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Comparative Functional Genomic Analysis Identifies Distinct and Overlapping Sets of Genes Required for Resistance to Monomethylarsonous Acid (MMA^{III}) and Arsenite (As^{III}) in Yeast

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Arsenic is a human toxin and carcinogen commonly found as a contaminant in drinking water. Arsenite (As^{III}) is the most toxic inorganic form, but recent evidence indicates that the metabolite monomethylarsonous acid (MMA^{III}) is even more toxic. We have used a chemical genomics approach to identify the genes that modulate the cellular toxicity of MMA^{III} and As^{III} in the yeast *Saccharomyces cerevisiae*. Functional profiling using homozygous deletion mutants provided evidence of the requirement of highly conserved biological processes in the response against both arsenicals including tubulin folding, DNA double-strand break repair, and chromatin modification. At the equitoxic doses of 150μM MMA^{III} and 300μM As^{III}, genes related to glutathione metabolism were essential only for resistance to the former, suggesting a higher potency of MMA^{III} to disrupt glutathione metabolism than As^{III}. Treatments with MMA^{III} induced a significant increase in glutathione levels in the wild-type strain, which correlated to the requirement of genes from the sulfur and methionine metabolic pathways and was consistent with the induction of oxidative stress. Based on the relative sensitivity of deletion strains deficient in GSH metabolism and tubulin folding processes, oxidative stress appeared to be the primary mechanism of MMA^{III} toxicity whereas secondary to tubulin disruption in the case of As^{III}. Many of the identified yeast genes have orthologs in humans that could potentially modulate arsenic toxicity in a similar manner as their yeast counterparts.

Key Words: **arsenic; arsenite; glutathione; monomethylarsonous acid; yeast.**

Arsenic (As) is a human carcinogen ubiquitous in the environment. Millions of people worldwide are exposed to this metalloid primarily through drinking water, where it is present as a natural contaminant. Chronic exposure to As is associated with several adverse effects on human health that range from alterations in skin pigmentation and non malignant pulmonary disease to cardiovascular disease and cancer of the skin, lung, bladder, liver, and kidney (Ferreccio *et al*., 2000; Mazumder *et al*., 2005; Smith *et al*., 1992, 1998; von Ehrenstein *et al*., 2005). The cancer risks estimated with lifetime exposure to high concentrations of As in water are very high and comparable with those for cigarette smoking and high concentrations of radon in homes (Smith *et al*., 1992). Therefore, the presence of As in the environment poses a serious risk to human health.

Some mammals, including humans, methylate the inorganic As (As^{III}) ingested in water to form the more toxic monomethylarsonous acid (MMA^{III}) (Mass *et al*., 2001; Petrick *et al*., 2000, 2001; Styblo *et al*., 2000). People that excrete high levels of monomethylated arsenicals in urine have a higher risk of As-induced cancer (Steinmaus *et al*., 2006). Moreover, humans excrete relatively more MMA^{III} in urine than any other animal species and are more sensitive to As carcinogenicity, suggesting that MMA^{III} may be central to As toxicity.

Various mechanisms have been proposed to explain arsenic's carcinogenicity, including spindle disruption, induction of chromosomal aberrations, formation of reactive oxygen species, inhibition of DNA repair, alteration in DNA methylation patterns, and promotion/progression in carcinogenesis (Kitchin, 2001; Kligerman and Tennant, 2007). Although the molecular mode of action of arsenic remains unclear, it is likely to involve several of these mechanisms.

The baker's yeast *Saccharomyces cerevisiae* shares many fundamental cellular processes with humans. Homozygous yeast deletion mutants of nonessential genes can be analyzed simultaneously to interrogate their growth phenotype and functionally profile the yeast genome under selective conditions of interest (Giaever *et al*., 2002). Previous studies have shown a low correlation between gene expression levels and genetic requirements for growth in several conditions. For example, very few DNA repair genes required for growth in the presence of DNA-damaging agents are upregulated in yeast (Birrell *et al*., 2002). Considering that growth is a better indicator for the requirement of a gene in the presence of

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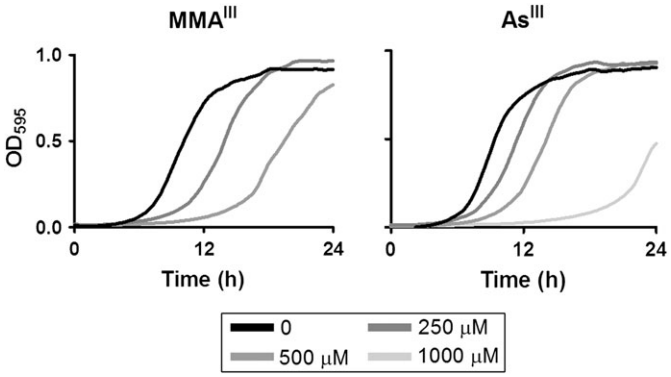

FIG. 1. Growth inhibition assays in the yeast BY4743 wild-type treated with increasing concentrations of MMA^{III} or As^{III}. Optical density at 595 nm (OD_{595}) was measured at 15-min intervals, with each of the points in the curves representing the average of at least three replicates in the same microplate. Standard error was omitted for clarity. Total growth was quantified by determining the area under the growth curves. From these data, the MMA^{III} and As^{III} concentrations required to inhibit growth by 20% were calculated to be 150 and 300μM, respectively.

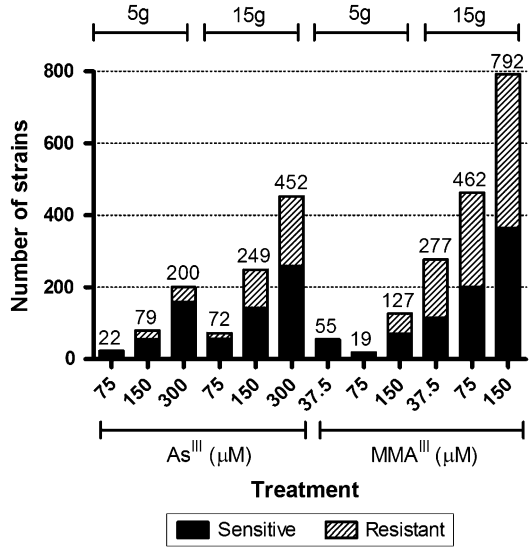

FIG. 2. Number of yeast sensitive and resistant strains (genes) identified by parallel analysis of deletion mutants in the different treatments for MMA^{III} and As^{III}. Exposures included three equitoxic concentrations equivalent to 25% IC_{20}, 50% IC_{20}, and the IC_{20} for 5g and 15g of growth, resulting in a total of six treatments per arsenical. The number of significant strains increased as the dose and number of generations increased.

a toxicant than its expression level, we conducted a functional profiling of the yeast genome to help determine the genes required for yeast's fitness in MMA^{III} and As^{III}, and gain insight into potential mechanisms underlying their toxicity.

MATERIALS AND METHODS

Yeast strains and culture. All yeast strains used in this study were of the BY4743 background (*MATa/MATα his3Δ1/his3Δ1*, *ura3Δ0/ura3Δ0*, *leu2Δ0/leu2Δ0*, *lys2Δ0/+*, *met15Δ0/+*). Growth was conducted in either liquid rich (1% yeast extract, 2% peptone, and 2% dextrose, YPD) or synthetic defined (SD) media, at 30°C with shaking at 200 rpm.

Arsenical exposures. Monomethylarsine oxide (MMA^{III}O) was a generous gift from Professor Miroslav Styblo. MMA^{III}O hydrolyzes to MMA^{III} in solution (Petrick *et al*., 2001). Sodium arsenite (Sigma-Aldrich, St Louis, MO) and MMA^{III}O stock solutions were prepared in sterile Milli-Q water (Millipore, Billerica, MA), protected from light and stored at −80°C until use. Under the growth conditions utilized, approximately 30 and 15% of the MMA^{III} was oxidized to MMA^{V} in the presence and absence of yeast cells, respectively. Likewise, about 60% of As^{III} was converted to the less toxic As^{V} when cells were present (Supplementary Table 1). There was no evidence of As^{III} or MMA^{III} methylation in yeast wild type.

TABLE 1
Arsenic Treatments Used in Functional Profiling Experiments

	Sodium arsenite (As^{III})		MMA^{III}	
Growth inhibitory concentration	5g	15g	5g	15g
25% IC_{20}	75μM	75μM	37.5μM	37.5μM
50% IC_{20}	150μM	150μM	75μM	75μM
IC_{20}	300μM	300μM	150μM	150μM

Note. Each MMA^{III} or As^{III} treatment was performed in triplicate for a total of 18 pools of homozygous mutants and compared with 12 rich media (YPD) controls in order to identify the strains that exhibit a significant change in growth.

Growth curve assays. Yeast strains were pregrown to mid-log phase, diluted to an optical density at 595 nm (OD_{595}) of 0.0165, and dispensed into different wells of a 48-well plate. Arsenical stock solutions were added to the desired final concentrations with at least three replicates per dose. Plates were incubated in a Tecan Genios spectrophotometer set to 30°C with intermittent shaking. OD_{595} measurements were taken at 15-min intervals for a period of 24 h. Raw absorbance data were averaged for all replicates, background corrected, and plotted as a function of time. The area under the curve (AUC) was calculated with Prism version 5.01 (GraphPad Software, La Jolla, CA), as a measure of growth, and expressed as a percentage of the control. AUCs were compared with either one- or two-way ANOVA, as appropriate, followed by Dunnett or Bonferroni post-tests, respectively.

Parallel analysis of yeast deletion mutants. Pool growth, genomic DNA extraction, barcode amplification, and hybridization were performed as previously described (Pierce *et al*., 2006), with minor modifications. Briefly, homozygous diploid deletion mutants ($n = 4607$) were grown in YPD at different arsenical concentrations for 5 and 15 generations (5g, 15g). Cells were collected and genomic DNA was extracted using the YDER kit (Pierce Biotechnology, Rockford, IL). The strain-specific barcodes in the DNA were amplified by PCR using a set of biotinylated primers, and reactions hybridized to TAG4 arrays (Affymetrix, Santa Clara, CA). Arrays were incubated overnight and then stained and scanned at an emission wavelength of 560 nm using a GeneChip scanner (Affymetrix). Raw and processed data files are available at the Gene Expression Omnibus (GEO) database.

Differential strain sensitivity analysis. Raw TAG4 array data were $\log_2$ transformed, corrected for signal saturation as previously described (Pierce *et al*., 2006), and corrected for mean chip background using robust location and scale estimators for $\log_2$-transformed intensities of null features (total of 18,000 equally distributed on the array). To account for variability in strain growth,

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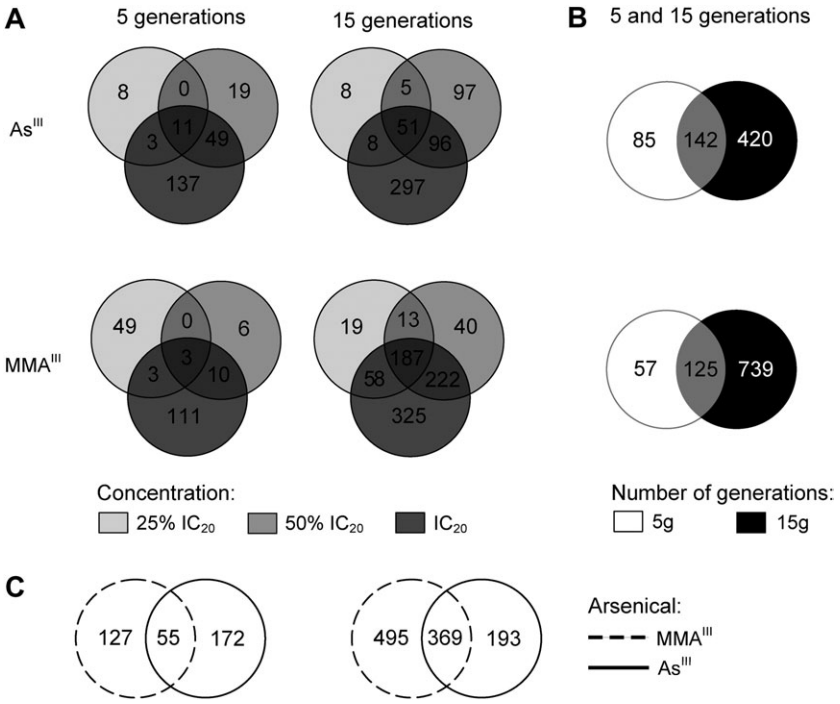

FIG. 3. Venn diagrams showing the number of overlapping and distinct genes between treatments. (A) Most genes at the highest MMA^{III} and As^{III} concentrations were not found at the lower concentrations. (B) Similarly, most genes were specific to 15g with some overlap with 5g. (C) MMA^{III} and As^{III} treatments identified different and overlapping sets of genes.

data from each treatment array were paired to data from 12 controls (5g or 15g) for analysis. Treatment-control pairs were normalized with locally weighted scatterplot smoothing (global normalization), and the differentially growing strains identified using an *alpha*-outlier approach (Loguinov *et al*., 2004). Data from three biological replicates were combined, resulting in 36 treatment-control data pairs per treatment group. Residual variances (with a robust scale estimator) of $\log_2$ (treatment/control) for each 36 pairs were inspected using box plots. The "effective pairs" were then determined by excluding pairs with abnormally high residual variance, or with suspected serial correlation in variability (regular patterns in the box plots) (see Supplementary Methods for sample plots).

Significant genes (strains) were statistically inferred using an exact binomial test, assuming that the outcomes for each gene in all effective treatment-control pairs were independent binary variables with the same probability of success ($p = 0.5$) for all trials (Bernoulli trials). For a particular gene n, outcomes were considered as "successful" if they were significant in the outlier analysis with q-values ≤ 0.05 in each of all effective pairs with $\log_2$ ratios of the same sign, simultaneously. The corresponding raw p values based on the exact binomial test were then corrected for multiplicity of comparisons using q-value approach and only the genes with q-value ≤ 0.05 were considered for further analysis. This approach does not apply a scale estimator and, as a result, it does not require between-chip pair normalization for the statistical inference.

Gene ontology scoring and network analysis. Data sets were verified for enrichment for any particular biological attribute by identifying significantly over represented Gene Ontology (GO) categories by a hypergeometric distribution using the Functional Specification resource, FunSpec (http://funspec.med.utoronto.ca/), with a p value cutoff of 0.01. Fitness data were visualized onto the yeast interaction (Kiemer *et al*., 2007) and regulatory networks (www.yeastract.com) (accessed on July 2007) using Cytoscape version 2.5.1 (www.cytoscape.org).

Reduced glutathione/oxidized glutathione measurements. Control and treated cultures were centrifuged and cell pellets washed in Milli-Q water. Samples were deproteinated by resuspension of the cell pellets in 5% metaphosphoric acid followed by incubation with shaking for 15 min at room temperature. The resulting cell lysates were then centrifuged and the supernatants saved for reduced glutathione (GSH) and oxidized glutathione (GSSG) quantitation. For liquid chromatography tandem mass spectrometry

TABLE 2
Genes with Significant $\log_2$ Ratios Identified in at Least Five of the Six MMA^{III} Treatments

		5g			15g		
		25% IC_{20}	50% IC_{20}	IC_{20}	25% IC_{20}	50% IC_{20}	IC_{20}
ORF	Standard name	75μM	150μM	300μM	75μM	150μM	300μM
YJL101C	*GSH1*	−3.45	−2.10	−4.45	−4.30		−4.90
YFR036W	*CDC26*	−1.7		−2.80	−4.15	−5.70	−3.90
YMR275C	*BUL1*	−1.4	−2.25	−3.15	−4.55		−4.50
YDR135C	*YCF1*	−0.80		−1.40	−2.85	−4.80	−4.20
YPL066W			−1.35	−1.70	−2.45	−4.75	−3.30
YJL179W	*PFD1*		−1.30	−2.00	−3.20	−4.50	−3.60
YPL241C	*CIN2*		−1.25	−1.45	−2.50	−3.60	−3.85
YCR068W	*ATG15*		−1.05	−1.75	−1.75	−3.05	−3.80
YJL120W			−1.10	−2.80	−4.15	−3.40	−4.75
YOR085W	*OST3*		1.30	1.75	1.70	2.50	3.50
YJL121C	*RPE1*	−2.00		−4.30	−3.20	−2.80	−2.60

Note. The requirement of a gene for optimal growth in MMA^{III} is quantified with a fitness score, defined as the normalized $\log_2$ ratio of the deletion strain's growth ($\log_2$ treatment − $\log_2$ control). A total of 11 (1%) of the identified genes were important for fitness in at least five of the six MMA^{III} treatments. Most genes were significant in one or two treatments and after 15g of growth. See Supplementary Table 2 for the list of all identified genes.

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TABLE 3
Genes with Significant $\log_2$ Ratios Identified in at Least Five of the Six As^{III} Treatments

		5g			15g		
ORF	Standard name	25% IC_{20} 75μM	50% IC_{20} 150μM	IC_{20} 300μM	25% IC_{20} 75μM	50% IC_{20} 150μM	IC_{20} 300μM
YJL179W	*PFD1*	−2.60	−2.85	−4.05	−4.70	−5.65	−4.80
YDR181C	*SAS4*	−1.90	−3.05	−4.00	−4.00	−4.50	−4.90
YOR213C	*SAS5*	−2.10	−2.40	−5.10	−3.90	−4.80	−5.55
YMR127C	*SAS2*	−1.75	−2.70	−3.90	−2.35	−3.80	−4.55
YPL241C	*CIN2*	−1.55	−1.95	−2.85	−4.35	−4.75	−5.15
YPR199C	*ARR1*	−1.65	−2.65	−5.35	−4.55	−5.90	−6.20
YEL003W	*GIM4*	−2.00	−1.70	−2.30	−1.45	−2.10	−3.30
YML095C-A		−2.30	−2.20	−3.10		−3.10	−3.10
YLR200W	*YKE2*	−2.30	−2.45	−3.05		−1.80	−3.60
YPR201W	*ARR3*	−2.00	−3.00	−4.25	−4.45		−5.90
YFR036W	*CDC26*		−2.90	−4.50	−3.65	−4.35	−3.45
YMR275C	*BUL1*		−2.30	−4.25	−3.60	−5.20	−3.40
YOR265W	*RBL2*		−1.40	−2.40	−1.00	−2.25	−4.00
YML124C	*TUB3*		−1.05	−200	−1.85	−2.45	−1.70
YNL025C	*SSN8*		−1.15	−2.50	−1.30	−2.85	−4.80
YCR081W	*SRB8*		−1.30	−2.85	−1.50	−2.25	−2.60
YLR006C	*SSK1*		−1.10	−2.80	−1.00	−1.35	−3.85
YJL128C	*PBS2*		−0.90	−3.00	−0.70	−1.40	−5.50
YOL081W	*IRA2*		−1.00	−1.30	−1.30	−1.75	−2.95
YCR068W	*ATG15*		−1.00	−2.30	−1.30	−2.05	−3.35
YGR133W	*PEX4*		−0.80	−1.40	−0.80	−1.00	−1.90
YGR057C	*LST7*		−1.20	−2.50	−1.95	−2.80	−4.70
YCL010C	*SGF29*		−1.10	−2.05	−1.30	−1.95	−2.90
YML041C	*VPS71*		−1.30	−2.10	−1.90	−2.10	−3.20
YNL329C	*PEX6*		−0.80	−1.60	−1.00	−1.00	−2.20
YDL082W	*RPL13A*		0.70	1.30	1.30	1.70	2.20

Note. The requirement of a gene for optimal growth in As^{III} is quantified with a fitness score, defined as the normalized $\log_2$ ratio of the deletion strain's growth ($\log_2$ treatment − $\log_2$ control). A total of 26 (4%) of the identified genes were important for fitness in at least five of the six As^{III} treatments. Most genes were significant in one or two treatments and after 15g of growth. See Supplementary Table 3 for the list of all identified genes.

(LC-MS-MS) quantitation of GSH, calibration standards, quality control samples and supernatant samples were precipitated with 2× volumes of ice-cold acetonitrile and centrifuged at 6100 × g for 30 min at 4°C. A volume of 10 μl of supernatant was injected to a 50 × 2 mm hydrophilic interaction liquid chromatography column and subsequently analyzed using a MDS SCIEX API 3000 Mass Spectrometer (Applied Biosystems, Foster City, CA) in MRM scan mode. Total GSH (GSH + 2*GSSG) was also determined using the Glutathione Assay Kit (Cayman Chemical, Ann Arbor, MI) following manufacturer's specifications. To determine GSSG concentration in this assay, 2-vinylpyridine was added to a final concentration of 10mM, incubated for 1 h to derivatize the GSH, and assayed for total GSH.

RESULTS

Functional Profiling of the Yeast Genome in MMA^{III} and As^{III}

MMA^{III} showed a higher potency than As^{III} to inhibit the growth of the wild-type yeast strain (Fig. 1). In order to study and compare the genetic requirements of yeast for growth in the presence of these arsenicals, we selected equitoxic concentrations that resulted in 20% growth inhibition (IC_{20}), as well as 50 and 25% of this IC_{20}. These doses corresponded to 150, 75, and 37.5μM for MMA^{III}, and 300, 150, and 75μM for As^{III}, respectively (Supplementary Figs. 1 and 2). We exposed pools of yeast homozygous deletion mutants for 5g and 15g of growth, totaling six treatments per arsenical, with three biological replicates each (Table 1).

We used a differential strain sensitivity analysis (as described in "Methods") to identify the strains that exhibit significant changes in growth after treatments with MMA^{III} and As^{III}, and determine the genes that influence yeast's fitness in their presence. The number of identified strains (genes) correlated directly both to the dose and to the number of generations of growth, ranging from few genes after 5g to several hundred after 15g (Fig. 2, Supplementary Tables 2 and 3), and showing different degrees of overlap (Figs. 3A and 3B).

Genetic Determinants of Yeast's Resistance to MMA^{III} and As^{III}

For each arsenical, we considered the genes that were significant in the majority of the six treatments as the ones having the most influence on yeast fitness. The genes that were essential for growth in at least five of the six MMA^{III} treatments (Table 2) were associated with cadmium ion response (*YCF1*, *GSH1*); multivesicular body membrane disassembly (*ATG15*); and response to mercury ion/hydrogen peroxide (*GSH1*). Similarly, genes identified in at least five of the six the As^{III} treatments (Table 3) belonged primarily to several biological processes including tubulin complex assembly (*GIM4*, *YKE2*, *RBL2*); protein import into peroxisome matrix, receptor recycling (*PEX4*, *PEX6*); postchaperonin tubulin folding pathway (*RBL2*, *CIN2*); histone exchange (*VPS71*, *SAS2*); chromatin modification (*SAS4*, *VPS71*, *SAS2*, *SAS5*); microtubule-based process (*TUB3*, *CIN2*); regulation of transcription, DNA dependent (*SGF29*, *SRB8*, *SAS4*, *SSK1*, *SAS2*, *SSN8*, *SAS5*, *ARR1*); arsenic resistance (*ARR1*, *ARR3*); chromatin silencing at telomere (*SAS4*, *SAS2*, *SAS5*); and protein ubiquitination (*PEX4*, *BUL1*).

About 15% of genes were common to the MMA^{III} and As^{III} treatments after 5g of growth and approximately 35% after 15g. In general, genes associated with cadmium ion transport, cytoskeleton organization and biogenesis, chromatin modeling, intracellular protein transport, regulation of transcription, and protein catabolism were common to both arsenical treatments, although there were also genes that belonged to these processes but were specific to one of them (Table 4). Glutathione biosynthesis, sulfur and methionine metabolism, and nicotinamide adenine nucleotide phosphate (reduced) (NADPH) regeneration were enriched for genes from the MMA^{III} treatments. Interestingly, the number of genes specific to MMA^{III} increased about fourfold from 5g to 15g, whereas the one for As^{III} was only slightly increased, as almost all of the newly identified genes in As^{III} overlapped with those of

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TABLE 4
Genes Required for Growth in the Presence of MMA^{III} and As^{III}, and their Associated Biological Processes

MMA^{III}		As^{III}	
GO biological process	Identified genes[a]	GO biological process	Identified genes[a]
		Arsenite transport*	*ARR3*
Glutathione biosynthesis*	*GSH1*		
Cadmium ion transport*	*YCF1*	Cadmium ion transport	*YCF1*
Chromatin modeling	*HTZ1*, *ZDS1*	Chromatin modeling*	*SAS2*, *SAS4*, *SAS5*, *SIN3*, *RSC1*, *RSC2*, *HTZ1*, *ZDS1*, *SAP30*, *SWD3*, *SUM1*, *ISW2*
Cytoskeleton organization and biogenesis	*CIN2*, *PFD1*, *GIM4*, *YKE2*	Cytoskeleton organization and biogenesis*	*CIN2*, *PFD1*, *TUB3*, *GIM4*, *YKE2*, *PAC10*, *CDC26*, *RBL2*, *BUD27*
Intracellular protein transport	*LST7*, *VPS71*, *TOM7*, *ARP6*, *MOG1*	Intracellular protein transport*	*LST7*, *VPS71*, *TOM7*, *ARP6*, *MOG1*
		Meiotic DNA DSB processing*	*SAE2*, *RAD50*
Methionine metabolism*	*MET3*, *MET14*, *MET1*, *MET22*, *MET16*		
NAPDH regeneration/metabolism*	*RPE1*, *TKL1*, *PYC1*, *TAL1*		
Regulation of transcription*	*TUP1*, *REG1*, *YAP3*, *MAL33*	Regulation of transcription*	*TUP1*, *REG1*, *ARR1*, *YAP3*, *MAL33*
Sulfur metabolism*	*MET1*, *MET3*, *MET14*, *MET16*, *MET22*, *MET32*, *GSH1*, *MET3*, *STR2*, *ECM17*, *MET14*, *MET1*, *MET22*, *MET16*		
Ubiquitin-dependent protein catabolism*	*CDC26*, *PEX4*, *BUL1*, *RPN4*	Ubiquitin-dependent protein catabolism*	*CDC26*, *PEX4*, *UBI4*, *BUL1*, *RPN4*, *UBP6*, *RPL40A*, *DOA1*, *UBI4*, *BUL1*, *UBP2*, *DEF1*, *CUE1*

Note. *Significantly enriched GO Biological Process in at least one treatment ($p < 0.01$).
[a]Genes were identified in at least one of the six treatments. This list is not comprehensive.

MMA^{III} (Fig. 3C, Supplementary Table 4). The genes specific to MMA^{III} were mainly associated with transcription and GSH metabolism after 5g and 15g of growth, respectively; whereas the ones found only in As^{III} were associated to DNA double-strand break (DSB) repair after both 5g and 15g (Table 5).

Pathways of MMA^{III} and As^{III} Detoxification in Yeast

We identified genes encoding known components of As^{III} resistance in yeast. Among these, the ARR genes (*ARR1*, transcription factor for arsenic resistance; *ARR2*, arsenate reductase; *ARR3*, As^{III} transporter) were specifically essential for growth in the presence of As^{III}. The identification of *ARR2* for resistance was likely due to As^{V} formed by oxidation of As^{III}. *YCF1*, encoding a vacuolar glutathione S-conjugate transporter, was essential for growth in both arsenicals. On the other hand, deletion of the aquaglyceroporin gene *FPS1*, whose product transports As^{III} into the cell, resulted in resistance not only to As^{III} but also to MMA^{III} (Fig. 4). The identification of *FPS1* and *YCF1* suggests a common entry path of arsenic into yeast cells and detoxification mechanism to the vacuole between these arsenicals, respectively.

Tubulin Folding Implicated in Arsenical Toxicity

Two important groups of genes identified as essential for growth with MMA^{III} and/or As^{III} were associated with tubulin folding (*CIN2*, *CIN4*, *RBL2*) and biogenesis (*PFD1*, *GIM4*, *PAC10*, *YKE2*). The genes in the later group encode subunits of the heterohexameric GIM/prefoldin complex, a component of the unfolded protein response that also participates in actin and tubulin biogenesis. Another related gene, *TUB3*, encodes for α-tubulin and was also essential for growth in the presence of As^{III} in the screen and to MMA^{III} only after growth assay (data not shown). Unlike the other tubulin genes *TUB1* and *TUB2*, *TUB3* is not required for viability; however, these results suggest that it plays an important role in the presence of arsenicals.

DNA DSB Repair/Homologous Recombination Modulate Toxicity

Biological processes associated with DNA DSB repair were important for resistance to As^{III} (Table 5). Members of the *RAD52* epistasis group (*RAD27*, *RAD50*, *RAD54*) were required for growth in at least one treatment with MMA^{III} or As^{III}. The proteins encoded by these genes are involved in homologous recombination and in the repair of DSBs. Rad50p is a subunit of the *M*re11p-*R*ad50p-*X*rs2p (MRX) complex, associated with several DNA repair processes. Individual strain analysis confirmed that deletion of any of the three subunits of this complex resulted in growth sensitivity to MMA^{III} and As^{III} (Fig. 5), providing evidence of its involvement in the response against As toxicity.

Chromatin Remodeling/Epigenetic Modifications Important in Arsenical Response

We identified multiple subunits of multimeric complexes including SAS, SAGA, SWR1, and Itc1p-Isw2p, associated to chromatin remodeling processes as involved in response to both arsenicals. We evaluated the phenotypes of the selected

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TABLE 5
Biological Processes Enriched with Genes that were Unique to MMA^{III} and As^{III} Treatments after 5g and 15g of Exposure

	MMA^{III}	As^{III}
5g	Ribophagy	Transcription
	Response to drug	Regulation of transcription, DNA dependent
	Transcription	Chromatin modification
	Regulation of transcription, DNA dependent	Protein deubiquitination
		Negative regulation of transposition, RNA mediated
		Response to nutrient
		Protein targeting to vacuole during ubiquitin-dependent protein catabolic process via the MVB pathway
		DSB repair via nonhomologous end joining
15g	Sulfate assimilation	G1/S transition of mitotic cell cycle
	Response to drug	DSB repair via break-induced replication
	Regulation of transcription from RNA polymerase I	Meiotic DNA DSB processing
	Promoter translation	Energy reserve metabolic process
	Cysteine biosynthetic process	
	Methionine biosynthetic process	
	Negative regulation of transposition, RNA mediated	

Note. Funspec cutoff p values were adjusted to obtain only few of the most significant biological processes for each individual treatment: MMA^{III} 5g, $p < 0.01$; MMA^{III} 15g, $p < 0.001$; As^{III} 5g, $p < 0.01$; As^{III} 15g < 0.005.

mutants *htz1*Δ, *itc1*Δ, *sas2*Δ, *swr1*Δ, and *yaf9*Δ, containing deletions of genes encoding key (and associated) components of these complexes (Fig. 5). Htz1p is a histone variant, H2AZ, involved in transcriptional regulation. Itc1p is part of the Itc1p-Isw2p chromatin remodeling complex, required for gene repression. Sas2p is the catalytic subunit of the SAS complex, which has histone 4 lysine 16 acetyltransferase activity. Swr1p is the catalytic subunit of the SWR1 complex, which replaces H2A-H2B by Htz1p-H2B histone dimers at specific chromosomal locations. Yaf9p is present in both the NuA4 histone H4 acetyltransferase and SWR1 complexes and contains a YEATS domain often found in subunits of chromatin-modifying complexes and in the human leukemogenic protein AF9. In addition, we tested the phenotype of *gcn5*Δ. Gcn5p is the catalytic subunit of the SAGA complex, which acetylates N-terminal lysine residues in histones H3 and H2B. Except for *sas2*Δ that was only sensitive to As^{III}, all the mutants tested were sensitive to both arsenicals. MMA^{III} was about twofold more potent than As^{III} in inhibiting mutant growth, suggesting a similarity of these compounds in toxicity mechanisms but differences in potency.

GSH is Essential for Yeast Growth in the Presence of MMA^{III}

Deletion of *GSH1* negatively affected yeast growth upon exposure to MMA^{III} but not As^{III} (Fig. 6A). *GSH1* encodes γ-glutamylcysteine synthetase, an enzyme that catalyzes the first step in GSH biosynthesis; thus, *gsh1*Δ mutants are deficient in GSH. In individual growth assay, this strain exhibited a significant decrease in growth at doses as low as 5μM of MMA^{III}, being approximately 30-fold more sensitive than the wild type (Supplementary Fig. 3). Although MMA^{V} was formed during incubation with MMA^{III}, treatment with MMA^{V} alone did not inhibit growth of wild type at 1mM (IC_{20} > 1mM), or *gsh1*Δ at the same MMA^{III} concentrations (data not shown). Therefore, the observed effects on growth are likely to be due to MMA^{III}. On the other hand, *gsh1*Δ was slightly more sensitive than wild type to As^{III} only when grown at concentrations above the IC_{50} (600μM) (Supplementary Fig. 4), being approximately 60-fold more sensitive to MMA^{III} than to As^{III} (IC_{20}'s of 5 and 300μM, respectively).

To further determine if the lack of GSH in *gsh1*Δ was the cause of its sensitivity to MMA^{III}, we treated the wild-type strain with buthionine sulfoximine (BSO), an inhibitor of Gsh1p and thus, GSH biosynthesis. In support of an important role of GSH in the protection against MMA^{III}, depletion of GSH significantly increased the sensitivity of the wild type to MMA^{III} but did not affect its growth even at As^{III} concentrations that were eightfold higher (Fig. 6B).

Ycf1p transports As^{III}-GSH conjugates into the vacuole and was essential for fitness not only in As^{III} but also in MMA^{III}. The strain *ycf1*Δ was able to grow in the presence of MMA^{III} but became significantly sensitive after treatment with BSO (Fig. 6C). Therefore, the requirement of GSH is unlikely to be associated with this detoxification pathway.

Treatment of the wild-type strain with MMA^{III} for 8 h resulted in a significant increase in total GSH of more than threefold at the highest exposure concentration of 150μM (Table 6). This increase was in agreement with the observed requirement of the transcription factors Met28p and Met32p, responsible for the induction of genes involved in the uptake of the sulfur and biosynthesis of the methionine utilized in GSH biosynthesis (Fig. 7A).

GSSG levels were increased after 8 h of MMA^{III} treatment when measured using an enzymatic-based method (Supplementary Table 5), and were in agreement with the observed sensitivity of *glr1*Δ to MMA^{III} (Fig. 6A). *GLR1* encodes GSH reductase, which is involved in the regeneration of GSH from its oxidized form GSSG. Other genes identified in our screen were *RPE1* and *TKL1*, encoding proteins involved in NADPH regeneration and thus, necessary in the reaction catalyzed by Glr1p.

Requirement of DNA Repair Genes for As^{III} Resistance in Yeast Despite Absence of DNA DSB

Because of the existing evidence that arsenicals are genotoxic, we further examined the role of *RAD27*, *RAD50*,

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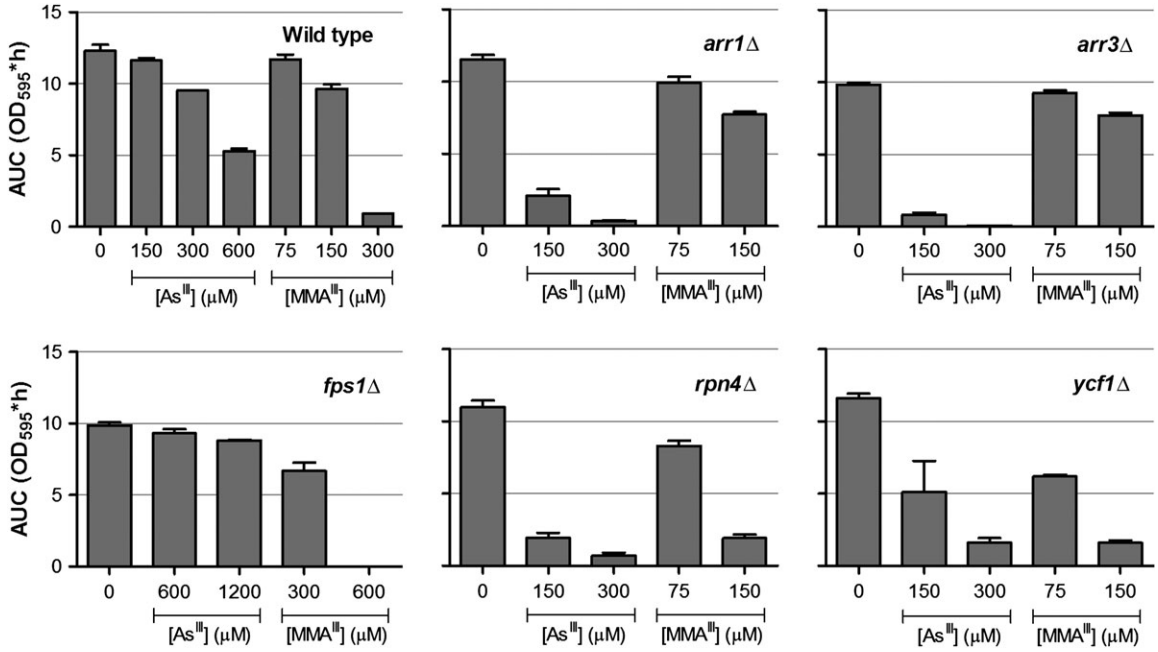

FIG. 4. Functional identification of known components of arsenic resistance in yeast. Growth assays were conducted for strains containing deletion in the identified genes associated with known As^{III}-resistance mechanisms. The AUC was calculated using growth data for 24 h of exposure to the IC_{20} and 50% IC_{20} of MMA^{III} or As^{III}. The bars represent the mean AUC of three technical replicates with standard error. Sensitivity was determined by comparing the AUC from the mutants to wild type, in the presence and absence of treatment. The detoxification pathways of MMA^{III} in yeast have not been studied in as much detail as those of As^{III}. The strains *rpn4*Δ and *ycf1*Δ displayed sensitivity to MMA^{III} and As^{III} but neither *arr1*Δ nor *arr3*Δ were affected by the former. The strain *fps1*Δ was resistant to both arsenicals.

and *XRS2* in the resistance to As^{III}. Deletion of any of these genes resulted in significant decrease in growth relative to the wild type, as shown by individual strain growth analysis (Fig. 5). Exposure to As^{III} concentrations as high as 1mM for 4 h did not show any apparent formation of DSBs in the wild-type or deletion strains after assessment with pulsed-field gel electrophoresis (Supplementary Fig. 5). In spite of the fact that 1mM of As^{III} inhibited wild-type growth by approximately 90% (Fig. 1), the absence of DSBs at this high concentration indicates that increased DNA damage is not the primary mechanism of As^{III} toxicity. The observed arsenical sensitivity of the RAD mutants could then be the result of other mechanisms of arsenic toxicity such as inhibition of DNA repair. Alternatively, the negative results could reflect a limitation of this method to detect low levels of this type of damage.

DISCUSSION

We identified the genetic requirements of yeast for resistance to MMA^{III} and As^{III} by using a genome-wide phenotypic analysis of yeast mutants containing deletions in nonessential genes. Functional profiling in yeast mutants has been previously reported for As^{III} (Haugen *et al.*, 2004; Jin *et al.*, 2008; Thorsen *et al.*, 2009; Vujcic *et al.*, 2007). Because MMA^{III} is more potent and formed in the human body after metabolism of As^{III}, the screening of As^{III} alone may not fully reflect the toxicity of As. Because of the poor overlap in identified gene sets between different As^{III} screens, possibly influenced by the different techniques used, we directly compared the genetic determinants for resistance to MMA^{III} and As^{III} in the same study. Comparison of the genes associated with the cellular response to these compounds could then provide insight into the adverse effects of As and identify conserved toxicity pathways in humans (Fig. 8).

Yeast cells are more resistant to arsenicals than human cells probably due to the presence of the As^{III} transporter, encoded by *ARR3*, not present in human cells, and/or the low transport rate of MMA^{III} into yeast cells (Liu *et al.*, 2006). In order to compare MMA^{III} and As^{III} treatments, we used concentrations that produced the same degree of growth inhibition in the BY4743 wild-type strain (20% inhibitory concentrations, IC_{20}, and 25 and 50% of this value). Further, we evaluated growth after 5g and 15g to account for the effect of the duration of exposure on the growth of certain strains and on overall fitness profiles. In some cases, the differences in fitness can be due to accumulation of cellular damage, such as DNA damage (Birrell *et al.*, 2002) or to the activation of general stress response pathways after 15g of growth. In addition, very small differences in growth between strains may not be detected after few generations but only after being amplified following many generations of growth.

Interestingly, our results showed that both arsenicals induced distinct but overlapping mutant sensitivity profiles, despite

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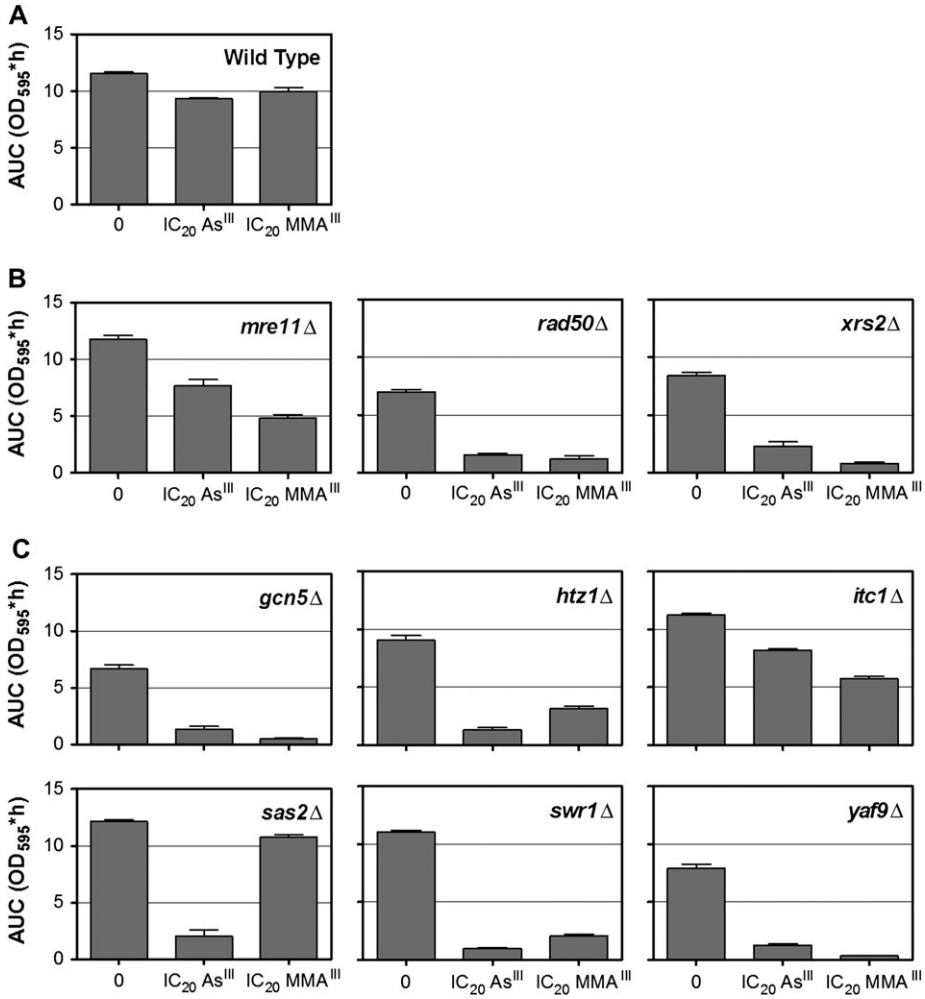

FIG. 5. Confirmation of novel As-resistance genes by growth curve analysis for selected yeast deletion mutants. The AUC was calculated for each strain after 24 h of exposure to the IC_{20} of MMA^{III} (150μM) or As^{III} (300μM). The bars represent mean AUC with standard error of at least three replicates. (A) Arsenical sensitivity was determined by comparison to the wild-type strain. (B) Genes encoding components of the MRX complex are essential for growth in the presence of As. (C) Genes associated with chromatin modifications are necessary for growth in As. Except for *sas2*Δ, the mutant strains tested exhibited sensitivity to both arsenicals.

yeast's inability to convert As^{III} to MMA^{III}. The absence of As^{III} metabolism to MMA^{III} in yeast allows dissecting their genetic requirements for resistance and studying their toxicity separately. However, those requirements arising from synergistic effects between them or from the metabolism of As^{III}, which may affect methyl donor or GSH pools, may not be identified in this organism. The genes found to be essential for fitness are associated with biological processes that are consistent with proposed mechanisms of arsenic toxicity. We identified tubulin metabolism, DNA repair, chromatin remodeling (epigenetic changes), and glutathione biosynthesis as key processes, as discussed below.

The requirement for multiple genes associated with tubulin assembly for growth in MMA^{III} and As^{III} can be related to the fact that As targets tubulin (Menzel *et al*., 1999; Zhang *et al*., 2007). Trivalent arsenicals have high affinity to cellular thiols due to the reactive pair of unshared electrons present in their outer shell. Among the many intracellular proteins that As binds, tubulin is probably one of the most common because of its abundance and high thiol content. In human cells, As blocks polymerization of tubulin by binding to vicinal cysteine residues and blocking the active site of GTP required for this process (Li and Broome, 1999; Zhang *et al*., 2007). The finding that genes involved in tubulin metabolism were also required in MMA^{III} was not unexpected, as MMA^{III} also binds to proteins (Styblo and Thomas, 1997) and is capable of inhibiting several enzymes more effectively than As^{III} (Lin *et al*., 2001; Petrick *et al*., 2001; Styblo *et al*., 1997). In yeast, deletion of *CIN2* and *CIN4* increased the rate of chromosome loss and sensitivity to the microtubule-disrupting fungicide benomyl (Hoyt *et al*., 1990; Stearns *et al*., 1990). These genes were also required in As, and provide further support that As targets microtubules and the cytoskeleton.

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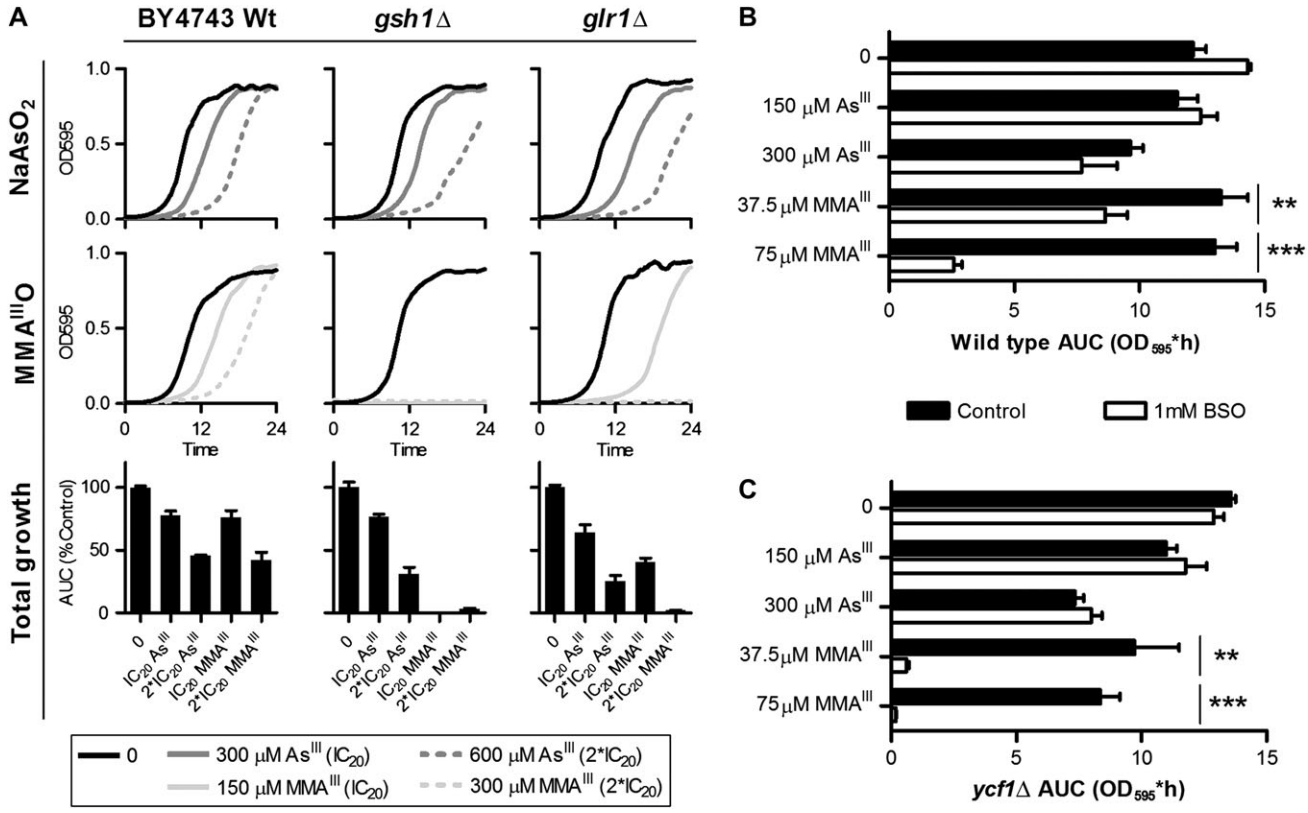

FIG. 6. Glutathione is required for optimal yeast growth in MMA^{III}. (A) The strains *gsh1*Δ and *glr1*Δ exhibited decreased growth in MMA^{III} relative to the wild type but not in As^{III}. Growth curves show average OD_{595} for 24 h of treatment with increasing concentrations of MMA^{III} and As^{III}, with error bars omitted for clarity. Bars represent the mean AUC with standard error expressed as percentage of controls. (B) Depletion of glutathione with BSO sensitized wild type to MMA^{III} but not to As^{III}. Strains were pre grown in SD media ± 1mM BSO and assayed for growth in As-containing SD media. BSO-pretreated cultures were grown with additional 1mM BSO, whereas nonpretreated cultures were grown without BSO. (C) Vacuolar sequestration of glutathione conjugates via Ycf1p is not the main pathway in the detoxification of MMA^{III}. The strain *ycf1*Δ is able to grow in MMA^{III} and becomes significantly sensitive after treatment with BSO.

Although the requirement of the MRX complex and some RAD genes in MMA^{III} and As^{III} suggested the induction of DNA damage, we did not observe any apparent increase in the formation of DSBs in several mutant strains after treatment with high doses of As^{III}. Arsenic has been shown to inhibit DNA repair enzymes and decrease DNA repair efficiency, possibly by disrupting the zinc fingers in these proteins (Piatek *et al.*, 2008; Takahashi *et al.*, 2000). Considering the occurrence of background levels of DSBs, the presence of As could disadvantage deletion strains that are already defective in DNA repair. Therefore, one explanation to our observations is that, by inhibiting DNA repair, As could stress DNA repair networks, resulting in sensitivity of certain RAD deletion mutants.

TABLE 6
Effects of Arsenical Treatments on GSH Levels in Wild-Type Strain

	20 min		8 h	
Treatment	Mean[a]	SD	Mean[a]	SD
Control	21.1	6.4	20.9	2.9
As^{III} 150μM	27.9	8.6	28.1	1.1
MMA^{III} 150μM	29.8	5.0	71.7	4.0

Note. GSH data represent mean of two biological replicates measured by LC-MS-MS. GSSG was not detected in these analyses. Enzymatic-based quantitation of GSH resulted in higher GSH levels than LC-MS-MS, although showing the same trend for MMA^{III} at 8 h (Supplementary Table 5).

[a]Expressed in picomoles per 10^6 yeast cells.

Epigenetic modifications are mediated by multimeric molecular complexes with highly conserved functions across species, including humans and yeast. Interestingly, several subunits of the yeast chromatin-modifying complexes SWR1, SAGA, SAS, and Itc1-Isw2, were essential for optimal growth in the presence of arsenicals (Supplementary Table 6), suggesting that their functions are necessary for the adaptation to As-induced stress. The histone Htz1p, which is incorporated into nucleosomes by SWR1, is expressed at higher levels after arsenic treatment (Dr W. Jo, unpublished data). The SWR1 complex genes and *HTZ1* are therefore needed for the induction of epigenetic changes associated with yeast's resistance to arsenicals. Because incorporation of Htz1p into chromatin is associated with rapid transcriptional activation under certain conditions (Zhang *et al.*, 2005), adaptation to

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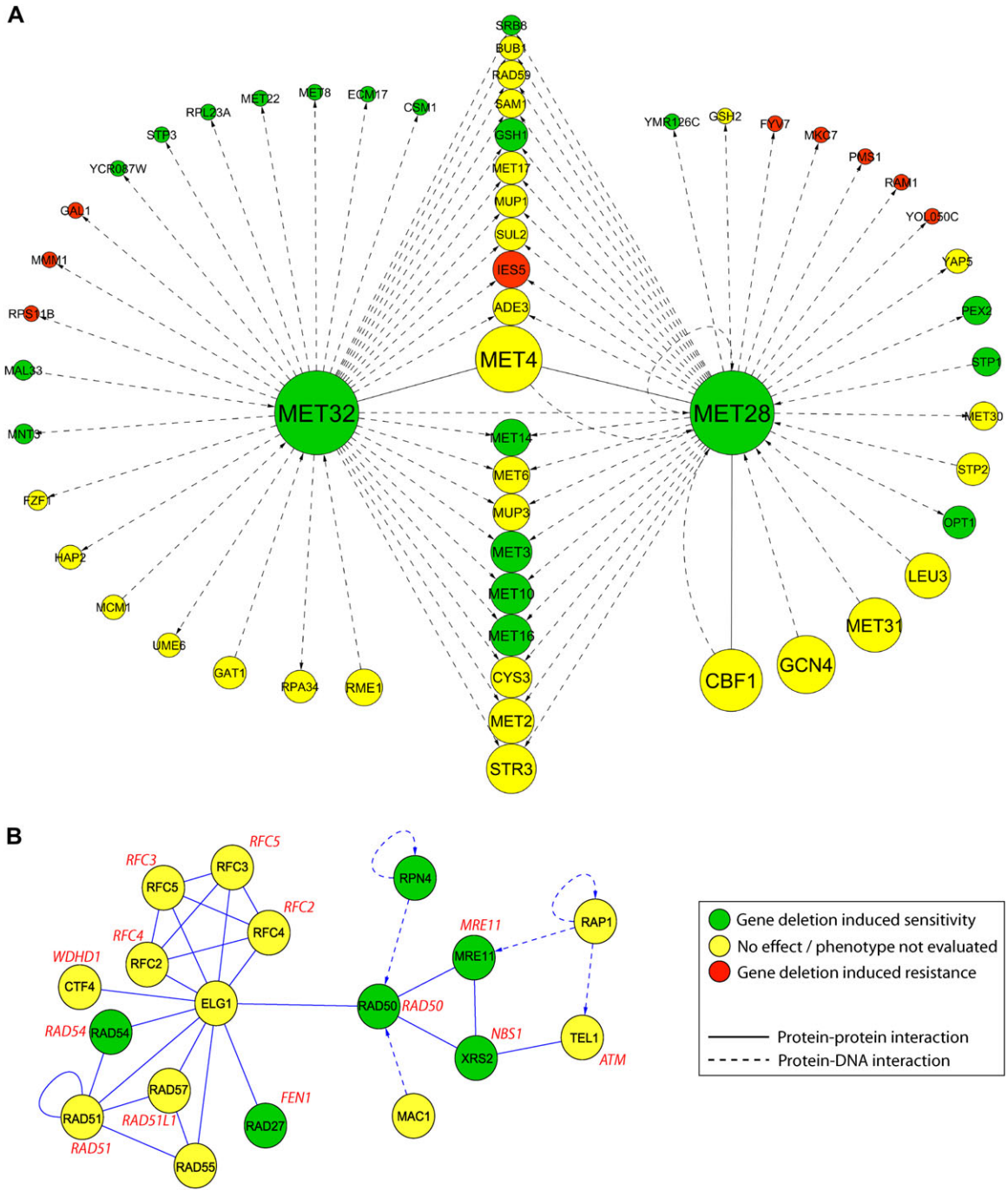

FIG. 7. Interaction networks of As sensitivity in yeast. (A) Requirement of genes from the sulfur metabolic pathway for growth in the presence of MMA^{III}. The transcription factor genes *MET28* and *MET32*, and several of their target genes involved in sulfur uptake and methionine metabolism are essential for growth after 15g of exposure to 150μM (IC_{20}) of MMA^{III}. The node size is directly proportional to its connectivity to other genes in the network, which were omitted for clarity purposes. Only the interactions between *MET32* and *MET28* with target genes are shown. From the genes shown, *YCR087W*, *CSM1*, *MAL33*, *MET32*, and *STP1* were also essential in As^{III}. (B) Network of As-responsive genes involved in DNA DSB repair. Genes identified as essential in at least one of the arsenical treatments are shown in green nodes. The homologous genes in humans (if any) are shown in red font next to the yeast gene. MRX interacts with the Atm-homolog Tel1p via Xrs2p, an interaction that is conserved in humans and is supportive of its important role in DNA repair.

arsenic may implicate differential expression of specific genes. In addition, these findings suggest that the other complexes mediate additional epigenetic changes upon arsenic exposure are also required for resistance.

At equitoxic low doses, the requirements for genes associated to GSH metabolism and related pathways differed between MMA^{III} and As^{III}. The increase in GSSG levels at high MMA^{III} concentrations, together with the requirement of

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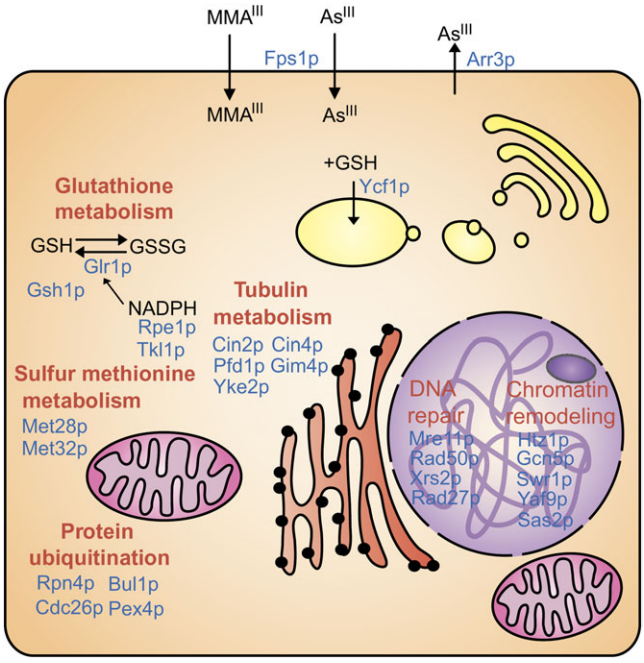

FIG. 8. Genes identified after MMA^{III} and As^{III} treatments in yeast by cellular localization. Genes essential for growth in these arsenicals are associated with GSH metabolism, tubulin metabolism, protein catabolism, DNA repair, and chromatin remodeling.

the GSH reductase gene, suggests that this arsenical promotes oxidation of GSH, which could be the result of its ability to inhibit GSH reductase *in vitro* (Styblo *et al.*, 1997) and/or produce reactive oxygen species (Liu *et al.*, 2001).

Several studies have reported the protective role of GSH against As^{III} (Han *et al.*, 2008; Ortiz *et al.*, 2009). Compared with previous yeast studies (Haugen *et al.*, 2004; Thorsen *et al.*, 2007, 2009), there was limited evidence in our data that As^{III} induces the GSH cellular response. Treatment with As^{III} slightly increased GSH levels but did not affect the growth phenotype of most mutants with a defect in GSH-related pathways. Based on the integrated phenotypic data and GSH measurements presented in this study, it can be concluded that the requirement of GSH in MMA^{III} is much higher than in As^{III}, suggesting that GSH is more important in the protection against MMA^{III}- than As^{III}-induced toxicity. Therefore, our results indicate that special attention must be given to MMA^{III} when assessing the effects of arsenic on GSH levels and susceptibility to arsenic toxicity. Because GSH is linked to cellular antioxidant status, oxidative stress may constitute an important component of MMA^{III} toxicity and may be responsible for its higher potency than As^{III}, at least in yeast.

The cellular responses against As^{III} at the concentrations tested demanded the function of genes primarily associated with tubulin metabolism, with minor involvement of those related to GSH metabolism. These findings are consistent with the hypothesis that reactive oxygen species are not involved in tubulin disruption and constitute another mechanism of arsenic toxicity (Kligerman and Tennant, 2007). Based on the relative sensitivity (at equitoxic doses) of deletion strains deficient in GSH metabolism and tubulin folding processes, oxidative stress appeared to be the primary mechanism of MMA^{III} toxicity and secondary to tubulin disruption in the case of As^{III}.

Because yeast and humans share in common many cellular metabolic pathways, a better understanding of the toxicity of arsenicals in yeast should help to understand their toxicity in humans and identify biomarkers of susceptibility. Several human orthologs to yeast genes associated with mitochondrial processes and involved in protection against arsenic-induced toxicity have been identified (Vujcic *et al.*, 2007). Similarly, several of the genes that we identified as essential in yeast's response to arsenic have at least one human homolog. These human genes could potentially modulate As toxicity in a similar way to their yeast counterparts and thus, influence susceptibility (Table 7). The MRX complex, from which we found its three subunits to be essential for yeast growth in arsenicals, is homologous to the mammalian Mre11-Rad50-Nbs1 (MRN)

TABLE 7
Yeast Genes Involved in Arsenic Resistance and their Orthologous Human Genes

Yeast gene[a]	Human gene homolog	Description for human protein
ELG1	*RFC1*	Subunit of the alternative replication factor C complex
GCN5	*PCAF*	Histone acetyltransferase P300/CBP-associated factor (PCAF). Catalytic subunit of the STAGA and SWR1 complexes
GLR1	*GSR*	Glutathione reductase
GSH1	*GCLC*	Catalytic subunit of glutamate-cysteine ligase
HTZ1	*H2AFV*	Histone variant H2AV (H2A.F/Z)
MRE11	*MRE11*	Subunit of MRN complex involved in DNA repair
RAD50	*RAD50*	DNA repair protein, subunit of the MRN complex
RAD57	*RAD51L1*	*RAD51*-like protein 1, DNA repair
SAS2	*MYST1*	H4K16 acetylation
SWR1	*SRCAP*	Snf2-related CBP activator protein. Catalytic subunit of the SWR1 complex that replaces H2A with the histone variant H2AZ at promoters
TUB3	*TUBA1A*	Tubulin alpha-1A chain
XRS2	*NBS1*	Nijmegen breakage syndrome gene, subunit of the MRN complex involved in DNA repair, activation of ATM
YAF9	*YEATS4*	Component of the NuA4 histone H4 acetyltransferase and SWR1 complexes and has homology to the human leukemogenic protein AF9
YKU70	*XRCC6*	70-kDa subunit of Ku antigen (KU70)

[a]Deletions of any of these genes increased the sensitivity of the mutant strain to MMA^{III} and/or As^{III} (listed in alphabetical order).

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complex. Among the components of the MRN complex, *RAD50* was found to be upregulated and Mre11 phosphorylated after treatment with As^{III} (Perez *et al*., 2008; Yuan *et al*., 2002), suggesting that this complex is involved in the response to As^{III} in human cells. Moreover, the E185Q polymorphism in *NBS1* has been associated with increased risk of basal cell carcinoma in men exposed to As (Thirumaran *et al*., 2006). Therefore, the functional conservation between the genes that we identified in this study and their human homologs raises the question whether or not the later could be necessary for As resistance in humans. Our findings in this study represent a starting point for more focused, confirmatory gene- or pathway-specific studies.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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