

Functional genomics analysis of n-alkyl sulfates toxicity in the yeast *Saccharomyces cerevisiae*

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Abstract

The n-alkyl sulfates (AS) are a class of anionic surfactants that are widely used in industry and in consumer products. In this study, the effects of AS on yeast growth and genome wide transcriptional profiles were analysed by DNA microarray technology. Induced genes were categorized by localization of gene products and by function according to accepted gene ontologies using the MIPS database. A number of genes whose products localized to the cell wall and peroxisome were significantly induced. Genes involved in energy metabolism (i.e., fatty acid β -oxidation pathway) were also significantly induced. To confirm the role of these functions, the sensitivity of selected single gene deletion strains to sodium dodecyl sulfate (SDS) was tested. Deletion strains of cell wall maintenance genes (Δ *GAS1*, Δ *KRE6*, and Δ *CHS5*) were found to be highly sensitive. Interestingly, mutants deleted for genes in the fatty acid β -oxidation pathway were not found to be sensitive. However, regulating genes in the fatty acid β -oxidation pathway were found to respond to SDS exposure in a dose-dependent manner and to be involved in H₂O₂ production. Here, we report a functional genomics analysis of genome-wide expression data to screen and evaluate AS toxicity in yeast. While the approach begins with a determination of highly-induced genes, its power lies in then determining the most relevant functions targeted by AS, and then assessing loss of key genes by evaluating AS sensitivity in the corresponding deletion mutants.

Key Words: Functional genomics, DNA microarray, Toxicity, n-alkyl sulfates, Yeast

Area of Interest: Genome Wide Experimental Data Analyses

1. Introduction

Toxicogenomics is the evolving science which measures global gene expression changes in biological samples exposed to toxic agents and investigates the complex interactions between genetic variability and environmental exposure on toxicological effects (1). DNA microarrays have become the most widely used and important method for measuring mRNA levels in toxicology studies (2). In toxicogenomics, analysis focuses on identification of toxicant-induced genes. While this approach is generally accepted, a bias towards the most highly induced genes generally exists, even though the most highly induced genes do not necessarily represent the most important with respect to response to a toxicological agent. Further, not all functional information for every gene is necessarily correct or complete or categorized properly (i.e., multifunctional genes) and therefore, the effects of a toxicant can be easily misinterpreted. It should be noted that functional analysis by yeast DNA microarray become a good screening tool, however this is not enough to convince and pointed out the important functions of selected genes. Thus, not only microarray technology can be an evaluating tool but also RT-PCR technique and deletion strain assay need to be incorporate with this method to support the toxicity assessment process. For example, on the biological effect by surfactant study (Kurita et al.)(3), which successful to explain the toxicity from transcriptional data but lack of the comparative experiment to confirm the important functions of the selected genes.

To avoid these biases, we suggest analyzing toxicogenomics data based on gene function with subsequent follow-up analysis of toxicant exposure of mutants deleted for key genes. As a first step, induced genes are categorized by function and localization of gene products using the gene ontologies assigned by the Munich International Centre for Protein Sequences (MIPS, <http://mips.gsf.de/genre/proj/yeast/index.jsp>). Second, specific functions presumed to be important for response to the toxicant within the induced categories are analyzed and selected. Third, the roles of these selected functions are then evaluated by assessing the sensitivity of key single gene deletion mutants.

To test this approach, n-alkyl sulfates (AS) (4), a widely used class of anionic surfactants were chosen as model toxicants due to their significant use in household cleaning products, personal care products, pesticide formulations, and in industrial processes (5). Understand of their basic unit and its effect such as short chain homologous series of AS which would provide fundamental information for study of their environment behavior. Specifically, we evaluated the toxic effects of a homologous series of AS species using a functional genomics approach in the well known model eukaryote, the yeast *Saccharomyces cerevisiae*.

2. Materials and Methods

2.1 Chemicals, strain, and growth conditions

Saccharomyces cerevisiae strain S288C (*Mat alpha SUC2 mal mel gal2 CUP1*) was grown in YPD medium (2% polypeptone, 1% yeast extract, 2% glucose) at 25°C. The homologous series of AS species: sodium n-dodecyl sulfate (SDS), sodium n-octyl sulfate (C8), sodium n-hexyl sulfate (C6), and sodium n-butyl sulfate (C4) were purchased from Wako Pure Chemical Industries, Ltd., Japan., Research Chemicals Ltd., England., Kanto Chemical Co., Inc., Japan.,

and Sigma-Aldrich Chemical Co., USA., respectively. Yeast growth was monitored following inoculation of a pre-culture into YPD supplemented with AS at different concentrations in 96-well plates at 25°C in a Biomek 2000 Laboratory Automation Workstation (Beckman Coulter, Inc., Fullerton, CA). A_{660} values were measured automatically every 2 h to obtain of growth curves for each treatment (6).

Treatments involved exposing exponentially-growing cultures ($A_{660} = 1.0$) to a selected concentration (IC_{50}) (7,8) of AS for 2 hr. Growth and survival were measured at A_{660} and by counting colony forming units (CFU) on YPD plates, respectively. After incubation for 2 hour, cells were harvested by centrifugation and pellets were washed three times with DEPC-treated H_2O before processing for RNA extraction. Under the screening proposal, a single experiment of yeast DNA microarray chip was performed for individual treatment.

Plate spot assays for growth (phenotypic assay) (9), were performed by suspending an 18-hr culture in H_2O , diluting in 10-fold steps, and plating 5 μ l of each dilution onto YPD agar plates containing AS.

2.2 DNA microarray procedure and analysis

Total RNA was extracted from 2 ml of yeast cells by using the RNeasy kit (QIAGEN, USA) following the manufacturer's instructions. Briefly, yeast cells were lysed enzymatically under isotonic conditions using lyticase (Sigma, USA). Spheroplasts were generated after incubation for 20 min at 30°C. The spheroplasts were collected following low speed centrifugation by careful removal of the supernatant. The spheroplasts were resuspended, mixed thoroughly with 70% ethanol, and then applied to an RNeasy mini column. Total RNA was then eluted in 30 μ l of RNase-free water and keep in -80°C. Total RNA was first converted to double-stranded cDNA, followed by synthesis of biotin-labeled cRNA using in vitro transcription as described (10,11) cRNA was then purified and fragmented. Hybridizations to chips (GeneFrontier Co., Tokyo, Japan) containing 24-mer oligonucleotide yeast probes were performed using single cRNAs derived from one biosource (one color hybridization). Hybridizations, washes, and scans were performed using standard protocols (NimbleGen System, Inc). Signals between each array were normalized using Robust Multiship Analysis normalization (12,13,14). Normalized data ratios >2-fold or <0.5-fold between treatment and control (control cells without chemicals treatment) were considered up- or down-regulated, respectively. In this study, the selection was carried out on the bases of biological experiments reported by Mizukami et al.(15). However, we did not use any statistical filter.

2.3 RT-PCR

Microarray-based expression data were confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR) (16,17) of selected genes using the StrataScript First-Strand Synthesis System (Stratagene, CA) for preparing a cDNA libraries solution following the PCR step. The PCR was performed using the TaKaRa Ex Taq HS enzyme (TaKaRa, Shiga, Japan) and a 10-fold dilution of the cDNA libraries solution. PCR conditions were: 95°C for 3 min, 25 cycle of 98°C 10 sec, 55°C 30 sec, and 72°C 1 min, and a final 72°C for 10 min. Gene names (systematic or common names), forward primer sequences, and reverse primer sequences are listed in table 1. The RT-PCR experiment was performed more than three times for individual gene checking to achieve and convince the reproducibility of selected genes and its expressions.

Table 1. Primers used in this study.

Gene	Forward primer	Reverse primer
ACT1	5'-ATTGCCGAAAGAATGCAAAAGG-3'	5'-CGCACAAAAGCAGAGATTAGAAACA-3'
GSC2	5'-GGATCTGGGTTGACGGGTACTT-3'	5'-TATAGCGAGAGGACCGAGCTTC-3'
SCW10	5'-AGGTTACTGTTGTCGTTTCCG-3'	5'-TGAGGATGAAGTCGAAGAACCA-3'
PIR3	5'-TGGTGCTATCTATGCTGCTGGT-3'	5'-TATGTGTGGGGAATGAGAGTGG-3'
CRH1	5'-TACGCCGGATACAGCTTTGG-3'	5'-AACAATACCGGTACCATTGGCA-3'
PHO5	5'-CCTGGCGACTATGGTATTTCTC-3'	5'-CCATTTCCAAATCATCGTCATCAC-3'
CHS5	5'-GTAAGTTGGATGCCTCATTGGC-3'	5'-CTGGGCTCTCCGGTTTATGG-3'
GAS1	5'-TCTACTCCAACAACGGTAGTCAG-3'	5'-TCAAAGCCTTCATACATTCGGAG-3'
KRE6	5'-ATGCTGGTCTCACAACCCCT-3'	5'-AGTTATCATTAGCCGTACTCACAG-3'
FAA2	5'-ACCACTGACCAGCTATCCCA-3'	5'-AATTGGCCTTGAACCTAAACAGTC-3'
POX1	5'-CTCCTATAGGTTACTTTGATGGCGATA-3'	5'-AAAGTCGCAAAACAGAGGGTTC-3'
FOX2	5'-GGTCAAGCTAATTATTCAGCAGCT-3'	5'-GACACTTTCGTACTTTCGTGTG-3'
POT1	5'-CAATCCCTTGGGCATGATCTC-3'	5'-ACTGGCAAATTGAGCCATCTG-3'
SPS19	5'-AAGACCGTAGAGAAGTTCGGT-3'	5'-AATAATGGAAAGTGGCAGAGACG-3'
ECI1	5'-TTCGGAACAAGCAAGTGGG-3'	5'-GCAAAGAGACCGTTGTACCAC-3'
GRE2	5'-ATTCTCCATGGAAGTTGTCCCAG-3'	5'-CTACACGTTCTACAGAATCAGCGG-3'
GPX1	5'-TCATTGTGCGTTCACACCTC-3'	5'-AACTTGTAGACGGGATCTTGC-3'
CTT1	5'-CATGTTTGGTGATAGAGGTACTCC-3'	5'-AATTGAGTGAACAGCTTTGCCTG-3'
GTT1	5'-TTCACCCATTAGGAAGATCTCCA-3'	5'-CAATCATTAAGGTGGTTGCAAGG-3'
GTT2	5'-TCTCAATGGCTGACATCAGATA-3'	5'-CCTCTGAATAGCTTCATGATTGG-3'
GSH1	5'-GTGAATTGCCAACAACAGCAA-3'	5'-TGTCAACTTTCTTTCACAACCGAAG-3'
GSH2	5'-CGAGCAAGTTTATCGAACGAA-3'	5'-AAGGGTGAAAGAAAGAAGGAATGAG-3'

3. Results and Discussion

3.1 Effects of n-alkyl sulfates on growth and genome wide transcription in yeast

As a first step, we evaluated the effect of AS treatments on yeast growth (figure 1). IC₅₀ concentrations (50% decrease in specific growth rate) for each chemical were 100 ppm SDS, 500 ppm C8, 47,000 ppm C6, and 15000 ppm C4, respectively. With reference to our previous report concerning anionic detergents (8), we isolated RNA in the present study from cells treated with IC₅₀ doses of the AS species. While doses that have no observable effect on growth can be useful for assessing tolerance, such doses will not be useful toxicological indicators. On the other hand, it is not possible to isolate sufficient mRNA from cells treated with lethal doses (15).

Transcription of a total of 402 (6.34%), 99 (1.56%), 104 (1.64%) and 775 (12.22%) ORFs was altered relative to untreated control cells for SDS (C12), octyl sulfate (C8), hexyl sulfate (C6), and butyl sulfate (C4), respectively. One hundred and eighty three (2.88%), 70 (1.10%), 81 (1.27%), and 348 (5.49%) ORFs were up-regulated and 219 (3.45%), 29 (0.45%), 23 (0.36%), and 427 (6.73%) ORFs were down-regulated for SDS (C12), octyl sulfate (C8), hexyl sulfate (C6), and butyl sulfate (C4), respectively.

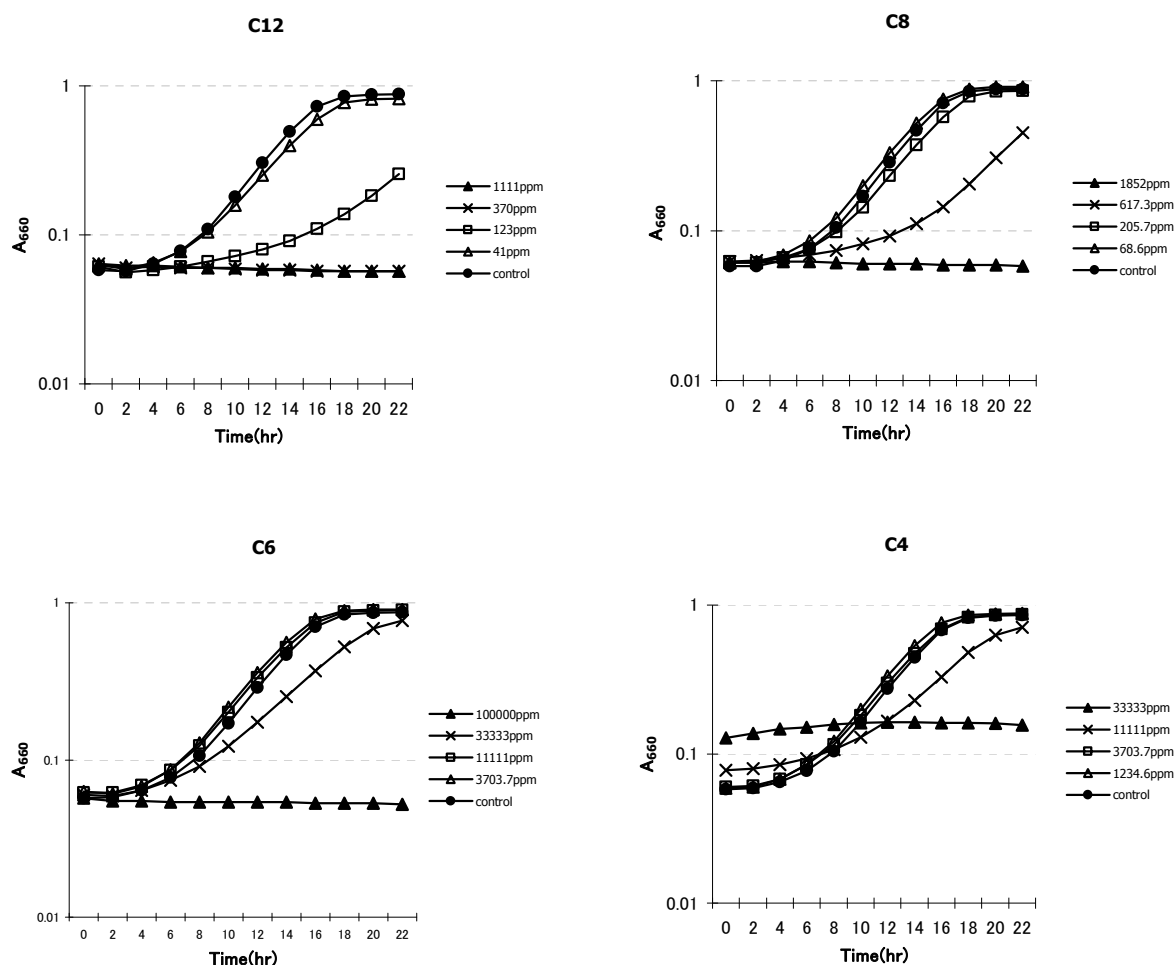


Figure 1. Growth profiles of yeast treated with different n-alkyl sulfates at the indicated concentrations.

3.2 Functional genomics deduced by clustering of genome wide transcription

By taking into account the average percentage of significant number of selected genes within annotated categories for all treatments, the expression data were classified by localization of gene products and by functional categories (Figure 2). The clustering profiles of the up- and down-regulated genes reveal the transcriptional response of yeast to AS treatments. The up-regulated genes comprise the following functional categories: “energy”, “cell rescue, defense and virulence”, and “C-compound and carbohydrate metabolism”. With respect to localization, genes which localize to the “cell wall”, “peroxisome”, and “extracellular” categories were found among the up-regulated genes. On the other hand, the functional categories associated with the down-regulated genes included “development (systemic)”, “protein synthesis”, and “transcription”. Genes that localized to “microsomes”, “extracellular”, and “cell wall” were found to be down-regulated. These results suggest that cells may overcome stress induced by exposure to AS by initiating a program of adaptive transcriptional responses, as described for the environmental stress response (ESR) whose goal appears to be to minimize cell damage to a variety of stresses (18). Based on the categories of down-regulated genes, development

(systemic), protein synthesis, and transcription, it appears that the yeast is slowing or halting growth, presumably to minimize cell damage and to conserve mass and energy until conditions are again favorable due to successful adaptation to the toxic environment (19).

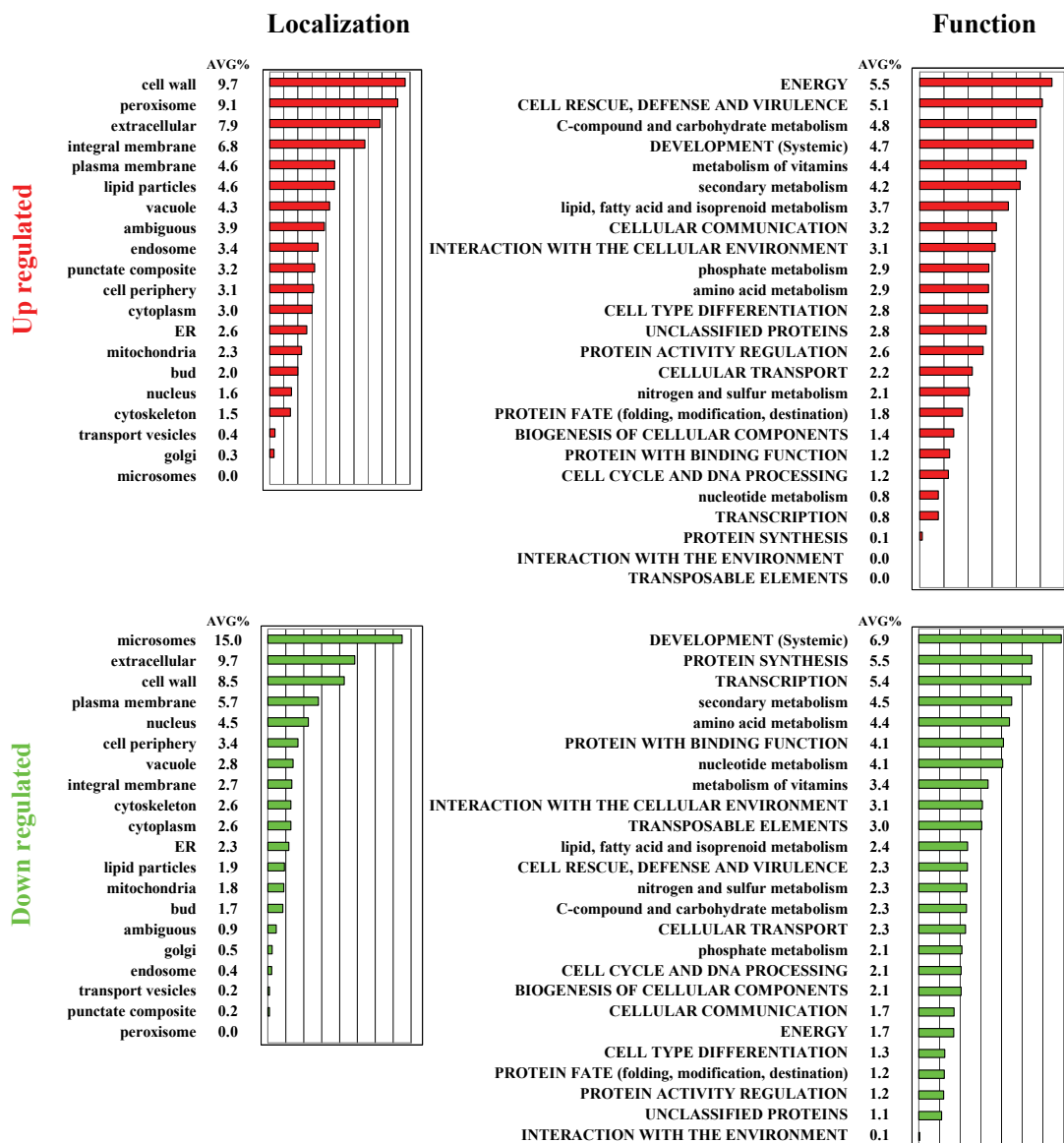


Figure 2. Up regulated (red) and down regulated (green) ORFs in response to treatment with alkyl sulfates. ORFs are classified into MIPS localization and functional categories.

3.3 AS induces genes involved in cell wall maintenance, oxidation of fatty acids and oxidative stress

To gain greater understanding of the changes in expression profiles, up- and down-regulated genes were grouped into sub-categories based on both function and localization of gene products (table 2). Table 2 lists 26 genes that are highly up-regulated and that localize to the “cell wall” and “biogenesis of cell wall”. Similarly, nine significantly up-regulated genes localized to the peroxisome membrane and peroxisome matrix. On the other hand, genes within the functional categories of “oxidation of fatty acids” (6 genes), “pentose phosphate pathway” (4 genes), “sugar, glucoside, polyol and carboxylate metabolism” (18 genes), “stress response” (53 genes), and “detoxification” (20 genes) were significantly up-regulated. Accordingly, up-regulated genes within these sub-categories were examined further. RT-PCR was used to confirm the microarray-based expression results and sensitivity phenotypes of key deletion mutants were assessed.

3.4 Genes associated with cell wall maintenance play an important role in cell survival

Yeast is surrounded by a cell wall that is a complex structure essential for maintenance of cell shape, prevention of lysis, and protection against certain harmful environmental conditions (20). A group of genes found to be induced by AS treatment are associated with the cell wall and with biogenesis of the cell wall, as show in figure 2 and table 2. For example, *PIR3* (Member of the Pir1p/Pir2p/Pir3p family), *CRH1* (Family of putative glycosidases) including a phosphatase precursor gene, *PHO5*, were significantly induced. Simultaneously, genes associated with biogenesis of the cell wall were also found to be highly up-regulated, i.e., *GSC2* (catalytic subunit of 1, 3-beta-glucan synthase) and *SCW10* (cell wall protein with similarity to glucanases). RT-PCR was performed on these cell wall-related genes (*PIR3*, *CRH1*, *PHO5*, *GSC2* and *SCW10*) using the housekeeping gene *ACT1* (17, 21, 22) as a control. The RT-PCR data (figure 3A) correlated with the microarray results. To further explore the coincident induction of cell wall-related genes and genes whose products localize to the cell wall, RT-PCR was performed on other genes known to function in cell wall biosynthesis but that were not found to be induced based on the microarray data. RT-PCR analysis of three such genes (figure 3A), *CHS5* (involved in chitin biosynthesis), *GAS1* (beta-1,3-gluconosyltransferase) and *KRE6* (protein required for beta-1,6 glucan biosynthesis) showed induction by AS. A phenotypic analysis of the corresponding deletion mutants was then undertaken. Mutants deleted for *CHS5*, *GAS1* and *KRE6* were all found to be highly sensitive to SDS (figure 3B). The observation that these three genes were co-induced by SDS suggests a possible relationship between cell wall biogenesis and carbon metabolism (figure 3C) and also suggests that the yeast cell wall may be a target for the toxicity of SDS.

The results of cell wall damage by alkyl sulfate give a significant awareness to the discharge of any kind of chemicals into an environment. Due to this kind of mechanism possible to introduce and make a synergistic effect with another chemical to penetrate more easily to intracellular of the living organism.

Table 2. Localization and functional categories of genes induced >2-fold by exposure to AS. Genes marked in red and black are annotated and uncharacterized, respectively. Genes are classified into sub-categories of localization and function are listed left to right in descending order of induction level. ND, No significant data.

Localization											
CELL WALL											
Subcategories	Gene name										
Cell wall bound protein	PIR3	CRH1	YJL171c	PHO5	DSE4	SPS100	FLO1	SCW10			
Biogenesis of cell wall	PIR3	CSR2	GSC2	ECM4	ROM1	DFG5	SPH1	MYO3	KTR2	SLT2	
	ECM13	ECM38	ECM7	MTL1	STF2	LAG2	FOX2	CAP2	YDL222c		
PEROXISOME											
Subcategories	Gene name										
Peroxisomal matrix	POX1	SPS19	FAT2	CIT2	FAA2	POT1	MLS1	FOX2			
Peroxisomal membrane	FAT2										
Function											
ENERGY											
Subcategories	Gene name										
Oxidation of fatty acids	POX1	SPS19	ECI1	POT1	TES1	FOX2					
Pentose-phosphate pathway	YGR043c	GND2	TKL2	ZWF1							
Fermentation	FDH1	YPL088w	ALD3	CYB2	YAL061w	AAD10	YFL057c	PDC5			
Energy conversion and regeneration	OYE3	MYO3	STF1	STF2	YEL020c						
Respiration	YKR046c	CYB2	YTP1	STF1	STF2	MBR1					
Glycolysis and gluconeogenesis	FBP26	GRE2	FYV10	MDH2	ZWF1	PGM2	PFK26	YMR323w	ERR2	ERR1	
Glyoxylate cycle	CIT2	YFL030w	MDH2	MLS1							
Electron transport and membrane-associated energy conservation	CYB2	STF1	SDH4	STF2							
Metabolism of energy reserves (e.g. glycogen, trehalose)	GSC2	PGM2	ATH1	TSL1	YGR287c	GDB1	GPH1				
Tricarboxylic-acid pathway (citrate cycle, Krebs cycle, TCA cycle)	CIT2	YMR118c	IDP3	SDH4	GDH1	CIT1	YLR164w				
Pyruvate dehydrogenase complex	ND [†]										
METABOLISM (C-compound metabolism)											
Subcategories	Gene name										
Sugar, glucoside, polyol and carboxylate metabolism	YGR043c	AMS1	FBP26	TKL2	DSE4	SCW10	ZWF1	PGM2	PFK26	ATH1	
	INO1	YJR096w	GRE3	TSL1	YGR287c	YPR1	CIT1	YOL155c			
Polysaccharide metabolism	GSC2	CHS1	SCW10	GDB1	YOL155c	GPH1					
Regulation of C-compound and carbohydrate metabolism	FBP26	YFL052w	MIG2	ROM1	FUN34	PFK26	NRG1	IMP2			
C-1 compound metabolism	FDH1										
C-2 compound and organic acid metabolism	SYM1										
Aminosaccharide metabolism	PGM2										
Aromate metabolism	ND										
Transfer of activated C-1 groups	ND										
CELL RESCUE, DEFENSE AND VIRULENCE											
Subcategories	Gene name										
Stress response	PIR3	YKL161c	DAK2	YDR533c	ALD3	PAI3	GRE2	CTT1	YJL144w	PRB1	
	HSP26	SIP18	GTT2	DDR2	HSP12	GCV1	PHO5	YRO2	YKR049c	YOR391c	
	YPL280w	GPX1	MYO3	ZWF1	HAL1	ATH1	ATX1	GRE3	SLT2	COS8	
	ORM2	UGA2	PTP2	NRG1	TSL1	YGP1	GTT1	YMR322c	HOR2	YCL033c	
	DDR48	HSP104	SNZ2	STF2	SSE2	CAP2	OXR1	GRE1	YAL028w	PPZ1	
	DAK1	SPL2	HSP78								
Detoxification	ARN1	GRE2	CTT1	ARN2	TPO4	GTT2	BOP1	GPX1	AZR1	HMX1	
	ZWF1	ATX1	SNG1	GTT1	CRS5	YFR022w	PDR5	QDR1	SIT1	ENB1	
Disease, virulence and defense	YOR1	CUP2	SNG1	FET3	PDR5	PDR16					
Degradation / modification of foreign (exogenous) compounds	ND										

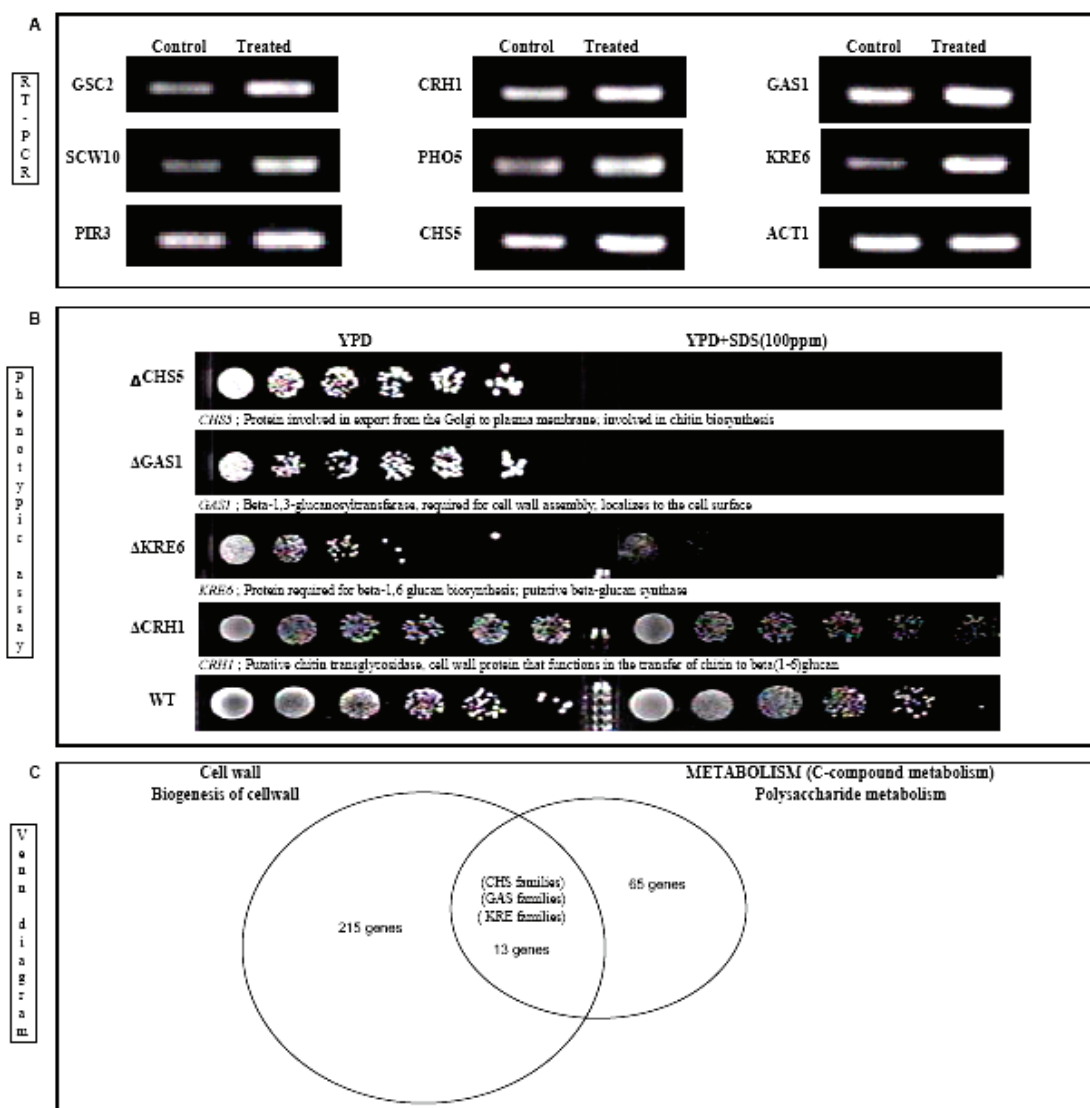


Figure 3. Analysis of cell wall-related genes. (A-B) Comparative RT-PCR and phenotypic assays are compared between control and SDS treatments. (C) The number of genes implicated in cell wall and metabolism of carbon compounds is shown in the Venn diagram.

3.5 Fatty acid β -oxidation pathway is involved in AS assimilation

Genes in the energy category were found to be highly up-regulated in response to AS treatment (figure 2). Among 11 sub-categories (table 2) within the category of energy, 67% of the genes involved in the fatty acid β -oxidation pathway were significantly up-regulated. Generally, because the energy yield of lipids is higher than for proteins or carbohydrates on a mass basis, the fatty acid β -oxidation pathway is one of the main sources of cellular energy. Due to the resemblance of AS to fatty acids, yeast may metabolize AS via the fatty acid β -oxidation pathway in the peroxisome. Indeed, others (23, 24) have reported that metabolism of n-alkyl sulfates-based surfactants such as LAS (linear alkyl benzene sulfonates) begins via oxidation of the terminal methyl group of the alkyl chain (ω - and β oxidation) (25).

In the present study, RT-PCR was used as a comparative method to confirm the microarray-based expression data. Interestingly, in the presence of SDS, every gene known to be involved in the fatty acid β -oxidation pathway was found to be up-regulated (figure 4A). This observation supports the notion that this pathway plays an important role under conditions of AS-induced stress. Moreover, *POX1*, which encodes the first and rate-limiting step in β -oxidation (26), exhibited a dose-dependent response to AS treatment (data not shown). However, deletion mutants lacking genes in this pathway were not found to be sensitive to SDS added to agar plates (data not shown). While it has been demonstrated that a *POX1* deletion mutant is unable to grow on fatty acids as sole carbon sources (27), our phenotypic analysis did not involve growth on AS as the sole carbon source. It is possible that the fatty acid β -oxidation genes do not constitute an efficient means of detoxifying AS species, but rather primarily play a role in energy production. Unlike in many other organisms, the fatty acid β -oxidation pathway in *S. cerevisiae* is restricted to the peroxisome, where short fatty acids are preferentially metabolized (28). The observation that this pathway is operative in a single organelle in *S. cerevisiae* makes yeast an attractive model organism for studying the degradation of fatty acids (28). It is of interest that in the present study, genes whose products localize to the peroxisome (figure 2) were found to be preferentially up-regulated. Taken together, our observations concerning fatty acid β -oxidation, the peroxisome and energy production suggest that AS species may be metabolized as sources of energy, and that AS induction of fatty acid β -oxidation occurs primarily for this purpose, not as a means of detoxification.

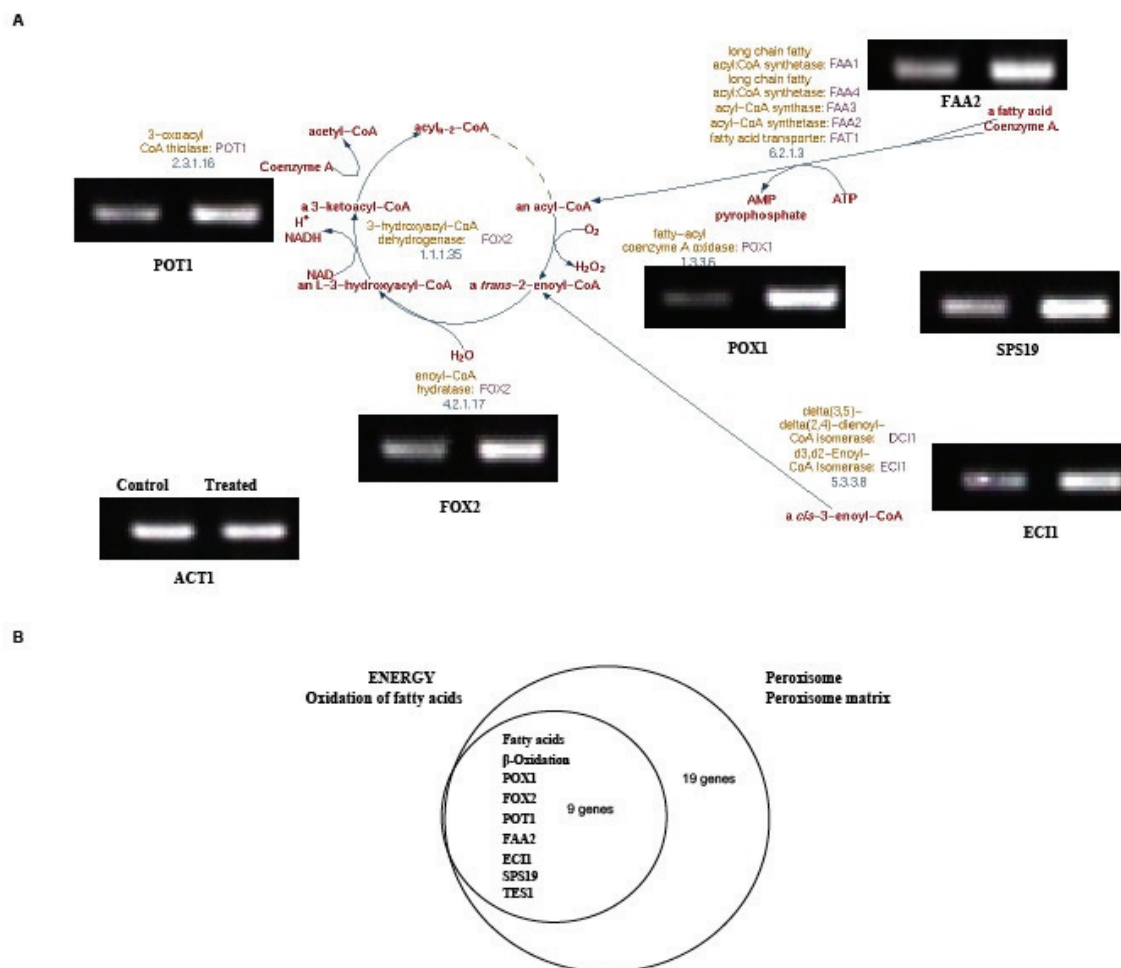


Figure 4. Fatty acid β -oxidation pathway. (A.) Expression intensity of genes in the fatty acid β -oxidation pathway after AS treatments are presented accompanied by a schematic of the pathway (Saccharomyces Genome Database) (B.) Genes in the fatty acid β -oxidation pathway and genes which localize to the peroxisome are shown in the Venn diagram.

3.6 Induction of genes associated with oxidative stress and hydrogen peroxide

The gene ontology category of cell rescue, defense and virulence is one of the most important with respect to functions required of cells under stress. Many detoxification processes can be predicted based on expression of genes in this category. Within sub-categories of stress response, 13 genes related to oxidative stress, and 10 related to osmotic and salt stress response were found to be highly induced (figure 5A). Further, within the sub-categories of detoxification, 4 genes that function in oxygen and radical detoxification were found to be up-regulated during treatment. Interestingly, within the sub-categories of oxidative stress response, a group of genes involved in oxidative stress related to H_2O_2 were found to be highly responsive. Oxidative stress refers to stress induced by various kinds of reactive oxygen species (ROS) such as hydroxyl radical (OH^\cdot), superoxide anion (O_2^\cdot) and hydrogen peroxide (H_2O_2) (29). Oxidative stress arises when the balance and metabolism of these ROS are perturbed (27). Thus, we measured induction of oxidative stress response genes including *GRE2*, *CTT1*, *GTT1*, *GTT2*, *GPXI*, *GSH1*, and *GSH2* by RT-PCR (figure 5B). The RT-PCR measurements were found to confirm the results of the microarray analysis that AS treatment induced expression of the above genes. One up-regulated gene, *GPXI*, whose function relates to relief of oxidative stress caused by H_2O_2 , detoxifies phospholipid hydroperoxides (30). Interestingly, a *GPXI* deletion mutant exhibited an SDS-sensitive phenotype as well (figure 5C).

A role for H_2O_2 -related stress during AS exposure may relate to the observation that β -oxidation of fatty acids produces H_2O_2 . While it is true that this may be one effect among many, the coincident induction and sensitivity results suggest that genes involved in H_2O_2 -induced oxidative stress may play a role in recovery from AS-induced stress.

3.7 Structure of n-alkyl sulfates and their toxicity

While many different mechanisms of toxicity exist for different types of surfactants, surfactants may also cause toxicity through multiple mechanisms (31). Recently, Garcia et.al reported that for LAS, a widely used alkyl sulfate compound, a highly significant relationship between toxicity and alkyl chain length was evident with toxicity increasing with increasing alkyl chain length (32). Based on similar gene expression profiles observed for induction by exposure to SDS, C8, C6, and C4 (table 3), it appears that these compounds may cause toxicity via similar mechanisms. In the present study, the fatty acid β -oxidation pathway was observed to be induced by AS species which resemble fatty acids. One H_2O_2 molecule is known to be generated from the reaction of fatty acyl coenzyme oxidase (28), for every two carbon atoms oxidized per cycle in the β -oxidation pathway (33). Thus, the amount of H_2O_2 is directly related to the number of carbon atoms in the structure of the oxidized compounds. While Hiltunen et al., reported that yeast has specific catalase genes (*CTA1*, *CTT1*) for the breakdown of H_2O_2 , catalase activity was found to be dispensable. It is possible that following AS treatment, the AS was modified by acyl CoA synthetase (*FAA2*) before being oxidized via rapid flux of the fatty acid β -oxidation pathway which would increase intracellular H_2O_2 levels relative to the untreated control. The amount of H_2O_2 produced is directly proportional to the chain length of the AS species—the longer, the more H_2O_2 produced. Thus, one possible cause of differential toxicity of different AS species is related to chain length. AS species of longer chain length are expected to be more toxic, all other considerations being equal.

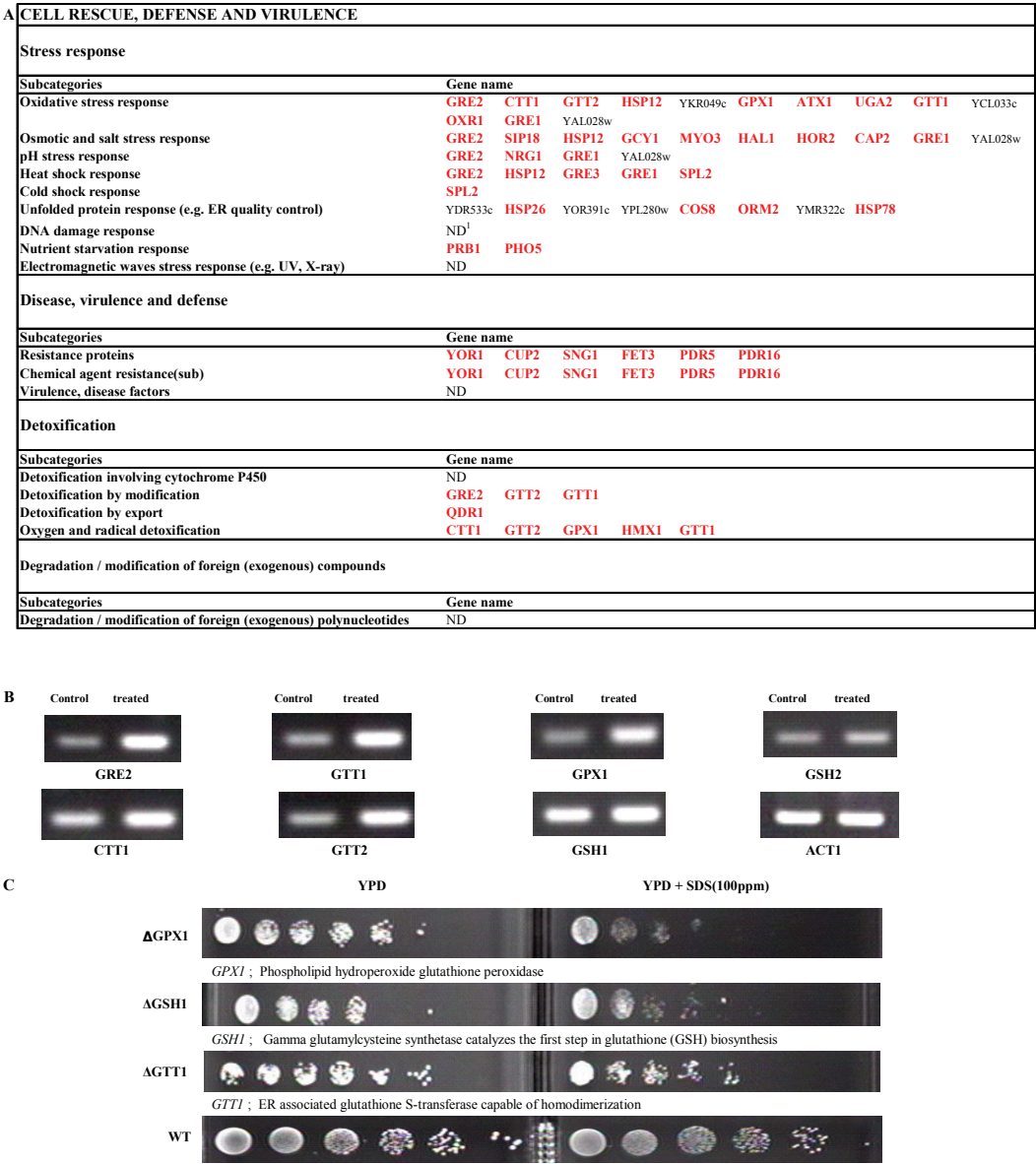


Figure footnote: 1ND, No significant data.

Figure 5. Significantly induced genes in the cell rescue, defense and virulence category. A. Genes marked in red and black are verified and uncharacterized, respectively, and are classified into sub-categories of function and are listed left to right in descending order of induction level. B. Induction of key genes as assayed by RT-PCR. C. Sensitivity of deletion mutants to SDS.

3.8 Functional genomics approach explains potential toxicity better than simple tabulation of highly up-regulated genes

Tabulating the highly up-regulated genes in a microarray experiment has been widely used as an approach for representing and describing toxicity in the field of toxicogenomics. Determining the most highly induced genes in a given experiment is fundamental to understanding the characteristics of toxicity.

We found that AS treatment induced high level expression of 26 genes (table 3). However, a number of deletion mutants missing these genes were not found to be sensitive to SDS, for example (deletion mutants were constructed for genes whose names are underlined, table 3). This result indicates that highly up-regulated genes do not necessarily promise to be important with respect to response to toxicity or cell survival. In the present study, highly up-regulated genes provided useful general information concerning the effects of cell exposure to AS, but alone they could not provide detailed information on the consequences of toxicity.

We believe that our functional genomics approach for extracting meaningful information from microarray analyses is superior to simple tabulation of lists of highly expressed genes for the following reasons. First, correlating significant localization categories for gene products based on exposure to AS, with significant functional gene categories increased the likelihood that the association was not due to chance. Second, confirming the microarray-based expression data with RT-PCR eliminated false positive genes. Third, seeking additional genes from among relevant sub-categories of implicated functions and testing their expression by RT-PCR led to new positive leads. Finally, testing the AS-sensitivity of a number of candidate deletion mutants provided additional evidence that the genes were indeed relevant to AS exposure.

Table 3. Highly up-regulated genes induced by all AS species. Genes are listed in descending order of induction level. Deletion mutants missing the underlined genes were tested for SDS sensitivity. Genes in bold font are annotated.

Gene	Localization	Function
POX1	Peroxisome	Fatty-acyl coenzyme A oxidase
SMP1	Nucleus	MADS-box transcription factor
PIR3	Cell wall	Member of the Pir1p/Pir2p/Pir3p family
OYE3	Cytoplasm	NAPDH dehydrogenase (old yellow enzyme), isoform 3
<u>YPL088W</u>	Cytoplasm	Similarity to aryl-alcohol dehydrogenases
YPS3	Plasma membrane	Gpi-anchored aspartic protease (Yapsin 3)
RTA1	Integral membrane	Involved in 7-amincholesterol resistance
<u>YLR194C</u>	ER	Protein of unknown function localised to ER
MLP1	Cytoplasm	Ser/thr-specific protein kinase, involved in protection against oxidative stress
PHM8	Cytoplasm	Protein of unknown function
YPK2	Cytoplasm	Ser/thr protein kinase
BAG7	Cytoskeleton	Rho GTPase activating protein
<u>YDR034W-B</u>	Cell periphery	Protein of unknown function
AFR1	Cytoskeleton	Protein involved in morphogenesis of the mating projection
YPC1	Integral membrane	Alkaline Ceramidase
<u>YML131W</u>	Cytoplasm	Putative hydroxydehydrogenase
HSP31	Cytoplasm	Member of the DJ-1/ThiJ/PfpI superfamily, chaperone and cysteine protease
<u>YHR209W</u>	Cytoplasm	Putative methyltransferase
FBP26	Cytoplasm	Fructose-2,6-bisphosphatase
<u>YHR138C</u>	Cytoplasm	Protein involved in vacular fusion
GRE2	Cytoplasm	Induced by osmotic stress; similar to dihydroflavonol 4-reductase from plants
PRM5	Mitochondria	Pheromone-regulated protein, induced during cell integrity signalling
CRH1	Cell wall	Family of putative glycosidases might exert a common role in cell wall organization
PRB1	Vacuole	Protease B, vacuolar
<u>YFL052W</u>	Unknown	Strong similarity to Maltose fermentation regulatory proteins Mal13p, Mal33p and Mal63p
PCA1	Integral membrane	P-type ATPase, controls intracellular cadmium level by enhancing cadmium efflux

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