Saccharomyces cerevisiae ACR2 gene encodes an arsenate reductase

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Abstract

The ACR2 gene of Saccharomyces cerevisiae was disrupted by insertion of a HIS3 gene. Cells with the disruption were sensitive to arsenate. This phenotype could be complemented by ACR2 on a plasmid. The ACR2 gene was cloned and expressed in Escherichia coli as a malE gene fusion with a C-terminal histidine tag. The combination of chimeric MBP-Acr2-6H protein and yeast cytosol from an ACR2-disrupted strain exhibited arsenate reductase activity.

Keywords: Yeast; Arsenate resistance

1. Introduction

The most frequent means of producing resistance to drugs and metals including arsenicals is via an extrusion mechanism, which lowers the amount of the metalloid to levels that are no longer toxic [1]. Bacterial resistance to arsenicals have been intensively studied [2–4]. In both Gram-positive and Gram-negative bacteria there are extrusion systems to eliminate arsenite, which contains As(III), a reduced form of arsenic. For resistance to arsenate (As(V)), a more oxidized form of arsenic, enzymatic reduction is required [5,6]. Bacterial arsenate reductases fall into two quite distinct families, the Gram-negative bacterial family typified by the ArsC enzyme of the Escherichia coli plasmid R773, which couples reduced glutathione and glutaredoxin to reduction of arsenate [7], and the Gram-positive bacterial family typified by the Staphylococcus aureus plasmid pI258-encoded arsC gene product, which uses thioredoxin as reductant [8].

Resistance to this metalloid in eukaryotes is less well characterized at the molecular level [9]. Recently a gene cluster on chromosome XVI of Saccharomyces cerevisiae termed ACR1, ACR2 and ACR3 has been reported to confer arsenical resistance in yeast [10,11]. While none of the three gene products show significant sequence similarity to the R773 or pI258 ars gene products, the ACR3 gene product exhibits 30% similarity to an open reading frame in an arsenical resistance operon from Bacillus subtilis [12], and Acr3p is most likely an arsenite exporter with a function similar to the bacterial ArsB proteins [11]. Expression of both ACR3 and ACR2 in wild-type yeast resulted in a slight increase in arsenate resistance,
leading to the suggestion that ACR2 might be an arsenate reductase [10].

In this communication we report that disruption of ACR2 produces sensitivity to arsenate but not to arsenite. The ACR2 gene was cloned and introduced into wild-type and ACR2-disrupted cells on a plasmid. The arsenate sensitive phenotype of ACR2-disrupted cells was complemented by expression of ACR2 on a plasmid. In wild-type cells expression of ACR2 on a plasmid increased resistance to arsenate. The ACR2 gene was fused to the bacterial malE gene at the 5' end and to six histidine codons at the 3' end. The gene fusion was expressed in E. coli and purified by a two-step procedure utilizing affinity chromatography for the two tags. Purified MBP-Acr2-6H alone reduced only small amounts of arsenate, but, when supplemented with cytosol from S. cerevisiae exhibited time dependent arsenate reduction. These results suggest that Acr2p is a eukaryotic equivalent of the bacterial ArsC arsenate reductases.

2. Materials and methods

2.1. Plasmids and strains

Plasmids and S. cerevisiae strains used in this study are described in Table 1. E. coli strain JM109 (recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi D(lac-proAB) F' [traD36 proAB lacI q lacZDM15]) was used for molecular cloning.

2.2. Media

S. cerevisiae strains were grown at 30°C in complete YPD [13] medium supplemented with 2% glucose. Alternatively, the minimal SD [13] medium with 2% glucose or galactose supplemented with auxotrophic requirements was used. E. coli cells were grown in LB medium [14] supplemented when necessary with 125 μg ml⁻¹ ampicillin or kanamycin.

2.3. DNA manipulations

Cloning procedures (plasmid purification, restriction digestion, endonuclease and exonuclease digestion, gel electrophoresis, polymerase chain reaction (PCR), ligation, dephosphorylation and E. coli trans-

formation) were carried out as described [14,15]. Transformation of yeast cells was carried out using a lithium acetate method [16]. Yeast genomic DNA was isolated using a kit from Qiagen.

2.4. ACR2 gene disruption

Disruption of the ACR2 gene was carried out by a one-step method [17] (Fig. 1). A 3.1-kb fragment of yeast genomic DNA containing ACR2 was amplified by PCR using a forward primer 5’-CGACA-TAACGTTATCTTGCC-3’ that hybridizes with a region 1998 bp upstream of ACR2 and a reverse primer 5’-GTCATTTGAGAGATTCGTACAGC-3’ that hybridizes to a region 729 bp downstream of ACR2. The fragment was ligated into vector pGEM-T. The resulting plasmid, carrying ACR3, ACR2 and a portion of ACR1, was digested at the unique PacI site in ACR2, made blunt with T4 DNA polymerase, and ligated with a 1.7-kb HIS3 gene. The HIS3 gene had been obtained from plasmid pUC18-HIS3-1 as a 1769-bp BamHI/SmaI fragment and made blunt with the Klenow fragment of DNA polymerase I. The plasmid was digested with EcoRI, and the 4769-bp fragment isolated and transformed into yeast strain W303-1B, producing the ACR2-disrupted strain RM1 by homologous recombination. Verification of the ACR2 disruption was confirmed by PCR using a forward primer 5’-GGCAACATCAAGTCAAGCCATG-3’ that hybridizes 363 bp upstream and a reverse primer 5’-GGAACATATTGAGGAGC-3’ that hybridizes 22 bp downstream of ACR2.

2.5. Arsenic resistance assays

Strains were grown overnight at 30°C in liquid SD medium with 2% glucose or galactose and the appropriate supplements. The cultures were diluted into minimal media to an OD₆₀₀ₙ₉₉ of 0.1 in presence of varying concentrations of sodium arsenate, incubated for an additional 24 h, following which the growth was estimated from OD₆₀₀ₙ₉₉.

2.6. Purification of the MBP-Acr2-6H chimeric protein

ACR2 from yeast strain W303-1B was cloned into
vector plasmid pGEM-T by PCR using forward primer 5'-CCATGGTAAGTTTCATAACGTC-3', introducing an NcoI site at the 5' end of ACR2, and reverse primer 5'-AAGCTTACCACTAA-CAATCAATTTAAGG-3', which both removed the stop codon of ACR2 and introduced a HindIII site at the 3' end of the gene. The ACR2 sequence was confirmed using an automated DNA sequencer (ALF-Express, Pharmacia). The ACR2 gene was cloned in-frame with the sequence for a C-terminal six histidine tag in pET28b, producing plasmid pACR2-1. The gene was excised from the pGEM-T construct by digestion with both NcoI and HindIII and ligated into vector plasmid pET28-b that had also been digested with both enzymes.

The ACR2-6H gene from pACR2-1 was amplified by PCR to introduce an EcoRI site at the 5' end and a PstI site at the 3' end. The forward primer was 5'-AATTCATGGTAAGTTTCATAACGTC-3', which introduced an EcoRI site at the 5' end of the fragment. The reverse primer was 5'-CTGCAGGCG-GCGCAGGCG-3', which introduced a PstI site at the 3'-end of the gene. The reverse primer started 50 bp within the pET28-b vector, so that the PCR product would contain the ACR2 gene with the six histidine codon tag and adjacent stop codon from pET28-b. The PCR fragment was ligated into pGEM-T and sequenced. It was then excised by digestion with both EcoRI and PstI and ligated into the pMAL-C2 vector that had been digested with both enzymes, creating an in-frame gene fusion of malE-ACR2-6H. The resulting construct was termed plasmid pACR2-2.

To construct plasmid pACR2-3, pACR2-2 was digested with PstI and made blunt by T4 DNA polymerase. The linearized plasmid was purified and digested with EcoRI, and the 496-bp fragment purified. Vector plasmid pYES2.0 was digested with NotI, made blunt with the Klenow fragment of DNA polymerase I. The linearized plasmid was purified and digested with EcoRI, and the 5.9-kb fragment purified. The two fragments were ligated together, producing plasmid pACR2-3, which was transformed into strains W303-1B and RM1.

For expression, cells of E. coli strain JM109 harboring plasmid pACR2-2 were grown overnight in 100 ml of LB supplemented with 125 µg/ml of ampicillin at 37°C. This culture was then diluted into 2 l of the same medium and incubated with shaking at 37°C until an OD600nm of 0.5 was reached. Isopropyl-1-thio-β-D-galactopyranoside (0.1 mM) was added, and the culture was incubated for an additional 12 h at 20°C to produce chimeric Acr2p. The cells were chilled on ice, harvested and suspended in
50 ml of buffer A (20 mM Tris, pH 7.4, 0.2 M NaCl, 1 mM EDTA and 10 mM β-mercaptoethanol). The cells were frozen at −20°C, thawed in cold water and disrupted by a single passing through French pressure cell (20,000 psi). Di-isopropylfluorophosphate (2.5 μg/g of wet cells) was added immediately to the lysate. The lysate was diluted to 80 ml with buffer A and centrifuged at 100,000 × g for 1 h at 4°C. The resulting supernatant solution was loaded at a flow rate of 1 ml min⁻¹ onto a 15-ml amylose column that had been pre-equilibrated with 300–400 ml of buffer A. The column was washed with 250 ml of buffer A. Chimeric Acr2p was eluted from the column with 200 ml of buffer A containing 10 mM maltose. Fractions containing the chimera were pooled and loaded onto a 5 ml Ni-NTA column pre-equilibrated with buffer B (50 mM MOPS, pH 7.5, 20% glycerol, 0.5 M NaCl, 10 mM β-mercaptoethanol and 20 mM imidazole) at a flow rate of 0.5 ml min⁻¹. The column was washed with 275 ml of buffer B. The chimeric protein was eluted with 125 ml of buffer B containing 200 mM imidazole. Fractions containing the protein were pooled, and concentrated using a Millipore Ultrafree-30 BIO-MAX-5K centrifugal filter at 2000 × g, and stored at −80°C until use. In each step the location of the chimeric Acr2p was identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [14]. The concentration of purified protein was estimated from the absorbance at 280 nm.

2.7. Assay of arsenate reductase activity

Arsenate reduction was assayed at 37°C in 0.02 ml of a reaction mixture consisting of 1 mM Tris-HCl, pH 7.5, 1 mM EGTA, 10% (w/v) glycerol (buffer C) and 30 μg of purified chimeric Acr2p as described previously [7]. Where indicated, cytosol from yeast strains W303-1B or RM1 was added to the reductase assay. Cytosol was prepared from 300-ml cultures grown in YPD medium with 2% glucose. At an OD₆₀₀nm of 0.5, 0.2 mM sodium arsenite was added as inducer. The cells were washed once with buffer C and suspended in 3 ml of a buffer consisting of 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1 mM EGTA, 5% sucrose and 10 mM β-mercaptoethanol. The cells were disrupted by grinding in a pestle and mortar with acid-washed glass beads (150–212 μm) (10 g wet cells) [18]. Disruption was verified using a phase contrast microscope. The suspension was diluted with 10–15 ml of buffer C and centrifuged at 2000 × g for 10 min at 4°C to remove the glass beads, unbroken cells and cell debris. The supernatant suspension was centrifuged at 100,000 × g at 4°C for 1 h, the pellet discarded, and the cytosol retained for assay. Protein concentration was estimated as described [19].

3. Results

3.1. Disruption of the ACR2 gene

The ACR2 gene was disrupted by insertion of a HIS3 gene, creating strain RM1 (Fig. 1). In the wild-type strain the PCR product migrated on an agarose gel with a mobility consistent with the predicted 0.76-kb size (Fig. 1 insert). PCR amplification of RM1 genomic DNA produced a 2.4-kb fragment, consistent with insertion of the 1.7-kb HIS3 gene.

RM1 lost the requirement for histidine compared with the parental type strain W303-1B (data not shown). It was still resistant to arsenite (data not shown) but exhibited sensitivity to arsenate (Fig. 1A). W303-1B was able to grow in sodium arsenate at 1 mM, while RM1 showed almost complete growth inhibition above 0.5 mM sodium arsenate.

To demonstrate that the arsenate sensitive phenotype was indeed the result of disruption of the ACR2 gene, strains W303-1B and RM1 were individually transformed with the multicopy E. coli-yeast shuttle vector pYES2.0 and with pACR2-3, which carries a wild-type ACR2 gene under control of the GAL1 promoter, with no sequence from the other ACR genes. The transformants were examined for arsenate resistance in presence of 2% galactose. ACR2 in the multicopy plasmid complemented the arsenate sensitivity (Fig. 2B). The growth of the wild-type and the complemented RM1 were identical. Interestingly, the wild-type transformed with pACR2-3 was slightly but reproducibly more resistant to arsenate than with only vector. Bobrowicz and coworkers [10] had found a similar result when both ACR3 and ACR2 were co-expressed on a plasmid in a wild-type yeast strain. The fact that ACR2 alone is suffi-
cient suggests that even in wild-type cells Acr2p is limiting for growth in the presence of arsenate.

3.2. Expression and enzymatic activity of ACR2

A *MalE-ACR2-6H* gene fusion was constructed, creating plasmid pACR2-2, and expression of the 57-kDa chimeric Acr2p in *E. coli* was induced with IPTG at 20°C. The MBP-Acr2-6H protein was purified by two successive chromatography steps. From the phenotype of the *ACR2* disrupted strain, it is likely that Acr2p is required for conversion of arsen-
Fig. 2. Phenotype of ACR2-disrupted S. cerevisiae and complementation with ACR2. A: Growth in liquid SD minimal medium in the presence of the indicated concentrations of sodium arsenate. Strains: ∅, W303-1B (wild-type); □, RM1 (ACR2::HIS3). B: Growth in liquid SD minimal medium containing 2% galactose in the presence of the indicated concentrations of sodium arsenate. Strains: W303-1B (□, ∅) and RM1 (♦, △) transformed with vector plasmid pYES2.0 (○, △) or pACR2-3 (□, ∅).
Fig. 3. Arsenate reductase activity of Acr2p. Reductase activity was assayed as described in Section 2. A: Reduction of arsenate was measured for 10 and 30 min with 5.6 μg cytosol from W303-1B, 4.5 μg purified Acr2 chimeric protein, or the combination of the two, as indicated. B: Reduction of arsenate was measured for the indicated times with 5.6 μg cytosol from RM1 (△); 4.5 μg purified Acr2 chimeric protein (○); 4.5 μg purified chimeric Acr2 and 5.6 μg RM1 cytosol (□).
ate to arsenite, suggesting that it may have arsenate reductase activity. Cytosol from the parental strain W303-1B (Fig. 3A) or from the ACR2-disrupted strain RM1 (Fig. 3B) reduced small amounts of arsenate. Similarly purified chimeric Acr2p reduced only small amounts of arsenate (Fig. 3A,B). When the purified protein was combined with cytosol from either strain, time dependent reductase activity was observed. Reduction was not increased by addition of NADPH, nor did reduced glutathione or reduced dithiothreitol specifically augment the activities of cytosols, protein or the combination of the two over the backgrounds produced by the reduced thiols alone (data not shown). However, the reduced thiols alone slowly reduced arsenate nonenzymatically, as thiols have been shown to do [20,21]. These results suggest that Acr2p is an arsenate reductase but that one or more cofactors present in yeast cytosol are required. ArsC encoded by the E. coli plasmid R773 requires reduced glutathione and glutaredoxin [7], while the S. aureus plasmid-encoded ArsC requires thioredoxin [8]. It is likely that reduction in yeast similarly requires a thiol-transfer protein and source of reductant. There are two glutaredoxins [22,23] and two thioredoxins in S. cerevisiae [24–26]. We examined a strain with disruptions in the two thioredoxin genes and found no effect on arsenate resistance (data not shown), suggesting that thioredoxin is not essential for Acr2p function. It is also possible that the actual reductase enzyme is not Acr2p but another cytosolic protein, and that Acr2p is an accessory protein to the actual reductase. Although we consider such a possibility unlikely, we cannot rule it out until reductase activity can be measured solely with purified Acr2p and reductant.

4. Discussion

Relatively little is known about the mechanisms of resistance to arsenicals in eukaryotes compared with the progress in bacteria. Recently three genes were reported to confer resistance to arsenate and arsenite in yeast [10,11]. Here we demonstrate that one of the genes, ACR2, is specifically required for resistance to arsenate but not to arsenite, and the data indicate that this gene encodes an arsenate reductase. There are several arguments that suggest an evolutionary relationship of the ACR2 gene product to other arsenate reductases. First, ACR2 is in a cluster of genes that includes a regulatory gene (ACR1) and the gene for a membrane protein that is probably an arsenite transporter (ACR3) [10,11]. On this point, the bacterial ars operons all have at least three genes, arsR encoding a transcriptional repressor, arsB coding for a membrane protein that transports arsenite, and arsC coding for an arsenate reductase [3]. Second, the protein encoded by ACR2 (130 residues), pI258 ArsC (131 residues), and R773 ArsC (141 residues) are all approximately the same size. Third, Acr2p shows a similar physiological and enzymatic role in arsenate resistance.

Interestingly, the ArsC enzymes of Gram-negative bacteria have a low but significant sequence similarity to reading frames in nif gene [27], cyanobacteria [28,29] and chloroplasts [30] clusters that may be reductases for compounds such as protochlorophyllide. The ArsC enzymes of Gram-positive bacteria exhibit low sequence similarity with a family of low molecular mass phosphatases [31,32]. Acr2p shows a low degree of sequence similarity to the Cdc25A phosphatase of S. cerevisiae and to another S. cerevisiae reading frame of unknown function, YG4E [33]. Thus it is possible that the three arsenate reducing enzymes are the result of convergent evolution.

Even if the reductases are not homologues, they may have common features in their mechanism. In particular these enzymes may all use redox-active cysteines in catalysis. The R773 ArsC Cys12 has been shown to be a catalytic residue [34]. Cys10 and Cys15 in the pI258 ArsC are conserved in the low molecular mass phosphatases. Cys76 in Acr2p aligns with the catalytic residue Cys430 of Cdc25A and Cys90 of YG4E [33]. This may suggest that there are some catalytic similarities between phosphatases and arsenate reductases. In addition, both bacterial reductases utilize small thiol transfer proteins, glutaredoxin with the R773 enzyme [7] and thioredoxin for the pI258 enzyme [8]. Although the source of reducing potential for the yeast enzyme is not yet known, a strain lacking both thioredoxins remains arsenite resistant. Although neither yeast glutaredoxin is required for viability, they are required for protection against oxidative stress [23]. Reduced glutathione is also an important antioxidant in yeast, and gsh1 mutants lacking GSH are...
sensitive to not only to oxidative stress but to cadmium [35,36]. Future work will be directed toward characterization of Acr2p activity and identification of the physiological electron donor(s) for arsenate reduction.

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References


