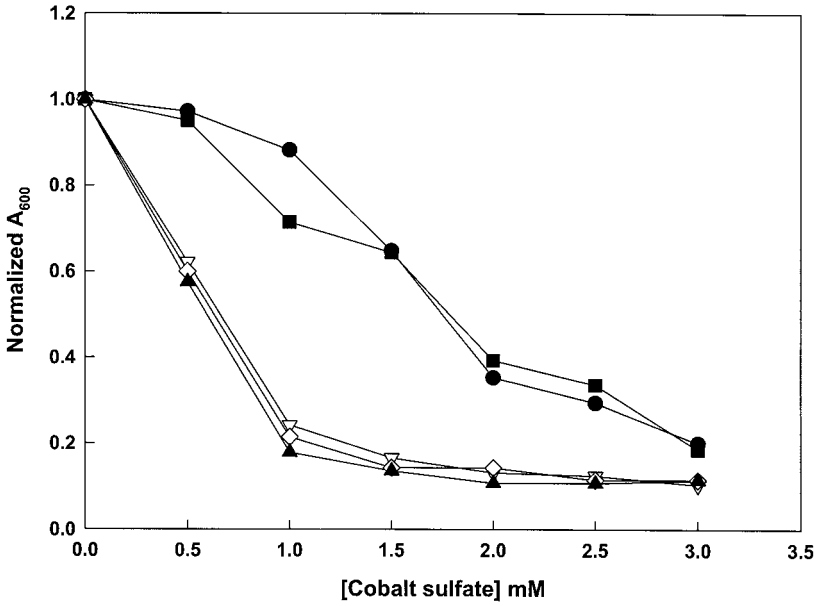

Figure 3. Co^{2+} ion sensitivity of *ARR4* disrupted *S. cerevisiae* at 37 °C. Cells were grown in liquid SD minimal medium with the indicated concentrations of cobalt sulfate. Strains used: (●), W303-1B (wild type); (▽), JSY-1 (*ARR4::HIS3*); (■), JSY-1 transformed with YEp352-*ARR4*. Dominant negative phenotype of cells expressing Arr4p[G30R] mutant either in the wild type or *ARR4* disrupted *S. cerevisiae.* (▲) W303-1B transformed with YEp352-*ARR4*[G30R]; and (◊) JSY-1 transformed with YEp352-*ARR4*[G30R].

cate that although the *S. cerevisiae ARR4* gene encodes an ATPase that exhibits significant identity to the bacterial ArsA ATPase, a subunit of an arsenic-resistance pump, the function of the yeast protein is probably not to confer resistance to a specific metal or metalloid, but may be as a component in a more general stress response.

Expression and biochemical characterization of Arr4p

Arr4p with a C-terminal six histidine tag was purified by metal chelate affinity chromatography, as described under *Materials and methods*. Approximately 5 mg of purified Arr4p could be purified per liter of *E. coli* culture. The protein could be purified to >95% homogeneity, as estimated from the intensity of Coomassie Blue staining of samples separated by SDS-PAGE (Figure 4, inset). The molecular mass of purified Arr4p was determined by gel filtration chromatography using a Sephacryl S-200 column (Figure 4). The nucleotide sequence of the *ARR4* gene with the C-terminal six-histidine tag indicates a predicted mass of 40,417-Da. The protein eluted as a ~80 kDa species from the Sephacryl S-200 column, suggesting an Arr4p homodimer.

Purified preparations of Arr4p-[His]6 exhibit Mg^{2+}-ATPase activity in the range of 100–140 nmoles/min/mg of protein. Maximal ATPase activity was observed at pH 7.5 and at 37 °C (data not shown). The Km for ATP was determined to be 2.8 mM. In contrast to the *E. coli* ArsA, whose ATPase activity is activated by As^{3+} or Sb^{3+} (Bhattacharjee *et al.* 1995), purified Arr4p preparations displays only basal ATPase activity that is not stimulated by either As^{3+}, Sb^{3+}, Cu^{2+}, or Co^{2+} (data not shown). The lack of effect of metal ions *in vitro* suggests that the additive effects of metal ions and elevated temperature *in vivo* is stress related rather than a direct effect of metals on Arr4p. Kurdi-Haidar, *et al.*, have reported that the human homolog, hASNA-1, shows basal ATPase activity that is stimulated 1.6-fold in the presence of As^{3+} but not in presence of Sb^{3+} (Kurdi-Haidar *et al.* 1998). The difference between the properties of the human and yeast homologues is unexplained.

Negative dominance

An Arr4p mutant exhibited negative dominance over the wild type gene. *E. coli* ArsA ATPase can be rendered catalytically inactive upon alteration of the conserved glycine residues at its nucleotide-binding consensus sequence (Karkaria *et al.* 1990; Kaur &

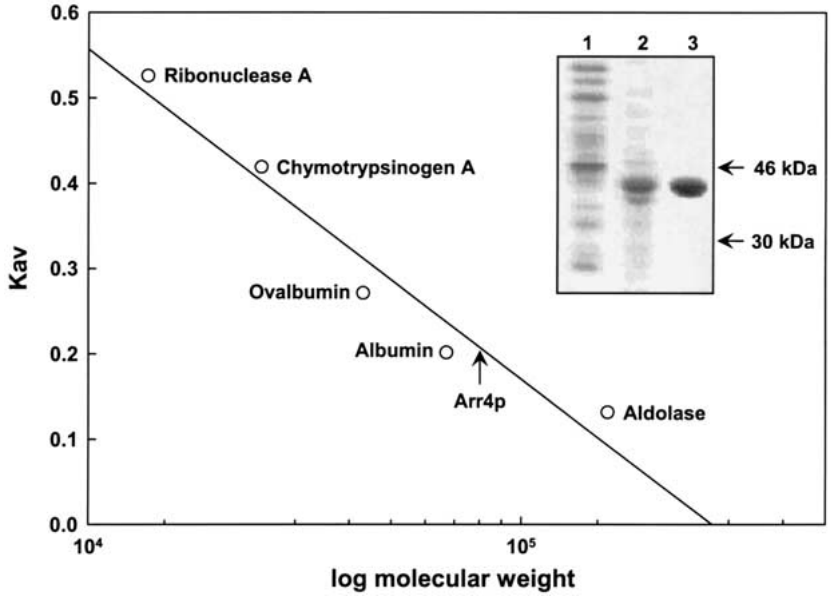

Figure 4. Purification of Arr4p and molecular mass estimation. Inset: *E. coli* strain BL21(DE3) harboring plasmid pArr4p-His6 was grown and induced as described in *Materials and methods*. Samples were prepared by treating the lysate with SDS sample buffer for 10 min at 37 °C, and analyzed by SDS-PAGE on a 12% polyacrylamide gel. *Lane 1*, cytosol from uninduced cells, *lane 2*, cytosol after induction with IPTG, *lane 3*, purified protein following ProBond metal chelate chromatography. The arrows indicate the position of migration of standard markers.

The molecular mass of Arr4p was determined from its elution position (arrow) following Sephacryl S-200 chromatography. The elution positions of the standard proteins are indicated for aldolase (158-kDa), albumin (67-kDa), ovalbumin (43-kDa), chymotrypsinogen A (25-kDa), and ribonuclease A (13.7-kDa).

Rosen 1992). A similar mutation in the *ARR4* gene was created by altering the Gly30 codon to Arg by site directed mutagenesis. Altered Arr4p with a G30R mutation was expressed in *E. coli* cultures at similar levels as the wild type (data not shown) and purified to homogeneity by metal chelate affinity chromatography. Purified Arr4p[G30R] exhibited no measurable Mg^{2+}-ATPase activity.

To examine the effect of the mutation *in vivo*, both W303 and JSY-1 strains were transformed with the plasmid YEp352-*ARR4*[G30R], which carries the altered *ARR4* gene under the control of its endogenous promoter. As shown in Figure 3, JSY-1 cells carrying the altered *ARR4*[G30R] gene was sensitive to Co^{2+} at 37 °C. JSY-1 cells transformed with the wild type *ARR4* gene exhibited similar levels of resistance as the wild type cells. On the other hand, wild type cells transformed with the altered gene exhibited a sensitive phenotype, indicating a dominant negative effect. Assuming that both subunits must be active, the negative dominance of the G30R mutation could be explained by formation of inactive wild type-mutant heterodimer. Alternatively, if *ARR4* is part of a larger complex, the mutant subunit could prevent interaction with a partner protein.

GFP-Arr4p localization under stress conditions

To examine the physiological role of Arr4p, the subcellular localization of the protein was determined. *ARR4*-disrupted *S. cerevisiae* strain JSY-1 was transformed with plasmid pUG36-*ARR4*. This plasmid expresses the yeast-enhanced green fluorescence protein (yEGFP3) fused to the N-terminus of Arr4p under control of the *MET25* promoter. Expression of GFP-Arr4p could be visualized by Western analysis after probing with a polyclonal antibody raised against Arr4p (data not shown). As predicted from the nucleotide sequence, the GFP-Arr4p fusion construct migrated as a 66-kDa species upon SDS-polyacrylamide gel electrophoresis. Expression of the fusion protein was repressed in the presence of 1.0 mM methionine.

Confocal laser scanning microscopy was used to monitor the localization of the cells expressing the GFP-Arr4p fusion construct, in cells grown in presence of 1 mM methionine to prevent protein overproduction. Cells grown under non-stress conditions displayed a diffused, green fluorescence throughout the cytosol. When the cells were grown at 37 °C in the presence of 0.5 mM Co^{2+} and 1 mM methionine, fluorescence from the GFP-Arr4p fusion protein

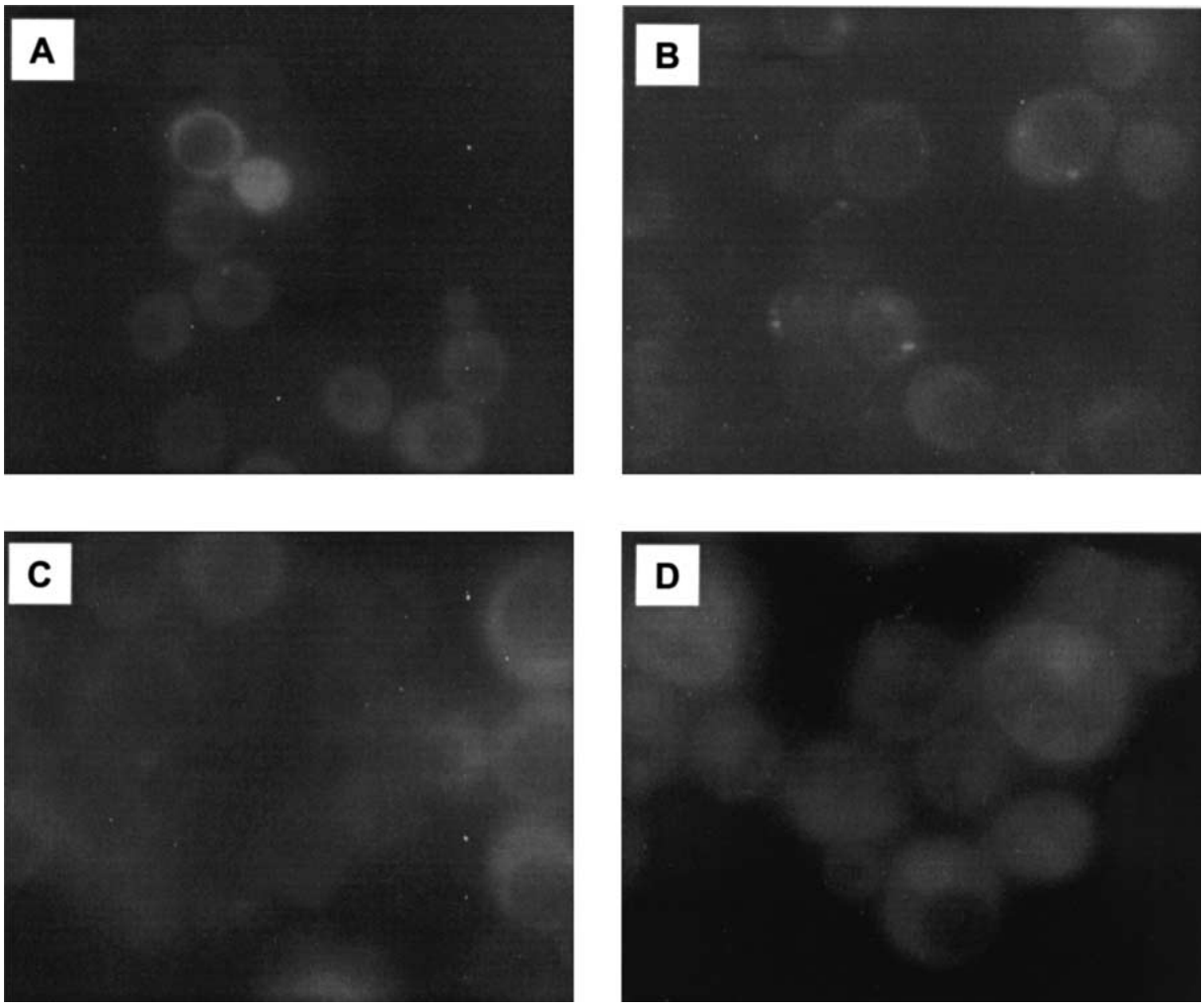

Figure 5. Cellular localization by confocal microscopy of GFP-Arr4p. GFP-Arr4p visualized in JSY-1 (*ARR4::HIS3*) cells bearing plasmid YEp352-GFP-*ARR4*, grown in absence (A) or presence (B) of 0.5 mM Co^{2+} at 37 °C. GFP-Arr4p[G30R] visualized in JSY-1 (*ARR4::HIS3*) cells bearing plasmid YEp352-GFP-*ARR4*[G30R], grown in absence (C) or presence (D) of 0.5 mM Co^{2+} at 37 °C.

was restricted to 1–2 punctate structures per cell. Punctate structures could also be observed when the GFP-Arr4p fusion construct was under the control of the *ARR4* endogenous promoter (Figure 5). The GFP-Arr4p fluorescent punctate structure is not localized in mitochondria (data not shown), as determined by staining the cells with rhodamine B hexyl ester, which selectively labels yeast mitochondria (Haugland 2001). Similarly, the results of DAPI (4′, 6-diamidino-2-phenylindole) staining (Haugland 2001) indicated that the punctate bodies are not localized in the nucleus (data not shown). The GFP-G30R-Arr4p fusion did not go into punctate bodies. The cellular localization of the punctate structure in a yet unidentified intracellular organelle is currently under investigation. The localization apparently requires the ATPase activity of Arr4p, since a GFP-G30R-Arr4p does not locate in punctate bodies but is, instead, observed diffusing throughout the cytosol. We propose that the Arr4p ATPase forms a complex with a membrane protein that is a component of a stress tolerance network.

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References

Bhattacharjee H, Rosen BP. 2000 Role of conserved histidine residues in metalloactivation of the ArsA ATPase. *BioMetals* **13**, 281–288.

Bhattacharjee H, Ghosh M, Mukhopadhyay R, Rosen BP. 1999 Arsenic transporters from *E. coli* to humans in Broome-Smith JK, Baumberg S, Sterling CJ, and Ward FB, eds, *Transport of molecules across microbial membranes*, Society for General Microbiology, pp. 58–79.

Bhattacharjee H, Ho YS, Rosen BP. 2001 Genomic organization and chromosomal localization of the Asna1 gene, a mouse homologue of a bacterial arsenic-translocating ATPase gene. *Gene* **272**, 291–299.

Bhattacharjee H, Li J, Ksenzenko MY, Rosen BP. 1995 Role of cysteinyl residues in metalloactivation of the oxyanion-translocating ArsA ATPase. *J Biol Chem* **270**, 11245–11250.

Bobrowicz P, Wysocki R, Owsianik G, Goffeau A, Ulaszewski S. 1997 Isolation of three contiguous genes, *ACR1*, *ACR2* and *ACR3*, involved in resistance to arsenic compounds in the yeast *Saccharomyces cerevisiae*. *Yeast* **13**, 819–828.

Bowman S, Ackerman SH, Griffiths DE, Tzagoloff A. 1991 Characterization of ATP12, a yeast nuclear gene required for the assembly of the mitochondrial F1-ATPase. *J Biol Chem* **266**, 7517–7523.

Bruhn DF, Li J, Silver S, Roberto F, Rosen BP. 1996 The arsenical resistance operon of IncN plasmid R46. *FEMS Microbiol Lett* **139**, 149–153.

Burke D, Dawson D, Stearns T. 2000 *Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual (2000 Edition)*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Davidson JF, Whyte B, Bissinger PH, Schiestl RH. 1996 Oxidative stress is involved in heat-induced cell death in Saccharomyces cerevisiae. *Proc Natl Acad Sci USA* **93**, 5116–5121.

Dey S, Dou D, Rosen BP. 1994 ATP-dependent arsenite transport in everted membrane vesicles of *Escherichia coli*. *J Biol Chem* **269**, 25442–25446.

Gatti D, Mitra B, Rosen BP. 2000 Escherichia coli soft metal ion-translocating ATPases. *J Biol Chem* **275**, 34009–34012.

Ghosh M, Shen J, Rosen BP. 1999 Pathways of As(III) detoxification in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA* **96**, 5001–5006.

Gill SC, von Hippel PH. 1989 Calculation of protein extinction coefficients from amino acid sequence data. *Anal Biochem* **182**, 319–326.

Haugland RP. 2001 *Handbook of Fluorescent Probes and Research Products*. Molecular Probes, Inc., Eugene, OR.

Hill JE, Myers AM, Koerner TJ, Tzagoloff A. 1986 Yeast/E. coli shuttle vectors with multiple unique restriction sites. *Yeast* **2**, 163–167.

Hsu CM, Rosen BP. 1989 Characterization of the catalytic subunit of an anion pump. *J Biol Chem* **264**, 17349–17354.

Karkaria CE, Chen CM, Rosen BP. 1990 Mutagenesis of a nucleotide-binding site of an anion-translocating ATPase. *J Biol Chem* **265**, 7832–7836.

Kaur P, Rosen BP. 1992 Mutagenesis of the C-terminal nucleotide-binding site of an anion- translocating ATPase. *J Biol Chem* **267**, 19272–19277.

Kurdi-Haidar B, Aebi S, Heath D, Enns RE, Naredi P, Hom DK, Howell SB. 1996 Isolation of the ATP-binding human homolog of the *arsA* component of the bacterial arsenite transporter. *Genomics* **36**, 486–491.

Kurdi-Haidar B, Heath D, Aebi S, Howell SB. 1998 Biochemical characterization of the human arsenite-stimulated ATPase (hASNA-I). *J Biol Chem* **273**, 22173–22176.

Mukhopadhyay R, Rosen BP. 1998 The *Saccharomyces cerevisiae ACR2* gene encodes an arsenate reductase. *FEMS Microbiol Lett* **168**, 127–136.

Rothstein RJ. 1983 One-step gene disruption in yeast. *Methods. Enzymol.* **101**, 202–211.

Sambrook J, Russell D. 2001 *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Suzuki K, Wakao N, Kimura T, Sakka K, Ohmiya K. 1998 Expression and regulation of the arsenic resistance operon of *Acidiphillium multivorum* AIU301 plasmid pKW301 in *Escherichia coli*. *Appl Environ Microbiol* **64**, 411–418.

Vogel G, Steinhart R. 1976 ATPase of *Escherichia coli*: purification, dissociation, and reconstitution of the active complex from the isolated subunits. *Biochemistry* **15**, 208–216.

Walker JE, Saraste M, Runswick MJ, Gay NJ. 1982 Distantly related sequences in the α- and β-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *Embo J* **1**, 945–951.

Walmsley AR, Zhou T, Borges-Walmsley MI, Rosen BP. 1999 The ATPase mechanism of ArsA, the catalytic subunit of the arsenite pump. *J Biol Chem* **274**, 16153–16161.

Weisman LS, Bacallao R, Wickner W. 1987 Multiple methods of visualizing the yeast vacuole permit evaluation of its morphology and inheritance during the cell cycle. *J Cell Biol* **105**, 1539–1547.

Zhou T, Rosen BP. 1997 Tryptophan fluorescence reports nucleotide-induced conformational changes in a domain of the ArsA ATPase. *J Biol Chem* **272**, 19731–19737.

Zhou T, Radaev S, Rosen BP, Gatti DL. 2000 Structure of the ArsA ATPase: the catalytic subunit of a heavy metal resistance pump. *Embo J* **19**, 4838–4845.

Zuniga S, Boskovic J, Jimenez A, Ballesta JP, Remacha M. 1999 Disruption of six *Saccharomyces cerevisiae* novel genes and phenotypic analysis of the deletants. *Yeast* **15**, 945–953.